

[0005] Given that the ligands determine the composition of the receptor dimers, and the intracellular kinase degeneracy of JAK/TYK and RTK enzymes, the number of cytokine and growth factor receptor dimer pairings that occur in nature only represents a small proportion of the total number of signaling-competent receptor pairings theoretically allowed by the system. For example, the human genome encodes for approximately forty different JAK/STAT cytokine receptors. In principle, approximately 1600 unique homo- and hetero-dimeric cytokine receptor pairs could be generated with the potential to signal through different JAK/TYK/STAT combinations. However, the human genome encodes for less than fifty different cytokine ligands, limiting the scope of cytokine receptor dimers to those that can be assembled by the natural ligands. A similar argument can be made for the RTK family of receptors and ligands. Furthermore, given that it has been shown that Death receptors are capable of signaling as dimers or trimers, this concept can also be extended to this family.

[0006] The ability to selectively activate signaling pathways of interest is of great interest. The present invention provides compositions and methods for this purpose.

SUMMARY

[0007] Engineered synthetic signaling molecules, herein termed "synthekines", are provided. Synthekines are genetically engineered, bi-specific ligands of cell surface receptors, where the synthekine specifically binds at high affinity to the extracellular domain(s) of at least one and frequently two different cell surface receptor polypeptides. The cell surface receptors are characterized by activation of signaling upon multimerization. In some examples, generation of a receptor multimer by binding to a synthekine results in intracellular trans-phosphorylation of the receptor. Synthekines include, without limitation, small organic molecules and polypeptides.

[0007a] In one aspect, the present disclosure refers to a synthekine comprising at least two binding domains, wherein a first binding domain specifically binds to the extracellular domain of a first receptor polypeptide and wherein a second binding domain specifically binds to the extracellular domain of a second receptor polypeptide, wherein said first and second receptor polypeptides are not naturally associated.

[0008] Synthekines may also be tri-specific ligands of cell surface receptors, or more, where the synthekine specifically binds at high affinity to the extracellular domain(s) of at least three different cell surface receptor polypeptides. The cell surface receptors are characterized by activation of signaling upon multimerization. In some examples, generation of a receptor multimer by binding to a synthekine results in intracellular trans-phosphorylation of the receptor. Synthekines include, without limitation, small organic molecules and polypeptides.

[0009] In some examples the cell surface receptor polypeptide is one or more of (i) a cytokine receptor that activates the JAK/STAT pathway in the cell; (ii) a receptor tyrosine kinase; or (iii) a TNFR superfamily member. In some examples each of the multimeric receptor polypeptides are naturally expressed in a targeted single cell. In some examples a target cell is engineered to express the one or more of multimeric receptor polypeptides.

[0010] In some examples a syntheKine specifically binds to two or more different cytokine receptors that, when activated by multimerization, trans-phosphorylate and signal through JAK/STAT, and other pathways, including but not limited to ERK, AKT, and other signaling messengers. In some examples the cytokine receptors are selected from, but not limited to β c, γ c, IL-3R α , β IL-3R, GM-CSFR α , IL-5R α , CNTF α , CRLF1, LIFR α , gp130, IL-6R α , IL-11R α , OSMR β , IL-2R α , IL-2R β , IL-2R γ , IL-4R α , IL-7R α , IL-9R α , IL-13R α , IL-15R α , IL-21R α , IFNAR2, IL-23R, EpoR, IL-12R β , IFNAR1, IFNAR2, G-CSFR, c-MPLR. In some specific examples, a syntheKine binds to two of such receptors, and activates JAK/STAT signaling. In some specific examples, a syntheKine binds to three of such receptors, and activates JAK/STAT signaling. Generally a syntheKine activates pathways distinct from those of a native cytokine that activates the receptor(s).

[0011] In some examples a syntheKine binds to two or more different receptor tyrosine kinase proteins that are activated by trans-phosphorylation when the proteins are multimerized. In the some examples the RTK receptors are selected from but not limited to EGFR, ErbB2, ErbB3, ErbB4, InsR, IGF1R, InsRR, PDGFR α , PDGFR β , CSF1R/Fms, cKit, Flt-3/Flk2, VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3, FGFR4, PTK7/CCK4, TrkA, TrkB, TrkC, Ror1, Ror2, MuSK, Met, Ron, Axl, Mer, Tyro3, Tie1, Tie2, EphA1-8, EphA10, EphB1-4, EphB6, Ret, Ryk, DDR1, DDR2, Ros, LMR1, LMR2, LMR3, ALK, LTK, SuRTK106/STYK1, Activin-R, BMP-R, TGF-beta-R, Noggin-R. In some specific examples, a syntheKine binds to two of such receptors, and activates signaling. Generally a syntheKine activates pathways distinct from those of a native ligand that activates the receptor(s).

[0012] In some examples a syntheKine binds to two or more different TNFR superfamily polypeptides that are activated when the proteins are multimerized. In the some examples the receptors are selected from TNFR1 (TNFRSF1A), TNFR2 (TNFRSF1B; TNFRSF2), 41-BB (TNFRSF9); AITR (TNFRSF18); BCMA (TNFRSF17), CD27 (TNFRSF7), CD30 (TNFRSF8), CD40 (TNFRSF5), Death Receptor 1 (TNFRSF10C), Death Receptor-3 (TNFRSF25), Death Receptor 4 (TNFRSF10A), Death Receptor 5 (TNFRSF10B), Death Receptor -6 (TNFRSF21), Decoy Receptor-3 (TNFRSF6B), Decoy Receptor 2 (TNFRSF10D), EDAR, Fas (TNFRSF6), HVEM (TNFRSF14), LT β -R (TNFRSF3), OX40 (TNFRSF4), RANK (TNFRSF11A), TACI (TNFRSF13B), Troy (TNFRSF19), XEDAR (TNFRSF27), Osteoprotegerin (TNFRSF11B), TWEAK receptor (TNFRSF12A), BAFF Receptor (TNFRSF13C), NGF receptor (TNFRSF16). In some specific examples, a syntheKine binds to two or more of such receptors, and activates signaling. Generally a syntheKine activates pathways distinct from those of a native ligand that activates the receptor(s).

- [0013] In other examples, a synthequine binds to two or more receptors of mixed classes, e.g. a JAK/STAT receptor combined with a TNFRSF and/or RTK receptor; an RTK receptor combined with a TNFRSF receptor, and the like.
- [0014] In some examples of the disclosure, the synthequine is a polypeptide, which can comprise separate or contiguous binding domains or elements that bind to each of the receptor extracellular domain (ECD) polypeptides. A polypeptide synthequine may be a single chain, dimer, or higher order multimer. The binding domain/element for each receptor may be directly joined, or may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, *etc.* In some examples the synthequine does not activate a native receptor configuration. For example, a synthequine binding domain may bind one chain of a native receptor, but be disabled from binding the second chain of a native receptor. Such binding domains include, without limitation, dominant negative mutants of cytokines.
- [0015] In polypeptide examples, the receptor binding domains may be selected from any domain that binds the desired receptor extracellular domain at high affinity, e.g. a K_d of not more than about 1×10^{-7} M, not more than about 1×10^{-8} M, not more than about 1×10^{-9} M, or not more than about 1×10^{-10} M. Suitable binding domains include, without limitation, de novo designed binding proteins, antibody derived binding proteins, e.g. scFv, Fab, *etc.* and other portions of antibodies that specifically bind to one or more receptor ECD sequences; nanobody derived binding domains; knottin-based engineered scaffolds; norrin and engineered binding fragments derived therefrom, naturally occurring binding domains, and the like. Naturally occurring binding domains, such as cytokines, growth factors and the like are generally engineered to prevent activity from activation of the native receptor. A binding domain may be affinity selected to enhance binding to a desired ECD; and/or mutagenized to prevent binding to an undesired ECD.
- [0016] A synthequine polypeptide can be fused, linked, or alternatively co-administered with an agent to enhance receptor activation. A synthequine can be fused, linked or alternatively co-administered with a cytokine, chemokine, or growth factor of interest.
- [0017] The binding domains may be contiguous within one globular domain, or separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, *etc.* The length of the linker, and therefore the spacing between the binding domains can be used to modulate the signal strength, and can be selected depending on the desired use of the synthequine. The enforced distance between binding domains can vary, but in certain examples may be less than about 100 angstroms, less than about 90 angstroms, less than about 80 angstroms, less than about 70 angstroms, less than about 60 angstroms, or less than about 50 angstroms.

- [0018] In some examples the linker is a rigid linker, in other examples the linker is a flexible linker. Where the linker is a peptide linker, it may be from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between binding domains. In some examples the linker comprises or consists of one or more glycine and/or serine residues.
- [0019] A synthequine can be multimerized, e.g. through an Fc domain, by concatenation, coiled coils, polypeptide zippers, biotin/avidin or streptavidin multimerization, and the like. The synthequine can also be joined to a moiety such as PEG, Fc, etc. as known in the art to enhance stability *in vivo*.
- [0019a] In one aspect, the present disclosure refers to a synthequine for any of the use as disclosed herein.
- [0019b] In one aspect, the present disclosure refers to a nucleic acid sequence encoding the synthequine as disclosed herein.
- [0019c] In one aspect, the present disclosure refers to a recombinant vector comprising the nucleic acid sequence as disclosed herein.
- [0019d] In one aspect, the present disclosure refers to a recombinant host cell comprising a nucleic acid sequence as disclosed herein.
- [0020] In one aspect, the present disclosure refers to a pharmaceutical composition comprising a synthequine as disclosed herein, and a pharmaceutically acceptable excipient. Compositions of interest include, without limitation, an effective dose of a synthequine in a pharmaceutically acceptable excipient. Compositions may comprise additional agents, e.g. adjuvants and the like. Synthequines may be produced synthetically; by various suitable recombinant methods, and the like, as known in the art.
- [0020a] In one aspect, the present disclosure refers to use of a synthequine that binds to the extracellular domains of two or more receptor polypeptides in a non-natural combination in the manufacture of a medicament for selective activation of a non-natural combination of receptor polypeptides in a cell, wherein contact of the cell with the synthequine causes multimerization of the receptors polypeptides and activation of signaling.
- [0021] In some examples of the disclosure, a method is provided for activating, increasing or enhancing selected JAK/STAT, DD, and/or RTK signaling in a cell. In such methods, a cell expressing cognate receptor polypeptides for a synthequine of interest is contacted with a concentration of a synthequine that is effective to increase signaling, e.g. to increase signaling by 25%, 50%, 75%, 90%, 95%, or more, relative to the signaling in the absence of the synthequine. Such signaling activation may induce JAK/STAT or RTK signaling pathways and include, without limitation, modulation of immune responses, growth factor responses, induction of death domain responses, and the like. In some methods, the receptor-expressing cell is contacted *in vitro*. In other examples, the receptor-expressing cell is contacted *in vivo*.
- [0022] In some examples of the disclosure, a method is provided for treating or preventing a disease or disorder in a subject in need thereof, the method comprising providing to the subject an effective amount of a synthequine. In particular examples, the subject has an immune disease or dysfunction.

BRIEF DESCRIPTION OF THE DRAWINGS.

- [0023] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

What is Claimed is:

1. Use of a synthequine that binds to the extracellular domains of two or more receptor polypeptides in a non-natural combination in the manufacture of a medicament for selective activation of a non-natural combination of receptor polypeptides in a cell, wherein contact of the cell with the synthequine causes multimerization of the receptors polypeptides and activation of signaling.

2. The use of claim 1, wherein the synthequine comprises a polypeptide.

3. The use of claim 1 or claim 2, wherein the receptor polypeptide(s) are one or more of (i) a cytokine receptor that activates the JAK/STAT pathway in the cell; (ii) a receptor tyrosine kinase; or (iii) a TNFR superfamily member.

4. The use of claim 3, where the cytokine receptors are selected from βc , γc , IL-3R α , β IL-3R, GM-CSFR α , IL-5R α , CNTF α , CRLF1, LIFR α , gp130, IL-6R α , IL-11R α , OSMR β , IL-2R α , IL-2R β , IL-2R γ , IL-4R α , IL-7R α , IL-9R α , IL-13R α , IL-15R α , IL-21R α , IFNAR2, IL-23R, EpoR, IL-12R β , IFNAR1, G-CSFR, c-MPLR.

5. The use of claim 4, wherein a synthequine binds to two of such receptors, and activates JAK/STAT signaling.

6. The use of claim 4, wherein a synthequine binds to three of such receptors and activates JAK/STAT signaling.

7. The use of claim 3, where the receptor tyrosine kinase proteins are selected from EGFR, ErbB2, ErbB3, ErbB4, InsR, IGF1R, InsRR, PDGFR α , PDGFR β , CSF1R/Fms, cKit, Flt-3/Fik2, VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3, FGFR4, PTK7/CCK4, TrkA, TrkB, TrkC, Ror1, Ror2, MuSK, Met, Ron, Axl, Mer, Tyro3, Tie1, Tie2, EphA1-8, EphA10, EphB1-4, EphB6, Ret, Ryk, DDR1, DDR2, Ros, LMR1, LMR2, LMR3, ALK, LTK, SuRTK106/STYK1.

8. The use of claim 7, wherein a synthequine binds to two of such receptors, and activates signaling.

9. The use of claim 3, where the TNFR superfamily member is selected from TNFR1 (TNFRSF1A), TNFR2 (TNFRSF1B; TNFRSF2), 41-BB (TNFRSF9); AITR (TNFRSF18); BCMA (TNFRSF17), CD27 (TNFRSF7), CD30 (TNFRSF8), CD40 (TNFRSF5), Death Receptor 1 (TNFRSF10C), Death Receptor-3 (TNFRSF25), Death Receptor 4 (TNFRSF10A), Death Receptor 5 (TNFRSF10B), Death Receptor -6 (TNFRSF21), Decoy Receptor-3 (TNFRSF6B), Decoy Receptor 2 (TNFRSF10D), EDAR, Fas (TNFRSF6), HVEM (TNFRSF14), LT α -R (TNFRSF3), OX40 (TNFRSF4), RANK (TNFRSF11A), TACI (TNFRSF13B), Troy (TNFRSF19), XEDAR (TNFRSF27), Osteoprotegerin (TNFRSF11B), TWEAK receptor (TNFRSF12A), BAFF Receptor (TNFRSF13C), NGF receptor (TNFRSF16).

10. The use of claim 9, wherein the syntheKine binds to two or more of such receptors and activates signaling.

11. The use of any of claims 1-10, wherein the syntheKine comprises binding domains with high affinity for two distinct extracellular domains of a receptor set forth in claim 3.

12. The use of claim 11, wherein the binding affinity is less than about 1×10^{-7} M.

13. The use of claim 11, wherein the binding domains are directly joined.

14. The use of claim 11, wherein the binding domains are joined through a linker.

15. The use of any of claims 1-14, wherein the binding domain is a mutated form of a native ligand.

16. The use of any of claims 1-14, wherein the binding domain is a de novo designed binding domain.

17. The use of any of claims 1-14, wherein the binding domain is an antibody derived binding protein.

18. The use of any of claims 1-14, wherein the binding domain is a nanobody derived binding domain.

19. The use of any of claims 1-14, wherein the binding domain is a knottin-engineered scaffold.

20. The use of any of claims 1-19 wherein the binding domains are joined by a peptide linker comprising from 2-100 amino acids.

21. The use of any of claims 1-19, wherein the binding domains are joined by a non-peptide linker.

22. A synthekine for use in any of claims 1-21.

23. A pharmaceutical composition comprising a synthequine of claim 22, and a pharmaceutically acceptable excipient.

24. A syntheKine comprising at least two binding domains, wherein a first binding domain specifically binds to the extracellular domain of a first receptor polypeptide and wherein a second binding domain specifically binds to the extracellular domain of a second receptor polypeptide, wherein said first and second receptor polypeptides are not naturally associated.

25. The syntheKine of claim 24 wherein the first receptor polypeptide and second receptor polypeptide are selected from of (i) a cytokine receptor that activates the JAK/STAT pathway in the cell; (ii) a receptor tyrosine kinase; or (iii) a TNFR superfamily member.

26. The syntheKine of any of claims 25 wherein the cytokine receptor is selected from the group consisting of: βc , γc , IL-3R α , β IL-3R, GM-CSFR α , IL-5R α , CNTF α , CRLF1, LIFR α , gp130, IL-6R α , IL-11R α , OSMR β , IL-2R α , IL-2R β , IL-2R γ , IL-4R α , IL-7R α , IL-9R α , IL-13R α , IL-15R α , IL-21R α , IFNAR2, IL-23R, EpoR, IL-12R β , IFNAR1, G-CSFR, and c-MPLR.

27. The syntheKine of any of claims 25 or 26 wherein the receptor tyrosine kinase protein is selected from the group consisting of: EGFR, ErbB2, ErbB3, ErbB4, InsR, IGF1 R, InsRR, PDGFR α , PDGFR β , CSF1 R/Fms, cKit, Flt-3/Flk2, VEGFR1 , VEGFR2, VEGFR3, FGFR1 , FGFR2, FGFR3, FGFR4, PTK7/CCK4, TrkA, TrkB, TrkC, Ror1, Ror2, MuSK, Met, Ron, Axl, Mer, Tyro3, Tie1, Tie2, EphA1-8, EphA1 O, EphB1-4, EphB6, Ret, Ryk, DDR1, DDR2,

Ros, LMR1, LMR2, LMR3, ALK, LTK, SuRTK106/STYK1, Acitivin-R, BMP-R, TGF-beta-R and Noggin-R.

28. The syntheKine of any of claims 25 or 26 wherein the TNFR superfamily member is selected from TNFR1 (TNFRSF1A), TNFR2 (TNFRSF1 B; TNFRSF2), 41-BB (TNFRSF9); AITR (TNFRSF18); BCMA (TNFRSF17), CD27 (TNFRSF7), CD30 (TNFRSF8), CD40 (TNFRSF5), Death Receptor 1 (TNFRSF10C), Death Receptor-3 (TNFRSF25), Death Receptor 4 (TNFRSF10A), Death Receptor 5 (TNFRSF10B), Death Receptor-6 (TNFRSF21), Decoy Receptor-3 (TNFRSF6B), Decoy Receptor 2 (TNFRSF10D), EDAR, Fas (TNFRSF6), HVEM (TNFRSF14), LT β R (TNFRSF3), OX40 (TNFRSF4), RANK (TNFRSF1 1A), TACI (TNFRSF13B), Troy (TNFRSF19), XEDAR (TNFRSF27), Osteoprotegerin (TNFRSF11 B), TWEAK receptor (TNFRSF12A), BAFF Receptor (TNFRSF13C), and NGF receptor (TNFRSF16).

29. The syntheKine of any of claims 24-28, wherein the at least first and second binding domains are directly joined.

30. The syntheKine of any of claims 24-28, wherein the at least first and second binding domains are joined via a linker.

31. The syntheKine of any of claims 24-30, wherein at least one binding domain is an antibody.

32. The syntheKine of claims 31 wherein the antibody is independently selected from intact IgG, IgE and IgM, bi-specific antibodies, multi-specific antibodies, single chain Fvs, Fabs, Small Modular ImmunoPharmaceuticals ("SMIPs™"), single chain or Tandem diabodies (TandAb®), VHHs, Anticalins®, Nanobodies®, minibodies, BiTE®s, ankyrin repeat proteins or DARPINs®, Avimers®, a DART, a TCR-like antibody, Adnectins®, Affilins®, Trans-bodies®, Affibodies®, a TrimerX®, MicroProteins, Fynomers®, Centyrins®, and a KALBITOR®.

33. The syntheKine of claim 31 wherein an antibody is a VHH.

34. The syntheKine of any of claims wherein the binding domain is native ligand IL-1 α , IL-1 β , IL-1 RA, IL-18, IL-2, IL-4, IL-7, IL-9, IL-13, IL-4, IL-15, IL-3, IL- 5, GM-CSF, IL-6, IL-

11, G-CSF, IL-12, LIF, OSM, IL-10, IL-20, IL-14, IL-16, IL-17, IFN- α , IFN- β , IFN- γ , LT- β , TNF- α , TNF- β , 4-1 BBL, CD70, CD153, CD178, TGF- β 1 , TGF- β 2, TGF- β 3, Epo, Tpo, Flt-3L, SCF, M-CSF, and MSP, where the binding domain does not activate the native receptor for the ligand.

35. A syntheKine of any of claims 24-34 wherein the syntheKine is a polypeptide.

36. A nucleic acid sequence encoding the syntheKine of claim 35.

37. A recombinant vector comprising the nucleic acid sequence of claim 36.

38. A recombinant host cell comprising a nucleic acid sequence of claim 36.