CLEAVABLE CONJUGATES OF TRANSTHYRETIN LIGAND AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/572,081 filed March 29, 2024, and U.S. Provisional Application No. 63/748,386 filed January 22, 2025, the entire contents of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] Aspects of the invention generally relate to a delivery system for active agents having a ligand (binder) that is selective for transthyretin (TTR) in the serum of a subject; and a cleavable linker configured for operatively attaching the ligand covalently to a drug to increase the plasma circulation time and duration of the drug in the serum.

BACKGROUND OF THE INVENTION

[0003] The poor pharmacokinetic profile of many drugs represents a major challenge in their continued development. This problem, in particular, has limited the ability of drugs to reach their tremendous therapeutic potential. In particular, the half-life of many drugs in the blood serum of a subject is of serious concern. Short half-life of a drug is usually due to (i) enzymatic degradation by serum proteases and/or (ii) fast renal clearance due to the molecular weight cutoff for peptides and proteins to be cleared through glomerular filtration being relatively high at approximately 30 kilodaltons. Those of skill in the art recognize that the short in vivo half-life of numerous drug candidates has limited their clinical potential by increasing the size and frequencies of doses needed to achieve the desired results, for example.

[0004] Accordingly, the art will appreciate having a delivery system that (i) improves the duration of action and plasma circulation of active drugs; (ii) avoids steric hindrance problems that affect binding affinity and potency of the active agents; (iii) avoids the renal tubular vacuolation caused by PEGylation; (iv) avoids the generation of antibodies that create immune response problems; and (v) has low manufacturing costs.

BRIEF SUMMARY OF THE INVENTION

[0005] It has been surprisingly discovered that the duration of action (DOA) of a drug can be significantly extended by covalently attaching the drug to a ligand that is selective for transthyretin (TTR) through a cleavable linker. Accordingly, the disclosure relates to a

fundamentally new prodrug approach for enhancing the plasma circulation and DOA of drugs without affecting their biological activity.

[0006] In one aspect, provided herein is a conjugate of formula (I):

$$T—L—L^B—E—D^1 \qquad (I)$$

or a pharmaceutically acceptable salt thereof, wherein:

T is a ligand selective for transthyretin TTR;

L is a linker;

L^B is absent or a click product formed via a Click reaction between a first Click handle and a second click handle;

E is a cleavable ester; and

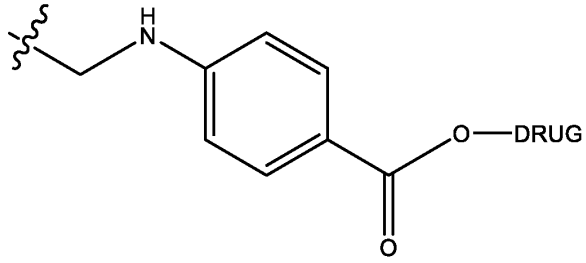
D^1 is a drug moiety.

[0007] In some embodiments, the cleavable ester moiety (E) of the conjugate of formula (I) comprises an aromatic ring. In some embodiments, the aromatic ring can be optionally substituted with one or more substituents as set forth herein. In some embodiments, para-amino benzoic acid (PABA) is used as a basic building block for forming an ester with a drug. In some embodiments, para-hydroxybenzoic benzoic acid (PHBA) is used as a basic building block for forming an ester with a drug moiety. In some embodiments, substituted PABA is used as a basic building block for forming an ester with a drug. In some embodiments, the aromatic ester group (e.g., PABA or PHBA) of the conjugate of formula (I) is hydrolyzed to a greater extent by carboxylesterase 2 (CES2) than by carboxylesterase 1 (CES1). In some embodiments, the aromatic ester group (e.g., PABA or PHBA) of the conjugate of formula (I) is hydrolyzed by CES2 but only to a minimal extent by CES1. In some embodiments, the aromatic ester group (e.g., PABA) of the conjugate of formula (I) is fully resistant to CES1.

[0008] Surprisingly, in some embodiments, it has been discovered that the TTR ligand influences the extent of hydrolysis and carboxylesterase selectivity of aromatic ester groups (e.g., PABA or PHBA groups) in the conjugate. For instance, in certain embodiments, TTR ligands (binders) of a conjugate of formula (I) are capable of increasing CES2 selectivity to conjugates of formula (I). Such CES2 selectivity provides for a more controlled or extended

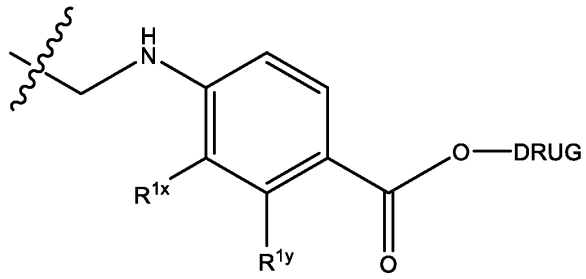
release of the drug from the conjugate. Additionally, the rate of hydrolysis can be effectively tuned (e.g., increased or decreased) by particular substitutions on the aromatic ester group (e.g., PABA or PHBA group) of the conjugate.

[0009] In some embodiments, the conjugate of formula (I) includes the following ester moiety:



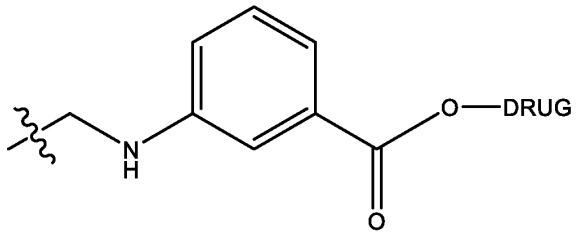
wherein ≈ represents the point of attachment to L or L^B.

[0010] In some embodiments, the conjugate of formula (I) includes the following ester moiety:



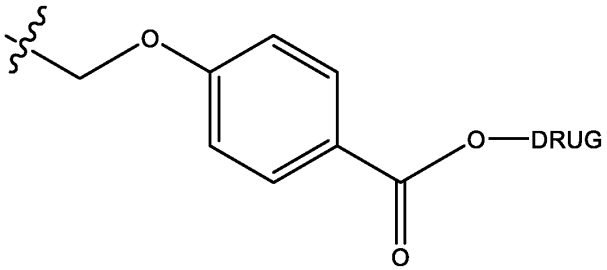
wherein ≈ represents the point of attachment to L or L^B, R^{1x} is H, F, Cl, NO_2, CF_3, or OMe, and R^{1y} is H or Me. In some embodiments, R^{1x} is H or Me and R^{1y} is H, F, Cl, Br, NO_2, CF_3, Me, or OMe. In some embodiments, R^{1x} is H and R^{1y} is Me.

[0011] In some embodiments, the conjugate of formula (I) includes the following ester moiety:



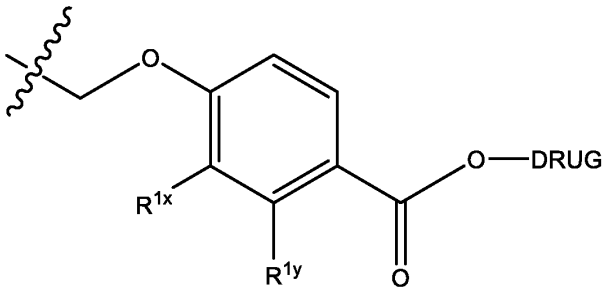
wherein ≀ represents the point of attachment to L or L^B.

[0012] In some embodiments, the conjugate of formula (I) includes the following ester moiety:



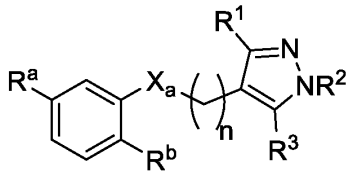
wherein ≀ represents the point of attachment to L or L^B.

[0013] In some embodiments, the conjugate of formula (I) includes the following ester moiety:



wherein ≀ represents the point of attachment to L or L^B, R^{1x} is H, F, Cl, NO_2, CF_3, or OMe, and R^{1y} is H or Me. In some embodiments, R^{1x} is H or Me and R^{1y} is H, F, Cl, Br, NO_2, CF_3, Me, or OMe. In some embodiments, R^{1x} is H and R^{1y} is Me.

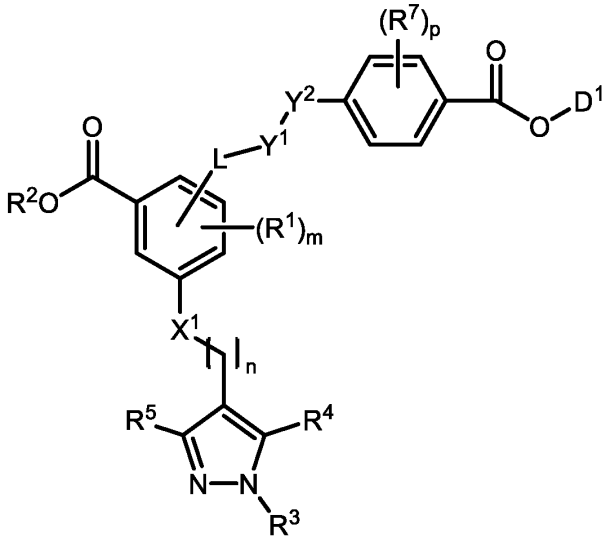
[0014] In some embodiments, the ligand selective for transthyretin TTR (T) is a compound of Formula (E)



(E)

or a pharmaceutically acceptable salt thereof, wherein the variables R^1, R^2, R^3, R^a, R^b, X and a are defined herein. In some embodiments, a conjugate of formula (I) comprises a ligand of formula (E) and the aromatic ester group (e.g., PABA or PHBA). In some embodiments, the compound of formula (E) increases CES2 selectivity to aromatic ester group (e.g., PABA or PHBA) in vivo.

[0015] In one embodiment, provided herein is a conjugate of formula (II):



(II),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0016] Any embodiments provided herein of a conjugate of formula (I) or formula (II), or pharmaceutically acceptable salts thereof, apply where applicable to any other formula detailed herein, the same as if each and every embodiment were specifically and individually listed. Thus, it is understood and described that each embodiment provided herein of a conjugate of formula (I) or formula (II) apply to conjugates of formulas (III)-(XII) or (III')-(VIII'), or variations thereof, or pharmaceutically acceptable salts thereof, the same as if each and every embodiment were specifically and individually listed. It is also understood and described that all such embodiments may be used in any of the pharmaceutical compositions, methods, kits, uses, or other aspects detailed herein.

[0017] In one aspect, provided herein are methods of increasing the plasma circulation time and DOA of a drug, said method comprising administering to a subject (e.g., a human patient) a therapeutically effective amount of a conjugate of the disclosure (e.g., conjugate of formula (I) or formula (II)). In some embodiments, administering the conjugate of the disclosure (e.g., conjugate of formula (I) or formula (II)) reduces the immunogenicity of the active agent relative to administering the drug alone in free form (nonconjugated form).

[0018] In some embodiments, a conjugate of the disclosure is administered parenterally to a subject in need thereof. For instance, a conjugate of the disclosure can be administered

subcutaneously, intravenously, intramuscularly or intrathecally. In other embodiments, the conjugates of the disclosure are administered orally.

[0019] In some embodiments where a conjugate of the disclosure is administered subcutaneously, the conjugates form a depot in the subcutaneous tissue. In some such embodiments, the solubility of the conjugate at physiological pH is less than the solubility of the unconjugated drug at physiological pH. As a result, the slow dissolution of the conjugate relative to the unconjugated drug slows the release of the drug into the systemic circulation, hence extending the duration of action of the drug. Subsequent binding of the conjugate to TTR in the systemic circulation further extends the duration of action of the drug. As shown in Example 11, in some embodiments, the ability of the conjugates to form depots at attributed to the zwitterionic nature of the conjugates at physiological pH. In some embodiments, despite a reduction of the solubility of the conjugate relative to the unconjugated drug, the conjugate is rapidly absorbed, resulting in a fast onset of action. Accordingly, conjugates of the disclosure provide drugs with both rapid onset and extended duration of action.

[0020] Conjugates of the disclosure, particularly conjugates of formula (II), show enhanced adsorption from tissue, particularly subcutaneous tissue, owing, in part, to the balanced hydrophilicity of the TTR selective ligand. Following adsorption from tissue (e.g., subcutaneous tissue), reversible binding of the TTR selective ligand of the conjugate results in significantly extended duration of action of the active agent. As set forth herein, cleavage of the linker between the TTR selective ligand and the active agent results in release of the active agent, hence allowing the active agent to exert its pharmacological effect.

[0021] Moreover, it has been discovered that release of the active agent can be effectively controlled by proper selection of a cleavable moiety in the conjugate. For instance, particular ester moieties, as described herein, hydrolyze slowly following administration, thereby releasing the active agent in a controlled manner for an extended period of time. The slower hydrolysis and the high degree of binding to TTR effectively extend the plasma circulation time and duration of action (DOA).

[0022] Therefore, the conjugates of the disclosure manifest several beneficial properties for a new prodrug approach. The balanced hydrophilicity of the conjugates allows for rapid adsorption into the blood from the tissue, the binding of TTR extends the plasma circulation time, and the cleavable moiety allows for modulation of release of the drug. The desired rate

of release of a particular active drug from a conjugate of the disclosure will vary from one active agent to another.

[0023] In some embodiments, D^1 is a peptide. As shown in the examples, conjugates of the disclosure comprising peptides as the active drug display markedly increased half-life duration of action relative to the free peptide drug, hence enabling reduced dosing of the peptide drugs.

[0024] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0025] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to treat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0026] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide) receptor agonist used to treat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0027] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0028] In some embodiments, the active drug (D^1) delivered by a conjugate of the disclosure is a small molecule, a nucleotide, an oligonucleotide, a polynucleotide, an amino acid, an oligopeptide, a polypeptides, or a protein. Active drugs, include. can include, but are not limited to, antiproliferatives, antineoplastics, antimitotics, anti-inflammatories, antiplatelets, anticoagulants, antifibrins, antithrombins, antibiotics, antiallergics, antioxidants.

[0029] In some embodiments, D^1 is a peptide, an oligopeptide, a polypeptide, a protein, an antibody, an oligonucleotide, a polynucleotide, a virus-like particle, a small molecule, an oligosaccharide, or an imaging agent.

[0030] In some embodiments, D^1 is a protein. In some embodiments, D^1 is an antibody.

[0031] In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0032] In some embodiments, D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0033] In some embodiments, D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0034] In some embodiments, D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0035] In some embodiments, following the administration of a conjugate of the disclosure (e.g., a conjugate of formula (I) or formula (II)), the plasma circulation and the duration of action (DOA) of the drug is at least 12 hours. In some such embodiments, following the administration of a conjugate the disclosure (e.g., a conjugate of formula (I) or formula (II)), the DOA of the drug is at least 20 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of drug is at least 96 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 1 week. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 2 weeks. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 1 month.

[0036] In some embodiments, the conjugates disclosed herein can provide a controlled and/or extended release of a drug. In some such embodiments, slow release of the drug from the conjugate can be administered safely.

[0037] In some embodiments, a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.005 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.01 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 10 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 10 mg/kg to about 20 mg/kg.

BRIEF DESCRIPTION OF FIGURES

[0038] **Fig. 1** shows that prodrugs of the disclosure bind selectively to TTR in human plasma and release naloxone, other drugs, and peptides in rat plasma. **Fig. 1a,** Chemical structures of naloxone, AG10-L, and prodrugs. AG10-L-E1-Naloxone is an alkyl ester prodrug. AG10-L-E2-Naloxone is a PABA aryl ester prodrug. C12-E2-Naloxone is a control fatty acid-conjugated PABA aryl ester prodrug. A number of other PABA-based prodrugs of small molecules and peptides are shown. **Fig. 1b**, Aqueous solubility of compounds (incubated at 100 μM) in potassium phosphate buffer (pH 7.4) and Log $D_{7.4}$ values of test compounds at physiological pH of 7.4 in potassium phosphate buffer (pH 7.4) and n-octanol. **Fig. 1c**, Binding affinity of compounds to purified human TTR in buffer was evaluated using fluorescence polarization assay. Compounds were tested at concentration range of 0.01 μM to

20 μM). The IC_{50} values were used to calculate the binding constant (K_d) using the Cheng–Prusoff equation. Data represent the mean ± s.d. (n = 3 independent replicates). **Fig. 1d**, Evaluation of the binding selectivity of compounds (tested at 10 μM) for TTR in human serum (concentration ~5 μM). The modification of TTR in human serum by covalent FPE probe was monitored for 6 h in the presence of FPE probe alone (DMSO) or probe and TTR ligands. Ligands that display lower fluorescence of FPE probe have higher binding selectivity to TTR. **Fig. 1e**, The 3 hr readings, relative to probe alone, were used to calculate the percent occupancy. Data represent the mean ± s.d. (n = 4 independent replicates). **Fig. 1f**, Plasma stability of prodrugs AG10-L-E1-Naloxone, AG10-L-E2-Naloxone, and C12-E2-Naloxone (tested at 5 μM) in human and **Fig. 1g**, rat plasma. The concentration remaining in respective media was determined at the indicated timepoints using HPLC. Bar graphs represent the mean of % compound remaining ± s.d. (n = 3 independent replicates).

[0039] **Fig. 2** shows that AG10-L-E2-Naloxone is bioactivated by CES2 enzyme hydrolysis. **Fig. 2a**, Chemical structures of AG10-L-E2-Naloxone and its hydrolysis products, AG10-L-E2-COOH and naloxone. **Fig. 2b**, Docking study of AG10-L-E2-Naloxone to both human TTR (pdb id: 4HIQ)[6] and mouse CES2 enzyme (pdb id: 8AXC). The linker length for AG10-L-E2-Naloxone (~29 Å) is long enough to allow AG10-L-E2-Naloxone to bind to both CES2 and TTR simultaneously. **Fig. 2c**, AG10-L-E2-Naloxone is hydrolyzed by purified CES2 in buffer but not with CES1. **Fig. 2d**, Metabolic bioactivation of AG10-L-E2-Naloxone in liver microsomes from human (HLM), rat (RLM), and monkey (MLM). HLM effectively produced naloxone from AG10-L-E2-Naloxone. The selective CES2 inhibitor, loperamide, significantly decreased the amount of naloxone released while there was no effect of the CES1 inhibitor, digitonin. The concentration of naloxone released from AG10-L-E2-Naloxone in HLM, MLM, and RLM was determined using validated LC-MS/MS method. **Fig. 2e**, Stability of AG10-L-E2-Naloxone (tested at 5 μM) in rat plasma in the absence and presence of CES2 inhibitor, loperamide, and CES1 inhibitor, digitonin. **Fig. 2f**, Stability of AG10-L-E2-Naloxone (tested at 5 μM) in monkey plasma (no CES1 or CES2 in plasma) and rabbit plasma (high abundance of CES1 and CES2 in plasma). The concentration remaining in respective media was determined at the indicated timepoints using HPLC. Bar graphs for **Fig. 2c** through **Fig. 2f** represent the mean of % compound remaining ± s.d. (n = 3 independent replicates).

[0040] **Fig. 3** shows pharmacokinetic/Pharmacodynamic evaluation of AG10-L-E2-Naloxone in rats. **Fig. 3a**, A single subcutaneous (s.c.) injection of 15 μmol kg^{-1} of test compound

(equivalent to 5 mg kg^{-1} of naloxone and 15.8 mg kg^{-1} of AG10-L-E2-Naloxone) were administered to male Sprague Dawley rats (*n = 3* for each group). The concentration of the test compounds in plasma was determined at different time points and expressed as means ± s.d. of three biological replicates. **Fig. 3b**, Concentration of brain and **Fig. 3c**, cerebrospinal fluid (CSF) naloxone (freely administered) and naloxone released from AG10-L-E2-Naloxone. Male Sprague Dawley rats (*n = 3* for each group) were dosed with a single subcutaneous (s.c.) injection of 15 µmol kg^{-1} of test compound (equivalent to 5 mg kg^{-1} of naloxone and 15.8 mg kg^{-1} of AG10-L-E2-Naloxone). The concentration of naloxone in brain tissue and CSF measured at 10 min and 30 min after dosing. Bar graphs show the respective mean ±s.d. of three biological replicates. **Fig. 3d**, Mortality data for morphine overdose and rescue by antagonists in male Sprague Dawley rats (*n = 9* biological replicates for each group). Dosing schedule of morphine (s.c. doses of 100 mg kg^{-1} at 0 hr and 50 mg kg^{-1} at 6 hr) and antagonists (naloxone and AG10-L1-E2-Naloxone; 15 µmol kg^{-1}; single s.c. dose at 5 min). **Fig. 3e**, Mortality data for fentanyl overdose and rescue by antagonists in male Sprague Dawley rats (*n = 8* biological replicates for each group). Dosing schedule of fentanyl (s.c. doses of 3 mg kg^{-1} at 0 hr and 3 mg kg^{-1} at 6 hr) and antagonists (naloxone and AG10-L1-E2-Naloxone; 15 µmol kg^{-1}; single s.c. dose at 5 min). **Fig. 3f** through **Fig. 3i**, Reversal of fentanyl OIRD in male Sprague Dawley rats (*n= 6* per group) by naloxone, AG10-L-E1-Naloxone, and AG10-L-E2-Naloxone, and C12-E2-Naloxone (15 µmol kg^{-1}; single s.c. doses at 1 min). **Fig. 3f**, Dosing schedule of fentanyl and antagonists. Reversal of OIRD is represented by Minute Volume relative to control (Minute Volume of rats before fentanyl injection was considered to be the baseline). Minute Volume (mean ± s.d.; *n= 6* biological replicates per group) is plotted as a function of time after **Fig. 3g**, first dose of fentanyl and antagonists, **Fig. 3h**, second fentanyl dose after 6 hr, and **Fig. 3i**, third fentanyl dose after 24 hr. Animals were monitored in plethysmography chamber for 90 min after each fentanyl dose. Data were analyzed using the GraphPad Prism V9.3.1.471 software (La Jolla, CA, USA).

[0041] Fig. 4 shows reversal of fentanyl OIRD by naloxone, nalmefene, and AG10-L-E2-Naloxone. **Fig. 4a**. A single subcutaneous (s.c.) injection of 60 µmol kg^{-1} of test compound (equivalent to 20 mg kg^{-1} of naloxone and 63 mg kg^{-1} of AG10-L-E2-Naloxone) were administered to male Sprague Dawley rats (*n = 3* for each group). The concentration of the test compounds in plasma was determined at different time points and expressed as means ± s.d. of three biological replicates. **Fig. 4b**, Dosing schedule of fentanyl and antagonists (60 µmol kg^{-1}). Reversal of OIRD is represented by Minute Volume relative to control (Minute

Volume of rats before fentanyl injection was considered to be the baseline). Minute Volume (mean ± s.d.; *n= 6* biological replicates per group) is plotted as a function of time after **Fig. 4c,** first dose of fentanyl and antagonists, **Fig. 4d**, second fentanyl dose after 6 hr, and **Fig. 4e**, third fentanyl dose after 24 hr. Animals were monitored in plethysmography chamber for 90 min after each fentanyl dose. **Fig. 4f**, A single intravenous (i.v.) injection of 60 μmol kg^{-1} of test compound (equivalent to 20 mg kg^{-1} of naloxone and 63 mg kg^{-1} of AG10-L-E2-Naloxone) were administered to male Sprague Dawley rats (*n = 3* for each group). The concentration of the test compounds in plasma was determined at different time points and expressed as means ± s.d. of three biological replicates. **Fig. 4g** through **Fig. 4i**, Reversal of fentanyl induced OIRD by intravenously (i.v.) administered naloxone and AG10-L-E2-Naloxone. Minute Volume (mean ± s.d.; *n= 6* biological replicates per group) is plotted as a function of time after **Fig. 4g**, First dose of fentanyl (3 mg kg^{-1}; i.v. dose) was followed by antagonist (60 μmol kg^{-1}; single i.v. dose) after 1 min. **Fig. 4h**, second dose of fentanyl (1.5 mg kg^{-1}; i.v. dose). **Fig. 4i**, third dose of fentanyl (1.5 mg kg^{-1}; i.v. dose). Animals were monitored in plethysmography chamber for 90 min after each fentanyl dose. The plethysmography data were analyzed as described above in **Fig. 3f** through **Fig. 3i**.

[0042] Fig. 5 shows preclinical evaluation and pharmacokinetic study of AG10-L-E2-Naloxone in monkey. **Fig. 5a** through **Fig. 5d**, Evaluating the efficacy of a mixture of AG10-L-E2-Naloxone and low dose of free naloxone against fentanyl induced OIRD in rats. A single s.c. dose combination of AG10-L-E2-Naloxone (60 μmol kg^{-1}; equivalent to 63 mg kg^{-1}) and naloxone (3 μmol kg^{-1}; equivalent to 1 mg kg^{-1}) resulted in excellent efficacy data with full reversals of OIRD after all three fentanyl doses. Minute Volume (mean ± s.d.; *n= 6* biological replicates per group). **Fig. 5e** through **Fig. 5g**, Evaluating the potential of using AG10-L-E2-Naloxone as a prophylactic agent against fentanyl in rats. Rats were first dosed with AG10-L-E2-Naloxone (60 μmol kg^{-1}) or naloxone (15 μmol kg^{-1} and 60 μmol kg^{-1}). After 6 hr, the rats were challenged with a single dose of fentanyl (1.5 mg kg^{-1}; s.c. dose). Minute Volume (mean ± s.d.; *n= 6* biological replicates per group). The plethysmography data were analyzed as described above in **Fig. 3f** through **Fig. 3i**. **Fig. 5h**, Effect of AG10-L-E2-Naloxone (60 μmol kg^{-1}; s.c. dose) administration on body weight of rats over four weeks. Three groups of rats were used. Group one was treated with a vehicle. Group two was treated with AG10-L-E2-Naloxone (60 μmol kg^{-1}). Group three was treated with fentanyl followed by AG10-L-E2-Naloxone (60 μmol kg^{-1}) and then doses of fentanyl at 6 hr and 24 hr. Body weigh represent mean ± s.d. (*n= 6* biological replicates per group). **Fig. 5i** through **Fig. 5k**, Assessment of the cytotoxicity of AG10-L-E2-Naloxone and its metabolite, AG10-L-E2-

COOH (at 100 μM), on a number of cell lines using the MTT assay after 72 hr. Doxorubicin was used as a positive control (at 10 μM). **Fig. 5i**, HeLa; cervical cancer cell line. **Fig. 5j**, Hep3B; human hepatoma cell line. **Fig. 5k**, MCF7; breast cancer cell line. Each bar shows mean ± s.d. (*n= 4* independent replicates). **Fig. 5l**, A single subcutaneous (s.c.) injection of AG10-L-E2-Naloxone (30 μmol kg^{-1}; equivalent to 31 mg $kg^{-1}$of) was administered to three male cynomolgus monkey. The concentration of the AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone in plasma for each monkey was determined at different time points and expressed as means ± s.d. of three biological replicates.

[0043] Fig. 6 shows calculated pKa values and predicted ionization for, AG10-L-E2-Naloxone (**Fig, 6a**), AG10-L-E2-COOH (**Fig, 6b**), and AG10-L-E2-Ethyl (**Fig, 6c**) in water (acidic ph ~3.5 obtained by dissolving the HCl salts of these conjugates in water) and physiological pH (7.4).

[0044] Fig. 7 shows docking study of AG10-L-E2-Naloxone and known CES2 selective prodrug esters with CES2 and CES1. The mouse CES2 enzyme (pdb id: 8AXC)[1] catalytic triad residues S230, E347, and H459 are represented as black sticks. Zoom in highlighting the distance between the nucleophilic S230 and the carbonyl ester of AG10-L-E2-Naloxone is 2.9 Å. The distance between S230 and the carbonyl ester of prodrugs irinotecan, procaine, and molnupiravir is 3.3 Å, 3.1 Å, and 3.5 Å, respectively. Docking of prodrugs with CES1 (pdb id: 1MX9)[2] showed longer distance of 9.2 Å, 6.8 Å, and 4.8 Å for irinotecan, procaine, and molnupiravir, respectively. Docking studies showed no binding of AG10-L-E2-Naloxone to CES1.

[0045] Fig. 8 shows chemical structures and stability of newly synthesized aryl-based naloxone prodrugs. **Fig. 8a**, Chemical structures of newly synthesized aryl-based naloxone prodrugs; BA-Naloxone, Boc-PABA-Naloxone, PABA-Naloxone and known CES2 selective prodrugs. Stability of prodrugs in **Fig. 8b** and **Fig. 8c** human plasma (tested at 100 μM), and **Figs. 8d-i**, rat plasma. Stability of compounds (tested at 100 μM) in rat plasma was performed in the absence and presence of CES2 inhibitor, loperamide, and CES1 inhibitor, digitonin. The known CES2 selective substrates procaine (local anesthetic), molnupiravir (COVID-19 therapeutic), and irinotecan (anticancer drug) were included for comparison. The acyl moieties of the esters are shown in red color. The concentration remaining in respective media was determined at the indicated time points using HPLC. Bar graphs represent the

mean of % compound remaining ± s.d. (n = 3 independent replicates). Statistical differences were determined using two-way ANOVA (mixed model) followed by Tukey's post hoc test.

[0046] Fig. 9 shows evaluations of the effect of TTR on the in vitro pharmacokinetics of AG10-L-E2-Naloxone**. Fig. 9a**, Stability of AG10-L-E2-Naloxone (tested at 5 μM) in rat plasma in the absence and presence of the selective TTR ligand, AG10 (10 μM). **Fig. 9b**, Metabolic bioactivation of AG10-L-E2-Naloxone (5 μM) in human liver microsomes (HLM), with and without human TTR (5 μM). **Fig. 9c**, Evaluating the effect of AG10-L-E2-Naloxone's (5 μM) binding to human TTR (5 μM) in buffer using rapid equilibrium dialysis with molecular weight cut-off (MWCO) of 8 kDa. Bar graphs for **Figs. 9a-c** represent the mean of % compound remaining/bound ± s.d. (n = 3 independent replicates). Statistical differences were determined using two-way ANOVA (mixed model) followed by Holm-Šídák test for a, b, and one-way ANOVA followed by Tukey's post hoc test for c. For a, $F(2,8) = 62.29$, ($^*P < 0.0001$ compared to AG10-L-E2-Naloxone). For b, $F(1,4) = 223.4$, ($^*P < 0.0001$ compared to AG10-L-E2-Naloxone). For c, $F(2,6) = 3216$, ($^*P < 0.0001$ compared to AG10-L-E2-Naloxone with no TTR or AG10).

[0047] Fig. 10 shows evaluation of the blood brain barrier penetration of AG10-L-E2-Naloxone and AG10-L-E2-COOH. **Fig. 10a**, The brain concentration to plasma concentration ratio and **Fig. 10b**, CSF to plasma concentration ratio of AG10-L-E2-Naloxone, AG10-L-E2-COOH, naloxone from AG10-L-E2-Naloxone, and freely administered naloxone. Male Sprague Dawley rats (n = 3 for each group) were dosed with a single s.c. injection of 15 μmol kg^{-1} of test compound (equivalent to 5 mg kg^{-1} of naloxone and 15.8 mg kg^{-1} of AG10-L-E2-Naloxone). Bar graphs show the respective mean ±s.d. of three biological replicates. Statistical differences were determined using two-way ANOVA followed by Holm-Šídák test ($^*P < 0.0001$ compared to naloxone). For the brain to plasma ratio experiment $F(1,4) = 59.34$, $P < 0.0001$ and for the CSF to plasma ratio experiment $F(1,4) = 35.87$, $P < 0.0001$.

[0048] Fig. 11 shows pharmacodynamic evaluation of repeated doses of AG10-L-E2-Naloxone in rats. Mortality data for fentanyl overdose and rescue by multiple small doses of naloxone in male Sprague Dawley rats (n = 8 biological replicates for each group). Dosing schedule of fentanyl (s.c. doses of 3 mg kg^{-1} at 0 hr and 3 mg kg^{-1} at 6 hr). Group1 (red line): dosed with vehicle. Group 2 (gray line): first dosed with a single s.c. dose of 3 μmol kg^{-1} of naloxone (equivalent to 1 mg kg^{-1}) 5 min after the first fentanyl dose followed by five smaller

doses of naloxone (0.6 μmol kg^{-1} of naloxone per dose; equivalent to 0.2 mg kg^{-1} per dose) at 1.5, 2.5, 3.5, 4.5, and 5.5 hr. Group 3 (blue line): first dosed with a single s.c. dose of 3 μmol kg^{-1} of naloxone (equivalent to 1 mg kg^{-1}) 5 min after the first fentanyl dose followed by ten smaller doses of naloxone (0.6 μmol kg^{-1} of naloxone per dose; equivalent to 0.2 mg kg^{-1} per dose) at 1.5, 2.5, 3.5, 4.5, 5.5., 6.5, 7.5, 8.5, 9.5, and 10.5 hr. Statistical differences were determined using Log-rank (Mantel-Cox) test followed by Bonferroni correction method for multiple comparisons, Bonferroni-corrected $\alpha = 0.016$. $^{*}P < 0.0001$ compared to vehicle and $^{**}P < 0.0026$ compared to vehicle.

[0049] Fig. 12 shows comparison of the pharmacokinetic profiles of the low and high doses of s.c. administered naloxone for up to 6 hr. The naloxone pharmacokinetic profiles (data from Fig. 3a and 5a) show that the higher naloxone dose (20 mg kg^{-1}, s.c.) has a slower clearance rate between 0.5 and 2 hr compared to the 5 mg kg^{-1} dose, likely due to temporary saturation of clearance pathways. After 2 hr, both doses exhibit similar clearance rates and linear kinetics, indicating limited tissue redistribution effects at the higher dose.

[0050] Fig. 13 shows reversal of intravenously (i.v.) administered fentanyl OIRD in male Sprague Dawley rats by multiple small doses of naloxone or AG10-L-E2-Naloxone. **Fig. 13a**, Dosing schedule of fentanyl and antagonists. Naloxone (0.6 μmol kg^{-1} per i.v. dose; equivalent to 0.2 mg kg^{-1}) was administered within 1 min of the first fentanyl dose (3 mg kg^{-1}; i.v. dose) followed by five small i.v. doses at 2, 3, 4, 5, 6 hr followed by a second dose of fentanyl (1.5 mg kg^{-1}; i.v. dose) at 6 hr ($n = 6$ per group). AG10-L-E2-Naloxone (15 μmol kg^{-1} per i.v. dose; equivalent to 5 mg kg^{-1}) was administered within 1 min of the first fentanyl dose (3 mg kg^{-1}; i.v. dose) followed by one dose at 4 hr. The second dose of fentanyl (1.5 mg kg^{-1}; i.v. dose) was administered at 6 hr. Reversal of OIRD is represented by Minute Volume relative to baseline (Minute Volume of rats before fentanyl injection was considered to be the baseline). Minute Volume (mean ± s.d.; $n = 6$ biological replicates per group) is plotted as a function of time after **Fig. 13b**, first dose of fentanyl and antagonists, **Fig. 13c**, second fentanyl dose after 6 hr. Animals were monitored in plethysmography chamber for 90 min after each fentanyl dose. Data were analyzed using the GraphPad Prism V9.3.1.471 software (La Jolla, CA, USA). A priori analysis revealed that to achieve a statistical power of 0.8 with alpha equal to 0.05, given our experimental s.d. values, the number of animals per group must be at least 3. The power analysis was performed by G*Power software (version 3.0.10). Statistical difference was determined using two-way ANOVA repeated measures followed by the Tukey's post hoc test. Significant differences were determined at (#) $P < 0.05$.

[0051] Figs. 14a-e show the results of rat plasma stability assays for AG10-L-E2-naloxone, AG10-L-PHBA-E-naloxone, PEG linker-E2-naloxone, AG10-short-L-E2-naloxone, and AG10-L-meta-amino-E-naloxone, respectively.

[0052] Fig. 15 shows the results of human plasma stability assays for AG10-L-E2-naloxone, AG10-L-PHBA-E-naloxone, PEG linker-E2-naloxone, AG10-short-L-E2-naloxone, and AG10-L-meta-amino-E-naloxone.

[0053] Figs. 16a-f show the results rat plasma stability assays for AG10-L-E2-paracetamol, AG10-L-E2-propofol, AG10-L-E2-estrone, AG10-L-E2-endomorphin-2, AG10-L-E2-Tyr-Ala, and AG10-L-E2-Dermorphin respectively.

[0054] Fig. 17 shows the results of human plasma stability assays for AG10-L-E2-paracetamol, AG10-L-E2-propofol, AG10-L-E2-estrone, AG10-L-E2-endomorphin-2, AG10-L-E2-Tyr-Ala, and AG10-L-E2-Dermorphin.

DETAILED DESCRIPTION OF THE INVENTION

[0055] Here we developed a fundamentally new prodrug approach for enhancing the DOA of drugs without affecting their biological activity. Our approach involves conjugating the drug through an ester moiety to a selective TTR ligand. The approach described herein can be used for a variety of drug moieties, including small molecules, peptides and proteins. Using selective TTR ligands, as set forth herein, to harness endogenous TTR represents a major advantage for a new prodrug approach. TTR is a 55 kDa homo-tetrameric protein that is mainly secreted from the liver into the blood and has a circulation half-life of approximately 2 days. The main function of TTR in humans (concentration in plasma ~5 μM) is to transport holo-retinol binding protein (RBP bound to retinol or vitamin A) in blood. Using orthogonal sites to those of holo-RBP, TTR acts as a backup carrier of thyroxine (<1% thyroxine bound).

[0056] Our approach has several unique features that would make it ideal for controlling the release and extending the DOA of drugs. First, the prodrugs are capable of binding to TTR, which could extend their DOA to 2 days or more. Second, the prodrugs have balanced hydrophilicity which is critical for rapid absorption from the subcutaneous tissue to provide sufficient concentration of antidote for effective rescue. Third, the balanced hydrophilicity, small size, and ease of synthesis of our conjugates (we are not using polymers) would make it ideal for subcutaneous (s.c.) administration, which has the advantages of ease of administration and at the same time would provide extension of duration of prodrugs

compared to the intravenous route. Fourth, the modular approach would allow us to generate prodrugs with tunable hydrolysis rates that can be applied for rescue, protection (prophylaxes), or both.

DEFINITIONS

[0057] "Individual" or "subject" refers to mammals and includes humans and non-human mammals. Examples of individuals include, but are not limited to, mice, rats, hamsters, guinea pigs, pigs, rabbits, cats, dogs, goats, sheep, cows, monkeys, and humans. In some embodiments, individual or subject refers to a human.

[0058] As used herein, "about" a parameter or value includes and describes that parameter or value per se. For example, "about X" includes and describes X per se.

[0059] "Treatment" or "treating" is an approach for obtaining beneficial or desired results including clinical results. Beneficial or desired results may include one or more of the following: decreasing one or more symptom resulting from the disease or condition; diminishing the extent of the disease or condition; slowing or arresting the development of one or more symptom associated with the disease or condition (*e.g.*, stabilizing the disease or condition, preventing or delaying the worsening or progression of the disease or condition); and relieving the disease, such as by causing the regression of clinical symptoms (*e.g.*, ameliorating the disease state, enhancing the effect of another medication, delaying the progression of the disease, increasing the quality of life, and/or prolonging survival).

[0060] As used herein, "delaying" development of a disease or condition means to defer, hinder, slow, retard, stabilize and/or postpone development of the disease or condition. This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease or condition.

[0061] As used herein, the term "therapeutically effective amount" or "effective amount" intends such amount of a compound of the disclosure or a pharmaceutically salt thereof sufficient to effect treatment when administered to an individual. As is understood in the art, an effective amount may be in one or more doses, *e.g.*, a single dose or multiple doses may be required to achieve the desired treatment endpoint. An effective amount may be considered in the context of administering one or more therapeutic agents, and a single agent may be

considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable or beneficial result may be or is achieved.

[0062] As used herein, "unit dosage form" refers to physically discrete units, suitable as unit dosages, each unit containing a predetermined quantity of active ingredient, or compound, which may be in a pharmaceutically acceptable carrier.

[0063] As used herein, by "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *e.g.*, the material may be incorporated into a pharmaceutical composition administered to an individual without causing significant undesirable biological effects.

[0064] As used herein, the term "half-life" can be used to refer to lose half of its activity, for example, its pharmacologic, physiologic, or radiologic activity. The term "half-life" can be used interchangeably with the terms "biological half-life" or "terminal half-life". In some embodiments, the half-life of a substance is the time it takes for a substance, for example, a metabolite, drug, signaling molecule, radioactive nuclide, or other substance, to lose half of its pharmacologic, physiologic, or radiologic activity. In some embodiments, the term half-life can be used to refer to the body's cleansing through the function of kidneys and liver in addition to excretion functions to eliminate a substance from the body. In some embodiments, the term "half-life" can be used to refer to the time it takes for the blood plasma concentration of a substance to halve (plasma half-life) its steady-state.

[0065] As used herein, the term "linker", "linkage" and "linking group" refers to a chemical linking moiety that connects two groups and has a backbone of 50 atoms or less in length. In some embodiments, the linker or linkage may be a covalent bond that connects two groups, or a chemical moiety having a chain of between 1 and 50 atoms in length, between 2 and 20 atoms in length, between 3 and 30 atoms in length, between 4 and 40 atoms in length, between 10 and 50 atoms in length, between 10 and 30 atoms in length, between 12 and 26 atoms in length, between 14 and 30 atoms in length, or any range therein in amounts of 1 atom in length. For example, a linker or a portion of a linker can have a length that is 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 45, or 50 carbon atoms in length, where the linker may be linear, branched, cyclic, or a single atom. In some embodiments, the linker may be 10 angstroms, 11 angstroms, 12 angstroms, 13 angstroms, 14 angstroms, 15 angstroms, 16 angstroms, 17 angstroms, 18 angstroms, 19 angstroms, 20 angstroms, 22 angstroms, 23 angstroms, 24 angstroms, 25 angstroms, 26 angstroms, 27

angstroms, 28 angstroms, 29 angstroms, 30 angstroms, 32 angstroms, 34 angstroms, 36 angstroms, 38 angstroms, 40 angstroms, 45 angstroms, 50 angstroms, or any amount therein in increments of 1 angstrom, in length. In some embodiments, one, two, three, four or five or more carbon atoms of a linker backbone may be optionally substituted with a sulfur, nitrogen or oxygen heteroatom. The bonds between backbone atoms may be saturated or unsaturated, usually not more than one, two, or three unsaturated bonds will be present in a linker backbone. The linker may include one or more substituent groups, for example with an alkyl, aryl or alkenyl group. A linker may include, without limitations, oligo(ethylene glycol); ethers, thioethers, tertiary amines, alkyls, which may be straight or branched, e.g., methyl, ethyl, n-propyl, 1-methylethyl (iso-propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), and the like. The linker backbone may include a cyclic group, for example, an aryl, a heterocycle or a cycloalkyl group, where 2 or more atoms, e.g., 2, 3 or 4 atoms, of the cyclic group are included in the backbone. A linker may be cleavable or non-cleavable.

[0066] The linking moiety may be conjugated to the TTR ligand and Y using any convenient functional groups (carboxylic acids, amines, alcohols, carbamates, esters, amide, ethers, thioethers, maleimides, and the like), and linking chemistries. For example, conjugation chemistry described by G. T. Hermanson ("Bioconjugate Techniques", Academic Press, Second Edition, 2008) may be readily adapted for use in preparing the subject heterobifunctional compounds and is hereby incorporated herein by reference in its entirety.

[0067] The term "alkyl", as used herein, refers to an unbranched or branched saturated univalent hydrocarbon chain. As used herein, alkyl has 1-20 carbons (*i.e.*, C_{1-20}alkyl), 1-16 carbons (*i.e.*, C_{1-16}alkyl), 1-12 carbons (*i.e.*, C_{1-12}alkyl), 1-10 carbons (*i.e.*, C_{1-10}alkyl), 1-8 carbons (*i.e.*, C_{1-8}alkyl), 1-6 carbons (*i.e.*, C_{1-6}alkyl), 1-4 carbons (*i.e.*, C_{1-4}alkyl), or 1-3 carbons (*i.e.*, C_{1-3}alkyl). Examples of alkyl groups include, but are not limited to, methyl, ethyl, propyl, *iso*-propyl, *n*-butyl, *sec*-butyl, *iso*-butyl, *tert*-butyl, pentyl, 2-pentyl, *iso*-pentyl, *neo*-pentyl, hexyl, 2-hexyl, 3-hexyl, and 3-methylpentyl. When an alkyl residue having a specific number of carbons is named by chemical name or molecular formula, all positional isomers having that number of carbon atoms may be encompassed—for example, "butyl" includes *n*-butyl, *sec*-butyl, *iso*-butyl, and *tert*-butyl; and "propyl" includes *n*-propyl and *iso*-propyl. Certain commonly used alternative names may be used and will be understood by those of ordinary skill in the art. For instance, a divalent group, such as a divalent "alkyl" group, may be referred to as an "alkylene".

[0068] The term "alkenyl", as used herein, refers to a branched or unbranched univalent hydrocarbon chain comprising at least one carbon-carbon double bond. As used herein, alkenyl has 2-20 carbons (*i.e.*, C_{2-20}alkenyl), 2-16 carbons (*i.e.*, C_{2-16}alkenyl), 2-12 carbons (*i.e.*, C_{2-12}alkenyl), 2-10 carbons (*i.e.*, C_{2-10}alkenyl), 2-8 carbons (*i.e.*, C_{2-8}alkenyl), 2-6 carbons (*i.e.*, C_{2-6}alkenyl), 2-4 carbons (*i.e.*, C_{2-4}alkenyl), or 2-3 carbons (*i.e.*, C_{2-3}alkenyl). Examples of alkenyl include, but are not limited to, ethenyl, prop-1-enyl, prop-2-enyl 1,2-butadienyl, and 1,3-butadienyl. When an alkenyl residue having a specific number of carbons is named by chemical name or molecular formula, all positional isomers having that number of carbon atoms may be encompassed—for example, "propenyl" includes prop-1-enyl and prop-2-enyl. Certain commonly used alternative names may be used and will be understood by those of ordinary skill in the art. For instance, a divalent group, such as a divalent "alkenyl" group, may be referred to as an "alkenylene".

[0069] The term "alkynyl", as used herein, refers to a branched or unbranched univalent hydrocarbon chain comprising at least one carbon-carbon triple bond. As used herein, alkynyl has 2-20 carbons (*i.e.*, C_{2-20}alkynyl), 2-16 carbons (*i.e.*, C_{2-16}alkynyl), 2-12 carbons (*i.e.*, C_{2-12}alkynyl), 2-10 carbons (*i.e.*, C_{2-10}alkynyl), 2-8 carbons (*i.e.*, C_{2-8}alkynyl), 2-6 carbons (*i.e.*, C_{2-6}alkynyl), 2-4 carbons (*i.e.*, C_{2-4}alkynyl), or 2-3 carbons (*i.e.*, C_{2-3}alkynyl). Examples of alkynyl include, but are not limited to, ethynyl, prop-1-ynyl, prop-2-ynyl, but-1-ynyl, but-2-ynyl, and but-3-ynyl. When an alkynyl residue having a specific number of carbons is named by chemical name or molecular formula, all positional isomers having that number of carbon atoms may be encompassed—for example, "propynyl" includes prop-1-ynyl and prop-2-ynyl. Certain commonly used alternative names may be used and will be understood by those of ordinary skill in the art. For instance, a divalent group, such as a divalent "alkynyl" group, may be referred to as an "alkynylene".

[0070] The term "alkoxy", as used herein, refers to an -O-alkyl moiety. Examples of alkoxy groups include, but are not limited to, methoxy, ethoxy, *n*-propoxy, *iso*-propoxy, *n*-butoxy, *tert*-butoxy, *sec*-butoxy, *n*-pentoxy, *n*-hexoxy, and 1,2-dimethylbutoxy.

[0071] The term "aryl", as used herein, refers to a fully unsaturated carbocyclic ring moiety. The term "aryl" encompasses monocyclic and polycyclic fused-ring moieties. As used herein, aryl encompasses ring moieties comprising, for example, 6 to 20 annular carbon atoms (*i.e.*, C_{6-20}aryl), 6 to 16 annular carbon atoms (*i.e.*, C_{6-16}aryl), 6 to 12 annular carbon

atoms (*i.e.*, C_{6-12}aryl), or 6 to 10 annular carbon atoms (*i.e.*, C_{6-10}aryl). Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, fluorenyl, and anthryl.

[0072] The term "cycloalkyl", as used herein, refers to a saturated or partially unsaturated carbocyclic ring moiety. The term "cycloalkyl" encompasses monocyclic and polycyclic ring moieties, wherein the polycyclic moieties may be fused, branched, or spiro. Cycloalkyl includes cycloalkenyl groups, wherein the ring moiety comprises at least one annular double bond. Cycloalkyl includes any polycyclic carbocyclic ring moiety comprising at least one non-aromatic ring, regardless of the point of attachment to the remainder of the molecule. As used herein, cycloalkyl includes rings comprising, for example, 3 to 20 annular carbon atoms (*i.e.*, a C_{3-20}cycloalkyl), 3 to 16 annular carbon atoms (*i.e.*, a C_{3-16}cycloalkyl), 3 to 12 annular carbon atoms (*i.e.*, a C_{3-12}cycloalkyl), 3 to 10 annular carbon atoms (*i.e.*, a C_{3-10}cycloalkyl), 3 to 8 annular carbon atoms (*i.e.*, a C_{3-8}cycloalkyl), 3 to 6 annular carbon atoms (*i.e.*, a C_{3-6}cycloalkyl), or 3 to 5 annular carbon atoms (*i.e.*, a C_{3-5}cycloalkyl). Monocyclic cycloalkyl ring moieties include, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. Polycyclic groups include, for example, bicyclo[2.2.1]heptanyl, bicyclo[2.2.2]octanyl, adamantyl, norbonyl, decalinyl, 7,7-dimethyl -bicyclo [2.2.1]heptanyl, and the like. Still further, cycloalkyl also includes spiro cycloalkyl ring moieties, for example, spiro[2.5]octanyl, spiro[4.5]decanyl, or spiro [5.5]undecanyl.

[0073] The term "halo", as used herein, refers to atoms occupying groups VIIA of The Periodic Table and includes fluorine (fluoro), chlorine (chloro), bromine (bromo), and iodine (iodo). Additionally, terms such as "haloalkyl" are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "C_{1-4}haloalkyl" is mean to include trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, difluoromethyl, and the like.

[0074] The term "heteroaryl", as used herein, refers to an aromatic (fully unsaturated) ring moiety that comprises one or more annular heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The term "heteroaryl" includes both monocyclic and polycyclic fused-ring moieties. As used herein, a heteroaryl comprises, for example, 5 to 20 annular atoms (*i.e.*, a 5- to 20-membered heteroaryl), 5 to 16 annular atoms (*i.e.*, a 5- to 16-membered heteroaryl), 5 to 12 annular atoms (*i.e.*, a 5- to 12-membered heteroaryl), 5 to 10 annular atoms (*i.e.*, a 5- to 10-membered heteroaryl), 5 to 8 annular atoms (*i.e.*, a 5- to 8-membered heteroaryl), or 5 to 6 annular atoms (*i.e.*, a 5- to 6-membered heteroaryl). Any monocyclic or polycyclic aromatic ring moiety comprising one or more

annular heteroatoms is considered a heteroaryl, regardless of the point of attachment to the remainder of the molecule (*i.e.*, the heteroaryl moiety may be attached to the remainder of the molecule through any annular carbon or any annular heteroatom of the heteroaryl moiety). Examples of heteroaryl groups include, but are not limited to, oxazolyl, thiazolyl, pyridinyl, pyrimidinyl, and pyridazinyl.

[0075] The term "heterocyclyl", as used herein, refers to a saturated or partially unsaturated cyclic moiety that encompasses one or more annular heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The term "heterocyclyl" includes both monocyclic and polycyclic ring moieties, wherein the polycyclic ring moieties may be fused, bridged, or spiro. Any non-aromatic monocyclic or polycyclic ring moiety comprising at least one annular heteroatom is considered a heterocyclyl, regardless of the point of attachment to the remainder of the molecule (*i.e.*, the heterocyclyl moiety may be attached to the remainder of the molecule through any annular carbon or any annular heteroatom of the heterocyclyl moiety). Further, the term heterocyclyl is intended to encompass any polycyclic ring moiety comprising at least one annular heteroatom wherein the polycyclic ring moiety comprises at least one non-aromatic ring, regardless of the point of attachment to the remainder of the molecule. As used herein, a heterocyclyl comprises, for example, 3 to 20 annular atoms (*i.e.*, a 3- to 20-membered heterocyclyl), 3 to 16 annular atoms (*i.e.*, a 3- to 16-membered heterocyclyl), 3 to 12 annular atoms (*i.e.*, a 3- to 12-membered heterocyclyl), 3 to 10 annular atoms (*i.e.*, a 3- to 10-membered heterocyclyl), 3 to 8 annular atoms (*i.e.*, a 3- to 8-membered heterocyclyl), 3 to 6 annular atoms (*i.e.*, a 3- to 6-membered heterocyclyl), 3 to 5 annular atoms (*i.e.*, a 3- to 5-membered heterocyclyl), 5 to 8 annular atoms (*i.e.*, a 5- to 8-membered heterocyclyl), or 5 to 6 annular atoms (*i.e.*, a 5- to 6-membered heterocyclyl). Examples of heterocyclyl groups include, *e.g.*, azetidinyl, piperidinyl, piperazinyl, pyrrolidinyl, pyrazolidinyl, thiazolidinyl, tetrahydrofuryl, or tetrahydropyranyl. Examples of spiro heterocyclyl rings include, but are not limited to, bicyclic and tricyclic ring systems, such as oxabicyclo[2.2.2]octanyl, 2-oxa-7-azaspiro[3.5]nonanyl, 2-oxa-6-azaspiro[3.4]octanyl, and 6-oxa-1-azaspiro[3.3]heptanyl. Examples of fused heterocyclyl rings include, but are not limited to, 5,6,7,8-tetrahydropyrido[4,3-d]pyrimidinyl, 6,7-dihydro-5H-pyrrolo[3,4-d]pyrimidinyl, 5,7-dihydrofuro[3,4-d]pyrimidinyl, and 5,7-dihydrothieno[3,4-d]pyrimidinyl, where the heterocyclyl can be bound via either ring of the fused system.

[0076] The term "oxo", as used herein, refers to a =O moiety.

[0077] The terms "optional" and "optionally", as used herein, mean that the subsequently described event or circumstance may or may not occur and that the description includes instances where the event or circumstance occurs and instances where it does not. Accordingly, the term "optionally substituted" infers that any one or more (*e.g.*, 1, 2, 1 to 5, 1 to 3, 1 to 2, etc.) hydrogen atoms on the designated atom or moiety or group may be replaced or not replaced by an atom or moiety or group other than hydrogen. By way of illustration and not limitation, the phrase "methyl optionally substituted with one or more chloro" encompasses $-CH_3$, $-CH_2Cl$, $-CHCl_2$, and $-CCl_3$ moieties.

[0078] It is understood that aspects and embodiments described herein as "comprising" include "consisting of" and "consisting essentially of" embodiments.

[0079] The term "pharmaceutically acceptable salt", as used herein, of a given compound refers to salts that retain the biological effectiveness and properties of the given compound and which are not biologically or otherwise undesirable. "Pharmaceutically acceptable salts" include, for example, salts with inorganic acids, and salts with an organic acid. In addition, if the compounds described herein are obtained as an acid addition salt, the free base can be obtained by basifying a solution of the acid salt. Conversely, if the product is a free base, an addition salt, particularly a pharmaceutically acceptable addition salt, may be produced by dissolving the free base in a suitable organic solvent and treating the solution with an acid, in accordance with conventional procedures for preparing acid addition salts from base compounds. *See*, *e.g.*, *Handbook of Pharmaceutical Salts Properties, Selection, and Use*, International Union of Pure and Applied Chemistry, John Wiley & Sons (2008), which is incorporated herein by reference in its entirety. Those skilled in the art will recognize various synthetic methodologies that may be used to prepare nontoxic pharmaceutically acceptable addition salts. Pharmaceutically acceptable acid addition salts may be prepared from inorganic or organic acids. Salts derived from inorganic acids include, *e.g.*, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like. Salts derived from organic acids include, *e.g.*, acetic acid, propionic acid, gluconic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluene-sulfonic acid, salicylic acid, trifluoroacetic acid, and the like. Likewise, pharmaceutically acceptable base addition salts can be prepared from inorganic or organic bases. Salts derived from inorganic bases include, by way of example only, sodium, potassium, lithium, aluminum, ammonium, calcium, and magnesium salts. Salts derived

from organic bases include, but are not limited to, salts of primary, secondary, and tertiary amines. Specific examples of suitable amines include, by way of example only, isopropylamine, trimethyl amine, diethyl amine, tri(*iso*-propyl) amine, tri(*n*-propyl) amine, ethanolamine, 2-dimethylaminoethanol, piperazine, piperidine, morpholine, N-ethylpiperidine, and the like.

[0080] Some of the compounds provided herein may exist as tautomers. Tautomers are in equilibrium with one another. By way of illustration, amide containing compounds may exist in equilibrium with imidic acid tautomers. Regardless of which tautomer is shown and regardless of the nature of the equilibrium among tautomers, the compounds of this disclosure are understood by one of ordinary skill in the art to comprise both amide and imidic acid tautomers. Thus, for example, amide-containing compounds are understood to include their imidic acid tautomers. Likewise, imidic-acid containing compounds are understood to include their amide tautomers.

[0081] The compounds of the present disclosure, or their pharmaceutically acceptable salts, may include an asymmetric center and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (*R*)- or (*S*)- (or as (D)- or (L)- for amino acids). The present disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms and mixtures thereof in any ratio. Optically active (+) and (-), (*R*)- and (*S*)-, or (D)- and (L)- isomers may be prepared using chiral synthons or chiral reagents, or may be resolved using conventional techniques, for example, chromatography and/or fractional crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or the resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high pressure liquid chromatography (HPLC), and chiral supercritical fluid chromatography (SFC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, unless specified otherwise, it is intended that the present disclosure includes both E and Z geometric isomers. Likewise, cis- and trans- are used in their conventional sense to describe relative spatial relationships.

CONJUGATES

[0082] In one aspect, provided herein is a conjugate of formula (I):

$$T—L—L^B—E—D^1 \quad (I)$$

or a pharmaceutically acceptable salt thereof, wherein:

T is a ligand selective for transthyretin TTR;

L is a linker;

L^B is absent or a click product formed via a Click reaction between a first Click handle and a second click handle;

E is a cleavable ester; and

D^1 is a drug moiety.

[0083] In some embodiments, the linker (L) has a size of from about 10 Å to about 40 Å. In some embodiments, the linker (L) has a size of from about 10 Å to about 40 Å. In some embodiments, the linker (L) has a size of from about 15 Å to about 30 Å. In some embodiments, the linker (L) has a size of from about 20 Å to about 30 Å. In some embodiments, the linker (L) has a size of about 20 Å. In some embodiments, the linker (L) has a size of about 25 Å. In some embodiments, the linker (L) has a size of about 30 Å.

[0084] In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 10 Å to about 50 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of from about 15 Å to about 45 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of from about 25 Å to about 40 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 20 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 25 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 30 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 35 Å. In some embodiments, the linker (L) and click product (L^B) taken together have a size of about 40 Å.

[0085] In some embodiments, L^B is the Click product of:

(i) a copper-catalyzed reaction between an azide and an alkyne;

(ii) a reaction between an azide and dibenzocyclooctene (DBCO);

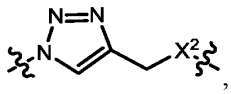
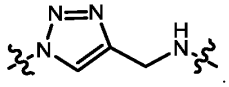
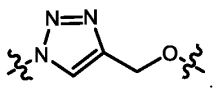
(iii) an inverse electron demand Diels-alder cycloaddition (IEDDA) between a trans-cyclooctene (TCO) moiety and a tetrazine ring, or

(iv) a Staudinger reaction between an azide and a phosphine.

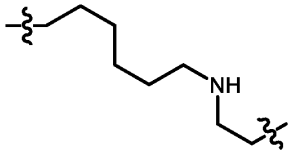
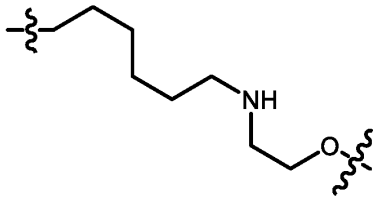
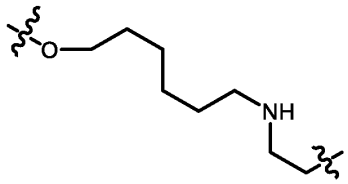
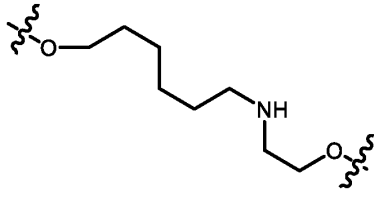
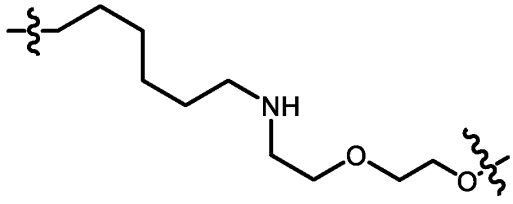
[0086] In some embodiments, the first click handle and the second click handle used to form click product L^B can be any suitable click chemistry pair (e.g., Azide-DBCO, Azide-BCN, Tz-TCO, meTz-TCO, etc.). In some embodiments, the click product can be formed using a copper-catalyzed click reaction. One such copper-catalyzed click reaction is a Huisgen 1,3-dipolar cycloaddition (CuAAC) between an azide and an alkyne (*see*, e.g., Tornøe et al., *J. Org. Chem.* 2002, 67 (9), 3057-3064 and Rostovtsev et al., *Angew. Chem. Int. Ed.* 2002, 41 (14), 2596-2599). In some embodiments, the click product is a triazole moiety. In some embodiments, the click product can be formed using a biorthogonal chemistry approach.

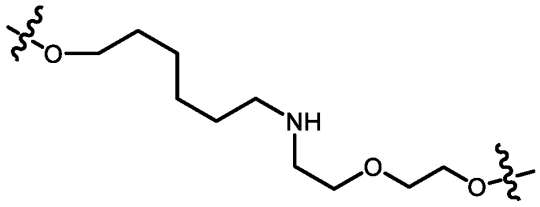
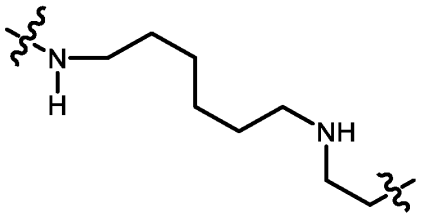
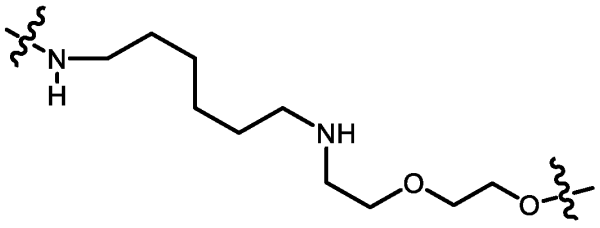
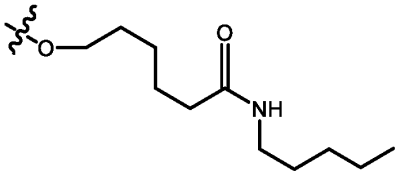
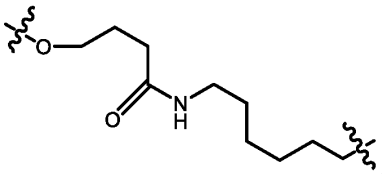
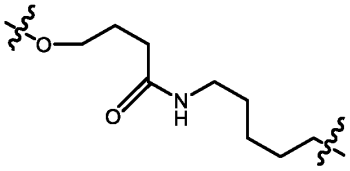
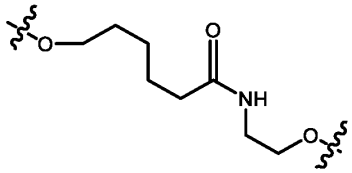
[0087] In some embodiments, the click product L^B can be formed from any suitable inverse electron demand Diels-Alder reaction. In some embodiments, the click product can be formed from an inverse electron demand Diels-Alder reaction between a trans-cyclooctene (TCO) moiety on the first or second click handle and a tetrazine (Tz) ring on the first or second click handle (*see*, e.g., Selvaraj et al., *Curr. Opin. Chem. Biol.* 2013, 17 (5), 753-760 and Rossin et al., *Bioconjugate Chem.* 2013, 24 (7), 1210-1217). In some embodiments, the first click handle comprises a tetrazine ring and the second click handle comprises a TCO moiety. In some embodiments, the tetrazine ring is unsubstituted. In some such embodiments, the tetrazine ring is methyltetrazine. In some embodiments, the tetrazine ring is a 6-methyl substituted tetrazine. In some embodiments, the click product is a dihydropyridazine moiety.

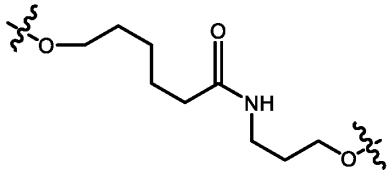
[0088] In some embodiments, the click product L^B is a triazine moiety. In some embodiments, the conjugate of formula (I) comprises one of the following moieties:

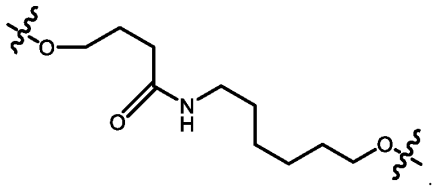
, wherein X^2 is $-CH_2-$, $-NR^x-$, -S- or -O-, wherein R^x is defined as elsewhere herein. In some embodiments, the conjugate comprises the moiety . In some embodiments, the conjugate comprises the moiety .

[0089] In some embodiments, L is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -N(CH_3)-, and -S-. In some embodiments, L is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -N(CH_3)-. In some embodiments, L is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

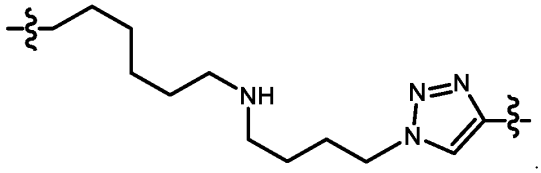
[0090] In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the

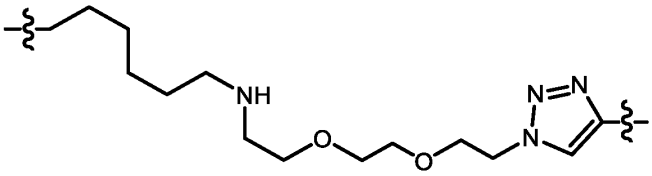
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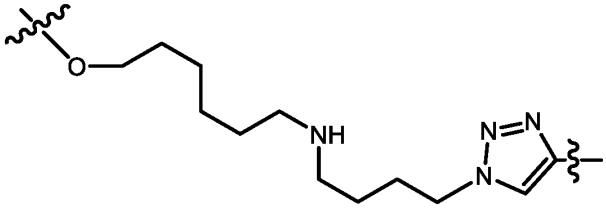

. In some embodiments, L comprises the moiety

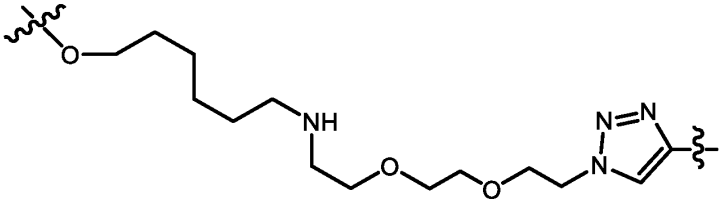

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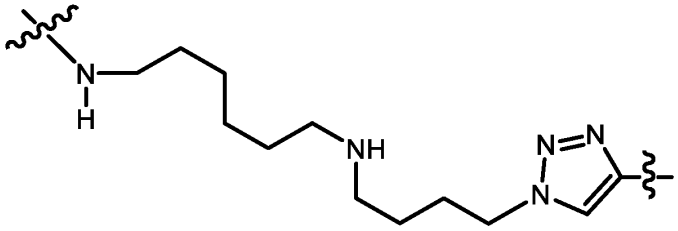
[0091] In some embodiments, L-L^B comprises the moiety

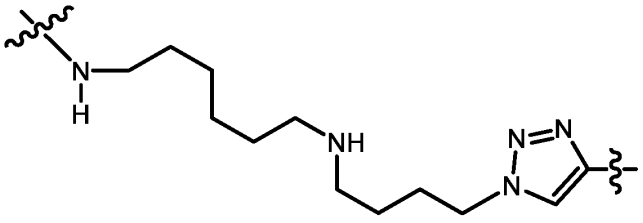

. In some embodiments, L-L^B comprises the moiety

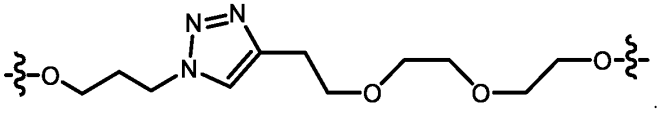

. In some embodiments, L-L^B comprises the moiety


. In some embodiments, L-L^B comprises the moiety

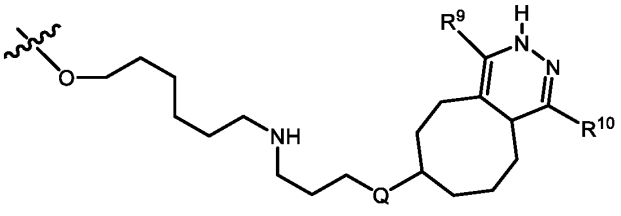

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. In some embodiments, L-L^B comprises the moiety

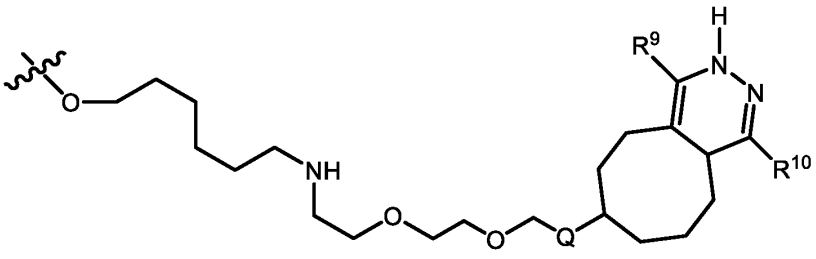


. In some embodiments, L-L^B comprises the moiety .

[0092] In some embodiments, L-L^B comprises the moiety

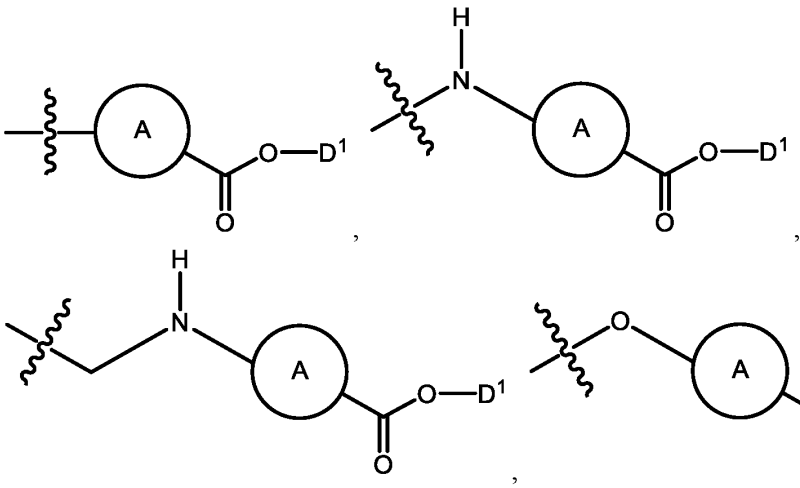


or



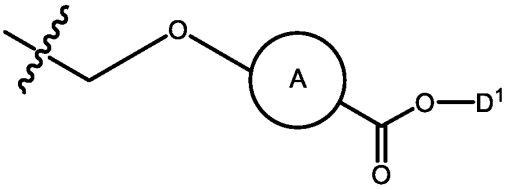
, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is $-CH_2-$, -O-, -S-, -NH-, or $-N(C_{1-4}alkyl)-$.

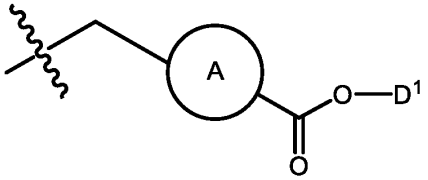
[0093] In some embodiments, the conjugate of formula (I) comprises the moiety

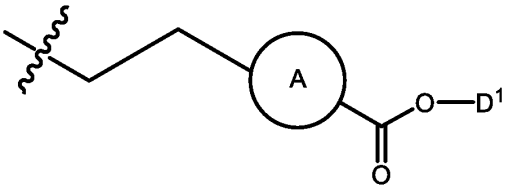


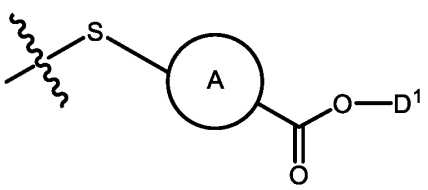
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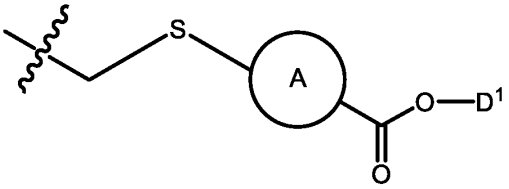
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, or


, wherein:

Ring A is C_{6-10}aryl or 5- to 10-membered heteroaryl, wherein

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more R^7, wherein each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NO_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl

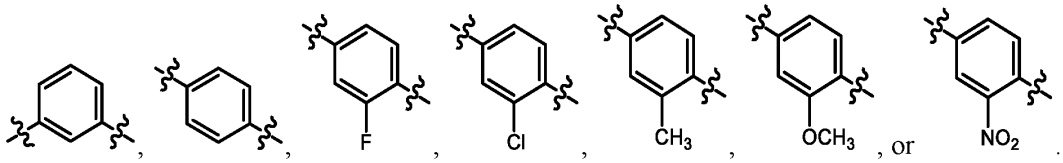
and ≀ indicates the point of attachment to the linker (L or L-L^B).

[0094] In some embodiments, Ring A is an optionally substituted 5- to 10-membered heteroaryl. Examples of heteroaryl rings include, but are not limited to, azetidinyl, pyrazyl, pyrrolyl, imidazolyl, pyrazolyl, pyridyl, pyrimidyl, pyridazyl, indolizyl, isoindolyl, indolyl, dihydroindolyl, indazolyl, purinyl, quinolizinyl, isoquinolinyl, quinolinyl, phthalazinyl, naphthylpyridinyl, quinoxalinyl, quinazolinyl, cinnolinyl, pteridinyl, carbazolyl, carbolinyl, phenanthridinyl, acridinyl, phenanthrolinyl, isothiazolyl, phenazinyl, isoxazolyl, phenoxazinyl, phenothiazinyl, imidazolidinyl, imidazolinyl, piperidinyl, piperazinyl, indolinyl, phthalimidyl, 1,2,3,4-tetrahydroisoquinolinyl, 4,5,6,7-tetrahydrobenzo[b]thiophenyl, thiazolyl, thiazolidinyl, thiophenyl, benzo[b]thiophenyl, morpholinyl, thiomorpholinyl, 1,1-dioxothiomorpholinyl, piperidinyl, pyrrolidinyl, and tetrahydrofuranyl. In some embodiments, Ring A is selected from the group consisting of phenyl, pyridyl, pyrimidinyl, oxazolyl, pyrazolyl, and thiazolyl, wherein the phenyl, pyridyl, pyrimidinyl, oxazolyl, pyrazolyl, or thiazolyl is optionally substituted with one or more C_{1-6} alkyl, C_{1-6} alkoxy, hydroxy, halo, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, -

$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{3-6} cycloalkyl, or -NO_2, wherein the C_{1-6} alkyl or C_{1-6} alkoxy is optionally substituted by one or more halo. In some embodiments, Ring A is selected from the group consisting of phenyl, pyridyl, and pyrimidinyl, wherein the phenyl, pyridyl, and pyrimidinyl is optionally substituted with one or more C_{1-6} alkyl, C_{1-6} alkoxy, hydroxy, halo, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{3-6} cycloalkyl, or nitro, wherein the C_{1-6} alkyl or C_{1-6} alkoxy is optionally substituted by one or more halo.

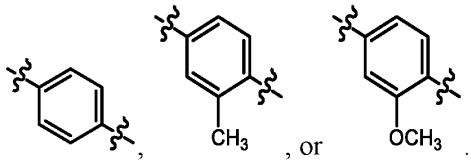
[0095] In some embodiments, Ring A is a phenyl group or a substituted phenyl group. In some such embodiments, Ring A is substituted with a methyl group, methoxy group, halo group (e.g., Cl, Br, F or I), nitro group, or CF_3 group.

[0096] In some embodiments, the conjugate of formula (I) comprises the moiety is



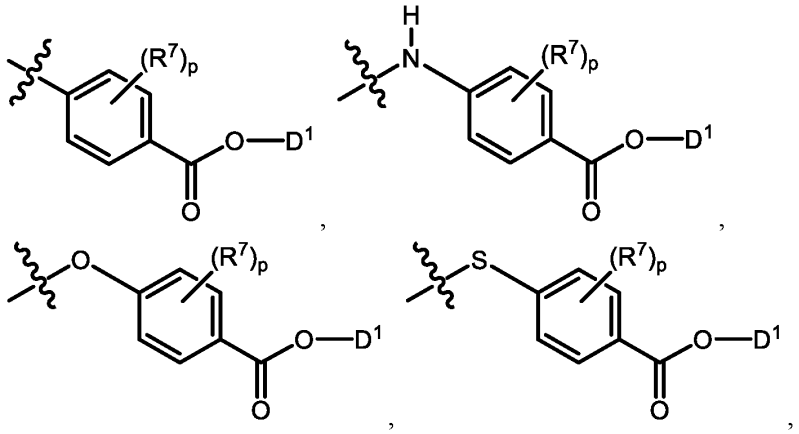
, or .

[0097] In some embodiments, the conjugate of formula (I) comprises the moiety is

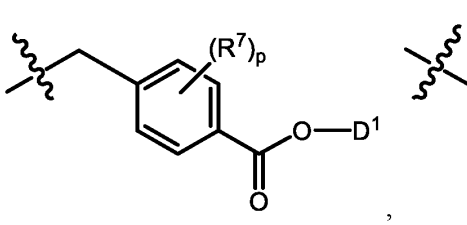
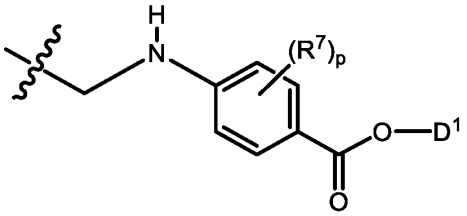
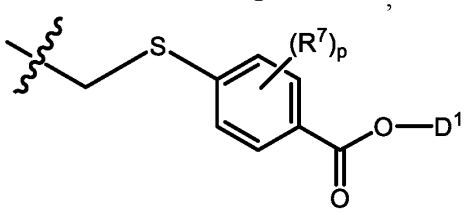
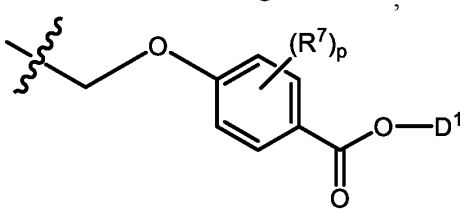
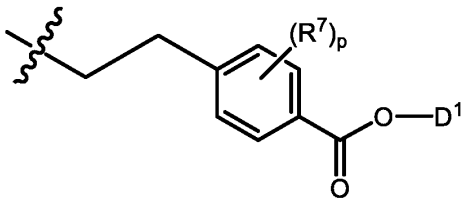


, or .

[0098] In some embodiments, the conjugate of formula (I) comprises the moiety

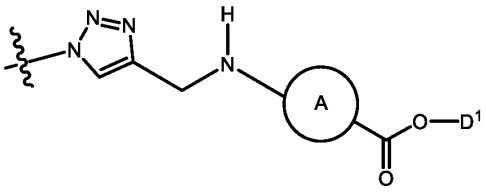
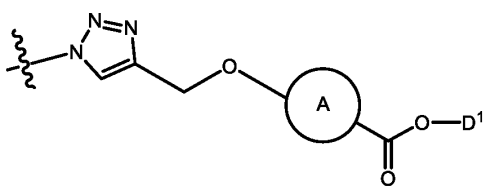
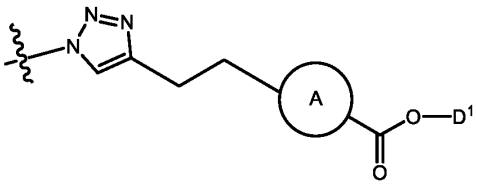
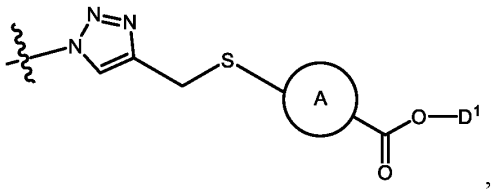


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, wherein:

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl, p is 0, 1, 2, or 3, and  indicates the point of attachment to the linker (L or L-L^B).

[0099] In some embodiments, the conjugate of formula (I) comprises the moiety


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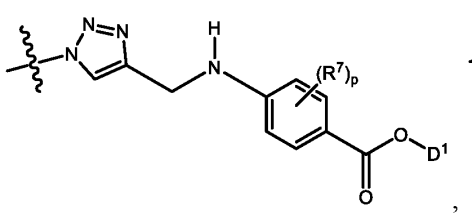
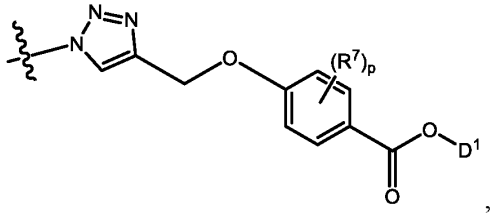
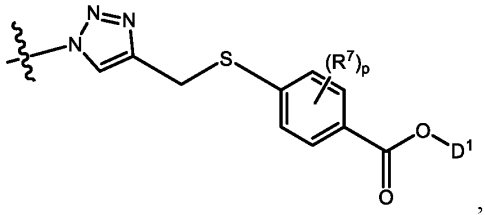
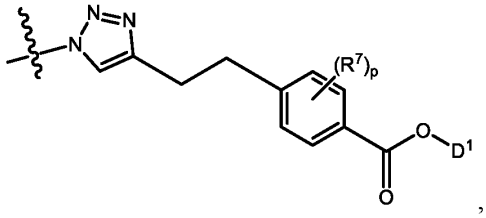
wherein:

Ring A is C_{6-10}aryl or 5- to 10-membered heteroaryl, wherein

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more R^7, wherein each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

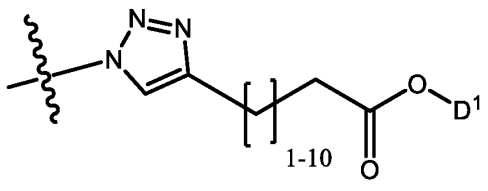
and ≈ indicates the point of attachment to the linker (L).

[0100] In some embodiments, the conjugate of formula (I) comprises the moiety

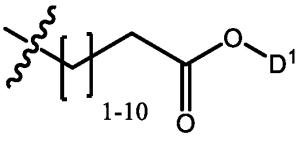

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wherein each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl, p is 0, 1, 2, or 3, and ≈ indicates the point of attachment to the linker (L).

[0101] In some embodiments, the conjugate of formula (I) comprises the moiety

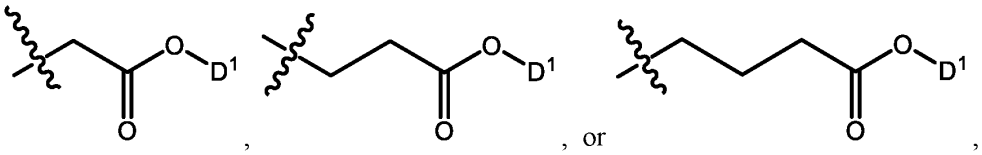

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[0102] In some embodiments, the conjugate of formula (I) comprises the moiety



, wherein each methylene group can be optionally substituted with halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy or C_{3-6} cycloalkyl.

[0103] In some embodiments, the conjugate of formula (I) comprises the moiety

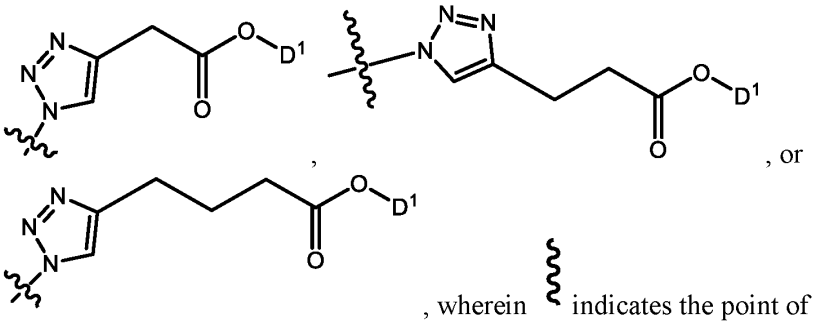


, , or ,

wherein each methylene group can be optionally substituted with halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy or C_{3-6} cycloalkyl.

and wherein  indicates the point of attachment to the linker (L or L-L^B), wherein  indicates the point of attachment to the linker (L).

[0104] In some embodiments, the conjugate of formula (I) comprises the moiety



, , , or , wherein indicates the point of attachment to the linker (L).

[0105] In some embodiments, D^1 is a peptide.

[0106] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some

embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0107] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0108] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0109] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0110] In some embodiments, D^1 is a protein.

[0111] In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some

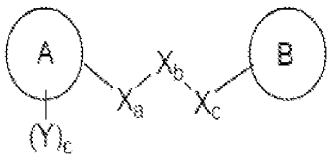
embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0112] In some embodiments, D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0113] In some embodiments, D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0114] In some embodiments, D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0115] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (A):



(A),

or a pharmaceutically acceptable salt thereof, wherein:

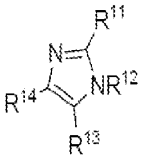
X_a, X_b and X_c are independently selected from $C(R^{8a})(R^{8b})$, O, N-R^{8b} or S; where R^{8a} and R^{8b} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

a ring is a 4 to 12-membered ring, in certain embodiments the 4 to 12-membered ring is an aromatic or heteroaromatic ring;

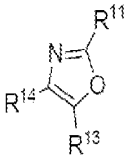
each Y is independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano;

c is an integer ranging from 0 to 5; and,

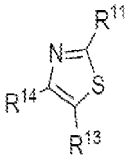
B ring is a heterocyclic ring selected from the following (h1-h30):



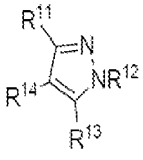
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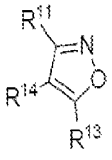
h2



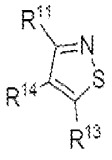
h3



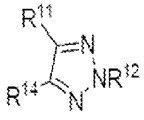
h4



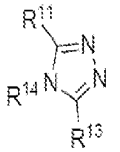
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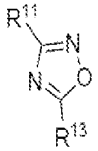
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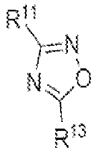
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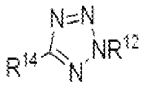
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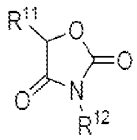
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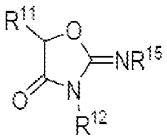
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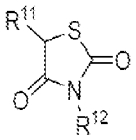
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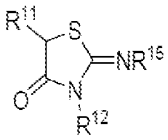
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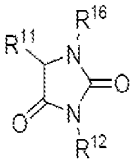
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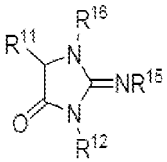
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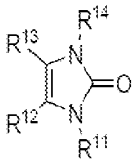
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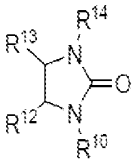
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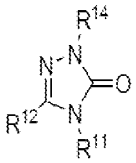
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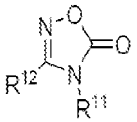
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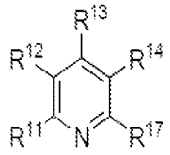
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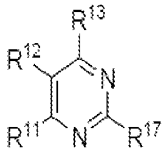
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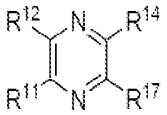
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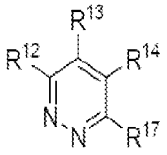
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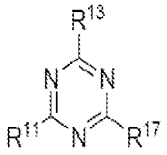
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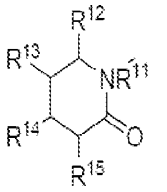
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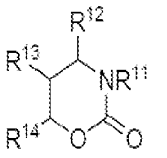
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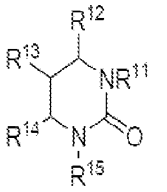
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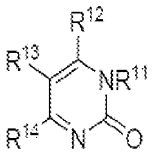
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h28



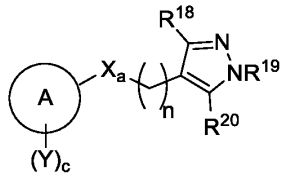
h28



h30

where R^{11}-$R^{16}$are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano; R^{17} is selected from a hydroxyl, alkyl, amino, and alkyl amino; and at least one of R^{11}-R^{16} is the linking group to X_c.

[0116] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (B):



(B),

or a pharmaceutically acceptable salt thereof, wherein:

n is an integer ranging from 0 to 8;

R^{18}, R^{19} and R^{20} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl,

aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano;

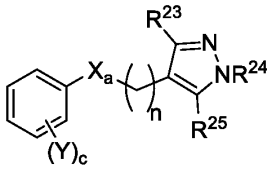
X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

A is a 5 to 12-membered ring, in certain embodiments the 5 to 12-membered ring is an aromatic or heteroaromatic ring;

each Y is independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano; and,

c is a number from zero to 5.

[0117] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (C):



(C).

or a pharmaceutically acceptable salt thereof, wherein:

n is an integer ranging from 1 to 4;

R^{23} is a short chain alkyl having 1 to 4 carbon atoms;

R^{24} is hydrogen;

R^{25} is a short chain alkyl having 1 to 4 carbon atoms;

X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl,

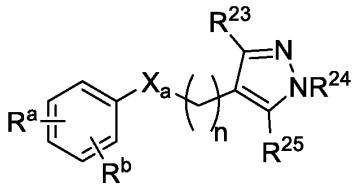
substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

each Y is independently selected from hydrogen, halogen, acyl, substituted acyl, carboxyl, heterocyclic group, alkoxycarbonyl sulfonamide, sulfonyl fluoride, thioester and substituted alkoxycarbonyl; and

c is 2.

[0118] In some embodiments, the ligand of Compound (C) is a structure in which R^{23} is methyl and R^{24} is methyl; Xa is O; and Y is fluoro or carboxyl.

[0119] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (D):



(D),

or a pharmaceutically acceptable salt thereof, wherein:

n is 1 to 8;

R^{23}, R^{24} and R^{25} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halo, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted

aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

R^a is CHO, COOH, $COOCH_3$, $COOR^6$, $CONR^7R^8$, tetrazolyl, CONHOH, $B(OH)_2$, $CONHSO_2Ar$, $CONHCH(R^9)COOH$, CF_3, hydrogen, halogen, alkyl, substituted alkyl, acyl, substituted acyl, carboxyl, heterocyclic group, sulfonamide, sulfonyl fluoride, thioester, alkoxycarbonyl or substituted alkoxycarbonyl;

R^b is CHO, COOH, $COOCH_3$, $COOR2^6$, $CONR2^7R2^8$, tetrazolyl, CONHOH, $B(OH)_2$, $CONHSO_2Ar$, $CONHCH(R2^9)COOH$, CF_3, hydrogen, halogen, alkyl, substituted alkyl, acyl, substituted acyl, carboxyl, heterocyclic group, sulfonamide, sulfonyl fluoride, thioester, alkoxycarbonyl or substituted alkoxycarbonyl;

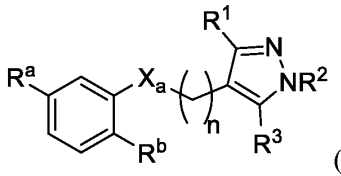
$R2^6$ is alkyl, haloalkyl, cycloalkyl, or heterocyclyl;

R^{27} and R^{28} are each independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, or heteroaryl; and,

R^{29} is the side chain of a naturally occurring α-amino carboxylic acid.

[0120] In some embodiments, the ligand of Compound (D) is a structure in which R^b is selected from bromo, chloro and fluoro.

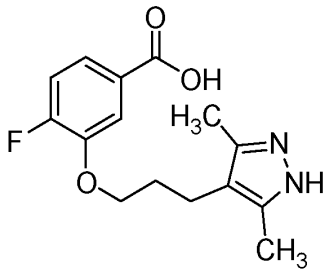
[0121] In some embodiments, the ligand of Compound (D) is a structure of Compound (E):



(E)

or a pharmaceutically acceptable salt thereof.

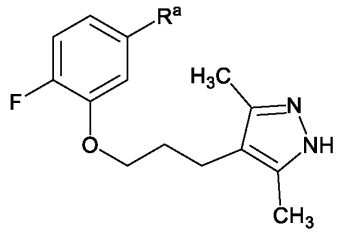
[0122] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (F):



(F)

or a pharmaceutically acceptable salt thereof.

[0123] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (G):



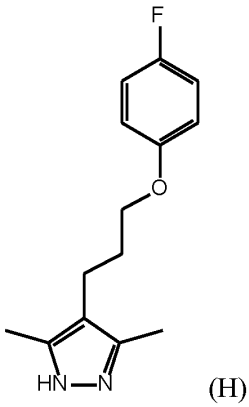
(G)

or a pharmaceutically acceptable salt thereof, wherein

R^a is OH, CHO, COOH, $CONH_2$, CONH(OH), $COOR^{30}$, $CONHR^{30}$;

R^{30} is straight of branched alkyl of 1-3 carbon atoms;

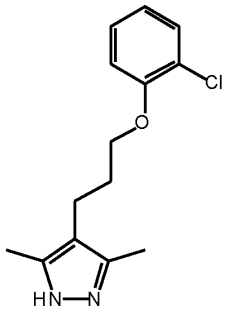
[0124] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (H):



(H)

or a pharmaceutically acceptable salt thereof.

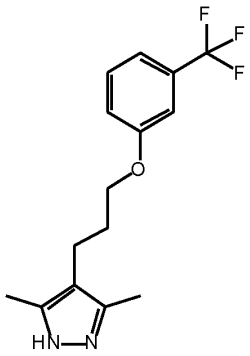
[0125] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (I):



(J)

or a pharmaceutically acceptable salt thereof.

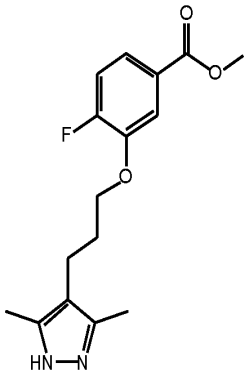
[0126] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (K):



(K)

or a pharmaceutically acceptable salt thereof.

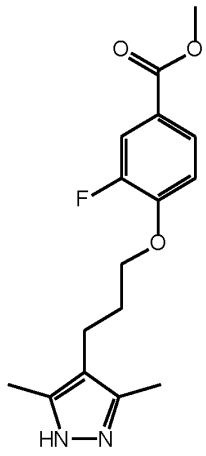
[0127] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (L):



(L)

or a pharmaceutically acceptable salt thereof.

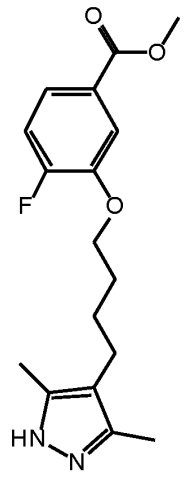
[0128] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (M):



(M)

or a pharmaceutically acceptable salt thereof.

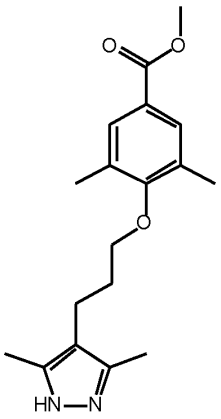
[0129] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (N):



(N)

or a pharmaceutically acceptable salt thereof.

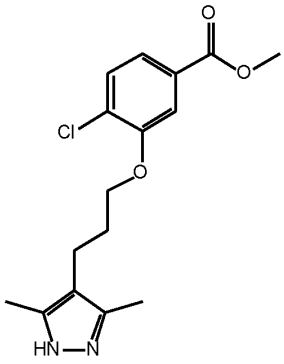
[0130] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (O):



(O)

or a pharmaceutically acceptable salt thereof.

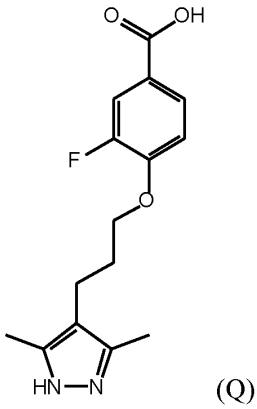
[0131] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (P):



(P)

or a pharmaceutically acceptable salt thereof.

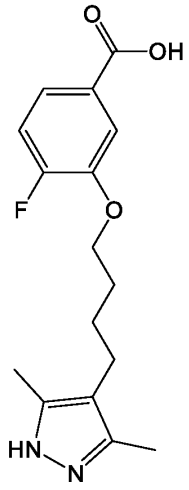
[0132] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (Q):



(Q)

or a pharmaceutically acceptable salt thereof.

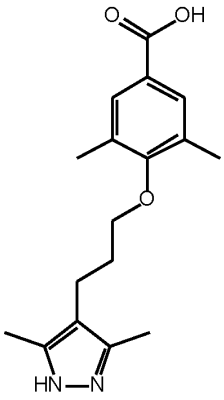
[0133] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (R):



(R)

or a pharmaceutically acceptable salt thereof.

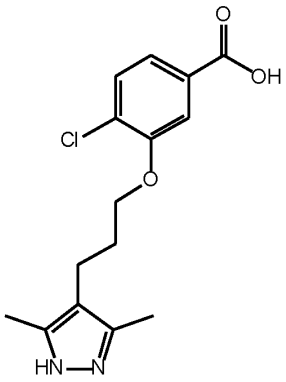
[0134] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (S):



(S)

or a pharmaceutically acceptable salt thereof.

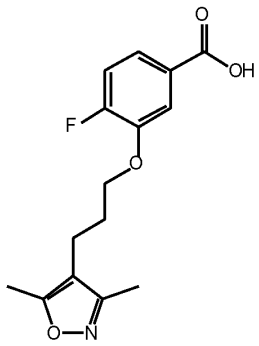
[0135] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (T):



(T)

or a pharmaceutically acceptable salt thereof.

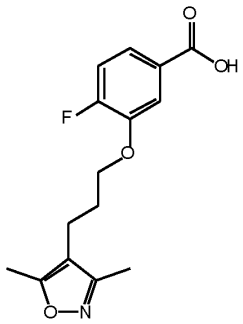
[0136] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (U):



(U)

or a pharmaceutically acceptable salt thereof.

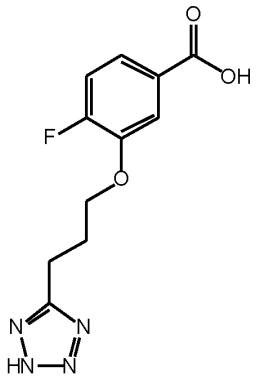
[0137] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (V):



(V)

or a pharmaceutically acceptable salt thereof.

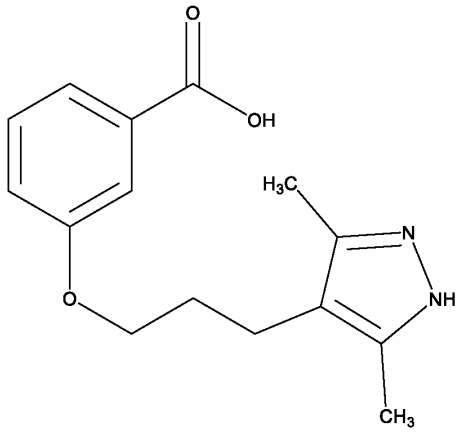
[0138] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (W):



(W)

or a pharmaceutically acceptable salt thereof.

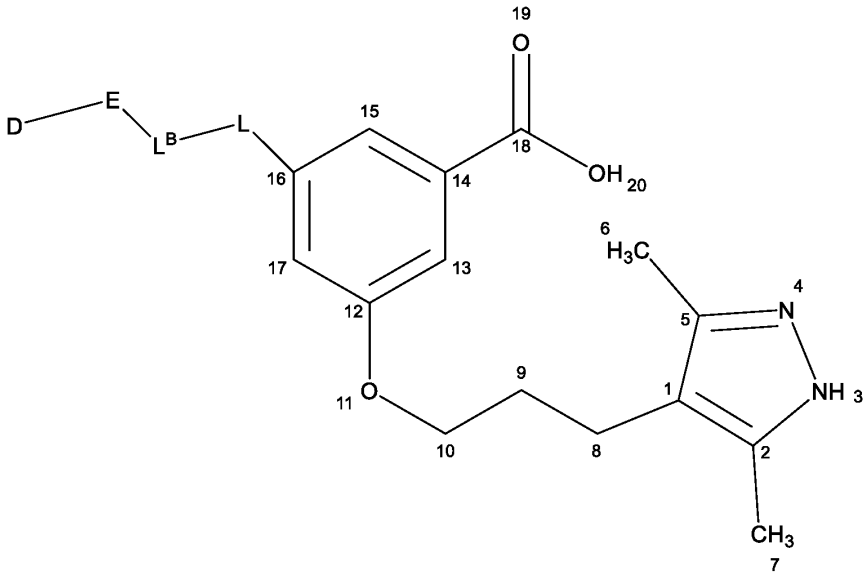
[0139] In some embodiments, T (a ligand selective for transthyretin TTR) has the structure of Compound (Y):



(Y)

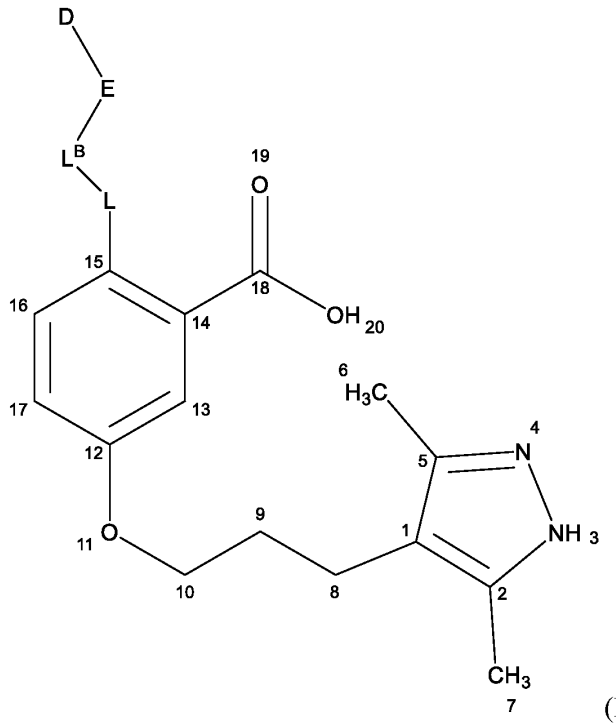
or a pharmaceutically acceptable salt thereof.

[0140] In some embodiments of Compound (Y), the linker (L) is attached to the ligand *meta* at C16 to the carboxy carbon at C14, such as conjugate of formula (Ia):



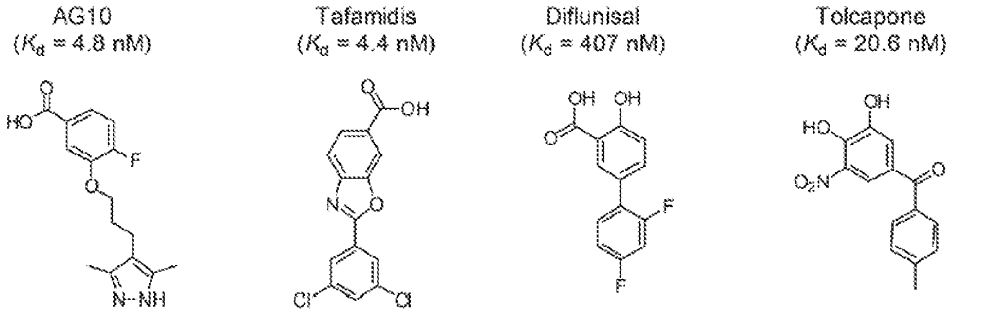
(Ia)

[0141] In some embodiments of Compound (Y), the linker (L) is attached to the ligand *ortho* at C15 to the carboxy carbon at C15, such as conjugate of formula (Ib):

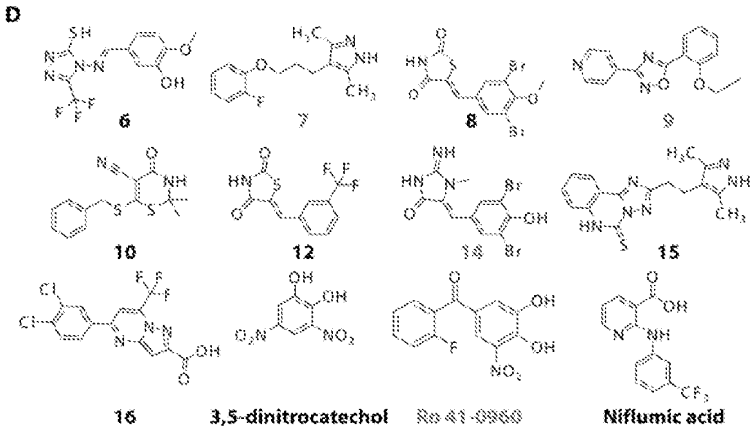


(Ib)

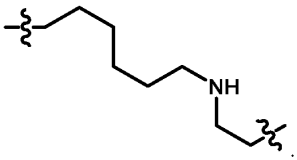
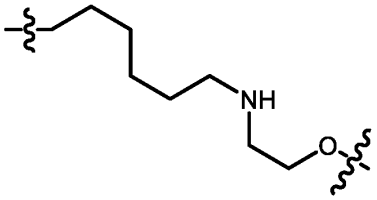
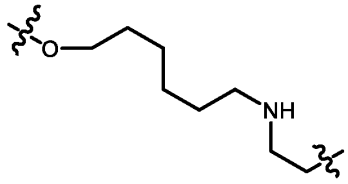
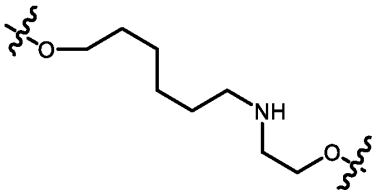
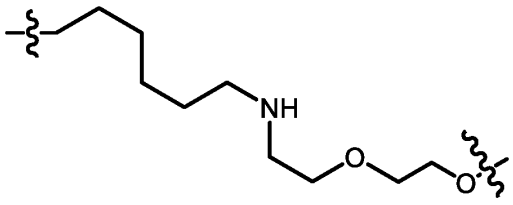
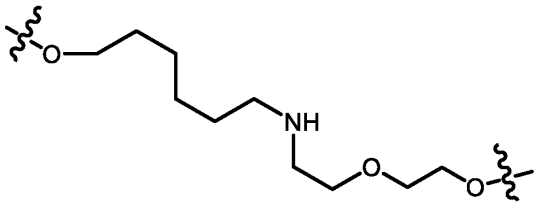
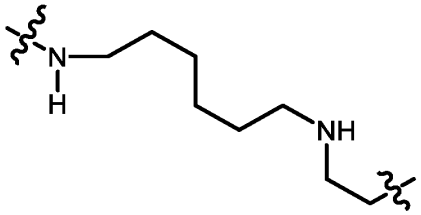
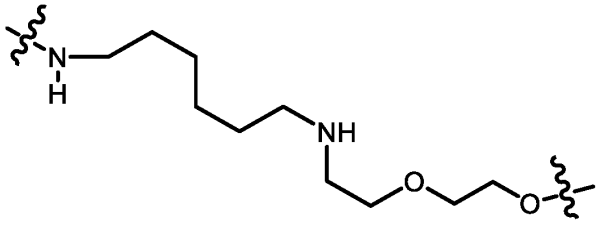
[0142] In some embodiments, T (a ligand selective for transthyretin TTR) has one of the following structures:

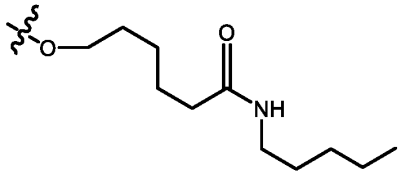
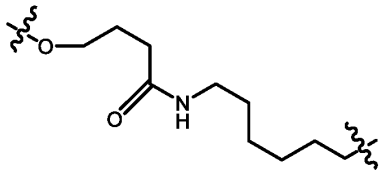
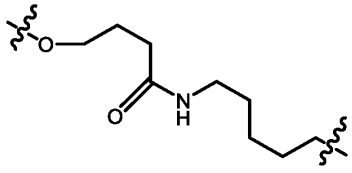
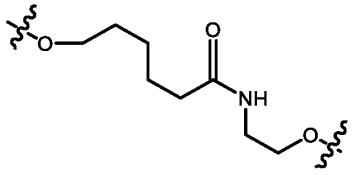
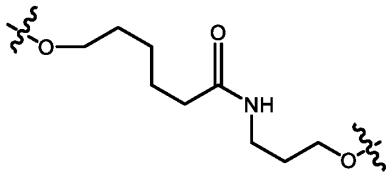
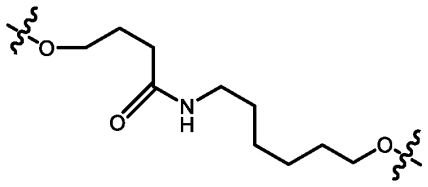


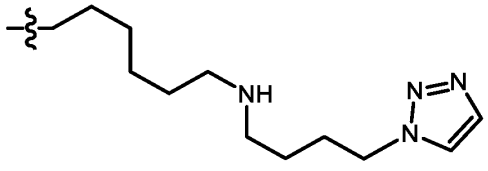
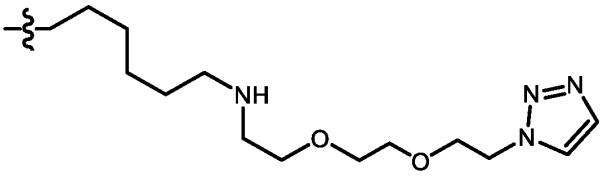
[0143] In some embodiments, T (a ligand selective for transthyretin TTR) has one of the following structures:

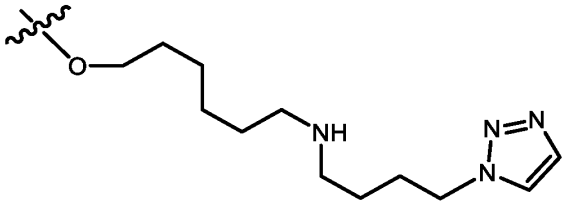


[0144] In some embodiments of a conjugate of formula (Ia) or formula (Ib), L is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo. In some embodiments, L is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -$N(CH_3)$-, and -S-. In some embodiments, L is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -$N(CH_3)$-. In some embodiments, L is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

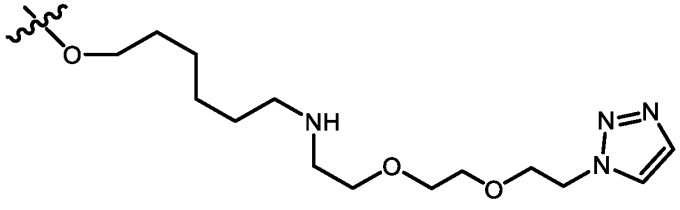
[0145] In some embodiments of formula (Ia) or formula (Ib), L comprises the moiety . In some embodiments, L comprises the moiety  . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety .

In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety .

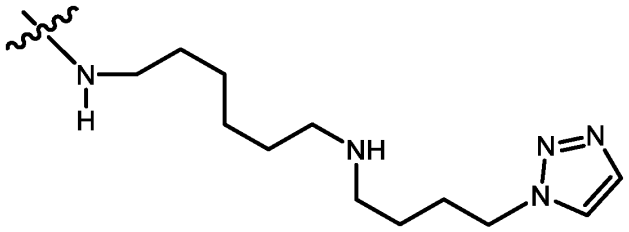
[0146] In some embodiments, L-L^B comprises the moiety . In some embodiments, L-L^B comprises the moiety . In some embodiments, L-L^B



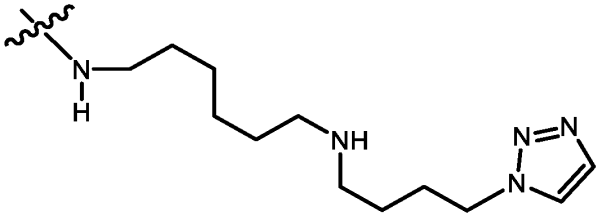
comprises the moiety . In some embodiments, L-L^B comprises the moiety



. In some embodiments, L-L^B comprises the moiety

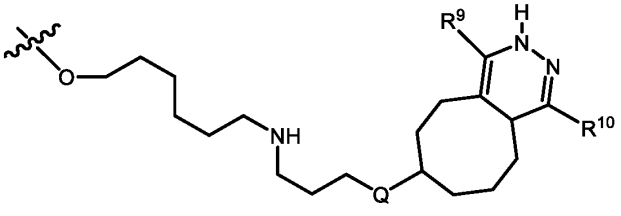


. In some embodiments, L-L^B comprises the moiety

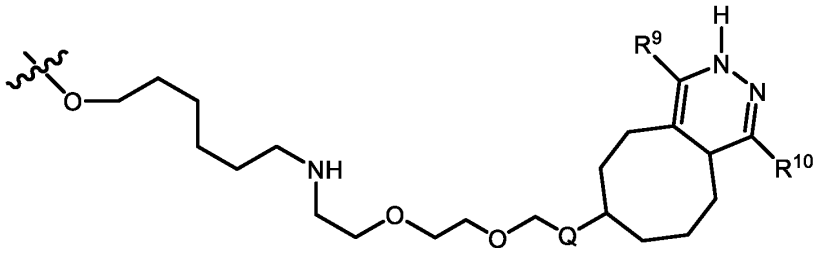


.

[0147] In some embodiments of formula (Ia) or formula (Ib), L-L^B comprises the moiety

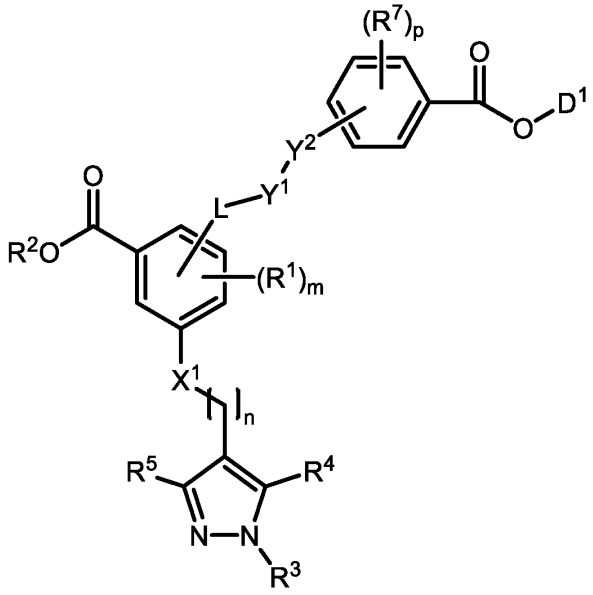


or



, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is -CH_2-, -O-, -S-, -NH-, or -N(C_{1-4}alkyl)-.

[0148] In one embodiment, provided herein is a conjugate of formula (II'):



(II'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O or S;

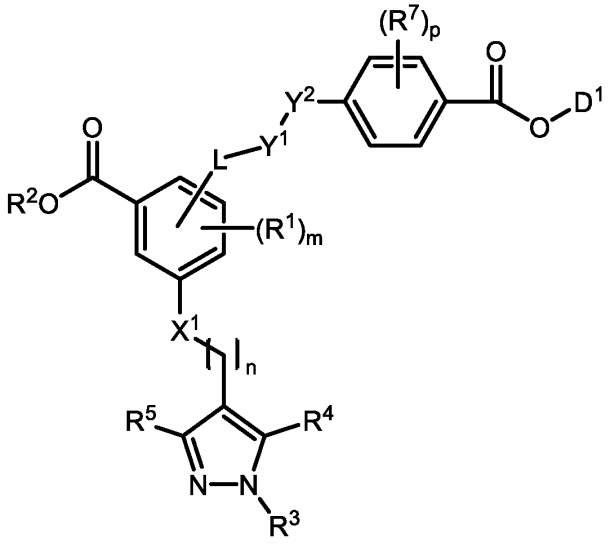
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, $O(C_{1-4}$ alkyl), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0149] In one embodiment, provided herein is a conjugate of formula (II):



(II),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O or S;

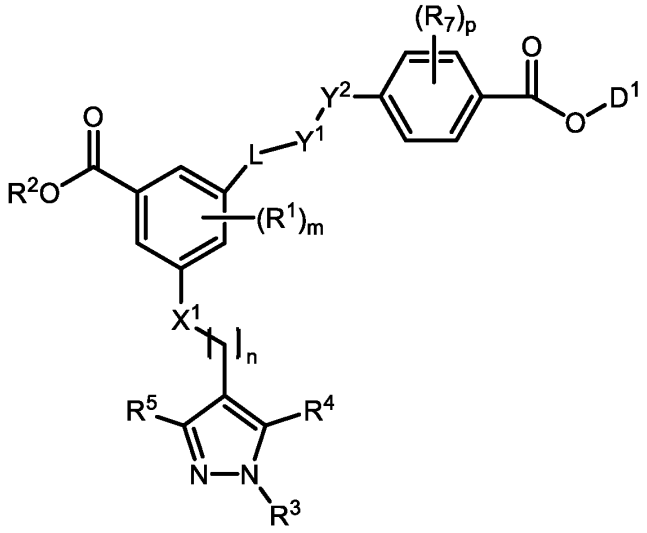
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

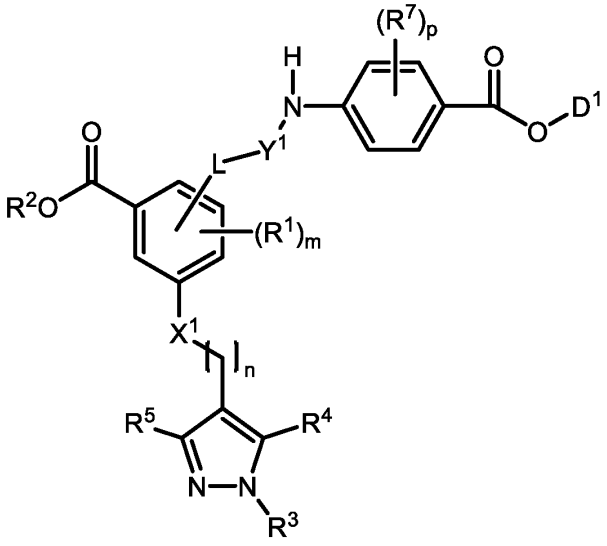
[0150] In some embodiments, the conjugate of formula (II) has the formula (IIa):



(IIa)

or a pharmaceutically acceptable salt thereof.

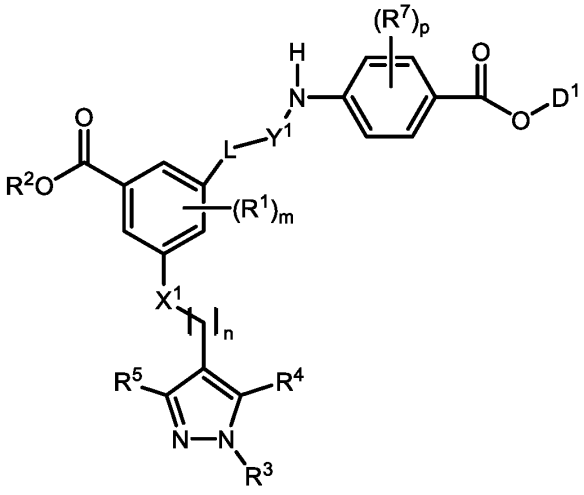
[0151] In some embodiments, the conjugate of formula (II) has the formula (IIb):



(IIb)

or a pharmaceutically acceptable salt thereof.

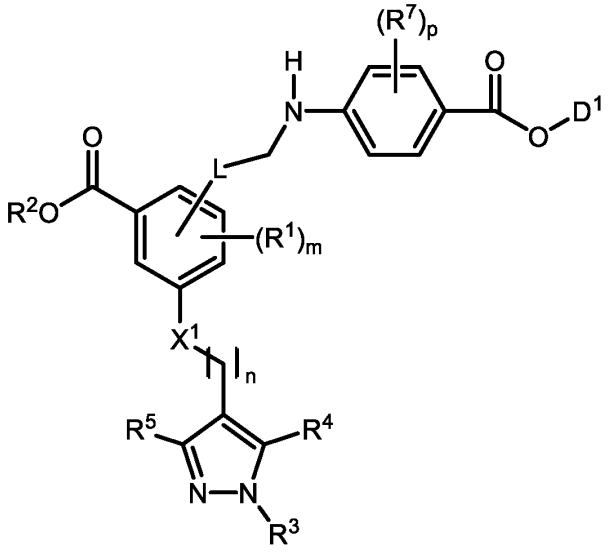
[0152] In some embodiments, the conjugate of formula (II) has the formula (IIc):



(IIc)

or a pharmaceutically acceptable salt thereof.

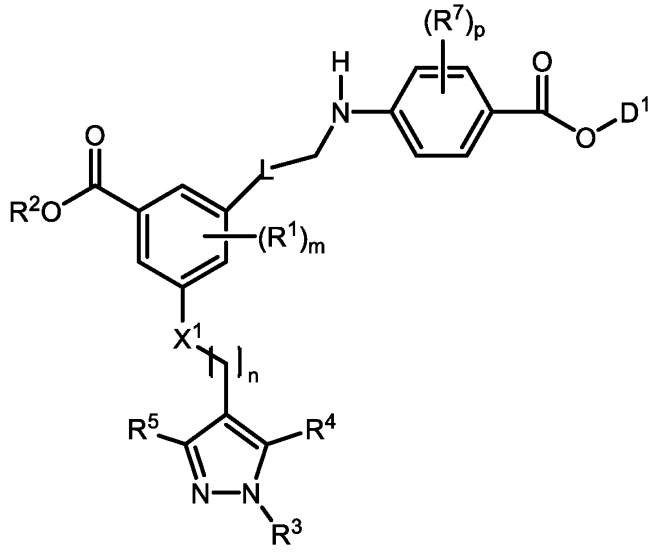
[0153] In some embodiments, the conjugate of formula (II) has the formula (IId):



(IId)

or a pharmaceutically acceptable salt thereof.

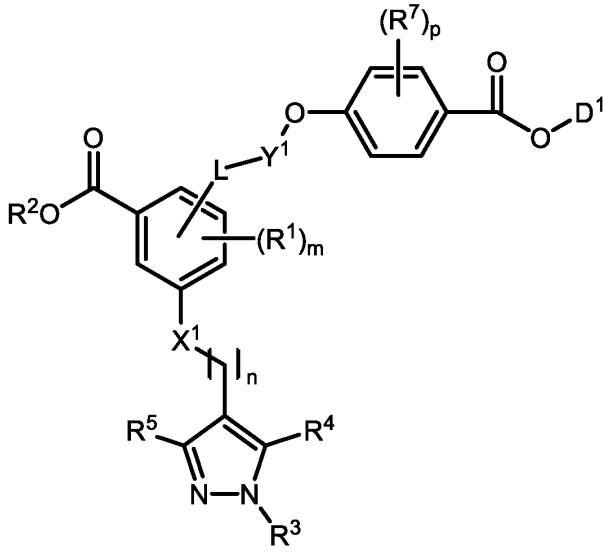
[0154] In some embodiments, the conjugate of formula (II) has the formula (IIe):



(IIe)

or a pharmaceutically acceptable salt thereof.

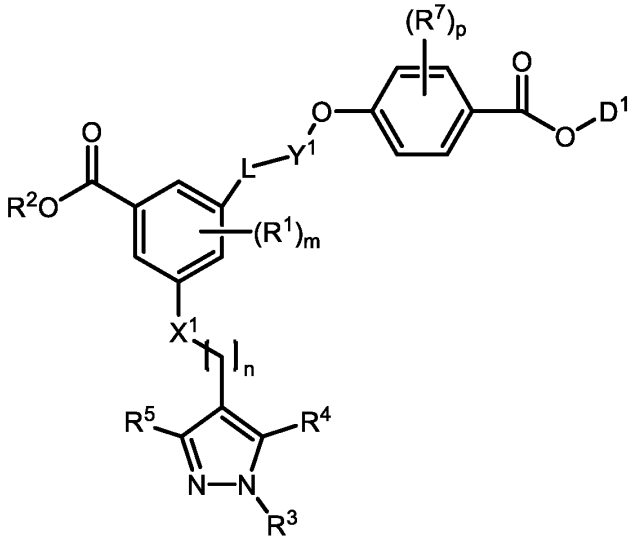
[0155] In some embodiments, the conjugate of formula (II) has the formula (IIf):



(IIf)

or a pharmaceutically acceptable salt thereof.

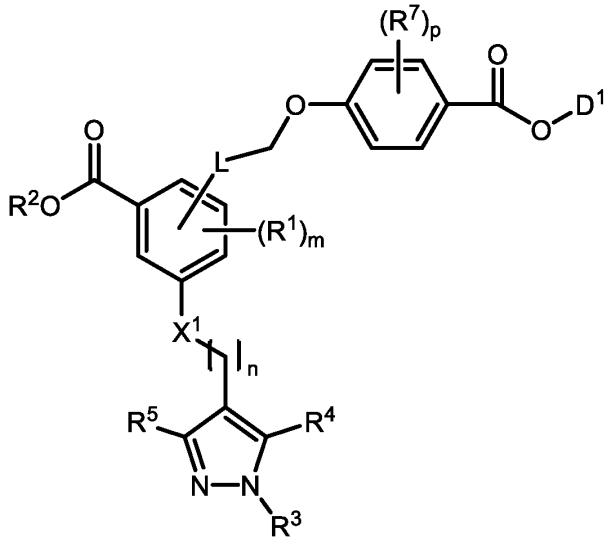
[0156] In some embodiments, the conjugate of formula (II) has the formula (IIg):



(IIg)

or a pharmaceutically acceptable salt thereof.

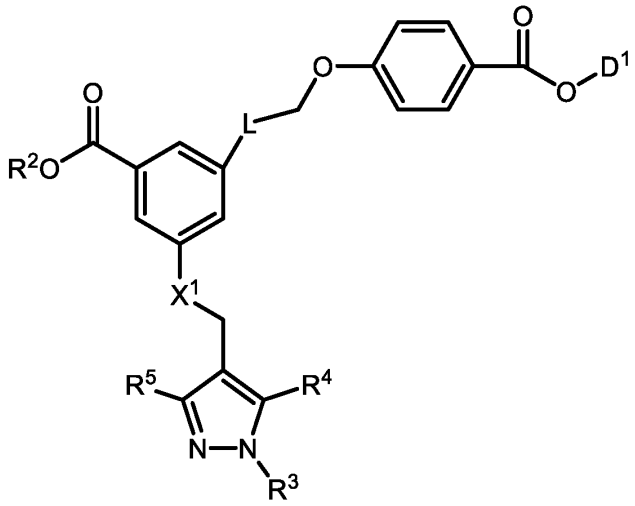
[0157] In some embodiments, the conjugate of formula (II) has the formula (IIh):



(IIh)

or a pharmaceutically acceptable salt thereof.

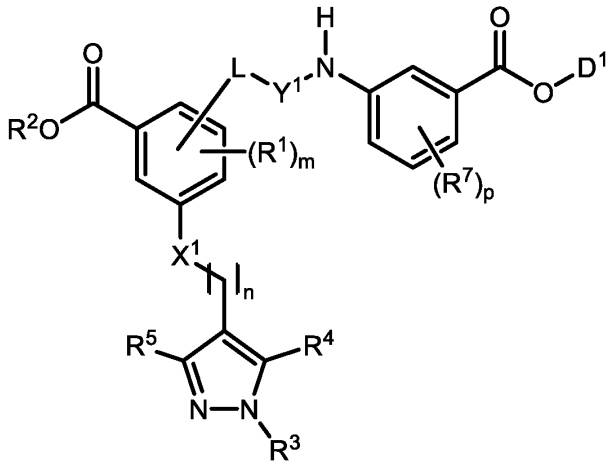
[0158] In some embodiments, the conjugate of formula (II) has the formula (IIi):



(IIi)

or a pharmaceutically acceptable salt thereof.

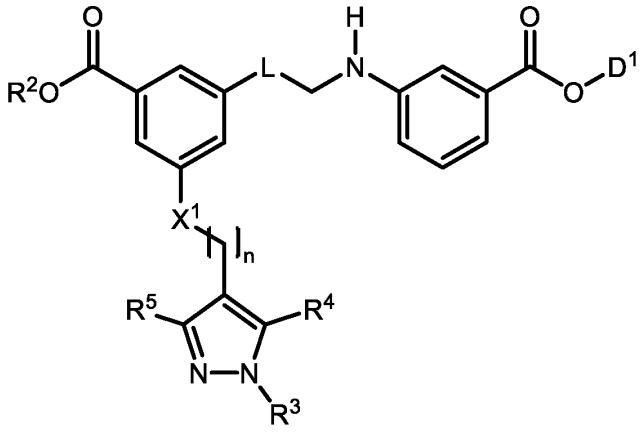
[0159] In some embodiments, the conjugate of formula (II') has the formula (IIj):



(IIj)

or a pharmaceutically acceptable salt thereof.

[0160] In some embodiments, the conjugate of formula (II') has the formula (IIk):



(IIk)

or a pharmaceutically acceptable salt thereof.

[0161] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0162] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0163] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

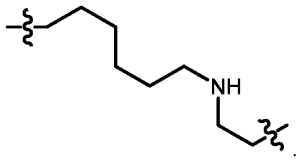
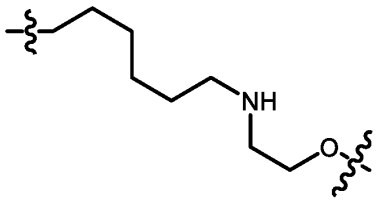
[0164] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or $-CH_3$. In some embodiments, R^5 is $-CH_3$.

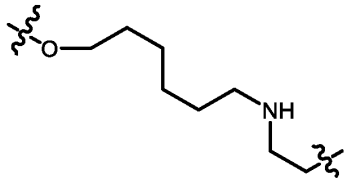
[0165] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIh), (IIj), or (IIk), n is 2 or 3. In some embodiments, n is 3.

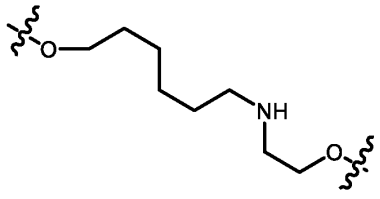
[0166] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIh), or (IIj), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

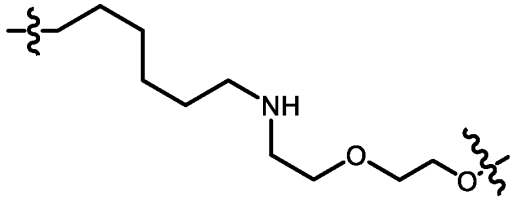
[0167] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIh), or (IIj), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

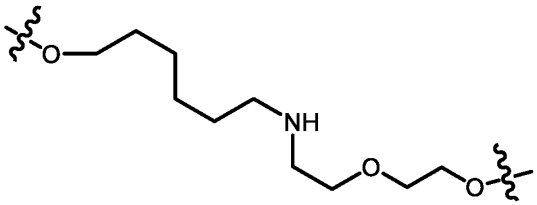
[0168] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), L is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, $-NR^x-$, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo. In some embodiments, L is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, $-N(CH_3)-$, and -S-. In some embodiments, L is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and $-N(CH_3)-$. In some embodiments, L is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

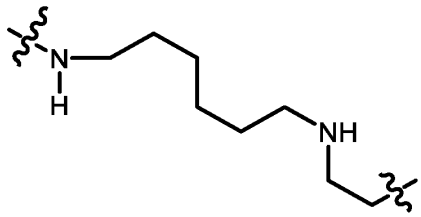
[0169] In some embodiments of the conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIi), L comprises the moiety . In some embodiments, L comprises the moiety 

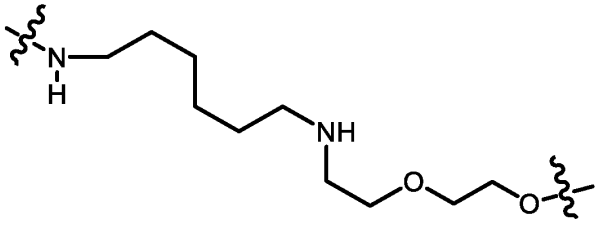

. In some embodiments, L comprises the moiety

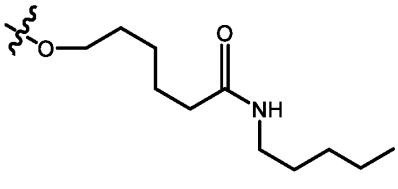

. In some embodiments, L comprises the moiety

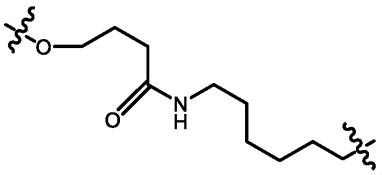
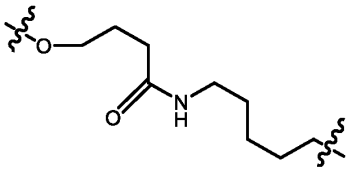
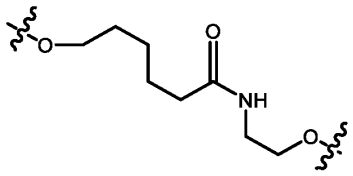
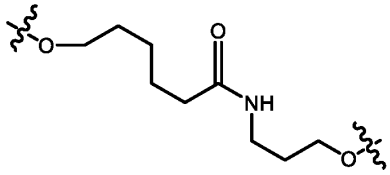
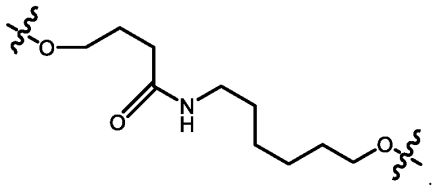

. In some embodiments, L comprises the moiety


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comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety . In some embodiments, L comprises the moiety .

[0170] In some embodiments of a conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0171] In some embodiments of a conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments,

D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0172] In some embodiments of a conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), D^1 is a peptide.

[0173] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0174] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

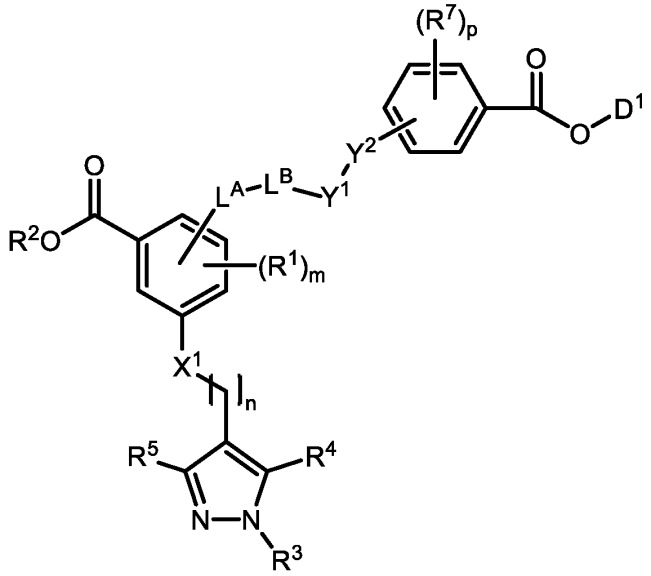
[0175] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0176] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0177] In some embodiments of a conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0178] In some embodiments of a conjugate of formula (II) or (II'), or any variation thereof, such as formula (IIa)-(IIk), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0179] In one embodiment, provided herein is a conjugate of formula (III'):



(III')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O, or S;

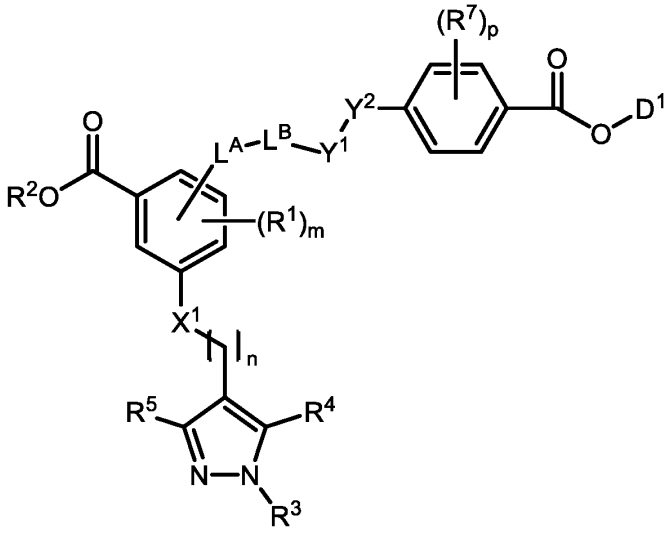
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0180] In one embodiment, provided herein is a conjugate of formula (III):



(III)

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O, or S;

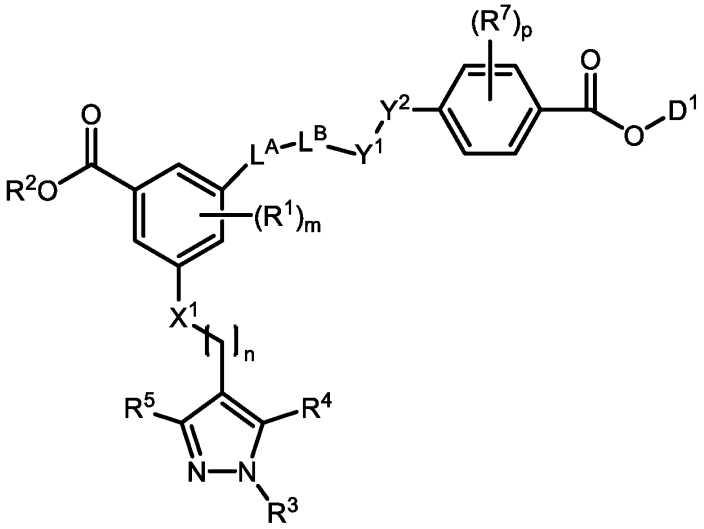
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

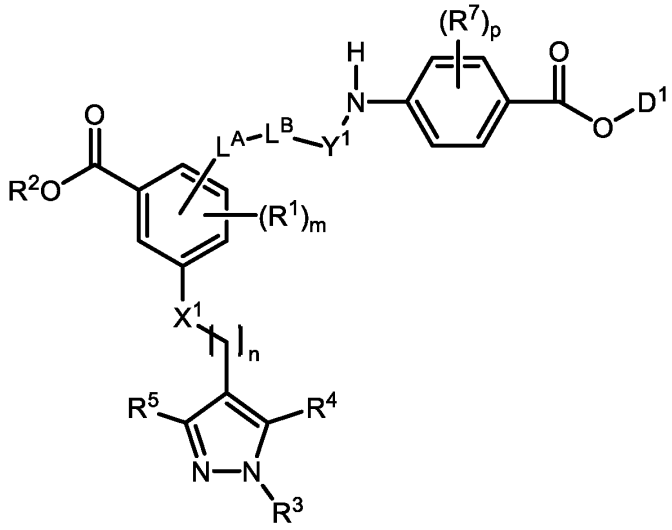
[0181] In some embodiments, the conjugate of formula (III) has the formula (IIIa):



(IIIa)

or a pharmaceutically acceptable salt thereof.

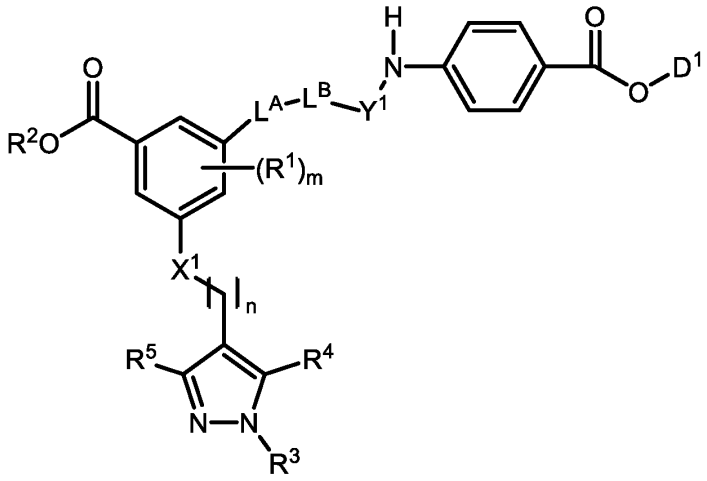
[0182] In some embodiments, the conjugate of formula (III) has the formula (IIIb):



(IIIb)

or a pharmaceutically acceptable salt thereof.

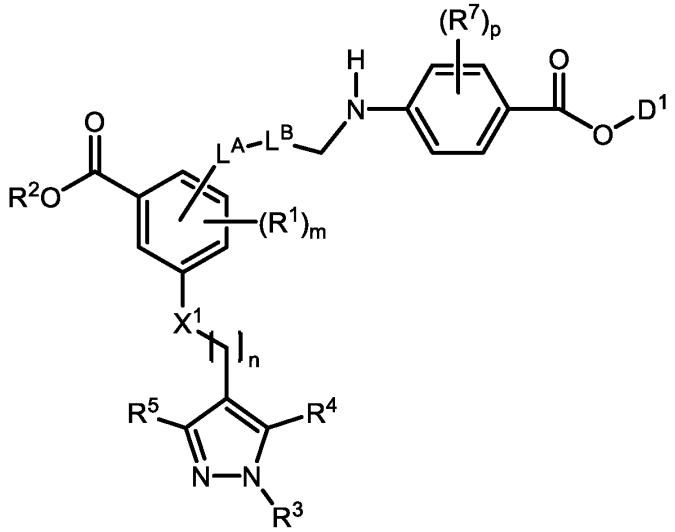
[0183] In some embodiments, the conjugate of formula (III) has the formula (IIIc):



(IIIc)

or a pharmaceutically acceptable salt thereof.

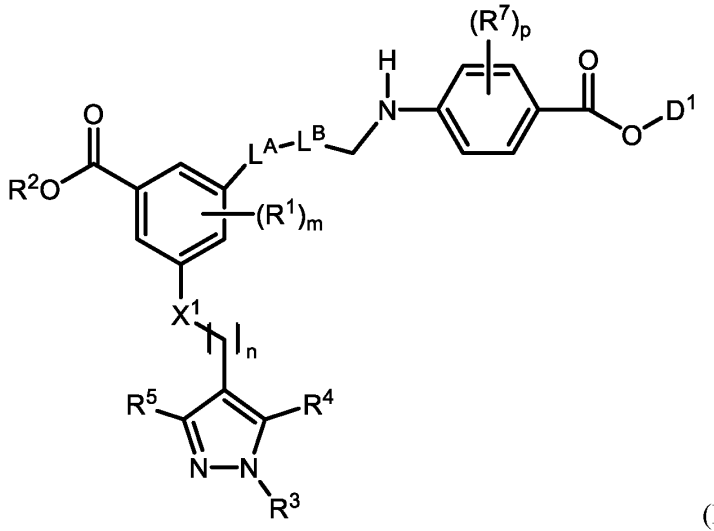
[0184] In some embodiments, the conjugate of formula (III) has the formula (IIId):



(IIId)

or a pharmaceutically acceptable salt thereof.

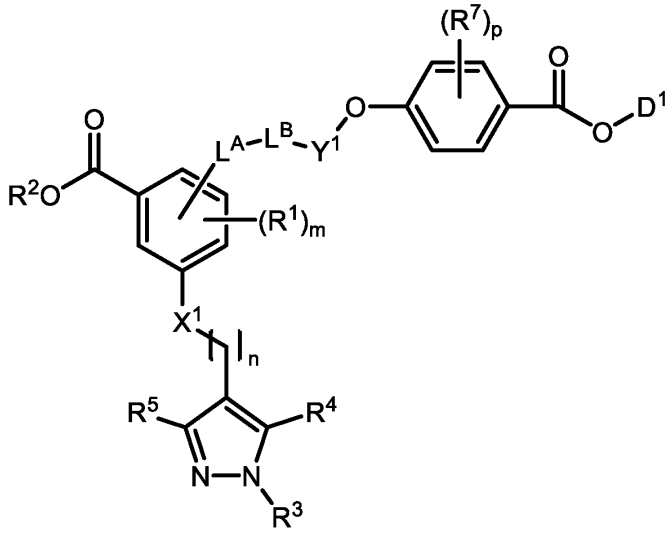
[0185] In some embodiments, the conjugate of formula (III) has the formula (IIIe):



(IIIe)

or a pharmaceutically acceptable salt thereof.

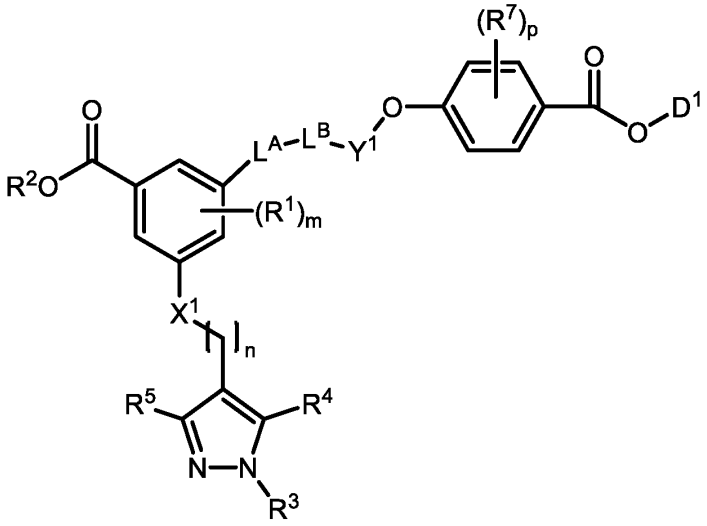
[0186] In some embodiments, the conjugate of formula (III) has the formula (IIIf):



(IIIf)

or a pharmaceutically acceptable salt thereof.

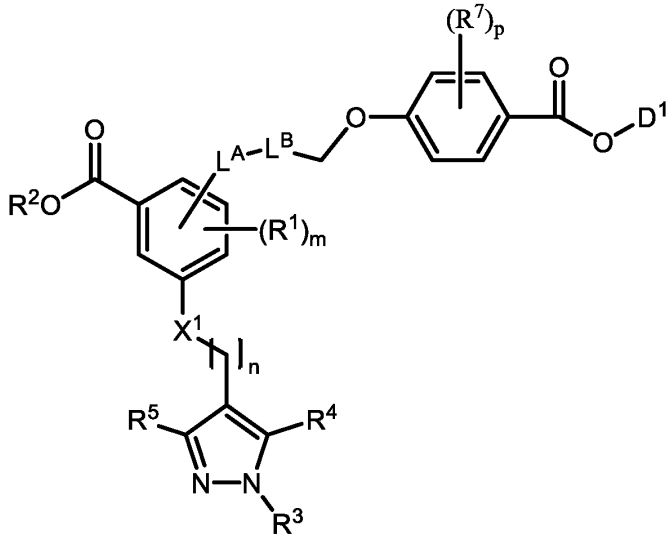
[0187] In some embodiments, the conjugate of formula (III) has the formula (IIIg):



(IIIg)

or a pharmaceutically acceptable salt thereof.

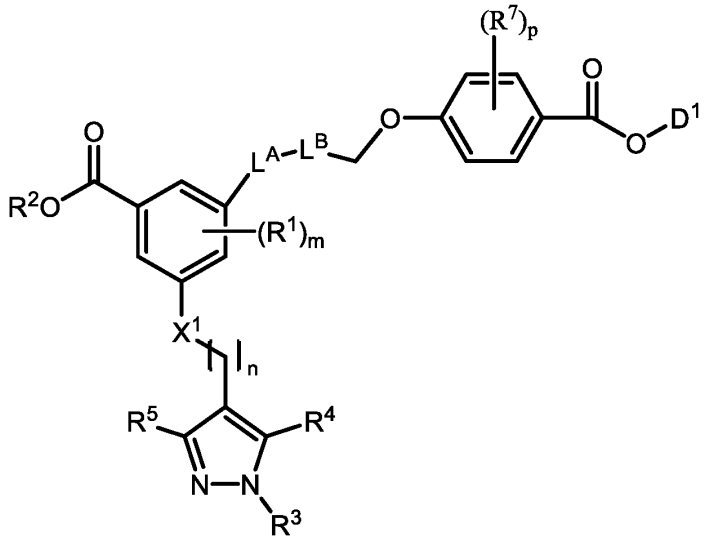
[0188] In some embodiments, the conjugate of formula (III) has the formula (IIIh):



(IIIh)

or a pharmaceutically acceptable salt thereof.

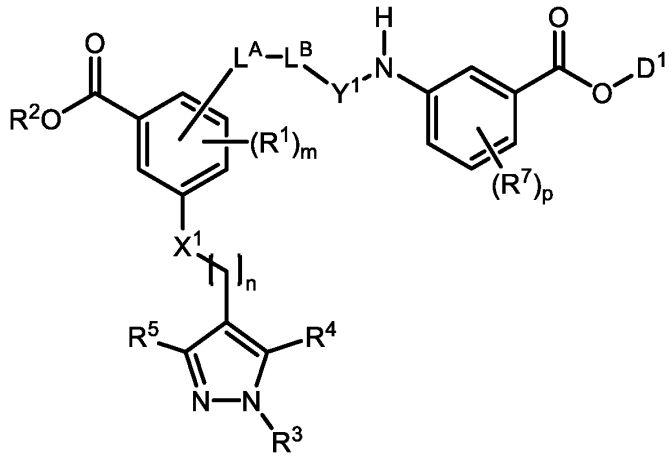
[0189] In some embodiments, the conjugate of formula (III) has the formula (IIIi):



(IIIi)

or a pharmaceutically acceptable salt thereof.

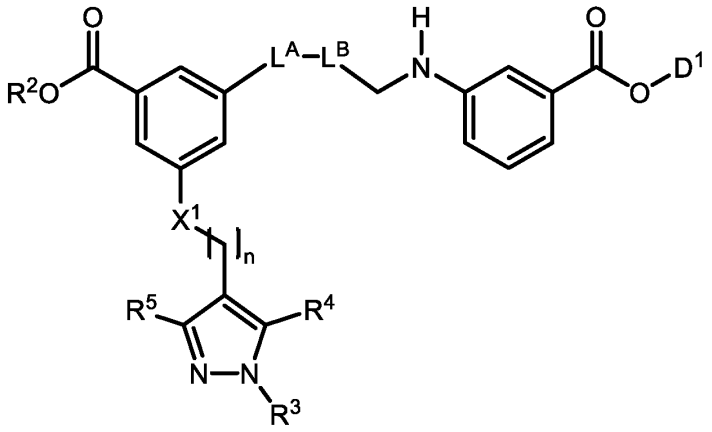
[0190] In some embodiments, the conjugate of formula (III') has the formula (IIIj):



(IIIj)

or a pharmaceutically acceptable salt thereof.

[0191] In some embodiments, the conjugate of formula (III') has the formula (IIIk):



(IIIk)

or a pharmaceutically acceptable salt thereof.

[0192] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0193] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0194] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

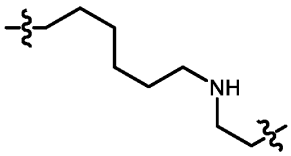
[0195] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or -CH_3. In some embodiments, R^5 is -CH_3.

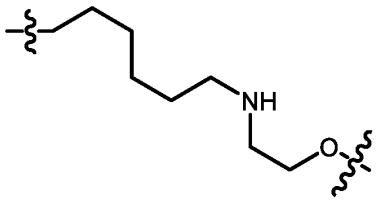
[0196] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), n is 2 or 3. In some embodiments, n is 3.

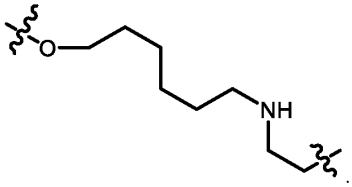
[0197] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIj), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

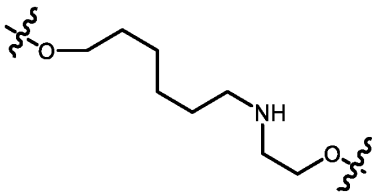
[0198] In some embodiments of the conjugate of formula (III) or (III'), or any variation thereof, such as formulas (IIIa), (IIIb), or (IIId)-(IIIj), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

[0199] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -$N(CH_3)$-, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -$N(CH_3)$-. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

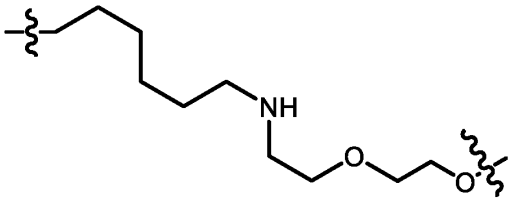
[0200] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), L^A comprises the moiety 

. In some embodiments, L^A comprises the moiety 

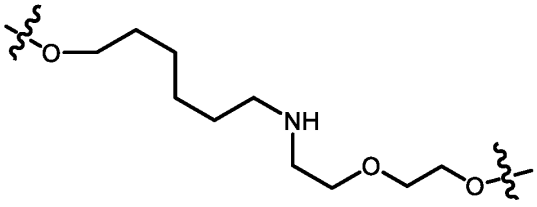
. In some embodiments, L^A comprises the moiety 

. In some embodiments, L^A comprises the moiety 

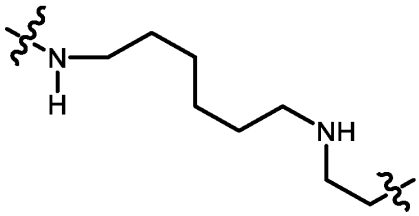
. In some embodiments, L^A comprises the moiety



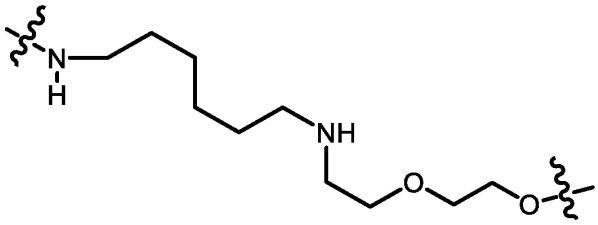
. In some embodiments, L^A comprises the



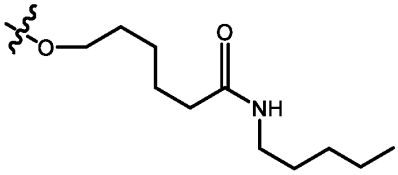
moiety . In some embodiments, L^A comprises



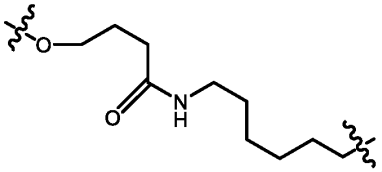
the moiety . In some embodiments, L^A comprises the



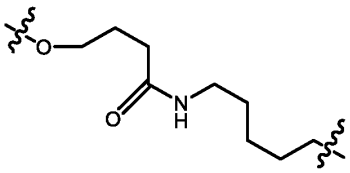
moiety . In some embodiments, L



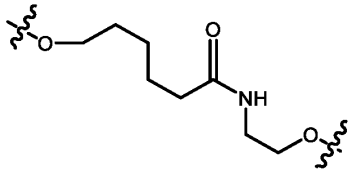
comprises the moiety . In some embodiments, L

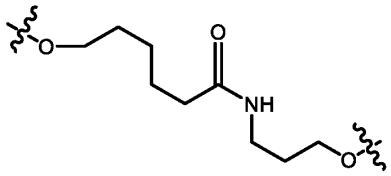


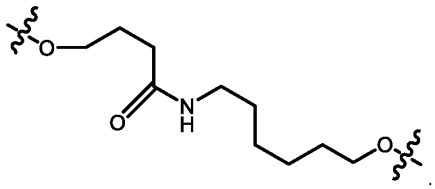
comprises the moiety . In some embodiments, L comprises



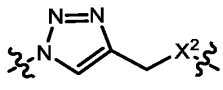
the moiety . In some embodiments, L comprises the moiety

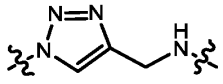

. In some embodiments, L comprises the moiety

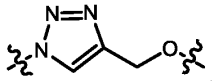

. In some embodiments, L comprises the moiety


.

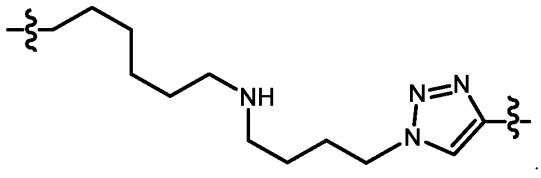
[0201] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), the click product L^B is a triazine moiety. In some embodiments, the conjugate of formula (III) or (III'), any of formula (IIIa)-(IIIi), comprises one of the following moieties:

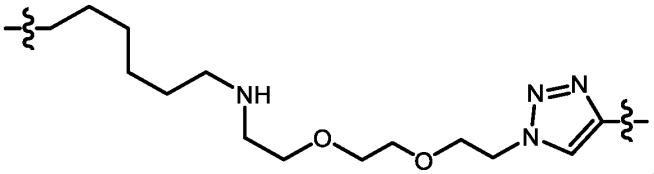
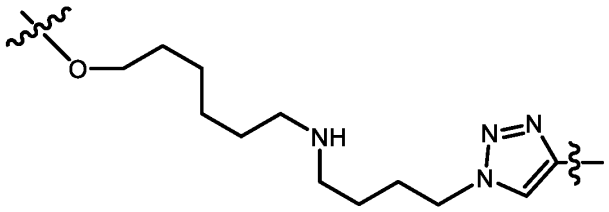
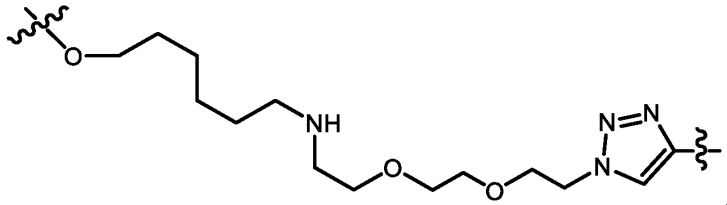
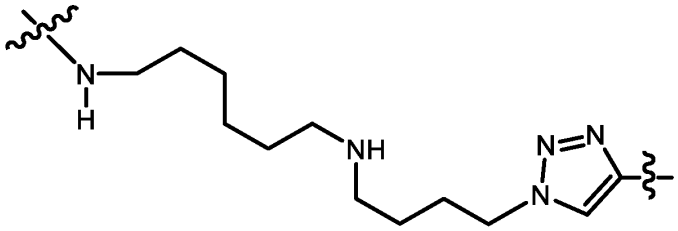
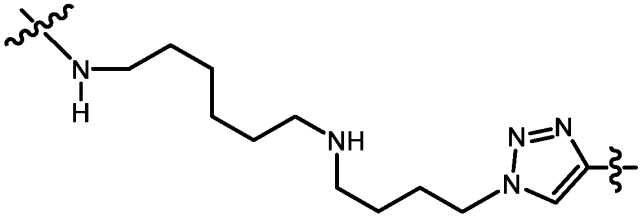

, wherein X^2 is $-CH_2-$, $-NR^x-$, -S- or -O-, wherein R^x is as defined elsewhere herein. In some embodiments, the conjugate comprises the moiety

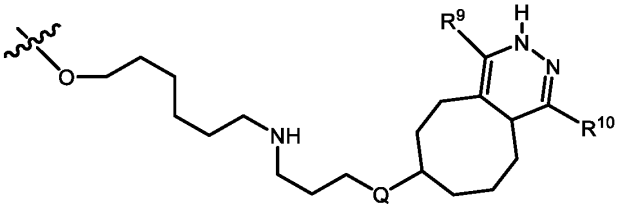

. In some embodiments, the conjugate comprises the moiety

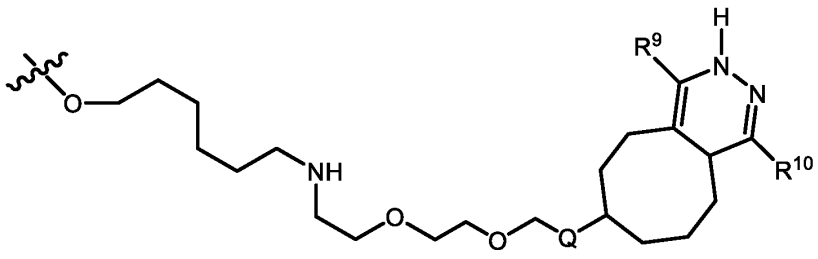

.

[0202] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), L^A-L^B comprises the moiety


. In some embodiments, L^A-L^B comprises the

moiety . In some embodiments, L^A-L^B comprises the moiety . In some embodiments, L^A-L^B comprises the moiety . In some embodiments, L^A-L^B comprises the moiety . In some embodiments, L^A-L^B comprises the moiety .

[0203] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), L^A-L^B comprises the moiety  or



, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is $-CH_2-$, -O-, -S-, -NH-, or $-N(C_{1-4}alkyl)-$.

[0204] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0205] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), D^1 is a peptide. In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0206] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0207] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

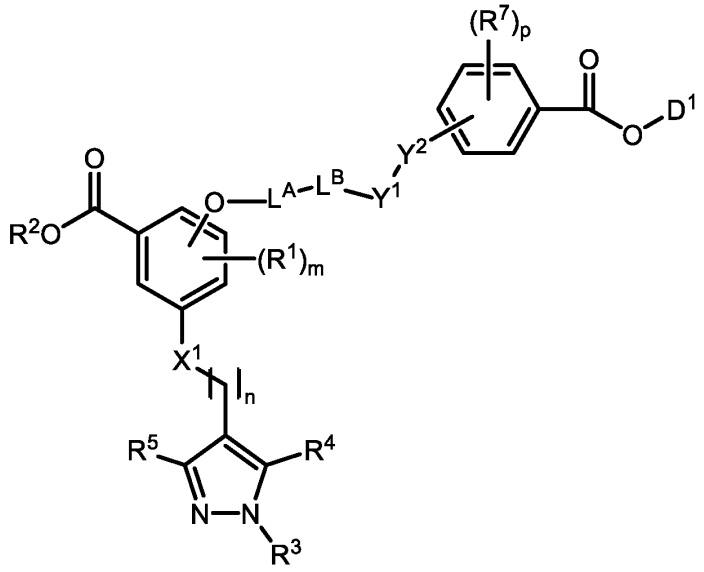
[0208] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0209] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0210] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0211] In some embodiments of a conjugate of formula (III) or (III'), or any variation thereof, such as formula (IIIa)-(IIIk), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0212] In one embodiment, provided herein is a conjugate of formula (IV'):



(IV')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

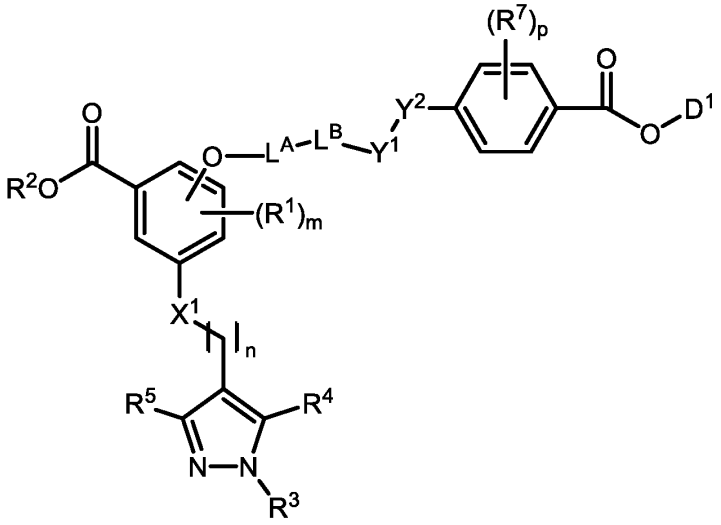
Y^2 is NR^6, O, or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0213] In one embodiment, provided herein is a conjugate of formula (IV):



(IV)

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

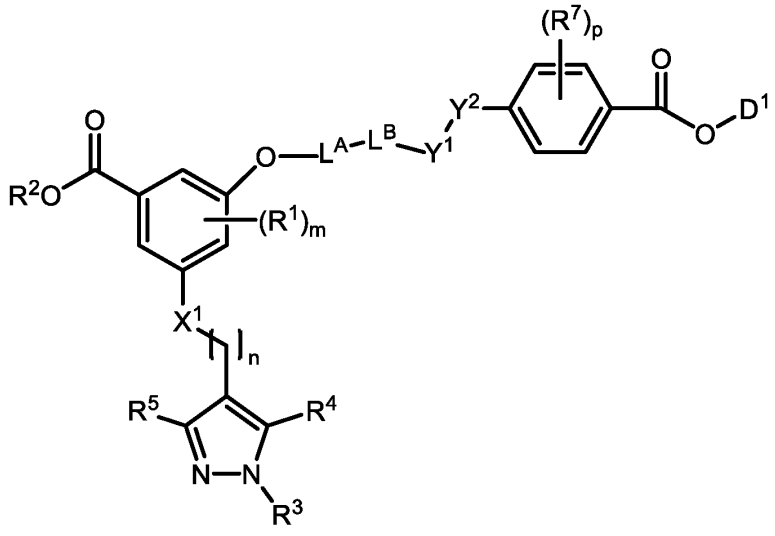
Y^2 is NR^6, O, or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

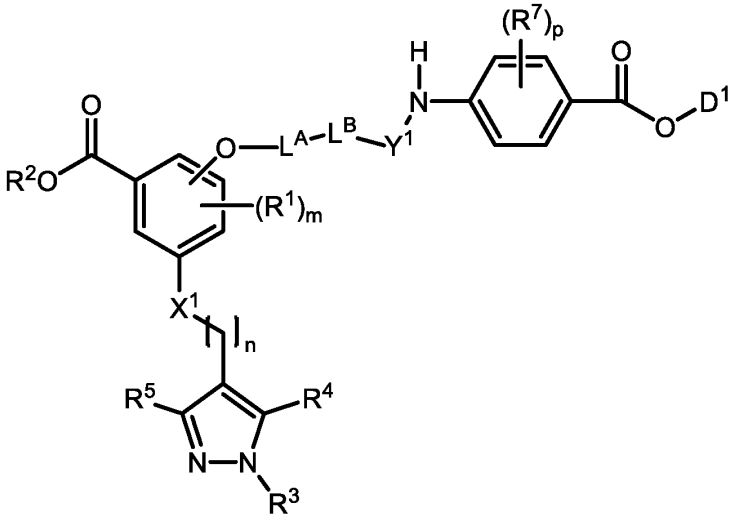
[0214] In some embodiments, the conjugate of formula (IV) has the formula (IVa):



(IVa)

or a pharmaceutically acceptable salt thereof.

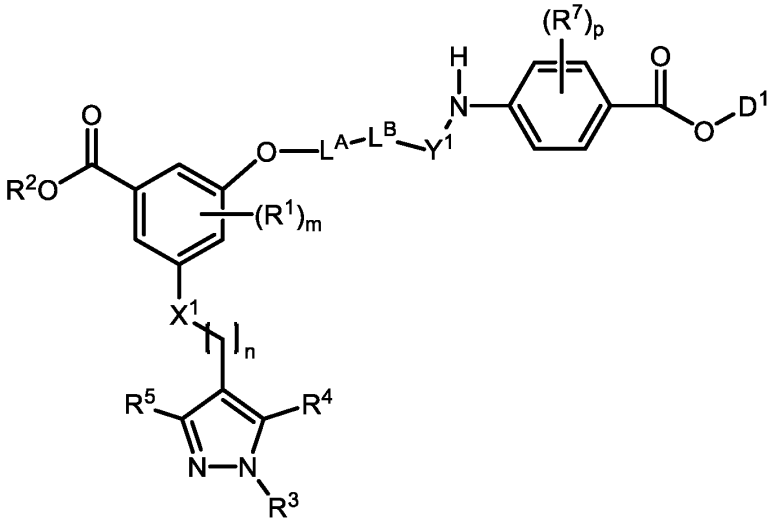
[0215] In some embodiments, the conjugate of formula (IV) has the formula (IVb):



(IVb)

or a pharmaceutically acceptable salt thereof.

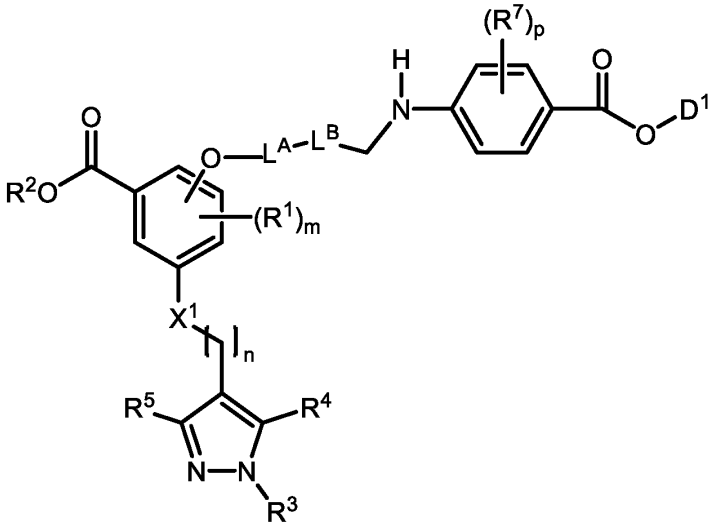
[0216] In some embodiments, the conjugate of formula (IV) has the formula (IVc):



(IVc)

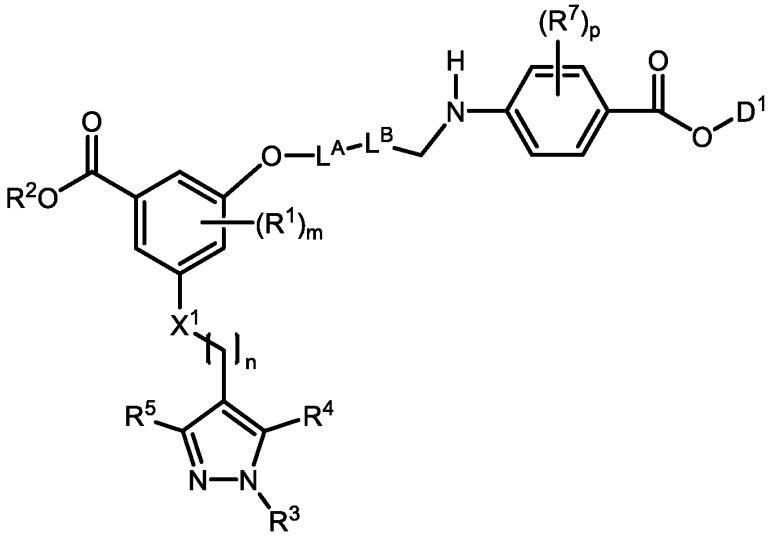
or a pharmaceutically acceptable salt thereof.

[0217] In some embodiments, the conjugate of formula (IV) has the formula (IVd):



or a pharmaceutically acceptable salt thereof.

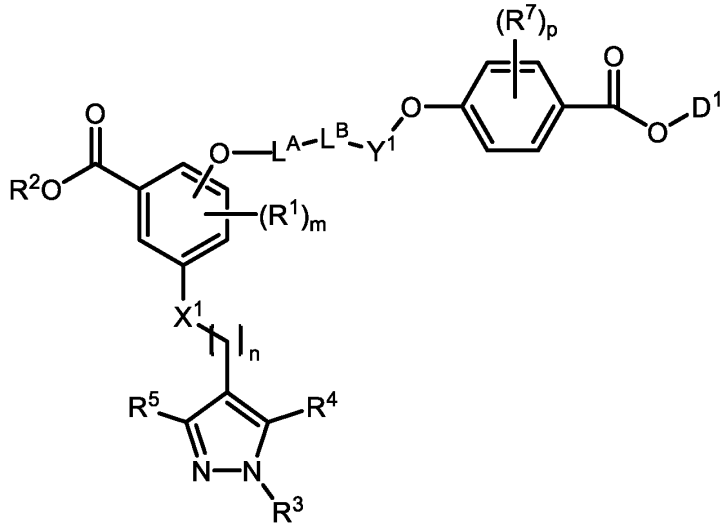
[0218] In some embodiments, the conjugate of formula (IV) has the formula (IVe):



(IVe)

or a pharmaceutically acceptable salt thereof.

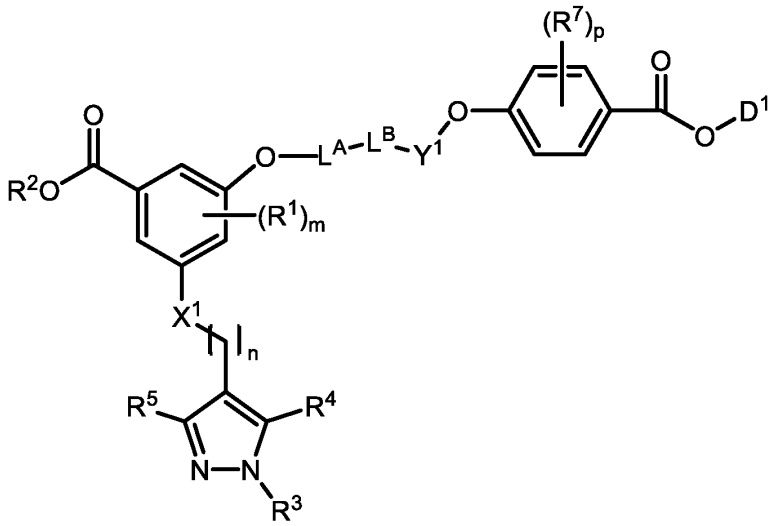
[0219] In some embodiments, the conjugate of formula (IV) has the formula (IVf):



(IVf)

or a pharmaceutically acceptable salt thereof.

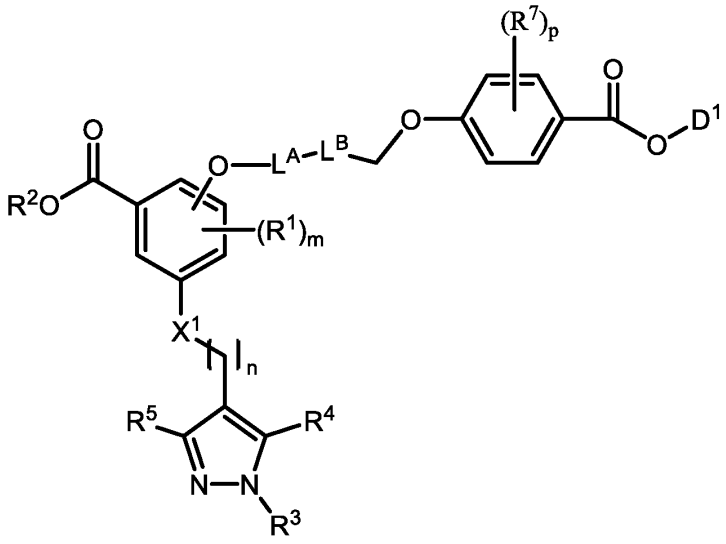
[0220] In some embodiments, the conjugate of formula (IV) has the formula (IVg):



(IVg)

or a pharmaceutically acceptable salt thereof.

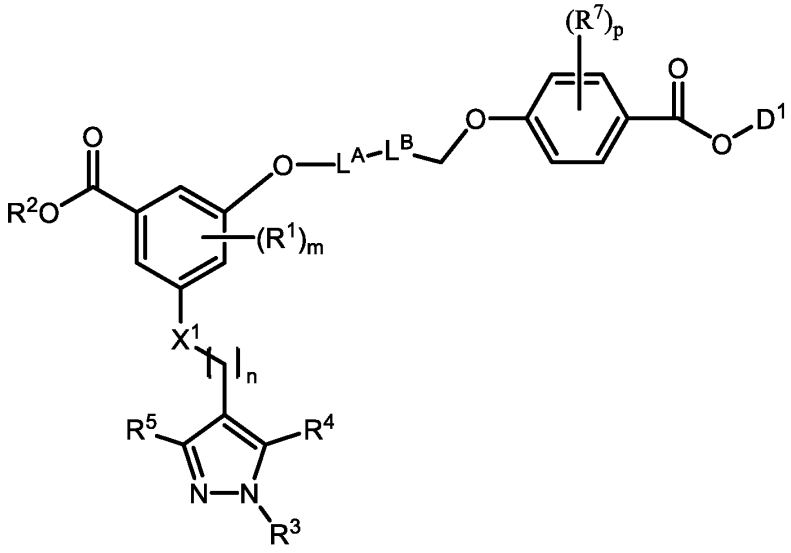
[0221] In some embodiments, the conjugate of formula (IV) has the formula (IVh):



(IVh)

or a pharmaceutically acceptable salt thereof.

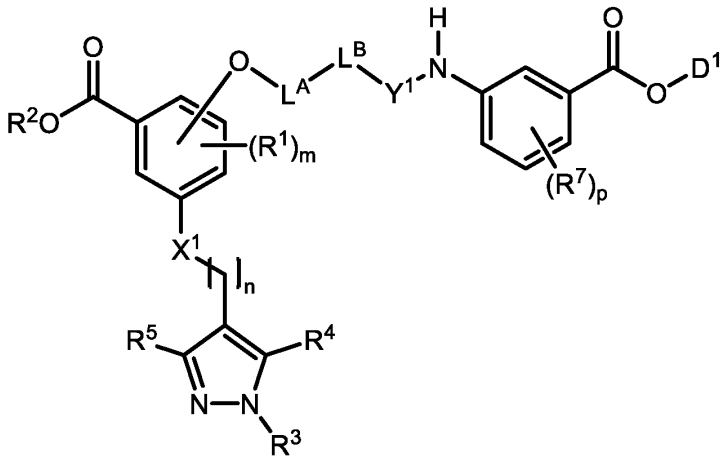
[0222] In some embodiments, the conjugate of formula (IV) has the formula (IVi):



(IVi)

or a pharmaceutically acceptable salt thereof.

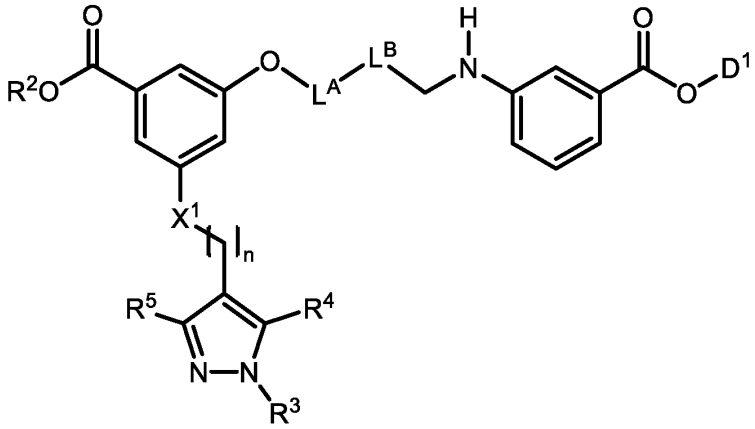
[0223] In some embodiments, the conjugate of formula (IV') has the formula (IVj):



(IVj)

or a pharmaceutically acceptable salt thereof.

[0224] In some embodiments, the conjugate of formula (IV') has the formula (IVk):



(IVk)

or a pharmaceutically acceptable salt thereof.

[0225] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0226] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0227] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

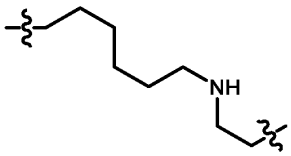
[0228] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or $-CH_3$. In some embodiments, R^5 is $-CH_3$.

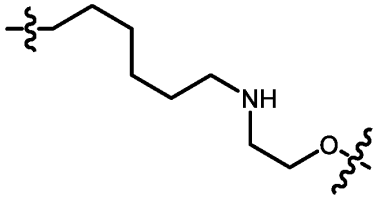
[0229] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), n is 2 or 3. In some embodiments, n is 3.

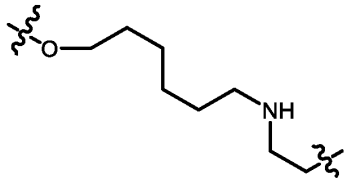
[0230] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVj), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

[0231] In some embodiments of the conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVj), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

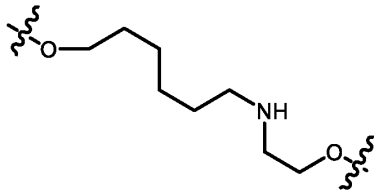
[0232] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, $-NR^x-$, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, $-N(CH_3)-$, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and $-N(CH_3)-$. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

[0233] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), L^A comprises the moiety 

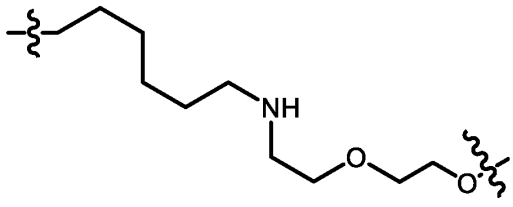
. In some embodiments, L^A comprises the moiety 



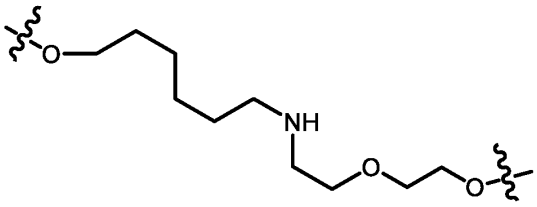
. In some embodiments, L^A comprises the moiety



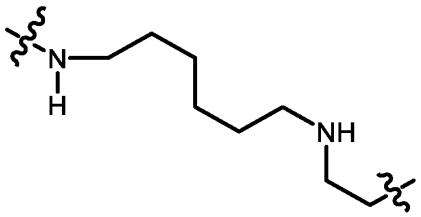
. In some embodiments, L^A comprises the moiety



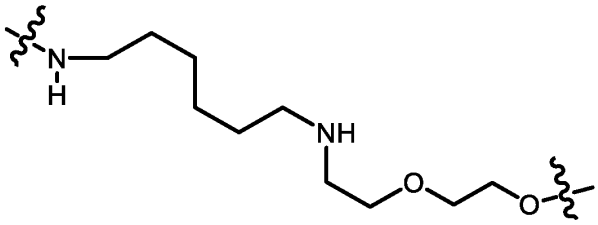
. In some embodiments, L^A comprises the



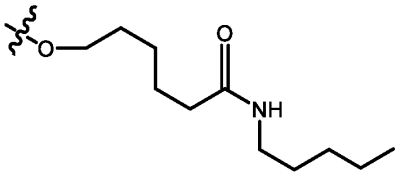
moiety . In some embodiments, L^A comprises



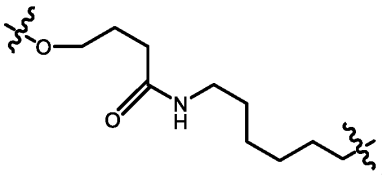
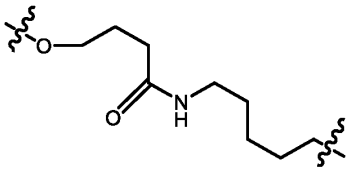
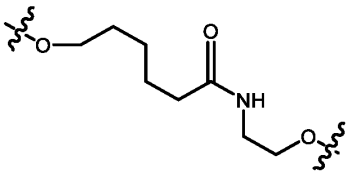
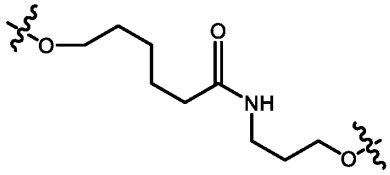
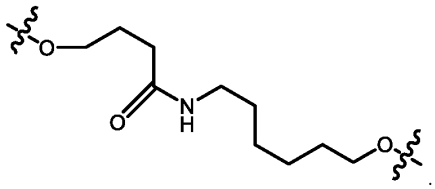
the moiety . In some embodiments, L^A comprises the



moiety . In some embodiments, L^A



comprises the moiety . In some embodiments, L^A

comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety .

[0234] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0235] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such

embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0236] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), D^1 is a peptide.

[0237] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0238] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

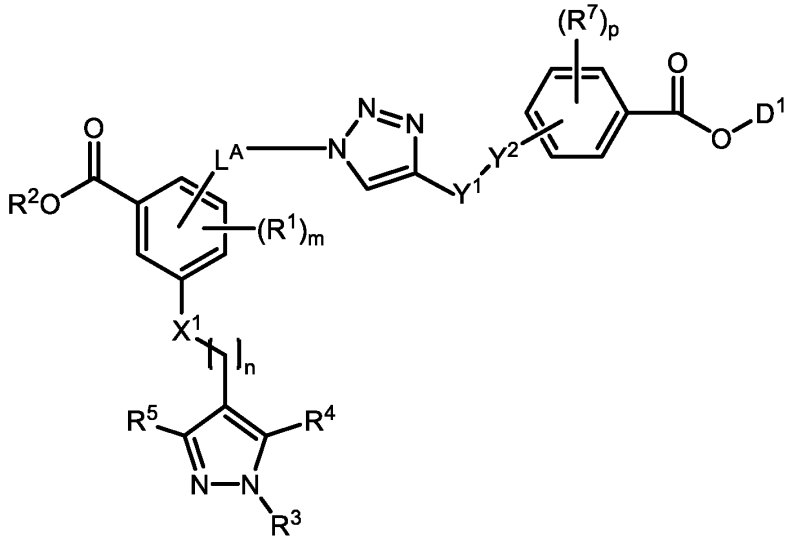
[0239] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0240] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0241] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0242] In some embodiments of a conjugate of formula (IV) or (IV'), or any variation thereof, such as formula (IVa)-(IVk), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0243] In some embodiments, provided herein is a conjugate of formula (V'):



(V'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

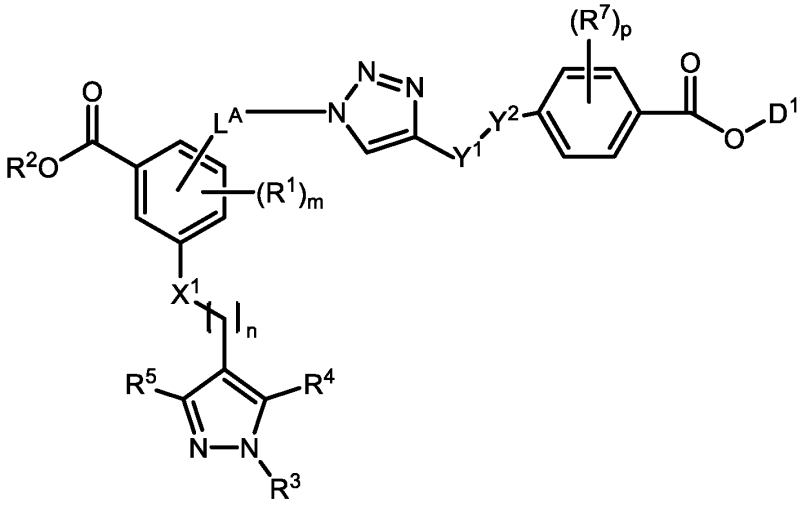
Y^2 is NR^6 , O or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0244] In some embodiments, provided herein is a conjugate of formula (V):



(V),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

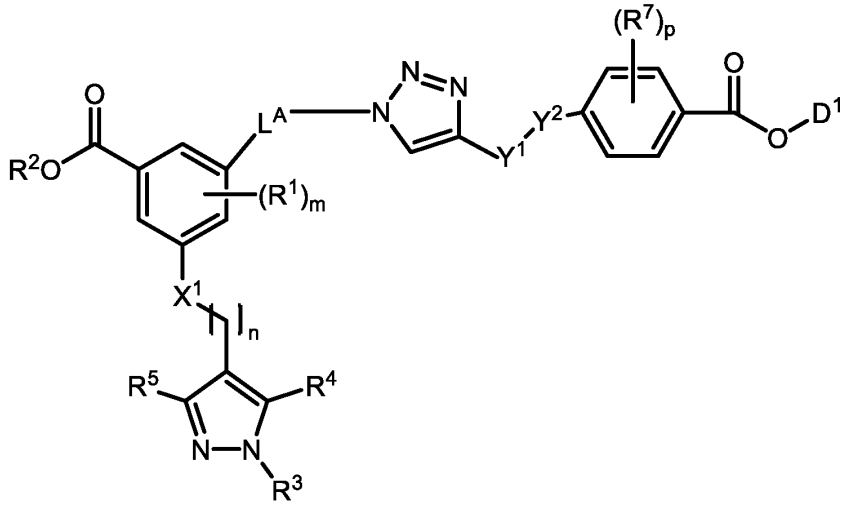
Y^2 is NR^6 , O or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

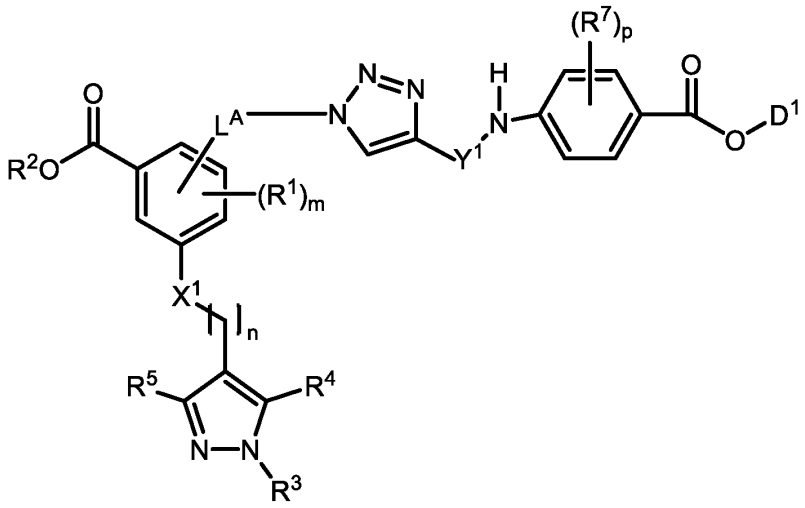
[0245] In some embodiments, the conjugate of formula (V) has the formula (Va):



(Va)

or a pharmaceutically acceptable salt thereof.

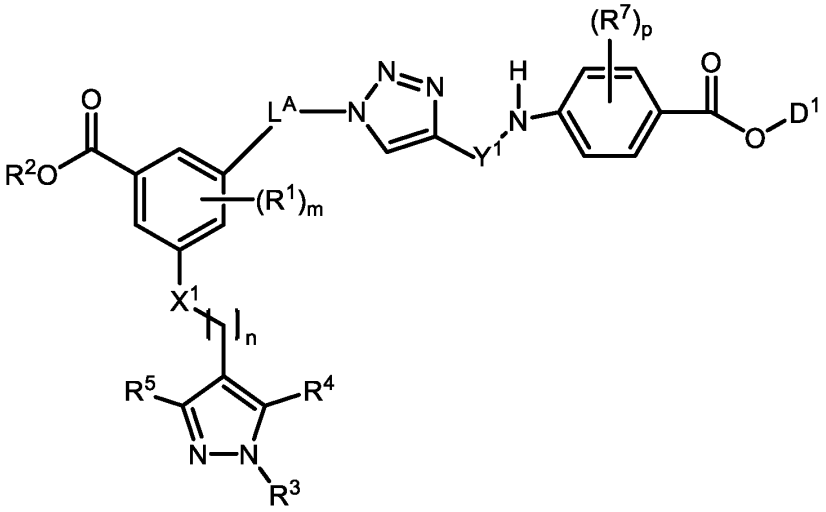
[0246] In some embodiments, the conjugate of formula (V) has the formula (Vb):



(Vb)

or a pharmaceutically acceptable salt thereof.

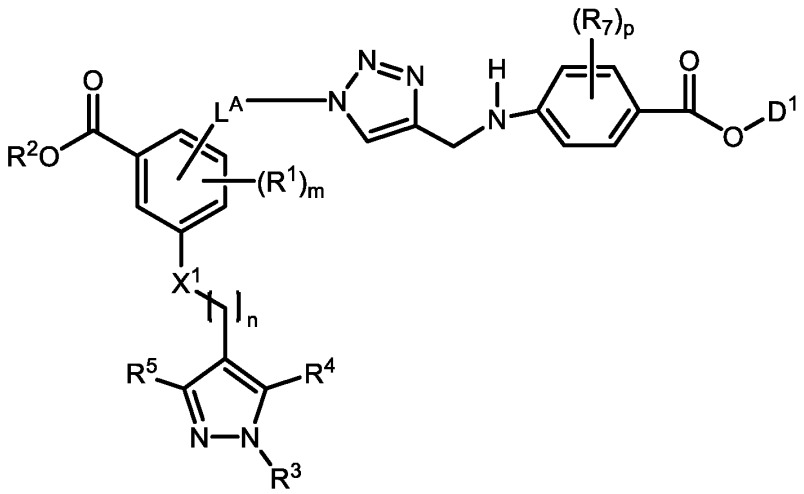
[0247] In some embodiments, the conjugate of formula (V) has the formula (Vc):



(Vc)

or a pharmaceutically acceptable salt thereof.

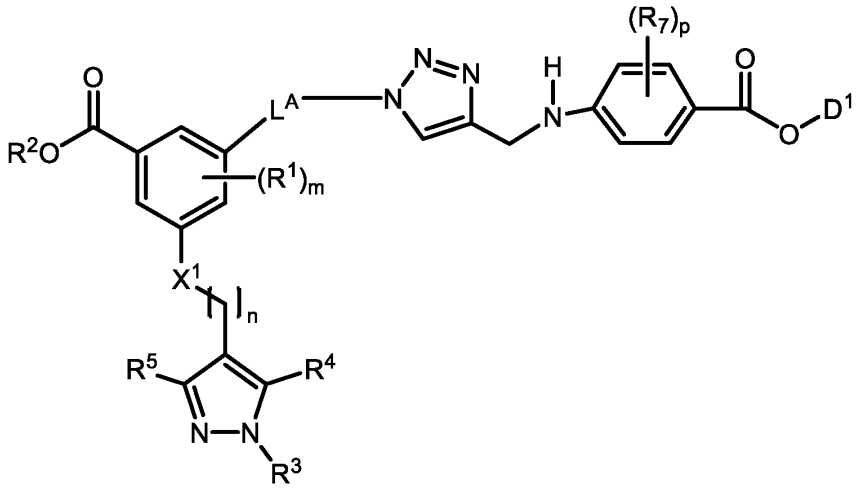
[0248] In some embodiments, the conjugate of formula (V) has the formula (Vd):



(Vd)

or a pharmaceutically acceptable salt thereof.

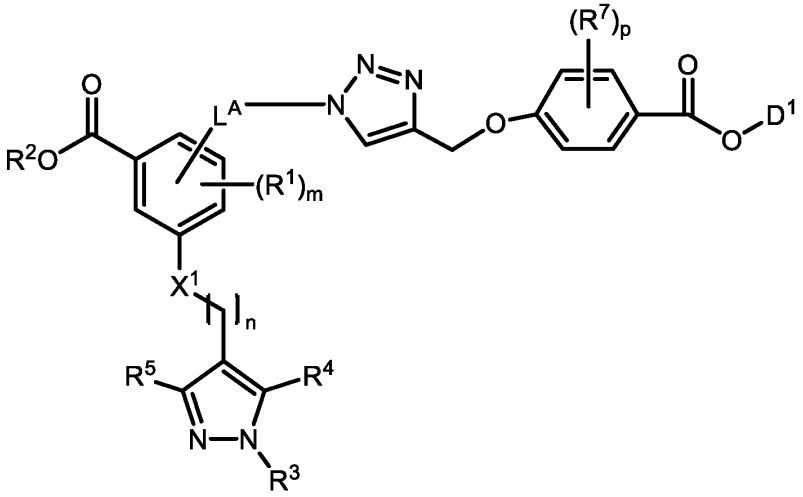
[0249] In some embodiments, the conjugate of formula (V) has the formula (Ve):



(Ve)

or a pharmaceutically acceptable salt thereof.

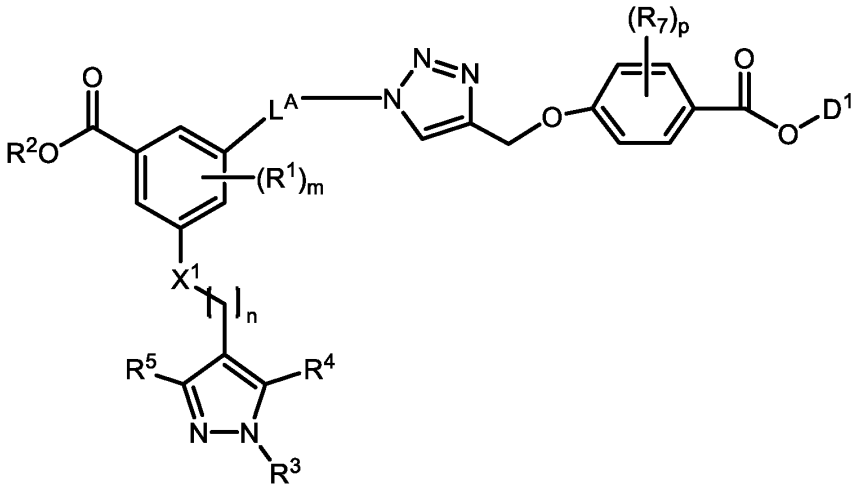
[0250] In some embodiments, the conjugate of formula (V) has the formula (Vf):



(Vf)

or a pharmaceutically acceptable salt thereof.

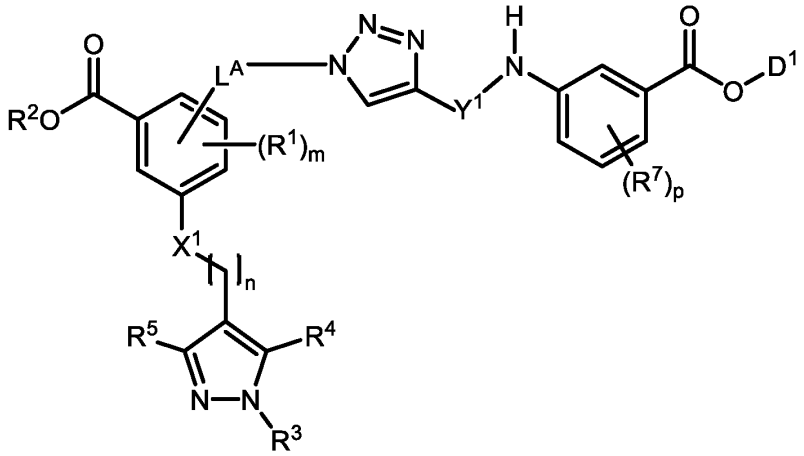
[0251] In some embodiments, the conjugate of formula (V) has the formula (Vg):



(Vg)

or a pharmaceutically acceptable salt thereof.

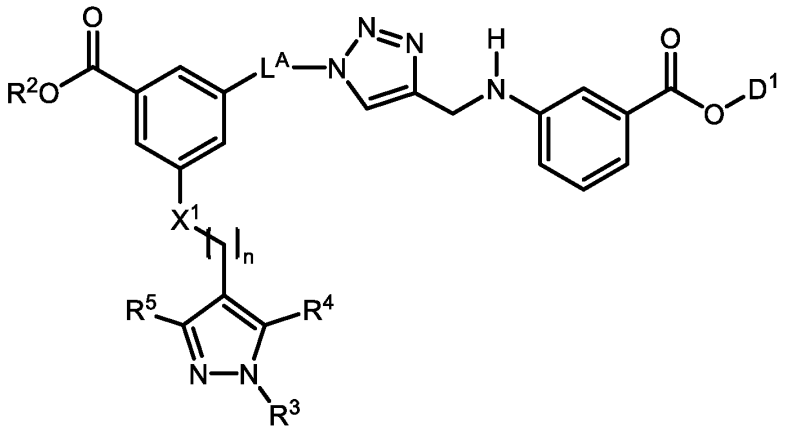
[0252] In some embodiments, the conjugate of formula (V') has the formula (Vh):



(Vh)

or a pharmaceutically acceptable salt thereof.

[0253] In some embodiments, the conjugate of formula (V') has the formula (Vi):



(Vi)

or a pharmaceutically acceptable salt thereof.

[0254] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0255] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0256] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

[0257] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or -CH_3. In some embodiments, R^5 is -CH_3.

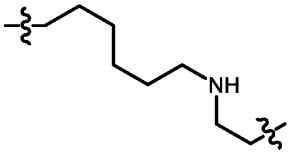
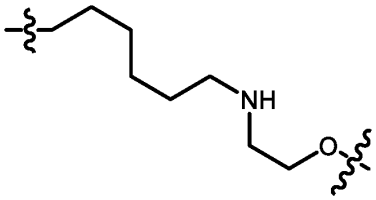
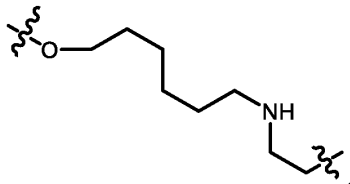
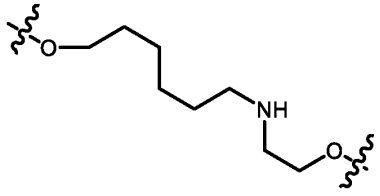
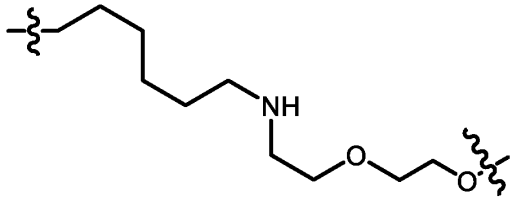
[0258] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), n is 2 or 3. In some embodiments, n is 3.

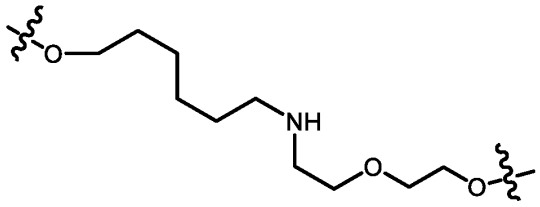
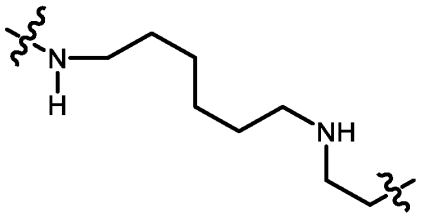
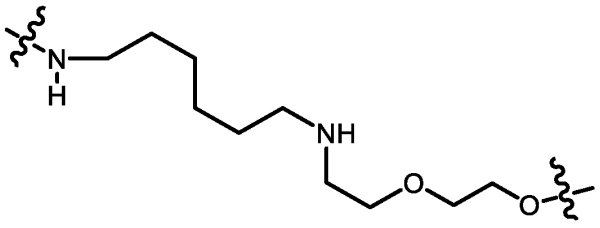
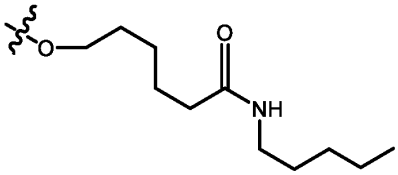
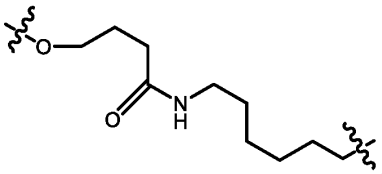
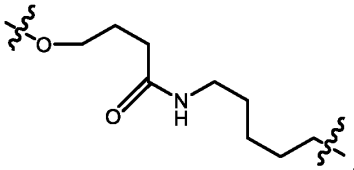
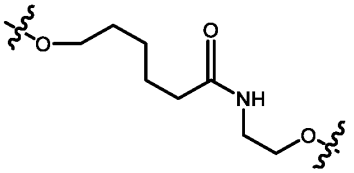
[0259] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vh), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

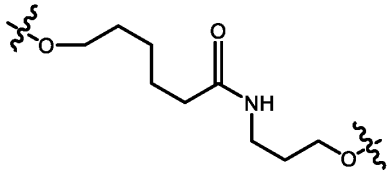
[0260] In some embodiments of the conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vh), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

[0261] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally

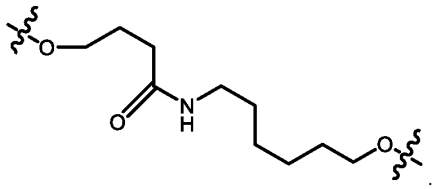
substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -N(CH_3)-, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -N(CH_3)-. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

[0262] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the

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[0263] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0264] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0265] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), D^1 is a peptide.

[0266] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some

embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0267] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

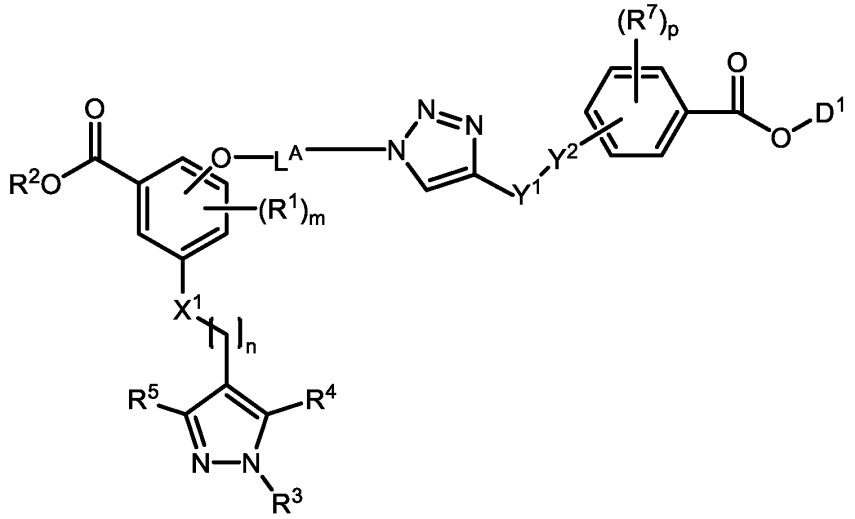
[0268] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0269] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0270] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0271] In some embodiments of a conjugate of formula (V) or (V'), or any variation thereof, such as formula (Va)-(Vi), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0272] In some embodiments, provided herein is a conjugate of formula (VI'):



(VI')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

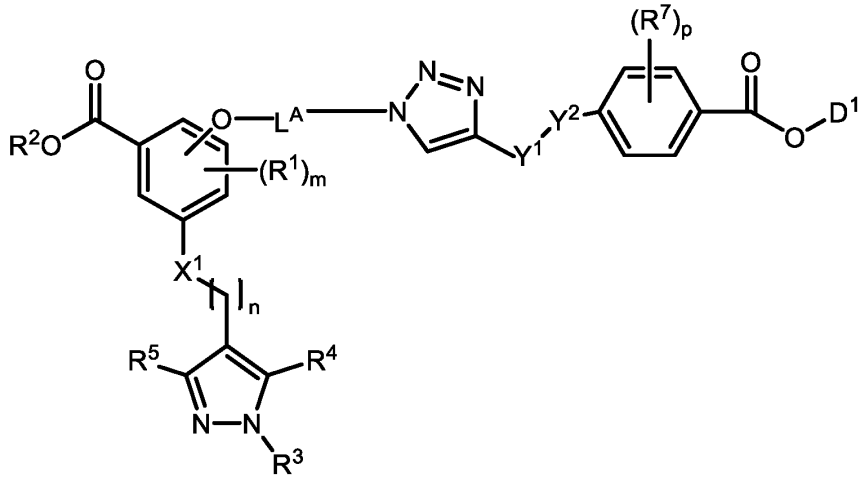
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

[0273] In some embodiments, provided herein is a conjugate of formula (VI):



(VI)

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

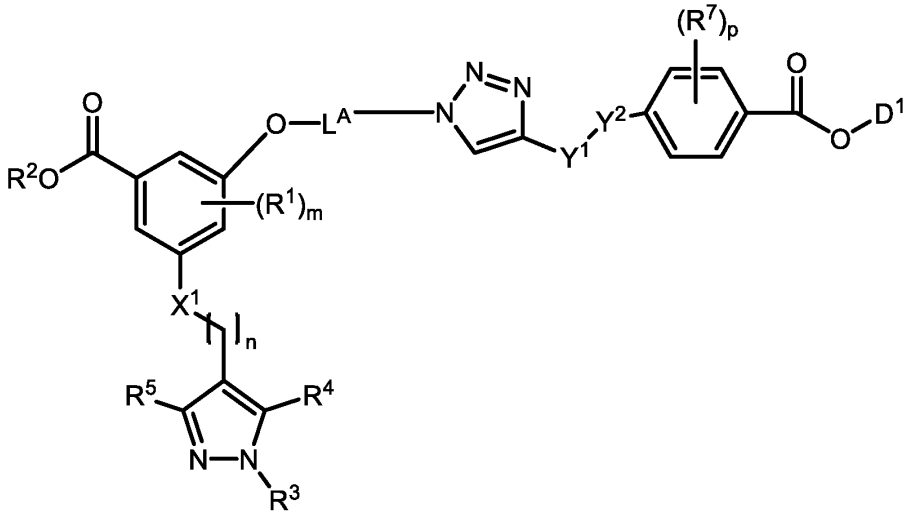
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

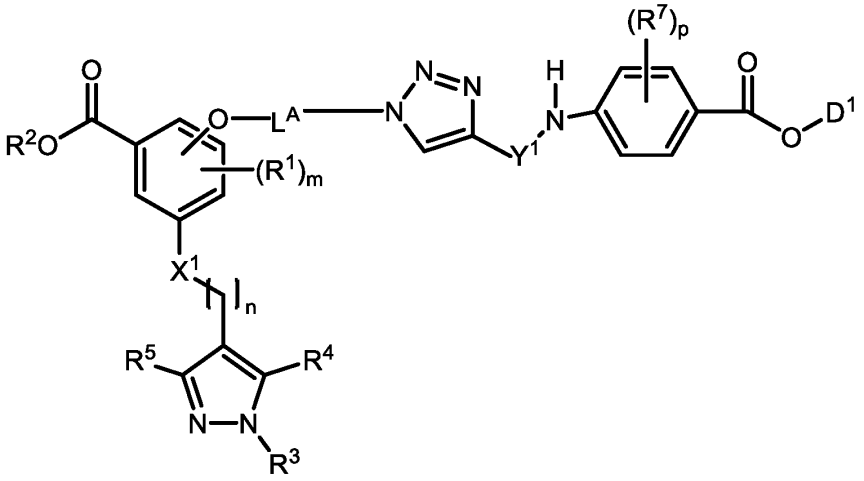
[0274] In some embodiments, the conjugate of formula (VI) has the formula (VIa):



(VIa)

or a pharmaceutically acceptable salt thereof.

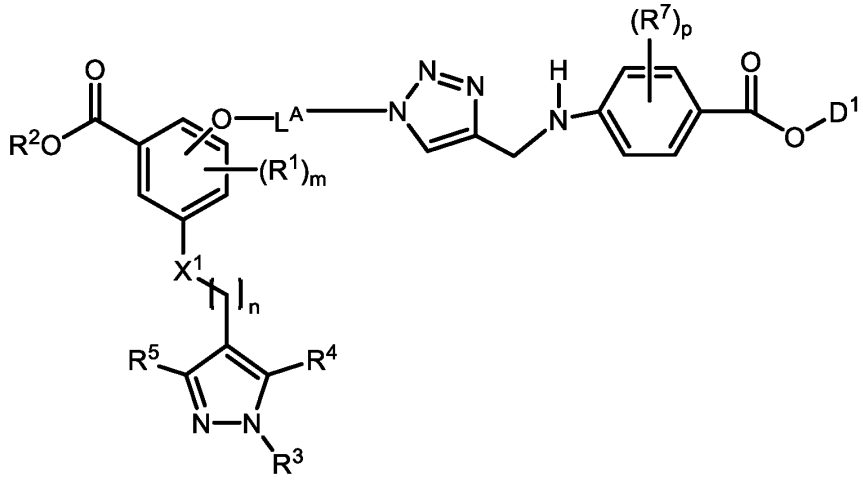
[0275] In some embodiments, the conjugate of formula (VI) has the formula (VIb):



(VIb)

or a pharmaceutically acceptable salt thereof.

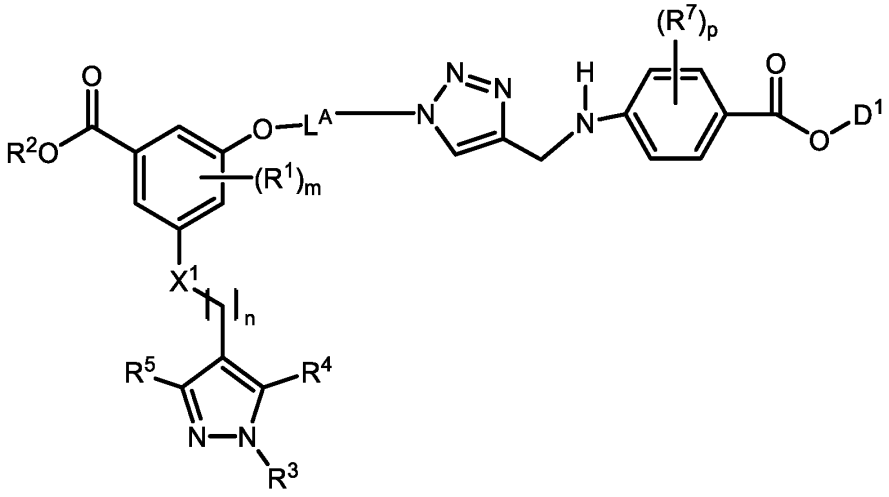
[0276] In some embodiments, the conjugate of formula (VI) has the formula (VIc):



(VIc)

or a pharmaceutically acceptable salt thereof.

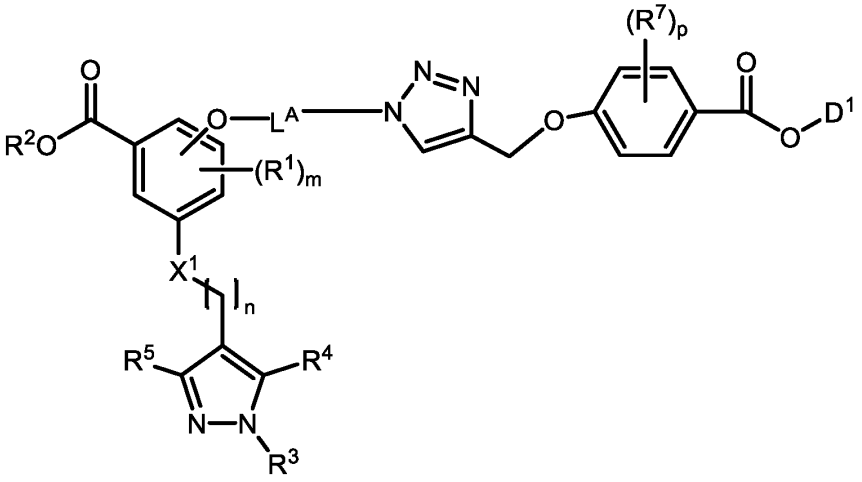
[0277] In some embodiments, the conjugate of formula (VI) has the formula (VId):



(VId)

or a pharmaceutically acceptable salt thereof.

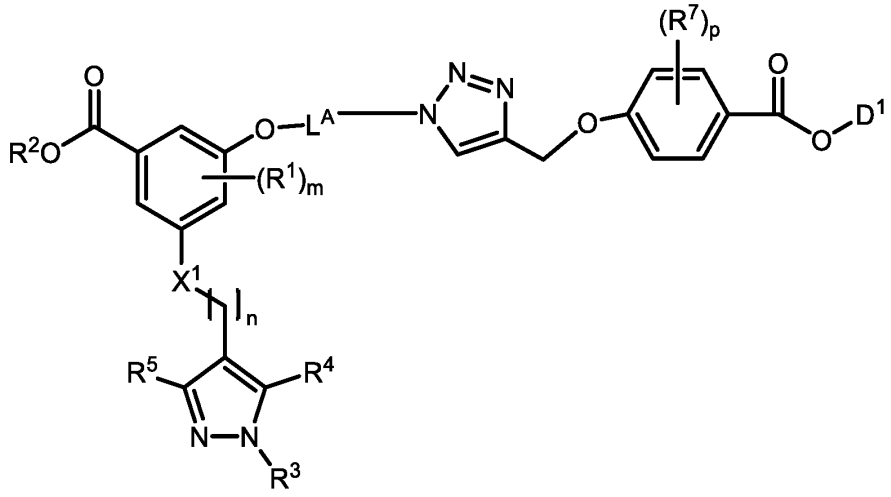
[0278] In some embodiments, the conjugate of formula (VI) has the formula (VIe):



(VIe)

or a pharmaceutically acceptable salt thereof.

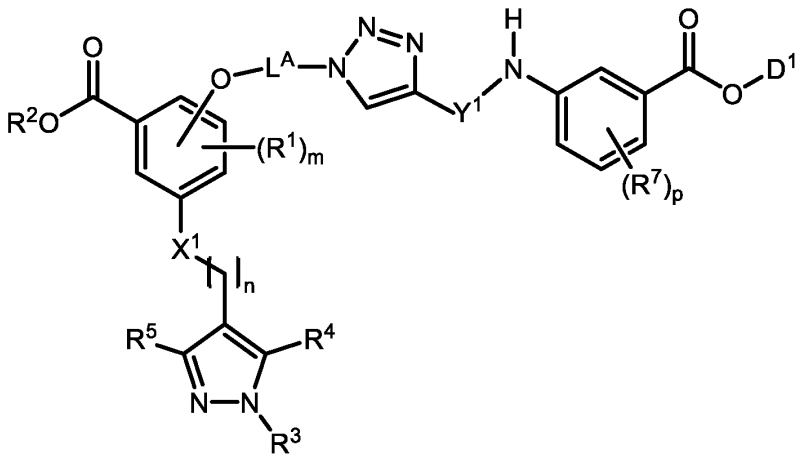
[0279] In some embodiments, the conjugate of formula (VI) has the formula (VIf):



(VIf)

or a pharmaceutically acceptable salt thereof.

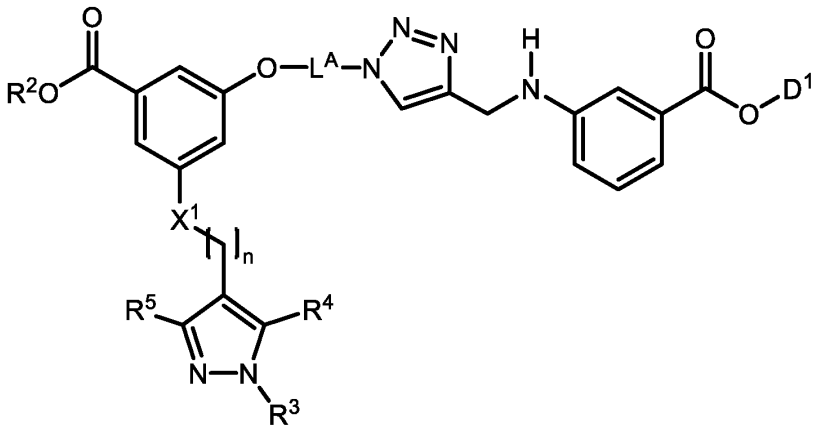
[0280] In some embodiments, the conjugate of formula (VI') has the formula (VIg):



(VIg)

or a pharmaceutically acceptable salt thereof.

[0281] In some embodiments, the conjugate of formula (VI') has the formula (VIh):



(VIh)

or a pharmaceutically acceptable salt thereof.

[0282] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0283] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0284] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

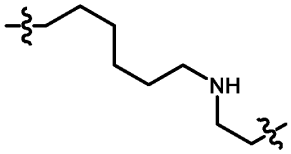
[0285] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or -CH_3. In some embodiments, R^5 is -CH_3.

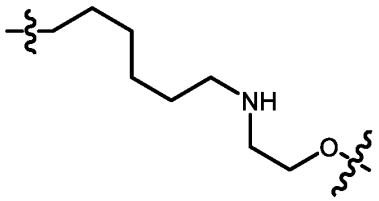
[0286] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), n is 2 or 3. In some embodiments, n is 3.

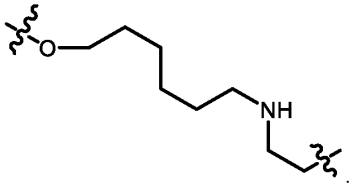
[0287] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIg), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

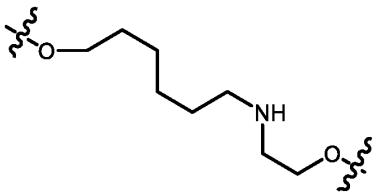
[0288] In some embodiments of the conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIg), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

[0289] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -$N(CH_3)$-, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -$N(CH_3)$-. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

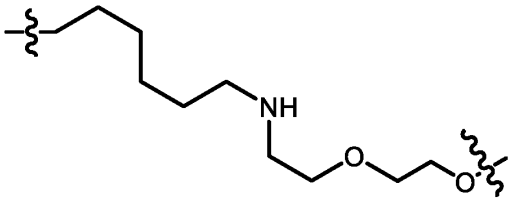
[0290] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), L^A comprises the moiety 

. In some embodiments, L^A comprises the moiety 

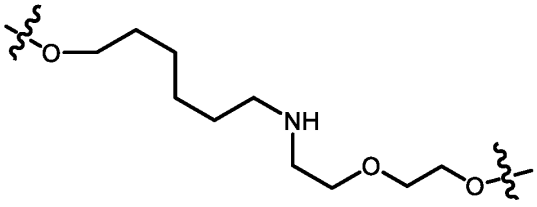
. In some embodiments, L^A comprises the moiety 

. In some embodiments, L^A comprises the moiety 

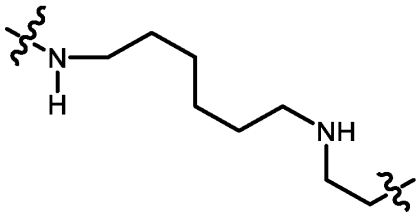
. In some embodiments, L^A comprises the moiety



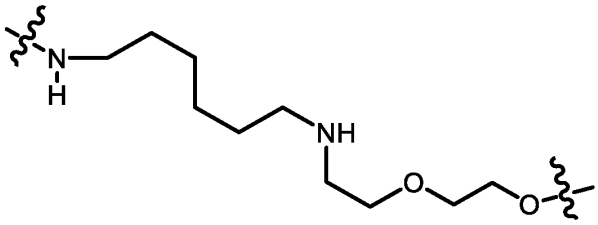
. In some embodiments, L^A comprises the



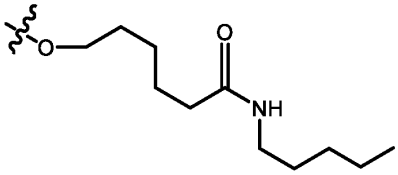
moiety . In some embodiments, L^A comprises



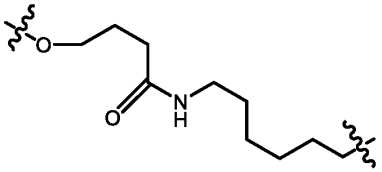
the moiety . In some embodiments, L^A comprises the



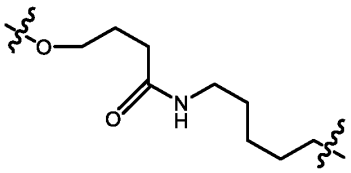
moiety . In some embodiments, L^A



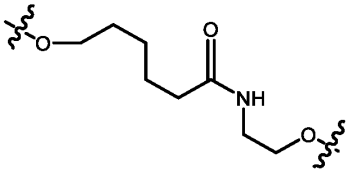
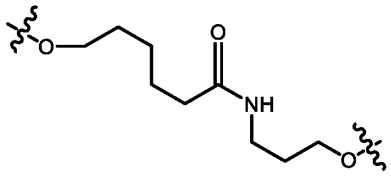
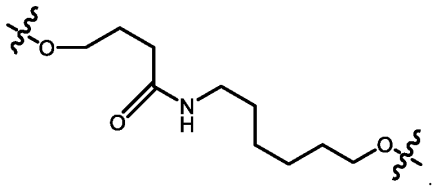
comprises the moiety . In some embodiments, L^A



comprises the moiety . In some embodiments, L^A



comprises the moiety . In some embodiments, L^A comprises

the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety .

[0291] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0292] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0293] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), D^1 is a peptide.

[0294] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone

(GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

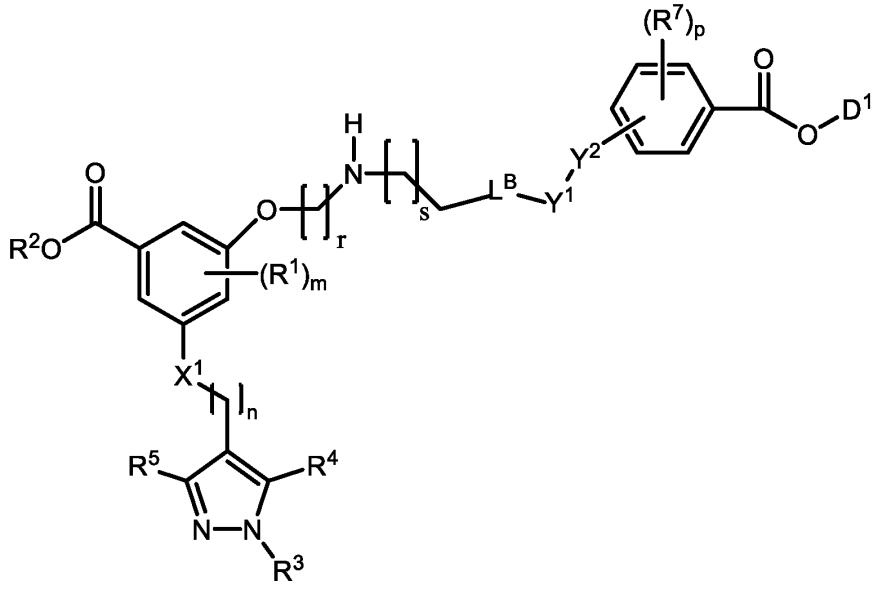
[0295] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0296] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0297] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0298] In some embodiments of a conjugate of formula (VI) or (VI'), or any variation thereof, such as formula (VIa)-(VIh), D^1 is a drug moiety. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphrine, or nalirphine.

[0299] In some embodiments, provided herein is a conjugate of formula (VII'):



(VII'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -

$CONHC_{1-4}$ alkyl, $-CON(C_{1-4}\text{ alkyl})_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

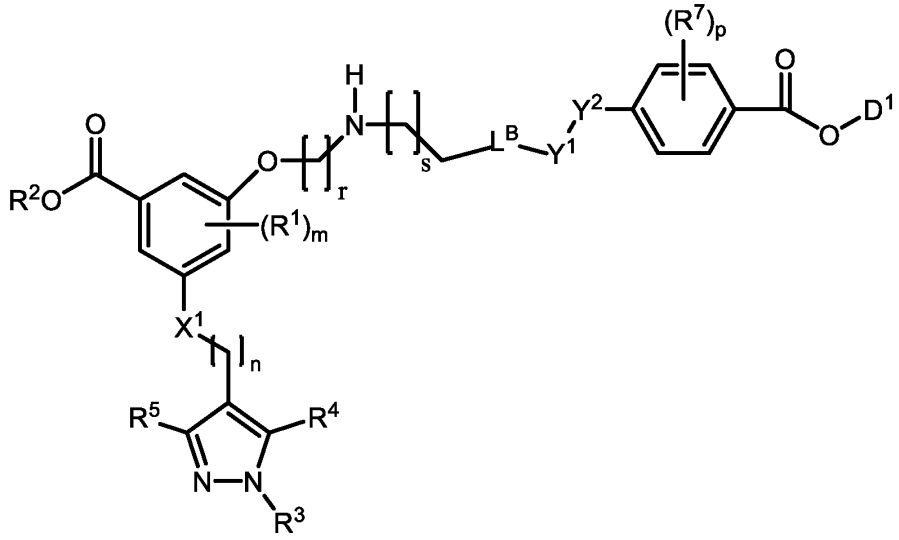
p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

s is 1, 2, 3, 4, 5, 6, or 7; and

D^1 is a drug moiety.

[0300] In some embodiments, provided herein is a conjugate of formula (VII):



(VII),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

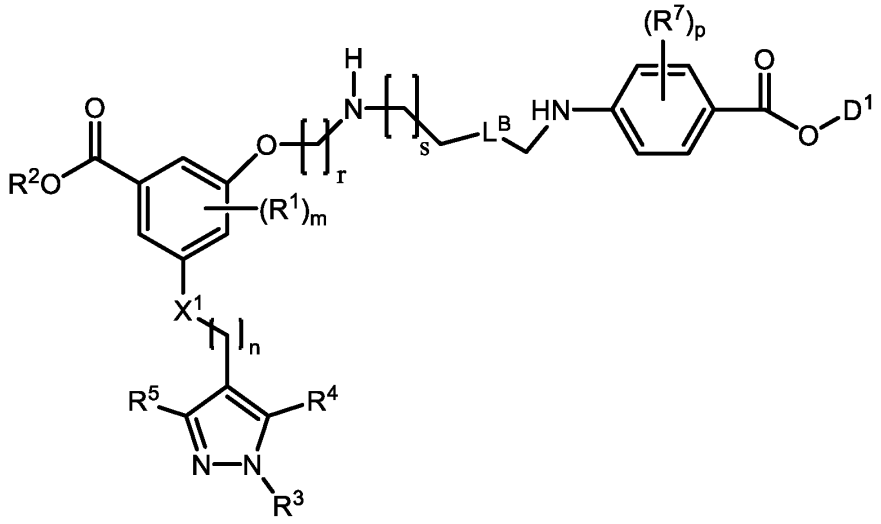
p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

s is 1, 2, 3, 4, 5, 6, or 7; and

D^1 is a drug moiety.

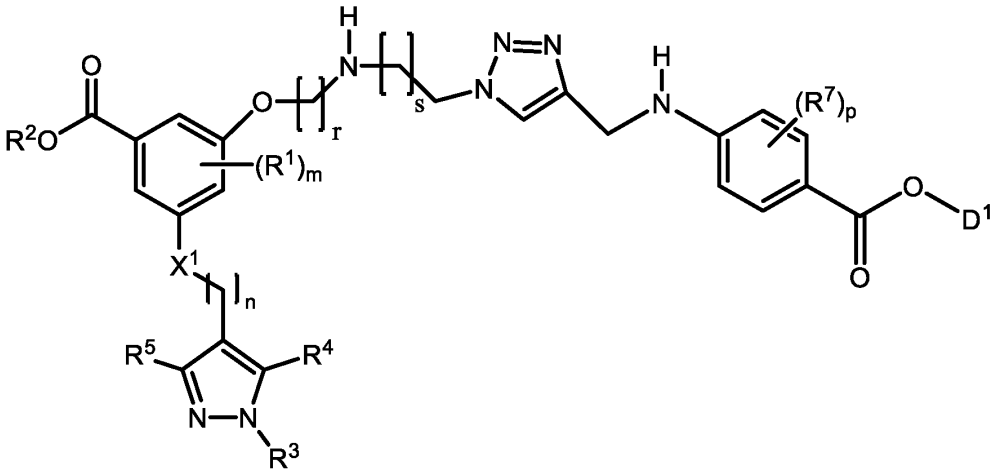
[0301] In some embodiments, the conjugate of formula (VII) has the formula (VIIa):



(VIIa)

or a pharmaceutically acceptable salt thereof.

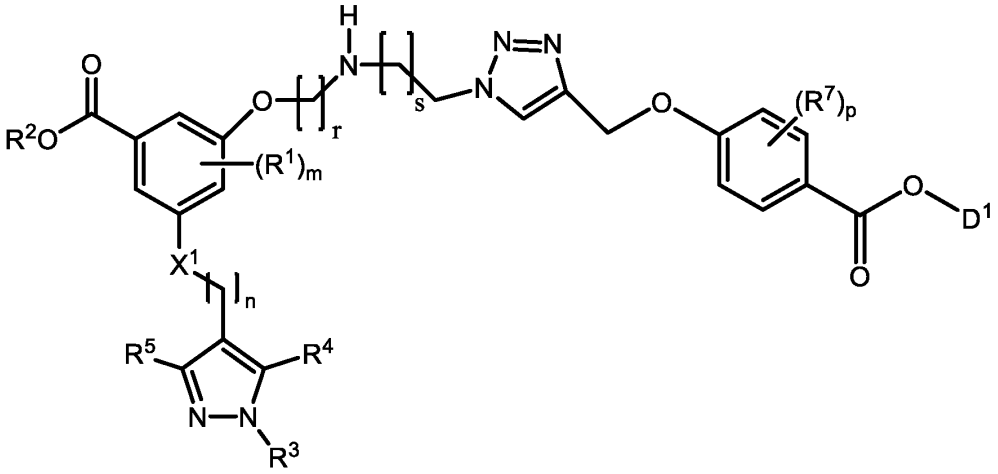
[0302] In some embodiments, the conjugate of formula (VII) has the formula (VIIb):



(VIIb)

or a pharmaceutically acceptable salt thereof.

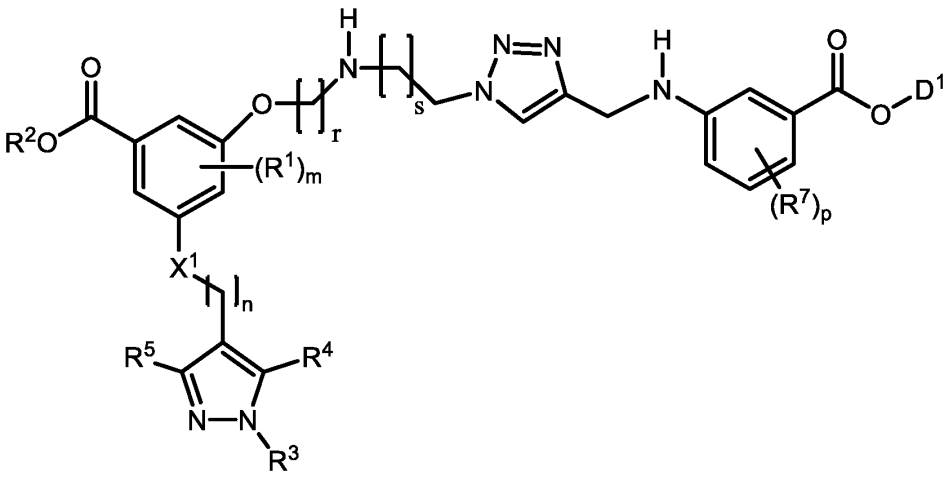
[0303] In some embodiments, the conjugate of formula (VII) has the formula (VIIc):



(VIIc)

or a pharmaceutically acceptable salt thereof.

[0304] In some embodiments, the conjugate of formula (VII') has the formula (VIId):



(VIId)

or a pharmaceutically acceptable salt thereof.

[0305] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0306] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0307] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

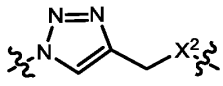
[0308] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or -CH_3. In some embodiments, R^5 is -CH_3.

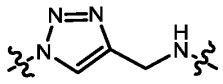
[0309] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), n is 2 or 3. In some embodiments, n is 3.

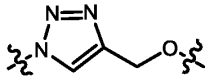
[0310] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

[0311] In some embodiments of the conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

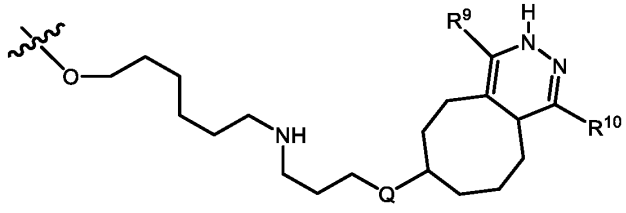
[0312] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), the click product L^B is a triazine moiety. In some embodiments, the conjugate of formula (VII) or (VII'), any of formula (VIIa)-(VIId), comprises one of the following moieties:

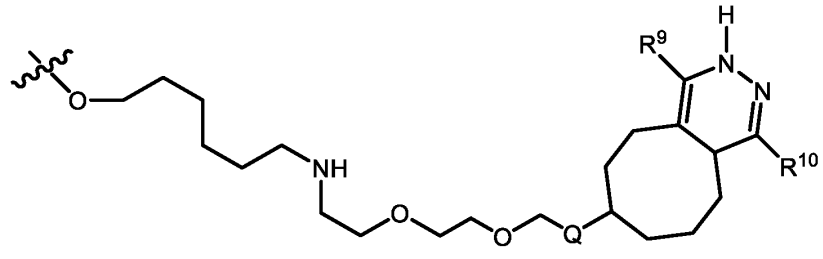

, wherein X^2 is $-CH_2-$, $-NR^x-$, -S- or -O-, wherein R^x is as defined elsewhere herein. In some embodiments, the conjugate comprises the moiety


. In some embodiments, the conjugate comprises the moiety


.

[0313] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), L^A-L^B comprises the moiety


or


, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is $-CH_2-$, -O-, -S-, -NH-, or $-N(C_{1-4}alkyl)-$.

[0314] In some embodiments of a conjugate of formula (VII) or (VII'), In some embodiments of a conjugate of formula (VII), or any variation thereof, such as formula (VIIa)-(VIId), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some

embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0315] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0316] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), D^1 is a peptide.

[0317] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0318] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

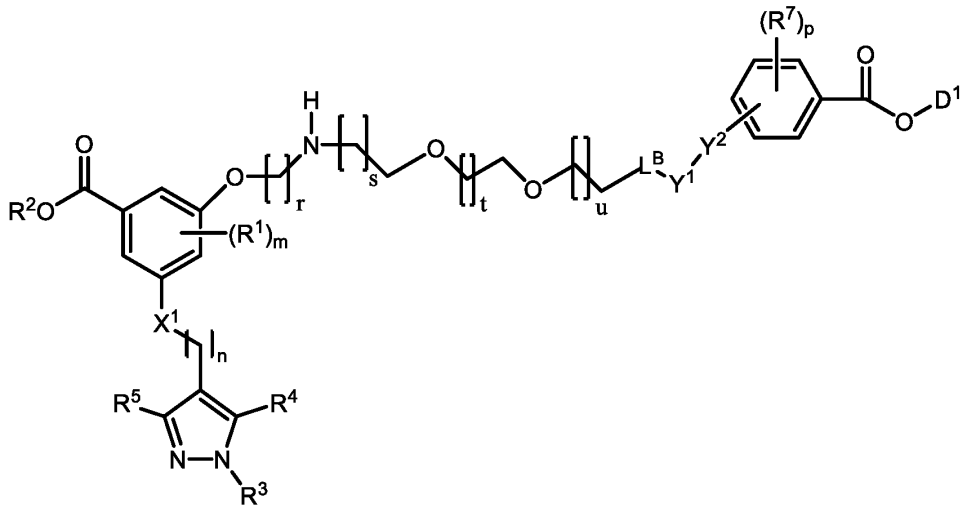
[0319] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0320] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0321] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0322] In some embodiments of a conjugate of formula (VII) or (VII'), or any variation thereof, such as formula (VIIa)-(VIId), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0323] In some embodiments, provided herein is a conjugate of formula (VIII'):



(VIII')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

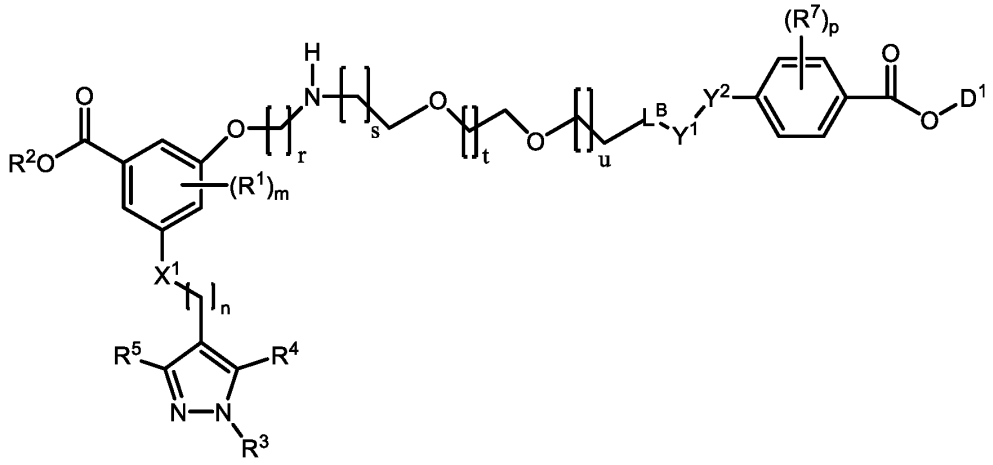
s is 1, 2, 3, 4, 5, 6, or 7;

t is 1, 2, 3, or 4;

u is 1, 2, 3, or 4; and

D^1 is a drug moiety.

[0324] In some embodiments, provided herein is a conjugate of formula (VIII):



(VIII)

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

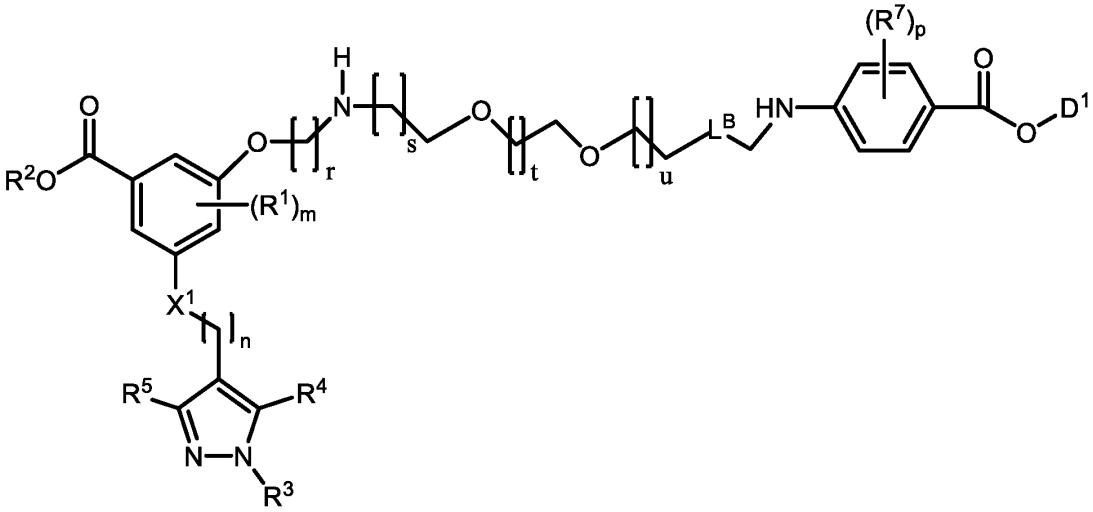
s is 1, 2, 3, 4, 5, 6, or 7;

t is 1, 2, 3, or 4;

u is 1, 2, 3, or 4; and

D^1 is a drug moiety.

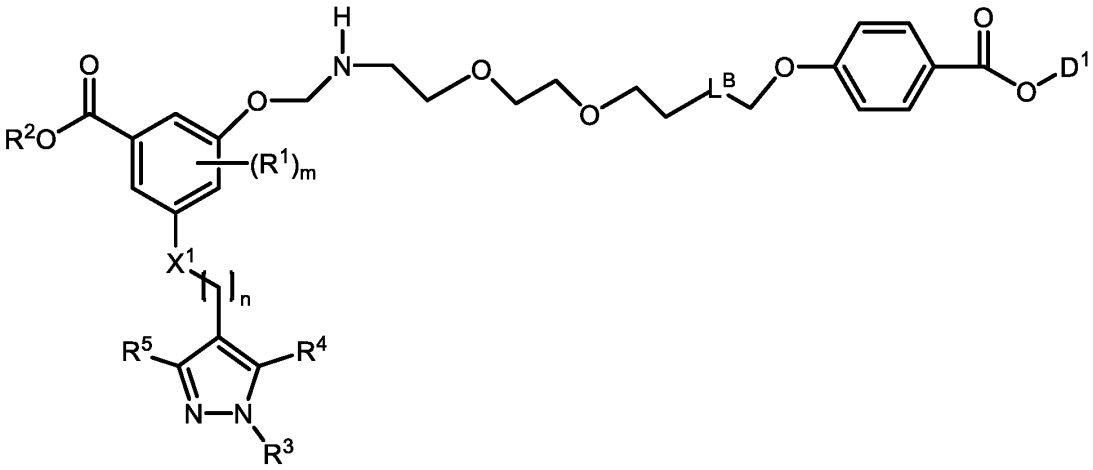
[0325] In some embodiments, the conjugate of formula (VIII) has the formula (VIIIa):



(VIIIa)

or a pharmaceutically acceptable salt thereof.

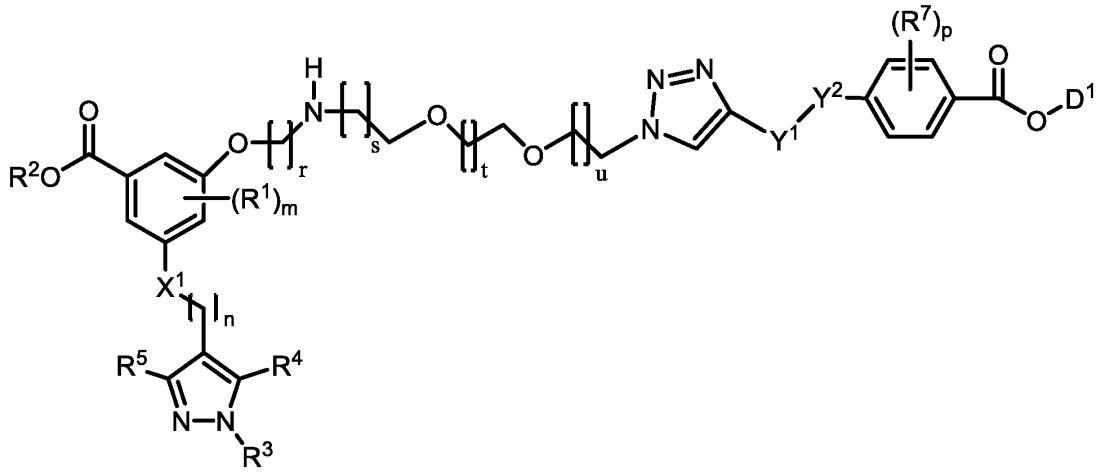
[0326] In some embodiments, the conjugate of formula (VIII) has the formula (VIIIb):



(VIIIb)

or a pharmaceutically acceptable salt thereof.

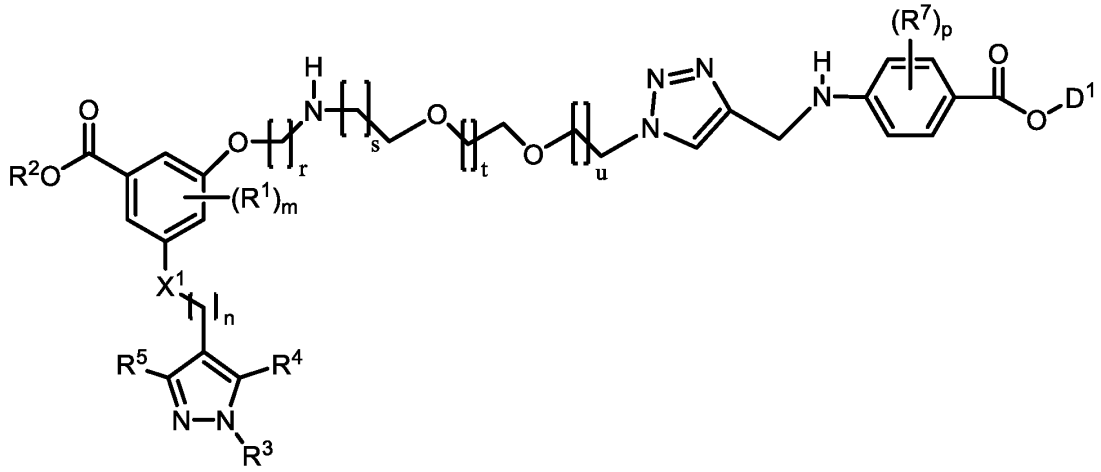
[0327] In some embodiments, the conjugate of formula (VIII) has the formula (VIIIc):



(VIIIc)

or a pharmaceutically acceptable salt thereof.

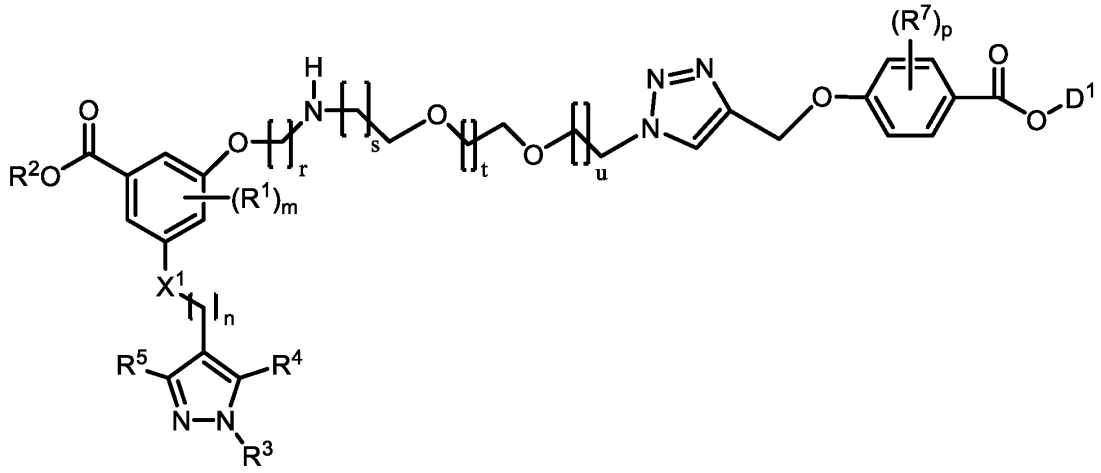
[0328] In some embodiments, the conjugate of formula (VIII) has the formula (VIIId):



(VIIId)

or a pharmaceutically acceptable salt thereof.

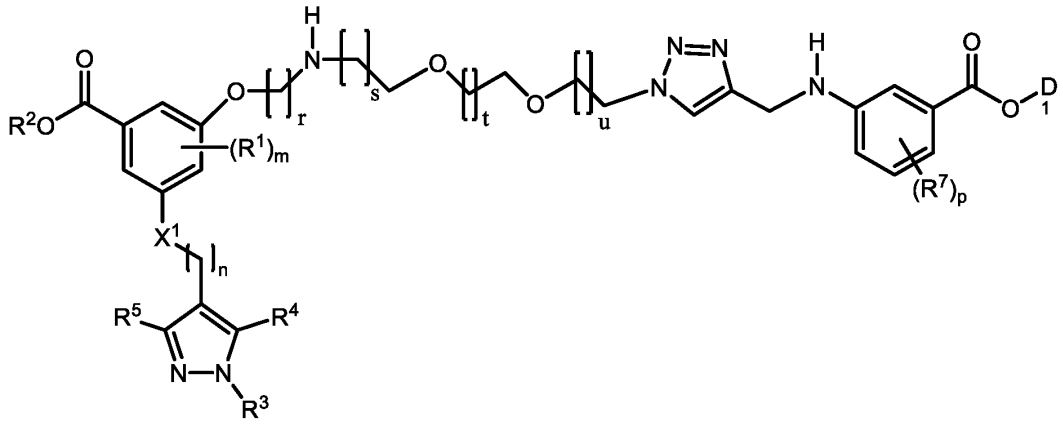
[0329] In some embodiments, the conjugate of formula (VIII) has the formula (VIIIe):



(VIIIe)

or a pharmaceutically acceptable salt thereof.

[0330] In some embodiments, the conjugate of formula (VIII') has the formula (VIIIf):



(VIIIf)

or a pharmaceutically acceptable salt thereof.

[0331] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0332] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

[0333] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or $-CH_3$. In some embodiments, R^4 is $-CH_3$.

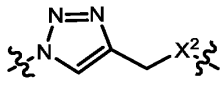
[0334] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or $-CH_3$. In some embodiments, R^5 is $-CH_3$.

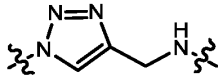
[0335] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), n is 2 or 3. In some embodiments, n is 3.

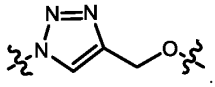
[0336] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

[0337] In some embodiments of the conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa), or (VIIIc)-(VIIIf), p is 0, 1, or 2. In some embodiments, p is 0 or 1. In some embodiments, p is 0. In some embodiments, p is 1.

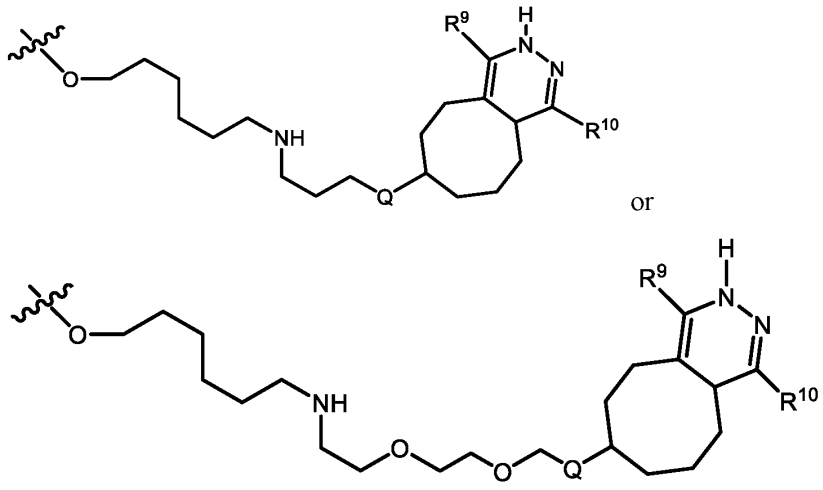
[0338] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIb), the click product L^B is a triazine moiety. In some embodiments, the conjugate of formula (VIII), any of formula (VIIIa)-(VIIIb), comprises one of the following moieties:


, wherein X^2 is $-CH_2-$, $-NR^x-$, -S- or -O-, wherein R^x is as defined elsewhere herein. In some embodiments, the conjugate comprises the moiety


. In some embodiments, the conjugate comprises the moiety


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[0339] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIb), L^A-L^B comprises the moiety



, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is $-CH_2-$, -O-, -S-, -NH-, or $-N(C_{1-4}alkyl)-$.

[0340] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0341] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0342] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), D^1 is a peptide.

[0343] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some

embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0344] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

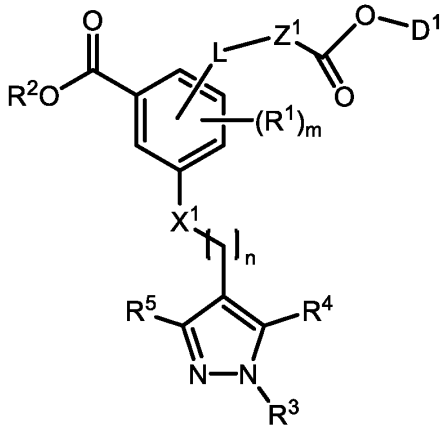
[0345] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0346] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0347] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0348] In some embodiments of a conjugate of formula (VIII) or (VIII'), or any variation thereof, such as formula (VIIIa)-(VIIIf), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0349] In some embodiments, provided herein is a conjugate of formula (IX):



(IX),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety.

[0350] In some embodiments of the conjugate of formula (IX), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or $-CH_3$. In some embodiments, R^2 is H.

[0351] In some embodiments of the conjugate of formula (IX), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or $-CH_3$. In some embodiments, R^3 is H.

[0352] In some embodiments of the conjugate of formula (IX), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or $-CH_3$. In some embodiments, R^4 is $-CH_3$.

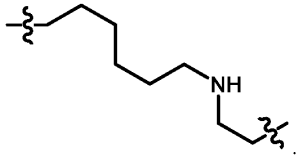
[0353] In some embodiments of the conjugate of formula (IX), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or $-CH_3$. In some embodiments, R^5 is $-CH_3$.

[0354] In some embodiments of the conjugate of formula (IX), n is 2 or 3. In some embodiments, n is 3.

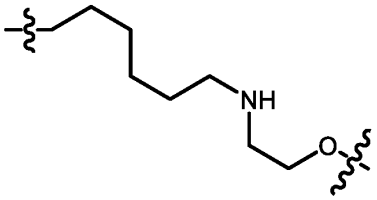
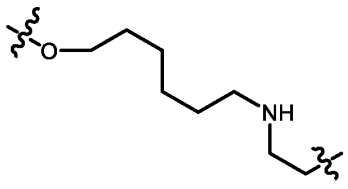
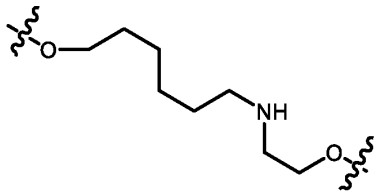
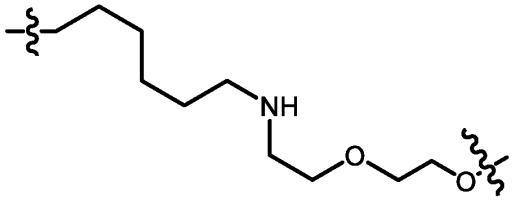
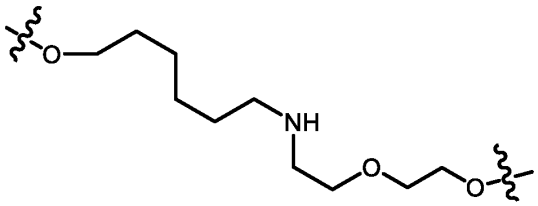
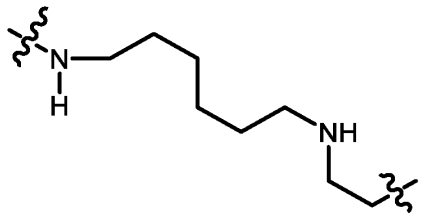
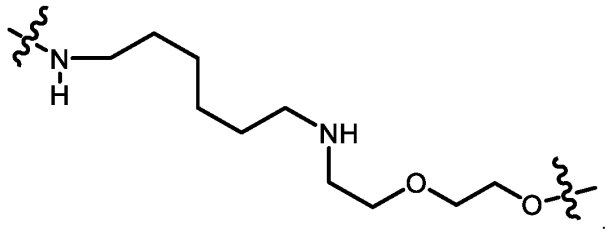
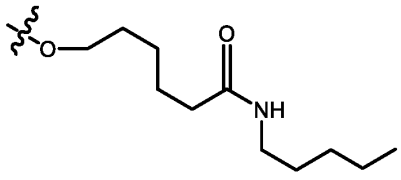
[0355] In some embodiments of the conjugate of formula (IX), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

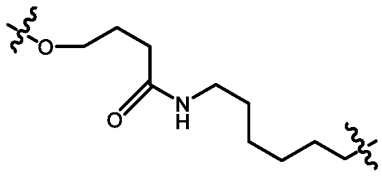
[0356] In some embodiments of the conjugate of formula (IX), L is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, $-NR^x$-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo. In some embodiments, L is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, $-N(CH_3)$-, and -S-. In some embodiments, L is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and $-N(CH_3)$-. In some embodiments, L is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

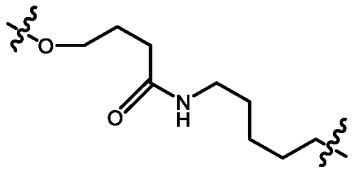
[0357] In some embodiments of the conjugate of formula (IX), L comprises the moiety

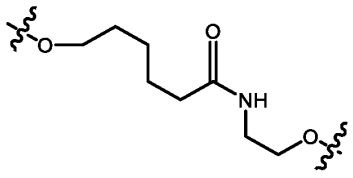


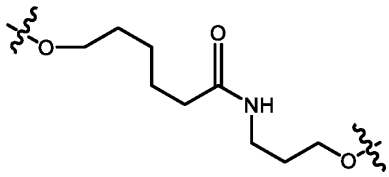
. In some embodiments, L comprises the moiety

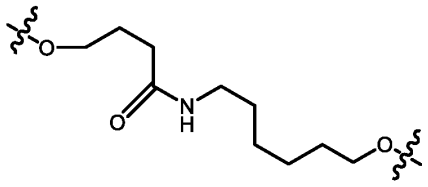


. In some embodiments, L comprises the moiety

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[0358] In some embodiments of a conjugate of formula (IX), or any variation thereof, D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0359] In some embodiments of a conjugate of formula (IX), or any variation thereof, D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such

embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0360] In some embodiments of a conjugate of formula (IX), or any variation thereof, D^1 is a peptide.

[0361] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0362] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

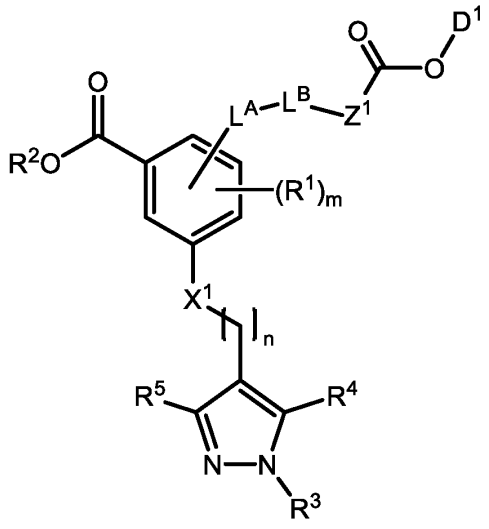
[0363] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0364] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0365] In some embodiments of a conjugate of formula (IX), or any variation thereof, D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0366] In some embodiments of a conjugate of formula (IX), or any variation thereof, D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0367] In some embodiments, provided herein is a conjugate of formula (X):



(X),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety.

[0368] In some embodiments of the conjugate of formula (X), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or -CH_3. In some embodiments, R^2 is H.

[0369] In some embodiments of the conjugate of formula (X), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or -CH_3. In some embodiments, R^3 is H.

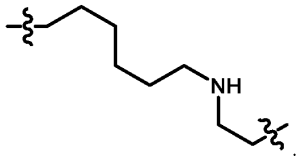
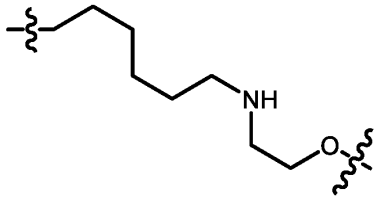
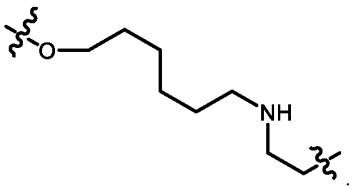
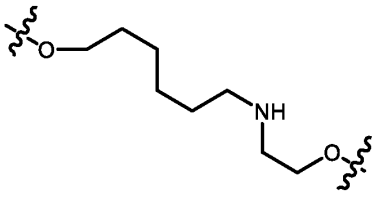
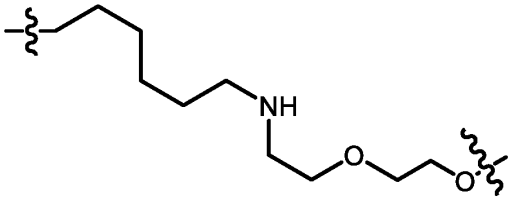
[0370] In some embodiments of the conjugate of formula (X), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or -CH_3. In some embodiments, R^4 is -CH_3.

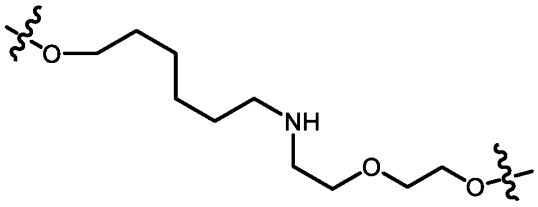
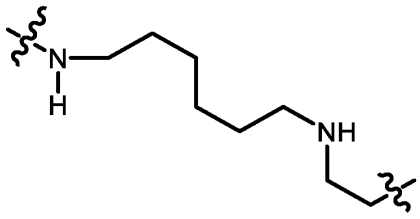
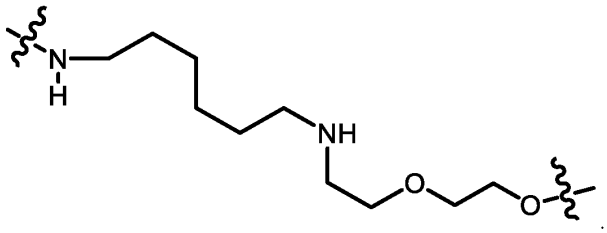
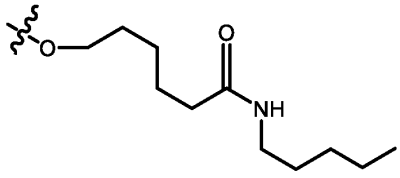
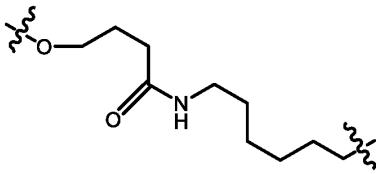
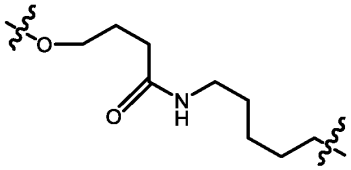
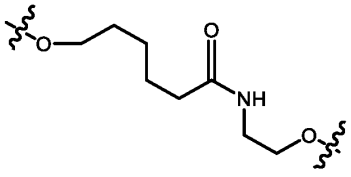
[0371] In some embodiments of the conjugate of formula (X), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or -CH_3. In some embodiments, R^5 is -CH_3.

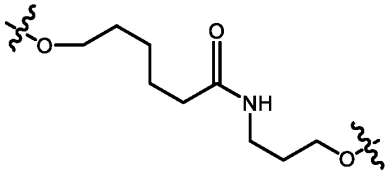
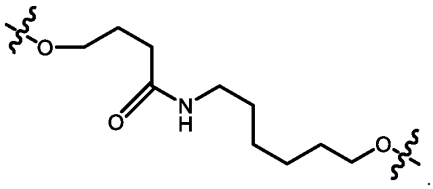
[0372] In some embodiments of the conjugate of formula (X), n is 2 or 3. In some embodiments, n is 3.

[0373] In some embodiments of the conjugate of formula (X), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

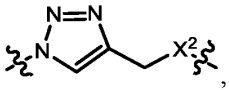
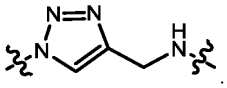
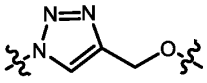
[0374] In some embodiments of a conjugate of formula (X), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NR^x-, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, -N(CH_3)-, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and -N(CH_3)-. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

[0375] In some embodiments of a conjugate of formula (X), L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments,

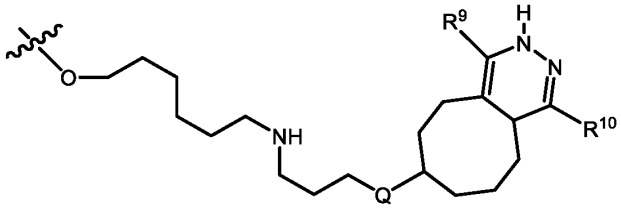
L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety .

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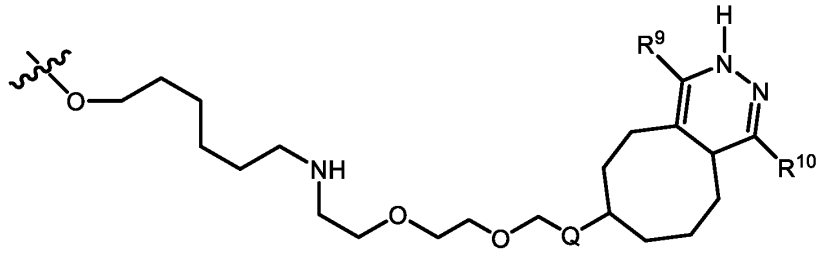
[0376] In some embodiments of a conjugate of formula (X), the click product L^B is a triazine moiety. In some embodiments, the conjugate of formula (X), comprises one of the following moieties:

, wherein X^2 is $-CH_2-$, $-NR^x-$, -S- or -O-, wherein R^x is as defined elsewhere herein. In some embodiments, the conjugate comprises the moiety . In some embodiments, the conjugate comprises the moiety .

[0377] In some embodiments of a conjugate of formula (X), L^A-L^B comprises the moiety



or

, wherein R^9 is H or C_{1-4} alkyl, R^{10} is H or C_{1-4} alkyl, and Q is $-CH_2-$, -O-, -S-, -NH-, or $-N(C_{1-4}alkyl)-$.

[0378] In some embodiments of a conjugate of formula (X), or any variation thereof, D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0379] In some embodiments of a conjugate of formula (X), or any variation thereof, D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0380] In some embodiments of a conjugate of formula (X), or any variation thereof, D^1 is a peptide.

[0381] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0382] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

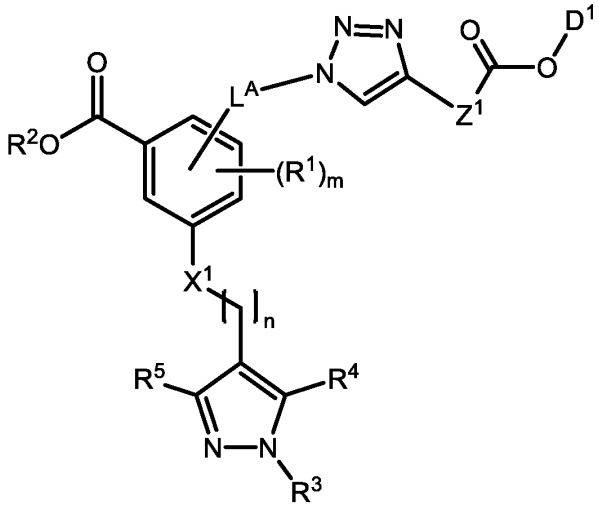
[0383] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0384] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0385] In some embodiments of a conjugate of formula (X), or any variation thereof, D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0386] In some embodiments of a conjugate of formula (X), or any variation thereof, D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0387] In some embodiments, provided herein is a conjugate of formula (XI):



(XI),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety.

[0388] In some embodiments of the conjugate of formula (XI), R^2 is H or C_{1-2} alkyl. In some embodiments, R^2 is H or $-CH_3$. In some embodiments, R^2 is H.

[0389] In some embodiments of the conjugate of formula (XI), R^3 is H or C_{1-2} alkyl. In some embodiments, R^3 is H or $-CH_3$. In some embodiments, R^3 is H.

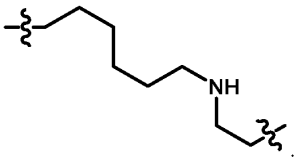
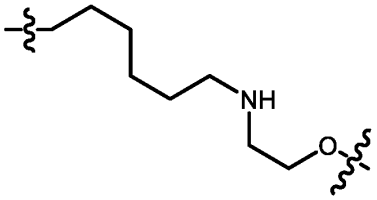
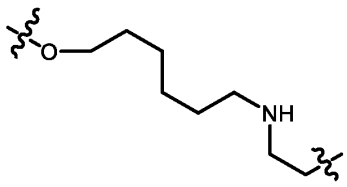
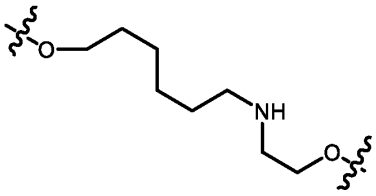
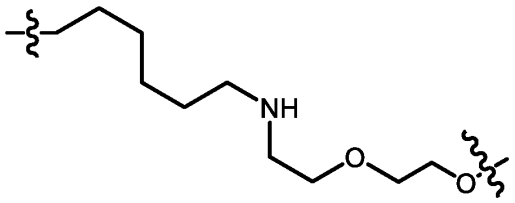
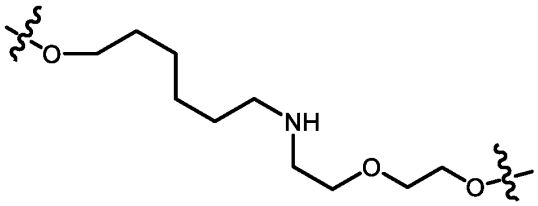
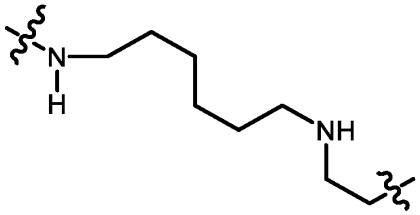
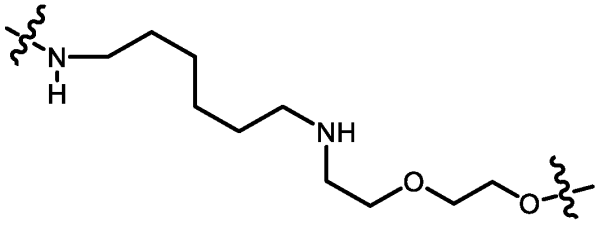
[0390] In some embodiments of the conjugate of formula (XI), R^4 is H or C_{1-2} alkyl. In some embodiments, R^4 is H or $-CH_3$. In some embodiments, R^4 is $-CH_3$.

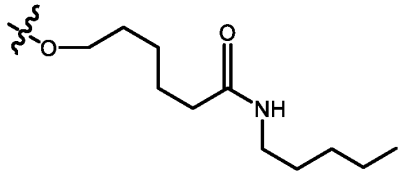
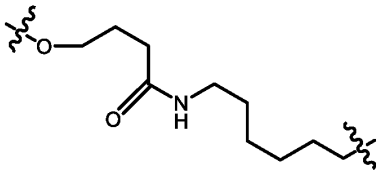
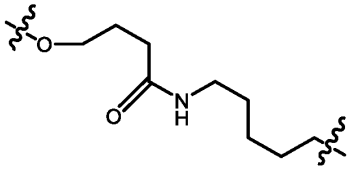
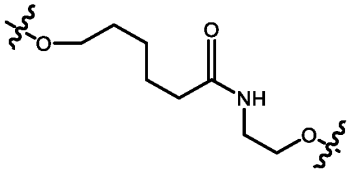
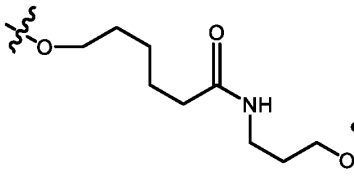
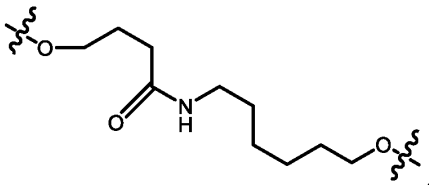
[0391] In some embodiments of the conjugate of formula (XI), R^5 is H or C_{1-2} alkyl. In some embodiments, R^5 is H or $-CH_3$. In some embodiments, R^5 is $-CH_3$.

[0392] In some embodiments of the conjugate of formula (XI), n is 2 or 3. In some embodiments, n is 3.

[0393] In some embodiments of the conjugate of formula (XI), m is 0, 1, or 2. In some embodiments, m is 0 or 1. In some embodiments, m is 0. In some embodiments, m is 1.

[0394] In some embodiments of a conjugate of formula (XI), L^A is C_{4-20}alkylene, wherein the C_{4-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, $-NR^x-$, and -S-, wherein L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo, and wherein R^x is H or C_{1-6}alkyl optionally substituted by halo. In some embodiments, L^A is C_{10-20}alkylene, wherein the C_{10-20}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, $-N(CH_3)-$, and -S-. In some embodiments, L^A is C_{10-15}alkylene, wherein the C_{10-15}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, -NH-, and $-N(CH_3)-$. In some embodiments, L^A is C_{5-10}alkylene, wherein the C_{5-10}alkylene is optionally substituted with one or more L^{1a}, and one or more alkylene units is optionally replaced with a group selected from -O-, and -NH-.

[0395] In some embodiments of a conjugate of formula (XI), L^A comprises the moiety . In some embodiments, L^A comprises the moiety  . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety .

In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety . In some embodiments, L^A comprises the moiety .

[0396] In some embodiments of a conjugate of formula (XI), or any variation thereof, D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is

Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0397] In some embodiments of a conjugate of formula (XI), or any variation thereof, D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0398] In some embodiments of a conjugate of formula (XI), or any variation thereof, D^1 is a peptide.

[0399] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0400] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0401] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or

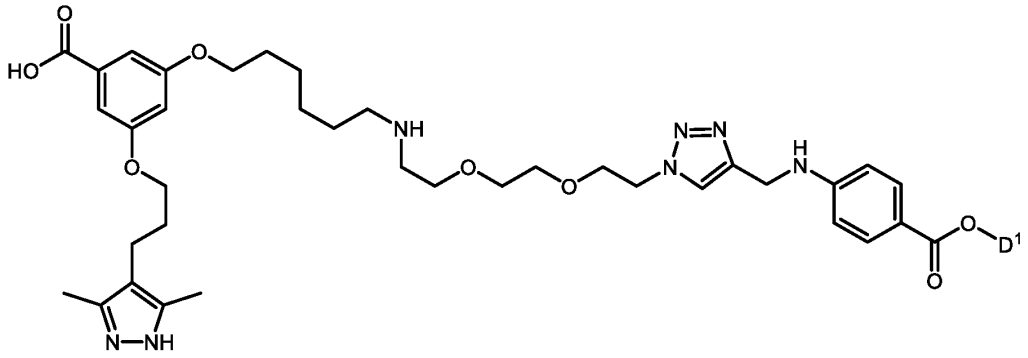
diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0402] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

[0403] In some embodiments of a conjugate of formula (XI), or any variation thereof, D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

[0404] In some embodiments of a conjugate of formula (XI), or any variation thereof, D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

[0405] In some embodiments, provided herein is a conjugate with a structure as shown:



(XII) ,

wherein D^1 is a drug moiety.

[0406] In some embodiments of a conjugate of formula (XII), D^1 is a protein. In some embodiments, D^1 is a cytokine. In some embodiments, the cytokine is Interleukin-2 (IL-2). In some embodiments, the cytokine is mutated IL-2, such as a cytokine described in US 9,206,943. In some embodiments, the cytokine is Interleukin-15 (IL-15). In some embodiments, the cytokine is mutated IL-15. In one such embodiment, the IL-15 is IL-15[N27D]. In some embodiments, the cytokine is Interleukin-7 (IL-7). In some embodiments, the cytokine is Interleukin-9 (IL-9). In some embodiments, the cytokine is

Interleukin-10 (IL-10). In some embodiments, the cytokine is Interleukin-21 (IL-21). In some embodiments, the cytokine is Interleukin-10 (IL-10).

[0407] In some embodiments of a conjugate of formula (XII), D^1 is an anti-cancer agent. In some such embodiments, D^1 is SN-38. In some such embodiments, D^1 is monomethyl auristatin E. In some such embodiments, D^1 is capecitabine. In some such embodiments, D^1 is irinotecan. In some such embodiments, D^1 is trifluridine. In some such embodiments, D^1 is doxorubicin. In some such embodiments, D^1 is docetaxel. In some such embodiments, D^1 is infigratinib.

[0408] In some embodiments of a conjugate of formula (XII), D^1 is a peptide.

[0409] In some embodiments, the peptide is RGK-MCA. In some embodiments, the peptide is Neurotensin. In some embodiments, the peptide is Gonadotropin-releasing hormone (GnRH). In some embodiments, the peptide is GIP (1-42), human peptide. In some embodiments, the peptide is D6-GnRH. In some embodiments, the peptide is insulin. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is calcitronin salmon. In some embodiments, the peptide is ipamorelin. In some embodiments, the peptide is vasopressin. In some embodiments, the peptide is glucagon. In some embodiments, the peptide is aclerastide. In some embodiments, the peptide is goserelin. In some embodiments, the peptide is leuprolide. In some embodiments, the peptide is teriparatide. In some embodiments, the peptide is lixisenatide. In some embodiments, the peptide is desmopressin. In some embodiments, the peptide is oxytocin. In some embodiments, the peptide is corticotropin. In some embodiments, the peptide is sermorelin. In some embodiments, the peptide is bremelanotide. In some embodiments, the peptide is abaloparatide. In some embodiments, the peptide is deslorelin.

[0410] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) receptor agonist used to trat obesity and/or diabetes. In some such embodiments the peptide drug is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide. Owing to the dramatic extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1 agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

[0411] In some embodiments, the peptide drug is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide receptor agonist used to trat obesity and/or diabetes. In some such embodiments, the peptide drug is tirzepatide. Owing to the dramatic

extension of duration of action of peptide drugs when incorporated into the conjugates of the disclosure, the GLP-1/GIP agonist conjugates can be administered on a reduced dosing schedule, such as once weekly, once biweekly, once every three weeks, or once monthly.

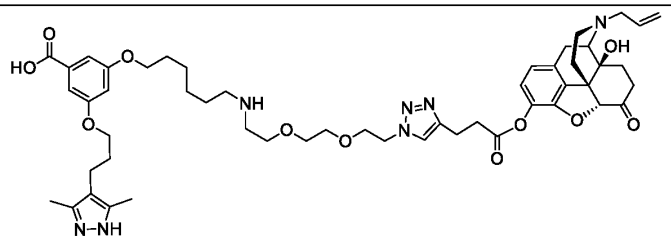
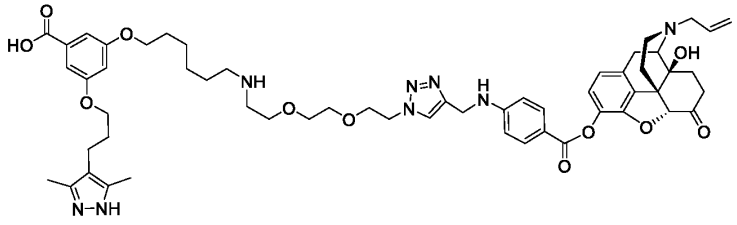
[0412] In some embodiments, the peptide is an endogenous opioid peptide. In some such embodiments, the peptide is endomorphin-1 or endomorphin-2. In other embodiments, the peptide is dermorphin.

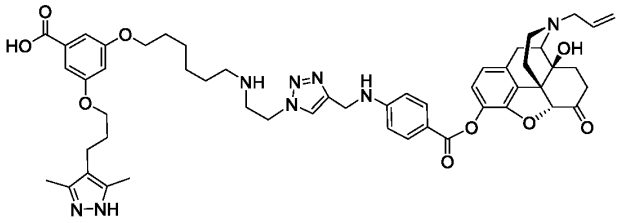
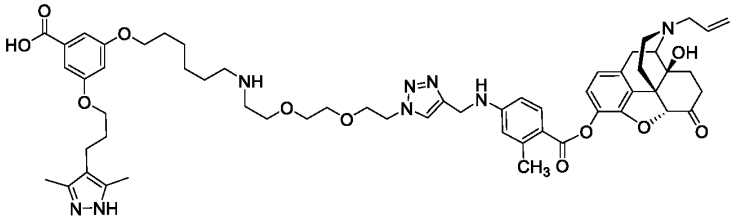
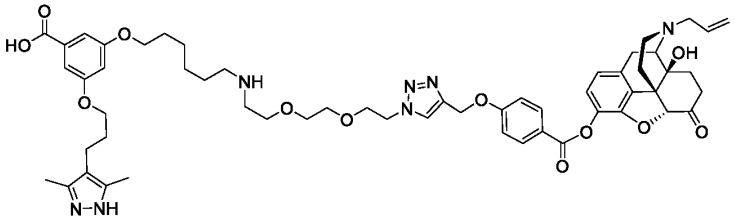
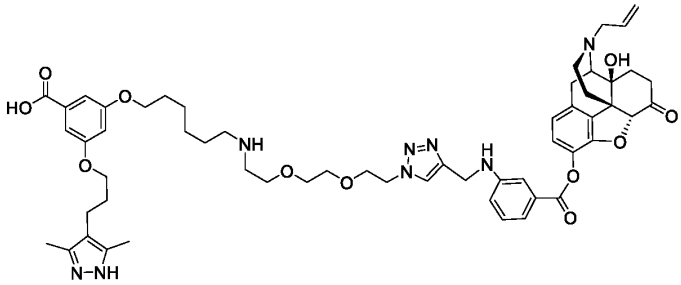
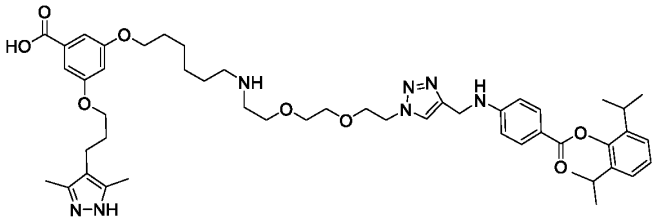
[0413] In some embodiments of a conjugate of formula (XII), D^1 is an opioid antagonist. In some embodiments, the opioid antagonist is naloxone, nalmefene, naltrexone, methylnaltrexone, nalbuphine, or nalorphine.

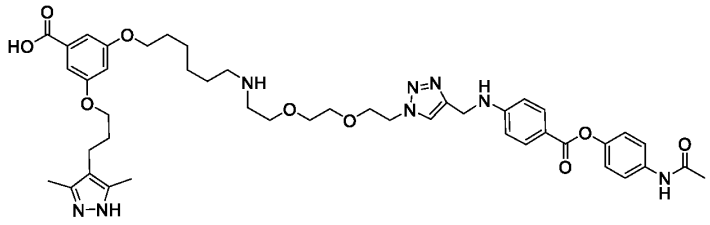
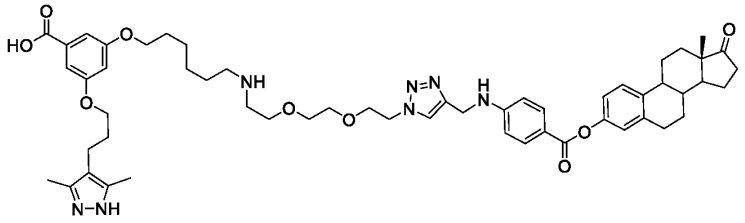
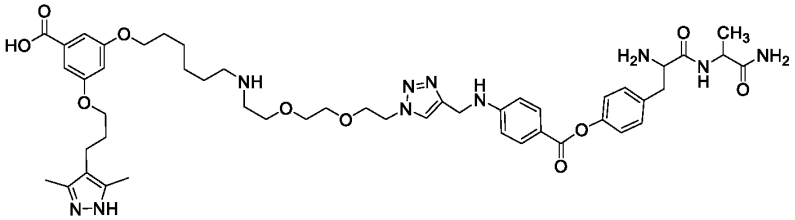
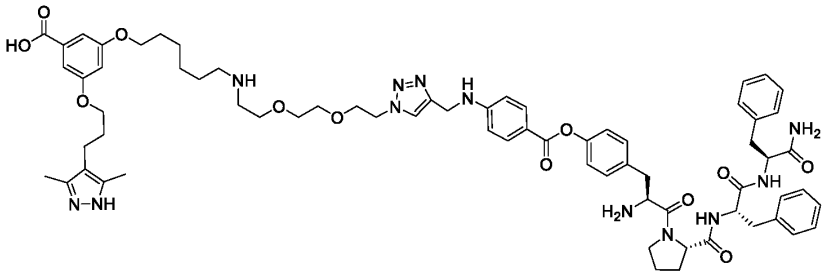
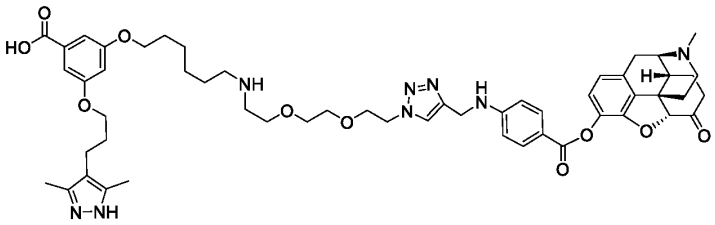
[0414] In some embodiments of a conjugate of formula (XII), D^1 is an opioid agonist. In some embodiments, the opioid agonist is morphine, oxycodone, hydromorphone, or methadone.

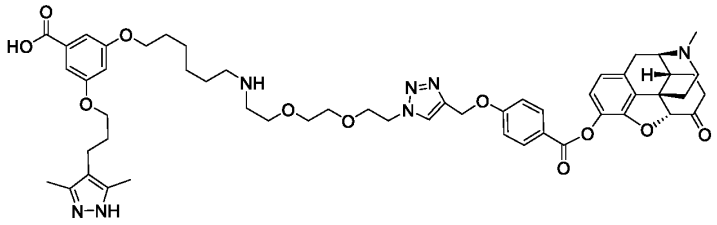
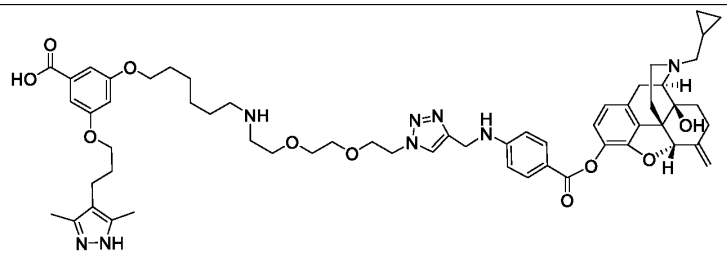
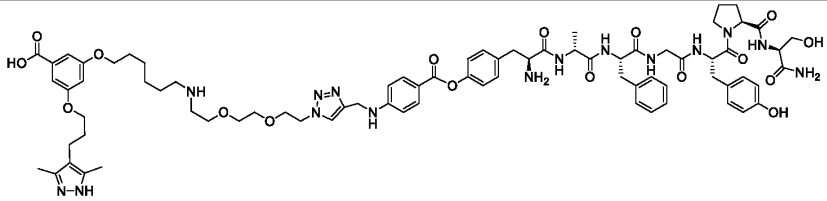
[0415] In some embodiments, the conjugate is selected from the conjugates of Table A, or a salt thereof. In some embodiments, the conjugate is selected from the conjugates of Table A.

Table A.

Conj.	Structure
AG10-L-E1-Naloxone	
AG10-L-E2-Naloxone	

Conj.	Structure
AG10-Short-L-E2-Naloxone	
AG10-L-E3-Naloxone	
AG10-L-PHBA-E-Naloxone	
AG10-L-meta-amino-E-Naloxone	
AG10-L-E2-Propofol	

Conj.	Structure
AG10-L-E2-Paracetamol	
AG10-L-E2-Estrone	
AG10-L-E2-Tyr-Ala	
AG10-L-E2-Endomorphin-2	
AG10-L-E2-Hydromorphone	

Conj.	Structure
AG10-L-PHBA-E-Hydromorphone	
AG10-L-E2-Nalmefene	
AG10-L-E2-Dermorphin	

PHARMACEUTICAL COMPOSITIONS

[0416] Provided herein are pharmaceutical compositions comprising one or more conjugates of the disclosure (e.g., conjugate of formula (I)-(XII) or (II')-(VIII'), or any variation or embodiment thereof, as described elsewhere herein, or a pharmaceutically acceptable salt of any of the foregoing. In some embodiments, provided herein is a pharmaceutical composition comprising (i) a compound of any one of formula (I)-formula (XII) or (II')-(VIII'), or any variation or embodiment thereof, as described elsewhere herein, or a pharmaceutically acceptable salt of any of the foregoing, and (ii) one or more pharmaceutically acceptable excipients.

[0417] Suitable pharmaceutically acceptable excipients may include, for example, fillers, diluents, sterile aqueous solutions and various organic solvents, permeation enhancers, solubilizers, and adjuvants. Various substances may be embraced by the term excipient, including without limitation any substance used as a binder, disintegrant, coating, compression/encapsulation aid, cream or lotion, lubricant, solutions for parenteral

administration, materials for chewable tablets, sweetener or flavoring, suspending/gelling agent, or wet granulation agent. Examples of suitable excipients are well-known to those skilled in the art. See, *e.g.*, *Handbook of Pharmaceutical Excipients*, Pharmaceutical Press (2017), which is incorporated herein by reference in its entirety. Such compositions are prepared in a manner well known in the pharmaceutical art. *See*, *e.g.*, *Remington's Pharmaceutical Sciences*, Academic Press, 23rd ed. (2020), which is incorporated herein by reference in its entirety.

METHODS OF TREATMENT

[0418] In one aspect, provided herein are methods of increasing the plasma circulation time of an active agent, comprising covalently attaching an active agent to a binder of a plasma protein to form a conjugate of the disclosure (e.g., a conjugate of Formula (I)-(XII) or (II')-(VIII'), or any variation thereof).

[0419] In one aspect, provided herein are methods of increasing the *in vivo* half-life of an active agent, comprising covalently attaching an active agent to a binder of a plasma protein to form a conjugate of the disclosure (e.g., a conjugate of Formula (I)-(XII) or (II')-(VIII'), or any variation thereof).

[0420] In one aspect, provided herein are methods of increasing the duration of action (DOA) of an active agent, comprising covalently attaching an active agent to a binder of a plasma protein to form a conjugate of the disclosure.

[0421] In one aspect, provided herein are methods reducing the immunogenicity of an active agent in a subject, the methods comprising obtaining a delivery system having a ligand with (i) a high selectivity for a plasma protein endogenous to the subject, the molecular weight of the plasma protein ranging from about 30 kDa to about 80 kDa; (ii) a high binding affinity, Kd, of at least 10−6 M for the plasma protein; and, (iii) a molecular weight ranging from about 200 Da to about 2000 Da; and, a linker that ranges in length from about 10 angstroms to about 50 angstroms, or from 10 atoms to 50 atoms. The method also includes covalently attaching the delivery system to an active agent to create a conjugated active agent; and, administering the conjugated active agent to the subject; wherein, the plasma protein shields the active agent from antibody generation in the subject after the administering.

[0422] In one aspect, provided herein are methods of treating a disease, disorder, or condition comprising administering to an individual in need thereof a conjugate of the disclosure (e.g., a conjugate of any one of formula (I)-formula (XII) or (II')-(VIII'), or any variation or embodiment thereof, or a stereoisomer or tautomer thereof, or a pharmaceutically acceptable

salt of any of the foregoing, or a pharmaceutical composition comprising a conjugate of the disclosure, or any variation or embodiment thereof, or a stereoisomer or tautomer thereof, or a pharmaceutically acceptable salt of any of the foregoing, and one or more pharmaceutically acceptable excipients.

[0423] In some embodiments, a therapeutically effective amount of the compound is administered.

[0424] In some embodiments, the half-life of the activity of the active agent (opioid antagonist or opioid agonist) can range from about 30 minutes to about 90 days when administered to a subject with the delivery systems provided herein. In some embodiments, the half-life of the activity of the active agent (opioid antagonist or opioid agonist) can range from about 30 minutes to about 30 days when administered to a subject with the delivery systems provided herein. For example, the half-life of the activity of the active agent can range from about 30 minutes to about 2 days when administered to a subject with a delivery system taught herein having a ligand that is highly selective for transthyretin, in some embodiments. Likewise, the half-life of the activity of the active agent can range from about 30 minutes to about 2 days when administered to a subject with a delivery system taught herein having a ligand that is highly selective for human serum albumin, in some embodiments. As such, in some embodiments the half-life of the activity of the active agent can be about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 6 hours, about 8 hours, about 10 hours, about 12 hours, about 18 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 10 days, about 12 days, about 16 days, about 20 days, about 30 days, or any amount therein in increments of 1 hour, when administered to a subject with a delivery system taught herein. One of skill will appreciate that the half-life of the activity of an active agent that is administered to a subject using a delivery system taught herein can range from about 30 minutes to about 2 days, from about 1 hour to about 36 hours, from about 2 hours to about 20 hours, from about 2 hours to about 10 hours, from about 3 hours to about 30 hours, from about 3 hours to about 15 hours, from about 4 hours to about 40 hours, from about 4 hours to about 20 hours, from about 5 hours to about 50 hours, from about 5 hours to about 25 hours, or any range or any amount therein in increments of 1 hour.

[0425] In one aspect, provided herein are methods of reducing the risk of recurrent respiratory depression, comprising administering to an individual in need thereof a conjugate of the disclosure prior to exposure to a drug.

[0426] In one aspect, provided herein are methods of prophylaxis, comprising administering to an individual in need thereof a conjugate of the disclosure prior to exposure to a drug.

[0427] In some embodiments, a conjugate of the disclosure is administered parenterally to a subject in need thereof. For instance, a conjugate of the disclosure can be administered subcutaneously, intravenously, intramuscularly or intrathecally.

[0428] Conjugates of the disclosure, particularly conjugates of formula (II)-(XII) or (II')-(VIII'), show enhanced adsorption from tissue, particularly subcutaneous tissue, owing, in part, to the hydrophilicity of the TTR selective ligand. Following adsorption from tissue (e.g., subcutaneous tissue), reversible binding of the TTR selective ligand of the conjugate results in significantly extended duration of action of the active agent. As set forth herein, cleavage of the linker between the TTR selective ligand and the active agent (opioid antagonist or opioid agonist) results in release of the active agent, hence allowing the active agent to exert its pharmacological effect.

[0429] Moreover, it has been discovered that release of the active agent can be effectively controlled by proper selection of a cleavable moiety in the conjugate. For instance, particular ester moieties (e.g., PABA or PHBA), as described herein, hydrolyze slowly following administration, thereby releasing the active agent in a controlled manner for an extended period of time. The slower hydrolysis and the high degree of binding to TTR effectively extend the plasma circulation time of the active agent.

[0430] Therefore, the conjugates of the disclosure manifest several beneficial properties for a new prodrug approach. The balanced hydrophilicity of the conjugates allows for rapid adsorption into the blood from the tissue, the binding of TTR extends the half life, and the cleavable moiety allows for modulation of release of the drug. The desired rate of release of a particular active drug from a conjugate of the disclosure will vary from one active agent to another.

[0431] In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 12 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 20 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 24 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 30 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 40 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 48 hours. In some such embodiments, following

the administration of a conjugate of the disclosure, the DOA of the drug is at least 72 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 96 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 1 week. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 2 weeks. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 3 weeks. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is at least 1 month. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is from about 12 hours to about 48 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is from about 24 hours to about 48 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is from about 48 hours to about 96 hours. In some embodiments, following the administration of a conjugate of the disclosure, the DOA of the drug is from about 24 hours to about 1 week.

[0432] In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 12 hours. In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 20 hours. In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 24 hours. In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 30 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 40 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 48 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 72 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 96 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 1 week. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 2 weeks. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 3 weeks. In some such embodiments,

following the administration of a conjugate of the disclosure, the plasma circulation of the drug is at least 1 month. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is from about 12 hours to about 48 hours. In some such embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is from about 24 hours to about 48 hours. In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is from about 48 hours to about 96 hours. In some embodiments, following the administration of a conjugate of the disclosure, the plasma circulation of the drug is from about 24 hours to about 1 week.

[0433] In some embodiments, the active drug (D^1) delivered by a conjugate of the disclosure is a small molecule, a nucleotide, an oligonucleotide, a polynucleotide, an amino acid, an oligopeptide, a polypeptides, or a protein. Active drugs, include. can include, but are not limited to, antiproliferatives, antineoplastics, antimitotics, anti-inflammatories, antiplatelets, anticoagulants, antifibrins, antithrombins, antibiotics, antiallergics, antioxidants.

[0434] Antiproliferatives include, for example, actinomycin D, actinomycin IV, actinomycin I1, actinomycin X1, actinomycin C1, and dactinomycin (Cosmegen.RTM., Merck & Co., Inc.). Antineoplastics or antimitotics include, for example, paclitaxel (TAXOL, Bristol-Myers Squibb Co.), docetaxel (TAXOTERE, Aventis S.A.), methotrexate, irinotecan, SN-38, azathioprine, vincristine, vinblastine, fluorouracil, doxorubicin hydrochloride (ADRIAMYCIN, Pfizer, Inc.) and mitomycin (MUTAMYCIN, Bristol-Myers Squibb Co.), and any prodrugs, codrugs, metabolites, analogs, homologues, congeners, derivatives, salts and combinations thereof. Antiplatelets, anticoagulants, antifibrin, and antithrombins include, for example, sodium heparin, low molecular weight heparins, heparinoids, hirudin, argatroban, forskolin, vapiprost, prostacyclin and prostacyclin analogues, dextran, D-phe-pro-arg-chloromethylketone (synthetic antithrombin), dipyridamole, glycoprotein IIb/IIIa platelet membrane receptor antagonist antibody, recombinant hirudin, and thrombin inhibitors (ANGIOMAX, Biogen, Inc.), and any prodrugs, codrugs, metabolites, analogs, homologues, congeners, derivatives, salts and combinations thereof. Cytostatic or antiproliferative agents include, for example, angiopeptin, angiotensin converting enzyme inhibitors such as captopril (CAPOTEN and CAPOZIDE, Bristol-Myers Squibb Co.), cilazapril or lisinopril (PRINVIL and PRINZIDE, Merck & Co., Inc.); calcium channel blockers such as nifedipine; colchicines; fibroblast growth factor (FGF) antagonists, fish oil (omega 3-fatty acid); histamine antagonists; lovastatin (MEVACOR, Merck & Co., Inc.); monoclonal antibodies including, but not limited to, antibodies specific for Platelet-Derived Growth Factor (PDGF)

receptors; nitroprusside; phosphodiesterase inhibitors; prostaglandin inhibitors; suramin; serotonin blockers; steroids; thioprotease inhibitors; PDGF antagonists including, but not limited to, triazolopyrimidine; and nitric oxide, and any prodrugs, codrugs, metabolites, analogs, homologues, congeners, derivatives, salts and combinations thereof. Antiallergic agents include, but are not limited to, pemirolast potassium (ALAMAST, Santen, Inc.), and any prodrugs, codrugs, metabolites, analogs, homologues, congeners, derivatives, salts and combinations thereof.

[0435] In some embodiments, a conjugate of the disclosure can be administered in combination with a second drug. For instance, in some embodiments, a conjugate of the disclosure can be administer in combination with a second drug (D^2) that is in unconjugated form. The second drug (D^2) in unconjugated form can be the same of different than the drug (D^1) of the conjugate. In some embodiments, both D^1 and D^2 are anti-cancer drugs.

[0436] In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.005 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.01 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.005 mg/kg to about 10 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.1 mg/kg to about 10 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 0.5 mg/kg to about 10 mg/kg. In some embodiments, a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 5 mg/kg to about 40 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 10 mg/kg to about 30 mg/kg. In some embodiments. a conjugate of the disclosure is administered subcutaneously to a human subject at a dose of from about 15 mg/kg to about 25 mg/kg.

KITS

[0437] The present disclosure further provides kits for carrying out the methods disclosed herein. The kits may comprise a conjugate of the disclosure or pharmaceutically acceptable salt thereof as described herein and suitable packaging. The kits may comprise one or more containers comprising any compound described herein. In one aspect, a kit includes a compound of the disclosure or a pharmaceutically acceptable salt thereof, and a label and/or

instructions for use of the compound in the treatment of a disease or disorder described herein. The kits may comprise a unit dosage form of the compound.

[0438] Provided herein is a kit, comprising (i) a conjugate of the disclosure, or a stereoisomer or tautomer thereof, or a pharmaceutically acceptable salt of any of the foregoing, or a pharmaceutical composition comprising a compound of formula (I), or any variation or embodiment thereof, or a stereoisomer or tautomer thereof, or a pharmaceutically acceptable salt of any of the foregoing, and one or more pharmaceutically acceptable excipients and (ii) instructions for use in treating

[0439] In some embodiments, the individual is a human.

[0440] Articles of manufacture are also provided, wherein the article of manufacture comprises a conjugate of the disclosure, or any variation or embodiment thereof, or a stereoisomer or tautomer thereof, or a pharmaceutically acceptable salt of any of the foregoing, in a suitable container

[0441] In some embodiments, the container may be a vial, jar, ampoule, preloaded syringe, or intravenous bag.

METHODS OF PREPARING

[0442] Provided herein are processes of preparing a conjugate of the disclosure, or any variation or embodiment thereof, or a pharmaceutically acceptable salt of any of the foregoing. The following synthetic methods, along with those that are detailed in the Examples, are merely illustrative of some of the methods by which the compounds of the present disclosure, or an embodiment or aspect thereof, can be synthesized. Various modifications to these synthetic reaction schemes can be made, as will be apparent to those of ordinary skill in the art. Although certain exemplary embodiments are depicted and described herein, the compounds of the present disclosure, or any variation or embodiment thereof, may be prepared using appropriate starting materials according to the methods described generally herein and/or by methods available to one of ordinary skill in the art.

[0443] When a specific stereoisomer, or an unspecified stereoisomer, or a mixture of stereoisomers is shown in the following general procedures, it is understood that similar chemical transformations can be performed on other specific stereoisomers, or an unspecified stereoisomer, or mixtures thereof.

[0444] Representative examples of making conjugates of the disclosure are provided in Example 1. In some embodiments, a hydroxyl group of a drug moiety can be subjected to an esterification reaction using methods known in the art. In some embodiments, the reactive hydroxyl group is an alkyl hydroxyl group on a small molecule, peptide or protein. In some

embodiments, the reactive hydroxyl group is a phenol hydroxyl group on a small molecule, peptide or protein. In some embodiments, a tyrosine residue of a peptide or protein serves as the site of esterification. In some embodiments, a serine residue on a peptide or protein serves as the site of esterification.

[0445] Where specific reagents or solvents are specified for reactions in the general procedures, the skilled artisan will recognize that other reagents or solvents can be substituted as desired.

EXAMPLES

[0446] The following synthetic reaction schemes, which are detailed in the Examples, are merely illustrative of some of the methods by which the compounds of the present disclosure, or an embodiment or aspect thereof, can be synthesized. Various modifications to these synthetic reaction schemes can be made, as will be apparent to those of ordinary skill in the art.

[0447] The starting materials and the intermediates of the synthetic reaction schemes can be isolated and purified if desired using conventional techniques, including, but not limited to, filtration, distillation, crystallization, chromatography, and the like. Such materials can be characterized using conventional means, including physical constants and spectral data.

[0448] Although certain exemplary embodiments are depicted and described herein, the compounds of the present disclosure, or any variation or embodiment thereof, may be prepared using appropriate starting materials according to the methods described generally herein and/or by methods available to one of ordinary skill in the art.

[0449] All publication, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entireties, to the same extent as if each were incorporated by reference individually.

Example 1: Synthesis and Characterization of Conjugates of the Disclosure

Materials and Methods

[0450] Materials and reagents. Naloxone hydrochloride (#N285000) was purchased from Toronto Research Chemicals, Toronto, Ontario. Nalmefene hydrochloride (#408550) was purchased from Tocris Bioscience™. Naloxone-D_5 100 µg/mL (#N-063) was purchased from Cerilliant. Morphine sulfate (CII) (#M1167) and Fentanyl citrate (CII) (#F1147) were purchased from Spectrum Chemical Manufacturing Corporation. Digitonin (#D0540) and Loperamide hydrochloride (# L0154) were purchased from Fisher Scientific. Dodecyl azide (#A1887) was purchased from Synthonix. Prealbumin from human plasma (human TTR)

(#P1742) and human serum (#H4522) were purchased from Sigma Aldrich. Innovative grade US origin rat plasma (#IGRTSDPLANAH), rabbit plasma (#IGRBPLANAH), monkey cynomolgus plasma (#IGMNCYPLANAH) and pooled human plasma (#IPLAWBNAH) were purchased from Innovative Research. XTreme 200 Mixed gender human liver microsomes (#1910096), Male IGS SD rat liver microsomes (#2210274), Male cynomolgus monkey liver microsomes (#2210248), and NADPH RapidStart Regeneration System for Extended Metabolism (#K5100-5) were purchased from Xenotech. Human carboxylesterase 1 (CES1, #E0287-1VL) and carboxylesterase 2 (CES2, #C4749) were purchased from Sigma Aldrich. RPMI-1640 Medium and minimum Eagle's medium (HyClone, Utah, USA), fetal bovine serum (Gemini), penicillin/streptomycin (100 unit/mL and 100 µg/mL (Gibco, NY, USA) and L-Glutamine (Glutamax-100X, #35-050-061, Gibco, NY, USA) were bought from the companies mentioned in the parentheses. CellBIND® 96- well clear plates (#CLS3340-50EA) were purchased from Corning®. CellTiter 96 non-radioactive cell proliferation assay kit (#G4000, Promega, WI, USA) was used to perform the MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5 diphenyltetrazolium bromide) assay for the test compounds in the cell lines.

Chemical synthesis and HPLC purity analysis

[0451] Chemistry general procedures. All reactions were carried out under an argon or nitrogen atmosphere using dry solvents under anhydrous conditions, unless otherwise noted. The solvents used were ACS grade from Fisher. Yields refer to chromatographically and spectroscopically (^{1}H NMR and ^{13}C NMR) homogeneous materials, unless otherwise noted. Reagents were purchased from Aldrich and Fisher and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on EMD Millipore® silica gel 60 plastic TLC plates coated with fluorescent indicator F_{254} TLC (#1.05735.0001), using UV light and iodine chamber as visualizing agents. Normal phase flash column chromatography was carried out using Combi Flash® Rf+ Lumen instrument (Teledyne ISCO) with High Performance Silica Flash Column (RediSep® Rf+ Gold), and preparative thin-layer chromatography (PTLC) separations were carried out on Analtech® 2mm (60F-254) (#P02015). ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM−ECA 600 spectrometer and calibrated using residual undeuterated solvent as an internal reference. High-resolution mass spectra (HRMS) were performed at the mass spectrometry facility at UC Berkley using electrospray ionization from solution. Coupling constants (J) were expressed in Hertz.

[0452] Preparative HPLC method for purification of the synthesized compounds. The purification was performed on a Waters 150Q LC System w/PDA and FlexInject system

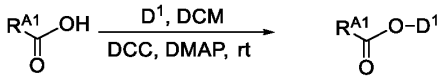
connected to a photodiode array detector operating between the UV ranges of 190-800 nm, using Waters Chromesope V2.1 software. The HPLC analysis was performed on an SunFire® Prep C18 OBD Column (19x250 mm, 5 μm) at ambient temperature upon injection of 5 mL of each sample to obtain the chromatogram at 310 nm UV absorbance. The mobile phase was composed of solvent A consisting of methanol-water (5:95, v/v) containing 0.1% trifluoroacetic acid and solvent B consisting of methanol-water (95:5, v/v) containing 0.1% trifluoroacetic acid and delivered at a flow of 20 mL min^{-1}. The HPLC program was an isocratic gradient for the first 4 min at 0% solvent B followed by a linear increase from 0-100% solvent B from 4-20 min followed by isocratic elution at 100% solvent B until 24 min followed by linearly going back down to 0% B at 26 min and then remaining at 0% solvent B for 4 min.

[0453] Analytical HPLC method for evaluating the purity of the synthesized compounds. Detailed HPLC information of key compounds (traces, retention times, and % purity) are included below. The analysis of the key compounds' purity (>95% for all compounds) was performed using C18 reverse-phase HPLC columns on Arc Premier Waters module HPLC system connected to a Waters 2998 photodiode array detector operating between the UV ranges of 190-800 nm and quantified using the Empower 3 software. The HPLC analysis was performed on a Waters™ XBridge C18 column (4.6x250 mm, 5μm), eluting at 1 mL min^{-1}, at ambient temperature upon injection of 20 μL of each sample to obtain the chromatogram at 310 nm UV absorbance. The mobile phase was composed of solvent A consisting of methanol-water (5:95, v/v) containing 0.1% ammonium hydroxide and solvent B consisting of methanol-water (95:5, v/v) containing 0.1% ammonium hydroxide. The HPLC program for the C18 column was a gradient method increasing linearly from 0-100 % solvent B at 0-20 min, followed by isocratic elution at 100% solvent B until 25 min, going back down to 0% of B at 27 min, and then remaining at 0% of B for 3 min.

General Procedure A. Preparation of naloxone free base.

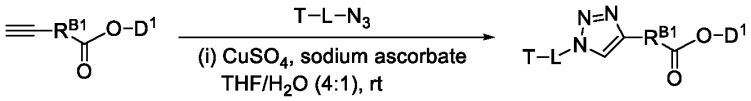
[0454] To a solution of naloxone hydrochloride (1 equivalent) in dichloromethane at ambient temperature, the tertiary amine base N,N-diisopropylethylamine (1.25 equivalents) is added. After stirring for a brief period (e.g., 5–10 minutes), the solvent is removed under reduced pressure to yield the corresponding free base of naloxone.

General Procedure B. Synthesis of esters



[0455] To the selected drug (D^1), a solution of the selected carboxylic acid (1.2 eq.) and a catalytic amount of an acylation catalyst (*e.g.*, 4-dimethylaminopyridine, 0.2 eq.) dissolved in dichloromethane was added under an inert atmosphere (e.g., N_2 or argon). The reaction mixture was stirred for a short period to allow pre-activation, followed by the addition of a coupling reagent such as N,N'-dicyclohexylcarbodiimide (approximately 1 eq.). The reaction mixture was stirred at ambient temperature for an extended period (e.g., 12–24 hrs) to ensure complete conversion. The mixture was then filtered and the solvent was removed under reduced pressure. The crude product was purified using chromatographic techniques (*e.g.*, flash column chromatography on silica gel using an appropriate gradient of ethyl acetate and hexane) to afford the target ester. The structure and purity of the product were confirmed using analytical methods such as nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS).

General Procedure C. Copper(I)-Catalyzed Azide–Alkyne Cycloaddition (CuAAC).



[0456] The click (CuAAC) coupling was carried out by coupling naloxone-alkyne/ drug-alkyne (1.2 equivalent), azide-linker-AG10/azide linker (1 equivalent), $CuSO_4.5H_2O$ (0.5 equivalent), and sodium ascorbate (1.2 equivalent) in a mixture of tetrahydrofuran/water (2:1). The reaction mixture was stirred at room temperature overnight. The tetrahydrofuran and water were removed under reduced pressure. The residues were either purified by silica gel chromatography or were dissolved in methanol and purified by preparative reverse phase HPLC to afford the desired product.

General Procedure D. Removal of Boc protecting groups

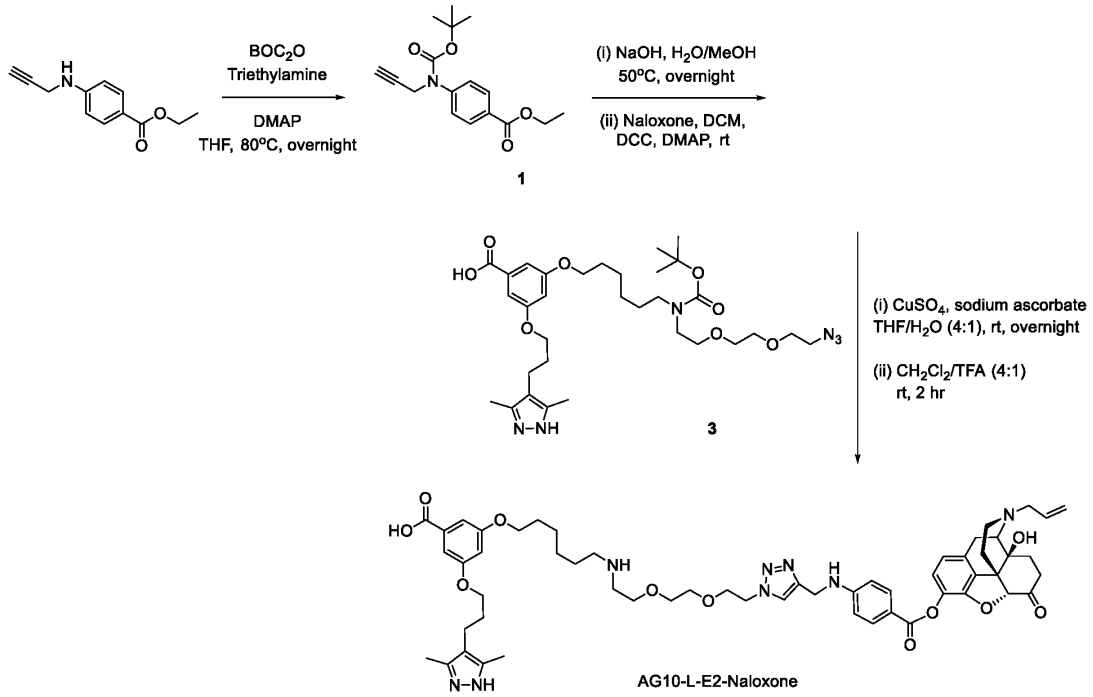
[0457] Boc-protected and conjugates and precursors were treated with a mixture of dichloromethane/trifluoroacetic acid (3:1). The reaction mixture was stirred at room temperature for 2 hr under inert atmosphere. Next, ammonium hydroxide dissolved in water (28-36%) was added slowly dropwise along with methanol. This solution was then concentrated under reduced pressure and purified by preparative reverse phase HPLC to afford the desired products.

General Procedure E. Fmoc peptide synthesis

[0458] . Rink amide resin was first swollen in dichloromethane (DCM) to prepare it for peptide synthesis. The Fmoc protecting group was removed using 20% piperidine in DMF for 25 minutes, followed by thorough washing with DMF. Coupling was carried out by preactivating the Fmoc-protected amino acid (3 equivalents) with HBTU (3 equivalents) and DIPEA (6 equivalents) in DMF for 3 minutes before adding the mixture to the resin and agitating for 40 minutes. Coupling and Fmoc deprotection were monitored using the Kaiser test to ensure completion. Upon completion of the synthesis, global cleavage from the resin and removal of acid-labile protecting groups (such as t-Boc and t-Bu) were achieved using a cocktail of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIPS) for 2 hours at room temperature. The crude peptide/peptide ester conjugate was then precipitated with cold diethyl ether, collected by centrifugation, washed, and dried. The final product was purified using preparative HPLC.

Synthesis of AG10-L-E2-Naloxone

[0459] Scheme 1 shows a schematic of the synthesis of AG10-L-E2-Naloxone.



Scheme 1. Synthesis of AG10-L-E2-Naloxone.

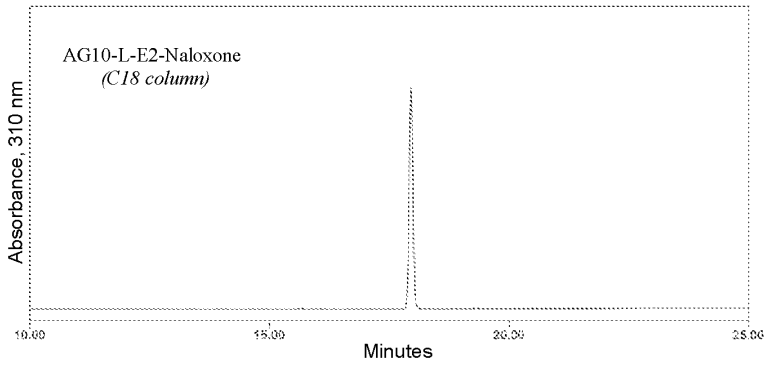
[0460] ethyl 4-((tert-butoxycarbonyl)(prop-2-yn-1-yl)amino)benzoate (1). To a solution of ethyl 4-(prop-2-yn-1-ylamino)benzoate (2 g, 9.84 mmol, 1 equiv), Di-tert-butyl dicarbonate (2.6 g, 11.9 mmol, 1.2 equiv), 4-dimethylaminopyridine (0.24 g, 1.96 mmol, 0.2 equiv), in dry tetrahydrofuran (12 mL) under a nitrogen atmosphere at room temperature, was added triethylamine (1.2 g, 1.64 mL, density of 0.73 g mL^{-1}, 11.86 mmol, 1.2 equiv). The reaction mixture is solution was stirred under overnight at 80°C. The reaction was allowed to cool to room temperature after which formic acid (906 mg, 0.742 mL, density of 1.22 g mL^{-1}, 19.68 mmol, 2 equiv) in 3 mL ethyl acetate was added dropwise into the stirring reaction vessel at room temperature. The solvent and excess formic acid were then dried under reduced pressure. The remaining residue was dissolved in ethyl acetate (30 mL), filtered, and the solvent was removed under reduced pressure. The resulting residue was purified by flash column chromatography (silica gel, 0-100% ethyl acetate/hexane) to afford compound **1** (2.76 g, 92.4% isolated yield) as a clear yellow viscous liquid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (d, 2H, *J* = 9), 7.38 (d, 2H, *J* = 9), 4.39 (s, 2H), 4.33 (q, 2H, *J* = 7.2), 2.24 (s, 1H), 1.44 (s, 9H), 1.35 (t, 3H, *J* = 7.2); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 166.12, 153.39, 146.16, 130.16, 127.75, 125.23, 100.00, 81.83, 79.64, 72.22, 61.02, 39.49, 28.29, 14.40. HRMS (DART) *m/z:* calculated for $C_{17}H_{21}NO_4$ $[M+Na]^+$ 326.1363; found: 326.1364.

[0461] (4aS,7aR,12bS)-3-allyl-4a-hydroxy-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl 4-((tert-butoxycarbonyl)(prop-2-yn-1-yl)amino)benzoate (2). To a stirring solution of **1** (2.76 g, 9.1 mmol, 1 equiv) in methanol (7.5 mL) at room temperature, was added Sodium Hydroxide (729 mg, 18.2 mmol, 2 equiv) in water (3.75 mL). This reaction mixture was stirred overnight at 50°C after which the water and methanol solvents were dried under reduced pressure. The remaining residue was re-dissolved with ethyl acetate (30 mL) containing formic acid (2.44 g, 2 mL, density of 1.22 g mL^{-1}, 53 mmol), filtered, and the solvent was removed under reduced pressure. The resulting residue was purified by flash column chromatography (silica gel, 0-100% ethyl acetate/hexane) to afford acid hydrolysis product of **1** (2.46 g) as a white powder which used directly in the next step. To a solution of Naloxone-hydrochloride (1.32 g, 3.63 mmol, 1 equiv) in CH_2Cl_2 (30 mL) at room temperature, was added N,N-Diisopropylethylamine (0.88 g, 1.2 mL, density of 0.742 g mL^{-1}, 4.5 mmol, 1.25 equiv) and stirred for five minutes. The CH_2Cl_2 was then removed under reduced pressure leaving a dried residue. To this residue, a solution of acid hydrolysis product of **1** (1.2 g, 4.36 mmol, 1.2 equiv) and 4-dimethylaminopyridine (89 mg, 0.72 mmol, 0.2 equiv) dissolved in CH_2Cl_2 (30 mL) at room temperature was added under nitrogen atmosphere. The reaction mixture was stirred for five

minutes followed by the addition of N-N'-dicyclohexylcarboiimide (0.75 g, 3.63 mL, 3.63 mmol, 1 equiv) in a 1 M solution in anhydrous CH_2Cl_2 under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 48 hr after which it was filtered and excess CH_2Cl_2 was removed under reduced pressure. The resulting residue was purified by flash column chromatography (silica gel, 0-100% ethyl acetate/hexane) to afford compound **2** (1.5 g, 70% isolated yield) a white powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.16 (d, 2H, *J* = 7.8), 7.44 (d, 2H, *J* = 8.4), 6.93 (d, 1H, *J* = 8.4), 6.71 (d, 1H, *J* = 8.4), 5.83-5.75 (m, 1H), 5.28-5.15 (m, 2H), 4.68 (s,1H), 4.41 (s,2H), 3.20-3.12 (m, 3H), 3.04-2.96 (m, 2H), 2.66-2.57 (m, 2H), 2.43-2.36 (m, 1H), 2.31-2.24 (m, 2H), 2.19-2.11 (m, 1H), 1.88-1.82 (m, 1H), 1.67-1.59 (m, 2H), 1.46 (s, 9H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 207.82, 163.73, 153.47, 148.11, 146.93, 135.19, 132.86, 131.27, 130.52, 130.34, 126.30, 125.43, 123.20, 119.63, 118.44, 100.00, 90.78, 82.08, 79.62, 72.59, 70.24, 62.06, 57.70, 50.80, 43.38, 39.51, 36.25, 31.34, 30.74, 28.42, 23.13; HRMS (DART) *m/z:* calculated for $C_{34}H_{36}N_2O_7$ $[M+H]^+$ 585.2595; found: 585.2593.

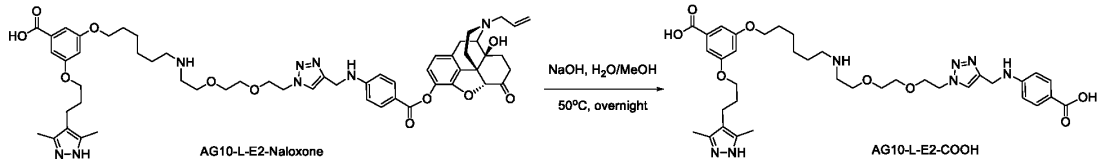
[0462] 3-((6-((2-(2-(2-(4-(((4-((((4aS,7aR,12bS)-3-allyl-4a-hydroxy-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl)oxy)carbonyl)phenyl)amino)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoic acid (AG10-L-E2-Naloxone). The click (CuAAC) coupling was carried out by coupling compound **2** (561 mg, 0.96 mmol, 1.2 equiv), compound **3** (515 mg, 0.8 mmol, 1 equiv; synthesized as reported earlier), $CuSO_4.5H_2O$ (100 mg, 0.4 mmol, 0.5 equiv), and sodium ascorbate (190 mg, 0.96 mmol, 1.2 equiv) in a) 9 mL mixture of tetrahydrofuran/water (2:1). The reaction mixture was stirred at room temperature overnight. The tetrahydrofuran and water were removed under reduced pressure. The residue was dissolved in water and extracted with CH_2Cl_2. The organic extracts were concentrated under reduced pressure and purified by flash column chromatography (silica gel, 0-100% CH_2Cl_2/ methanol) to afford the di-Boc protected AG10-L-E2-Naloxone intermediate which was directly treated with a 4 mL mixture of CH_2Cl_2/trifluoroacetic acid (3:1). The reaction mixture and stirred at room temperature for 2 hr under inert atmosphere. Next, ammonium hydroxide dissolved in water (28-36%) was added slowly dropwise along with 3 mL of methanol until no hydrogen gas was seen to be forming. This solution was then concentrated under reduced pressure and purified by preparative reverse phase HPLC to afford AG10-L-E2-Naloxone (633 mg, 64% isolated yield) as a sticky solid; ^{1}H NMR (600 MHz, CD_3OD) δ 7.84-7.79 (m, 3H), 7.03-6.99 (m, 2H), 6.95-6.87 (m, 1H), 6.84-6.76 (m, 1H), 6.65-6.60 (m,

2H), 6.59-6.54 (m, 1H), 5.90-5.77 (m, 1H), 5.64-5.49 (m, 2H), 4.80 (s, 1H), 4.48-4.42 (m, 1H), 4.40-4.37 (m, 1H), 3.94-3.80 (m, 6H), 3.79-3.72 (m, 2H), 3.56-3.34 (m, 6H), 3.23 (s, 1H), 3.1-2.96 (m, 4H), 2.92-2.82 (m, 3H), 2.75-2.59 (m, 2H), 2.59-2.5 (m, 3H), 2.16 (s, 6H), 1.94-1.27 (m, 16H); ^{13}C-NMR (150 MHz, CD_3OD) δ 207.09, 167.44, 163.90, 160.28, 160.22, 153.67, 148.33, 145.07, 133.37, 133.27, 132.33, 128.84, 128.72, 128.17, 125.69, 124.44, 123.78, 120.62, 114.76, 111.96, 107.85, 107.80, 106.17, 89.73, 89.69, 70.17, 70.07, 69.88, 69.25, 68.15, 67.12, 66.13, 61.90, 55.64, 52.98, 49.85, 48.99, 47.38, 46.69, 46.10, 38.21, 35.16, 30.86, 29.64, 28.85, 27.72, 26.15, 25.76, 25.49, 23.38, 18.86, 10.62; HRMS (DART) *m/z:* calculated for $C_{56}H_{70}N_8O_{11}$ $[M+H]^+$ 1031.5237; found: 1031.5241.



Synthesis of AG10-L-E2-COOH

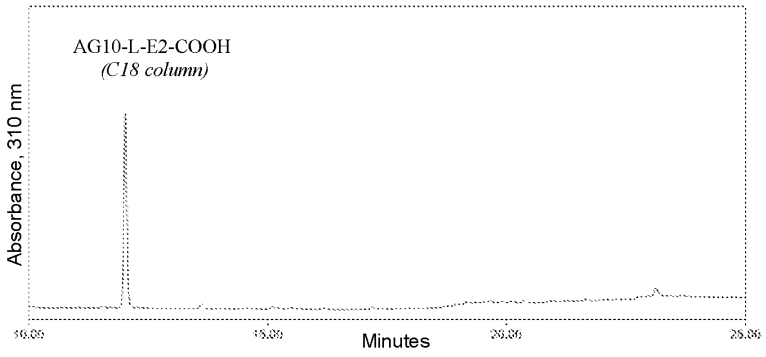
[0463] Scheme 2 shows a schematic of the synthesis of AG10-L-E2-COOH



Scheme 2. Synthesis of AG10-L-E2-COOH.

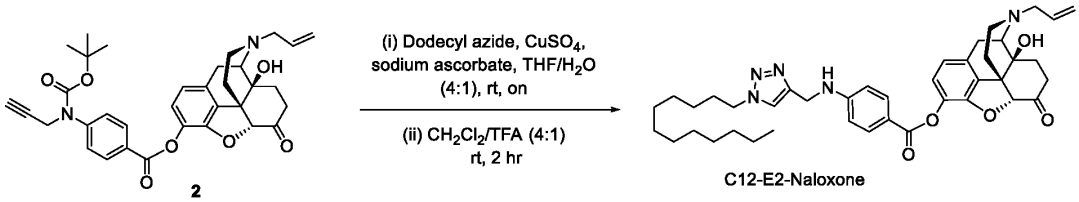
[0464] 3-((6-((2-(2-(2-(4-(((4-carboxyphenyl)amino)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoic acid (AG10-L-E2-COOH). To a solution of AG10-L-E2-Naloxone (1000 mg, 0.97 mmol, 1 equiv) in methanol (4 mL), was added sodium hydroxide (77.6 mg, 1.94 mmol, 2 equiv) in water (1 mL). The solution was stirred overnight at 50°C. The solvent was then removed under reduced pressure. The remaining residue was purified by preparative

reverse phase HPLC to afford AG10-L-E2-COOH (420 mg, 60% isolated yield); HRMS (DART) *m/z:* calculated for $C_{37}H_{51}N_7O_8$ $[M+H]^+$ 722.3872; found: 722.3869.



Synthesis of C12-L-E2-Naloxone

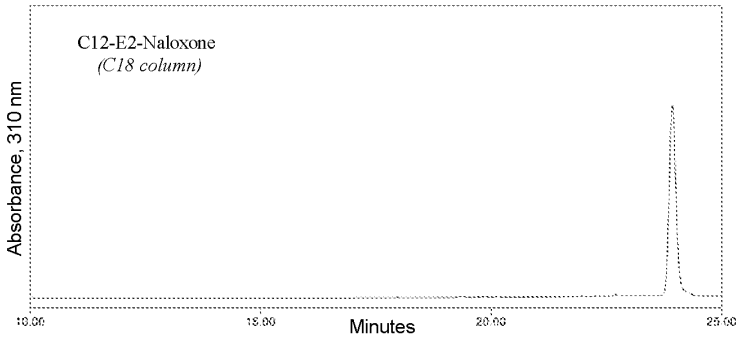
[0465] Scheme 3 shows a schematic of the synthesis of AG10-L-E2-COOH



Scheme 3. Synthesis of C12-E2-Naloxone.

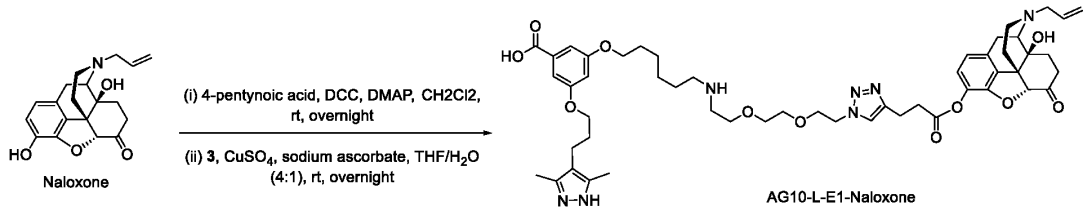
[0466] (4aS,7aR,12bS)-3-allyl-4a-hydroxy-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl 4-(((1-dodecyl-1H-1,2,3-triazol-4-yl)methyl)amino)benzoate (C12-E2-Naloxone). The click (CuAAC) coupling was carried reacting compound **2** (500 mg, 0.86 mmol, 1 equiv), Dodecyl azide (182 mg, 0.85 mmol, 1 equiv), $CuSO_4.5H_2O$ (106.7 mg, 0.425 mmol, 0.5 equiv), and sodium ascorbate (169.4 mg, 0.855 mmol, 1 equiv) in a 3 mL mixture of THF/H_2O (4:1). The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure. The residue was extracted by ethyl acetate. The organic extract was concentrated under reduced pressure to afford an intermediate and was used for the next step directly. Then, to a solution of the intermediate, was added a mixture containing TFA and CH_2Cl_2, (1:4 ratio) (1 mL), and the reaction mixture was stirred at room temperature for 2 hr. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound C12-E2-Naloxone. (255 mg, 43% yield over two steps); ^1H NMR (600 MHz, CD_3OD) δ 7.87-7.78 (m, 3H), 6.94 (t, 1H, J = 8.4), 6.85-6.78 (m, 1H), 6.61 (s, 2H), 5.91-5.79 (m, 1H), 5.64-5.52 (m,

2H), 4.82 (s, 1H), 4.58-4.34 (m, 1H), 4.27 (t, 2H, J = 7.2), 3.92-3.66 (m, 2H), 3.57-3.32 (m, 2H), 3.17-2.50 (m, 3H), 2.21-1.46 (m, 9H), 1.26-1.06 (m, 18H), 0.79 (t, 3H, J = 7.2); ^{13}C-NMR (150 MHz, CD_3OD) δ 206.56, 164.97, 148.36, 133.63, 133.04, 131.95, 128.21, 127.65, 126.50, 125.52, 125.30, 124.27, 123.05, 120.21, 119.64, 111.58, 97.09, 93.49, 89.43, 69.73, 62.55, 55.47, 50.14, 49.02, 46.31, 33.42, 31.73, 29.89, 29.39, 29.19, 28.69, 27.59, 26.97, 26.08, 25.41, 24.73, 22.93, 22.40, 13.12; HRMS (DART) *m/z:* calcd for $C_{41}H_{53}N_5O_5$ $[M+H]^+$ 696.4119; found: 696.4121.



Synthesis of AG10-L-E1-Naloxone

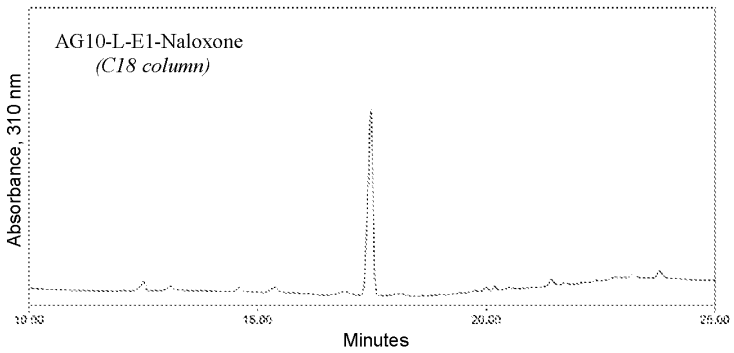
[0467] Scheme 4 shows a schematic of the synthesis of AG10-L-E1-Naloxone



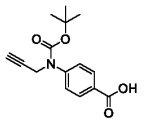
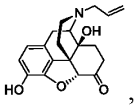
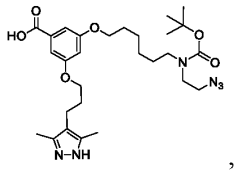
Scheme 4. Synthesis of AG10-L-E1-Naloxone.

[0468] 3-((6-((2-(2-(2-(4-(3-(((4aS,7aR,12bS)-3-allyl-4a-hydroxy-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl)oxy)-3-oxopropyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoic acid (AG10-L-E1-Naloxone). To a solution of naloxone hydrochloride (500 mg, 1.374 mmol, 1 equiv) in CH_2Cl_2 (4 mL) at room temperature, was added N,N-Diisopropylethylamine (0.24 mL ,1.374 mmol, 1 equiv) and stirred for 10 min. The solution was concentrated under reduced pressure to afford naloxone, which was used

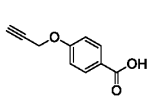
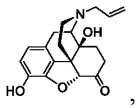
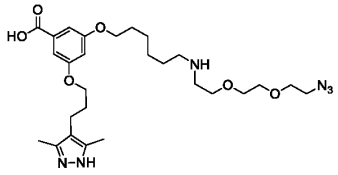
directly in the next step. 4-pentynoic acid (134.8 mg, 1.374 mmol, 1 equiv), DMAP (33.6 mg, 0.275 mmol, 0.2 equiv), was added to naloxone in CH_2Cl_2 (3 ml) under argon atmosphere. DCC (0.46 ml, 2.748 mmol, 2 equiv) was then added to the solution under argon atmosphere and reaction was stirred overnight at room temperature. The reaction was purified by flash column chromatography (silica gel, 0-100% ethyl acetate/hexane) and the intermediate was used directly in the next step. The click (CuAAC) coupling was carried out by coupling the residue and de-Boc compound **3** (300 mg, 0.549 mmol, 1 equiv; reported earlier), $CuSO_4.5H_2O$ (68.5 mg, 0.2745 mmol, 0.5 equiv), and sodium ascorbate (108.8 mg, 0.549 mmol, 1 equiv) in a 3 mL mixture of THF/H_2O (4:1). The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound AG10-L-E1-Naloxone. (250 mg, 47% yield over two steps); ^{1}H NMR (600 MHz, DMSO-d6) δ 7.95 (s, 1H), 7.05-6.95 (m, 3H), 6.84 (d, 1H, J = 8.4), 6.72-6.68 (m, 1H), 5.95-5.87 (m, 1H), 5.62-5.54 (m, 2H), 5.11 (s, 1H), 4.52-4.46 (m, 2H), 4.03-3.91 (m, 5H), 3.86-3.76 (m, 3H), 3.65 (d, 1H, J = 6), 3.64-3.61 (m, 2H), 3.59-3.49 (m, 5H), 3.24-3.16 (m, 1H), 3.12-3.04 (m, 3H), 3.04-2.80 (m, 7H), 2.70-2.56 (m, 1H), 2.5 (s, 1H), 2.29-2.08 (m, 8H), 2.06-1.91 (m, 1H), 1.87-1.80 (m, 2H), 1.75-1.66 (m, 2H), 1.65-1.57 (m, 2H), 1.55-1.15 (m, 7H); ^{13}C-NMR (150 MHz, DMSO-d6) δ 206.93, 170.46, 167.45, 160.28, 158.82, 158.60, 147.92, 145.46, 133.38, 132.69, 129.15, 128.96, 128.14, 125.67, 124.18, 123.07, 120.70, 107.81, 106.19, 89.91, 70.13, 70.07, 69.91, 69.28, 68.15, 67.10, 66.14, 61.85, 55.63, 55.44, 49.81, 49.03, 47.38, 46.65, 46.06, 35.16, 33.41, 30.88, 29.49, 28.86, 27.79, 26.15, 25.81, 25.49, 23.38, 21.18, 18.73, 10.42. HRMS (DART) *m/z:* calcd for $C_{51}H_{67}N_7O_{11}$ $[M+H]^+$ 954.4971; found: 954.4971.



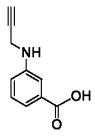
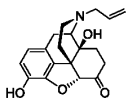
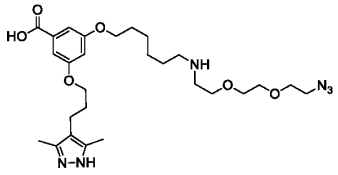
Synthesis of AG10-Short-L-E2-Naloxone

[0469] AG10-Short-L-E2-Naloxone was prepared according to **General Procedure B** using  and , followed by **General Procedure C** using , following by **General Procedure D**.

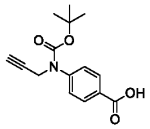
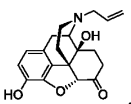
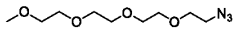
Synthesis of AG10-L-PHBA-E-Naloxone

[0470] AG10-L-PHBA-E-Naloxone was prepared according to **General Procedure B** using  and , followed by **General Procedure C** using , following by **General Procedure D**.

Synthesis of AG10-L-meta-amino-L-Naloxone

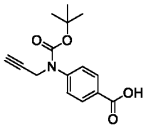
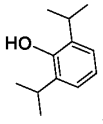
[0471] AG10-L-meta-amino-E-Naloxone was prepared according to **General Procedure B** using  and , followed by **General Procedure C** using .

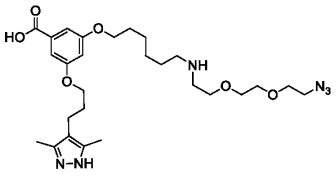
Synthesis of PEG Linker-E2-Naloxone

[0472] PEG-Linker-E2-Naloxone was prepared according to **General Procedure B** using  and , followed by **General Procedure C** using , followed by **General Procedure D**.

Synthesis of AG10-L-E2-Propofol

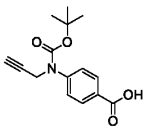
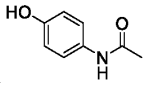
[0473] AG10-L-E2-Propofol was prepared according to **General Procedure B** using

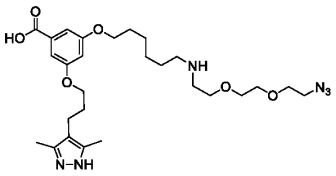
 and , followed by **General Procedure C** using

, followed by **General Procedure D**.

Synthesis of AG10-L-E2-Paracetomol

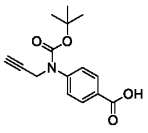
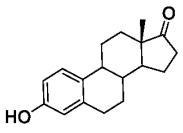
[0474] AG10-L-E2-Paracetomol was prepared according to **General Procedure B** using

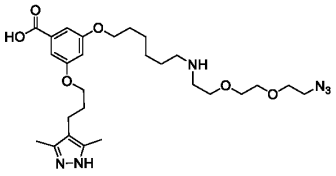
 and , followed **General Procedure C** using

, followed by **General Procedure D**.

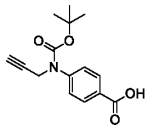
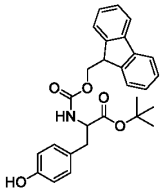
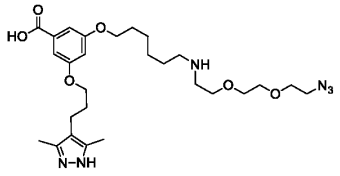
Synthesis of AG10-L-E2-Estrone

[0475] AG10-L-E2-Estrone was prepared according to **General Procedure B** using

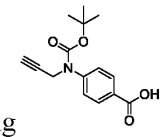
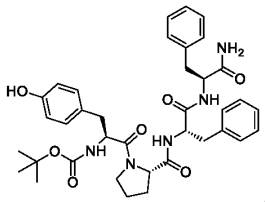
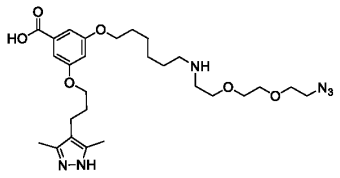
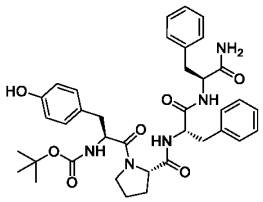
 and , followed **General Procedure C** using

, followed by **General Procedure D**.

Synthesis of AG10-L-E2-Tyr-Ala

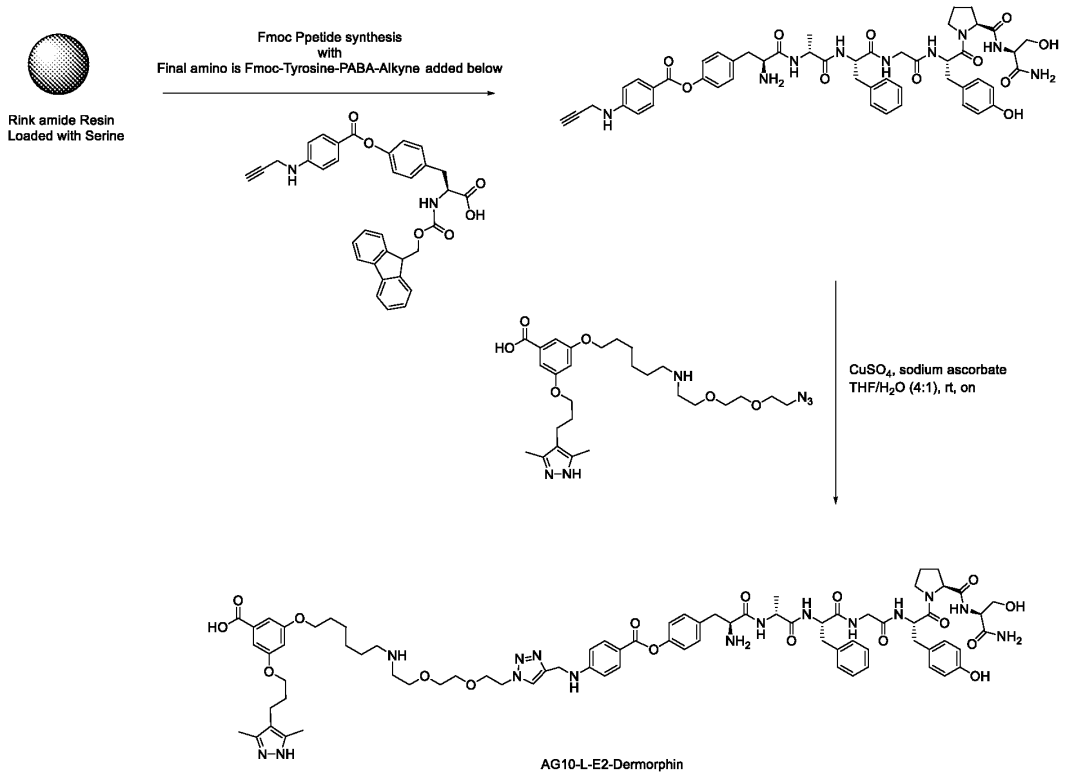
[0476] AG10-L-E2-Tyr-Ala was prepared according to **General Procedure B** using  and , followed by **General Procedure D**, followed by **General Procedure C** using .

Synthesis of AG10-L-E2-Endomorphin-2

[0477] Endomorphin-2 was prepared according to **General Procedure B** using  and , followed by **General Procedure D**, followed by **General Procedure C** using   was prepared using endomorphin-2 and di-tert-butyl dicarbonate in the presence of triethylamine and DMAP in THF at about 80°C.

Synthesis of AG10-L-E2-Dermorphin

[0478] AG10-L-E2-Dermorphin was prepared according to **General Procedure E.** Scheme 5 shows the synthesis of AG10-L-E2-Dermorphin.



Scheme 5. Synthesis of AG10-L-E2-Dermophin

[0479] Analytical HPLC method. Test compounds were analyzed using Waters™ XBridge C18 column (4.6x250 mm, 5 µm) on Arc Premier Waters module HPLC system connected to a Waters 2998 photodiode array detector operating between the UV ranges of 190-800 nm. HPLC analysis was performed using a gradient method increasing linearly from 0-100% solvent B in 25 min. The mobile phase was composed of solvent A consisting of methanol-water (5:95, v/v) containing 0.1% ammonium hydroxide and solvent B consisting of methanol-water (95:5, v/v) containing 0.1% ammonium hydroxide, at a flow rate of 1.0 mL min^{-1}.

[0480] Solubility of compounds in potassium phosphate buffer (pH 7.4). Stocks solutions (10 mM) of test compounds were prepared in DMSO and stored at -20°C. Compounds (200 µM) were incubated in 50 mM potassium phosphate buffer (pH 7.4) at 37°C for 72 hr. The samples were vortexed, spun for 5 min at 12000 rpm, and supernatant was filtered. A sample aliquot (100 µL) was then removed and quenched by adding 200 µL of 0.1% formic acid in methanol. The mixture was centrifuged again at 12000 rpm for 5 minutes. Clear supernatant was then analyzed by analytical HPLC as described method. Calibration curves for prodrugs

were generated by dissolving DMSO solution of test compounds in 0.1% formic acid in methanol (concentrations at 200, 100, 50, 25, 12.5, and 6.25 μM) and then analyzed by HPLC. Calibration curves were used to quantitate the concentration of soluble test compounds in the potassium phosphate buffer. Data represent the mean ± s.d. (n = 3 independent replicates) and a representative HPLC trace is shown.

[0481] Log $D_{7.4}$ determination using shake-flask. An aqueous potassium phosphate buffer (10 mM, pH 7.4) solution has been prepared and then saturated with n-octanol. Likewise, n-octanol was saturated with the pH 7.4 potassium aqueous phosphate buffer. Both solutions have been heavily vortexed and then left resting to ensure complete separation of the two phases. The shake-flask experiments were carried out at room temperature. Once the phases have been prepared, 1 mL of 1-octanol and 1 mL of the aqueous potassium phosphate buffer are added to a glass vial. 20 μL of test compound stock solution (10 mM stock solution in DMSO; 100 μM final concentration in the pre-saturated mixture) is added and the vial is shaken for one hour at room temperature. After one hour, the layers were allowed to separate and aliquots (100 μL) of each of the n-octanol and aqueous buffer layers were processed by adding 200 μL of 0.1% formic acid in methanol. The mixture was centrifuged again at 12,000 rpm for 5 minutes. Clear supernatant was then analyzed by analytical HPLC as described method. Calibration curves were used to quantitate the concentration of test compounds. Data represent the mean ± s.d. (n = 3 independent replicates).

The following equation was used to calculate Log $D_{7.4}$.

$$Log\ D_{7.4} = Log\ \frac{concentration\ of\ compound\ in\ octanol\ phase}{concentration\ of\ compound\ in\ aqueous\ phase}$$

Example 2: Evaluation of Binding Affinities of Conjugates

[0482] Evaluation of binding affinity of test compounds to TTR in buffer. The binding affinity of AG10-L, AG10-L-E2-COOH, AG10-L-E2-Naloxone, and AG10-L-E1-Naloxone to TTR in buffer was determined by their ability to displace FP probe from TTR using a Fluorescence Polarization (FP) assay. Serial dilutions of compounds (0.010 μM to 20 μM) were added to a solution of FP-probe (50 nM) and TTR (300 nM) in assay buffer (PBS pH 7.4, 0.01% TritonX100, 1% DMSO in 25 μL final volumes) in a 384-well plate. The samples were allowed to equilibrate by agitation on a plate shaker for 20 minutes at room temperature. Fluorescence polarization (excitation λ 485 nm, emission λ 525 nm, cutoff λ 515 nm)

measurements were taken using a SpectraMax M5 Microplate Reader (Molecular Devices). We used SoftMax® Pro software v5.4.1 (Molecular Devices, Inc.) to collect the fluorescence data. The IC50 values were obtained by fitting the data to the following equation [y = (A-D)/(1 + (x/C)^B) + D], where A = maximum FP signal, B = slope, C = apparent binding constant (K_{app}), and D = minimum FP signal. The binding constant (K_d) values were calculated using the Cheng–Prusoff equation from the IC_{50} values. Data represent the mean ± s.d. (n = 3 independent replicates).

[0483] Evaluation of binding affinity and selectivity of test compounds to TTR in human Serum. The binding affinity and selectivity of AG10-L, AG10-L-E2-COOH, AG10-L-E2-Naloxone, and AG10-L-E1-Naloxone to TTR were determined by their ability to compete with the binding of a fluorescent probe exclusion (FPE probe) binding to TTR in human serum. AG10 and Tafamidis were used as controls. An aliquot (98 μL) of human serum was mixed with 1 μL of test compounds (1.0 mM stock solution in DMSO; 10 μM final concentration in serum) and 1 μL of FPE probe (0.36 mM stock solution in DMSO; 3.6 μM final concentration in serum). The fluorescence changes (λ_{ex} = 328 nm and λ_{em} = 384 nm) were monitored every 15 min using a SpectraMax M5 microplate reader for 6 h at 25 °C. Data represent the mean ± s.d. (n = 4 independent replicates).

[0484] Results: The binding affinity of prodrugs AG10-L-E1-Naloxone and AG10-L-E2-Naloxone were evaluated using fluorescence polarization (FP) binding assay (K_d = 394.3 nM and 258.8 nM) respectively; **Fig. 1c**). This binding affinity is similar to the binding affinity of AG10-L and AG10-L-E2-COOH (K_d = 262.9 nM and 213 nM, respectively). We then evaluated the selectivity of prodrugs binding to TTR over > 4,000 other human serum proteins) using a well-established TTR serum fluorescent probe exclusion (FPE) selectivity assay. Our data showed that all test compounds maintained very good binding selectivity to TTR in human serum (~50 to 70% TTR occupancy) (**Fig. 3d,e**). The performance of A AG10-L-E2-COOH 48.1% TTR occupancy) was similar to that of the TTR stabilizer, tafamidis (an approved drug for TTR amyloidosis; 49.2% TTR occupancy).

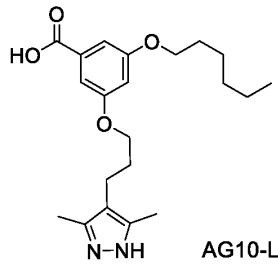
Example 3: Stability Analysis

[0485] Human and rat plasma stability assay of test compounds. Test compounds (5 μM) were incubated in human or rat plasma at 37°C. 100 μL plasma samples were analyzed at 0 hr, 0.25 hr, 2 hr, 4 hr, and 24 hr. All the samples were processed by adding 200 μL of quenching buffer (0.1% formic acid in methanol) followed by centrifuging at 15,000 rpm for

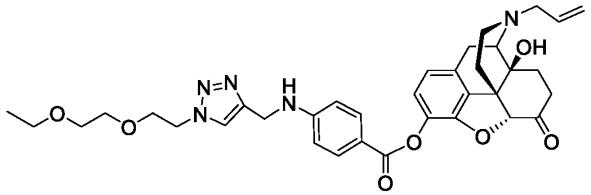
5 minutes. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 15,000 rpm for another 5 minutes. The supernatant was analyzed by analytical HPLC as described method. Data represent the mean ± s.d. (n = 3 independent replicates).

[0486] Results: We first evaluated the stability of the prodrugs in human plasma. AG10-L-E1-Naloxone was unstable (0% remaining after 1 min of serum incubation, **Fig. 1f**), which fits with the instability of alkyl-based ester prodrugs. In contrast, the PABA-based esters (AG10-L-E2-Naloxone and C12-E2-Naloxone) were fully protected from hydrolysis in human plasma. While aryl-based esters are more stable than their alkyl counterparts, we were surprised by the full stability of these prodrugs in human plasma. Our modeling studies showed that the linker we used is long enough to extend the PABA ester of the TTR T4 binding pocket, which should in principle allow plasma esterase to target our prodrugs even if there are bound to TTR (**Fig. 2b**). To investigate this observation, we evaluated the stability of the prodrugs in rat serum. As expected, the alkyl ester prodrug (AG10-L-E1-Naloxone) was hydrolyzed the fastest (0% remaining after 1 min of serum incubation) (**Fig. 1g**). The stability was higher for the PABA ester, AG10-L-E2-Naloxone (60% remaining after 15 min and 3.8% remaining after 2 hr). The hydrolysis rate of C12-E2-Naloxone (34.2% remaining after 15 min and 0% remaining after 2 hr) was slightly faster than AG10-L1-E2-Naloxone. The disparity in prodrugs hydrolysis between human and rat plasma pointed to the carboxylesterase enzymes 1 and 2 (CES1 and CES2), which are present in rat plasma but absent in human plasma.

[0487] Docking with TTR and CES2. In silico docking simulation of mouse Ces2c with AG10-L-E2-Nalxone was carried out using Dock6. Crystal structures of TTR (pdb id: 4HIQ) and Ces2c (pdb id: 8AXC) were obtained from RCSB.org protein data bank site. The geometry optimizations of AG10-L-E2-Nalxone were carried out at the B3LYP/6-311G* level using Gaussian09. For Docking with TTR and Ces2c, AG10-L-E2-Nalxone was split into two, AG10-Linker and Naloxone-Linker. Geometry optimizations of both ligands with shorter linkers were carried out at the same B3LYP/6-311G* level. Molden was used to analyze ligand geometries and to prepare ligand files for docking. UCSF Chimer program was for docking preparation of receptors and for analysis of docking results. AG10-L_A and L_B-E2-Nalxone are docked to TTR and Ces2c respectively. The docking complexes of TTR---- AG10-L_A and Ces2c--- L_B-E2-Nalxone are superimposed onto the optimized structure of AG10-L-E2-Nalxone to see the possibility of forming a ternary complex of TTR- AG10-L-E2-Nalxone -Ces2c.



AG10-L_A



L_B-E2-Naloxone

Example 4: Stability of Conjugates to Hydrolysis by Carboxylesterases

Materials and Methods

[0488] Metabolic Study of AG10-L-E2-Naloxone in recombinant human carboxylesterases. The reaction mixture, consisting of CES1 (30 μg mL^{-1} or 100 μg mL^{-1}) or CES2 (30 μg mL^{-1}) in 50 mM potassium phosphate buffer was incubated for 5 min at 30°C. The reaction was initiated by adding AG10-L-E2-Naloxone at a final concentration of 5 μM (or DMSO as a negative control). After incubation, 100 μL samples from the reaction mixture were quenched at 0 hr and 1 hr by adding 100 μL of quenching buffer (0.1% formic acid in methanol) to the samples to terminate the reaction at each designated time point. Samples were then centrifuged at 12,000 rpm for 5 minutes at 4°C. Supernatants (150 μL) were further diluted by adding 75 μL of 0.1 % formic acid in water. Samples were stored at −20°C until analysis by HPLC as described above. Data represent the mean ± s.d. (*n = 3* independent replicates).

[0489] Microsomal stability assay. 2 μL of AG10-L-E2-Naloxone Stock (1 mM in DMSO) were added to microcentrifuge tubes containing 3.2 μL acetonitrile and 194.8 μL potassium phosphate buffer (50 mM, pH 7.4, containing 3 mM $MgCl_2$). The assay tubes were preincubated at 37°C for 10 min before liver microsomes addition. Human, monkey, or rat liver microsomes and NADPH regeneration solution (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase; concentration of 2 mg mL^{-1} and 2 mM, 10 mM, and 2 Unit mL^{-1}, respectively) were added to potassium phosphate buffer (water replaced NADPH in negative control). The reaction mixtures were pre-warmed at 37°C for 10 min. The NADPH was generated using the rapidSTART NADPH regenerating system (Xenotech), by adding 0.7 mL of high purity water to the frozen components in the RapidStart vial, vortexing, and then adding the activated RapidStart solution to the incubation. 200 μL of this NADPH regeneration solution were added to each pre-incubated assay tube already containing 200 μL AG10-L-E2-Naloxone in phosphate buffer. The final reaction concentrations for AG10-L-E2-Naloxone, microsomes, NADP, glucose-6-phosphate, and

glucose-6-phosphate dehydrogenase are 5 μM, 1 mg mL^{-1}, 1 mM, 5 mM, and 1 Unit mL^{-1}, respectively. The vials were incubated at 37°C. At 0 hr and 1 hr, 100 μL aliquots were taken and the microsomal reaction was terminated by addition of 100 μL of quenching buffer (0.1% formic acid in methanol containing 15 ng mL^{-1} Naloxone-D_5 as the internal standard). Samples were centrifuged at 12,000 rpm for 5 min and supernatants (150 μL) were further diluted by adding 75 μL of 0.1 % formic acid in water. Samples were stored at −20°C until analysis using validated LC-MS/MS method using Triple Quadrupole mass spectrometer (QQQ). Data represent the mean ± s.d. (*n* = *3* independent replicates).

[0490] Microsomal stability assay with Loperamide, or Digitonin. The microsomal assay described above was also performed in HLMs in presence of Loperamide or Digitonin (final concentration 100 μM). Loperamide or Digitonin were added to the microsomal buffer before addition into the test compound.

[0491] Rat plasma stability assay of AG10-L-E2-Naloxone in presence of CES inhibitors. Rat plasma was pre-incubated with either of CES2 inhibitor, loperamide (100 μM), and CES1 inhibitor, digitonin (100 μM), or DMSO (negative control) for 5 minutes at room temperature. After that, AG10-L-E2-Naloxone (5 μM) was added to the incubation mixtures. Samples were assayed at 0 hr, 0.5 hr, and 1 hr. All the samples were processed by adding 200 μL of quenching buffer (0.1% formic acid in methanol) followed by centrifuging at 15,000 rpm for 5 minutes. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 15,000 rpm for another 5 min. The supernatant was analyzed by analytical HPLC as described method. Data represent the mean ± s.d. (*n* = 3 independent replicates).

[0492] Monkey and rabbit plasma stability assay of AG10-L-E2-Naloxone. AG10-L-E2-Naloxone (5 μM) was incubated in monkey or rabbit plasma at 37°C. 100 μL samples were analyzed at 0 hr and 24 hr. All the samples were processed by adding 200 μL of quenching buffer (0.1% formic acid in methanol) followed by centrifuging at 15,000 rpm for 5 minutes. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 15,000 rpm for another 5 min. The supernatant was analyzed by analytical HPLC as described method. Data represent the mean ± s.d. (*n* = 3 independent replicates).

Results

[0493] Carboxylesterase (CES) enzymes play a critical role in the hydrolytic inactivation of many clinically used ester drug and in the bioactivation of prodrugs. CES1 and CES2 are the two most significant forms of CESs in humans. Human CES1 plays a major role in bioactivation of many prodrugs, while CES2 plays a minor role. There is a marked species

differences in the tissue distribution and catalytic activity of CESs, making human translation challenging. CES1 is highly expressed in the liver, while CES2 is mainly found in the small intestine, but also present in the colon, kidney, and liver (10-15 times lower protein levels than CES1).

[0494] We investigated the potential of CES1/CES2 role in the bioactivation of AG10-L-E2-Naloxone. C12-E2-Naloxone was insoluble in buffer (**Fig. 1b**) which prohibited us from evaluating its CES selectivity. Our in vitro data with purified humans CESs clearly showed that AG10-L-E2-Naloxone is a specific substrate for CES2 (**Fig. 2c**). The CES2 *specificity* for AG10-L-E2-Naloxone was very surprising since only a few prodrugs (e.g., procaine and irinotecan) are *selectively* bioactivated by CES2 (they are also bioactivated by CES1 and other plasma proteins). For example, in addition to liver CES2, procaine undergoes rapid hydrolysis by butyrylcholinesterase in human plasma. In contrast, the bioactivation of irinotecan by CES2 is very slow (less than 2% of the dose is bioactivated by CES2). To further investigate the CES2 specificity and evaluate the potential translation to humans, we evaluated the metabolic bioactivation of AG10-L-E2-Naloxone in liver microsomes from human, rat, and monkey (**Fig. 2d**). Human liver microsomes (HLM) effectively produced naloxone from AG10-L-E2-Naloxone. The selective CES2 inhibitor, loperamide, significantly decreased the amount of naloxone released while there was no effect of the CES1 inhibitor, digitonin. The amount of naloxone released from AG10-L-E2-Naloxone in the rat liver microsomes (RLM) was lower than that form HLM, mainly due to higher metabolism (de-alkylation by CYP) of naloxone released by RLM compared to HLM. The expression of CES enzymes in monkey and human is similar and therefore we evaluated the bioactivation of AG10-L-E2-Naloxone in monkey liver microsomes (MLM). The amount of naloxone released MLM was similar to HLM, suggesting that monkey could be a reasonable animal model evaluating AG10-L-E2-Naloxone.

[0495] The specificity of CES2 for bioactivation of AG10-L-E2-Naloxone was also confirmed by performing the rat plasma stability with CES1 and CES2 inhibitors (**Fig. 2e**). We also confirmed our results by evaluating the stability of AG10-L-E2-Naloxone in monkey plasma (no CES1 or CES2 in plasma; 100% stability after 24 hr) and rabbit plasma (high abundance of CES1 and CES2 in plasma; 0% remaining after 24 hr).

[0496] While the crystal structure of human CES1 is available, the first crystal structure for a CES2 form mouse was recently reported as a potential ortholog of human CES2. The overall structure of mouse CES2 resembles that of the human CES1 with differences in the access to the active site. We performed a docking study of AG10-L-E2-Naloxone with both human

CES1 and mouse CES2. There was no binding for AG10-L-E2-Naloxone in CES1. In contrast, AG10-L-E2-Naloxone fits very well in the active site of CES2 and the nucleophilic Serine (S230) is in close proximity to the carbonyl of the ester groups in AG10-L-E2-Naloxone (**Fig. 2b**). This data represent the first docking experiment of a specific CES2 substrate with a CES2 enzyme. Insights form the binding mode and selectivity of CES2 for AG10-L-E2-Naloxone represent could be helpful in designing additional CES2 specific prodrugs. The lower concentration of liver CES2 in comparison to CES1 provides a unique CES2 bioactivation for AG10-L-E2-Naloxone. This could be advantageous to our controlled slow and continuous release of naloxone from AG10-L-E2-Naloxone (discussed below in the Example 10 monkey study).

Example 5: Subcutaneous administration of AG10-L-E2-Naloxone to rats releases naloxone in an extended and controlled manner without spikes in plasma and brain concentration

Materials and Methods

[0497] Evaluation of subcutaneous and intravenous pharmacokinetic profile of AG10-L-E2-Naloxone and Naloxone in rats. Jugular vein cannulated male Sprague Dawley rats (225–250 g; 7-8 weeks old) from Charles River were used for both subcutaneous (s.c) and intravenous (i.v) pharmacokinetic (PK) studies. For the i.v pk study, each animal received one i.v bolus dose of either naloxone, or AG10-L-E2-Naloxone (60 μmol kg^{-1}; equivalent to 20 mg kg^{-1} naloxone) in 500 μL dosing solution (10% DMSO, 20% PEG, 70% H2O) followed by an injection of 200 μL sterile saline to flush the jugular vein cannula (n = 3 rats per group). 200 μL blood samples were collected from each rat, via jugular vein cannula, in heparinized tubes at predetermined time points (0.033,0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hr postdosing), and the volume was replaced with sterile normal saline. For the s.c pk study, 500 μL dosing solution (per 250 g rat) in the scruff area was administered of either naloxone 15 μmol kg^{-1} or 60 μmol kg^{-1}, AG10-L-E2-Naloxone 15 μmol kg^{-1} or 60 μmol kg^{-1}. In each of the four groups (n = 3 rats per group), blood samples were collected from each rat via jugular vein cannula, in heparinized tubes at predetermined time points (0.033, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h postdosing), and the same volume was replaced with sterile normal saline. The plasma samples were prepared directly after collection of blood by centrifugation at 1,500 g for 10 min at 4°C. Next, an aliquot of 50 μL plasma sample was precipitated with 2 volumes of extraction buffer (0.1% formic acid in methanol containing with 15 ng/mL naloxone-D_5). Samples were vortexed for 30 sec, and then kept at -20°C for 5 min. Then, the

samples were centrifuged at 15,000 rpm for 5 min; the supernatant was collected and kept in the -20°C freezer for 5 min and centrifuged again at 15,000 rpm for another 5 min. The samples were analyzed on the same day of study using LC–MS/MS method using Triple Quadrupole mass spectrometer (QQQ) to quantitate the concentration of naloxone and AG10-L-E2-Naloxone in all groups.

[0498] Sample preparation and LC-MS/MS analysis of the experimental compounds. **Sample preparation.** Stock solutions of naloxone (20 mM), and AG10-L-E2-Naloxone (10 mM) were prepared in DMSO solution. The working solutions were prepared by serial dilution of the DMSO solution mentioned above. The calibration curve standards were prepared by spiking aliquots (1 µL) of each working solution to 49 µL blank rat plasma. Quality control (QC) samples prepared at low, medium, and high concentrations were extracted at the same time with the animal samples. An aliquot of 50 µL plasma was precipitated with 2 volumes of extraction buffer (0.1% formic acid in methanol containing with 15 ng mL^{-1} naloxone-D_5). The corresponding double blank samples were extracted with the extraction buffer without the internal standard i.e., 0.1% formic acid in methanol. The samples were processed same way described above. For the quantification of naloxone, 20 µL of samples were injected for LC-MS/MS analysis. A triple quadrupole mass spectrometer (QQQ) was used to quantitate the analytes in the plasma samples. The mobile phase was composed of solvent A (0.1% formic acid in water containing 0.5 mM ammonium formate.) and solvent B (0.1% formic acid in methanol), at a flow rate of 0.5 mL min^{-1}. LC-MS/MS analysis was performed on a WatersTM XBridge C18 (4.6x150 mm, 5 µm). The turbo spray ion source was set in the positive ionization mode. Fragmentation pattern and peak areas were used to identify and quantitate the test compounds, respectively. LC-MS/MS data were collected from Agilent masshunter software. Naloxone-D_5 was used as the internal standard. The gas flow, nebulizer, sheath gas temperature, sheath gas flow, capillary voltage, nozzle voltage and gas temperature were set as 7 l/min, 30 psi, 250°C, 12 l/min, 3500 V, 500 V and 300°C. LC-MS/MS analysis was performed using a gradient method increasing linearly from 0-100% solvent B in 0-3 min, stayed at 100% solvent B for 5.5 min, then went down from 100-0% solvent B in 0.5 min and finally stayed at 0% solvent B for 1 min. The retention time for naloxone and naloxone-D_5 was 4.62 and 4.62 min respectively. The total run was 7 min. Based on the calibration curves for naloxone in plasma and the internal standard generated by the LC−MS/MS analyst, mean ± s.d. concentrations of naloxone were determined. Samples were diluted when the concentration of the samples was outside the calibration curve range. The detailed mass spectrometer conditions for multiple reaction monitoring (MRM) of each

compound are listed in the Table below. The lower limit of quantitation (LLOQ) of naloxone was 1 nM. The linear ranges were calculated based on the regression analysis between the analyte and internal standard peak area ratio and the analyte concentration. The accuracy was between 80% and 120% in every case.

Compound Name:	Precursor ion	Product ion	Dwell	Frag mentor	Collision energy	Cell accelerator voltage	Polarity
Naloxone-D_5 (ISTD):	333.2	315.1	200	116	18	4	Positive
Naloxone- D_5 (ISTD):	333.2	212.1	200	116	40	4	Positive
Naloxone	328.2	310.1	200	136	18	4	Positive
Naloxone	328.2	212.1	200	136	40	4	Positive

[0499] Evaluation of the brain uptake of AG10-L-E2-Naloxone and Naloxone in rats. Jugular vein cannulated male Sprague Dawley rats were used for this study. Animals were randomized in four treatment groups ($n = 3$ animals per group). Each animal received one s.c 15 μmol kg^{-1} dose of naloxone or AG10-L-E2-Naloxone, in 500 μL dosing solution (10% DMSO, 20% PEG400 and 70% sterile deionized water). 10 and 30 min after the dosing, the rats were anesthetized with an intraperitoneal injection of 90 mg kg^{-1} ketamine and 9 mg kg^{-1} xylazine. The CSF samples were collected from the cisterna magna with a 22-gauge needle. The blood was collected from the aortic exsanguination with a 20-gauge needle followed by decapitation and brain collection. *Sample preparation and LC-MS/MS analysis of the experimental compounds.* Sample preparation. The brain was immediately snapped frozen in the liquid nitrogen. Plasma samples were processed and analyzed the same way as plasma samples described in the pharmacokinetic study. The CSF and brain samples were stored at -80 °C for further analysis. Stock solutions of naloxone (20 mM), and AG10-E2-Naloxone (10 mM) were prepared in DMSO solution. The working solutions were prepared by serial dilution of the DMSO solution mentioned above. The calibration curve standards were prepared by spiking aliquots (1 μL) of each working solution to 49 μL artificial CSF (125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl2, and 1.18 mM MgCl2), or blank rat brain homogenate. Quality control (QC) samples prepared at low, medium, and high concentrations were extracted at the same time with the animal samples. Brain homogenate was prepared by homogenizing the brain tissue with 2 volumes (w:v) of homogenizing solution (PBS buffer). An aliquot of 50 μL brain homogenate, or CSF sample was precipitated 2 volumes of

extraction buffer (methanol-water (95:5, v/v) containing 0.1% formic acid with 15 ng mL^{-1} naloxone-D_5). The corresponding double blank samples were extracted with the extraction buffer without the internal standard i.e., methanol-water (95:5, v/v) containing 0.1% formic acid. The CSF and brain homogenate samples were vortexed for 30 seconds, and then kept at -20°C for 5 minutes. Then, the samples were centrifuged at 15,000 rpm for 5 minutes; the supernatant was collected and kept in the -20°C freezer for 5 min and centrifuged again at 15,000 rpm for another 5 min. The samples were stored in -80°C for further analysis. LC-MS/MS analysis was also performed as described for the pharmacokinetic study of these molecules. The LLOQ of naloxone and AG10-L-E2-Naloxone in brain and CSF matrices is 1 nM. The ratio of the brain (ng g^{-1}) versus plasma concentration (ng mL^{-1}) is expressed as the percentage brain to plasma ratio. The ratio of the CSF (ng mL^{-1}) versus plasma concentration (ng mL^{-1}) is expressed as the percentage CSF to plasma ratio.

[0500] Survival of rats after LD_{50} doses of Fentanyl or Morphine. Male Sprague Dawley rats (225–250 g; 7-8 weeks old) were obtained from Charles River Laboratories. Rats were randomly assigned to three groups (vehicle, naloxone, and AG10-L-E2-Naloxone). Rats first received a s.c. dose of 100 mg kg^{-1} of morphine ($n = 9$ per group). After 5 min of morphine dose, rats were administered a single s.c. dose of vehicle (10% DMSO, 20% PEG400, and 70% H_2O), naloxone (15 µmol kg^{-1}, equivalent to 5 mg kg^{-1}), or AG10-L-E2-Naloxone (15 µmol kg^{-1}, equivalent to 15.8 mg kg^{-1}). Naloxone and AG10-L-E2-Naloxone were dissolved in the same vehicle dosing solution. Morphine was dissolved in water. Volume of injections for morphine and antagonists were 200 and 500 µL, respectively. Rats were then put back into their cages under routine conditions. The number of rats that died and their approximate time of death was recorded. After 6 hr of the first morphine dose, all rats that survived received a second s.c. dose of 50 mg kg^{-1} morphine. Rats were placed back into their cages and observed to record any death. The total number of rats that died was recorded and survival rate was calculated in each group.

[0501] For the fentanyl mortality study, rats first received a s.c. dose 3 mg kg^{-1} of fentanyl ($n = 8$ per group). After 5 min of fentanyl dose, rats were administered a single s.c. dose of vehicle (10% DMSO, 20% PEG400, and 70% H_2O), naloxone (15 µmol kg^{-1}, equivalent to 5 mg kg^{-1}), or AG10-L-E2-Naloxone (15 µmol kg^{-1}, equivalent to 15.8 mg kg^{-1}). Naloxone and AG10-L-E2-Naloxone were dissolved in the same vehicle dosing solution. Fentanyl was dissolved in water. Volume of injections for fentanyl and antagonists were 200 µL and 500 µL, respectively. Rats were then put back into their cages under routine conditions. The number of rats that died and their approximate time of death was recorded. After 6 hr of the

first morphine dose, all rats that survived received a second s.c. dose of 3 mg kg^{-1} fentanyl. Rats were placed back into their cages and observed to record any death. The total number of rats that died was recorded and survival rate was calculated in each group.

[0502] Reversal and prevention of fentanyl induced respiratory depression in rats. The efficacy of antagonists to reverse fentanyl induced respiratory depression in rats was evaluated using whole body plethysmography apparatus from DSI Buxco (St. Paul, MN; with FinePointe software). Sprague-Dawley rats (225– 250 g; 7-8 weeks old) (*n = 6* per group) were habituated for 30 min to the plethysmography chambers located inside sound-attenuating closed room under a continuous flow of fresh air (2.2 l/min). Rats were awake and free moving in the testing chamber and during the monitoring period. For baseline measurements, rat respiration was recorded for 20 min prior to injections. Rats were then removed from the chambers and sequentially treated with fentanyl (3 mg kg^{-1}, single s.c. or i.v. dose) followed by a single s.c. or i.v. dose of antagonist one min later (15 µmol kg^{-1} or 60 µmol kg^{-1}). Rats were placed back in the chambers and the respiratory parameters were measured for 90 min. Rats were challenged with two additional s.c. or i.v. doses of fentanyl (1.5 mg kg^{-1} each) at 6 hr and 24 hr post antagonist dosing. The respiratory parameters were measured for 90 min after each fentanyl injection. Rat ventilation was measured by recording changes in chamber pressure which is then processed and digitized. For each rat, the software calculated the average Tidal Volume (V_T: milliliter per breath), Ventilatory Frequency (f: breaths per minute), and Minute Volume ($V_E = V_T * f$: milliliter per minute). Since it is the product of V_T and f, Minute Volume was reported as it considered a quantifiable net physiological average that is scientifically relevant. Each rat's baseline Minute Volume data were averaged and specified as 100% of baseline values. Minute Volume readings after dosing were then normalized and reported for each rat in 5 min intervals based on the following equation: Minute Volume (% of control baseline) = Minute Volume at specific time/Minute Volume baseline * 100.

[0503] For the prophylactic administration of antagonist to counter fentanyl induced respiratory depression: rats (*n = 6* per group) were habituated for 30 min to the plethysmography chambers, and the baseline measurements were recorded as described above. Rats received a single s.c. dose vehicle, naloxone (15 µmol kg^{-1} and 60 µmol kg^{-1}), or AG10-L-E2-Naloxone (60 µmol kg^{-1}) and the respiratory parameters were measured for 90 min. After 6 hr, rats were challenged with single s.c. doses of fentanyl (1.5 mg kg^{-1}) and were placed back in the plethysmography chambers. The respiratory parameters were measured for 90 min and the data were processed and reported as described above. The treatment doses for

morphine and fentanyl were determined in preliminary studies and were also based on published literature. The dose of morphine used (100 mg kg^{-1}) is within the reported LD50 of 64–140 mg kg^{-1} for morphine in rats. For fentanyl, we used the reported value of 3 mg kg^{-1}. These doses were confirmed in preliminary experiments where we started with lower doses of morphine and fentanyl without observing any lethality. The overdose studies with morphine and fentanyl were approved by the University of the Pacific's IACUC.

[0504] Preliminary evaluation of AG10-L-E2-Naloxone toxicity in rats. The in vivo toxicity of AG10-L-E2-Naloxone was evaluated in rats. Adult male Sprague-Dawley rats (Charles River), body weight ranging 225-250 g, were used for the study. Three groups of rats were used. Group one was treated with a 500 µL of vehicle (10% DMSO, 20% PEG and 70% H_2O). Group two was treated with AG10-L-E2-Naloxone (60 µmol kg^{-1}; s.c. dose) dissolved in 500 µL of vehicle. Group three was treated with fentanyl (3 mg kg^{-1}; s.c. dose) followed by AG10-L-E2-Naloxone (60 µmol kg^{-1}) and then two doses of fentanyl (1.5 mg kg^{-1}; each s.c. dose) at 6 hr and 24 hr. The animal weights were monitored weekly for four weeks. Body weigh represent mean ± s.d. (*n= 6* biological replicates per group).

Results

AG10-L1-E2-Naloxone prevented mortality in rats after two doses of morphine or fentanyl

[0505] We evaluated the pharmacokinetic (PK) properties of naloxone and AG10-L-E2-Naloxone in rats (**Fig. 3a**). Both compounds (15 µmol kg^{-1}equivalent to a naloxone dose of 5 mg kg^{-1}) were administered as single s.c. doses to rats and blood samples were withdrawn from a jugular vein cannula at pre-determined time points (ranging from 5 min to 72 hr) (**Fig. 3a**). Consistent with our hypothesis, the PK profiles of AG10-L-E2-Naloxone was markedly different than naloxone. Both compounds were absorbed rapidly (naloxone was detected in plasma after 2 min of dosing free naloxone or AG10-L-E2-Naloxone) and T_{max} was ~15 min. The C_{max} for freely administered naloxone (1360 nM) was significantly higher than C_{max} for naloxone released from AG10-L-E2-Naloxone (16.9 ± 2.1nM). However, while there was no measurable amount of free naloxone 4 hr after dosing, AG10-L-E2-Naloxone and its released naloxone were still present even after 72 hr (**Fig. 3a**). The PK profile of naloxone displayed the typical spike profile (ranging from C_{max} ~1360 nM at 15 min to C_{min} of 34 nM at 4 hr). Strikingly, the release of naloxone from AG10-L-E2-Naloxone followed a flatter profile (ranging from C_{max} ~17 nM at 15 min to C_{min} of 2.4 nM at 72 hr), which we believe will be advantageous over the early concentration spike observed for naloxone (potentially causing withdrawal symptoms). Importantly, human data show that the C_{max} for naloxone (3.5 nM for

2 mg dose and 17 nM for 5 mg does dose check IM or SC dose) after s.c. dosing is ~10 nM. To our knowledge, this is the first example of PK profile that displayed this flat steady release of therapeutic molecule without the presence or burst effect or typical peaks and toughs observed with multiple doing. This data strongly support and validate our novel approach in which hydrophilicity of our prodrugs controls the release of these molecules from the subcutaneous tissue. This slow release in combination to TTR binding resulted a slow and steady plasma concentration of released of naloxone in rats.

[0506] We then evaluated the blood–brain barrier (BBB) penetration for AG10-L-E2-Naloxone (5 mg/kg; s.c.) in rats (**Fig. 3b**). The concentrations of naloxone released from AG10-L1-E2-Naloxone in brain (7.8 nM and 30.1 nM after 10 min and 30 min, respectively) were ~30 to 60-fold lower (after 10 min and 30 min, respectively) than those of freely administered naloxone (236.3 nM and 1721.7 nM after 10 min and 30 min, respectively). Similar data for both compounds were observed in the cerebrospinal fluid (CSF) (**Fig. 3c**). This clearly shows that AG10-L1-E2-Naloxone releases a significant amount of naloxone that crosses the BBB. There was no detectable concentration of the parent AG10-L1-E2-Naloxone in the brain or CSF. These data support our hypothesis that the hydrophilicity and selective binding to TTR limit the ability of the AG10-conjugates to cross the BBB.. The percentage brain to plasma ratio for naloxone released from AG10-L1-E2-Naloxone (250.3% after 30 min,) and the CSF to plasma ratio (173.3% after 30 min) were very similar to that of freely administered naloxone. This is significantly higher than 2% (cut-off for BBB penetration; it is well established that molecules having < 2% of its plasma concentration in the brain or CSF are not considered to cross the BBB).

[0507] We evaluated the potential of our prodrugs as antidotes for rescue against mortality by opioid overdose. Two opioid, morphine and fentanyl were evaluated. While the half-life ($t_{1/2}$) of fentanyl (~7 hr) and morphine (2 to 3 hr) are relatively short, the duration of overdose could last for up to 24 hr for fentanyl and 120 hr for morphine (depending on the opioid dose that is often unknown). The treatment doses for morphine and fentanyl were determined in preliminary studies and were also based on published literature. The dose for morphine (100 mg kg^{-1}) is within the reported LD_{50} of 64–140 mg kg^{-1} for morphine in rats. For fentanyl, we used the reported value of 3 mg kg^{-1}. Three groups of rats (n= *9* per group) were dosed with morphine (100 mg kg^{-1}; single s.c. dose). after 5 min: group 1 was dosed with vehicle; group 2 was dosed with naloxone (15 μmol kg^{-1}, equivalent to 5 mg kg^{-1}; single s.c. dose), and group 3 was dosed with molar equivalent dose of AG10-L-E2-Naloxone (15 μmol kg^{-1}, equivalent to 15.8 mg kg^{-1}; single s.c. dose). The 15 μmol kg^{-1} doses for naloxone and AG10-

L-E2-Naloxone chosen is higher than what has been used in the literature (1 mg kg^{-1}, equivalent to 3 μmol kg^{-1};) to make sure that the respiratory depression of the morphine and fentanyl dose is almost completely reversed by the slower release of naloxone from our prodrugs. All control groups dosed with vehicle died after the first morphine dose, while naloxone and AG10-L-E2-Naloxone groups remained alive (**Fig. 3d**). After 6 hr, a second dose of morphine (50 mg kg^{-1}; single s.c. dose) was administered to the naloxone and AG10-L-E2-Naloxone treated animals to mimic re-narcotization. All of the naloxone group was dead within 2 hr after the second morphine dose. Remarkably, all the AG10-L1-E2-Naloxone group were alive and remained alive for a few weeks after dosing.

[0508] We also performed a similar experiment by challenging rats with two doses of fentanyl (3 mg kg^{-1} each, s.c., at time = 0 hr and 6 hr; $n = 8$ per group) (**Fig. 3e**). Six rats in the vehicle group died after the first fentanyl does and the remaining rats died after the second dose. In the naloxone groups, all 8 rats died after the second doss of fentanyl. Importantly, only one rat out of eight died in the AG10-L-E2-Naloxone treated group after the second dose of fentanyl (**Fig. 3e**). The data clearly demonstrate the higher efficacy of AG10-L1-E2-Naloxone vs. naloxone in lowering mortality in rats administered with two high fentanyl doses. Due to the urgent need to develop effective antidotes against fentanyl, the following studies will focus on evaluating our prodrugs against fentanyl overdose.

Reversal of opioid-induced respiratory depression (OIRD) in rats

[0509] Systemic side effects of opioid toxicity include life-threatening OIRD that can result in a potentially deadly outcome for the patient. OIRD is also a main obstacle for the safe administration of morphine for acute pain after injury or trauma during emergencies. OIRD can be managed appropriately when monitored by trained medical personnel and when appropriate equipment is available. However, this is difficult in emergency situations where these options are not available (e.g., battlefield environment or weaponized synthetic opioids). Although tolerance to respiratory depression develops with chronic dosing, its potential for mortality in opioid-naïve patients means that it is the side effect most feared by clinicians in post-surgical settings. We tested the ability of our prodrugs (15 μmol kg^{-1} which is equivalent to 5 mg kg^{-1} of naloxone; single s.c. dose, $n = 6$) to reverse the OIRD of high dose of fentanyl (LD_{50} of 3 mg kg^{-1}; single s.c. dose) using non-invasive whole-body plethysmography (**Figs. 3f-i**).

[0510] After the first fentanyl dose (**Fig. 3g**), no reversal of OIRD was observed with s.c. dosing of vehicle or the lipidated naloxone prodrug containing the PABA ester, C12-E2-Naloxone (despite hydrolysis in rat serum; **Fig. 1g**). This is predicted since lipidation slows

the release of conjugates from s.c. tissue. We also had difficulty formulating C12-E2-Naloxone in the dosing vehicle, necessitating doubling the volume of the dosing vehicle. Two out of six rats in each of the vehicle and C12-E2-Naloxone treatment groups died after the first fentanyl dose, and therefore we did not proceed with testing the C12-E2-Naloxone prodrug. As expected, naloxone and the alkyl ester AG10-L-E1-Naloxone provided rapid reversal of OIRD after 10 min (demonstrated by rapid recovery in the Minute Volume; **Fig. 3g**). For AG10-L-E2-Naloxone, there was a gradual recovery in the Minute Volume of rats post dosing, but the Minute Volume did not reach levels close to those of normal rats (Minute Volume at 90 min was 52.5 ± 4.5% of control).

[0511] To test the ability of prodrugs to prevent fentanyl-induced ventilatory depression after the initial reversal (i.e., analogous to re-narcotization), rats were challenged with a second dose of fentanyl (1.5 mg kg^{-1}; single s.c. dose) 6 hr after dosing of naloxone or prodrugs (**Fig. 3h**). As expected, naloxone was not effective in reversing OIRD and four out of six rats in the naloxone group died after the second fentanyl dose. AG10-L-E1-Naloxone was also not effective (confirmed above by rat serum stability showing rapid hydrolysis of the alkyl ester; **Fig. 1g**). AG10-L-E2-Naloxone was the only prodrug that displayed significant reversal of OIRD after the second dose of fentanyl at 6 hr (**Fig. 3h**). After 24 hr of dosing of naloxone or prodrugs, rats were challenged with a third dose of fentanyl (1.5 mg kg^{-1}; single s.c. dose). The higher efficacy of AG10-L-E2-Naloxone in preventing mortality and reversing OIRD was again demonstrated over naloxone and AG10-L-E1-Naloxone (one out of six rats in the AG10-L-E1-Naloxone group died after the third fentanyl dose) (**Fig. 3i**). The slow onset of efficacy for AG10-L-E2-Naloxone suggested that the initial concentration of released naloxone from the prodrug needs to be enhanced to obtain effective rescue from the first fentanyl dose. However, this slow-release profile might be helpful in certain therapeutic application such as treating recovering form analgesia and neonates (discussed below)

Example 6: AG10-L1-E2-Naloxone is more effective than naloxone and nalmefene in preventing re-narcotization by fentanyl

[0512] Our data showed that low dose of AG10-L-E2-Naloxone was effective in reducing mortality after two doses of fentanyl (3 mg kg^{-1} each dose). However, the plethysmography results (**Fig. 3g**) suggested the need to increase the initial concentration of released naloxone. Therefore, we evaluated the pharmacokinetic profile of a higher dose of AG10-L-E2-Naloxone (4-fold higher; 60 µmol kg^{-1}; equivalent to 63 mg kg^{-1}) (**Fig. 4a**). For this very high dose, the C_{max} for freely administered naloxone (6498 ± 2.15 nM) was significantly higher

than C_{max} for naloxone released from AG10-L-E2-Naloxone (~30 nM, **Fig. 4a**). In contrast to naloxone, and as observed for the lower dose, the release of naloxone from AG10-L-E2-Naloxone followed a similar flatter profile (ranging from C_{max} ~30 nM at 30 min to C_{min} of 15 nM at 96 hr). (very nice 96 hr to support monkey). We then tested the efficacy of AG10-L-E2-Naloxone at the higher dose (60 μmol kg^{-1}; equivalent to 63 mg kg^{-1}) (**Figs. 4b-e**). We also included a very high dose of the two FDA-approved antidotes, naloxone (60 μmol kg^{-1}; equivalent to 20 mg kg^{-1}) and nalmefene (60 μmol kg^{-1}; equivalent to 21 mg kg^{-1}) as controls. After the first fentanyl dose, nalmefene was effective in reversing OIRD within 2 min of dosing (fits well with reported rapid absorption; T_{max} ~20 min) (**Fig. 4c**). However, there was significant hyperventilation and some convulsion or seizure in rats dosed with nalmefene, which indicate the precipitation of opioid withdrawal symptoms. 3 rats in the fentanyl control died. The efficacy of higher dose of AG10-L-E2-Naloxone (60 μmol kg^{-1}, reversal within 20 min) was better than that for lower dose (15 μmol kg^{-1}reversal within 50 min) (**Fig. 4c**). After challenging the rats with a second fentanyl dose, nalmefene and AG10-L-E2-Naloxone were equally effective in reversing OIRD, which demonstrates the presence of effective levels of released naloxone after 6 hr of dosing AG10-L-E2-Naloxone (**Fig. 4d**). The naloxone control was not effective in reversing OIRD after administering the second fentanyl dose (i.e., 6 hr after naloxone dosing; 2 rats in the naloxone control groups died). We then challenged the rats with a third dose of fentanyl after 24 hr of dosing the antagonist (to mimic re-narcotization) (**Fig. 4e**). Remarkably, while nalmefene was not effective in reversing fentanyl-induced OIRD, AG10-L-E2-Naloxone effectively counteracted OIRD. This data demonstrate that we developed what we believe as the longest acting mu-opioid antagonist prodrug (could be effective as a reversal agent and potentially an ideal prophylactic agent).

Example 7: High dose of naloxone does not provide additional efficacy vs lower dose

[0513] The lack of correlation between the pharmacokinetics and pharmacodynamics profile of naloxone was surprising. Pharmacokinetics data show that there is a significant plasma concentration of free naloxone at 6 hr (25 nM), which is similar to that of naloxone released form AG10-L-E2-Naloxone (21 nM) (**Fig. 4a**). However, rats treated with naloxone did not perform well in plethysmography experiments after the second fentanyl dose and two rats died despite the presence of sufficient naloxone in circulation (Fig 4a and 4d). This data suggest that, in comparison to the low and sustained levels for naloxone from AG10-L-E2-Naloxone (Conc. at 15 min = 20 nM), the initial very high levels of naloxone (conc. at 15 min

= 6498 nM) could indeed be harmful and may make the animals more vulnerable to the toxicity of the second fentanyl dose.

[0514] To investigate this, we performed another experiment where both fentanyl and antagonist (naloxone and AG10-L-E2-Naloxone) were administered intravenously (60 µmol kg^{-1}; equivalent to 20 mg kg^{-1}, single i.v. dose) **(Fig. 4f)**. Pharmacokinetics data show that both naloxone (conc. at 15 min = 20597 nM) and naloxone and AG10-L-E2-Naloxone (conc. at 15 min = 1599 nM) are very high at early timepoints after dosing. While both treatment groups reversed OIRD after the first fentanyl dose, there was significant hyperventilation in the naloxone treated rats and the animals experienced severe convulsions/seizures. No hyperventilation or seizures was observed for the AG10-L-E2-Naloxone treated animals. The pharmacokinetics data show that there is a significantly high plasma concentration for both free naloxone and naloxone released from AG10-L-E2-Naloxone at 6 hr (750 nM and 60 nM, respectively) and at 24 hr (395 nM and 44 nM, respectively) **(Fig. 4f)**. However, while AG10-L-E2-Naloxone was mildly active after the second fentanyl dose, neither treatment groups showed effective protection against OIRD after the second (6 hr) and third (24 hr) doses of fentanyl (**Figs. 4g-i**). This data indicated that exposing rats to a very high doses of naloxone after fentanyl dosing might indeed cause some initial harm to the animals or cause changes in the mu/other receptors that make the animals less protected by naloxone at later timepoints. There were reports of pulmonary edema after high/rapid reversal. However, this warrants further investigation.

Example 8: AG10-L1-E2-Naloxone is a potential preventive and prophylactic agent against fentanyl induced mortality and OIRD

[0515] To compensate for the lower efficacy of AG10-L-E2-Naloxone in the first 30 min after dosing, we tested the feasibility of combining the prodrug with a small amount of free naloxone. A single s.c. dose combination of AG10-L-E2-Naloxone (60 µmol kg^{-1}; equivalent to 63 mg kg^{-1}) and naloxone (3 µmol kg^{-1}; equivalent to 1 mg kg^{-1}) resulted in excellent efficacy data with full reversals of OIRD after all three fentanyl doses (**Figs. 5a-d**). Importantly, no rats experience severe hyperventilation or convulsion/seizure indicating low risk for AG10-L-E2-Naloxone to develop opioid-induced withdrawal symptoms.

[0516] We finally explored the potential of using AG10-L-E2-Naloxone as a prophylactic agent against fentanyl (**Figs. 5e-g**). Rats (n = *6* per treatment group) were first dosed with AG10-L-E2-Naloxone (60 µmol kg^{-1}) or naloxone (15 µmol kg^{-1} and 60 µmol kg^{-1}). There was not change in the Minute Volume compared to vehicle treated animals. After 6 hr, the

rats were challenged with a single dose of fentanyl (1.5 mg kg^{-1}; s.c. dose). AG10-L-E2-Naloxone was the only antidote that resulted in significant protection for the rats against OIRD and all animals (*n = 6*) survived. In contrast, both naloxone doses were not effective in preventing OIRD or mortality (4 animals died in the vehicle group; 3 animals died in 15 μmol kg^{-1} dose of naloxone; and 2 animals died in 60 μmol kg^{-1} dose of naloxone). This data support the observation of low efficacy of higher doses of naloxone despite the presence of a significant concentration of naloxone in circulation (Fig 4) and highlight the potential challenges of using higher doses on mu-opioid antagonist for counteracting fentanyl overdose.

Example 9: Preclinical evaluation of AG10-L1-E2-Naloxone – Rat Studies

[0517] We evaluated the effect of AG10-L-E2-Naloxone on the body weight of three groups of rats (*n = 6* per group). Group one was treated with a vehicle. Group two was treated with AG10-L-E2-Naloxone (60 μmol kg^{-1}). Group three was treated with fentanyl followed by AG10-L-E2-Naloxone (60 μmol kg^{-1}) and then doses of fentanyl at 6 hr and 24 hr (same protocol as above). Rats were observed for four weeks, and no signs of toxicity or mortality were observed. All animals gained the expected body weight throughout the whole study period and no significant differences were observed between the AG10-L-E1-Nalxone compared to vehicle treated group (**Fig. 5h**).

[0518] We also tested the effect of high concentration of AG10-L-E2-Naloxone (at 100 μM, which is 100-fold higher than C_{max} in rat of 0.8 μM) and its metabolite, AG10-L-E2-COOH, on viability and proliferation of three cell lines. Cell viability (MTT) assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay. HeLa and MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 unit mL^{-1} and 100 μg/mL^{-1}, respectively), and 1% l-glutamine under the humidified atmosphere of 5% CO2 and 95% air at 37°C. Hep3B was cultured in minimum Eagle's medium with 10% FBS. The cells were grown to about 75% confluence and seeded into 96-well plates at ~5000 cells/well for HeLa and MCF-7 cells and at ~8000 cells/well for Hep3B and then treated with naloxone, AG10-L-E2-Naloxone and AG10-L-E2-COOH (tested at 100 μM) or Doxorubicin (10 μM) as a positive control. Control cells were also treated with the appropriate concentration of the vehicle (DMSO). Cell viability was determined following the standard CellTiter 96 Non-Radioactive Cell Proliferation Assay protocol after 72 hr incubation at 37°C and 5% CO_2.

[0519] Both compounds showed no cytotoxic effects toward any of the cell lines that were tested (Fig 5i-k). We also confirmed that AG10-L-E2-COOH was stable in aqueous solution for at least two months at room temperature.

Example 10: Preclinical evaluation of AG10-L1-E2-Naloxone – Monkey Studies

Materials and Methods

[0520] A single subcutaneous (s.c.) injection of AG10-L-E2-Naloxone (30 μmol kg^{-1}; equivalent to 31 mg $kg^{-1}$of) was administered to three male cynomolgus monkey. The concentration of the AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone in plasma for each monkey was determined at different time points and expressed as means ± s.d. of three biological replicates.

Results

[0521] To evaluate the potential for translating our data to human, we evaluated the pharmacokinetic profile of AG10-L-E2-Naloxone in non-human primates, cynomolgus monkey (*n = 3*) (Fig 5l). Monkey represented a potential model since it lacks CES enzymes in plasma (**Fig. 2f**) and the release naloxone form AG10-L-E2-Naloxone in Monkey liver microsomes is similar to that of human (**Fig. 2d**). Monkey was also used as a preclinical model to test the efficacy of antidotes against fentanyl and carfentanil. AG10-L-E2-Naloxone was administered a single s.c. dose of (30 μmol kg^{-1}; equivalent to 31 mg kg^{-1}; which is half the dose in rats) and the plasma concentration of parent AG10-L-E2-Naloxone and naloxone released were measured over 72 hr. The enhanced pharmacokinetic profile of the parent AG10-L-E2-Naloxone was similar to that in rat (compared to typical 2 mg IM dose of naloxone; Cmax and disappear in 4 hr). AG10-L-E2-Naloxone was rapidly absorbed (plasma conc. At 5 min = 309 nM). The C_{max} (2392 nM) was also achieved rapidly within 15 min of dosing. Importantly, the concentration of AG10-L-E2-Naloxone from 4 hr to 72 hr (the last timepoint tested) was within a narrow range (303 nM to 141 nM). The pharmacokinetic profile of the naloxone released from AG10-L-E2-Naloxone was remarkable, ranging from 3 nM at 15 min post dosing to 4.5 nM after 72 hr. The C_{max} at 8 hr was 12.5 nM. The data on the lower concentration of naloxone relative to its parent prodrug, agree with in vitro data which is highlighted the stability of the prodrug in monkey plasma but allows the prodrug to release naloxone by CES2 in liver and potentially other organs with CES2 activity. This slow and extended release of steady concentration of naloxone is what believe provided with the excellent efficacy profile in rats. We predict that the AG10-L-E2-Naloxone and naloxone

released will be extended to a longer time in monkey, which is supported by the pharmacokinetic study in rats (at least 96 hr; Fig 4a).

Example 11: Development and preclinical testing of a naloxone prodrug depot for extended protection against opioid overdose

Introduction

[0522] In 2023, the US reported more than 80,000 opioid overdose deaths, the majority of which were caused by synthetic opioids like fentanyl[1]. Among high school students, an average of 22 adolescents died weekly from fentanyl-laced counterfeit pills in 2022[2]. Fentanyl, with a lethal dose of about two milligrams, poses significant risks, especially for opioid-naïve individuals. While the half-life of fentanyl (~7 hr) is relatively short, the duration of overdose could last for up to 24 hr[3]. Naloxone, a μ-opioid receptor antagonist used for reversing overdoses, has a short duration of action (DOA ~1 hr), requiring repeated doses and continuous monitoring[4]. This highlights the need for long-acting opioid antagonists. Emerging approaches include pseudo-irreversible antagonists, covalent naloxone nanoparticles, and antibody-based strategies[4-7]. However, these approaches do not address the main challenges: rapid reversal of opioid-induced respiratory depression and subsequent protection from re-narcotization. Developing extended-release opioid antagonists is crucial, and one promising approach involves using prodrugs that slowly release the active drug over time[8-11]. However, this prodrug approach has had limited success with naloxone so far.

[0523] Our strategy involves endowing naloxone prodrugs with a hydrophilic derivative of the small molecule, AG10 (Alhamadsheh-Graef molecule 10 or acoramidis)[12], which binds reversibly to the plasma protein transthyretin (TTR). Acoramidis, discovered by our group, is the most potent and selective transthyretin (TTR) ligand and has recently received FDA approval for treating transthyretin amyloid cardiomyopathy (ATTR-CM)[13]. This example introduces a naloxone prodrug delivery system leveraging the ionizable properties of our hydrophilic AG10 derivative. Upon subcutaneous administration of the fully soluble prodrug solution, the prodrug forms a zwitterion at physiological pH, reducing the prodrug's solubility and creating a depot that extends the release and duration of action of naloxone. The prodrug is specifically bioactivated by carboxylesterase 2 (CES2) enzyme present in human liver, allowing controlled and extended naloxone release. Additionally, its balanced hydrophilicity and ability to bind to TTR in plasma reduce passive diffusion across the blood-brain barrier (BBB), potentially lowering the risk of precipitated opioid withdrawal symptoms and central nervous system (CNS) toxicity.

[0524] In this example we demonstrate the potential of our approach by developing a naloxone prodrug that displays extended DOA compared to naloxone against fentanyl and morphine overdose in rats. The consistent pharmacokinetic profile of naloxone released from our prodrug in male rats and cynomolgus monkeys, combined with superior efficacy (in rats) and a promising safety profile and chemical stability, highlights the potential of translating this prodrug to humans. This simple and practical naloxone prodrug system could significantly advance the treatment of opioid overdoses and potentially other opioid-related disorders.

Results

Concept of the prodrug delivery system

[0525] The degree of ionization and solubility of ionizable drugs is dependent on the pH of the drug solution. The absorption rate of a drug from the subcutaneous (s.c.) tissue is also affected by the physiological pH of the s.c. interstitial fluid (pH ~7.4). One example is insulin glargine (Lantus®), which is a long-acting insulin analog (~24 hr DOA after s.c. injection). The incorporation of two arginine residues shifts its isoelectric point from pH 5.4 to 6.7, making it soluble in slightly acidic solution (pH 4)[14]. Upon injection into s.c. tissue, the acidic solution is neutralized by the interstitial fluid, forming a depot of micro-precipitates that dissolve slowly to release insulin glargine over time. The slow absorption rate of insulin glargine, acting as the rate-limiting step, influences its pharmacokinetic profile by controlling the gradual reduction in insulin plasma levels. This behavior exemplifies flip-flop kinetics, where the terminal phase of concentration decline is more dependent on release and absorption from the injection site than on systemic clearance[15]. However, the low levels of soluble free insulin glargine, along with the slow dissolution of the depot, result in delayed absorption and a slower onset of action compared to regular insulin. This delayed absorption is a drawback for long-acting lipid or polymer depots[16], especially for applications requiring rapid onset, such as opioid antidotes. It's important to note that non-polymeric drugs that form a depot after injection, similar to insulin glargine, are quite rare.

[0526] We recently developed a hydrophilic derivative of AG10 that enhances the DOA and limits BBB penetration of non-cleavable covalently linked opioid agonists and antagonists in rats[17]. Surprisingly, these highly hydrophilic covalent conjugates were rapidly absorbed after subcutaneous dosing, appearing in rat plasma within 2 minutes, despite their expected limited

membrane permeability. We hypothesize that these conjugates form a zwitterion at physiological pH, reducing the hydrophilicity/solubility of the conjugates in interstitial fluid, and forming a depot similar to insulin glargine. This led us to investigate utilizing the potential zwitterionic nature of the AG10 derivative to create a prodrug delivery system for extending the DOA of subcutaneously administered drugs, focusing on the opioid antagonist naloxone.

Design of naloxone prodrugs

[0527] Our prodrug approach involves conjugating a linker modified-hydrophilic derivative of AG10 to naloxone using esters with varying stability. Modeling studies indicated that AG10-Linker can extend out of TTR, allowing naloxone conjugation via different ester moieties. Esters, common in prodrugs, can be activated by hydrolysis through enzymatic or chemical means, with esterases present throughout the body, including in blood, liver, kidneys, adipose tissue, and brain[18].

[0528] We initially explored esterifying naloxone with aliphatic acid derivatives (AG10-L-E1-Naloxone, **Fig. 1a**), but found the hydrolysis rate to be very rapid (discussed below). Recognizing that the metabolic fate of esters depends on their steric and electronic properties, we switched to using the aromatic para-amino benzoic acid (PABA) for forming the ester with naloxone (AG10-L-E2-Naloxone, **Fig. 1a**). PABA was chosen for its stabilizing steric (aromatic moiety) and electronic (electron donating para-amino group) effects on the ester moiety. PABA is naturally occurring in humans, making it largely non-toxic (LD_{50} in dogs and rat >2 g kg^{-1})[19]. We also synthesized the acyl hydrolysis product of AG10-L-E2-Naloxone (i.e., AG10-L-E2-COOH, **Fig. 1a**). As control for fatty acid conjugation (established half-life extension approach for peptides, through binding to albumin), we synthesized a lipidated naloxone prodrug containing the PABA ester, C12-E2-Naloxone. All compounds were synthesized as HCl salts (utilizing the presence of multiple basic amines in the conjugates).

[0529] To test our zwitterion hypothesis, we evaluated the aqueous solubility of test compounds in water and phosphate buffer (pH 7.4, to mimic the pH of s.c. tissue). Dissolving the prodrugs' HCl salts in water resulted in an acidic pH of ~3.5. All compounds, except C12-E2-Naloxone (5 μM), were soluble in water at 200 μM (**Fig. 1b**). The solubility of naloxone at pH 7.4 in phosphate buffer was slightly lower (170 μM) than water. However, AG10-L-

E2-Naloxone's solubility dropped significantly at pH 7.4 (44 μM) compared to water (200 μM), supporting our hypothesis that the zwitterionic form of AG10-Linker lowers solubility at physiological pH (**Fig. 6a**). AG10-L-E2-COOH was fully soluble in both water and buffer (200 μM), indicating no zwitterion formation (**Fig. 6b**). Conversely, AG10-L-E2-Ethyl, which forms a zwitterion (**Fig. 6c**), showed lower solubility at pH 7.4 (97 μM) compared to pH 3.5 (200 μM). The log$D_{7.4}$ values further supported the hypothesis, showing a major decrease in hydrophilicity for zwitterionic AG10-L-E2-Ethyl (log$D_{7.4}$ = 1.2) compared to AG10-L-E2-COOH (log$D_{7.4}$ = -1.6). AG10-L-E2-Naloxone (log$D_{7.4}$ = 1.5) demonstrated a balanced hydrophilicity/lipophilicity, compared to both naloxone (log$D_{7.4}$ = 1.2) and the lipidated C12-E2-Naloxone (log$D_{7.4}$ = 2.0), enhancing its s.c. absorption. Achieving this balance is important, as it enabled the formation of a depot while maintaining a significant free soluble fraction (44 μM, **Fig. 1b**), which is essential for efficient and rapid prodrug absorption.

Prodrugs bind selectively to TTR in buffer and in human serum

[0530] The binding affinity of prodrugs AG10-L-E1-Naloxone and AG10-L-E2-Naloxone to TTR were evaluated using fluorescence polarization (FP) binding assay[20] (K_d = 394.3 nM and 258.8 nM, respectively; **Fig. 1c**). This is comparable to AG10-L-E2-COOH (K_d = 213 nM). The selectivity of these compounds for TTR over more than 4,000 other human serum proteins was assessed using a TTR serum fluorescent probe exclusion (FPE) selectivity assay[21]. All test compounds demonstrated strong binding selectivity to TTR in human serum (~50 to 70% TTR occupancy) (**Fig. 1d, e**). AG10-L-E2-Naloxone showed a TTR occupancy of 48.1%, similar to the approved TTR stabilizer, tafamidis (49.2%).

AG10-L-E2-Naloxone and C12-E2-Naloxone prodrugs are stable in human plasma but release naloxone in rat plasma

[0531] The in vivo efficacy studies of the prodrugs was evaluated in rats. Initially, we assessed the hydrolysis of the prodrug esters in human and rat plasma, given their similar TTR plasma concentrations (~ 5 μM) and the high sequence homology (~80%)[22-24]. The TTR thyroxine binding sites, where AG10 and prodrugs bind, are conserved between rat and human[23]. In human plasma, the alkyl-based AG10-L-E1-Naloxone prodrug was unstable, degrading completely within 1 min, while the PABA-based esters (AG10-L-E2-Naloxone and C12-E2-Naloxone) were fully stable at 24 hr (**Fig. 1f**). This unexpected stability was

surprising since modeling studies showed that the PABA ester extends out of the TTR binding pocket, allowing esterase access (**Fig. 2b**). To investigate this observation, we evaluated the stability of the prodrugs in rat plasma. AG10-L-E1-Naloxone degraded rapidly, while AG10-L-E2-Naloxone showed moderate stability (60% remaining after 15 min, 3.8% after 2 hr). C12-E2-Naloxone had slightly lower stability than AG10-L-E2-Naloxone. This disparity in hydrolysis rates pointed to carboxylesterase enzymes 1 and 2 (CES1 and CES2), which are present in rat plasma but absent in human plasma[25].

AG10-L-E2-Naloxone is specifically bioactivated by CES2 enzyme

[0532] Carboxylesterase (CES) enzymes, CES1 and CES2, are crucial for the hydrolytic inactivation of many ester drugs[25]. Human CES1 is primarily responsible for the bioactivation of several prodrugs, while CES2 plays a minor role[26,27]. CES1 is highly expressed in the liver, whereas CES2 is primarily found in the small intestine, kidney, and liver. The expression level of CES2 in human liver is ~9-fold lower than CES1[27]. We investigated the roles of CES1 and CES2 in the bioactivation of AG10-L-E2-Naloxone. Due to its insolubility in buffer (**Fig. 1b**), we could not evaluate the CES selectivity for C12-E2-Naloxone. In vitro studies with recombinant human CESs showed AG10-L-E2-Naloxone is a specific substrate for CES2 (**Fig. 2c**), which is surprising since few prodrugs, like procaine and irinotecan, are selectively hydrolyzed by CES2. Procaine undergoes rapid hydrolysis by butyrylcholinesterase in human plasma in addition to liver CES2, while irinotecan is slowly hydrolyzed by both CES1 and CES2, with less than 5% of the dose bioactivated[27,28]. To further investigate CES2 specificity and the potential for human translation, we assessed the metabolic bioactivation of AG10-L-E2-Naloxone in liver microsomes from humans, rats, and cynomolgus monkeys (**Fig. 2d**). Human liver microsomes (HLM) effectively produced naloxone from AG10-L-E2-Naloxone, and the CES2 inhibitor loperamide significantly decreased naloxone release, while the CES1 inhibitor digitonin had no protection. Rat liver microsomes (RLM) produced less naloxone due to higher metabolism and de-alkylation by CYP3A4. Given the similar CES2 expression in monkeys and humans[25], monkey liver microsomes (MLM) showed naloxone release comparable to HLM, suggesting monkeys as suitable models for evaluating the efficacy of AG10-L-E2-Naloxone. The specific CES2 bioactivation of AG10-L-E2-Naloxone was confirmed using rat plasma with CES1 and CES2 inhibitors (**Fig. 2e**). Additionally, AG10-L-E2-Naloxone showed 100% stability in

cynomolgus monkey plasma (lacking CES1 and CES2)[25] and 0% stability in rabbit plasma (high CES1 and CES2 levels) after 24 hr (**Fig. 2f**).

[0533] The 9-fold lower liver concentration of CES2 compared to CES1[27] provides unique bioactivation for AG10-L-E2-Naloxone. Additionally, CES2 is absent from s.c. adipose tissue while CES1 is present[29], reducing the potential burst release of naloxone from the prodrug depot. While the crystal structure of human CES1 is available[30], the first crystal structure of mouse CES2, a potential ortholog of human CES2, was recently reported[31]. The mouse CES2 structure resembles human CES1, with differences in active site access. Docking studies showed no binding of AG10-L-E2-Naloxone to CES1, but it fit well in the active site of CES2, with the nucleophilic Serine (S230) in close proximity (2.9 Å) to the carbonyl carbon of the ester group in AG10-L-E2-Naloxone (**Fig. 2b** and **Fig. 7**). These data were supported by performing a docking study of the three known CES2 selective prodrugs irinotecan, procaine, and molnupiravir to both CES1 and CES2 (**Fig. 7**). The three prodrugs showed very good binding to CES2 with a favorable distance between the nucleophilic S230 and the carbonyl carbon of prodrugs (3.3 Å, 3.1 Å, and 3.5 Å for irinotecan, procaine, and molnupiravir, respectively). While there was no binding between AG10-L-E2-Naloxone and CES1, the three other prodrugs displayed modest to weak binding with distance between the nucleophilic S230 and the carbonyl carbon of prodrugs (9.2 Å, 6.8 Å, and 4.8 Å for irinotecan, procaine, and molnupiravir, respectively). Therefore, our study represents the first docking experiment of a CES2-specific substrate, offering insights for designing additional CES2-specific prodrugs.

The specific hydrolysis of AG10-L-E2-Naloxone by CES2 is driven by conjugation to AG10-Linker

[0534] We investigated whether the specific hydrolysis of AG10-L-E2-Naloxone by CES2 is driven by the PABA ester or a combination of PABA and AG10-Linker. We synthesized three aryl-based naloxone prodrugs without the AG10-Linker. The benzoic acid naloxone ester (BA-Naloxone) hydrolyzed the fastest in human plasma, while the Boc-PABA-Naloxone and PABA-Naloxone were more stable (**Fig. 8**). This highlights the stabilizing effect of the electron donating amine group in PABA on the ester moiety. All three compounds were effectively hydrolyzed in human plasma, indicating they are substrates for esterases other than CES1 or CES2. CES2 selectivity was demonstrated in rat plasma stability studies with CES2 and CES1 inhibitors. Our data suggest that the specific hydrolysis of

AG10-L-E2-Naloxone by CES2 is driven by conjugating the PABA ester to AG10-Linker. We also tested procaine, molnupiravir, and irinotecan, known CES2 substrates, finding they are not specific to CES2 (**Fig. 8**). To our knowledge, AG10-L-E2-Naloxone is the first prodrug specifically bioactivated by CES2.

[0535] The specific hydrolysis of AG10-L-E2-Naloxone by CES2 challenges our current understanding of the structural features required for CES2 substrates[27]. It has been proposed that compounds with a larger acyl moiety are hydrolyzed by CES1, while CES2 preferentially hydrolyze compounds with a small acyl moiety, such as procaine and molnupiravir. Our data indicate that compounds with a much larger acyl moieties, like AG10-L-E2-COOH, can also be hydrolyzed by CES2. These insights could aid in designing new prodrugs with higher specificity for CES2 hydrolysis.

Evaluating the effect of TTR on the in vitro pharmacokinetics of AG10-L-E2-Naloxone

[0536] While AG10-L-E2-Naloxone was stable in human plasma, the presence of CES2 enzymes in rat plasma hindered the accurate evaluation of TTR's role in the pharmacokinetics of AG10-L-E2-Naloxone. Therefore, we conducted in vitro experiments to investigate the role of TTR in our system (**Fig. 9**). We assessed the stability of AG10-L-E2-Naloxone (tested at 5 μM) in rat plasma, both in the absence and presence of the selective TTR ligand AG10 (10 μM) (**Fig. 9a**). We observed a small but significant reduction in the amount of AG10-L-E2-Naloxone remaining when AG10 was present, indicating that TTR provides some protection against CES2 in *rat* plasma. This outcome was anticipated, as we designed the linker in AG10-L-E2-Naloxone (~29 Å) to be sufficiently long to allow binding to both CES2 and TTR simultaneously (see **Fig. 2b**). The protective effect of TTR was further supported by evaluating the metabolic stability of AG10-L-E2-Naloxone in human liver microsomes, with and without human TTR (**Fig. 9b**).

[0537] It is important to note that the primary impact of TTR on enhancing the pharmacokinetic profile of AG10-L-E2-Naloxone would stem from its ability to decrease renal filtration, a significant factor that reduces the duration of action of hydrophilic molecules. The instability of AG10-L-E2-Naloxone to CES2 in rat plasma precludes the in vivo evaluation of TTR effect on PK. To simulate this effect in vitro, we used rapid equilibrium dialysis to mimic TTR's influence on decreasing AG10-L-E2-Naloxone renal filtration (**Fig. 9c**). The data show that binding to human TTR significantly limited the ability

of AG10-L-E2-Naloxone to passively traverse the dialysis membrane. This finding is supported by our previous in vivo work on non-cleavable linkers, which demonstrated the effect of TTR on reducing renal filtration of these conjugates in rats[32,33]. Therefore, we believe that the effect of TTR on enhancing the pharmacokinetic profile of AG10-L-E2-Naloxone would be demonstrated in humans, as we observed in cynomolgus monkeys below.

Subcutaneous administration of AG10-L-E2-Naloxone to rats releases naloxone in an extended and controlled manner without spikes in plasma and brain concentration

[0538] To investigate the effect of the zwitterionic nature and TTR binding of AG10-Linker on the DOA of the prodrugs, we evaluated the pharmacokinetic properties of naloxone and AG10-L-E2-Naloxone in male rats. Both compounds (15 μmol kg^{-1}equivalent to a naloxone dose of 5 mg kg^{-1}) were administered as single s.c. doses, with blood samples collected from a jugular vein cannula at predetermined time points (2 min to 72 hr) (**Fig. 3a** and **Table 2**). Consistent with our hypothesis, the pharmacokinetic profile of AG10-L-E2-Naloxone differed significantly from naloxone. Both were rapidly absorbed (detected in plasma 2 min after dosing), with a T_{max} of ~15 min. The C_{max} for freely administered naloxone (1,390 ± 187 nM) was much higher than for naloxone released from AG10-L-E2-Naloxone (16.9 ± 1.5 nM). The cutoff time for all time points was 72 hr. Freely administered naloxone levels at the 6 hr timepoint and beyond were below the lower limit of quantification (LLOQ) of 1 nM. AG10-L-E2-Naloxone and its released naloxone were present even after 72 hr. Free naloxone displayed a typical spike/peak profile (C_{max} ~1,390 nM at 15 min to C_{min} of 31 nM at 4 hr), whereas AG10-L-E2-Naloxone followed a flatter, peakless profile (C_{max} ~17 nM at 15 min to C_{min} of 2.4 nM at 72 hr) (**Fig. 3a**), suggesting flip-flop kinetics. This could potentially prevent early withdrawal symptoms. Importantly, human data show that the C_{max} for naloxone after s.c. administration is ~10 nM for a 2 mg dose[34]. Therefore, our findings provide the first example of a pharmacokinetic profile displaying flat and steady release of a therapeutic small molecule without burst effects or typical peaks and troughs seen with multiple dosing or polymeric vehicles[35,36]. These results validate our approach, demonstrating that the balanced hydrophilicity/lipophilicity of our prodrugs controls its release from s.c. tissue. This slow release, combined with TTR binding (reducing renal filtration) and CES2 bioactivation, resulted in a controlled and steady plasma concentration of released naloxone in rats.

[0539] We then evaluated the BBB penetration for AG10-L-E2-Naloxone in rats (15 μmol kg^{-1}; s.c. **Fig. 3b**). The brain concentrations of naloxone released from AG10-L-E2-Naloxone

(7.8 ± 1.7 nM at 10 min and 30.1 ± 2.1 nM at 30 min) were 30-fold to 60-fold lower than those from freely administered naloxone (236.3 ± 50 nM at 10 min and 1721.7 ± 280 nM at 30 min). Similar results were observed in the cerebrospinal fluid (CSF) (**Fig. 3c**). The brain-to-plasma ratio for naloxone from AG10-L-E2-Naloxone (250.3 ± 24.1% at 30 min) and the CSF-to-plasma ratio (137.7 ± 29.7% at 30 min) were similar to freely administered naloxone. No detectable levels of AG10-L-E2-Naloxone or its hydrolysis product, AG10-L-E2-COOH, were found in the brain or CSF (**Fig. 10**; molecules having < 2% of its plasma concentration in the brain or CSF are not considered to cross the BBB). These findings support our hypothesis that the hydrophilicity and selective TTR binding limit the ability of AG10-conjugates to cross the BBB.

AG10-L-E2-Naloxone prevents mortality in rats after two doses of morphine or fentanyl

[0540] We evaluated AG10-L-E2-Naloxone as an antidote for opioid (morphine and fentanyl) overdose. The opioid doses were based on preliminary studies and literature, with morphine at 100 mg kg^{-1} (within reported LD_{50} of 64–140 mg kg^{-1})[37] and fentanyl at 3 mg kg^{-1} (reported LD_{50})[38]. Three groups of male rats (n = 9 per group) received morphine (100 mg kg^{-1}, s.c.). After 5 min: group 1 received vehicle; group 2 received naloxone (15 µmol kg^{-1}, equivalent to 5 mg kg^{-1}, s.c.); group 3 received AG10-L-E2-Naloxone (15 µmol kg^{-1}, s.c.). All vehicle-dosed controls died after the first morphine dose, while naloxone and AG10-L-E2-Naloxone groups survived (**Fig. 3d**). After 6 hr, a second morphine dose (50 mg kg^{-1}, s.c.) was given to naloxone and AG10-L-E2-Naloxone groups to mimic re-narcotization. All naloxone-treated rats died within 2 hr. Notably, all rats treated with AG10-L-E2-Naloxone survived.

[0541] In a similar experiment with fentanyl (two doses of 3 mg kg^{-1} each, s.c., at 0 hr and 6 hr; n = 8 per group), six vehicle-treated rats died after the first dose, and the remaining two died after the second (**Fig. 3b**). In the naloxone group, all rats died after the second fentanyl dose. The higher survival rate for the two rats in the vehicle group compared to the naloxone group after the second fentanyl dose likely indicates acute tolerance, which develops quickly, often within hours or days, and can occur with a single administration of a high dose or short-term repeated doses[39,40]. This rapid tolerance could be due to receptor desensitization or internalization, temporarily reducing opioid receptor responsiveness. Acute tolerance differs

from chronic tolerance, which develops over extended use over time and involves longer-lasting receptor downregulation (decreased receptor numbers) and gene expression changes.

[0542] In the AG10-L-E2-Naloxone group, only one rat died after the second fentanyl dose. These results demonstrate the higher efficacy of AG10-L-E2-Naloxone over naloxone in reducing mortality in rats given very high doses of morphine and fentanyl. The efficacy data also correlate well with the pharmacokinetic study using the same dose of antagonist (**Fig. 3a**; no freely administered naloxone was detected at 6 hr, whereas 9 nM of naloxone from AG10-L-E2-Naloxone was present). These results suggest that the extended release of naloxone from AG10-L-E2-Naloxone provided protection against re-narcotization and death in rats. To confirm that the short half-life of naloxone is responsible for its lack of efficacy in the mortality study, we conducted an experiment to replicate clinical settings (**Fig. 11**). Instead of a single 5 mg kg^{-1} s.c. dose, two groups of rats received an initial loading s.c. dose of 1 mg kg^{-1} naloxone. Group 1 subsequently received five smaller doses of naloxone (0.2 mg kg^{-1} per s.c. dose every hour, totaling 1 mg kg^{-1} over 6 hr). Group 2 received ten smaller doses of naloxone (0.2 mg kg^{-1} per s.c. dose every hour, totaling 2 mg kg^{-1} over 12 hr). The data demonstrate that continuous administration of smaller doses of naloxone effectively reduced mortality. While continuous s.c. administration of naloxone for up to 12 hr reduced mortality after a second fentanyl dose at 6 hr (only 2 out of 8 rats died, blue line in **Fig. 11**, discontinuing naloxone dosing 30 minutes before the second fentanyl dose at 6 hr resulted in a significant increase in deaths (6 out of 8 rats died, gray line in **Fig. 11**). This outcome highlights the challenges of requiring continuous naloxone administration in clinical settings, as discussed in detail below. Importantly, the data in **Fig. 3e** illustrate the potential clinical utility and advantage of a single s.c. dose of AG10-L-E2-Naloxone in providing protection against re-narcotization. The following studies will focus on evaluating AG10-L-E2-Naloxone against fentanyl overdose due to the urgent need for effective antidotes.

Reversal of opioid-induced respiratory depression (OIRD) in rats

[0543] Systemic side effects of opioid toxicity include life-threatening opioid-induced respiratory depression (OIRD), which can be fatal, especially in opioid-naïve patients[3,41,42]. We tested the ability of our prodrugs (15 µmol kg^{-1}; single s.c. dose, $n = 6$) to reverse OIRD caused by a high dose of fentanyl (LD_{50} of 3 mg kg^{-1}; single s.c. dose) in naïve male rats using non-invasive whole-body plethysmography (**Fig. 3f-i**). This high dose of fentanyl, over

10 times higher than literature reports[5-7], was used to mimic the OIRD of potent synthetic opioid analogues. After the first fentanyl dose (**Fig. 3g**), neither the vehicle nor the lipidated naloxone prodrug, C12-E2-Naloxone, reversed OIRD (despite showing hydrolysis in rat plasma, **Fig. 1g**). This was expected as lipidation slows the release of prodrug from s.c. tissue. We also had difficulty formulating C12-E2-Naloxone, necessitating doubling the dosing vehicle volume. Two out of six rats in each group died, and therefore we did not proceed with testing the C12-E2-Naloxone prodrug. Naloxone and the alkyl ester AG10-L-E1-Naloxone provided rapid reversal of OIRD within 5 min. For AG10-L-E2-Naloxone, recovery began at 65 min, but Minute Volume did not reach normal levels (55.2 ± 16.6% of baseline at 90 min).

[0544] To test the prevention of fentanyl-induced respiratory depression after initial reversal (re-narcotization), rats received a second fentanyl dose (1.5 mg kg^{-1}; s.c.) 6 hr after naloxone or prodrug dosing (**Fig. 3h**). Naloxone failed to prevent OIRD, with four out of six rats dying. AG10-L-E1-Naloxone was also ineffective in preventing OIRD. AG10-L-E2-Naloxone significantly prevented OIRD after the second fentanyl dose. After 24 hr, rats received a third fentanyl dose (1.5 mg kg^{-1}; s.c.). AG10-L-E2-Naloxone again demonstrated higher efficacy in preventing mortality and reversing OIRD compared to naloxone and AG10-L-E1-Naloxone (one out of six rats died in the AG10-L-E1-Naloxone group) (**Fig. 3i**). The slow onset of AG10-L-E2-Naloxone suggests that enhancing the initial naloxone release from the prodrug may be needed for effective rescue from the first fentanyl dose. However, this slow-release profile might be helpful in certain therapeutic applications (discussed below).

AG10-L-E2-Naloxone is more effective than naloxone and nalmefene in preventing re-narcotization by fentanyl

[0545] Our data showed that a low dose of AG10-L-E2-Naloxone effectively reduced mortality after two doses of fentanyl (3 mg kg^{-1} each). However, plethysmography results suggested a need to increase the initial concentration of released naloxone. Therefore, we evaluated the pharmacokinetic profile of a higher dose of AG10-L-E2-Naloxone (4-fold higher; 60 µmol kg^{-1}, equivalent to 63 mg kg^{-1}, single s.c. dose) (**Fig. 4a** and **Table 3**). The cutoff time for all time points was 96 hr. The plasma concentrations of freely administered naloxone were below the limit of quantification (BLQ) at the corresponding 72 hr and 96 hr. The C_{max} for freely administered naloxone (6,498 nM) was significantly higher than that for naloxone released from AG10-L-E2-Naloxone (36 nM). The pharmacokinetic profile of the

higher AG10-L-E2-Naloxone dose followed a similar flatter release profile to the lower dose (C_{max} ~30 nM at 30 min to C_{min} of 15 nM at 96 hr). This pharmacokinetic profile of AG10-L-E2-Naloxone strongly supports a flip-flop pharmacokinetics as demonstrated for other s.c. administered drugs such as insulin glargine and buprenorphine[15,43].

[0546] While there was no freely administered naloxone detected at 6 hr in the low 5 mg kg^{-1} s.c. dose, we hypothesize that the low levels of naloxone observed at later time points following the high 20 mg kg^{-1} s.c. dose may result from residual naloxone precipitating in the subcutaneous tissue post-administration. This hypothesis is supported by the data in **Fig. 1b**, which show that naloxone has lower solubility at physiological pH (170 μM) compared to the slightly acidic dosing solution (200 μM). Following administration, the small insoluble fraction of naloxone at physiological pH will gradually dissolve and absorb at a slower rate than the initial major soluble fraction, leading to a steady profile at later time points.

[0547] Another possible explanation for this observation is the redistribution of naloxone from the tissue reservoir following the initial high plasma concentration. To investigate this, we compared the pharmacokinetic parameters for the 5 mg kg^{-1} and 20 mg kg^{-1} s.c. doses (see **Tables 2 and 3**). Increasing the dose by 4-fold resulted in approximately 4.5-fold and 6-fold increases in C_{max} and AUC_{inf}, respectively. To eliminate the influence of later time points on the AUC, we compared the $AUC_{0\text{-}6hr}$ between the 5 mg kg^{-1} and 20 mg kg^{-1} s.c. doses (**Fig. 12**). The $AUC_{0\text{-}6hr}$ for the 20 mg kg^{-1} dose was also 6-fold higher than that of the 5 mg kg^{-1} dose, indicating dose non-linearity at the higher naloxone dose due to a decrease in clearance, likely resulting from enzyme saturation. This is reflected by the slower slope from 0.5 hr to 2 hr in the 20 mg kg^{-1} s.c. dose compared to the 5 mg kg^{-1} s.c. dose, indicating reduced clearance (Fig. 12). Importantly, the clearance rate of naloxone for both doses followed linear kinetics after 2 hr, suggesting that the effect of tissue redistribution at later time points is minimal, and that the dissolution of precipitated naloxone is a more plausible explanation.

[0548] We then tested the efficacy of the higher dose (60 μmol kg^{-1}) of AG10-L-E2-Naloxone and included high doses of the FDA-approved antidotes, naloxone and nalmefene (60 μmol kg^{-1}), as controls (**Fig. 4b-e**). After the first fentanyl dose, nalmefene reversed OIRD within 2 min but caused hyperventilation and mild convulsions due to withdrawal symptoms[44,45] (**Fig. 4c**). Three vehicle control rats died. The higher dose of AG10-L-E2-Naloxone was more effective than the lower dose. After a second fentanyl dose, nalmefene and AG10-L-E2-Naloxone were equally effective in reversing OIRD, indicating effective

levels of released naloxone 6 hr after dosing AG10-L-E2-Naloxone (**Fig. 4d**). Naloxone was ineffective after the second fentanyl dose, with two rats dying. After a third fentanyl dose at 24 hr (to mimic re-narcotization), AG10-L-E2-Naloxone effectively counteracted OIRD, while nalmefene was not effective, with one rat dying (**Fig. 4e**). To our knowledge, these data suggest that AG10-L-E2-Naloxone is the longest-acting μ-opioid antagonist prodrug reported, with no observed withdrawal symptoms, potentially serving as both a reversal and prophylactic agent.

High dose of naloxone does not provide additional efficacy vs. lower dose

[0549] The lack of correlation between the pharmacokinetics and pharmacodynamics of high-dose naloxone was surprising. Despite significant plasma concentrations of naloxone at 6 hr (25 nM), similar to that from AG10-L-E2-Naloxone (21 nM), rats treated with the high naloxone dose performed poorly in plethysmography experiments after a second fentanyl dose, with two rats dying (**Fig 4a and 4d**). These data suggest that high initial levels of naloxone (6,498 nM at 15 min) may be harmful, making animals more vulnerable to the toxicity of the second fentanyl dose. To investigate further, we performed an efficacy study in which we administered fentanyl and high doses of antagonists (naloxone and AG10-L-E2-Naloxone) intravenously (60 μmol kg^{-1}; single i.v. dose) (**Fig. 4g-i**). Before starting our efficacy study, we conducted a pharmacokinetic study on high i.v. doses of naloxone and AG10-L-E2-Naloxone (**Fig. 4f**; 60 μmol kg^{-1}; single i.v. dose) (**Table 4**). The data showed high early concentrations of both naloxone (20,597 nM at 15 min) and naloxone released from AG10-L-E2-Naloxone (1,599 nM at 15 min). The cutoff time for all time points in the pharmacokinetic study was 24 hr. For the 20 mg kg^{-1} doses, the C_{max} ratios of i.v./s.c. were 8-fold for naloxone and 500-fold for AG10-L-E2-Naloxone, indicating a significant depot effect for AG10-L-E2-Naloxone after s.c. dosing due to flip-flop kinetics caused by slower dissolution and gradual absorption (see **Tables 3 and 4**). The high AUC ratios of i.v./s.c. for AG10-L-E2-Naloxone (3-fold for naloxone and 50-fold for AG10-L-E2-Naloxone) further support this. To explain the high naloxone concentration at the 24 hr cutoff for the 20 mg kg^{-1} i.v. dose, we calculated the steady-state volume of distribution (V_{ss}) and found it to be 12.4 L kg^{-1}, which is 10-fold higher than that of a smaller dose (1.6 mg kg^{-1}, V_{ss} = 1.23 L kg^{-1})[17]. This suggests nonlinear pharmacokinetics, with more naloxone distributed to tissues at higher doses. Nonlinear pharmacokinetics was also indicated by the reduced clearance (CL = 1.3 L/hr/kg for high dose vs. 3.4 L/hr/kg for low dose), resembling toxicokinetic profiles seen in opioid overdose cases, where zero-order elimination dominates. In zero-order elimination, a

small dose increase in opioids can disproportionately raise plasma levels, leading to intoxication, as a constant amount of the drug is eliminated over time. This contributes to prolonged and severe opioid toxicity where standard pharmacokinetic parameters often become irrelevant[3].

[0550] For the efficacy study (**Fig. 4g-i**; antagonists administered at 60 μmol kg^{-1}; single i.v. dose), while both treatments reversed OIRD after the first fentanyl dose, naloxone-treated rats experienced significant hyperventilation and severe convulsions, unlike AG10-L-E2-Naloxone-treated animals (**Fig. 4g**). 4 rats died in the vehicle group. Both treatments showed high plasma naloxone concentrations at 6 hr (750 nM for naloxone and 60 nM for naloxone released from AG10-L-E2-Naloxone) and at 24 hr (395 nM and 44 nM, respectively). However, neither treatment effectively protected against OIRD after the second (6 hr) and third (24 hr) fentanyl doses (**Fig. 4h, i**). These findings suggest that very high initial naloxone levels may harm animals or alter the μ-opioid receptors, reducing the effectiveness of naloxone over time. There have been rare reports of fatal naloxone-induced pulmonary edema in humans at high doses[46,47]. We believe prodrugs like AG10-L-E2-Naloxone could help investigate these effects further.

AG10-L-E2-Naloxone is a potential preventive and prophylactic agent against fentanyl induced mortality and OIRD

[0551] To compensate for the lower efficacy of AG10-L-E2-Naloxone in the first 30 min after dosing, we combined the prodrug with a small amount of free naloxone. A single s.c. dose of AG10-L-E2-Naloxone (60 μmol kg^{-1}) and naloxone (3 μmol kg^{-1}; equivalent to 1 mg kg^{-1}) provided excellent efficacy, fully reversing OIRD after all three fentanyl doses without causing severe hyperventilation or convulsions, indicating a low risk of withdrawal symptoms (**Fig. 5a-d**).

[0552] We also explored AG10-L-E2-Naloxone as a prophylactic against fentanyl. Rats received AG10-L-E2-Naloxone (60 μmol kg^{-1}; s.c.) or naloxone (15 μmol kg^{-1} and 60 μmol kg^{-1}; s.c.) (**Fig. 5e-g**). Minute Volume remained unchanged compared to vehicle-treated animals (**Fig. 5f**). After 6 hr, rats were challenged with fentanyl (1.5 mg kg^{-1}; s.c.). AG10-L-E2-Naloxone significantly protected rats against OIRD, with all animals surviving. In contrast, both naloxone doses were ineffective, with multiple deaths in each group (**Fig. 5g**). This data further supports our hypothesis that higher doses of naloxone negatively impact efficacy and increase vulnerability to fentanyl toxicity, even with significant amounts of

naloxone present. These results highlight the challenges of using higher doses of naloxone for fentanyl overdose and support the potential of AG10-L-E2-Naloxone as a more effective antidote.

Multiple small doses of naloxone provide better efficacy against fentanyl-induced OIRD

[0553] Chronic opioid antagonist treatment leads to an increase in μ-opioid receptors density (receptor upregulation) without changing receptor affinity, resulting in heightened sensitivity to opioid agonists[48]. In one study, a 7-day naloxone infusion in mice showed dose-dependent receptor upregulation. While low s.c. doses (0.1–1 mg/kg/day) caused minimal changes, a high dose of 10 mg/kg/day resulted in an 80% increase in μ-opioid receptor density. This upregulation also boosted morphine's analgesic potency, indicating functional super-sensitivity[49]. In addition, animal studies suggest that chronic naltrexone treatment can lead to μ-opioid receptor upregulation, which may heighten overdose susceptibility, though clinical evidence remains inconclusive[50,51].

[0554] In our prophylaxis study (**Fig. 5e-g**), rats received s.c. doses of AG10-L-E2-Naloxone (60 μmol kg^{-1}) or naloxone (15 or 60 μmol kg^{-1}) and were then challenged with fentanyl after 6 hr. While neither dose of naloxone prevented respiratory depression at the six-hour mark, AG10-L-E2-Naloxone successfully did, despite having similar naloxone levels to the 60 μmol kg^{-1} dose of naloxone (**Fig. 4a**). The low naloxone dose (15 μmol kg^{-1}) was ineffective, likely due to the absence of naloxone at 6 hr (**Fig. 3a**). However, the lack of efficacy for the high naloxone dose (60 μmol kg^{-1}) was surprising. We hypothesize that the high initial concentration observed in high naloxone dose (C_{max} = 6,640 nM; **Table 3**) may cause acute μ-opioid receptor upregulation, leading to increased sensitivity to fentanyl. In contrast, AG10-L-E2-Naloxone provides a steady, low naloxone level (C_{max} = 36 nM; concentration ~10–30 nM over 96 hr), potentially avoiding such receptor alterations and preventing super-sensitivity. This is supported by data in **Fig. 4f-i** and **Table 4**, which show that very high intravenous (i.v.) doses (60 μmol kg^{-1}) of naloxone (C_{max} = 54,396 nM) and AG10-L-E2-Naloxone (C_{max} for released naloxone = 2,026 nM) were effective after the initial fentanyl dose but failed to provide protection against subsequent second and third fentanyl doses. These results are unexpected since μ-opioid receptor upregulation has been only reported when opioid antagonists are chronically administered.

[0555] These findings suggest that effective overdose prevention may require both: avoiding high-naloxone plasma concentration spikes and importantly maintaining consistent naloxone levels to counteract opioid effects. This hypothesis was supported by our studies with multiple smaller i.v. doses of naloxone and AG10-L-E2-Naloxone (**Fig. 13**). Treating rats with six small i.v. doses of naloxone (0.2 mg kg^{-1} per dose) or two i.v. doses of AG10-L-E2-Naloxone (15 μmol kg^{-1}) effectively protected the rats against respiratory depression after both the first and second fentanyl doses. Comparing this with data from 100-fold higher i.v. doses of naloxone (**Fig. 4g-i**), it's evident that low doses provide superior efficacy, likely because they avoid major receptor upregulation. This finding is consistent with the mortality study using multiple small s.c. doses (**Fig. 11**) and support our hypothesis that for effective protection against re-narcotization, two factors are essential: the presence of naloxone and minimal receptor upregulation (potentially due to spikes in naloxone concentration), reducing vulnerability to opioid toxicity. We hypothesize that this is uniquely achievable with AG10-L-E2-Naloxone depot, as it sustains efficacy without triggering receptor upregulation often associated with traditional naloxone use.

Preclinical evaluation of AG10-L-E2-Naloxone

[0556] We evaluated the effect of AG10-L-E2-Naloxone on body weight in three groups of rats (**Fig. 5h**). Group one received a vehicle, group two received AG10-L-E2-Naloxone (60 μmol kg^{-1}), and group three received fentanyl followed by AG10-L-E2-Naloxone and additional fentanyl doses at 6 hr and 24 hr. Over four weeks, all animals gained expected body weight without toxicity or mortality, showing no significant differences between the AG10-L-E2-Naloxone and vehicle groups. High concentrations of AG10-L-E2-Naloxone and its metabolite AG10-L-E2-COOH (100 μM) showed no cytotoxic effects on three cell lines (**Fig. 5i-k**). We also confirmed that AG10-L-E2-Naloxone was stable in aqueous solution for at least two months at room temperature.

[0557] The off-target pharmacology of AG10-L-E2-Naloxone (tested at 10 μM; which ~12-fold higher than the C_{max} of the highest s.c. dose in rats) was assessed using a panel of 87 targets (Eurofins SafetyScreen87 panel). This panel included receptors, ion channels, enzymes, or transporters involved in a wide range of biological processes. AG10-L-E2-Naloxone displayed excellent selectivity with IC_{50} >10 μM for 82 targets. It bound moderately to μ-opioid (IC_{50} = 0.43 μM) and κ-opioid (IC_{50} = 1.1 μM) receptors (which is much weaker binding than naloxone; IC_{50} ~1 nM), and weakly to M2 (IC_{50} = 8.8 μM) and

M4 (IC_{50} = 5.5 μM) muscarinic receptors, with moderate binding to M1 (IC_{50} = 0.46 μM) (**Table 1**). Since neither AG10-L-E2-Naloxone nor its metabolite (AG10-L-E2-COOH) cross the BBB (**Fig. 3b,c**), no issues are expected with binding to the M1 muscarinic receptor, which is mainly expressed in the brain[52].

[0558] To evaluate the potential for translating our data to humans, we evaluated AG10-L-E2-Naloxone in non-human primates (cynomolgus monkeys, n = 3). Monkeys are suitable models for predicting human pharmacokinetics for CES substrates, as they, like humans, lack CES enzymes in their blood[25] (**Fig. 2f**). Monkey CES enzymes have high sequence similarity (86-97%) and similar liver expression levels to humans[25]. Our results showed that naloxone release from AG10-L-E2-Naloxone in monkey liver microsomes is similar to humans (**Fig. 2d**). Monkeys were also used to test antidote efficacy against potent opioids like fentanyl and carfentanil[6,53]. Three monkeys received single s.c. doses of AG10-L-E2-Naloxone (35 μmol kg^{-1}; equivalent to 34 mg kg^{-1}; which is ~half the dose in rats), and plasma concentrations were measured over 72 hr. AG10-L-E2-Naloxone showed rapid absorption (C_{max} of 2,392 nM at 15 min) with a steady concentration range (303 nM to 141 nM) from 4 to 72 hr (**Fig. 5l** and **Table 5**). The released naloxone from AG10-L-E2-Naloxone showed a steady profile (3 nM at 15 min to C_{max} of 12.5 nM at 8 hr), maintaining around 5 nM from 24 hr to 72 hr. The lower concentration of released naloxone compared to its parent prodrug, AG10-L-E2-Naloxone, fits with our in vitro data. This highlights the prodrug's stability in monkey plasma while allowing naloxone release through CES2 activity in the liver and potentially other CES2-active organs. These concentrations align with clinically used naloxone formulations (intramuscular and s.c. doses of 0.4 mg to 2 mg results in C_{max} of 2.6 nM and 11 nM, respectively). These data indicate that AG10-L-E2-Naloxone is an excellent prophylactic antidote. Combining AG10-L-E2-Naloxone with a small amount of free naloxone could be an ideal reversal and prophylactic opioid antidote.

Tables

Table 1: AG10-L-E2-Naloxone interactions with 87 primary molecular targets

ASSAY NAME	IC50
Specific binding	
mu (MOP) (h) (agonist radioligand)	**0.43 µM**
M1 (h) (antagonist radioligand)	**0.46 µM**
kappa (h) (KOP) (agonist radioligand)	**1.1 µM**
M4 (h) (antagonist radioligand)	**5.5 µM**
M2 (h) (antagonist radioligand)	**8.8 µM**
mGluR5 (h) (agonist radioligand)	>10 µM
5-HT transporter (h) (antagonist radioligand)	>10 µM
5-HT1A (h) (agonist radioligand)	>10 µM
5-HT1B (h) (antagonist radioligand)	>10 µM
5-HT2A (h) (agonist radioligand)	>10 µM
5-HT2B (h) (agonist radioligand)	>10 µM
5-HT2C (h) (antagonist radioligand)	>10 µM
5-HT3 (h) (antagonist radioligand)	>10 µM
A1 (h) (antagonist radioligand)	>10 µM
A2A (h) (agonist radioligand)	>10 µM
Adenosine Guinea Pig Transporter Mass Spectrometry Binding (Antagonist Ligand) Assay, Cerep	>10 µM
alpha1A (h) (antagonist radioligand)	>10 µM
alpha1B (h) (antagonist radioligand)	>10 µM
alpha1D (h) (antagonist radioligand)	>10 µM
alpha2A (h) (antagonist radioligand)	>10 µM
alpha2B (h) (antagonist radioligand)	>10 µM
AMPA (agonist radioligand)	>10 µM
AR(h) (agonist radioligand)	>10 µM
AT1 (h) (antagonist radioligand)	>10 µM
B2 (h) (agonist radioligand)	>10 µM
beta1 (h) (agonist radioligand)	>10 µM
beta2 (h) (antagonist radioligand)	>10 µM
BZD (central) (agonist radioligand)	>10 µM
Ca2+ channel (L dihydropyridine site) (antagonist radioligand)	>10 µM
Ca2+ channel (L diltiazem site) (benzothiazepines) (antagonist radioligand)	>10 µM
Ca2+ channel (L verapamil site) (phenylalkylamine) (antagonist radioligand)	>10 µM
Ca2+ channel (N) (antagonist radioligand)	>10 µM
CB1 (h) (agonist radioligand)	>10 µM
CB2 (h) (agonist radioligand)	>10 µM
CCK1 (CCKA) (h) (agonist radioligand)	>10 µM
CCK2 (CCKB) (h) (agonist radioligand)	>10 µM
CCR1 (h) (agonist radioligand)	>10 µM
Cl- channel (GABA-gated) (TBOB site) (antagonist radioligand)	>10 µM
CXCR2 (IL-8B) (h) (agonist radioligand)	>10 µM
CysLT1 (LTD4) (h) (agonist radioligand)	>10 µM
D1 (h) (antagonist radioligand)	>10 µM
D2L (h) (antagonist radioligand)	>10 µM
D2S (h) (agonist radioligand)	>10 µM
delta (DOP) (h) (agonist radioligand)	>10 µM
dopamine transporter (h) (antagonist radioligand)	>10 µM

ASSAY NAME	IC50
Specific binding	
mu (MOP) (h) (agonist radioligand)	0.43 μM
M1 (h) (antagonist radioligand)	0.46 μM
kappa (h) (KOP) (agonist radioligand)	1.1 μM
M4 (h) (antagonist radioligand)	5.5 μM
M2 (h) (antagonist radioligand)	8.8 μM
mGluR5 (h) (agonist radioligand)	>10 μM
5-HT transporter (h) (antagonist radioligand)	>10 μM
5-HT1A (h) (agonist radioligand)	>10 μM
5-HT1B (h) (antagonist radioligand)	>10 μM
5-HT2A (h) (agonist radioligand)	>10 μM
5-HT2B (h) (agonist radioligand)	>10 μM
5-HT2C (h) (antagonist radioligand)	>10 μM
5-HT3 (h) (antagonist radioligand)	>10 μM
A1 (h) (antagonist radioligand)	>10 μM
A2A (h) (agonist radioligand)	>10 μM
Adenosine Guinea Pig Transporter Mass Spectrometry Binding (Antagonist Ligand) Assay, Cerep	>10 μM
alpha1A (h) (antagonist radioligand)	>10 μM
alpha1B (h) (antagonist radioligand)	>10 μM
alpha1D (h) (antagonist radioligand)	>10 μM
alpha2A (h) (antagonist radioligand)	>10 μM
alpha2B (h) (antagonist radioligand)	>10 μM
AMPA (agonist radioligand)	>10 μM
AR(h) (agonist radioligand)	>10 μM
AT1 (h) (antagonist radioligand)	>10 μM
B2 (h) (agonist radioligand)	>10 μM
beta1 (h) (agonist radioligand)	>10 μM
beta2 (h) (antagonist radioligand)	>10 μM
BZD (central) (agonist radioligand)	>10 μM
Ca2+ channel (L dihydropyridine site) (antagonist radioligand)	>10 μM
Ca2+ channel (L diltiazem site) (benzothiazepines) (antagonist radioligand)	>10 μM
Ca2+ channel (L verapamil site) (phenylalkylamine) (antagonist radioligand)	>10 μM
Ca2+ channel (N) (antagonist radioligand)	>10 μM
CB1 (h) (agonist radioligand)	>10 μM
CB2 (h) (agonist radioligand)	>10 μM
CCK1 (CCKA) (h) (agonist radioligand)	>10 μM
CCK2 (CCKB) (h) (agonist radioligand)	>10 μM
CCR1 (h) (agonist radioligand)	>10 μM
Cl- channel (GABA-gated) (TBOB site) (antagonist radioligand)	>10 μM
CXCR2 (IL-8B) (h) (agonist radioligand)	>10 μM
CysLT1 (LTD4) (h) (agonist radioligand)	>10 μM
D1 (h) (antagonist radioligand)	>10 μM
D2L (h) (antagonist radioligand)	>10 μM
D2S (h) (agonist radioligand)	>10 μM
delta (DOP) (h) (agonist radioligand)	>10 μM
dopamine transporter (h) (antagonist radioligand)	>10 μM

ER Alpha Human Estrogen NHR Binding (Agonist radioligand) Assay, Cerep	>10 μM
ETA (h) (agonist radioligand)	>10 μM
GABA transporter (antagonist radioligand)	>10 μM
GABAA1 (h) (alpha1,beta2,gamma2) (agonist radioligand)	>10 μM
GABAB(B1b/B2) Human GABA B GPCR Mass Spectrometry Binding (Antagonist Ligand) Assay, Cerep	>10 μM
Glutamate (Non-Selective) Rat Ion Channel Glycine (Strychnine-Insensitive),Mass Spectrometry Binding	>10 μM
GR (h) (agonist radioligand)	>10 μM
H1 (h) (antagonist radioligand)	>10 μM
H2 (h) (antagonist radioligand)	>10 μM
kainate (agonist radioligand)	>10 μM
KV channel (antagonist radioligand)	>10 μM
M3 (h) (antagonist radioligand)	>10 μM
MAO-A (antagonist radioligand)	>10 μM
MC1 (agonist radioligand)	>10 μM
MC4 (h) (agonist radioligand)	>10 μM
N muscle-type (h) (antagonist radioligand)	>10 μM
N neuronal alpha4beta2 (h) (agonist radioligand)	>10 μM
NK1 (h) (agonist radioligand)	>10 μM
NMDA (antagonist radioligand)	>10 μM
Non-Selective Rat Glycine Ion Channel Strychnine Mass Spectrometry Binding Assay, Cerep	>10 μM
norepinephrine transporter (h) (antagonist radioligand)	>10 μM
PAF (h) (agonist radioligand)	>10 μM
PCP (antagonist radioligand)	>10 μM
Potassium Channel hERG (human)- [3H] Dofetilide	>10 μM
PPARgamma (h) (agonist radioligand)	>10 μM
PR (h) (agonist radioligand)	>10 μM
RARalpha (h) (agonist radioligand)	>10 μM
Sodium Channel Site2 (Non-selective) Rat Ion Channel Batrachotoxin Mass Spectrometry Binding	>10 μM
V1a (h) (agonist radioligand)	>10 μM
Y1 (h) (agonist radioligand)	>10 μM
Enzymatic activity	
ACE (h)	>10 μM
acetylcholinesterase (h)	>10 μM
ATPase (Na+/K+)	>10 μM
cathepsin G (h)	>10 μM
COX1(h)	>10 μM
COX2(h)	>10 μM
IR (INSR) Human RTK Kinase Enzymatic Radiometric [Km ATP]	>10 μM
Lck Human TK Kinase Enzymatic Radiometric Assay [Km ATP]	>10 μM
MAO-B (h) recombinant enzyme	>10 μM
PDE3A (h)	>10 μM
PDE4D2 (h)	>10 μM
PKCalpha Human PKC Kinase Enzymatic Radiometric [Km ATP]	>10 μM

Table 2: Rat pharmacokinetic parameters of naloxone, AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone (single subcutaneous dose of each compound at 15 μmol kg^{-1}). Statistical differences were determined using one-way ANOVA followed by Holm-Šídák multiple comparison test. For $t_{1/2}$, $F(2, 6) = 41.50$, $P = 0.0003$ (*$P < 0.02$, **$P < 0.0002$ compared to Naloxone); for C_{max}, $F(2, 6) = 83.01$, $P < 0.0001$ (*$P < 0.0001$, **$P < 0.0001$ compared to Naloxone) ; for $AUC_{0\text{-}24h}$, $F(2, 6) = 49.33$, $P < 0.0002$ (*$P < 0.002$, **$P < 0.002$

compared to Naloxone); for AUC_{last}, F(2, 6) = 50.50, P < 0.0002 (*P < 0.001, **P < 0.02 compared to Naloxone). Data are presented as mean (± s.d.) (n = 3 rats per group).

	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (nM)	AUC_{0-24h} (nM.h)	AUC_{last} (nM.h)
Naloxone	0.63 ± 0.03	0.67 ± 0.29	1390 ± 186.82	1822.50 ± 219.95	1794.04 ± 216.97
AG10-L-E2-Naloxone	11.96 ± 0.40*	0.5 ± 0.43	441.65 ± 136.66*	3361.52 ± 626.30*	4531.50 ± 825.30*
Naloxone from AG10-L-E2-Naloxone	33.99 ± 7.89**	1.5 ± 2.17	16.90 ± 1.50**	252.70 ± 15.74**	584.73 ± 21.44**

Table 3: Rat pharmacokinetic parameters of naloxone, AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone (single subcutaneous dose of each compound at 60 μmol kg^{-1}). Statistical differences were determined using one-way ANOVA followed by Holm-Šídák multiple comparison test. For C_{max}, F(2,6) = 113.1, P < 0.0001 (*P < 0.0001, **P < 0.0001 compared to Naloxone); for AUC_{0-24h}, F(2,6) = 58.69, P < 0.0001 (*P < 0.0002, **P < 0.0001 compared to Naloxone); for AUC_{last}, F(2,6) = 52.62, P < 0.0002 (**P < 0.0001 compared to Naloxone). Data are presented as mean (± s.d.) (n = 3 rats per group).

	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (nM)	AUC_{0-24} (nM.h)	AUC_{last} (nM.h)
Naloxone	159.52 ± 208.66	0.33 ± 0.14	6640.80 ± 1010.81	10844.56 ± 1662.81	11164.10 ± 1627.24
AG10-L-E2-Naloxone	44.92 ± 14.82	0.43 ± 0.51	910.25 ± 45.60*	4077.51 ± 590.11*	8856.04 ± 1220.35
Naloxone from AG10-L-E2-Naloxone	189.02 ± 167.84	2.33 ± 1.53	36.20 ± 10.40**	2293.40 ± 85.76**	1721.29 ± 85.76**

Table 4: Rat pharmacokinetic parameters of naloxone, AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone (single intravenous dose of each compound at 60 μmol kg^{-1}). Statistical differences were determined using one-way ANOVA followed by Holm-Šídák multiple comparison test. For C_{max}, F(2,6) = 24.65, P < 0.001 (*P < 0.002 compared to Naloxone); for AUC_{0-24h}, F(2,6) = 77.96, P < 0.0001 (*P < 0.0001 compared to Naloxone); for AUC_{last}, F(2,6) = 77.96, P < 0.0001 (*P < 0.0001 compared to Naloxone). data are presented as mean (± s.d.) (n = 3 rats per group).

	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (nM)	AUC_{0-24h} (nM.h)	AUC_{last} (nM.h)
Naloxone	30.32 ± 25.94	0.03 ± 0.0	54395.99 ± 17006.60	34687.08 ± 11427.64	34687.08 ± 11427.64
AG10-L-E2-Naloxone	19.51 ± 14.37	0.03 ± 0.0	459973 ±150486.60*	202874.20 ± 34561.75*	202874.20 ± 34561.75*
Naloxone from AG10-L-E2-Naloxone	11.05 ± 4.70	0.10 ± 0.13	2026.22 ± 450.84	3707.41 ± 903.11	3707.41 ± 903.11

Table 5: Monkey pharmacokinetic parameters of AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone (single subcutaneous dose of 35 mg kg^{-1}; equivalent to 34 μmol kg-^{1}). Data are presented as mean (± s.d.) (n = 3 monkeys per group).

	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (nM)	AUC_{0-24hr} (hr*ng/ml)	AUC_{last} (hr*ng/ml)
AG10-L-E2-Naloxone	44.55 ± 10.96	0.33 ± 0.14	2603.33 ± 207.44	8750 ± 2028.82	18533.33 ± 2000.83
Naloxone from AG10-L-E2-Naloxone	67.93 ± 14.95	13.33 ± 9.24	4.25 ± 2.35	70.97 ± 21.73	168.67 ± 17.10

Table 6: Mass spectrometer conditions for multiple reaction monitoring (MRM) of the tested compounds.

Compound Name	Precursor ion	Product ion	Dwell	Fragmentor (volts)	Collision energy (volts)	Cell accelerator voltage (volts)	Polarity
Naloxone-D5 (ISTD)	333.2	315.1	200	116	18	4	Positive
Naloxone	328.2	310.1	200	136	18	4	Positive
AG10-L-E2-Naloxone	1031.5	704.4	200	190	50	4	Positive
AG10-L-E2-COOH	722.4	557.3	200	165	40	4	Positive

Discussion

[0559] Naloxone is an essential tool for reversing opioid overdoses. On the street, it is typically administered via nasal spray or intramuscular/subcutaneous injection by bystanders or first responders. Due to naloxone's short half-life of 30 to 90 minutes, its effectiveness is often limited, necessitating multiple doses to prevent re-narcotization by fentanyl and its synthetic analogues. Patients generally require ongoing monitoring, and naloxone should be administered every 2 to 3 minutes for patients who relapse[34]. Failure to monitor the patient until emergency medical services (EMS) arrive, especially after high fentanyl or synthetics overdose, could result in death. For patients who relapse after multiple doses, EMS can provide oxygen and additional intravenous doses (if i.v. access is quickly available), with the dosage adjusted based on the patient's response during transport to the hospital.

[0560] In a hospital setting, patients receive close monitoring to address potential re-narcotization, especially with long-acting opioids. The precise naloxone dose needed for opioid reversal varies based on the amount and type of opioid ingested, its receptor affinity, the patient's weight and age, comorbidities, and any polysubstance use. Because this information is often unknown, an empirical dosing approach is necessary. For adults, initial doses range from 0.4 mg to 2 mg, with increases every 2 to 3 minutes as needed, up to a total of 10 mg. Patients with rebound toxicity are often moved to the intensive care unit (ICU), where they are administered continuous naloxone infusions. This approach extends ICU stays, increases costs, and adds to healthcare resource demands, as patients typically require several hours or even overnight observation to ensure the effects of the overdose do not re-emerge once naloxone dissipates.

[0561] A promising alternative to traditional naloxone would be a single-dose extended-release naloxone (e.g., AG10-L-E2-Naloxone). AG10-L-E2-Naloxone or its analogues could offer sustained opioid antagonism, minimizing the need for frequent re-dosing and continuous infusions. It would also be highly advantageous in rural or remote areas, as it allows time for EMS assistance to arrive without requiring urgent, continuous naloxone administration. The extended-release format of AG10-L-E2-Naloxone offers flexibility for both opioid-naive and opioid-dependent patients. Opioid-naive individuals, who lack tolerance, typically require higher naloxone doses (0.4 to 2 mg) for effective reversal, which could be potentially addressed by co-administering small dose of free naloxone with AG10-L-E2-Naloxone in a single s.c. injection. In contrast, opioid-dependent individuals benefit from lower naloxone doses (0.1 to 0.2 mg) to avoid severe withdrawal symptoms and therefore AG10-L-E2-Naloxone could be particularly advantageous for opioid-dependent groups, as the concentration of released naloxone is low and steady. Extended-release naloxone can potentially heighten withdrawal symptoms (something we did not observe with our efficacy studies in rats) but it does offer sustained protection against re-narcotization, which is crucial for overdose management. In essence, withdrawal might be unavoidable, but steady, extended naloxone dosing can offer better overdose protection.

[0562] By bridging the gap in pre-hospital settings (and potentially in hospitals), a single s.c. dose of AG10-L-E2-Naloxone can provide prolonged protection and reduce healthcare burdens, ultimately improving overdose management for diverse populations especially in mass casualty scenarios where quick, personalized care and continuous monitoring isn't feasible. A multi-day extended-release naloxone that does not require refrigeration could also provide crucial opioid protection for military personnel and high-risk groups, reducing the need for frequent dosing and immediate medical support.

[0563] Managing OIRD is feasible with trained medical personnel and appropriate equipment but challenging in emergencies like battlefields or rural areas. Naloxone and nalmefene are difficult to deploy effectively in mass casualty situations involving synthetic opioids, prompting the NIH to call for stronger, longer-acting antagonists[4]. Current long-acting injectable strategies, such as extended-release naltrexone (Vivitrol), require deep intramuscular administration by healthcare professionals and have slow drug release and absorption, making them impractical for opioid overdose scenarios[54]. Despite their long in vivo half-life, antibodies face challenges as opioid antidotes due to their limited ability to cross the blood-brain barrier, delayed onset, high production costs, and the need for

refrigeration. These issues restrict their practicality in emergencies where rapid, central nervous system action is crucial[55].

[0564] Our AG10-based prodrug has several unique features making it an effective opioid antidote. It forms a s.c. depot and binds to TTR, extending its DOA for at least 4 days. The balanced hydrophilicity/lipophilicity ensures rapid absorption and effective rescue. It avoids brain spikes of free naloxone, reducing life-threatening withdrawal symptoms. The modular approach allows for tunable hydrolysis rates for rescue, protection (prophylaxis), or both. Its low cost, ease of synthesis, and chemical stability offer a practical solution with extended shelf-life and no need for refrigeration (a requirement for Vivitrol and antibody-based therapeutics). The Department of Defense has approved a high-dose naloxone (10 mg) autoinjector for non-medically trained individuals to protect service members from synthetic opioids like fentanyl. The benefit versus risk of increasing the naloxone dose has been debated[34,56,57]. Our prodrugs could be useful mechanistic probes to investigate the potential harm of higher naloxone doses. A limitation of this study is the exclusive use of male rats and monkeys, which may not fully capture potential sex-specific differences in pharmacokinetics and efficacy.

[0565] Our strategy extends beyond opioid antidotes. For example, the prodrugs can replace naloxone in opioid abuse deterrent drugs like suboxone. Controlled and slow release of antagonists can reduce withdrawal symptoms, benefiting neonates, children, the elderly, and those with cardiovascular issues[3]. It is also useful in post-surgical recovery where gradual analgesia loss is preferred. This is critical when the analgesic is morphine, which has a half-life of 2-3 hr but can result in prolonged overdose durations of up to 120 hr[3]. The extended duration of a single s.c. injection would also reduce staff burden and medical costs, such as the need for 24-hr hospital monitoring for children with opioid overdose[3]. Overall, our strategy could significantly improve therapeutic outcomes for various opioid antagonists and agonists.

Methods

Study design and ethical approval

[0566] Our research complies with all ethical regulations, and all animal study protocols were approved prior to study initiation. Approximately 6-8 weeks old normal male Sprague-

Dawley rats (226-250 g each) from Charles River Laboratories Inc., Hollister, CA, USA were used for different animal studies. For the jugular vein cannulated rats, pre and post-operative care were performed according to the Charles River Institutional Animal Care and Use Committee (IACUC) policy. Rats were housed individually in standard polycarbonate disposable rat cages with pre-bedded corn cob and wood shavings. The animal room environment was maintained at 64-79°F, 50-70% relative humidity, and automatic 12-hr light / 12-hr dark cycle (light hours: 7:00 am-7:00 pm). All the animals were acclimated for 5 days upon arrival. They were provided with a standard laboratory diet and normal tap water ad libitum. The animal studies were conducted in accordance with National Institutes of Health (NIH) guidelines for the care and use of live animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at University of the Pacific. The IACUC protocol 24R03, 22R02, and 21R06 were used in this study.

[0567] The cynomolgus monkey pharmacokinetic study was outsourced to Illinois Institute of Technology Research Institute (IITRI), a preclinical contract research organization (CRO) that provides services that support FDA submissions. No animals were euthanized at the end of the study, and the study did not have humane endpoints. Three male non-human primates (cynomolgus monkey) from the IITRI holding colony were used for this study. The animals were originally obtained from Worldwide Primates, Inc. (Miami, FL). All cynomolgus monkeys were approximately 6-13 years old at dosing initiation. Body weights used for dose calculations for the three animals assigned to the study were 8.76, 6.86 and 7.42 kg. Each animal was identified by a letter and/or number combination that was unique within the study. This identification was tattooed on the ear of each cynomolgus monkey. Positive identification was required, at a minimum, after each cage change, dosing and blood sampling. Cynomolgus monkeys were individually housed in stainless steel cages that were equipped with automatic watering systems. The cages were cleaned daily. The cynomolgus monkeys were kept in a temperature- and humidity-controlled room. As specified in the relevant facility Standard Operating Procedure, animal rooms were held within a temperature range of approximately 18-29°C and a relative humidity range of approximately 30-70% during this study. Fluorescent lighting in the animal rooms was provided on a cycle of 12 hours of light followed by 12 hours of darkness. Procedures for animal care and housing were in accordance with Animal Welfare Standards (Title 9, Code of Federal Regulations, Part 3, 1991 Revision) and standards set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Certified Primate Diet (Inotiv Teklad Certified Primate Diet, #2055C [Indianapolis/West Lafayette, IN]) was provided to the animals twice

daily. City of Chicago municipal water was available ad libitum. Based on analysis reports for the diet provided by the vendor and external water analysis reports, no contaminants were known to be present in the food or water at levels expected to interfere with the outcome of the study. To maintain their psychological well-being, animals were periodically offered toys and additional types of food (e.g., fresh fruit). The NHP work for our cynomolgus monkey study was approved by the IIT Research Institute (IITRI) Institutional Animal Care and Use Committee (IACUC).

Solubility of test compounds in potassium phosphate buffer (pH 7.4) and water

[0568] Stocks solutions (10 mM) of test compounds were prepared in DMSO and stored at -20°C. Compounds (200 µM) were incubated in 50 mM potassium phosphate buffer (pH 7.4) or water at 37°C for 72 hr. The samples were vortexed, spun for 5 min at 1600 x g, and supernatant was filtered. A sample aliquot (100 µL) was then removed and quenched by adding 200 µL of methanol containing 0.1% formic acid. The mixture was centrifuged again at 1600 x g for 5 minutes. Clear supernatant was then analyzed by analytical HPLC. Calibration curves for test compounds were generated by dissolving DMSO solution of test compounds in methanol containing 0.1% formic acid (concentrations at 200, 100, 50, 25, and 12.5 µM) and then analyzed by HPLC. Calibration curves were used to quantitate the concentration of soluble test compounds in the potassium phosphate buffer and water. Data represents the mean ± s.d. (n = 3 independent replicates).

Log $D_{7.4}$ determination using shake-flask assay

[0569] An aqueous potassium phosphate buffer (10 mM, pH 7.4) solution has been prepared and then saturated with n-octanol. Likewise, n-octanol was saturated with potassium aqueous phosphate buffer (pH 7.4). Both solutions were heavily vortexed and then left resting to ensure complete separation of the two phases. The shake-flask experiments were carried out at room temperature. Once the phases have been prepared, 1 mL of 1-octanol and 1 mL of the aqueous potassium phosphate buffer were added to a glass vial. 20 µL of test compound stock solution (10 mM stock solution in DMSO; 100 µM final concentration in the pre-saturated mixture) is added and the vial is shaken for one hour at room temperature. After one hour, the layers were allowed to separate and aliquots (100 µL) of each of the n-octanol and aqueous buffer layers were processed by adding 200 µL of methanol containing 0.1% formic acid. The mixture was centrifuged again at 1600 x g for 5 minutes. Clear supernatant was

then analyzed by analytical HPLC as described method. Calibration curves were used to quantitate the concentration of test compounds. Data represents the mean ± s.d. (n = 3 independent replicates).

[0570] The following equation was used to calculate Log $D_{7.4}$.

$$\text{Equation 1: Log } D_{7.4} = \text{Log } \frac{\text{concentration of compound in octanol phase}}{\text{concentration of compound in aqueous phase}}$$

Evaluation of binding affinity of test compounds to TTR in buffer

[0571] Prealbumin from human plasma (human TTR) (#P1742) was purchased from Sigma Aldrich. The binding affinity of AG10-L-E2-COOH, AG10-L-E2-Naloxone, and AG10-L-E1-Naloxone to TTR in buffer was determined by their ability to displace FP probe from TTR using a Fluorescence Polarization (FP) assay[20]. Serial dilutions of compounds (0.010 µM to 20 µM) were added to a solution of FP-probe (50 nM) and TTR (300 nM) in assay buffer (PBS pH 7.4, 0.01% TritonX100, 1% DMSO in 25 µL final volumes) in a 384-well plate. The samples were allowed to equilibrate by agitation on a plate shaker for 20 minutes at room temperature. Fluorescence polarization (excitation λ 485 nm, emission λ 525 nm, cutoff λ 515 nm) measurements were taken using a SpectraMax M5 Microplate Reader (Molecular Devices). SoftMax® Pro software v5.4.1 (Molecular Devices, Inc.) was used to collect fluorescence data. The IC50 values were obtained by fitting the data to the following Equation 2 [y = (A-D)/(1 + (x/C)^B) + D], where A = maximum FP signal, B = slope, C = apparent binding constant (K_{app}), and D = minimum FP signal. The binding constant (K_d) values were calculated using the Cheng–Prusoff equation from the IC_{50} values. Data represents the mean ± s.d. (n = 3 independent replicates).

Evaluation of binding affinity and selectivity of test compounds to TTR in human serum

[0572] Human serum (#H4522) was purchased from Sigma Aldrich. The binding affinity and selectivity of AG10-L-E2-COOH, AG10-L-E2-Naloxone, and AG10-L-E1-Naloxone to TTR were determined by their ability to compete with the binding of a fluorescent probe exclusion (FPE probe) binding to TTR in human serum. AG10 and tafamidis were used as controls. An aliquot (98 µL) of human serum was mixed with 1 µL of test compounds (1.0 mM stock

solution in DMSO; 10 μM final concentration in serum) and 1 μL of FPE probe (0.36 mM stock solution in DMSO; 3.6 μM final concentration in serum). The fluorescence changes (λ_{ex} = 328 nm and λ_{em} = 384 nm) were monitored every 15 min using a SpectraMax M5 microplate reader for 6 h at 25 °C. Data represent the mean ± s.d. (*n* = 4 independent replicates).

Human and rat plasma stability assay of test compounds

[0573] Test compounds (5 μM) were incubated in human or rat plasma at 37°C. 100 μL plasma samples were analyzed at 0 hr, 0.25 hr, 2 hr, 4 hr, and 24 hr. All the samples were processed by adding 200 μL of quenching buffer (methanol containing 0.1% formic acid) followed by centrifuging at 1600 x g for 5 minutes at 4°C. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 1600 x g for another 5 minutes at 4°C. The supernatant was analyzed by analytical HPLC. Data represents the mean ± s.d. (*n* = 3 independent replicates).

Metabolic study of AG10-L-E2-Naloxone by recombinant human carboxylesterases (CES) 1 and 2

[0574] The reaction mixture, consisting of CES1 (30 μg mL^{-1} or 100 μg mL^{-1}) or CES2 (30 μg mL^{-1}) in 50 mM potassium phosphate buffer, was incubated for 5 min at 30°C. The reaction was initiated by adding AG10-L-E2-Naloxone at a final concentration of 5 μM (or DMSO as a negative control). After incubation, 100 μL samples from the reaction mixture were quenched at 0 hr and 1 hr by adding 100 μL of quenching buffer (methanol containing 0.1% formic acid) to the samples to terminate the reaction at each designated time point. Samples were then centrifuged at 1600 x g for 5 minutes at 4°C. Supernatants (150 μL) were further diluted by adding 75 μL of water containing 0.1% formic acid. Samples were stored at −20°C until analysis by HPLC. Data represents the mean ± s.d. (*n* = *3* independent replicates).

Microsomal stability assay

[0575] AG10-L-E2-Naloxone Stock (2 μL of 1 mM in DMSO) were added to microcentrifuge tubes containing 3.2 μL acetonitrile and 194.8 μL potassium phosphate buffer (50 mM, pH 7.4, containing 3 mM $MgCl_2$). The assay tubes were preincubated at 37°C

for 10 min before liver microsomes addition. Human, monkey, or rat liver microsomes and NADPH regeneration solution (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase; concentration of 2 mg mL^{-1} and 2 mM, 10 mM, and 2 Unit mL^{-1}, respectively) were added to potassium phosphate buffer. The reaction mixtures were pre-warmed at 37°C for 10 min. The NADPH was generated using the rapidSTART NADPH regenerating system (Xenotech), by adding 0.7 mL of high purity water to the frozen components in the RapidStart vial, vortexing, and then adding the activated RapidStart solution to the incubation. 200 µL of NADPH regeneration solution were added to each pre-incubated assay tube already containing 200 µL AG10-L-E2-Naloxone in phosphate buffer. The final reaction concentrations for AG10-L-E2-Naloxone, microsomes, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase are 5 µM, 1 mg mL^{-1}, 1 mM, 5 mM, and 1 Unit mL^{-1}, respectively. The vials were incubated at 37°C. At 0 hr and 1 hr, 100 µL aliquots were taken and the microsomal reaction was terminated by addition of 100 µL of quenching buffer (methanol containing 0.1% formic acid and 15 ng mL^{-1} Naloxone-D_5 as the internal standard). Samples were centrifuged at 1600 x g for 5 min and supernatants (150 µL) were further diluted by adding 75 µL of water containing 0.1% formic acid. Samples were stored at −20°C until analysis using validated LC-MS/MS method using Triple Quadrupole mass spectrometer (QQQ). Data represents the mean ± s.d. (*n = 3* independent replicates).

Microsomal stability assay in presence of CES inhibitors

[0576] The microsomal assay described above was also performed in HLMs in presence of loperamide, selective CES2 inhibitor, or Digitonin, selective CES1 inhibitor (final concentration 100 µM). Loperamide or Digitonin were added to the microsomal buffer before addition into the test compound.

Rat plasma stability assay of AG10-L-E2-Naloxone in presence of CES inhibitors

[0577] Rat plasma was pre-incubated with either loperamide (100 µM), or digitonin (100 µM), or DMSO (negative control) for 5 minutes at room temperature. After that, AG10-L-E2-Naloxone (5 µM) was added to the incubation mixtures. Samples were assayed at 0 hr, 0.5 hr, and 1 hr. All the samples were processed by adding 200 µL of quenching buffer (methanol containing 0.1% formic acid) followed by centrifuging at 1600 x g for 5 minutes at 4°C. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 1600 x g for

another 5 min at 4°C. The supernatant was analyzed by analytical HPLC as described method. Data represents the mean ± s.d. (n = 3 independent replicates).

Monkey and rabbit plasma stability assay of AG10-L-E2-Naloxone

[0578] AG10-L-E2-Naloxone (5 µM) was incubated in cynomolgus monkey or rabbit plasma at 37°C. 100 µL samples were analyzed at 0 hr and 24 hr. All the samples were processed by adding 200 µL of quenching buffer (methanol containing 0.1% formic acid) followed by centrifuging at 1600 x g for 5 minutes at 4°C. The supernatant was kept in the -20°C freezer for 5 minutes and centrifuged again at 1600 x g for another 5 min at 4°C. The supernatant was analyzed by analytical HPLC. Data represents the mean ± s.d. (n = 3 independent replicates).

Human and rat plasma stability assay of newly synthesized aryl-based naloxone prodrugs, procaine, molnupiravir, and irinotecan in presence of CES inhibitors

[0579] Test compounds were pre-incubated (final concentration; 100 µM) with loperamide (100 µM) or digitonin (100 µM) as described above. Samples were assayed at 0 hr, 0.5 hr, and 1 hr in rat plasma with CES inhibitors and at 0 hr, 4 hr, and 24 hr in human plasma. All samples were processed and analyzed as mentioned above.

Rapid equilibrium dialysis (RED) of AG10-L-E2-Naloxone

[0580] A RED device with a 48-well single-use plate and 8K MWCO inserts (#90006) was purchased from Thermo Fisher Scientific. The assay was performed following the manufacturer's instructions. Briefly, 300 µL of AG10-L-E2-Naloxone (final concentration 5 µM), with or without human transthyretin (hTTR, final concentration 5 µM), was added to each sample chamber, and 550 µL of 100 mM potassium phosphate buffer (pH 7.4) was added to each buffer chamber. To confirm the effect of hTTR, AG10 (final concentration 10 µM) was added to one group in the sample chamber. The plate was sealed, incubated at 37 ± 1°C, and agitated at 250 rpm on an orbital shaker for 18 hr. After incubation, 100 µL from both the sample and buffer chambers were quenched with 100 µL of quenching buffer (methanol with 0.1% formic acid) and centrifuged at 1600 x g for 5 minutes at 4°C. The supernatant was analyzed by analytical HPLC method described. Data represents the mean± s.d. (n = 3 independent replicates).

[0581] The following equation was used to calculate the percentage of the test compound bound:

% Free = (Concentration buffer chamber/Concentration sample chamber) x 100%
% Bound = 100% – % Free

Overall survival of rats after LD_{50} doses of fentanyl or morphine

[0582] Male Sprague Dawley rats (225–250 g; 7-8 weeks old) were obtained from Charles River Laboratories. Rats were randomly assigned to three groups (vehicle, naloxone, and AG10-L-E2-Naloxone). Rats first received a s.c. dose of 100 mg kg^{-1} of morphine (*n = 9* per group). After 5 min of morphine dose, rats were administered a single s.c. dose of vehicle, naloxone (15 µmol kg^{-1}, equivalent to 5 mg kg^{-1}), or AG10-L-E2-Naloxone (15 µmol kg^{-1}, equivalent to 15.8 mg kg^{-1}). Naloxone and AG10-L-E2-Naloxone were dissolved in the same vehicle dosing solution. Morphine was dissolved in water. Volume of injections for morphine and antagonists were 200 and 500 µL, respectively. Rats were then put back into their cages under routine conditions. The number of rats that died and their approximate time of death was recorded. After 6 hr of the first morphine dose, all rats that survived received a second s.c. dose of 50 mg kg^{-1} morphine. Rats were placed back into their cages and observed to record any death. The total number of rats that died was recorded and survival rate was calculated in each group.

[0583] For the fentanyl mortality study, rats first received a s.c. dose 3 mg kg^{-1} of fentanyl (*n = 8* per group). After 5 min of fentanyl dose, rats were administered a single s.c. dose of vehicle, naloxone (15 µmol kg^{-1}, equivalent to 5 mg kg^{-1}), or AG10-L-E2-Naloxone (15 µmol kg^{-1}, equivalent to 15.8 mg kg^{-1}). Naloxone and AG10-L-E2-Naloxone were dissolved in the same vehicle dosing solution. Fentanyl was dissolved in water. Volume of injections for fentanyl and antagonists were 200 µL and 500 µL, respectively. Rats were then put back into their cages under routine conditions. The number of rats that died and their approximate time of death was recorded. After 6 hr of the first fentanyl dose, all rats that survived received a second s.c. dose of 3 mg kg^{-1} fentanyl. Rats were placed back into their cages and observed to record any death. The total number of rats that died was recorded and survival rate was calculated in each group.

Reversal and prevention of fentanyl induced respiratory depression in rats

[0584] The efficacy of antagonists to reverse fentanyl induced respiratory depression in rats was evaluated using whole body plethysmography apparatus from DSI Buxco (St. Paul, MN; with FinePointe software). Sprague-Dawley rats (225– 250 g; 7-8 weeks old) (*n = 6* per group) were habituated for 30 min to the plethysmography chambers located inside sound-attenuating closed room under a continuous flow of fresh air (2.2 l/min). Rats were awake and free moving in the testing chamber and during the monitoring period. For baseline measurements, rat respiration was recorded for 20 min prior to injections. Rats were then removed from the chambers and sequentially treated with fentanyl (3 mg kg^{-1}, single s.c. or i.v. dose) followed by a single s.c. or i.v. dose of antagonist one min later (15 µmol kg^{-1} or 60 µmol kg^{-1}). Rats were placed back in the chambers and the respiratory parameters were measured for 90 min. Rats were challenged with two additional s.c. or i.v. doses of fentanyl (1.5 mg kg^{-1} each) at 6 hr and 24 hr post antagonist dosing. The respiratory parameters were measured for 90 min after each fentanyl injection. Rat ventilation was measured by recording changes in chamber pressure which is then processed and digitized. For each rat, the software calculated the average Tidal Volume (V_T: milliliter per breath), Ventilatory Frequency (f: breaths per minute), and Minute Volume ($V_E = V_T$ * f: milliliter per minute). Since it is the product of V_T and f, Minute Volume was reported as it considered a quantifiable net physiological average that is scientifically relevant. Each rat's baseline Minute Volume data were averaged and specified as 100% of baseline values. Minute Volume readings after dosing were then normalized and reported for each rat in 5 min intervals based on the following equation: Minute Volume (% of control baseline) = Minute Volume at specific time/Minute Volume baseline * 100.

Prophylactic administration of antagonist to counter fentanyl induced respiratory depression

[0585] Rats (*n = 6* per group) were habituated for 30 min to the plethysmography chambers, and the baseline measurements were recorded as described above. Rats received a single s.c. dose vehicle, naloxone (15 µmol kg^{-1} and 60 µmol kg^{-1}), or AG10-L-E2-Naloxone (60 µmol kg^{-1}) and the respiratory parameters were measured for 90 min. After 6 hr, rats were challenged with single s.c. doses of fentanyl (1.5 mg kg^{-1}) and were placed back in the plethysmography chambers. The respiratory parameters were measured for 90 min and the data were processed and reported as described above. The treatment doses for morphine and fentanyl were determined in preliminary studies and were also based on published literature. The dose of morphine used (100 mg kg^{-1}) is within the reported LD_{50} of 64–140 mg kg^{-1} for morphine in rats. For fentanyl, we used the reported value of 3 mg kg^{-1}. These doses were

confirmed in preliminary experiments where we started with lower doses of morphine and fentanyl without observing any lethality. The overdose studies with morphine and fentanyl were approved by the University of the Pacific's IACUC.

Preliminary evaluation of AG10-L-E2-Naloxone toxicity in rats

[0586] The in vivo toxicity of AG10-L-E2-Naloxone was evaluated in rats. Adult male Sprague-Dawley rats (Charles River), body weight ranging 225-250 g, were used for the study. Three groups of rats were used. Group one was treated with a 500 µL of vehicle. Group two was treated with AG10-L-E2-Naloxone (60 µmol kg^{-1}; s.c. dose) dissolved in 500 µL of vehicle. Group three was treated with fentanyl (3 mg kg^{-1}; s.c. dose) followed by AG10-L-E2-Naloxone (60 µmol kg^{-1}) and then two doses of fentanyl (1.5 mg kg^{-1}; each s.c. dose) at 6 hr and 24 hr. The animal weights were monitored weekly for four weeks. Body weights represent mean ± s.d. (n= *6* biological replicates per group).

MTT cytotoxicity assay of test compounds against HeLa, Hep3B, and MCF-7 cells

[0587] Cell viability (MTT) assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay. HeLa and MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100-unit mL^{-1} and 100 µg/mL^{-1}, respectively), and 1% l-glutamine under the humidified atmosphere of 5% CO2 and 95% air at 37°C. Hep3B was cultured in minimum Eagle's medium with 10% FBS. The cells were grown to about 75% confluence and seeded into 96-well plates at ~5000 cells/well for HeLa and MCF-7 cells, and at ~8000 cells/well for Hep3B and then treated with naloxone, AG10-L-E2-Naloxone and AG10-L-E2-COOH (tested at 100 µM) or Doxorubicin (10 µM) as a positive control. Control cells were also treated with the appropriate concentration of the vehicle (DMSO). Cell viability was determined following the standard CellTiter 96 Non-Radioactive Cell Proliferation Assay protocol after 72 hr incubation at 37°C and 5% CO_2.

Off-target selectivity of AG10-L-E2-Naloxone

[0588] The off-target pharmacology of AG10-L-E2-Naloxone (tested at 10 µM) was assessed using a broad in vitro panel of 87 targets (Eurofins SafetyScreen87 panel, Cerep, France). This panel included receptors, ion channels, enzymes, or transporters involved in a wide range of biological processes (**Table 1**). The screen uses validated radio-ligand competition binding assays and enzymatic inhibition assays. Experimental conditions for each of these 87 assays are available at Eurofins web page. Effects of AG10-L-E2-Naloxone were evaluated

alongside known receptor ligands or substrates, with positive results defined as >50% inhibition of ligand binding.

Pharmacokinetic evaluation of subcutaneous dose of AG10-L-E2-Naloxone in male cynomolgus monkey

[0589] A single subcutaneous dose of 35 mg kg^{-1}; (equivalent to 34 µmol kg^{-1}) was administered on Day 1 in one shot on the lower back of each animal, using a constant dosing volume of 0.88 mL kg^{-1} body weight. Animals were observed at least twice daily for mortality or evidence of moribundity prior to initiation of dosing and following dose administration. Any abnormal clinical signs were to be recorded. Animals were weighed prior to dosing on Day 1. Blood samples (approximately 2-3 mL) for determinations of plasma levels were collected from the femoral vein of each animal on Day 1 at approximately 0.08-, 0.25-, 0.5-, 1-, 2-, 4-, 8-, 24-, 48- and 72-hr post-dose. EDTA was used as an anticoagulant. Following collection, blood samples were held on ice until processed to plasma within 30 minutes of collection by centrifugation and mixed with a 1:1 ratio of formic acid (10%) to stabilize. The resulting plasma samples were stored frozen (at approximately -70°C) until analyzed at IITRI using LC-MS/MS for concentration of the analytes AG10-L-E2-Naloxone and naloxone released from AG10-L-E2-Naloxone. The advanced LC-MS instruments at IITRI allowed for a lower limit of quantification (LLOQ) of 0.3 nM. Fluconazole and Naloxone-d5 were used as internal standards for AG10-L-E2-Naloxone and Naloxone, respectively. For the determination of AG10-L-E2-Naloxone and released Naloxone in plasma, a 100 µL aliquot from each sample was added to the appropriate well of a 96-well plate, followed by addition of 300 µL of internal standard-mix working solution (20 ng mL^{-1} each of Naloxone-d5 and Fluconazole in acetonitrile) to all samples for protein precipitation, except for the double blank and solvent blank where 300 µL acetonitrile was added for protein precipitation. The plate was sealed and vortex-mixed for two minutes, followed by centrifugation at 400 x g for 10 minutes at 4 ± 4°C. A portion of the resulting supernatant (200 µL) was transferred into another 96-well plate and evaporated to dryness under a stream of N2 at 40 ℃ followed by reconstitution with 150 µL of 20% acetonitrile in water. The plate was sealed and vortex-mixed for two minutes prior to instrumental analysis. Freshly prepared blanks, calibrators and QC samples were analyzed along with the study samples. Instrument calibrators and QC samples were prepared in acidified blank non-human primate plasma (sourced from BioIVT, Hicksville, NY; K2EDTA anticoagulant; stored frozen at -20°C; documentation is maintained with the study records; acidified with 10% formic acid at a 1:1 ratio of plasma:10% formic acid) using an appropriate working solution consisting of AG10-

L-E2-Naloxone and Naloxone. Nominal calibrator concentrations ranged from 1.00 to 1000 ng mL^{-1} and from 0.200 to 200 ng mL^{-1} for AG10-L-E2-Naloxone and Naloxone, respectively, while QC samples were prepared at nominal concentrations of 3.00, 400 and 800 ng mL^{-1} for AG10-L-E2-Naloxone and at 0.600, 80.0 and 160 ng mL^{-1} for Naloxone. Blank, calibrator and QC samples were processed for analysis following the extraction procedure described above. Samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to a method qualified at IITRI. Calibration curves (separate for AG10-L-E2-Naloxone and Naloxone) were calculated from the linear regression (weighting factor of 1/x2) of the analyte to internal standard peak area ratios versus the analyte concentrations. Concentrations of the analytes (AG10-L-E2-Naloxone and Naloxone) in the samples were determined using the peak area ratios and the regression parameters of the appropriate calibration curve. Plasma drug level concentrations were used to calculate PK parameters using the noncompartmental model for extravascular administration using Phoenix WinNonlin software (Version 8.3; Certara, Princeton, NJ). Nominal dose levels and blood collection times were used for PK analysis.

Example 12: Stability of Additional Naloxone Conjugates to Hydrolysis by Carboxylesterases

[0590] Additional conjugates comprising naloxone were prepared as described in Example 1. Rat plasma stability assay of the conjugates in presence of CES inhibitors was evaluated as described in Examples 4 and 11. **Figs. 14a-e** show the results of the stability assays for AG10-L-E2-naloxone, AG10-L-PHBA-E-naloxone, PEG linker-E2-naloxone, AG10-short-L-E2-naloxone, and AG10-L-meta-amino-E-naloxone, respectively. Similar to results with conjugates evaluated in prior examples, the hydrolysis all of the conjugates evaluated in this example were inhibited by the selective CES2 inhibitor loperamide. The extent of inhibition was dependent on the nature of the linker, linker length, and the orientation of the linker with respect to the ester moiety (e.g., meta vs. para substitution). None of the conjugates evaluated in the example were inhibited by the selective CES1 inhibitor digitonin.

[0591] The five conjugates evaluated in the rat plasma stability assay were also evaluated in a human plasma stability assay as described in Examples 4 and 11. The results are shown in **Fig. 15**. All of the para-aminobenzoic acids (PABA) ester conjugates were stable in human plasma, further substantiating that the conjugates are CES2 specific. Moreover, the p-hydroxybenzoic acid (PHBA) and meta-amino-benzoic acid were also somewhat CES2

selective. However. hydrolysis of these esters was observed at six hours, suggesting that hydrolysis is catalyzed by other enzymes in human plasma.

Example 13: Stability of Conjugates that Comprise Small Molecule and Peptide Drugs to Hydrolysis by Carboxylesterases

[0592] Additional conjugates comprising small molecule or peptide drugs were prepared as described in Example 1. Rat plasma stability assay of the conjugates in presence of CES inhibitors was evaluated as described in Examples 4 and 11. **Figs. 16a-f** show the results of the stability assays for AG10-L-E2-Paracetamol, AG10-L-E2-Propofol, AG10-L-E2-Estrone, AG10-L-E2-Endomorphin-2, AG10-L-E2-Tyr-ala, and AG10-L-E2-Dermorphin respectively. Hydrolysis of all of the conjugates evaluated in this example were inhibited by the selective CES2 inhibitor loperamide to a certain extent. None of the conjugates evaluated in the example were inhibited by the selective CES1 inhibitor digitonin.

[0593] The five conjugates evaluated in the rat plasma stability assay were also evaluated in a human plasma stability assay as described in Examples 4 and 11. The results are shown in **Fig. 17**. All of the conjugates were stable in human plasma, further substantiating that the conjugates are CES2 specific. Remarkably, minimal or no hydrolysis of the conjugates comprising the small molecules or peptides was observed even after six hours. Th results provide strong evidence that the methods disclosed herein are applicable to peptide drugs and small molecule drugs.

Example 14: Solubility of Conjugates at Physiological pH Levels

[0594] As set forth in Example 11, we evaluated the aqueous solubility of particular conjugates of the disclosure in water and phosphate buffer (pH 7.4, to mimic the pH of s.c. tissue). In this example, we evaluated the solubility of other conjugates in water and pH 7.4 buffer, including conjugates comprising small molecule or peptide drugs. The results of the solubility studies are shown in **Table 7**.

Table 7: Solubility of test compounds in water and in buffer.

Compound	Solubility in Water (pH 3.5) (μM)	Solubility in Buffer (pH 7.4) (μM)
Naloxone	200	170
AG10-L-E2-Naloxone	200	44
AG10-Short-L-E2-Naloxone	200	5
AG10-L-meta-amino-E-Naloxone	200	40

AG10-L-PHBA-E-Naloxone	200	42
PEG linker-E2-Naloxone	200	192
Propofol	3	3
AG10-L-E2-Propofol	18.5	0.4
Estrone	6	6
AG10-L-E2-Estrone	6.4	0.4
Paracetamol	175	192
AG10-L-E2-Paracetamol	153	14
Tyr-Ala	200	157
AG10-L-E2-Tyr-Ala	178	112
Endomorphin-2	166	159
AG10-L-E2-Endomorphin-2	187	10
Dermorphin	174	80
AG10-L-E2-Dermorpgin	190	20

[0595] The results in **Table 7** indicate that conjugating the zwitterionic AG10-linker to different classes of drugs reduces the solubility of drugs at pH 7.4. Without being bound by theory, the results suggest that the drug conjugates form precipitates at physiological pH. As a result, the conjugates effectively form a depot following subcutaneous administration, resulting in a prolonged action of the drugs.

REFERENCES

1 *National Institute on Drug Abuse. Drug overdose death rates. https://nida.nih.gov/research-topics/trends-statistics/overdose-death-rates.*

2 Friedman, J. & Hadland, S. E. The Overdose Crisis among U.S. Adolescents. *N Engl J Med* **390**, 97-100 (2024). https://doi.org/10.1056/NEJMp2312084

3 Boyer, E. W. Management of opioid analgesic overdose. *N Engl J Med* **367**, 146-155 (2012). https://doi.org/10.1056/NEJMra1202561

4 France, C. P. *et al.* Countermeasures for Preventing and Treating Opioid Overdose. *Clin Pharmacol Ther* **109**, 578-590 (2021). https://doi.org/10.1002/cpt.2098

5 Kassick, A. J. *et al.* Covalently Loaded Naloxone Nanoparticles as a Long-Acting Medical Countermeasure to Opioid Poisoning. *ACS Pharmacol Transl Sci* **4**, 1654-1664 (2021). https://doi.org/10.1021/acsptsci.1c00168

6 Bremer, P. T., Burke, E. L., Barrett, A. C. & Desai, R. I. Investigation of monoclonal antibody CSX-1004 for fentanyl overdose. *Nat Commun* **14**, 7700 (2023). https://doi.org/10.1038/s41467-023-43126-0

7 Jimenez, V. M., Jr., Castaneda, G. & France, C. P. Methocinnamox Reverses and Prevents Fentanyl-Induced Ventilatory Depression in Rats. *J Pharmacol Exp Ther* **377**, 29-38 (2021). https://doi.org/10.1124/jpet.120.000387

8 Voronkov, M. *et al.* Modifying naloxone to reverse fentanyl-induced overdose. *Int J Pharm* **611**, 121326 (2022). https://doi.org/10.1016/j.ijpharm.2021.121326

9 Wallisch, M. *et al.* Naloxone pro-drug rescues morphine induced respiratory depression in Sprague-Dawley rats. *Respir Physiol Neurobiol* **180**, 52-60 (2012). https://doi.org/10.1016/j.resp.2011.10.009

10 Huang, B. *et al.* Human plasma-mediated hypoxic activation of indolequinone-based naloxone pro-drugs. *Bioorg Med Chem Lett* **19**, 5016-5020 (2009). https://doi.org/10.1016/j.bmcl.2009.07.061

11 Zhang, W. *et al.* On-Demand Opioid Effect Reversal with an Injectable Light-Triggered Polymer-Naloxone Conjugate. *Nano Lett* **23**, 10545-10553 (2023). https://doi.org/10.1021/acs.nanolett.3c03426

12 Penchala, S. C. *et al.* AG10 inhibits amyloidogenesis and cellular toxicity of the familial amyloid cardiomyopathy-associated V122I transthyretin. *Proc Natl Acad Sci U S A* **110**, 9992-9997 (2013). https://doi.org/10.1073/pnas.1300761110

13 Gillmore, J. D. *et al.* Efficacy and Safety of Acoramidis in Transthyretin Amyloid Cardiomyopathy. *N Engl J Med* **390**, 132-142 (2024). https://doi.org/10.1056/NEJMoa2305434

14 McKeage, K. & Goa, K. L. Insulin glargine: a review of its therapeutic use as a long-acting agent for the management of type 1 and 2 diabetes mellitus. *Drugs* **61**, 1599-1624 (2001). https://doi.org/10.2165/00003495-200161110-00007

15 Yáñez, J. A., Remsberg, C. M., Sayre, C. L., Forrest, M. L. & Davies, N. M. Flip-flop pharmacokinetics--delivering a reversal of disposition: challenges and opportunities during drug development. *Ther Deliv* **2**, 643-672 (2011). https://doi.org/10.4155/tde.11.19

16 Tran, J. T., Binger, K. J. & Miles, T. M. Assessment of oral overlap with antipsychotic long-acting injectables initiated in an inpatient setting. *Ment Health Clin* **13**, 147-151 (2023). https://doi.org/10.9740/mhc.2023.06.147

17 Tuhin, M. T. H. *et al.* Peripherally restricted transthyretin-based delivery system for probes and therapeutics avoiding opioid-related side effects. *Nat Commun* **13**, 3590 (2022). https://doi.org/10.1038/s41467-022-31342-z

18 Rautio, J. *et al.* Prodrugs: design and clinical applications. *Nat Rev Drug Discov* **7**, 255-270 (2008). https://doi.org/10.1038/nrd2468

19 Scott, C. C., & Robins E. B. . Toxicity of p-Aminobenzoic Acid. *Experimental Biology and Medicine* **49** (1942). https://doi.org/doi.org/10.3181/00379727-49-13507

20 Alhamadsheh, M. M. *et al.* Potent kinetic stabilizers that prevent transthyretin-mediated cardiomyocyte proteotoxicity. *Sci Transl Med* **3**, 97ra81 (2011). https://doi.org/10.1126/scitranslmed.3002473

21 Choi, S., Connelly, S., Reixach, N., Wilson, I. A. & Kelly, J. W. Chemoselective small molecules that covalently modify one lysine in a non-enzyme protein in plasma. *Nat Chem Biol* **6**, 133-139 (2010). https://doi.org/10.1038/nchembio.281

22 Schreiber, G. The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis. *J Endocrinol* **175**, 61-73 (2002). https://doi.org/10.1677/joe.0.1750061

23 Sundelin, J. *et al.* The primary structure of rabbit and rat prealbumin and a comparison with the tertiary structure of human prealbumin. *J Biol Chem* **260**, 6481-6487 (1985).

24 Dickson, P. W., Howlett, G. J. & Schreiber, G. Metabolism of prealbumin in rats and changes induced by acute inflammation. *Eur J Biochem* **129**, 289-293 (1982). https://doi.org/10.1111/j.1432-1033.1982.tb07051.x

25 Di, L. The Impact of Carboxylesterases in Drug Metabolism and Pharmacokinetics. *Curr Drug Metab* **20**, 91-102 (2019). https://doi.org/10.2174/1389200219666180821094502

26 Wang, D. *et al.* Human carboxylesterases: a comprehensive review. *Acta Pharm Sin B* **8**, 699-712 (2018). https://doi.org/10.1016/j.apsb.2018.05.005

27 Nagaoka, M., Sakai, Y., Nakajima, M. & Fukami, T. Role of carboxylesterase and arylacetamide deacetylase in drug metabolism, physiology, and pathology. *Biochem Pharmacol* **223**, 116128 (2024). https://doi.org/10.1016/j.bcp.2024.116128

28 de Man, F. M., Goey, A. K. L., van Schaik, R. H. N., Mathijssen, R. H. J. & Bins, S. Individualization of Irinotecan Treatment: A Review of Pharmacokinetics, Pharmacodynamics, and Pharmacogenetics. *Clin Pharmacokinet* **57**, 1229-1254 (2018). https://doi.org/10.1007/s40262-018-0644-7

29 Lian, J., Nelson, R. & Lehner, R. Carboxylesterases in lipid metabolism: from mouse to human. *Protein Cell* **9**, 178-195 (2018). https://doi.org/10.1007/s13238-017-0437-z

30 Bencharit, S., Morton, C. L., Xue, Y., Potter, P. M. & Redinbo, M. R. Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat Struct Biol* **10**, 349-356 (2003). https://doi.org/10.1038/nsb919

31 Eisner, H. *et al.* The Crystal Structure of Mouse Ces2c, a Potential Ortholog of Human CES2, Shows Structural Similarities in Substrate Regulation and Product Release to Human CES1. *Int J Mol Sci* **23** (2022). https://doi.org/10.3390/ijms232113101

32 Penchala, S. C. *et al.* A biomimetic approach for enhancing the in vivo half-life of peptides. *Nat Chem Biol* **11**, 793-798 (2015). https://doi.org/10.1038/nchembio.1907

33 Liu, F., Ul Amin, T., Liang, D., Park, M. S. & Alhamadsheh, M. M. Enhancing the Pharmacokinetic Profile of Interleukin 2 through Site-Specific Conjugation to a Selective Small-Molecule Transthyretin Ligand. *J Med Chem* **64**, 14876-14886 (2021). https://doi.org/10.1021/acs.jmedchem.1c01426

34 Moss, R. B. & Carlo, D. J. Higher doses of naloxone are needed in the synthetic opiod era. *Subst Abuse Treat Prev Policy* **14**, 6 (2019). https://doi.org/10.1186/s13011-019-0195-4

35 Huang, X. & Brazel, C. S. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *J Control Release* **73**, 121-136 (2001). https://doi.org/10.1016/s0168-3659(01)00248-6

36 Bakhrushina, E. O. *et al.* Burst Release from In Situ Forming PLGA-Based Implants: 12 Effectors and Ways of Correction. *Pharmaceutics* **16** (2024). https://doi.org/10.3390/pharmaceutics16010115

37 Strandberg, J. J. *et al.* Toxicological analysis in rats subjected to heroin and morphine overdose. *Toxicol Lett* **166**, 11-18 (2006). https://doi.org/10.1016/j.toxlet.2006.05.007

38 Vardanyan, R. S. & Hruby, V. J. Fentanyl-related compounds and derivatives: current status and future prospects for pharmaceutical applications. *Future Med Chem* **6**, 385-412 (2014). https://doi.org/10.4155/fmc.13.215

39 Borgland, S. L. Acute opioid receptor desensitization and tolerance: is there a link? *Clin Exp Pharmacol Physiol* **28**, 147-154 (2001). https://doi.org/10.1046/j.1440-1681.2001.03418.x

40 Raehal, K. M., Schmid, C. L., Groer, C. E. & Bohn, L. M. Functional selectivity at the μ-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev* **63**, 1001-1019 (2011). https://doi.org/10.1124/pr.111.004598

41 Algera, M. H. *et al.* Tolerance to Opioid-Induced Respiratory Depression in Chronic High-Dose Opioid Users: A Model-Based Comparison With Opioid-Naïve Individuals. *Clin Pharmacol Ther* **109**, 637-645 (2021). https://doi.org/10.1002/cpt.2027

42 Britch, S. C. & Walsh, S. L. Treatment of opioid overdose: current approaches and recent advances. *Psychopharmacology (Berl)* **239**, 2063-2081 (2022). https://doi.org/10.1007/s00213-022-06125-5

43 Jones, A. K., Ngaimisi, E., Gopalakrishnan, M., Young, M. A. & Laffont, C. M. Population Pharmacokinetics of a Monthly Buprenorphine Depot Injection for the Treatment of Opioid Use Disorder: A Combined Analysis of Phase II and Phase III Trials. *Clin Pharmacokinet* **60**, 527-540 (2021). https://doi.org/10.1007/s40262-020-00957-0

44 Stolbach, A. I., Mazer-Amirshahi, M., Nelson, L. S. & Cole, J. B. American College of Medical Toxicology and the American Academy of Clinical Toxicology Position Statement: Nalmefene Should Not Replace Naloxone as the Primary Opioid Antidote at This Time. *J Med Toxicol* **20**, 64-67 (2024). https://doi.org/10.1007/s13181-023-00981-z

45 Donnerstag, N. *et al.* Severe opioid withdrawal syndrome after a single dose of nalmefene. *Eur J Clin Pharmacol* **71**, 1025-1026 (2015). https://doi.org/10.1007/s00228-015-1884-1

46 Elkattawy, S., Alyacoub, R., Ejikeme, C., Noori, M. A. M. & Remolina, C. Naloxone induced pulmonary edema. *J Community Hosp Intern Med Perspect* **11**, 139-142 (2021). https://doi.org/10.1080/20009666.2020.1854417

47 Rzasa Lynn, R. & Galinkin, J. L. Naloxone dosage for opioid reversal: current evidence and clinical implications. *Ther Adv Drug Saf* **9**, 63-88 (2018). https://doi.org/10.1177/2042098617744161

48 Lahti, R. A. & Collins, R. J. Chronic naloxone results in prolonged increases in opiate binding sites in brain. *Eur J Pharmacol* **51**, 185-186 (1978). https://doi.org/10.1016/0014-2999(78)90343-6

49 Sirohi, S., Kumar, P. & Yoburn, B. C. Mu-opioid receptor up-regulation and functional supersensitivity are independent of antagonist efficacy. *J Pharmacol Exp Ther* **323**, 701-707 (2007). https://doi.org/10.1124/jpet.107.127019

50 Binswanger, I. A. & Glanz, J. M. Potential Risk Window for Opioid Overdose Related to Treatment with Extended-Release Injectable Naltrexone. *Drug Saf* **41**, 979-980 (2018). https://doi.org/10.1007/s40264-018-0705-8

51 Jain, P. *et al.* Evaluation of Opioid Overdose Reports in Patients Treated with Extended-Release Naltrexone: Postmarketing Data from 2006 to 2018. *Drug Saf* **44**, 351-359 (2021). https://doi.org/10.1007/s40264-020-01020-4

52 Jiang, S. *et al.* M1 muscarinic acetylcholine receptor in Alzheimer's disease. *Neurosci Bull* **30**, 295-307 (2014). https://doi.org/10.1007/s12264-013-1406-z

53 Langston, J. L., Moffett, M. C., Makar, J. R., Burgan, B. M. & Myers, T. M. Carfentanil toxicity in the African green monkey: Therapeutic efficacy of naloxone. *Toxicol Lett* **325**, 34-42 (2020). https://doi.org/10.1016/j.toxlet.2020.02.008

54 Alanis-Hirsch, K. *et al.* Extended-Release Naltrexone: A Qualitative Analysis of Barriers to Routine Use. *J Subst Abuse Treat* **62**, 68-73 (2016). https://doi.org/10.1016/j.jsat.2015.10.003

55 Martinez, S. *et al.* The potential role of opioid vaccines and monoclonal antibodies in the opioid overdose crisis. *Expert Opin Investig Drugs* **32**, 181-185 (2023). https://doi.org/10.1080/13543784.2023.2187286

56 Payne, E. R., Stancliff, S., Rowe, K., Christie, J. A. & Dailey, M. W. Comparison of Administration of 8-Milligram and 4-Milligram Intranasal Naloxone by Law Enforcement During Response to Suspected Opioid Overdose - New York, March 2022-August 2023. *MMWR Morb Mortal Wkly Rep* **73**, 110-113 (2024). https://doi.org/10.15585/mmwr.mm7305a4

57 Lemen, P. M. *et al.* High-dose naloxone formulations are not as essential as we thought. *Harm Reduct J* **21**, 93 (2024). https://doi.org/10.1186/s12954-024-00994-z

CLAIMS

What is claimed is:

1. A conjugate of formula (I):

$$T—L—L^B—E—D^1 \quad (I)$$

or a pharmaceutically acceptable salt thereof, wherein:

T is a ligand selective for transthyretin TTR;

L is a linker;

L^B is absent or a click product formed via a Click reaction between a first Click handle and a second click handle;

E is a cleavable ester; and

D^1 is a drug moiety.

2. The conjugate of claim 1, wherein:

L^B is the Click product of:

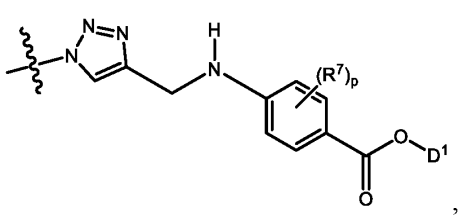
(i) a copper-catalyzed reaction between an azide and an alkyne;

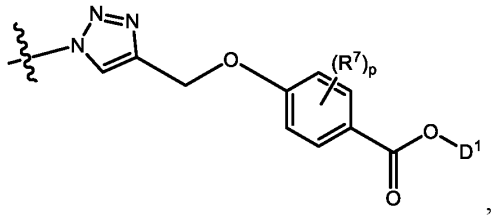
(ii) a reaction between an azide and dibenzocyclooctene (DBCO);

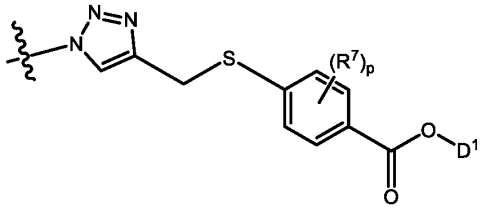
(iii) an inverse electron demand Diels-alder cycloaddition (IEDDA) between a trans-cyclooctene (TCO) moiety and a tetrazine ring, or

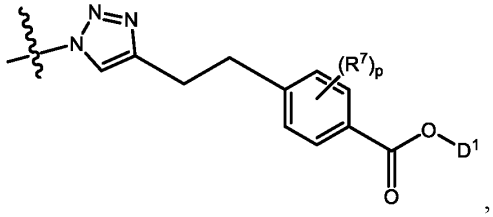
(iv) a Staudinger reaction between an azide and a phosphine.

3. The conjugate of claim 1, comprising the moiety:


,


,

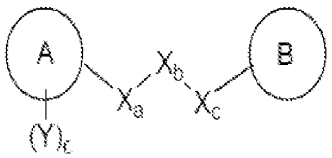

, or


,

wherein each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, -

$CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl, p is 0, 1, 2, or 3, and ≈ indicates the point of attachment to the linker (L).

4. The conjugate of claim 1, wherein T has the structure of Compound (A):



(A),

or a pharmaceutically acceptable salt thereof, wherein:

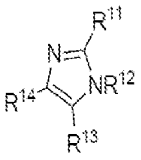
X_a, X_b and X_c are independently selected from $C(R^{8a})(R^{8b})$, O, $N\text{-}R^{8b}$ or S; where R^{8a} and R^{8b} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

a ring is a 4 to 12-membered ring, in certain embodiments the 4 to 12-membered ring is an aromatic or heteroaromatic ring;

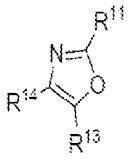
each Y is independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano;

c is an integer ranging from 0 to 5; and,

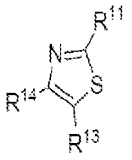
B ring is a heterocyclic ring selected from the following (h1-h30):



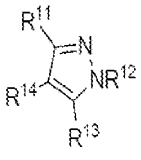
h1



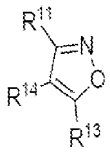
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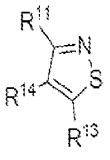
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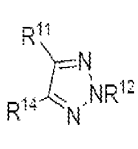
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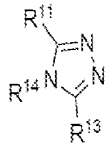
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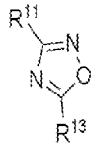
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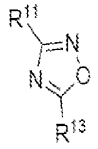
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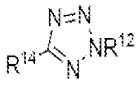
h8



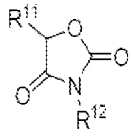
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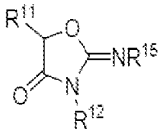
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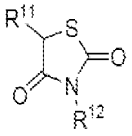
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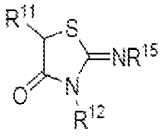
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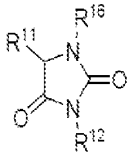
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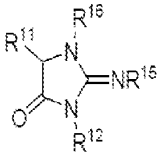
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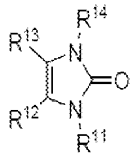
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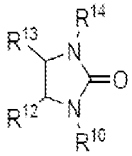
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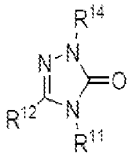
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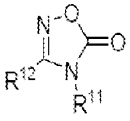
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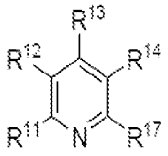
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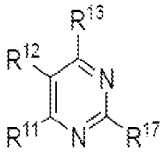
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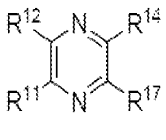
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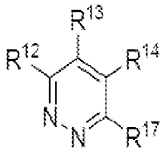
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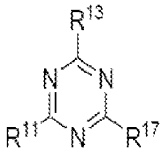
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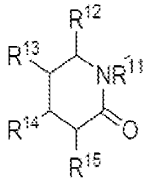
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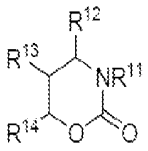
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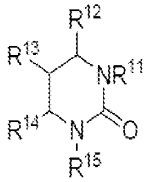
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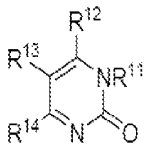
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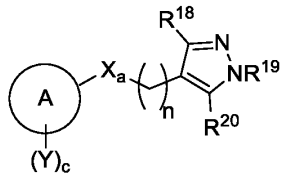
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h30

where R^{11}-$R^{16}$are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano; R^{17} is selected from a hydroxyl, alkyl, amino, and alkyl amino; and at least one of R^{11}-R^{16} is the linking group to X_c.

5. The conjugate of claim 1, wherein T has the structure of Compound (B):



(B),

or a pharmaceutically acceptable salt thereof, wherein:

n is an integer ranging from 0 to 8;

R^{18}, R^{19} and R^{20} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano;

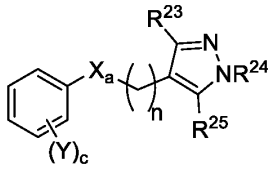
X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

A is a 5 to 12-membered ring, in certain embodiments the 5 to 12-membered ring is an aromatic or heteroaromatic ring;

each Y is independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, sulfonamide, sulfonyl fluoride, thioester and cyano; and,

c is a number from zero to 5.

6. The conjugate of claim 1, wherein T has the structure of Compound (C):



(C).

or a pharmaceutically acceptable salt thereof, wherein:

n is an integer ranging from 1 to 4;

R^{23} is a short chain alkyl having 1 to 4 carbon atoms;

R^{24} is hydrogen;

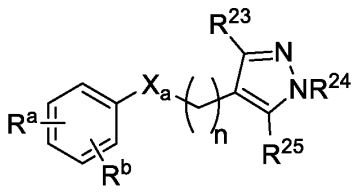
R^{25} is a short chain alkyl having 1 to 4 carbon atoms;

X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

each Y is independently selected from hydrogen, halogen, acyl, substituted acyl, carboxyl, heterocyclic group, alkoxycarbonyl sulfonamide, sulfonyl fluoride, thioester and substituted alkoxycarbonyl; and

c is 2.

7. The conjugate of claim 1, wherein T has the structure of Compound (D):



(D),

or a pharmaceutically acceptable salt thereof, wherein:

n is 1 to 8;

R^{23}, R^{24} and R^{25} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halo, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl,

aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

X_a is $C(R^{21})(R^{22})$, O, N-R^{22} or S; where R^{21} and R^{22} are independently selected from hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, alkoxy, aryloxy, hydroxyl, heterocyclic group, halogen, nitro, acyl, substituted acyl, carboxyl, alkoxycarbonyl, substituted alkoxycarbonyl, aminoacyl, substituted aminoacyl, amino, substituted amino, acylamino, substituted acylamino, and cyano;

R^a is CHO, COOH, $COOCH_3$, $COOR^6$, $CONR^7R^8$, tetrazolyl, CONHOH, $B(OH)_2$, $CONHSO_2Ar$, $CONHCH(R^9)COOH$, CF_3, hydrogen, halogen, alkyl, substituted alkyl, acyl, substituted acyl, carboxyl, heterocyclic group, sulfonamide, sulfonyl fluoride, thioester, alkoxycarbonyl or substituted alkoxycarbonyl;

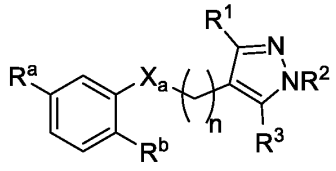
R^b is CHO, COOH, $COOCH_3$, $COOR2^6$, $CONR2^7R2^8$, tetrazolyl, CONHOH, $B(OH)_2$, $CONHSO_2Ar$, $CONHCH(R2^9)COOH$, CF_3, hydrogen, halogen, alkyl, substituted alkyl, acyl, substituted acyl, carboxyl, heterocyclic group, sulfonamide, sulfonyl fluoride, thioester, alkoxycarbonyl or substituted alkoxycarbonyl;

$R2^6$ is alkyl, haloalkyl, cycloalkyl, or heterocyclyl;

R^{27} and R^{28} are each independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, or heteroaryl; and,

R^{29} is the side chain of a naturally occurring α-amino carboxylic acid.

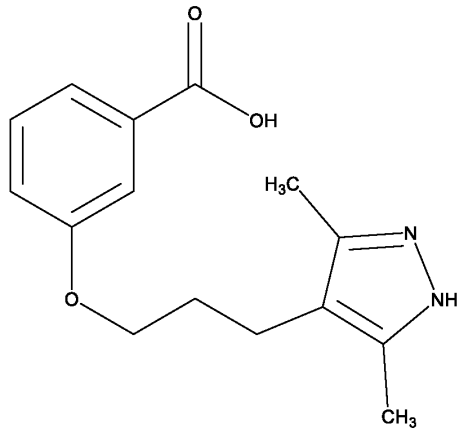
8. The conjugate of claim 7, wherein T has the structure of Compound (E):



(E)

or a pharmaceutically acceptable salt thereof.

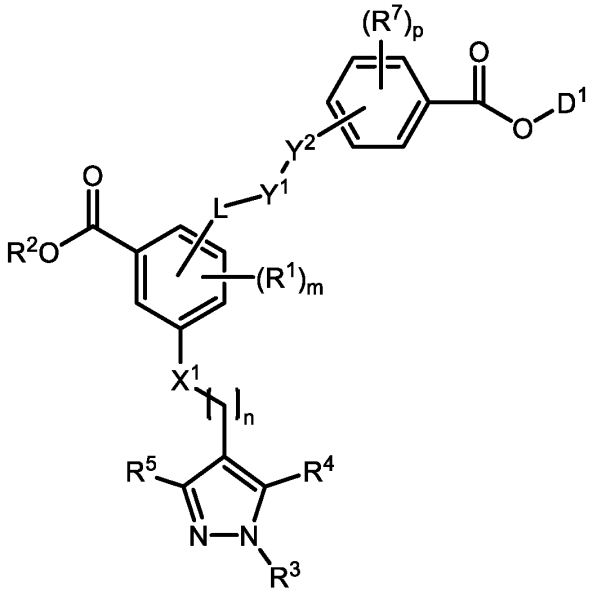
9. The conjugate of claim 7, wherein T has the structure of Compound (Y):



(Y)

or a pharmaceutically acceptable salt thereof.

10. A conjugate of formula (II'):



(II'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O or S;

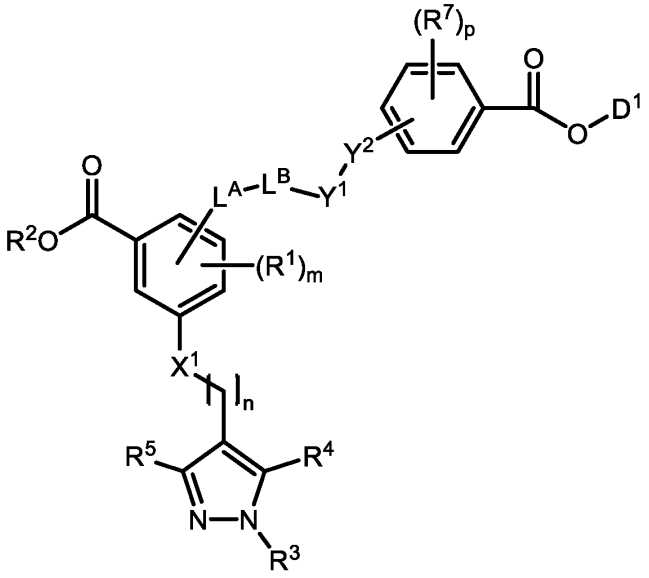
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

11. A conjugate of formula (III'):



(III')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6, O, or S;

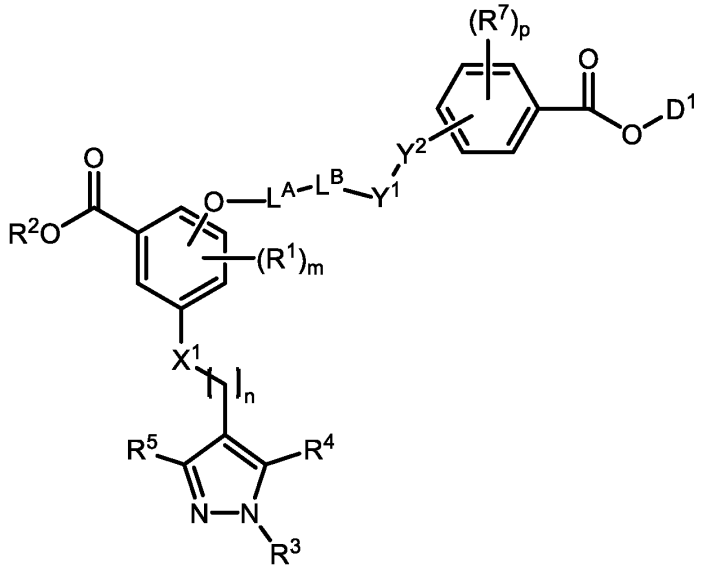
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

12. A conjugate of formula (IV'):



(IV')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, 3, or 4;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Y^1 is a bond or C_{1-4}alkylene;

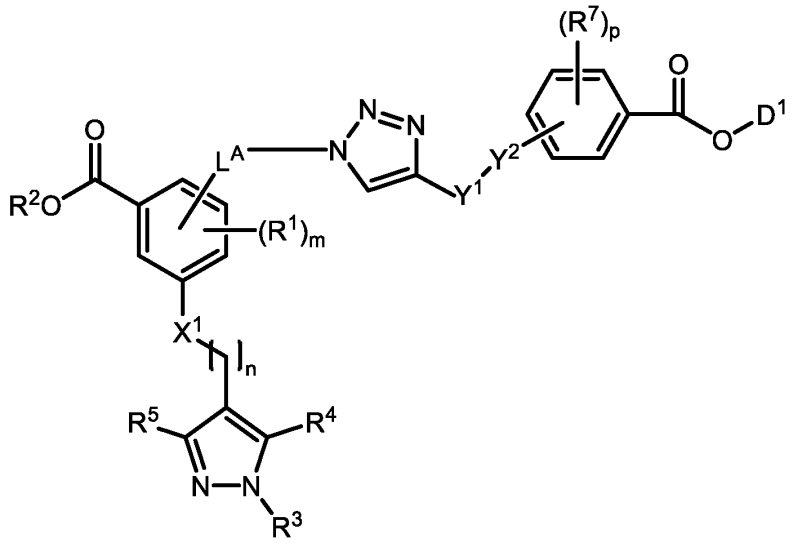
Y^2 is NR^6, O, or S;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), NO_2, CF_3, OMe, O(C_{1-4} alkyl), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -$CONHC_{1-4}$ alkyl, -$CON(C_{1-4}$ alkyl$)_2$, -$NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

13. A conjugate of formula (V'):



(V'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

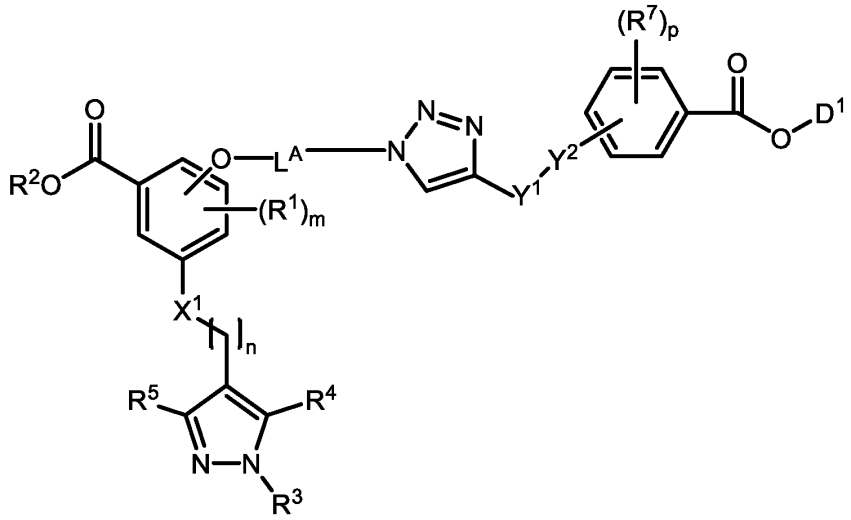
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, -OCF_3, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$, -$CONH_2$, -

$CONHC_{1-4}$ alkyl, $-CON(C_{1-4}\text{ alkyl})_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl; p is 0, 1, 2, or 3; and

D^1 is a drug moiety.

14. A conjugate of formula (VI'):



(VI')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, $-NR^y$-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

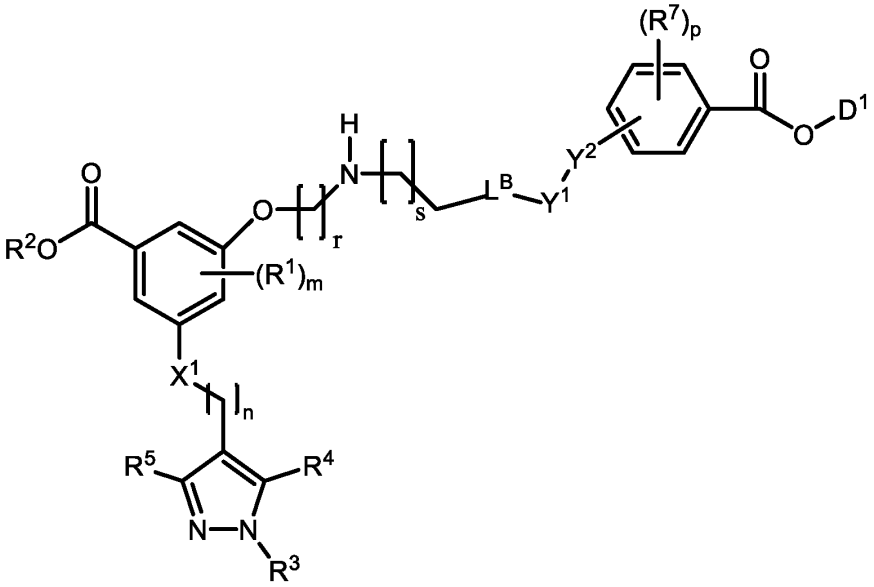
R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl

p is 0, 1, 2, or 3; and

D^1 is a drug moiety (opioid agonist or opioid antagonist).

15. A conjugate of formula (VII'):



(VII'),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

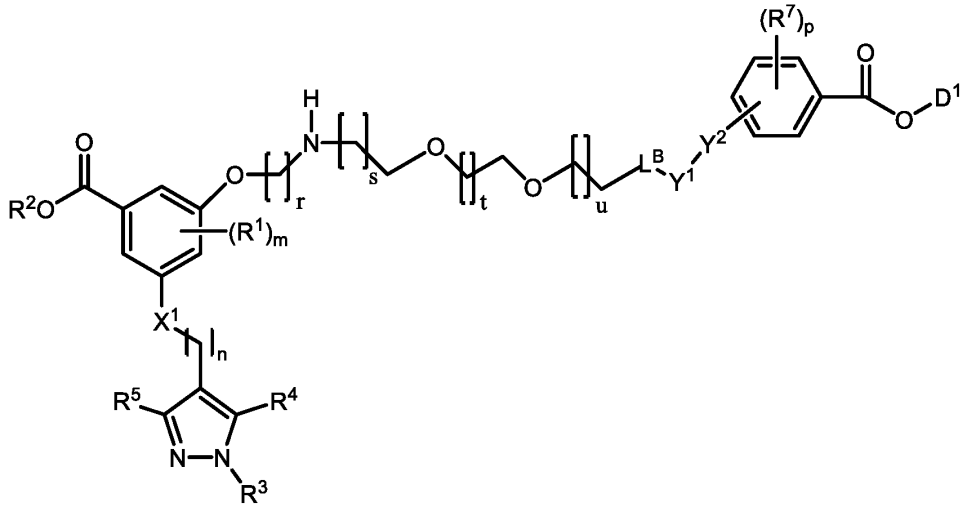
p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

s is 1, 2, 3, 4, 5, 6, or 7; and

D^1 is a drug moiety.

16. A conjugate of formula (VIII'):



(VIII')

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

Y^1 is a bond or C_{1-4}alkylene;

Y^2 is NR^6 , O or S;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

R^6 is H or C_{1-4}alkyl;

each R^7 is independently selected from the group consisting of hydrogen, halogen atoms (e.g., Cl, Br or F), hydroxy, $-OCF_3$, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, $-CONH_2$, $-CONHC_{1-4}$ alkyl, $-CON(C_{1-4}$ alkyl$)_2$, $-NHCOC_{1-4}$ alkyl, C_{1-6} alkyl, C_{1-6} alkoxy and C_{3-6} cycloalkyl;

p is 0, 1, 2, or 3;

r is 1, 2, 3, 4, 5, 6, or 7;

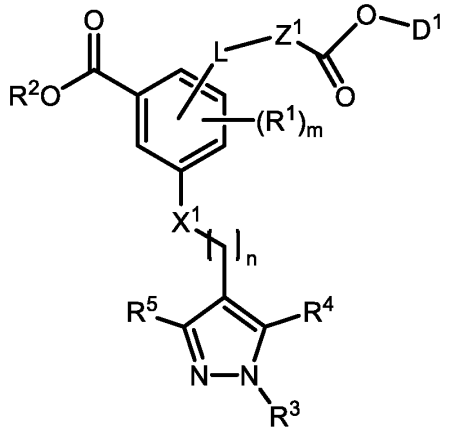
s is 1, 2, 3, 4, 5, 6, or 7;

t is 1, 2, 3, or 4;

u is 1, 2, 3, or 4; and

D^1 is a drug moiety.

17. A conjugate of formula (IX):



(IX),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or $-NR^x$-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L is a linker;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

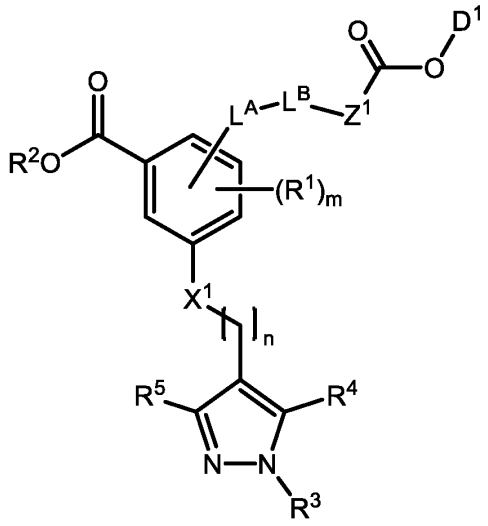
the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety (opioid agonist or opioid antagonist).

18. A conjugate of formula (X):



(X),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

L^B is a click product formed via a Click reaction between a first Click handle and a second click handle;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

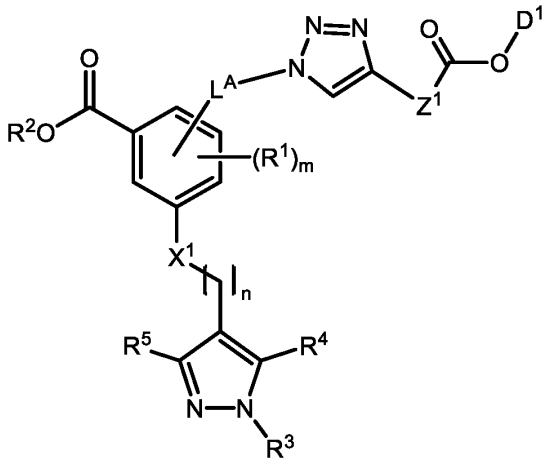
the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety.

19. A conjugate of formula (XI):



(XI),

or a pharmaceutically acceptable salt thereof, wherein:

each R^1 is independently halo or C_{1-4}alkyl;

m is 0, 1, 2, or 3;

R^2 is H or C_{1-4}alkyl;

X^1 is -O- or -NR^x-;

n is 0, 1, 2, or 3;

R^3 is H or C_{1-4}alkyl;

R^4 is H or C_{1-4}alkyl;

R^5 is H or C_{1-4}alkyl;

R^x is H or C_{1-4}alkyl;

L^A is C_{3-20}alkylene, wherein

the C_{3-20}alkylene is optionally substituted with one or more L^{1a}, and

one or more alkylene units is optionally replaced with a group selected from -O-, -NR^y-, and -S-;

each L^{1a} is halo, C_{1-6}alkyl, or C_{1-6}alkyl substituted by halo;

each R^y is H or C_{1-4}alkyl;

Z^1 is C_{1-6}alkyl, C_{6-10}aryl, or 5- to 10-membered heteroaryl, wherein

the C_{1-6}alkyl is optionally substituted with one or more Z^{1a}, and

the C_{6-10}aryl or 5- to 10-membered heteroaryl is optionally substituted with one or more Z^{1b};

each Z^{1a} or Z^{1b} is independently halo, C_{1-6}alkoxy, or C_{1-6}alkyl optionally substituted by halo; and

D^1 is a drug moiety.

20. The conjugate of any one of claims 1-19, wherein D^1 is a peptide.

21. The conjugate of any one of claims 1-19, wherein D^1 is a small molecule, a nucleotide, an oligonucleotide, a polynucleotide, an amino acid, an oligopeptide, a polypeptides, or a protein.

22. The conjugate of any one of claims 1-19, wherein D^1 is a cytokine.

23. The conjugate of claim 22, wherein the cytokine is Interleukin-2 (IL-2).

24. The conjugate of claim 22, wherein the cytokine is Interleukin-15 (IL-15).

25. The conjugate of any one of claims 1-19, wherein D^1 is an anti-cancer agent.

26. The conjugate of claim 25, wherein the anti-cancer agent is SN-38, irinotecan, capecitabine, monomethyl auristatin E, trifluridine, docetaxel, or infigratinib.

27. The conjugate of any one of claims 1-19, wherein D^1 is insulin.

28. The conjugate of any one of claims 1-19, wherein D^1 is a glucagon-like-peptide-1 (GLP-1) agonist.

29. The conjugate of claim 28, wherein the GLP-1 agonist is semaglutide, liraglutide, dulaglutide, albiglutide, or exenatide.

30. The conjugate of any one of claims 1-19, wherein D^1 is a glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide) receptor agonist.

31. The conjugate of claim 30, wherein glucagon-like-peptide-1 (GLP-1) and GIP (glucose-dependent insulinotropic polypeptide) receptor agonist tirzepatide.

32. A method of treating a disease, comprising administering a conjugate if any one of claims 1-31.

33. The method of claim 32, further comprising administering a second drug (D^2) in free (unconjugated) form.

34. The method of claim 33, wherein D^1 is the same as D^2.

35. The method of claim 33, wherein D^1 is different than D^2.

36. A method of treating cancer, comprising administering a conjugate of claim 25 or claim 26.

37. The method of claim 36, further comprising administering a second drug (D^2) in free (unconjugated) form.

38. The method of claim 37, wherein D^1 is the same as D^2.

39. The method of claim 37, wherein D^1 is different than D^2.

40. A method of treating obesity comprising administering a conjugate of any one of claims 28-31.

41. A method of treating diabetes comprising administering a conjugate of any one of claims 28-31.