

Conjugates of Glucagon and AMPK activators

Technical Field

- 5 The present invention relates generally to the field of therapeutic conjugates and more specifically to conjugates having glucagon receptor activity and an AMP-activated protein kinase (AMPK) activator.

Background Art

- 10 Metabolic disorders are any of the diseases or disorders that disrupt normal metabolism, the process of converting food to energy on a cellular level. The development of a metabolic disorder may be caused by different deficiencies such as for example genetic (inherited) deficiencies, deficiencies in certain hormones or enzymes or consumption of too much of certain foods. If left un-
- 15 treated, the metabolic disorders may progress into several life-threatening diseases, including but not limited to fatty liver diseases, such as Non-Alcoholic Steatohepatitis (NASH), type I or II diabetes, heart diseases, dyslipidemia and obesity.

- Obesity is the most prevalent nutritional disease of humans and do-
- 20 mestic animals such as dogs and cats in affluent societies, exceeding by far the number of nutritional deficiency diseases. As alternatives to bariatric surgery, attempts have been made to design weight-lowering drugs for the treatment of obesity. This has resulted in drugs that act by preventing the absorption of fats by acting as lipase inhibitors in the gut, or by inhibiting food intake via
- 25 selective serotonin receptor 2C agonism in the hypothalamus.

- Non-Alcoholic Steatohepatitis (NASH) is the second stage of non-alcoholic fatty liver diseases (NAFLD) and occurs when the build-up of fat in the liver cells is accompanied with inflammation. If left untreated, NASH progresses into fibrosis and further into cirrhosis, culminating in the need for a liver trans-
- 30 plant. NASH is heavily influenced by lifestyle, e.g., chronic excessive calorie intake and sedentary activity, and is distinct from other fatty liver diseases caused by alcohol abuse or medication side effects. To date, no drug therapy has been FDA-approved for NASH and thus weight loss and a change in

lifestyle remains the first line treatment. In response to a growing need for new treatment regimens to NASH, several NASH drugs are being developed and tested, e.g. obeticholic acid, Elafibranor and Aramchol. However, since these drugs have been associated with adverse effects, including liver toxicity and a
5 rise in LDL cholesterol, there remains a need for drugs suitable for treatment of NASH.

Glucagon is a 29 amino acid peptide hormone which is derived from the tissue-specific posttranslational processing of the proglucagon peptide. Glucagon is predominantly secreted from the alpha cells of the pancreas and
10 has a proven role in glucose metabolism, lipolysis, ketogenesis, energy expenditure, appetite and food intake. Glucagon offers many benefits for metabolic diseases independent from its glycemic effects, including lowering lipids in circulation and in the liver. The use of glucagon for treatment of metabolic diseases is known from the prior art. US 2020352900 A1 describes the use of
15 hormone peptides, including glucagon, in the treatment of various disorders associated with insulin resistance and obesity. US 2006014670 A1 describes the use of glucagon and insulin to achieve therapeutically effective control of diabetes but also to prevent hypoglycemia in diabetic patients. However, the use of glucagon in treatment of metabolic diseases is halted by the effect of
20 glucagon on increasing blood glucose levels.

AMP-activated protein kinase (AMPK) is a central regulator of energy homeostasis, which coordinates metabolic pathways and thus balances nutrient supply with energy demand. Due to the favourable physiological outcomes of AMPK activation on metabolism, AMPK has been considered to be an im-
25 portant therapeutic target for controlling human diseases including metabolic syndrome and cancer. In recent years, several companies have undertaken medicinal chemistry campaigns on developing AMPK activators, thus AMPK activators are known from the prior art. A list of recently identified indirect and direct AMPK activators is provided in Steinberg *et al.* (Nat Rev Drug Dis 2019;
30 18(7): 527-55141). Myers *et al.* (Science 2017; 357(6350): 507-511) describes an orally available AMPK activator, (3*R*,3*aR*,6*R*,6*aR*)-6-((6-([1,1'-biphenyl]-4-yl)-7-chloro-3*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol

also known as MK-8722, effective in insulin-independent glucose uptake and glycogen synthesis, with resultant improvements in glycemia. However, the use of the compound is halted by the induction of cardiac hypertrophy caused by MK-8722. The clinical use of AMPK activators is thus, to this date, hampered
5 by adverse effects of the compounds on metabolic pathways.

Thus, there exist a growing need for novel treatment regimens suitable in the treatment of metabolic diseases with greater efficacy, high safety (low toxicological effect), which also offers convenient and safe administration options.

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Summary of the invention

In view of the above, it is therefore an object of the present invention to provide an effective and safe therapeutic agent useful in the treatment of metabolic diseases, including obesity and NASH, in subjects in need thereof.

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Accordingly, a first aspect of the present invention relates to a conjugated molecule comprising a peptide displaying at least 0.1 % activity of native glucagon at the glucagon receptor, and an AMP-activated protein kinase (AMPK) activator, wherein the peptide is covalently bonded to the AMPK activator either directly or through a linker.

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The inventors have surprisingly found that conjugation of peptides with glucagon agonism and AMPK activation represents a novel medicinal strategy for effectively reversing obesity, lowering plasma cholesterol and blood glucose levels in obese subjects. Conjugates based on this strategy are superior in causing a reduction body weight relative to the glucagon or MK-8722 alone, as shown in Fig. 3. Furthermore, the significant reduction in body weight achieved
25 by the conjugates of the present inventive is independent of food intake, as shown in Figs. 4 and 5. Also, the conjugates are effective in lowering blood glucose and cholesterol levels, as shown in Figs. 6-8. The inventors have found that the ability of AMPK to improve glucose homeostasis is effective in cancel-
30 ling the diabetic liabilities of glucagon, while the specific targeting achieved by glucagon restricts the activation of AMPK in cardiac tissue. Consequently, the conjugates of the present invention harvest the beneficial pharmacological

effects on lipid metabolism from each of the individual components, while circumventing hallmark adverse effects. Without being bound by any particular theory, the inventors speculate that this effect is achieved by the peptide hormone which serves a bifunctional role as a lipolysis stimulating drug and as a targeting agent, allowing for site-selective delivery of the otherwise non-specific AMPK activator to the liver. Also, it has been shown that conjugates based on an alternative glucagon peptide variant (SEQ ID NO: 3) and alternative AMPK activators display comparable or improved AMPK activities as compared to single, standard AMPK activators known to the clinic, see Figs 20 and 21. It is thus expected that these additional conjugates will show positive results in terms of reversing obesity, lowering plasma cholesterol and blood glucose levels in obese subjects. This hypothesis is at least supported by the further findings that co- and tri-agonists according to SEQ ID NO:4-6 conjugated with AMPK activator MK-8722 show improved effect on blood glucose in mice after a compound tolerance test, see Fig. 21. Furthermore, the developed conjugates have a dual functionality, in that they combine AMPK activators with glucagon agonists, and is capable of targeting two distinct pathways involved in hepatic fatty acid oxidation.

A peptide will have an amino terminus and a carboxyl terminus. In the context of the invention, the amino terminus and the carboxyl terminus may also be referred to as the N-terminus and the C-terminus, respectively, and corresponding derived forms.

The peptide may consist of amino acids encoded by the genetic code or it may contain amino acids encoded by the genetic code and natural amino acids, which are not encoded by the genetic code, such as hydroxyproline, γ -carboxyglutamate, ornithine, phosphoserine, D-alanine (dAla), and D-glutamine. Further, the peptide may incorporate synthetic amino acids such as D-alanine, and D-leucine, or α -aminoisobutyric acid (Aib), d-Serine (dSer), N-methyl-serine.

The peptide may be obtained from any source or the peptide may be produced as desired. For example, the peptide may be isolated from a tissue, or the peptide may be produced recombinantly or synthesized by methods that

are well known to the person skilled in the art.

The conjugated molecule comprises a peptide, the peptide (in its free form) displaying at least 0.1% activity of native glucagon at the glucagon receptor. In the context of the present invention, glucagon receptor activity, which
5 may also be referred to as glucagon activation, can be measured in an *in vitro* assay by measuring cAMP induction in HEK293 cells over-expressing the glucagon receptor (GCCR). The assay may be carried out as described in, for example, Finan *et al.*, Cell 167.3: 843-857 (2016), which is incorporated herein by reference. In an embodiment, the peptide of the conjugate displays at least
10 1% activity of native glucagon, such as at least 5%, 10%, 15%, 20%, or 30% activity.

The peptide of the conjugated molecule may be any peptide having at least 0.1% activity of native glucagon at the glucagon receptor. In an embodiment, the peptide of the conjugate is of the glucagon-superfamily. The glucagon-superfamily is a group of peptides related in structure in their N-terminal and C-terminal regions (see, for example, Sherwood *et al.*, Endocrine Reviews 21: 619-670 (2000), which is incorporated herein by reference). Members of this group include glucagon related peptides, including, but not limited to, modified glucagon (SEQ ID NO: 1) and unmodified glucagon (SEQ ID NO: 2), and
15 analogues, derivatives or conjugates with up to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications relative to the native peptide. Also contemplated is a modified glucagon analogue according to SEQ ID NO: 3. Such peptides preferably retain the ability to interact with receptors of the glucagon receptor superfamily, preferably the glucagon receptor.

25 Also contemplated are peptides with co- or tri-agonist activities which display the ability to bind to different receptors of the glucagon receptor superfamily. In an embodiment, such co- or tri-agonist can be selected from Glucagon-like peptide 1 (GLP-1)/glucagon receptor co-agonist (SEQ ID NO: 4), Gastric inhibitory polypeptide (GIP)/glucagon receptor co-agonist (SEQ ID NO: 5)
30 and GLP-1/GIP/glucagon receptor tri-agonist (SEQ ID NO: 6).

In an embodiment, the peptide of the conjugate has at least 80% amino acid sequence identity to SEQ ID NO:1. For example, the peptide may

have at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than about 97% identity to SEQ ID NO:1. In one embodiment, the peptide has an amino acid sequence with at least 80% identity to SEQ ID NO:1. In another embodiment, the peptide has an amino acid sequence with at least 95% identity to SEQ ID NO:1. In a specific embodiment, the peptide has the amino acid sequence of SEQ ID NO:1. Such a peptide may have a significantly greater glucagon activity at the glucagon receptor compared to native glucagon at the glucagon receptor. As such, the peptide may, if conjugated to an AMPK activator, accumulate at a greater rate at the site of glucagon receptors, which in turn may lead to a greater efficacy of the AMPK activator. An example of a peptide of the conjugate having at least 80% amino acid sequence identity to SEQ ID NO: 1 is shown in Fig. 22, showing the amino acid sequence alignments of glucagon peptide of SEQ ID NO: 1 with the co-agonist GLP-1/glucagon (SEQ ID NO: 4) and the tri-agonist GLP-1/GIP/glucagon (SEQ ID NO: 6). As seen from Fig. 22, the amino acid difference between glucagon of SEQ ID NO:1 and the co- and tri-agonist of SEQ ID NO: 4 and 6, respectively, are six amino acids, thus, giving rise to 80 % and 85 % sequence identity, respectively.

The peptide of the conjugated molecule will have a length sufficient for the peptide (in its free form), to display at least 0.1 % activity of native glucagon at the glucagon receptor. In general, this can be observed for peptides comprising at least 10 amino acids, but the activity may not be displayed when the peptide comprises more than 60 amino acids. Thus, in an embodiment, the peptide has a length in the range of 10 to 60 amino acids, e.g. 20 to 50 amino acids. Amino acid sequences of the present invention that are identical to other peptides sequences to a certain percentage should comprise enough of the amino acid sequence of a peptide, e.g. at least 10 amino acids, to afford putative identification of that peptide, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool) (for a review see Altschul, *et al.*, Meth Enzymol. 266: 460, 1996; and Altschul, *et al.*, Nature Genet. 6: 119, 1994).

In the context of the present invention, peptide may differ in %-identity by having substitutions, insertions of natural or synthetic amino acids and/or having amino acid deletions.

- In an embodiment, the peptide is modified by acetylation, fatty acid
5 conjugation, diacid conjugation, albumin conjugation, small-molecule albumin binders, and/or PEG conjugation. Also contemplated are peptides modified by linking to carrier proteins, such as antibodies. The modifications are preferably at position 16, 17, 20, 21, 24, and 29-40 of the peptide in SEQ ID NO:1 (counted from the N-terminal), within a C-terminal region, or at the C-terminal amino acid.
- 10 Since none of the amino acids in positions 29 to 40 of SEQ ID NO:1 are directly involved in binding of peptide to the glucagon receptor, these amino acids may be exchanged and modified without influencing the binding of the peptide to the glucagon receptor. The conjugation may be made by any suitable linker, such as by disulfide, maleimide, alpha-ketone, or click-chemistry based conjugation.
- 15 The skilled person knows how to prepare such conjugates. Preferably, PEG molecules may be larger than 1 kDa and fatty acids and diacids may contain more than 12 carbon atoms. It is generally preferred to add a spacer between the modification (PEG/fatty acid/diacid) and the peptide, the linker preferably being a gamma-Glu linker, a short PEG chain.
- 20 The conjugated molecule comprises an AMPK activator. Any AMPK activator may be used with the conjugate. However, it is preferred that the AMPK activator is a small molecule, e.g up to 900 kDa. For example, in one embodiment, the AMPK activator is selected from 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-([1,1'-biphenyl]-4-yl)-7-chloro-3*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (MK-8722), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-chloro-5-(4-(1-(hydroxymethyl)cyclopropyl)phenyl)-1*H*-benzo[*d*]imidazol-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (PF-739), 6-Chloro-5-[4-(1-hydroxycyclobutyl)phenyl]-1*H*-indole-3-carboxylic acid (PF-06409577), 1,1-Dimethylbiguanide hydrochloride (metformin), 4-Hydroxy-3-(2'-hydroxy-1,1'-biphenyl-4-yl)-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-5-carbonitrile (A-769662), 2-Chloro-5-[[5-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-4-oxo-2-thiazolyl]amino]benzoic acid (PT-1), 2-
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[[2-(2-Bromo-4-methylphenoxy)ethyl]thio]-pyrimidine (ZLN024), 2-[[4-(Diethyl-amino)-2-hydroxyphenyl]methylene]hydrazide-4-pyridinecarboxylic acid (RSVA-405), and analogues thereof. MK-8722, PF-739 and PF-06409577 are preferred. In another embodiment, the AMPK activator is a simplified analogue
5 of PF-06409577. The synthesis of such simplified analogue of PF-06409577 is exemplified by the compounds 14a-14e reported in Example 3 and shown in Fig. 17, Scheme 1.

The peptide of the invention and the AMPK activator are covalently bonded. In the context of the present invention, the conjugated molecule may
10 also be referred to as a peptide-drug-conjugate (PDC). The peptide and the AMPK activator may be bonded directly to each other. For example, the AMPK activator may be bonded covalently through an ether bond, an ester bond, a carbamate bond, a carbonate bond, a triazole bond, a maleimide bond and an amide bond.

15 In the context of the present invention, being directly covalently bonded means that the peptide has a covalent bond with the AMPK activator, e.g. there are no additional chemical groups between the two molecules, such as a linker group.

In one embodiment, the AMPK activator is covalently bonded at the C-
20 terminal region of the peptide. In the context of the invention, the C-terminal region may be up to 50% of the amino acids counted from the C-terminus, such as up to 40%, 30%, 25%, 20%, or 10% of the amino acids counted from the C-terminus. For instance, the C-terminal region of SEQ ID NO:1 may be amino acids 15 to 40, 26 to 40, or 30 to 40 (numbers counted from N-terminal). Thus,
25 the AMPK activator may be bonded, either directly or via a linker, to any one of the 10 amino acids counted from the C-terminus. Thereby the AMPK activator produces little or no steric hindrance at the N-terminal of the peptide. It is also contemplated that more than one AMPK activator may be bonded to the same peptide molecule.

30 The peptide and the AMPK activator may be bonded through a linker. Any linker may be used. However, it is generally preferred that the linker has a length of up to 30 atoms. A longer chain may have the advantage of distancing

the AMPK activator from the peptide, such that the AMPK activator is of no or little steric hindrance to the peptide, when the peptide interacts with a glucagon receptor, and vice versa. No or low steric hindrance of the peptide affords a greater affinity towards the glucagon receptor. A conjugate with a greater affinity towards the glucagon receptor is likely to have a greater accumulation at the site of glucagon receptors.

In one embodiment the AMPK activator is covalently bonded to the peptide via a cleavable chemical linker, the cleavable chemical linker being selected from acid-cleavable linkers, enzyme-cleavable linkers, peptide-cleavable linkers, and linkers comprising a disulfide group. Such linkers are generally well-known in the art for their use in peptide-drug conjugates. Examples of such cleavable linkers are compounds comprising glucuronide, beta-galactoside, disulfide, hydrazone and/or which compounds are cleavable by galactosidases, glucuronidases, pyrophosphatases, phosphatases, arylsulfatases, proteases, or esterases. The linkers preferably release the AMPK activator in its free form (i.e. native form), which may be achieved by many different linker chemistries such as the disulfide linkers disclosed herein. These linker chemistries and additional linker chemistries are well-known by the skilled person.

In a preferred embodiment, the AMPK activator is covalently bonded to the peptide via a chemical linker comprising a disulfide group. A disulfide group allows for the release of AMPK activator from the peptide when chemically reduced. A chemical linker comprising a disulfide group, also known as a disulfide linker, ensures that the peptide and the AMPK activator of the conjugate remain conjugated for an extended period during systemic circulation. The disulfide group of the disulfide linker may be reduced in a reducing environment, such as an intracellular environment, resulting in the conjugate being cleaved such that the peptide part of the conjugate is separated from the AMPK activator part of the conjugate. The reduction may be through disulfide exchange with e.g. a thiol, such as glutathione or reductases such as intracellular protein disulfide-isomerase enzymes.

The chemical linker may be chosen from chemical linkers known in the art with the general formula $R'-S-S-R''$, in which the R' and R'' groups may be

identical or different from each other. Advantageously, the conjugate may accumulate at and/or close to the sites of glucagon receptors in the body due to the affinity of the peptide towards glucagon receptors, and the AMPK activator may be released at the sites and/or close to the sites of the glucagon receptors.

- 5 When free from peptide part of the conjugate, the AMPK activator may have a suitably effect as site-specific AMPK activator. It is speculated by the inventors that the conjugate may be cleaved in the extracellular environment immediately adjacent to cells harbouring glucagon receptors, or that the conjugate may be internalized by the cells harbouring glucagon receptors and cleaved in the re-
- 10 ducing environment of the cells.

In one embodiment, the conjugated molecule is conjugated via a chemical linker, wherein the chemical linker has the formula $R_1-R_3-S-S-R_4-R_5-O-CO-R_2$, wherein R_1 is the peptide, R_2 the AMPK activator, R_3 is optional and when present is selected from $C(CH_3)_2$, CH_2-CH_2 , or CH_2 , bonded to a side

15 chain of the peptide or to a carbon atom of the backbone chain of the peptide, R_4 is $(CH_2)_n$ or C_6H_4 , R_5 is optional and when present is selected from $C(CH_3)_2$, $CH-CH_3$, CH_2-CH_2 , or CH_2 , and n is 1, 2, or 3. When the chemical linker is reduced, the liberated AMPK activator part of the conjugate undergoes intramolecular cyclisation which leads to the release of the AMPK activator into its

20 free form.

In one embodiment, the chemical linker has the formula $R_1-R_3-S-S-(CH_2)_n-NH-CO-R_2$, wherein R_1 is the peptide, R_2 the AMPK activator, R_3 is optional and when present is selected from $C(CH_3)_2$, CH_2-CH_2 , or CH_2 , bonded to a side chain of the peptide or to a carbon atom of the backbone chain of the

25 peptide, and n is 1, 2, or 3.

In one embodiment, the chemical linker has the formula $R_1-R_4-R_3-S-S-(CH_2)_n-NH-CO-R_2$, wherein R_1 is the peptide, R_2 the AMPK activator, R_3 is optional and when present is selected from $CH(CH_3)_2$, CH_2-CH_2 , or CH_2 , bonded to a side chain of the peptide or to a carbon atom of the backbone chain

30 of the peptide, R_4 is optional and when present is selected from $CH(CH_3)_2$, CH_2-CH_2 , or CH_2 , bonded to a side chain of the peptide or to a carbon atom of the backbone chain of the peptide and n is 1, 2, or 3.

In an embodiment, the second radical bond is to the backbone of the peptide of the invention.

In another embodiment, the second radical bond is to a side chain of the peptide of the invention.

5 In the context of the present invention, when R_1 is bonded to the backbone chain of the peptide, $C(CH_3)_2$ (L-penicillamine) may be referred to as Pen, CH_2-CH_2 (L-homocysteine) may be referred to as hCys, and CH_2 (L-Cysteine) may be referred to as Cys.

As used herein, the first and the second radical bond is used to state
10 the presence of at least two free bonds in the chemical linkers disclosed herein.

In another embodiment, the AMPK activator is covalently bonded to the peptide via a non-cleavable linker, wherein the non-cleavable linker is selected from polyethylene glycol (PEG) linkers, carbon linkers, SMCC and mc with conjugation chemistries of maleimides, ethers, amides, triazoles, disulfide,
15 and thioether.

The present invention facilitates the design and synthesis of a library of conjugated molecules comprising a peptide and an AMPK activator appended via chemical linkers. Fig. 1 shows how such conjugated molecules, may be designed. As shown in Fig. 1, the conjugate may be prepared by chemically bonding an AMPK activator (MK-8722 in Fig. 1) to a peptide. The skilled
20 person will appreciate that a vast number of different chemical linkers may be prepared by the methods disclosed herein and by other methods reported in literature, and these chemical linkers may be used to append peptides and AMPK activator to the methods disclosed herein and as reported elsewhere in
25 the known art.

The inventors have surprisingly found that the peptide of the present invention may serve a bifunctional role as a weight lowering drug and a targeting agent, allowing for site-selective delivery of otherwise non-specific small-molecules, such as AMPK activators, to the liver. It is understood, that the targeting properties of the peptide of the present invention may also facilitate delivery of the AMPK activators to other sites, such as for example the endocrine
30 pancreas.

The conjugated molecule disclosed herein provides selectivity and also up-concentrates drug action in the targeted region. This targeting enabled by the conjugated molecule allows for an improved therapeutic index, i.e. a lower minimum effective concentration. Furthermore, the coupling allows to
5 add-on another layer of metabolic drug action to the efficacy of glucagon receptor targeting medicines..

The inventors have demonstrated a surprising synergistic effect of the conjugates of the invention on reduction on body weight and this is significantly greater in comparison to the effect obtained with the administration of peptide
10 or the drug alone, see Fig. 3. Interestingly, the food intake shows that the weight loss is uncoupled from appetite suppression, suggesting its effect is purely driven by increased energy expenditure, see Figs. 4 and 5.

The inventors have further demonstrated a surprising synergistic effect of the conjugates of the invention on lowering blood glucose and plasma cholesterol, and this is significantly greater in comparison to the effect obtained
15 with the administration of the peptide or the drug alone, see Figs. 6 to 8. The surprising synergistic effect of the conjugates of the invention are further supported by the findings shown in Fig. 20, which shows AMPK activities of conjugates of glucagon according to SEQ ID NO:3 and AMPK activators, the synthesis of which are disclosed in Example 3, and the findings shown in Fig. 21,
20 which shows the effect of different co- and tri-agonist peptides (SEQ ID NOs:4-6)/MK8722 conjugates on blood glucose in mice after a compound tolerance test.

In an embodiment of the present invention the conjugated molecule is
25 for use in therapy.

In an embodiment, the conjugated molecule of the present invention is for use in the treatment of obesity, type 2 diabetes, hyperinsulinemia, insulin resistance, impaired glucose tolerance, hypercholesterolaemia, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and
30 dyslipidaemiae.

Another aspect of the present invention relates to a pharmaceutical composition comprising the conjugated molecule according to the invention,

and a pharmaceutically acceptable carrier. Any embodiment of the conjugated molecule may be used in the pharmaceutical composition.

The invention relates to the use of the conjugated molecule according to the invention in the manufacture of a pharmaceutical composition. In particular, the pharmaceutical composition is for use in the treatment of obesity, type 2 diabetes, hyperinsulinemia, insulin resistance, impaired glucose tolerance, hypercholesterolaemia, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and dyslipidemia. Any embodiment of the conjugated molecule may be used in the manufacture of the pharmaceutical composition.

The data disclosed in the present invention have been obtained in studies of mice, but the conclusions are equally relevant for humans, since the major hormonal pathways governing energy metabolism are similar between mice and humans as they display comparable receptor expression profiles.

The conjugate of the present invention may be administered in the form of a pharmaceutical composition. Accordingly, the present invention further provides a pharmaceutical composition, which comprises a conjugate of the present invention or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. The pharmaceutical formulations may be prepared by conventional techniques. Briefly, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more excipients which may also act as diluents, solubilizers, lubricants, suspending agents, binders, preservatives, wetting agents, tablet disintegrating agents or an encapsulating material.

The conjugate comprised in the pharmaceutical formulation may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilisation from solution for constitution before use with a suitable vehicle, e.g., sterile, pyrogen-free water.

In one embodiment, the pharmaceutical composition is suited for subcutaneous administration, intramuscular administration, intraperitoneal administration, intravenous administration or for oral administration. Accordingly, the

compositions of the present invention may be provided in unit dose form in ampoules, pre-filled syringes, small volume infusion or in multi-dose containers, optionally with an added preservatives. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles.

- 5 In accordance with the current disclosure, pharmaceutical compositions are provided wherein the weight lowering effects of peptides with glucagon activity are combined with AMPK activator in a single modality. Active delivery via peptides with glucagon activity to the liver avoid unwanted adverse effects, such as for examples cardiac hypertrophy caused by AMPK activation.
- 10 Positive metabolic effects of AMPK activators may include improvements in glucose uptake, fatty acid oxidation, mitochondrial biogenesis and insulin sensitivity, which may be beneficial for reducing obesity and obesity-related metabolic disorders in humans or mammal.

 Thus, the therapeutic utility of a peptide of the invention and AMPK
15 activator pairing offers a new approach for the treatment of obesity and its associated metabolic disorders.

 A further aspect of the present invention relates to a method of reducing body weight in a mammal comprising administering the conjugated molecule of the invention or the pharmaceutical composition of the invention.

- 20 The conjugated molecule or the pharmaceutical composition may be administered subcutaneously, orally, intramuscularly, intraperitoneally, or intravenously.

 The conjugated molecule, and thus also the pharmaceutical composition, is effective in reducing body weight. Therefore, the conjugated molecule
25 and the pharmaceutical composition may be used in the treatment of obesity at any level. Obesity may be described in terms of the body mass index (BMI), which is defined as the body mass divided by the square of the body height, e.g. as expressed in units of kg/m^2 . Without being bound by theory, the present inventors consider that the BMI can be used to define a limit between patho-
30 genic obesity and non-pathogenic obesity. For example, in the context of the invention, a BMI of 30 kg/m^2 may be interpreted as the limit between pathogenic obesity and non-pathogenic obesity. However, other values of BMI can

also be considered to define the limit between pathogenic obesity and non-pathogenics obesity. Thus, for example, BMI values of 24 kg/m², 25 kg/m², 26 kg/m², 27 kg/m², 28 kg/m², 29 kg/m², 30 kg/m², 31 kg/m², 32 kg/m², 33 kg/m², 34 kg/m², and 35 kg/m² are considered to define the limit between
5 pathogenic obesity and non-pathogenic obesity.

In a further aspect, the present invention relates to a non-therapeutic treatment of mammals for reducing body weight comprising orally administering to said mammal the conjugated molecule according to the invention. For example, the mammal may have a non-pathogenic BMI. In particular, the
10 method may comprise orally administering the conjugated molecule to a subject having a BMI below the limit defining non-pathogenic obesity.

In the above, the invention has mainly been described with reference to a few embodiments. However, as is readily appreciated by a person skilled in the art, other embodiments than the ones disclosed above are equally possible within the scope of the invention.
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Other aspects and advantageous features of the present invention are described in detail and illustrated by non-limiting working examples below.

Generally, all terms used herein are to be interpreted according to their ordinary meaning in the technical field, and applicable to all aspects and embodiments of the invention, unless explicitly defined or stated otherwise. All
20 references to "a/an/the [conjugate, molecule, linker, peptide, etc.]" are to be interpreted openly as referring to at least one instance of said conjugate, agent, molecule, linker, peptide, etc., unless explicitly stated otherwise.

In the context of the present invention, the term glucagon means a
25 peptide of the glucagon-superfamily. The peptide of the invention may also be considered to be weight regulating hormone peptides and to function as an active delivery agent of the conjugated molecule of the present invention to the liver.

In the context of the present invention, the term "peptide" means a
30 compound composed of stretch of 10 to 60 amino acids connected by peptide bonds.

The term "analogue" as used herein in relation to glucagon peptide

and AMPK activators means a peptide or compound or drug having an analogous or similar biological property and effect.

The term "derivative" as used herein in relation to a peptide or an amino acid means a chemically modified peptide or amino acid, wherein at least one substituent is not present in the unmodified peptide or amino acid or analogues thereof, i.e. a peptide or an amino acid which has been covalently modified. Typical modifications are amides, carbohydrates, alkyl groups, acyl groups, esters and the like.

In the context of the present invention, the term "percentage identity" or "% identity" means % of identical amino acids between two compared peptides, in particular using the BLAST algorithm.

The term "AMPK activator" as used herein means a compound which is an activator or agonist or stimulant of AMP-activated protein kinase (AMPK). Examples of AMPK activators include, but are not limited to, 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-((1,1'-biphenyl)-4-yl)-7-chloro-3*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (MK-8722), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-chloro-5-(4-(1-(hydroxymethyl)cyclopropyl)phenyl)-1*H*-benzo[*d*]imidazol-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (PF-739), 6-Chloro-5-[4-(1-hydroxycyclobutyl)phenyl]-1*H*-indole-3-carboxylic acid (PF-06409577), 1,1-Dimethylbiguanide hydrochloride (metformin), 4-Hydroxy-3-(2'-hydroxybiphenyl-4-yl)-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-5-carbonitrile (A-769662), 2-Chloro-5-[[5-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-4-oxo-2-thiazolyl]amino]benzoic acid (PT-1), 2-[[2-(2-Bromo-4-methylphenoxy)ethyl]thio]-pyrimidine (ZLN024), 2-[[4-(Diethylamino)-2-hydroxyphenyl]methylene]hydrazide-4-pyridinecarboxylic acid (RSVA-405).

Brief description of figures

The above, as well as additional objects, features, and advantages of the present invention is better understood through the following illustrative and non-limiting detailed description of embodiments of the present invention, with reference to the appended drawings, wherein:

Fig. 1 shows an example of a peptide and AMPK activator conjugate,

Fig. 2 displays the mechanism by which MK-8722 is released from the conjugate of Fig. 1,

Fig. 3 shows the weight-lowering effect of a conjugate of a peptide of
5 SEQ ID NO:1 and MK-8722 (Glucagon Pen40/MK-8722),

Fig. 4 shows the effect of Glucagon Pen40/MK-8722 conjugate on daily food intake in mice,

Fig. 5 shows the effect of Glucagon Pen40/MK-8722 conjugate on cumulative food intake in mice,

10 Fig. 6 shows the effect of Glucagon Pen40/MK-8722 conjugate on blood glucose in mice after a compound tolerance test (CTT),

Fig. 7 shows the effect of Glucagon Pen40/MK-8722 conjugate on blood glucose in mice after an intraperitoneal glucose tolerance test (IpGTT),

Fig. 8 shows the effect of Glucagon Pen40/MK-8722 conjugate on
15 plasma cholesterol in mice,

Fig. 9 shows the weight-lowering effect of a conjugate of a peptide of SEQ ID NO:1 and AICAR (Glucagon Pen40/AICAR),

Fig. 10 shows the effect of Glucagon Pen40/AICAR conjugate on daily food intake in mice,

20 Fig. 11 shows the effect of Glucagon Pen40/AICAR conjugate on cumulative food intake in mice,

Fig. 12 shows the effect of Glucagon Pen40/AICAR conjugate on blood glucose in mice after a compound tolerance test (CTT),

Fig. 13 shows the effect of Glucagon Pen40/AICAR conjugate on
25 blood glucose in mice after an intraperitoneal glucose tolerance test (IpGTT),

Fig. 14 shows the effect of Glucagon Pen40/AICAR conjugate on plasma cholesterol in mice,

Fig. 15 shows a synthesis route of a chemical linker derivatized AICAR,

30 Fig. 16 shows a synthesis route for chemical linker derivatized MK-8722,

Fig. 17 shows part of a synthesis route for conjugates of glucagon

peptide of SEQ ID NO:3 and AMPK activator compounds synthesized according to Example 3,

Fig. 18 shows a further part of a synthesis route for conjugates of glucagon peptide of SEQ ID NO:3 and AMPK activator compounds synthesized according to Example 3,

Fig. 19 shows a further part of a synthesis route for conjugates of glucagon peptide of SEQ ID NO:3 and AMPK activator compounds synthesized according to Example 3,

Fig. 20 shows the results from an AMPK activity assay testing the conjugates of glucagon peptide of SEQ ID NO: 3 and AMPK activators as synthesized according to Example 3,

Fig. 21 shows the effect of different co- and tri-agonist peptides of SEQ ID NOs:4-6 conjugated with AMPK activator MK8722 on blood glucose in mice after a compound tolerance test, and

Fig. 22 shows an amino acid sequence alignment between co- and tri-agonists peptides of SEQ ID NOs: 4-6 and the glucagon peptide of SEQ ID NO: 1.

Detailed description

Fig. 1 shows an example of a peptide and AMPK activator conjugate 100, which consists of MK-8722 101 chemically appended to a C-terminal cysteine 102 of the peptide of SEQ ID NO:1 103 through a chemical linker 104, the chemical linker 104 comprising a disulfide group 105. A side chain 106 of the C-terminal cysteine 102, may optionally be derivatised, such that length n of the side chain 106 is 1 or 2 carbon atoms. A modification called hCys40 of the side chain 106 has length n = 2 carbon atom and R = hydrogen. A modification called hCys40 of the side chain 106 has length n = 1 carbon atom and R = methyl. Regular cysteine is called Cys40.

Fig. 2 displays the mechanism by which MK-8722 is released from the conjugate 100 of Fig. 1. The chemical linker 104 comprising a disulfide group 105 is self-immolative and may be reduced in a reducing environment (not shown) such as an intracellular environment to produce thiol groups, separating

the peptide part 103 of the conjugate 100 from the MK-8722 part 101 of the conjugate. On the MK-8722 part 101 of the molecule, a liberated nucleophilic thiol 109 undergoes spontaneous intramolecular cyclization to release MK-8722 as the native unmodified MK-8722 drug (free form of MK-8722).

5 Figs 3-14 display the results of the *in vivo* mice studies disclosed in Example 2.

Fig. 3 shows the weight-lowering effect of a conjugate of a peptide of SEQ ID NO:1 and MK-8722 chemically appended via the linker shown in Fig. 1 and 2 (Glucagon-Pen40/MK-8722) (100nmol/kg) and equimolar doses of the peptide of SEQ ID NO:1 (Glucagon Pen40) or MK-8722 measured in body weight percentage (BW %) of diet-induced obesity (DIO) mice treated for 7 days. Data is expressed as mean $\pm$ SEM and N is 8 per group. Mice treated with monotherapies of either Glucagon Pen40 or MK-8722 showed a slight reduction in body weight, appr. 4 % BW % reduction. The highest reduction in BW % was observed in DIO mice treated with Glucagon Pen40/MK-8722, the conjugate giving rise to approximately 10 % BW % reduction after 7 days of treatment. Additionally, based on the slope of the curve, it would seem that a further reduction in body weight could be expected for the Glucagon Pen40/MK-8722 if treatment was extended.

20 Fig. 4 shows the effect of Glucagon Pen40/MK-8722 (100nmol/kg) or equimolar doses of Glucagon Pen40 or MK-8722 on daily food intake (gram per day) in DIO mice treated for 7 days. Data is expressed as mean $\pm$ SEM and N = 8 per group. During the 7 days of treatment, mice treated with Glucagon Pen40, MK-8722 or Glucagon Pen40/MK-8722 showed similar daily food intake as compared to the control group of mice (Vehicle, i.e. saline). At the end of the study, mice treated with Glucagon Pen40 showed a slight reduction in food intake compared to the control group (vehicle).

Fig. 5 shows the effect of Glucagon Pen40/MK-8722 and equimolar doses of Glucagon Pen40 or MK-8722 on cumulative food intake (Cumulative FI, gram per day) in DIO mice treated for 6 days. Data is expressed as mean $\pm$ SEM and N = 8 per group. Over the course of the treatment, mice treated with Glucagon Pen40, MK-8722 or Glucagon Pen40/MK-8722 showed similar

cumulative food intake as compared to the control group of mice (Vehicle, i.e. saline).

Figs 6 and 7 show the effect of Glucagon Pen40/MK-8722 and equimolar doses of Glucagon Pen40 or MK-8722 compared to a control group (vehicle, i.e. saline) on blood glucose level (mmol/L) in DIO mice subjected to either a compound tolerance test (CTT) (Fig. 6) or Intraperitoneal glucose tolerance test (ipGTT) (Fig. 7) on day 7 of the treatment course. The blood glucose levels were measured over a course of 40 hours (CTT, Fig. 6) and 2 hours (IpGTT, Fig. 7). Data is expressed as mean $\pm$ SEM, N = 5 to 6 per group. In general, Glucagon Pen40/MK872 results in a significantly lower initial increase in blood glucose levels (immediately after stressing, >1h) compared to the control group.

Fig. 8 shows the effect of Glucagon Pen40/MK-8722 and equimolar doses of Glucagon Pen40 or MK-8722 compared to a control group (vehicle, i.e. saline) on plasma cholesterol level (mg/dL) in DIO mice treated for 7 days. Data is expressed as mean $\pm$ SEM, N = 5 to 6 per group. A significant reduction in plasma cholesterol level was observed for mice treated with Glucagon Pen40/MK-8722. Furthermore, mice treated with the conjugate not only displayed a much lower plasma cholesterol level compared to the vehicle 100 mg/dL vs. 200 mg/dL), but the conjugate also clearly outperformed MK-8722 and Glucagon Pen40 administered as monotherapies.

Fig. 9 shows the weight-lowering effect of a conjugate of a peptide of SEQ ID NO:1 and AICAR chemically appended via the linker shown in Fig. 1 and 2 (Glucagon-Pen40/AICAR) (100nmol/kg) and equimolar doses of the peptide of SEQ ID NO:1 (Glucagon Pen40) or AICAR measured in body weight percentage (BW %) of diet induced (DIO) mice treated for 7 days. Data is expressed as mean $\pm$ SEM and N is 8 per group. Mice treated with monotherapies of either Glucagon Pen40 or AICAR or the conjugate Glucagon Pen40/AICAR showed only a slight reduction in body weight, appr. 4 % BW % reduction, compared to control group (Vehicle, i.e. saline). The highest reduction in BW % was observed in DIO mice treated with either Glucagon Pen40 alone or the conjugate Glucagon Pen40/AICAR, both giving rise to approximately 5 % BW %

reduction after 7 days of treatment.

Fig. 10 shows the effect of Glucagon Pen40/AICAR (100nmol/kg) or equimolar doses of Glucagon Pen40 or AICAR on daily food intake (gram per day) in DIO mice treated for 7 days. Data is expressed as mean $\pm$ SEM and N = 8 per group. During the 7 days of treatment, mice treated with Glucagon Pen40, AICAR or Glucagon Pen40/AICAR showed similar daily food intake as compared to the control group of mice (Vehicle, i.e. saline). At the end of the study, mice treated with Glucagon Pen40 or Glucagon Pen40/AICAR showed a slight reduction in food intake compared to the control group (vehicle) and AICAR. Based on the slope of the curve, it would seem that a negative trend in food intake could be expected for the Glucagon Pen40/AICAR or Glucagon Pen40 if treatment was extended.

Fig. 11 shows the effect of Glucagon Pen40/AICAR and equimolar doses of Glucagon Pen40 or AICAR on cumulative food intake (Cumulative FI, gram per day) in DIO mice treated for 6 days. Data is expressed as mean $\pm$ SEM and N = 8 per group. Over the course of the treatment, mice treated with Glucagon Pen40, AICAR or Glucagon Pen40/AICAR showed similar cumulative food intake as compared to the control group of mice (Vehicle, i.e. saline).

Figs 12 and 13 show the effect of Glucagon Pen40/AICAR and equimolar doses of Glucagon Pen40 or AICAR compared to a control group (vehicle, i.e. saline) on blood glucose level (mmol/L) in DIO mice subjected to either CTT (Fig. 12) or ipGTT (Fig. 13) on day 7 of the treatment course. The blood glucose levels were measured over a course of 40 hours (CTT, Fig. 12) and 2 hours (IpGTT, Fig. 13). Data is expressed as mean $\pm$ SEM, N = 5 to 6 per group. In both stress tests, Glucagon Pen40/AICAR results in a significantly lower initial increase in blood glucose levels (immediately after stressing, >1h) compared to the control group.

Fig. 14 shows the effect of Glucagon Pen40/AICAR and equimolar doses of Glucagon Pen40 or AICAR compared to a control group (vehicle, i.e. saline) on plasma cholesterol level (mg/dL) in DIO mice treated for 7 days. Data is expressed as mean $\pm$ SEM, N = 5 to 6 per group. The highest reduction in plasma cholesterol level was observed for mice treated with Glucagon Pen40

or Glucagon Pen40/AICAR. Mice treated with AICAR alone showed similar plasma cholesterol levels as the control group, and it was concluded that the effect AICAR on plasma cholesterol is limited..

Fig. 20 shows the results of AMPK activity assays (Example 4) of the synthesized conjugate compounds 22a-22d and 23a-23e of AMPK activator compounds and glucagon peptide of SEQ ID NO: 3 (Example 3). Results show that the synthesized conjugate compounds 22a-22d and 23a-23e display comparable or improved AMPK activities as compared to the control compounds PF-06409577 (PF) and MK-8722 (Glilx lab; GLXC-11445).

Fig. 21 shows the effect of different conjugates of co- and tri-agonist peptide(SEQ ID NOs:4-6) and MK8722 compared to a control group (vehicle, i.e. saline) on blood glucose level (mM) in DIO mice subjected to an acute compound tolerance test. The blood glucose levels were measured 24 hours after subcutaneous injection. Data is expressed as mean $\pm$ SEM, N = 8 per group. In general, the three conjugates glucagon/GLP-1/MK8722, glucagon/GIP/MK8722 and glucagon/GLP-1/GIP/MK8722 (depicted "Triagonist" in Fig. 21) result in significantly lower blood glucose levels compared to the groups treated with vehicle or the co- and tri-agonist peptides alone.

Fig. 22 shows an amino acid sequence alignment between co- and tri-agonists peptides of SEQ ID NOs: 4-6 and the glucagon peptide of SEQ ID NO: 1 used in the drug conjugates, wherein X₁ is D-alanine, D-serine, alpha-aminoisobutyric acid, N-methyl-serine, glycine or valine, and X₂ is cysteine (hCys40/Cys40) or L-penicillamine (Pen40).

Conclusion

The presented data demonstrate that chemical conjugation of a Glucagon analogue and an AMPK activator represents a novel medicinal strategy for effectively reversing metabolic disorders, including obesity, diabetes and NASH. Conjugates based on this strategy are superior in lowering body weight and cholesterol levels relative to the glucagon control and are not flawed with adverse central effects of AMPK activator.

Examples

Example 1: Preparation of peptides and peptide-AMPK activator conjugates.

5 **Materials:** All solvents and reagents were purchased from commercial sources and used without further purification. H-Rink amide ChemMatrix® resin was used for peptide elongation. Unless otherwise stated Fmoc-protected (9-fluorenylmethyl carbamate) amino acids were purchased from Iris-Biotech or Gyros Protein Technologies, and H-Rink amide ChemMatrix® resin, 35-100
10 mesh; loading of 0.40 – 0.60 mmol/g from Sigma Aldrich. The commercially available *N*^α-Fmoc amino acid building blocks were purchased as the following sidechain protected analogs: Arg, Pmc; Asp, O^tBu; Cys, Trt; Gln, Trt; His, Trt; Lys, Trt; Ser, ^tBu; and Trp, Boc (Pmc = 2,2,5,7,8-pentamethylchoman-6-sulfonyl, O^tBu = *tert*-butyl ester, Trt = trityl, Boc = *tert*-butyloxycarbonyl, and ^tBu =
15 *tert*-butyl ether).

 All peptides and conjugates of peptides and AMPK activators were characterized by analytical reverse phase ultra-performance liquid chromatography (RP-UPLC) (Waters) and electrospray ionization liquid chromatography mass spectrometry (ESI-LCMS) coupled to a Agilent 6410 Triple Quadrupole
20 Massfilter with a C18 column (Zorbax Eclipse, XBD-C18, 4.6 × 50 mm). The ESI-LCMS was eluting with a binary buffer system composed of H₂O:MeCN:TFA (A: 95:5:0.1, B: 5:95:0.1) at a flow rate of 0.75 mL/min. Purities were determined by RP-UPLC equipped with a C18 column (Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm) eluting with a binary buffer system composed
25 of H₂O:MeCN:TFA (A: 95:5:0.1, B: 5:95:0.1) at a flow rate of 0.45 mL/min.

Automated peptide synthesis protocol for Fmoc-protection scheme: Peptides were prepared as their C-terminally amidated derivatives using a Prelude X, induction heating assisted, peptide synthesizer (Gyros Protein Technologies, Tucson, AZ, USA) with 10 mL glass vessels. All reagents
30 were freshly prepared as stock solutions in DMF: Fmoc-protected amino acid (0.2 M), HCTU (0.5 M), DIPEA (1.0 M) and piperidine (20 % v/v). Peptide elongation was achieved by consecutive synthetic manipulations using the

following protocol: Deprotection (2 × 2 min, RT, 300 rpm shaking) and coupling (2 × 5 min, 75°C, 300 rpm shaking, for Arg and His 2 × 5 min, 50°C, 300 rpm shaking). Peptides were prepared using double and triple couplings consisting of AA/HCTU/DIPEA (ratio 1:1.25:2.5) in 5-fold excess compared to the resin.

5 **Purification:** The crude peptide or conjugates of peptides and AMPK activator was analyzed by RP-UPLC and ESI-LCMS or MALDI-TOF mass spectrometry prior to purification. Purifications were performed with a reverse-phase high-performance liquid chromatography (RP-HPLC) system (Waters) equipped with a reverse phase C18 column (Zorbax, 300 SB-C18, 21.2 × 250
10 mm) and eluting with a linear gradient (flow rate 20 mL/min) using a binary buffer system of H₂O:MeCN:TFA (A: 95:5:0.1; B: 5:95:0.1). Fractions were collected at intervals of 0.3 minutes and characterized ESI-LCMS. Purity was determined by RP-UPLC at 214 nm, and fractions with purities >95% were pooled and lyophilized. The final lyophilized products were used in further experiments.

15 **Conjugation protocol for assembly of conjugates of peptides and AMPK activators:** The pure peptide and the pure thiopyridyl-activated AMPK activator conjugate was dissolved in a binary solvent system (A: DMF; 6 M Guanidine, 1.5 M Imidazole in H₂O at pH = 8) (ratio 7:1) and agitated for at least 2 hours. The crude reaction mixture was monitored by analytical RP-
20 UPLC and ESI-LCMS. Upon completion, the reaction mixture was diluted with buffer A and buffer B and purified directly using RP-HPLC eluting with a linear gradient.

Desalting: All peptides were desalted prior to biological experiments. Desalting was performed by consecutively re-dissolving the peptide or the con-
25 jugate of a peptide and an AMPK activator in dilute aqueous 0.01 M HCl followed by lyophilization, repeated 3 times. The purity of the peptide or the conjugate was monitored by RP-UPLC and ESI-LCMS before being used for in vivo or in vitro experiments.

2-(pyridin-2-yl)disulfaneyl)ethan-1-amine hydrochloride. In a dry
30 schlenk round-bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere, 2'-aldrithiol (5.00 g, 22.70 mmol, 3.0 equiv.) was dissolved in dry MeOH (20 mL) followed by addition of cysteamine hydrochloride (859.5 mg,

7.57 mmol, 1.0 equiv.). The reaction was stirred for 2 hours at room temperature and subsequently concentrated *in vacuo*. The crude yellow oil was purified by either silica gel flash column chromatography (CH₂Cl₂:MeOH 17:3) or redissolved in MeOH and precipitated with cold ether to afford 2-(pyridin-2-yl-disulfaneyl)ethan-1-amine hydrochloride as a white crystalline (1.39 g, >95 %).
5 R_f = 0.22; UPLC/MS (ESI): m/z calcd. for C₇H₁₀N₂S₂ [M+H]⁺ = 187.0, found 187.2; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.51 (dt, J = 4.9, 1.4 Hz, 1H), 8.23 (s, 3H), 7.84 (td, J = 7.7, 1.9 Hz, 1H), 7.78 – 7.72 (m, 1H), 7.30 (ddd, J = 7.4, 4.8, 1.1 Hz, 1H), 3.14 – 3.06 (m, 4H); ¹³C NMR (101 MHz, DMSO) δ 158.05, 149.83,
10 137.91, 121.64, 120.08, 37.67, 34.79..

5-amino-1-((3a*R*,4*R*,6*R*,6a*R*)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-1*H*-imidazole-4-carboxamide. In a flame-dried schlenk round bottomed flask equipped with a magnetic stirring bar, Perchloric acid (70 %, 2.38 mL, 27.69 mmol, 1.3 equiv.) was added to a stirring
15 suspension of Aicar (5.50 g, 21.3 mmol, 1.0 equiv) in dry acetone (360 mL). After stirring for 3h at room temperature, the reaction was quenched by dropwise addition of ammonium hydroxide (4.04 mL, 29.83 mmol, 1.4 equiv.) at 0 °C. The solid obtained was filtered and dried to afford 5-amino-1-((3a*R*,4*R*,6*R*,6a*R*)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3, 4-*d*][1,3]
20 dioxol-4-yl)-1*H*-imidazole-4-carboxamide as a white crystalline (4.26 g, 67 %). Purity >95 % (HPLC, 0-50B in 20 min), R_t = 16.60 min; UPLC/MS (ESI): m/z calcd. for C₁₂H₁₉N₄O₅ [M+H]⁺ = 299.1, found 299.4; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.37 (s, 1H), 6.74 (d, J = 50.9 Hz, 2H), 5.93 (s, 2H), 5.75 (d, J = 3.6 Hz, 1H), 5.27 (t, J = 4.9 Hz, 1H), 5.08 (dd, J = 6.3, 3.7 Hz, 1H), 4.87 (dd, J = 6.4,
25 2.9 Hz, 1H), 4.10 (q, J = 3.7 Hz, 1H), 3.52 (tq, J = 11.7, 6.1, 4.7 Hz, 2H), 3.31 (s, 1H), 1.53 (s, 3H), 1.32 (s, 3H); ¹³C NMR (101 MHz, DMSO) δ 166.60, 142.75, 128.16, 113.38, 112.82, 88.58, 84.97, 82.31, 80.53, 61.00, 26.96, 25.20.

((3a*R*,4*R*,6*R*,6a*R*)-6-(5-amino-4-carbamoyl-1*H*-imidazol-1-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl (2-(pyridin-2-yl-disulfaneyl)ethyl)carbamate. In a flame-dried schlenk round-bottomed flask
30

equipped with a magnetic stirring bar and under N₂-atmosphere, 5-amino-1-((3aR,4R,6R,6aR)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-1H-imidazole-4-carboxamide (600.0 mg, 2.01 mmol, 1.0 equiv.) was dissolved in dry DMF (20 mL) followed by addition of N,N-Carbonyldiimidazole (391.0 mg, 2.41 mmol, 1.2 equiv.) and dry pyridine (488 µL, 6.03 mmol, 3.0 equiv.). The reaction was continuously monitored by UPLC-MS. Upon full conversion to the activated carbamate, 2-(pyridin-2-ylidisulfaneyl)ethan-1-amine hydrochloride (468.4 mg, 3.02 mmol, 1.5 equiv.) was added and the reaction heated to 45 °C with an oil bath and stirred for 22h. Compound 2-(pyridin-2-ylidisulfaneyl)ethan-1-amine hydrochloride was solubilized as the reaction progressed. Upon completion (monitored by UPLC-MS), the reaction was transferred to a separatory funnel with CH₂Cl₂ (75 mL) and washed with water (3x50 ml) and brine (2x 50 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo*. The crude oil was purified by silica gel flash column chromatography using a gradient (CH₂Cl₂ -> CH₂Cl₂:MeOH 19:1 -> CH₂Cl₂:MeOH 9:1) to afford ((3aR,4R,6R,6aR)-6-(5-amino-4-carbamoyl-1H-imidazol-1-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl (2-(pyridin-2-ylidisulfaneyl)ethyl) carbamate as a white crystalline solid (916 mg, 89 %); Purity >95 % (HPLC, 0-50B in 20 min), R_t = 16.60 min; UPLC/MS (ESI):

m/z calcd. for C₂₀H₂₇N₆O₆S₂ [M+H]⁺ = 511.1, found 511.2; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.51 – 8.28 (m, 1H), 7.86 – 7.73 (m, 2H), 7.51 (t, *J* = 5.7 Hz, 1H), 7.39 (s, 1H), 7.24 (ddd, *J* = 7.3, 4.8, 1.2 Hz, 1H), 6.76 (d, *J* = 31.7 Hz, 2H), 5.90 (s, 2H), 5.78 (d, *J* = 3.5 Hz, 1H), 5.17 (dd, *J* = 6.3, 3.5 Hz, 1H), 4.84 (dd, *J* = 6.3, 3.0 Hz, 1H), 4.24 (q, *J* = 4.4 Hz, 1H), 4.11 – 3.97 (m, 2H), 3.28 (q, *J* = 6.5 Hz, 2H), 2.90 (t, *J* = 6.1 Hz, 2H), 2.52 (d, *J* = 2.3 Hz, 1H), 1.53 (s, 3H), 1.32 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 166.42, 159.01, 155.61, 149.58, 142.90, 137.79, 127.51, 121.19, 119.29, 113.72, 112.47, 87.60, 82.49, 82.27, 80.52, 63.70, 37.53, 26.87, 25.19.

(2R,3S,4R,5R)-5-(5-amino-4-carbamoyl-1H-imidazol-1-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (2-(pyridin-2-ylidisulfaneyl)ethyl)carbamate. In a 10 mL vial with a screw cap, ((3aR,4R,6R,6aR)-6-(5-amino-4-carbamoyl-1H-imidazol-1-yl)-2,2-dimethyltetrahydrofuro[3,4-

d][1,3]dioxol-4-yl)methyl(2-(pyridin-2-yl)disulfaneyl)ethyl)carbamate (80.0 mg, 0.16 mmol, 1.0 equiv.) was dissolved in a 1:3 mixture of MeCN and 1.0 M aqueous HCl and left at the shaker for 3h (Until HPLC and UPLC-MS showed full conversion). Subsequently, the reaction mixture was lyophilized to afford
 5 (2*R*,3*S*,4*R*,5*R*)-5-(5-amino-4-carbamoyl-1*H*-imidazol-1-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl(2-(pyridin-2-yl)disulfaneyl)ethyl)carbamate as the dihydrochloride salt (84 mg, >95 %). Purity >95 % (HPLC, 0-50B in 20 min), R_t = 11.62 min; UPLC/MS (ESI): m/z calcd. for $C_{17}H_{23}N_6O_6S_2$ $[M+H]^+$ = 471.1, found 471.4; ^1H NMR (600 MHz, DMSO- d_6) δ 8.82 (s, 1H), 8.46 (dd, J = 5.0, 1.8 Hz, 1H), 7.85 (td, J = 7.8, 1.9 Hz, 1H), 7.79 (d, J = 8.0 Hz, 1H), 7.60 (t, J = 5.8 Hz, 1H), 7.26 (ddd, J = 7.5, 4.8, 1.1 Hz, 1H), 5.78 (d, J = 4.8 Hz, 1H), 4.37 (t, J = 5.0 Hz, 1H), 4.19 (m, 2H), 4.03 (m, 1H), 3.29 (q, J = 6.4 Hz, 2H), 2.92 (m, 2H); ^{13}C NMR (151 MHz, DMSO) δ 162.32, 161.07, 158.92, 155.79, 149.35, 142.90, 138.13, 127.63, 121.31, 119.51, 103.12, 88.46, 83.31, 73.73, 69.93, 63.80,
 15 37.58.

(3*S*,3*aS*,6*S*,6*aS*)-6-((5-([1,1'-biphenyl]-4-yl)-6-chloro-1*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-yl (2-(pyridin-2-yl)disulfaneyl)ethyl)carbamate. In a flame-dried round-bottomed flask under Argon atmosphere, MK-8722 (12 mg, 0.027 mmol, 1.0 equiv.), *N,N*-carbonyl diimidazole (5.2 mg, 0.032 mmol, 1.2 equiv.) and dry pyridine (6.5 μ L, 0.08 mmol, 3.0 equiv.) were dissolved in dry DMF (1 mL) and left on a shaker (500 rpm) overnight at ambient temperature. The formation of the activated carbamate was confirmed by UPLC-MS; UPLC/MS (ESI): m/z calcd. for $C_{28}H_{24}ClN_5O_5$ $[M+2H]^{2+}$ = 272.6 found 272.4. Upon full conversion as monitored by UPLC-MS, 2-(pyridin-2-yl)disulfaneyl)ethan-1-amine hydrochloride (7.48 mg, 0.040 mmol, 1.5 equiv.) in dry DMF (1 mL) was added via a syringe and the temperature increased to 50 °C and left overnight. The reaction mixture was diluted with H₂O:MeCN (1:1) and purified by preparative HPLC (eluting with a gradient from 0-100% B, over 20 mL/min) followed by lyophilization affording
 25 (3*S*,3*aS*,6*S*,6*aS*)-6-((5-([1,1'-biphenyl]-4-yl)-6-chloro-1*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-yl
 30 (2-(pyridin-2-yl)disulfaneyl)ethyl)carbamate as a white solid (11.6 mg, 66 %); Purity >95 % (HPLC),

$R_T = 16.77$ min; UPLC-MS; UPLC/MS (ESI): m/z calcd. for $C_{32}H_{30}ClN_5O_5S_2$ $[M+2H]^{2+} = 331.6$ found 331.5; ^1H NMR (600 MHz, DMSO- d_6) δ 8.49 – 8.43 (m, 1H), 7.95 (s, 1H), 7.83 (td, $J = 7.7, 1.9$ Hz, 1H), 7.81 – 7.72 (m, 7H), 7.59 (t, $J = 5.7$ Hz, 1H), 7.50 (t, $J = 7.7$ Hz, 2H), 7.43 – 7.37 (m, 1H), 7.32 – 7.21 (m, 1H), 5.48 (q, $J = 5.9$ Hz, 1H), 4.94 (td, $J = 6.9, 5.4$ Hz, 1H), 4.87 (t, $J = 5.2$ Hz, 1H), 4.65 (t, $J = 5.2$ Hz, 1H), 4.13 (dd, $J = 9.5, 6.1$ Hz, 1H), 3.93 (ddd, $J = 16.9, 9.2, 6.3$ Hz, 2H), 3.68 (dd, $J = 8.8, 7.3$ Hz, 1H), 3.35 – 3.22 (m, 2H), 2.92 (t, $J = 6.8$ Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 160.21, 159.06, 155.38, 149.57, 146.90, 139.69, 139.63, 137.88, 137.85, 130.09, 129.01, 127.65, 126.70, 126.09, 121.79, 121.22, 119.31, 116.22, 114.30, 80.44, 80.07, 78.61, 73.33, 70.12, 69.79, 37.59.

Preparation of GlucagonPen40/AICAR (Penicillamine linked): A

Glucagon peptide derivative was synthesized using the Fmoc protocol disclosed above and conjugated with a chemical linker derivatized AICAR analog.

The chemical synthesis of the chemical linker derivatized AICAR was performed via the synthetic route disclosed in Fig. 15.

Glucagon Pen40/MK-8722: The conjugate was prepared using the

Conjugation protocol disclosed above and by the chemical reaction shown in Fig. 16, the chemical reaction being performed in 6M guanidine, 1.5M imidazole buffer at room temperature for 2 hours. RP-UPLC and ESI-LCMS analyses determined the purity to >95%.

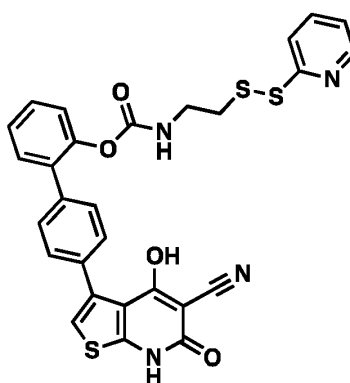
Example 2: *In vivo* pharmacology studies in diet-induced obesity (DIO) mice.

C57BL6J male mice, in the following referred to as diet-induced obesity (DIO) mice, were maintained on a high-fat diet (58 % energy from fat) and had, for each study, an average body weight of more than 45 gram prior to study start. Mice were either housed individually or double-housed. The mice were maintained on a 12 h dark-light cycle at 21-23°C. Compounds were administered subcutaneously once daily (between 2pm-5pm) and food intake (FI) and body weight (BW) measured at the corresponding time. For body composition, measures of fat and lean mass were performed prior to the study (1-3 days prior to study start) and on the final day of the study using an MRI scanner

(EchoMRI). The group of mice injected with a vehicle (saline) served as the control group.

Example 3: Preparation of conjugates of modified glucagon (SEQ ID NO: 3) and AMPK activator

4'-(5-cyano-4-hydroxy-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridin-3-yl)-[1,1'-biphenyl]-2-yl (2-(pyridin-2-yl)disulfaneyl)ethyl carbamate (402)

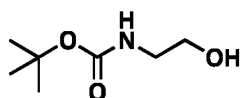


In a flame-dried round-bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere, A-769662 (100 mg, 0.28 mmol, 1.0 equiv.) and dry Et₃N (116 μ L, 0.83 mmol, 3.0 equiv.) were dissolved in dry CH₂Cl₂ (5 mL). The reaction mixture was cooled to 0 °C in an ice-water bath before nitrophenyl chloroformate (62 mg, 0.31 mmol, 1.1 equiv.) was added. The reaction was monitored by UPLC-MS. Upon completion, **137** (57 mg, 0.31 mmol, 1.1 equiv.) was added and the reaction heated to 50 °C and left stirring overnight. The reaction mixture was diluted with H₂O:MeCN (1:1) and purified by preparative HPLC (eluting with a gradient of 0-100% B, over 20 mL/min) followed by lyophilization to afford **402** as a white solid (78 mg, 49 %); **LC/MS** (ESI): m/z calcd. for C₂₈H₂₁N₄O₄S₃ [M+H]⁺ = 573.1, found 573.1; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.57 (s, 1H), 8.49 – 8.42 (m, 1H), 7.95 (t, *J* = 5.8 Hz, 1H), 7.77 (td, *J* = 7.7, 1.9 Hz, 1H), 7.76 – 7.70 (m, 1H), 7.52 – 7.45 (m, 2H), 7.48 – 7.40 (m, 2H), 7.43 – 7.35 (m, 2H), 7.34 (td, *J* = 7.5, 1.3 Hz, 1H), 7.26 – 7.21 (m, 1H), 7.19 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.05 (s, 1H), 3.30 (q, *J* = 6.4 Hz, 2H), 2.84 (t, *J* = 6.7 Hz, 2H); **¹³C NMR** (151 MHz, DMSO) δ 162.47, 159.49, 155.03, 150.06,

30

148.39, 138.22, 137.32, 136.67, 135.54, 134.44, 130.86, 129.82, 128.96,
128.22, 126.34, 124.18, 121.66, 119.73, 116.35, 40.41, 37.89.

***tert*-butyl (2-hydroxyethyl)carbamate (**2**) (Fig. 17, Scheme 1)**

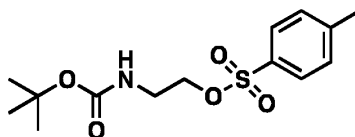


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In a flame-dried round-bottomed flask equipped with a magnetic stirring bar under N₂-atmosphere, Di-*tert*-butyl dicarbonate (7.86 g, 36.01 mmol, 1.10 equiv.) was added to a stirring solution of 2-ethanol amine (1.98 mL, 32.74 mmol, 1.0 equiv.) and dry Et₃N (6.70 mL, 49.11 mmol, 1.5 equiv.) in anhydrous CH₂Cl₂ (30 mL) and left overnight. The reaction mixture was concentrated *in vacuo*, redissolved in EtOAc (100 mL) and the organic layer washed with a solution of 0.5 N aqueous HCl (2x 50 mL) and brine (50 mL), dried over MgSO₄, filtered and concentrated *in vacuo* to afford **2** as a colorless oil (5.21 g, >95 %). ¹H NMR (400 MHz, Chloroform-*d*) δ 5.06 (s, 1H), 3.66 (t, *J* = 5.1 Hz, 2H), 3.25 (q, *J* = 5.4 Hz, 2H), 1.42 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 156.94, 79.72, 62.54, 46.15, 28.49.

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2-((*tert*-butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (3**) (Fig. 17, Scheme 1)**



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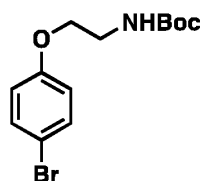
In a flame-dried round-bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere, **2** (5.28 g, 32.74 mmol, 1.0 equiv.) and dry Et₃N was dissolved in anhydrous CH₂Cl₂ (100 mL), added *p*-toluene sulfonyl chloride (9.36 g, 49.12 mmol, 1.5 equiv.) and left overnight. The reaction was transferred to a separatory funnel with CH₂Cl₂ (100 mL) and the combined organic layer washed with H₂O (3x50 mL) and brine (50 mL), dried over Na₂SO₄ and concentrated *in vacuo*. Purification by CombiFlash (eluting with a gradient 0-50 in 45 minutes) yielded **3** as a brown oil (8.98 g, 87 %). *R_f* = 0.61 (1:1,

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EtOAc:Heptanes); $^1\text{H NMR}$ (600 MHz, Chloroform-*d*) δ 7.90 – 7.69 (m, 2H), 7.40 – 7.33 (m, 2H), 4.85 (s, 1H), 4.06 (t, J = 5.1 Hz, 2H), 3.37 (q, J = 5.5 Hz, 2H), 2.44 (s, 3H), 1.40 (s, 9H); $^{13}\text{C NMR}$ (151 MHz, CDCl_3) δ 155.73, 145.14, 132.83, 130.08, 128.05, 79.89, 69.57, 39.88, 28.40, 21.75.

5

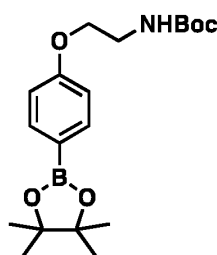
***tert*-butyl (2-(4-bromophenoxy)ethyl)carbamate (4) (Fig. 17, Scheme 1)**



In a flame dried round-bottomed flask equipped with a magnetic stirring
 10 bar and under N_2 -atmosphere, **3** (2.5 g, 13.92 mmol, 1.0 equiv.), 4-bromophenol (2.65 g, 15.31 mmol, 1.1 equiv.) and oven-dried K_2CO_3 (5.76 g, 41.2 mmol, 3.0 equiv.) was suspended in dry DMF (20 mL) at 60 °C overnight. Then, water was added to the reaction mixture and the aqueous layer extracted with EtOAc (3x 100 mL). The combined organic layers were washed with with
 15 0.1 N aqueous NaOH (100 mL) and brine (100 mL), dried over MgSO_4 , filtered and concentrated *in vacuo* to afford **4** (4.09 g, 93 %); **UPLC/MS (ESI):** m/z calcd. for $\text{C}_9\text{H}_{10}\text{BrNO}_2$ $[\text{M}+\text{H}-\text{tBu}]^+ = 260.0$, found 260.1; $^1\text{H NMR}$ (600 MHz, Chloroform-*d*) δ 7.62 – 7.28 (m, 2H), 6.90 – 6.63 (m, 2H), 4.98 (s, 1H), 3.97 (t, J = 5.2 Hz, 2H), 3.51 (dt, J = 5.5 Hz, 2H), 1.44 (s, 9H); $^{13}\text{C NMR}$ (151 MHz,
 20 CDCl_3) δ 157.82, 155.98, 132.44, 116.38, 116.38, 113.34, 79.73, 67.54, 40.16, 28.51.

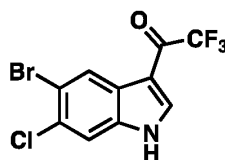
***tert*-butyl (2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)ethyl)carbamate (5) (Fig. 17, Scheme 1)**

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In a flame-dried round-bottomed flask equipped with a magnetic stirring bar, **4** (2.04 g, 6.5 mmol, 1.0 equiv.), bis(pinacolato)diboron (3.30 g, 13.02 mmol, 2.0 equiv.) AcOK (3.44 g, 35 mmol, 5.4 equiv.), and Pd(dppf)Cl₂ · CH₂Cl₂ (508 mg, 0.65 mmol, 0.1 equiv.) were mixed with dry degassed DMSO (30 mL) and the reaction mixture quickly degassed before heating to 80 °C for 13h. The reaction mixture was filtered through a pad of Celite®, the filtrate transferred to a separatory funnel with water (100 mL) and the aqueous layer extracted with EtOAc (3x 75 mL). The combined organic layers were washed with H₂O (3x 100 mL) and brine (2x 100 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude black oil was purified by silica gel flash column chromatography eluting with a gradient (Heptanes:EtOAc, 20:1 to 5:1), affording **5** as a colorless oil (1.73 g, 73 %). **UPLC/MS (ESI)**: m/z calcd. for C₁₅H₂₃BNO₅ [M+H-tBu]⁺ 308.2, found 308.3; **¹H NMR** (400 MHz, Chloroform-d) δ 7.93 – 7.34 (m, 2H), 6.93 – 6.67 (m, 2H), 4.04 (t, *J* = 5.1 Hz, 2H), 3.53 (q, *J* = 5.2 Hz, 2H), 1.44 (s, 9H), 1.32 (s, 12H);

1-(5-bromo-6-chloro-1*H*-indol-3-yl)-2,2,2-trifluoroethan-1-one (7)
(Fig. 17, Scheme 1)

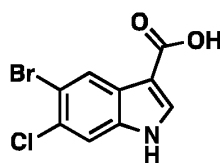


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In a flame-dried round bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere 5-bromo-6-chloro-1*H*-indole (2.22 g, 9.54 mmol,

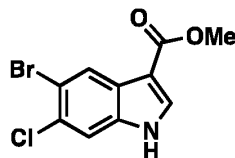
1.0 equiv.) was dissolved in anhydrous DMF (50 mL) and cooled to 0 °C in an ice-bath. Trifluoroacetic acid anhydride (5.3 mL, 38.2 mmol, 4.0 equiv.) was added dropwise to the stirring solution. The reaction was left stirring before being quenched with saturated aqueous Na₂CO₃ leading to precipitation. The precipitate was filtered and collected to afford **7** (quant.). **UPLC/MS (ESI):** m/z calcd. for C₁₀H₅BrClF₃NO [M+H]⁺ = 327.9, found 328.0; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.33 (d, *J* = 1.4 Hz, 1H), 8.27 (dp, *J* = 3.9, 2.0 Hz, 1H), 7.93 (s, 1H), 7.70 (d, *J* = 1.8 Hz, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 171.26 (q, CO-CF₃), 147.33, 145.16, 129.68, 125.47, 118.14 (q, CO-CF₃), 117.85, 115.23, 114.20, 108.97.

5-bromo-6-chloro-1*H*-indole-3-acid (8**) (Fig. 17, Scheme 1)**



In a round bottomed flask equipped with a magnetic stirring bar, **7** (3.11 g, 9.54 mmol, 1.0 equiv.) was suspended in 3.0 N aqueous NaOH (50 mL) and refluxed for 16h. The reaction mixture was allowed to cool to room temperature before being acidified to pH 1-2 using 5.0 N aqueous HCl and extracted with EtOAc (3x 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford the intermediate carboxylic acid **8** as a brown solid (quant.). **UPLC/MS (ESI):** m/z calcd. for C₁₀H₄BrClNO₂ [M-H]⁻ = 273.9, found 273.9.

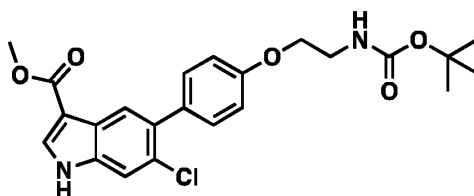
methyl 5-bromo-6-chloro-1*H*-indole-3-carboxylate (9**) (Fig. 17, Scheme 1)**



In a flame-dried round bottomed flask equipped with a magnetic stirring bar, crude **8** (2.34 g, 7.71 mmol, 1.0 equiv.) was dissolved in dry MeOH (50 mL) and added fuming sulfuric acid (1.0 mL) dropwise before heating to reflux for 12 hours. The reaction mixture was concentrated *in vacuo* to afford the methyl ester **9** as a reddish solid (1.90 g, 86 %). **UPLC/MS (ESI):** *m/z* calcd. for C₁₀H₆BrClNO₂ [M-H]⁻ = 287.9, found 288.0; ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.18 (s, 1H), 8.27 (s, 1H), 8.19 (s, 1H), 7.75 (s, 1H), 3.82 (s, 3H); ¹³C NMR (151 MHz, DMSO) δ 164.59, 136.31, 135.24, 126.96, 126.48, 125.00, 114.51, 114.37, 106.51, 51.45.

10

methyl 5-(4-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-6-chloro-1*H*-indole-3-carboxylate (10) (Fig. 17, Scheme 1)

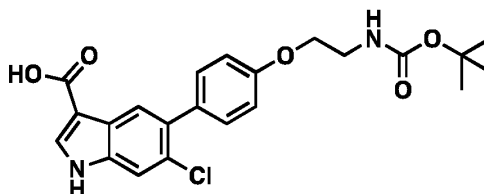


In a round-bottomed flask equipped with a magnetic stirring bar and under N₂ atmosphere, **9** (1g, 3.47 mmol, 1.0 equiv.) and **5** (1.22 g, 3.47 mmol, 1.0 equiv.) were dissolved in toluene (66 mL). K₂CO₃ (3.02 g, 21.85 mmol, 7.0 equiv.) was dissolved in H₂O (33 mL) and the solution added to reaction mixture followed by degassing by bubbling N₂ through the reaction mixture for 10 minutes. Pd(dppf)Cl₂·CH₂Cl₂ (247 mg, 0.34 mmol, 0.1 equiv.) was added and the reaction refluxed for 3 hours. The reaction mixture was concentrated *in vacuo*, redissolved in EtOAc (100 mL) and filtered through a pad of celite®. Purification by CombiFlash (eluting with a gradient 0-50 in 50 minutes) afforded **10** as a brown solid (1.33 g, 93 %). *R_f* = 0.61 (1:1, EtOAc:Heptanes); **UPLC/MS (ESI):** *m/z* calcd. for C₁₈H₁₈ClN₂O₃ [M+H-Boc]⁺ = 345.1, found 345.4; ¹H NMR (600 MHz, Chloroform-*d*) δ 9.00 – 8.91 (s, 1H), 8.10 (s, 1H), 7.93 (s, 1H), 7.53 (s, 1H), 7.43 – 7.37 (d, *J* = 8.6 Hz, 2H), 6.95 (d, *J* = 8.7, 2H), 5.06 (s, 1H), 4.08 (t, *J* = 5.7 Hz, 2H), 3.90 (s, 3H), 3.57 (t, *J* = 5.8 Hz, 2H), 1.47 (s, 9H); ¹³C NMR (151 MHz, CDCl₃) δ 165.38, 157.98, 135.75, 134.60, 133.32, 132.18, 131.31,

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128.31, 127.94, 124.94, 123.77, 114.89, 113.97, 112.55, 109.15, 77.37, 77.16, 76.95, 67.33, 51.46, 51.34, 28.56, 25.01, 24.74.

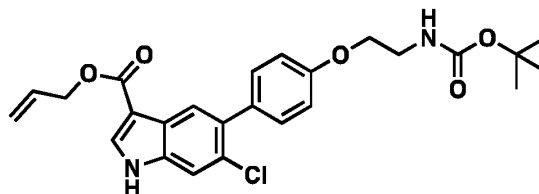
5-(4-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-6-chloro-1*H*-indole-3-carboxylic acid (11) (Fig. 17, Scheme 1)



In a round-bottomed flask equipped with a magnetic stirring bar, **10** (685 mg, 1.54 mmol, 1.0 equiv.) was dissolved in a solvent system consisting of THF:MeOH:H₂O (12 mL, 1:1:1) and added NaOH (185 mg, 4.62 mmol, 3.0 equiv.) and refluxed overnight. Then, the reaction was concentrated *in vacuo* followed by acidification to pH = 3-4. The aqueous layer was extracted with EtOAc (3x 50 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by CombiFlash (eluting with a gradient 50-100% EtOAc in Heptanes) afforded **11** as a brownish solid (597 mg, 90 %). *R_f* = 0.43 (EtOAc);

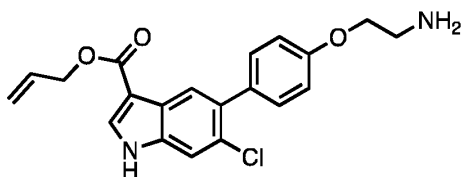
UPLC/MS (ESI): *m/z* calcd. for C₂₂H₂₂ClN₂O₅ [M-H]⁻ = 429.1, found 429.3; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.09 (s, 1H), 11.91 (d, *J* = 2.8 Hz, 1H), 8.07 (d, *J* = 2.8 Hz, 1H), 7.93 (s, 1H), 7.61 (s, 1H), 7.34 (d, *J* = 8.7 Hz, 2H), 7.01 (d, *J* = 8.7 Hz, 2H), 4.01 (t, *J* = 6.2 Hz, 2H), 3.33 (t, 6, 1 2H), 1.40 (s, 9H); **¹³C NMR** (151 MHz, DMSO) δ 165.55, 157.63, 155.68, 135.89, 133.75, 132.78, 132.44, 130.74, 125.79, 125.24, 122.68, 114.01, 112.83, 107.56, 77.76, 73.49, 66.41, 28.22.

allyl 5-(4-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-6-chloro-1*H*-indole-3-carboxylate (12) (Fig. 17, Scheme 1)



In a flame-dried round bottomed flask equipped with a magnetic stirring bar and under N₂ atmosphere, **11** (500 mg, 1.16 mmol, 1.0 equiv.) and NaHCO₃ (157 mg, 1.86 mmol, 1.6 equiv.) was suspended in dry DMF (10 mL) followed by addition of allyl bromide (151 μ L, 1.74 mmol, 1.5 equiv.) via syringe. The reaction mixture was heated to 50 °C and stirred for 12 hours. The reaction was quenched with H₂O (20 mL) and transferred to a separatory funnel with CH₂Cl₂ (50 mL). The organic layer was washed with H₂O (5x 30 mL) and brine (2x 30 mL), dried over MgSO₄, filtered and concentrated *in vacuo* to yield **12** as a brown solid (543 mg, >95 %). **UPLC/MS (ESI):** m/z calcd. for C₂₅H₂₆ClN₂O₅ [M-H]⁻ = 469.2, found 469.4; **¹H NMR** (600 MHz, Chloroform-*d*) δ 8.95 – 8.92 (m, 1H), 8.13 (d, *J* = 2.8 Hz, 1H), 7.95 (d, *J* = 2.9 Hz, 1H), 7.54 (s, 1H), 7.44 – 7.38 (m, 2H), 6.98 – 6.93 (m, 2H), 6.04 (ddt, *J* = 17.3, 10.9, 5.6 Hz, 1H), 5.39 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.26 (dq, *J* = 10.5, 1.4 Hz, 1H), 5.05 (s, 1H), 4.82 (tt, *J* = 6.8, 1.5 Hz, 2H), 4.08 (t, *J* = 5.1 Hz, 2H), 3.57 (q, *J* = 5.2 Hz, 2H), 1.47 (s, 9H); **¹³C NMR** (151 MHz, CDCl₃) δ 164.50, 157.97, 156.12, 135.75, 134.64, 133.30, 132.89, 132.24, 131.31, 128.33, 125.06, 123.80, 118.02, 113.97, 112.57, 109.07, 79.73, 77.37, 77.16, 76.95, 67.34, 64.77, 40.35, 28.57.

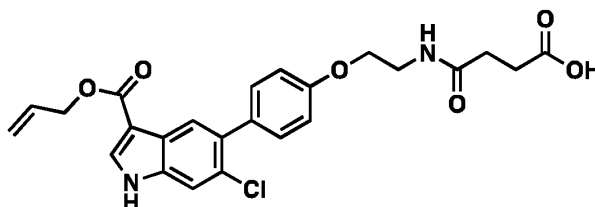
allyl 5-(4-(2-aminoethoxy)phenyl)-6-chloro-1H-indole-3-carboxylate (13) (Fig. 17, Scheme 1)



In a round-bottomed flask equipped with a magnetic stirring bar, **12** (500 mg, 1.06 mmol, 1.0 equiv.) was dissolved in CH₂Cl₂ (25 mL) and cooled to 0 °C using an ice-water bath followed by dropwise addition of 50 % TFA in CH₂Cl₂ (8 mL). The reaction was stirred at room temperature for 2 hours before being concentrated *in vacuo*. The oil was co-evaporated with multiple rounds of ether to afford **13** as a brown solid (385 mg, > 95 %). **UPLC/MS (ESI):** m/z calcd. for C₂₀H₂₀ClN₂O₃ [M+H]⁺ = 371.1, found 371.4; **¹H NMR** (400 MHz,

DMSO-*d*₆) δ 12.13 (d, *J* = 3.0 Hz, 1H), 8.20 (d, *J* = 3.0 Hz, 1H), 8.03 (s, 3H), 7.95 (d, *J* = 2.7 Hz, 1H), 7.93 (s, 1H), 7.66 (s, 1H), 7.46 – 7.32 (m, 2H), 7.20 – 7.01 (m, 2H), 6.03 (ddt, *J* = 17.2, 10.5, 5.3 Hz, 1H), 5.38 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.23 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.76 (dt, *J* = 5.3, 1.6 Hz, 2H), 4.23 (t, *J* = 5.1 Hz, 2H), 3.27 (h, *J* = 4.7 Hz, 2H); ¹³C NMR (101 MHz, DMSO) δ 162.28, 157.10, 135.96, 133.27, 133.00, 132.98, 130.83, 126.05, 124.91, 122.43, 117.28, 114.23, 113.10, 106.33, 64.42, 63.64, 38.43.

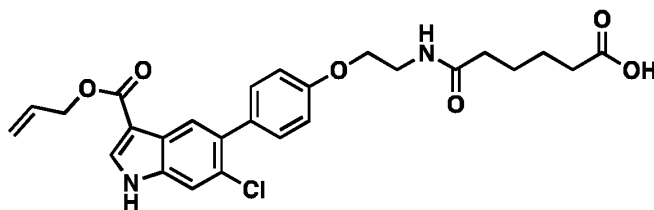
4-((2-(4-(3-((allyloxy)carbonyl)-6-chloro-1*H*-indol-5-yl)phenoxy)ethyl)amino)-4-oxobutanoic acid (14a) (Fig. 17, Scheme 1)



In a flame-dried flask equipped with a magnetic stirring and under N₂-atmosphere, **13** (75 mg, 0.20 mmol, 1.0 equiv.) and dry Et₃N (83 μ L, 0.60 mmol, 3.0 equiv.) was dissolved in dry CH₂Cl₂ (5 mL) before adding methyl succinyl chloride (75 μ L, 0.4 mmol, 2.0 equiv.) via syringe. The reaction was stirred for 2 hours before being evaporated to dryness (Formation of double acylated intermediate). The crude reaction mixture was redissolved in a mixture of THF and H₂O (1:1, 4 mL) and added NaOH (30 mg, 0.7 mmol, 3.5 equiv.) and left stirring for 4 hours. The reaction mixture was concentrated *in vacuo*, adsorbed on silica and purified by flash chromatography (EtOAc + 1% AcOH) yielding **14a** as a white solid (43 mg, 45 %). *R*_f = 0.11 (EtOAc + 1% AcOH); UPLC/MS (ESI): *m/z* calcd. for C₂₄H₂₂ClN₂O₆ [M-H]⁻ = 469.9, found 469.4; ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.09 (s, 1H), 8.19 (s, 1H), 8.15 (q, *J* = 5.6, 4.7 Hz, 1H), 7.92 (s, 1H), 7.64 (s, 1H), 7.36 – 7.33 (m, 2H), 7.07 – 7.00 (m, 2H), 6.03 (ddt, *J* = 17.3, 10.5, 5.3 Hz, 1H), 5.38 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.23 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.76 (dt, *J* = 5.3, 1.6 Hz, 2H), 4.04 (t, *J* = 5.7 Hz, 2H), 3.45 (q, *J* = 5.6 Hz, 2H), 2.44 (dd, *J* = 7.3, 6.1 Hz, 2H), 2.36 (t, *J* = 6.7 Hz, 2H); ¹³C NMR (151 MHz, DMSO) δ 173.84, 171.34, 163.58, 157.67, 135.90, 134.19, 133.27,

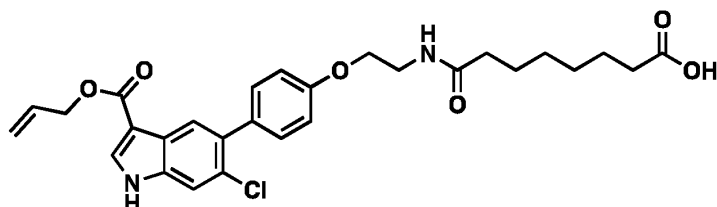
133.13, 132.39, 130.74, 126.11, 124.87, 122.45, 117.31, 114.07, 113.05, 106.37, 66.38, 63.66, 38.30, 29.93, 29.13.

6-((2-(4-(3-((allyloxy)carbonyl)-6-chloro-1H-indol-5-yl)phenoxy)ethyl)amino)-6-oxohexanoic acid (14b) (Fig. 17, Scheme 1)



In a flame-dried flask equipped with a magnetic stirring and under N₂-atmosphere, **13** (75 mg, 0.20 mmol, 1.0 equiv.) and dry Et₃N (83 μL, 0.60 mmol, 3.0 equiv.) was dissolved in dry CH₂Cl₂ (5 mL) before adding methyl adipoyl chloride (67 μL, 0.4 mmol, 2.0 equiv.) via syringe. The reaction was stirred for 2 hours before being evaporated to dryness (Formation of double acylated product). The crude reaction mixture was redissolved in a mixture of THF and H₂O (1:1, 4 mL) and added NaOH (30 mg, 0.7 mmol, 3.5 equiv.) and left stirring for 4 hours. The reaction mixture was concentrated *in vacuo*, adsorbed on silica and purified by flash chromatography (39:1 EtOAc:MeOH + 1% AcOH) yielding **14b** as a white solid (40.3 mg, 40 %). *R_f* = 0.23 (39:1 EtOAc:MeOH + 1% AcOH); **UPLC/MS** (ESI): *m/z* calcd. for C₂₆H₂₆ClN₂O₆ [M-H]⁻ = 398.0, found 397.4; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.08 (d, *J* = 2.7 Hz, 1H), 11.98 (s, 1H), 8.19 (d, *J* = 2.6 Hz, 1H), 8.07 (t, *J* = 5.6 Hz, 1H), 7.92 (s, 1H), 7.64 (s, 1H), 7.37 – 7.32 (m, 2H), 7.05 – 7.00 (m, 2H), 6.03 (ddt, *J* = 17.2, 10.5, 5.3 Hz, 1H), 5.38 (dq, *J* = 17.3, 1.7 Hz, 1H), 5.23 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.76 (dt, *J* = 5.3, 1.6 Hz, 2H), 4.04 (t, *J* = 5.7 Hz, 2H), 3.45 (q, *J* = 5.6 Hz, 2H), 2.20 (t, *J* = 6.9 Hz, 2H), 2.11 (t, *J* = 7.0 Hz, 2H), 1.56 – 1.43 (m, 4H); **¹³C NMR** (151 MHz, DMSO) δ 174.35, 172.25, 163.58, 157.67, 135.89, 134.19, 133.27, 133.13, 132.37, 130.73, 126.10, 124.86, 122.44, 117.31, 114.06, 113.04, 106.37, 66.36, 63.65, 38.18, 34.96, 33.40, 24.76, 24.12.

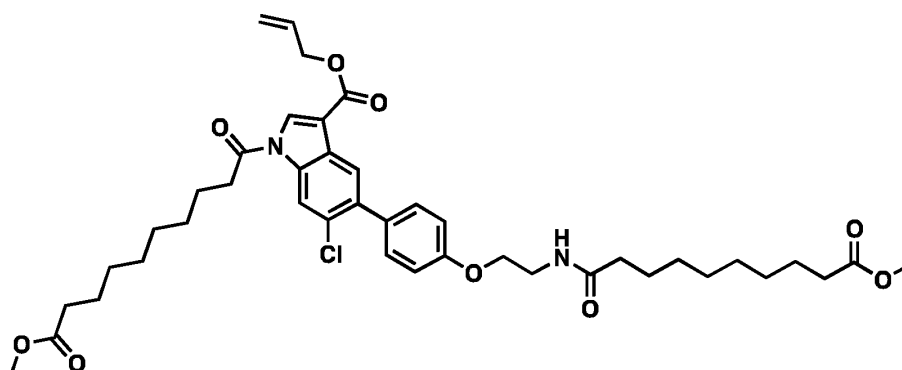
8-((2-(4-(3-((allyloxy)carbonyl)-6-chloro-1*H*-indol-5-yl)phenoxy)ethyl)amino)-8-oxooctanoic acid (14c) (Fig. 17, Scheme 1)



In a flame-dried flask equipped with a magnetic stirring and under N₂-
 5 atmosphere, **13** (75 mg, 0.20 mmol, 1.0 equiv.) and dry Et₃N (83 μ L, 0.60 mmol, 3.0 equiv.) was dissolved in dry CH₂Cl₂ (5 mL) before adding methyl octanoate chloride (121 μ L, 0.4 mmol, 2.0 equiv.) via syringe. The reaction was stirred for 2 hours before being evaporated to dryness (Formation of double acylated intermediate). The crude reaction mixture was redissolved in a mixture of THF
 10 and H₂O (1:1, 4 mL) and added NaOH (30 mg, 0.7 mmol, 3.5 equiv.) and left stirring for 4 hours. The reaction mixture was concentrated *in vacuo*, adsorbed on silica and purified by flash chromatography (EtOAc + 1% AcOH) yielding **14c** as a white solid (43 mg, 45 %). *R_f* = 0.31 (EtOAc + 1% AcOH); **UPLC/MS** (ESI): *m/z* calcd. for C₂₈H₃₀ClN₂O₆ [M-H]⁻ = 525.2, found 525.5; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.49 (s, 1H), 8.17 (s, 1H), 8.14 (t, *J* = 5.6 Hz, 1H), 7.91 (s, 1H), 7.68 (s, 1H), 7.52 – 7.25 (m, 2H), 7.25 – 6.94 (m, 2H), 6.02 (ddt, *J* = 17.3, 10.5, 5.3 Hz, 1H), 5.37 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.23 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.75 (dt, *J* = 5.3, 1.6 Hz, 2H), 4.03 (t, *J* = 5.8 Hz, 2H), 3.43 (q, *J* = 5.7 Hz, 2H), 2.08 (dt, *J* = 17.5, 7.4 Hz, 4H), 1.53 – 1.39 (m, 2H), 1.24 (m, 4H); **¹³C NMR**
 15 (151 MHz, DMSO) δ 175.64, 172.52, 163.61, 157.66, 135.98, 134.10, 133.28, 133.05, 132.39, 130.74, 125.98, 124.86, 122.39, 117.28, 114.04, 113.15, 106.22, 66.32, 63.62, 38.14, 35.27, 28.60, 28.49, 25.19, 25.11.

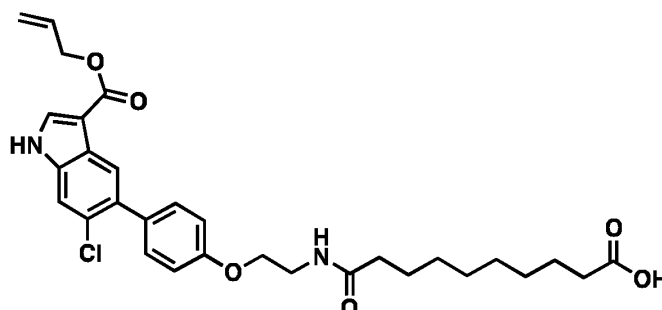
allyl 6-chloro-5-(4-(2-(10-methoxy-10-oxodecanamido)ethoxy)phenyl)-1-(10-methoxy-10-oxodecanoyl)-1*H*-indole-3-carboxylate (14d') (Fig. 17, Scheme 1)

25



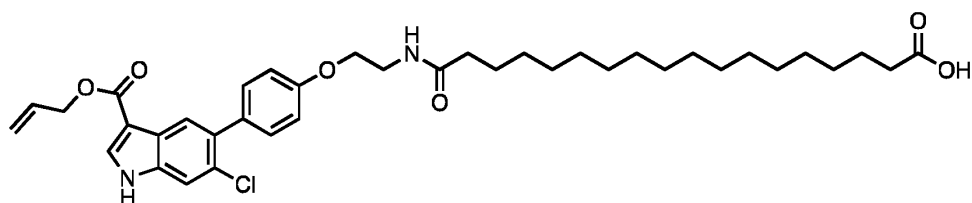
In a flame-dried round bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere, **13** (75 mg, 0.20 mmol, 1.0 equiv.) HBTU (118 mg, 0.30 mmol, 1.5 equiv.) monomethyl sebacate (70 mg, 0.3 mmol, 1.5 equiv.) were suspended in dry DMF (5 mL) followed by addition of dry Et₃N (83 μ L, 0.6 mmol, 3.0 equiv.). The reaction was left stirring for 4 hours. Then, the reaction mixture was transferred to a separatory funnel with EtOAc (50 mL). The organic layer was washed with saturated aqueous NaHCO₃ (3x 50 mL) and brine (50 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. The crude residue was evaporated on silica and purified by flash chromatography (1:1 EtOAc:Heptanes + 1% AcOH) yielding **14d'** - the double acylated intermediate - as a white solid (90 mg, 54 %). *R_f* = 0.15 (1:1 EtOAc:Heptanes + 1% AcOH); ¹H NMR (600 MHz, Chloroform-*d*) δ 8.64 (s, 1H), 8.18 (s, 1H), 8.10 (s, 1H), 7.43 – 7.39 (m, 2H), 7.02 – 6.77 (m, 2H), 6.10 – 5.98 (m, 1H), 5.96 (t, *J* = 5.7 Hz, 1H), 5.41 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.30 (dq, *J* = 10.4, 1.3 Hz, 1H), 4.85 (dt, *J* = 5.8, 1.4 Hz, 2H), 4.11 (t, *J* = 5.0 Hz, 2H), 3.71 (q, *J* = 5.3 Hz, 2H), 3.67 (s, 3H), 3.65 (s, 3H), 2.97 (t, *J* = 7.3 Hz, 2H), 2.30 (dt, *J* = 17.0, 7.5 Hz, 4H), 2.22 (t, *J* = 7.6 Hz, 2H), 1.86 (p, *J* = 7.4 Hz, 2H), 1.62 (dq, *J* = 20.4, 7.2 Hz, 4H), 1.52 – 1.43 (m, 2H), 1.42 – 1.25 (m, 16H); ¹³C NMR (151 MHz, CDCl₃) δ 174.44, 174.40, 171.58, 163.43, 158.12, 137.16, 135.38, 132.82, 132.29, 131.43, 131.22, 131.00, 126.32, 123.57, 118.83, 117.90, 114.07, 113.59, 67.05, 65.48, 51.61, 51.58, 39.13, 36.85, 35.70, 34.22, 34.20, 29.32, 29.27, 29.24, 29.19, 29.13, 25.79, 25.03, 24.47.

10-((2-(4-(3-((allyloxy)carbonyl)-6-chloro-1H-indol-5-yl)phenoxy)ethyl)amino)-10-oxodecanoic acid (14d) (Fig. 17, Scheme 1)



In a round bottomed flask equipped with a magnetic stirring bar, **14d'**
 5 (90 mg, 0.13 mmol, 1.0 equiv.) was dissolved in a solvent system consisting of THF and H₂O (6 mL, 1:1) followed by addition of NaOH (17.6 mg, 0.46 mmol, 3.5 equiv.). The reaction was left stirring for 6 hours before being concentrated *in vacuo*. The crude residue was evaporated on silica and purified by flash chromatography (EtOAc + 1% AcOH) yielding **14d** as a white solid (36 mg, 52
 10 %). **R_f** = 0.19 (EtOAc + 1% AcOH); **UPLC/MS** (ESI): *m/z* calcd. for C₃₀H₃₄ClN₂O₆ [M-H]⁻ = 553.2, found 553.5; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.09 (s, 1H), 8.19 (s, 1H), 8.04 (t, *J* = 5.6 Hz, 1H), 7.92 (s, 1H), 7.64 (s, 1H), 7.34 (d, *J* = 8.7 Hz, 1H), 7.04 – 6.96 (m, 2H), 6.03 (ddt, *J* = 17.3, 10.5, 5.3 Hz, 1H), 5.38 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.23 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.76 (dt, *J*
 15 = 5.3, 1.6 Hz, 2H), 4.04 (t, *J* = 5.7 Hz, 2H), 3.44 (q, *J* = 5.6 Hz, 2H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.09 (t, *J* = 7.4 Hz, 2H), 1.52 – 1.40 (m, 4H), 1.23 (m, 8H); **¹³C NMR** (151 MHz, DMSO) δ 174.50, 172.47, 163.57, 157.70, 135.90, 134.19, 133.27, 133.12, 132.36, 130.73, 126.09, 124.86, 122.44, 117.29, 114.04, 113.05, 106.36, 66.37, 63.65, 38.19, 35.28, 33.75, 28.64, 28.62, 28.58, 28.53,
 20 25.22, 24.50.

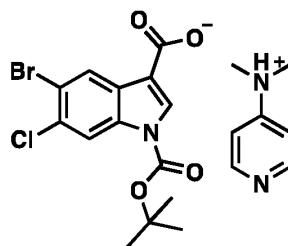
20-((2-(4-(3-((allyloxy)carbonyl)-6-chloro-1H-indol-5-yl)phenoxy)ethyl)amino)-20-oxoicosanoic acid (14e) (Fig. 17, Scheme 1)



In a flame-dried round bottomed flask equipped with a magnetic stirring bar and under N_2 atmosphere, **13** (75 mg, 0.20 mmol, 1.0 equiv.), Octadecanedioic acid mono-*tert*-butyl ester (121 mg, 0.30 mmol, 1.5 equiv.) and HBTU (115 mg, 0.30 mmol, 1.5 equiv.) were suspended in dry DMF (5 mL) before addition of dry Et_3N (83 μ L, 0.60 mmol, 3.0 equiv.). The reaction was left stirring for 2 hours before being diluted with CH_2Cl_2 (25 mL) and the organic layer washed with $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude intermediate was redissolved in CH_2Cl_2 (10 mL), cooled to 0 °C using an ice-water bath and treated with 50 % TFA in CH_2Cl_2 (4 mL). The reaction was allowed to warm to room temperature and left stirring for 2 hours before being concentrated in vacuo. Purification by flash chromatography (1:1 EtOAc:heptanes + 1% AcOH) afforded **14e** as a white solid (44.5 mg, 32 %). R_f = 0.28 (1:1 EtOAc:Heptanes + 1% AcOH); **UPLC/MS** (ESI): m/z calcd. for $C_{38}H_{51}ClN_2O_5$ $[M+H]^+$ = 667.3, found 667.1; **^1H NMR** (600 MHz, $DMSO-d_6$) δ 12.07 (d, J = 3.0 Hz, 1H), 11.97 – 11.90 (m, 1H), 8.19 (d, J = 2.9 Hz, 1H), 8.04 (t, J = 5.6 Hz, 1H), 7.92 (s, 1H), 7.64 (s, 1H), 7.37 – 7.31 (m, 2H), 7.06 – 6.99 (m, 2H), 6.09 – 5.97 (m, 1H), 5.38 (dq, J = 17.2, 1.6 Hz, 1H), 5.23 (dddq, J = 10.4, 5.9, 4.4, 1.5 Hz, 1H), 4.76 (dt, J = 5.3, 1.6 Hz, 2H), 4.04 (t, J = 5.6 Hz, 2H), 3.44 (q, J = 5.6 Hz, 2H), 2.16 (t, J = 7.0 Hz, 3H), 2.09 (t, J = 7.3 Hz, 2H), 1.54 – 1.41 (m, 6H), 1.30 – 1.15 (m, 26H); **^{13}C NMR** (151 MHz, $DMSO$) δ 174.45, 172.48, 163.56, 157.72, 135.89, 133.26, 130.71, 126.08, 124.88, 122.46, 117.27, 114.02, 113.03, 106.38, 66.40, 63.64, 48.62, 38.20, 35.29, 33.64, 29.02, 29.00, 28.96, 28.94, 28.87, 28.79, 28.70, 28.57, 28.52, 25.25, 24.46.

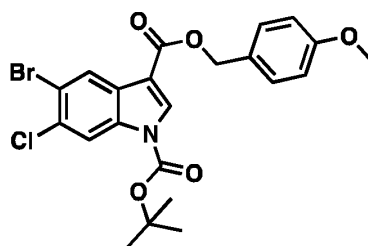
***N,N*-dimethylpyridin-4-aminium 5-bromo-1-(*tert*-butoxycarbonyl)-6-chloro-1*H*-indole-3-carboxylate (**15**) (Fig. 18, Scheme 2)**

43



In a flame-dried round-bottomed flask equipped with a magnetic stirring bar and under N₂-protection, **7** (3.71 g, 16.05 mmol, 1.0 equiv.) and *N,N*-dimethyl aminopyridine (0.98 g, 8.03 mmol, 0.5 mmol) were suspended in dry CH₂Cl₂ (100 mL) before adding tert butyl dicarbonate (3.85 g, 17.67 mmol, 1.1 equiv.) in one portion and the resulting mixture stirred for 6 hours. The reaction was quenched with water and extracted with EtOAc (200 mL). The combined organic layer was washed with H₂O (3x 100 mL) and brine (100 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. The crude solid was recrystallized from EtOAc to afford **15** as the DMAP salt (2.51 g, 64 %). **UPLC/MS** (ESI): *m/z* calcd. for C₁₄H₁₂BrClNO₄ [M-H]⁻ = 374.0, found 374.2; **¹H NMR** (400 MHz, DMSO-*d*₆) δ 8.39 (s, 1H), 8.23 (s, 1H), 8.17 – 8.12 (m, 2H), 8.12 (s, 1H), 6.69 – 6.64 (m, 2H), 2.99 (s, 6H), 1.64 (s, 9H); **¹³C NMR** (101 MHz, DMSO) δ 164.67, 154.56, 147.95, 147.08, 134.34, 132.49, 128.96, 128.19, 125.67, 116.27, 116.24, 113.40, 106.70, 85.79, 27.44.

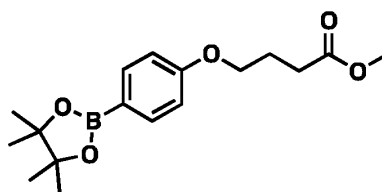
1-(*tert*-butyl) 3-(4-methoxybenzyl) 5-bromo-6-chloro-1*H*-indole-1,3-dicarboxylate (16**) (Fig. 18, Scheme 2)**



In a flame-dried round bottomed flask equipped with a magnetic stirring bar under N₂ atmosphere, **15** (2.50 g, 5.04 mmol, 1.0 equiv.), *p*-methoxybenzyl chloride (1.03 mL, 7.56 mmol, 1.5 equiv.) and anhydrous K₂CO₃ (1.39 g, 10.08 mmol, 2.0 equiv.) were suspended in dry DMF (20 mL). The reaction was

heated to 50 °C and stirred for 3 hours. Then, the reaction was transferred to a separatory with CH₂Cl₂ (200 mL). The organic layer was washed with H₂O (5x100 mL) and brine (2x100 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by CombiFlash (eluting with 10 % EtOAc in Heptanes over 45 minutes) afforded **16** as a white solid (2.49 g, >95 %). *R_f* = 0.35; **UPLC/MS** (ESI): *m/z* calcd. for C₂₂H₂₀BrClNO₅ [M-H]⁻ = 494.0, found 494.2; **¹H NMR** (600 MHz, Chloroform-*d*) δ 8.42 (s, 1H), 8.33 (s, 1H), 8.21 (s, 1H), 7.44 – 7.38 (m, 2H), 6.96 – 6.86 (m, 2H), 5.33 (s, 2H), 3.82 (s, 3H), 1.67 (s, 9H); **¹³C NMR** (151 MHz, CDCl₃) δ 163.49, 159.92, 148.46, 134.92, 133.26, 131.24, 130.41, 130.37, 129.56, 128.18, 127.62, 126.29, 118.13, 117.05, 114.21, 113.95, 111.62, 86.25, 66.38, 55.47, 28.18.

methyl 4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)butanoate (18) (Fig. 18, Scheme 2)

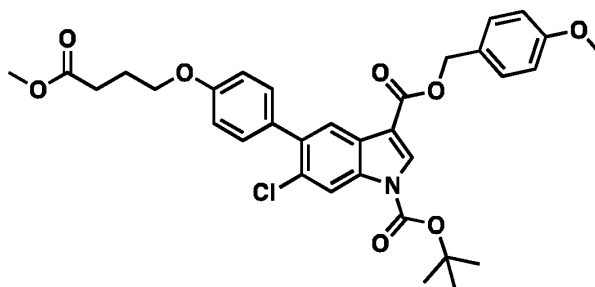


15

In a flame dried round-bottomed flask equipped with a magnetic stirring bar and under N₂-atmosphere, methyl 4-bromobutanoate (1.8 mL, 13.63 mmol, 1.2 equiv.), 4-Hydroxyphenylboronic acid pinacol ester (2.50 g, 11.36 mmol, 1.0 equiv.) and oven-dried K₂CO₃ (5.65 g, 40.89 mmol, 3.0 equiv.) was suspended in dry DMF (40 mL) at 50 °C overnight. Then, water was added to the reaction mixture and the aqueous layer extracted with EtOAc (3x 100 mL). The combined organic layer was washed with with H₂O (3x100 mL) and brine (100 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by CombiFlash (eluting with a gradient 20% EtOAc in Heptanes over 50 minutes) afforded **18** as a transparent oil (3.21 g, 88 %). *R_f* = 0.21 (1:4, EtOAc:Heptanes); **¹H NMR** (600 MHz, Chloroform-*d*) δ 7.88 – 7.55 (m, 2H), 6.99 – 6.73 (m, 2H), 4.03 (t, *J* = 6.1 Hz, 2H), 3.68 (s, 3H), 2.53 (t, *J* = 7.3 Hz, 2H), 2.14 – 2.08 (m, 2H), 1.33 (s, 12H); **¹³C NMR** (151 MHz, CDCl₃) δ 173.77, 161.54, 136.66, 113.97, 83.69, 66.61, 51.77, 30.67, 25.00, 24.74.

25

1-(*tert*-butyl) 3-(4-methoxybenzyl) 6-chloro-5-(4-(4-methoxy-4-oxobutoxy)phenyl)-1*H*-indole-1,3-dicarboxylate (19**) (Fig. 18, Scheme 2)**



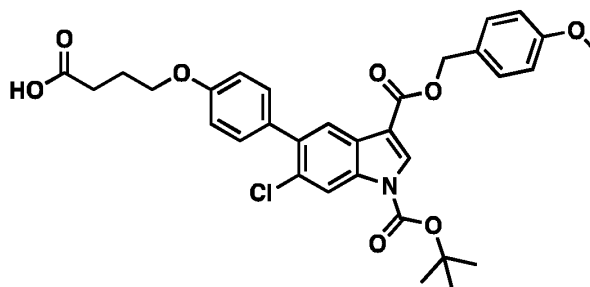
5 In a round-bottomed flask equipped with a magnetic stirring bar and under N₂ atmosphere, **16** (804 mg, 1.56 mmol, 1.0 equiv.) and **18** (500 mg, 1.56 mmol, 1.0 equiv.) were dissolved in toluene (30 mL). K₂CO₃ (1.51 g, 10.93 mmol, 7.0 equiv.) was dissolved in H₂O (15 mL) and the solution added to reaction mixture followed by degassing by bubbling N₂ through the reaction

10 mixture for 10 minutes. Pd(dppf)Cl₂·CH₂Cl₂ (128 mg, 0.16 mmol, 0.1 equiv.) was added and the reaction refluxed for 3 hours. The reaction mixture was concentrated *in vacuo*, redissolved in EtOAc (100 mL) and filtered through a pad of celite®. The filtrate was transferred to a separatory funnel with EtOAc (100 mL). The combined organic layer was washed with H₂O (3x100 mL) and

15 brine (100 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by CombiFlash (eluting with a gradient 20% EtOAc in Heptanes over 50 minutes) afforded **19** as a white solid (907 mg, >95 %). *R_f* = 0.21 (1:4, EtOAc:Heptanes); **UPLC/MS** (ESI): *m/z* calcd. for C₃₃H₃₆ClNO₈ [M+H]⁺ = 608.2, found 607.6; **¹H NMR** (400 MHz, Chloroform-*d*) δ 8.30 (s, 1H), 8.24 (s, 1H), 8.11 (s, 1H), 7.45 – 7.33 (m, 4H), 6.98 – 6.92 (m, 2H), 6.90 (d, *J* = 8.7 Hz, 2H), 5.31 (s, 2H), 4.07 (t, *J* = 6.1 Hz, 2H), 3.81 (s, 3H), 3.71 (s, 3H), 2.57 (t, *J* = 7.3 Hz, 2H), 2.22 – 2.10 (m, 2H), 1.69 (s, 9H); **¹³C NMR** (101 MHz, CDCl₃) δ 173.83, 163.84, 159.82, 158.42, 148.77, 136.51, 134.90, 132.82, 132.39, 131.12, 130.26, 128.38, 126.67, 123.89, 116.54, 114.15, 114.05, 112.37,

20 85.84, 66.85, 66.17, 55.45, 51.80, 30.75, 28.22, 24.85.

4-(4-(1-(*tert*-butoxycarbonyl)-6-chloro-3-(((4-methoxybenzyl)oxy)carbonyl)-1*H*-indol-5-yl)phenoxy)butanoic acid (20)
(Fig. 18, Scheme 2)



5 In a round-bottomed flask equipped with a magnetic stirring bar, methyl ester **19** (300 mg, 0.49 mmol, 1.0 equiv.) was dissolved in a H₂O:THF (1:1) mixture and added NaOH (22 mg, 0.54 mmol, 1.1 equiv.) and stirred for 6 hours. Upon completion as monitored by UPLC-MS, the reaction was concentrated *in vacuo*, re-dissolved in EtOAc (50 mL) and washed with 0.05 N aqueous HCl (1x 20 mL), dried over MgSO₄, filtered, and concentrated *in vacuo*. Purification by silica gel flash chromatography (eluting with a gradient from 50% EtOAc in heptanes to EtOAc) afforded **20** as a white solid (139 mg, 47 %). **UPLC/MS** (ESI): *m/z* calcd. for C₃₂H₃₃ClNO₈ [M+H]⁺ = 594.2, found 593.6; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 12.16 (s, 1H), 8.26 (s, 1H), 8.22 (s, 1H), 7.97 (s, 1H), 7.44 – 7.39 (m, 2H), 7.38 – 7.32 (m, 2H), 7.05 – 7.00 (m, 2H), 6.97 – 6.91 (m, 2H), 5.29 (s, 2H), 4.05 (t, *J* = 6.4 Hz, 2H), 3.75 (s, 3H), 3.74 (d, *J* = 11.0 Hz, 1H), 2.42 (t, *J* = 7.3 Hz, 2H), 1.99 (h, *J* = 6.9, 6.5 Hz, 2H), 1.65 (s, 9H); **¹³C NMR** (151 MHz, DMSO) δ 174.07, 162.72, 159.21, 158.08, 147.97, 135.65, 134.14, 132.95, 131.12, 130.62, 130.02, 128.73, 127.98, 126.12, 123.13, 115.91, 114.13, 113.89, 111.14, 86.06, 66.65, 65.61, 55.09, 30.13, 27.45, 24.25.

Glucagon analogue; C-terminal lysine homolog (21) (Fig. 19, Schemes 3 and 4)

H—H-D^{*}Ser^{*}-Q-G-T-F-T-S^{*}-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-G-G-P-S-S-G-A-P-P-S-K—NH₂
* triple coupling

The resin-bound peptide (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis. Coupling reactions were performed as double coupling and triple couplings as specified with asterisks.

- 5 The C-terminal lysine was introduced with a Mtt protecting group to allow orthogonal deprotection for continued synthesis. Some of the peptide was cleaved using cleavage cocktail A to be used as control in assays. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded glucagon analogue **21** as a white powder after lyophilization (16.6 mg, 5.3 %). Analytical HPLC
10 purity: >95% (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{193}H_{288}N_{56}O_{61}S^{4+}$ 1101.1; found 1101.2.

Modified glucagon and AMPK activator conjugate; PEG-linker: 1 unit (22a) (Figure 19, Scheme 3).

- 15 The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple
20 couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Fmoc-amino-EG-CH₂CH₂COOH was coupled to the liberated amine using general procedure for manual HBTU coupling, followed by Fmoc-deprotection using standard procedure for manual Fmoc deprotection. Finally, **20** was double coupled using
25 standard procedure for HBTU coupling. The conjugate was cleaved using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **22** as a white powder after lyophilization (5.1 mg, 3.4 %). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{217}H_{315}ClN_{58}O_{67}S^{4+}$ 1218.8; found 1218.8.

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Modified glucagon and AMPK activator conjugate. PEG-linker: 2 units (22b) (Figure 19, Scheme 3).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Fmoc-amino-PEG₂-CH₂CH₂COOH was coupled to the liberated amine using general procedure for manual HBTU coupling, followed by Fmoc-deprotection using standard procedure for manual Fmoc deprotection. Finally, **20** was double coupled using standard procedure for HBTU coupling. The conjugate was cleaved using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **22** as a white powder after lyophilization (6.9 mg, 4.3%). Analytical HPLC purity: 98 % (λ = 214 nm). LCMS *m/z*: [M+4H]⁴⁺ Calcd. for C₂₁₉H₃₁₉ClN₅₈O₆₈S⁴⁺ 1229.6; found 1229.7.

Modified glucagon and AMPK activator conjugate. PEG-linker: 3 units (22c) (Figure 19, Scheme 3).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Fmoc-amino-PEG₃-CH₂COOH was coupled to the liberated amine using general procedure for manual HBTU coupling, followed by Fmoc-deprotection using standard procedure for manual Fmoc deprotection. Finally, **20** was double coupled using standard procedure for HBTU coupling. The conjugate was cleaved using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **22** as a white powder after lyophilization (1.8 mg,

1.5%). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$
Calcd. for $C_{220}H_{320}ClN_{58}O_{69}S^{4+}$ 1237.1; found 1237.2.

**Modified glucagon and AMPK activator conjugate. PEG-linker: 4
5 units (22d) (Figure 19, Scheme 3).**

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine
10 in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Fmoc-PEG₄-CH₂COOH was coupled to the liberated amine using general procedure for manual HBTU coupling, followed by Fmoc-deprotection using standard procedure for manual
15 Fmoc deprotection. Finally, **20** was double coupled using standard procedure for HBTU coupling. The conjugate was cleaved using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **22** as a white powder after lyophilization (3.7 mg, 2.6%). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$
20 Calcd. for $C_{222}H_{325}ClN_{58}O_{70}S^{4+}$ 1248.1; found 1248.2.

**Modified glucagon and AMPK activator conjugate. C4 Linker (23a)
(Figure 19, Scheme 4).**

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05
25 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved
30 using general procedure for Mmt deprotection. Compound **14a** was coupled to the liberated amine using general procedure for PyBOP coupling. The allyl ester was deprotected using general procedure for allyl deprotection, and the

conjugate liberated from the resin conjugate using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **23a** as a white powder after lyophilization (9.1 mg, 6.9%). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$

5 Calcd. for $C_{214}H_{309}ClN_{58}O_{66}S^{4+}$ 1204.2; found 1202.0.

Modified glucagon and AMPK activator conjugate. C6 Linker (23b)
(Figure 19, Scheme 4).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the

10 general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved

15 using general procedure for Mmt deprotection. Compound **14b** was coupled to the liberated amine using general procedure for PyBOP coupling. The allyl ester was deprotected using general procedure for allyl deprotection, and the conjugate liberated from the resin conjugate using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded

20 acylated GLP-1 homolog **23b** as a white powder after lyophilization (2.3 mg, 3.1%). Analytical HPLC purity: >95% (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{216}H_{313}ClN_{58}O_{66}S^{4+}$ 1211.3; found 1211.3.

Modified glucagon and AMPK activator conjugate. C8 Linker (23c)
25 (Figure 19, Scheme 4).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine

30 in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Compound **14c** was coupled to

the liberated amine using general procedure for PyBOP coupling. The allyl ester was deprotected using general procedure for allyl deprotection, and the conjugate liberated from the resin conjugate using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **23c** as a white powder after lyophilization (4.7 mg, 3.0%). Analytical HPLC purity: >95% (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{218}H_{313}N_{58}O_{66}S^{4+}$ 1218.3; found 1218.6.

Modified glucagon and AMPK activator conjugate. C10 Linker (23d) (Figure 19, Scheme 4).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Compound **14d** was coupled to the liberated amine using general procedure for PyBOP coupling. The allyl ester was deprotected using general procedure for allyl deprotection, and the conjugate liberated from the resin conjugate using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **23d** as a white powder after lyophilization (4.6 mg, 4.0%). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{220}H_{321}N_{58}O_{66}S^{4+}$ 1225.3; found 1225.4.

25

Modified glucagon and AMPK activator conjugate. C18 Linker (23e) (Figure 19, Scheme 4).

The resin-bound peptide **21** (SEQ ID NO: 3) was synthesized on 0.05 mmol scale on ChemMatrix® Rinkamide resin (0.50 mmol/g) following the general procedure for automated peptide synthesis, except Fmoc-Lys(Mmt)-OH was used for the lysine in position 40 and Boc-His(Trt)-OH for the histidine in position 1. Coupling reactions were performed as double couplings and triple

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couplings as specified with asterisks. On-resin Mmt deprotection was achieved using general procedure for Mmt deprotection. Compound **14e** was coupled to the liberated amine using general procedure for PyBOP coupling. The allyl ester was deprotected using general procedure for allyl deprotection, and the conjugate liberated from the resin conjugate using cleavage cocktail A. Purification by preparative HPLC (C18, 10-60% B over 50 min) afforded acylated GLP-1 homolog **23e** as a white powder after lyophilization (5.5 mg, 3.7%). Analytical HPLC purity: >95 % (λ = 214 nm). LCMS m/z : $[M+4H]^{4+}$ Calcd. for $C_{228}H_{337}ClN_{58}O_{86}S^{4+}$ 1253.1; found 1253.5.

10

Example 4: Test of conjugates of modified glucagon (SEQ ID NO: 3) and AMPK activator in AMPK activity assay.

AMPK activity was assessed by using 50 ng recombinant heterotrimeric AMPK $\alpha 1\beta 1\gamma 1$ (Signalchem P47-110GH added to a 35 μ l reaction containing assay buffer (50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, and 1 mM DTT), 200 μ M synthetic peptide substrate (AMARA), and 100 μ M $[\gamma\text{-}^{32}P]\text{-ATP}$ (PerkinElmer), in the presence of 1 μ M MK-8722 (positive control), 1 μ M PF-06409577 (positive control), or PF-06409577-glucagon conjugates (0–1000 nM). Reactions were performed at 30°C and terminated after 30 min by spotting 35 μ l onto P81 phosphocellulose paper (Whatman). Radioactivity of each sample was measured by Cherenkov counting.

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Example 5: Glucose tolerance and compound tolerance test.

Glucose tolerance: Mice were fasted for five hours before being challenged with intraperitoneal injections of 1.75 g/kg of glucose dissolved in isotonic saline. Tail blood glucose concentrations were measured using a handheld glucometer (Contour XT, Bayers) 0, 15, 30, 60, 90 and 120 minutes after injection.

25

Compound tolerance: Compound tolerance was assessed by subcutaneous injection of the experimental compound followed by measurements of tail blood glucose concentrations using a handheld glucometer (Contour XT, Bayers) 0, 1, 2, 4 and 24 hours after injection unless

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otherwise specified. For Fig 21, blood glucose levels of co- and tri-agonist compounds were measured after 24 hours.

C L A I M S

1. A conjugated molecule comprising a peptide displaying at least 0.1 % activity of native glucagon at the glucagon receptor, and an AMP-activated protein kinase (AMPK) activator, wherein the peptide is covalently bonded to the AMPK activator either directly or through a linker.
2. The conjugated molecule according to claim 1, wherein the peptide is of the glucagon-superfamily.
3. The conjugated molecule according to claim 1 or 2, wherein the peptide has at least 80% amino acid sequence identity to SEQ ID NO:1.
4. The conjugated molecule according to any one of the preceding claims, wherein the peptide consists of at least 10 amino acids and no more than 60 amino acids.
5. The conjugated molecule according to any one of the preceding claims, wherein the AMPK activator is covalently bonded to the peptide via a cleavable chemical linker, the cleavable chemical linker being selected from acid-cleavable linkers, enzyme-cleavable linkers, peptide-cleavable linkers, and linkers comprising a disulfide group.
6. The conjugated molecule according to claim 5, wherein the chemical linker has the formula $R_1-R_3-S-S-R_4-R_5-NH-CO-R_2$, wherein R_1 is the peptide, R_2 the AMPK activator, R_3 is optional and when present is selected from $C(CH_3)_2$, CH_2-CH_2 , or CH_2 , bonded to a side chain of the peptide or to a carbon atom of the backbone chain of the peptide, R_4 is $(CH_2)_n$ or C_6H_4 , R_5 is optional and when present is selected from $C(CH_3)_2$, $CH-CH_3$, CH_2-CH_2 , or CH_2 , and n is 1, 2, 3 or 4.
7. The conjugated molecule according to claims 1 to 4, wherein the AMPK activator is covalently bonded to the peptide via a non-cleavable linker,

wherein the non-cleavable linker is selected from polyethylene glycol linkers, carbon linkers, SMCC and mc with conjugation chemistries of maleimides, ethers, amides, triazoles, disulfide, and thioether.

5 8. The conjugated molecule according to any one of the preceding claims, wherein the AMPK activator is selected from 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-([1,1'-biphenyl]-4-yl)-7-chloro-3*H*-imidazo[4,5-*b*]pyridin-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (MK-8722), (3*R*,3*aR*,6*R*,6*aR*)-6-((6-chloro-5-(4-(1-(hydroxymethyl)cyclopropyl)phenyl)-1*H*-benzo[*d*]imidazol-2-yl)oxy)hexahydrofuro[3,2-*b*]furan-3-ol (PF-739), 6-Chloro-5-[4-(1-hydroxycyclobutyl)phenyl]-1*H*-indole-3-carboxylic acid (PF-06409577), 1,1-Dimethylbiguanide hydrochloride (metformin), 4-Hydroxy-3-(2'-hydroxybiphenyl-4-yl)-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-5-carbonitrile (A-769662), 2-Chloro-5-[[5-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-4-oxo-2-thiazolyl]amino]benzoic acid (PT-1), 2-[[2-(2-Bromo-4-methylphenoxy)ethyl]thio]-pyrimidine (ZLN024), 2-[[4-(Diethylamino)-2-hydroxyphenyl]methylene]hydrazide-4-pyridinecarboxylic acid (RSVA-405), and analogues thereof.

20 9. The conjugated molecule according to any one of the preceding claims for use in therapy.

10. The conjugated molecule according to any one of the preceding claims for use in the treatment of obesity, type 2 diabetes, hyperinsulinemia, 25 insulin resistance, impaired glucose tolerance, hypercholesterolaemia, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and dyslipidemia.

11. A pharmaceutical composition comprising the conjugated molecule according to any one of the claims 1 to 8 or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

12. A method of reducing body weight of a mammal comprising administering the conjugated molecule according to any one of claims 1 to 9 or the pharmaceutical composition according to claim 11 to the mammal.

5 13. A non-therapeutic treatment of a mammal for reducing body weight comprising orally administering the conjugated molecule according to any one of claims 1 to 8 or the pharmaceutical composition according to claim 11 to the mammal.

10 14. The non-therapeutic treatment of a mammal for reducing body weight according to claim 13, wherein the mammal has a non-pathogenic body mass index (BMI).

15 15. A non-therapeutic treatment of a mammal for reducing plasma cholesterol comprising orally administering the conjugated molecule according to any one of claims 1 to 8 or the pharmaceutical composition according to claim 11 to the mammal.