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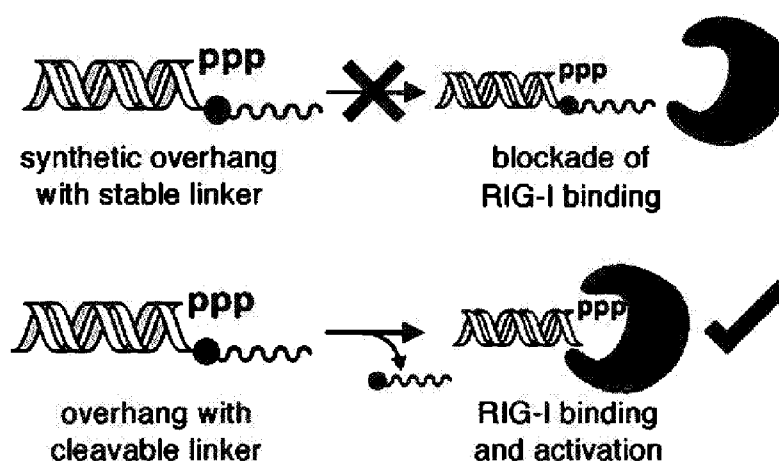


FIG. 1

(57) Abstract: Provided herein are a selective pattern recognition receptor (PRR) agonist and a method of selectively activating a PRR. The selective PRR agonist includes a nucleic acid agonist and a macromolecule conjugated to the nucleic acid agonist. The method includes administering the selective PRR agonist to a subject, and cleaving at least a portion of the macromolecule conjugated to the nucleic acid agonist, the cleaving of at least a portion of the macromolecule permitting the agonist to bind a PRR.



**PATTERN RECOGNITION RECEPTOR AGONIST PRODRUGS
AND METHODS OF USE THEREOF**

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/615,370, filed January 9, 2018, the entire disclosure of which is incorporated herein by this reference.

GOVERNMENT INTEREST

[0002] This invention was made with government support under grant numbers T32DK101003 and 5R21AI121626 awarded by the National Institutes of Health (NIH), grant number W81XWH-16-1-0063 awarded by the Department of Defense (DOD) Congressionally Directed Medical Research Programs (CDMRP), and grant number CBET-1554623 awarded by the National Science Foundation (NSF). The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. The ASCII copy of the Sequence Listing, which was created on January 8, 2019, is named 11672N-18075W.txt and is 1 kilobyte in size.

TECHNICAL FIELD

[0004] The presently-disclosed subject matter relates to pattern recognition receptor (PRR) agonist prodrugs and methods of use thereof. In particular, the presently-disclosed subject matter relates to nucleic acid PRR agonists with macromolecules conjugated thereto and methods of use thereof.

BACKGROUND

[0005] The innate immune system plays a critical role in defense against pathogen infection, immune recognition of tumors, tissue repair and regeneration, and the pathogenesis of autoimmunity. Accordingly, there has been considerable interest in therapeutic strategies that modulate innate immune signaling pathways, including activation of the innate immune system as a strategy to combat cancer or improve the efficacy of vaccines. Upon microbial infection, tissue damage, or aberrant cellular behavior (*e.g.*, tumor growth), the innate immune system initiates and coordinates a localized inflammatory response that typically restrains systemic inflammation and resultant toxicity and pathology. By contrast,

administration of many molecular activators of the innate immune system results in systemic biodistribution and inflammation that limits the therapeutic window and/or restricts use to certain applications or administration routes (*e.g.*, topical, intratumoral).

[0006] Pattern recognition receptors (PRRs) recognize specific molecular patterns associated with pathogen invasion to trigger an inflammatory response that is critical to mounting an appropriate immune response to clear the infection. A diversity of nucleic acid PRR agonists (*e.g.*, CpG ODN, poly(I:C)) have been widely explored to activate innate immunity for applications in cancer immunotherapy and vaccine adjuvants. One important PRR involved in the detection of viruses is retinoic acid-inducible gene I (RIG-I, also known as DDX58), which resides in the cytosol and recognizes short, double-stranded RNA that contains a triphosphate group at the 5' end (pppRNA). Activation of RIG-I triggers a multifaceted anti-viral innate immune response that shares significant homology with responses that are associated with productive anti-tumor immunity (*e.g.*, type I interferons, T cell chemokines). Additionally, activation of RIG-I in cancer cells has been shown to increase their immunogenicity as well as induce immunogenic cell death. Hence, agonists of the RIG-I pathway have recently emerged as a promising class of antiviral agents, vaccine adjuvants, and cancer immunotherapeutics, and are currently being evaluated in a clinical trial (NCT03065023).

[0007] However, unlike many other PRRs, which are expressed primarily in hematopoietic cells and often restricted to specific subsets of immune cells, RIG-I is present in the cytosol of virtually all cell types. Due to this ubiquity of RIG-I expression, the systemic administration of RIG-I agonists risks induction of systemic inflammation. Additionally, the risk for induction of system inflammation may carry with it possible dose-limiting toxicities.

[0008] Accordingly, there is a need for articles and methods for selective RIG-I pathway activation.

SUMMARY

[0009] The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

[0010] This summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This summary is merely exemplary of the numerous and varied embodiments. Mention of one or

more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this summary or not. To avoid excessive repetition, this summary does not list or suggest all possible combinations of such features.

[0011] In some embodiments, the presently-disclosed subject matter includes a selective pattern recognition receptor (PRR) agonist, comprising a nucleic acid agonist and a macromolecule conjugated to the nucleic acid agonist. In some embodiments, the nucleic acid agonist includes a nucleic acid duplex having at least two phosphoryl groups attached to a 5' end thereof. In one embodiment, the nucleic acid duplex comprises a double stranded nucleic acid molecule. In one embodiment, the nucleic acid duplex comprises a single-stranded nucleic acid molecule. In another embodiment, the single-stranded nucleic acid molecule comprises a hairpin loop RNA molecule. In one embodiment, the at least two phosphoryl groups are selected from the group consisting of a diphosphate group and a triphosphate group.

[0012] In some embodiments, the macromolecule has a molecular weight of at least 550 Da. In some embodiments, the selective PRR agonist further comprises an environmentally selective linker conjugating the macromolecule to the nucleic acid agonist. In one embodiment, the environmentally selective linker is selected from the group consisting of reactive oxygen species (ROS) sensitive linking agents, pH sensitive linking agents, redox sensitive linking agents, enzymatically cleavable linking agents, light sensitive linking agents, and combinations thereof. In another embodiment, the redox sensitive linking agents are selected from the group consisting of glutathione sensitive linkers, nitroreductase/NADH sensitive linkers, and combinations thereof. In another embodiment, the pH sensitive linking agents are selected from the group consisting of hydrazone, silyl ethers, other low pH sensitive linking agents, and combinations thereof. In another embodiment, the enzymatically cleavable linking agents are selected from the group consisting of matrix metalloproteinases, dipeptide/*p*-aminobenzyl alcohol systems, lysosomal, beta-glucuronidase, intracellular esterases, and combinations thereof.

[0013] In some embodiments, the macromolecule is conjugated to a 3' end of the nucleic acid duplex, the 3' end that the macromolecule is conjugated to and the 5' end that the at least two phosphoryl groups are attached to being at a single terminus of the nucleic acid duplex. In one embodiment, the agonist is a retinoic acid-inducible gene I (RIG-I) agonist. In some

embodiments, the macromolecule is conjugated to a phosphoryl group attached to the 5' end of the nucleic acid duplex. In some embodiments, removal of at least a portion of the macromolecule permits binding of the agonist to a PRR.

[0014] Also provided herein, in some embodiments, is a method of selectively activating a pattern recognition receptor (PRR), the method comprising administering a selective PRR agonist to a subject; and cleaving at least a portion of the macromolecule conjugated to the nucleic acid duplex; wherein the cleaving of at least a portion of the macromolecule permits the agonist to bind a PRR. In some embodiments, the method further comprises an environmentally selective linker conjugating the macromolecule to the nucleic acid agonist. In some embodiments, the cleaving step comprises passively or actively subjecting the selective PRR agonist to an environmental stimulus corresponding to the environmentally selective linker. In some embodiments, the method further comprises a carrier system attached to the agonist.

[0015] Further features and advantages of the presently-disclosed subject matter will become evident to those of ordinary skill in the art after a study of the description, figures, and non-limiting examples in this document.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The presently-disclosed subject matter will be better understood, and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

[0017] FIG. 1 shows a schematic illustration depicting the use of synthetic polymer overhangs to block activity of 5' ppp-RNA agonists of RIG-I, and that activity can be restored in response to environmental stimuli if synthetic overhangs are conjugated via a cleavable linker.

[0018] FIG. 2 shows a schematic illustration depicting the synthesis of PEG-pppRNA conjugates according to an embodiment of the disclosure.

[0019] FIGS. 3A-C show images depicting characterization of PEG-pppRNA conjugates. **(A)** Agarose gel electrophoresis demonstrating reduced migration distance with increased overhang molecular weight. UC = unconjugated pppRNA. PEG molecular weight and linker chemistry of PEG-pppRNA conjugates indicated above each lane. **(B)** Unconjugated

pppRNA (left) and 550-mal-pppRNA (right) after 2% agarose gel electrophoresis at 148 V for 75 min. (C) Agarose gel electrophoresis of unconjugated cRNA (UC) and cRNA conjugates with PEG_{5k} overhangs and indicated linker chemistry. Conjugates incubated with glutathione are indicated with '+GSH.'

[0020] FIG. 4 shows a graph illustrating dose-response curves comparing normalized Interferon Regulatory Factor (IRF) response in A549-Dual reporter cells elicited by unmodified pppRNA without a thiol group (unmodified), unconjugated, thiol-containing pppRNA (unconjugated), and vehicle (Lipofectamine 2000) control.

[0021] FIGS. 5A-D show graphs illustrating the effect of PEG synthetic overhang molecular weight, linker cleavability, and conjugation site on ppp-RNA mediated activation of interferon regulatory factor (IRF) pathway. (A) Dose-response curve comparing the response to unconjugated pppRNA and those conjugated with maleimide-linked PEG-pppRNA at indicated molecular weights. (B) Dose-response curves comparing the response to unconjugated pppRNA and pppRNA conjugated to PEG_{5k}-mal on the side opposite the 5' ppp motif. (C) Dose-response curve comparing the response to unconjugated pppRNA and those conjugated to PEG_{5k} overhangs with indicated linker chemistry. (D) Bar graph comparing the EC₅₀ of all groups tested. (o) denotes conjugation on the side opposite the 5' ppp motif. *** $p = 0.0003$ and **** $p < 0.0001$ by one-way ANOVA with Tukey's test.

[0022] FIG. 6 shows a graph comparing relative IRF response in A549-Dual reporter cells to cRNA lacking the 5' ppp group, pppRNA, and 5 kDa PEG-cRNA conjugates of indicated linker chemistry. Cells treated at 20 nM concentrations of indicated treatment.

[0023] FIG. 7 shows a graph illustrating concentration of hIFN- β 1 secreted by A549-Dual cells treated with PEG_{5k}-pppRNA conjugates (at 20 nM RNA) with indicated linker chemistry. ** $p < 0.01$ **** $p < 0.0001$ one-way ANOVA with Tukey's test.

[0024] FIG. 8 shows a schematic illustrating selective immune response in the presence of an appropriate stimulus, according to an embodiment of the disclosure.

[0025] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and are herein described below in detail. It should be understood, however, that the description of specific embodiments is not intended to limit the disclosure to cover all modifications, equivalents and alternatives falling within the spirit and scope of the disclosure as defined by the appended claims.

DEFINITIONS

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present disclosure, including the methods and materials are described below.

[0027] Following long-standing patent law convention, the terms “a,” “an,” and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a cell” includes a plurality of cells, and so forth.

[0028] The terms “comprising,” “including,” and “having” are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[0029] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[0030] As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, concentration, percentage, or the like is meant to encompass variations of in some embodiments $\pm 50\%$, in some embodiments $\pm 40\%$, in some embodiments $\pm 30\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

[0031] As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. It is also understood that there are a number of values disclosed herein, and that unless stated otherwise each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0032] All combinations of method or process steps as used herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

DETAILED DESCRIPTION

[0033] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[0034] The presently-disclosed subject matter relates to pattern recognition receptor (PRR) agonists and methods of use thereof. In some embodiments, the PRR agonist includes a selective nucleic acid agonist including at least one macromolecule conjugated thereto. Suitable nucleic acid agonists include any molecularly-defined nucleic acid agonist of a PRR. As will be appreciated by those skilled in the art, the specific PRR of interest will determine the nucleic acid agonist that is selected.

[0035] In some embodiments, the nucleic acid agonist includes a phosphorylated nucleic acid duplex. As used herein, the term “phosphorylated nucleic acid duplex” refers to any molecule including ribonucleic acid (RNA) and/or deoxyribonucleic acid (DNA) base pairing between complementary nucleotide sequences in the same or separate nucleic acid strands, and at least two phosphoryl groups (e.g., diphosphate or triphosphate) at a 5' end of at least one strand. Various PRRs, such as, but not limited to, retinoic acid-inducible gene I (RIG-I), recognize the phosphoryl groups attached to these phosphorylated nucleic acid duplexes, resulting in binding thereto.

[0036] In one embodiment, the phosphorylated nucleic acid duplex includes a double-stranded nucleic acid molecule with base pairing between separate, complementary strands. In another embodiment, the phosphorylated nucleic acid duplex includes a double-stranded RNA molecule, which may be comprised entirely of RNA bases or a combination of RNA bases and at least one DNA base. In a further embodiment, one or more of the RNA and/or DNA bases may be modified, such as, for example, by including a phosphothioate linkage.

These double-stranded nucleic acid molecules include at least 8 base pairs, at least 10 base pairs, between 10 and 1000 base pairs, between 10 and 750 base pairs, between 10 and 500 base pairs, between 10 and 250 base pairs, between 10 and 100 base pairs, between 10 and 50 base pairs, between 10 and 40 base pairs, between 10 and 30 base pairs, between 10 and 20 base pairs, between 10 and 14 base pairs, 8 base pairs, 10 base pairs, 12 base pairs, 14 base pairs, 16 base pairs, 18 base pairs, 20 base pairs, 22 base pairs, 24 base pairs, 26 base pairs, 28 base pairs, 30 base pairs, more than 30 base pairs, or any combination, sub-combination, range, or sub-range thereof. In some embodiments, each double-stranded nucleic acid molecule includes two termini, each terminus including the 5' end of one strand and the 3' end of the other, complementary strand. In such embodiments, the 5' end of at least one strand is phosphorylated with a diphosphate or triphosphate group.

[0037] In an alternate embodiment, the nucleic acid duplex includes a single-stranded nucleic acid molecule with base pairing between complementary regions within the same strand. These single-stranded nucleic acid duplexes have a single terminus including both the 5' and 3' end of the single strand. In such embodiments, the 5' end of the single strand is phosphorylated with a diphosphate or triphosphate group. In one embodiment, the single-stranded nucleic acid molecule includes at least 8 base pairs, at least 10 base pairs, between 8 and 100 base pairs, between 8 and 80 base pairs, between 8 and 60 base pairs, between 8 and 50 base pairs, between 8 and 40 base pairs, between 8 and 30 base pairs, between 10 and 30 base pairs, between 10 and 20 base pairs, between 10 and 14 base pairs, 8 base pairs, 10 base pairs, 12 base pairs, 14 base pairs, 16 base pairs, 18 base pairs, 20 base pairs, 22 base pairs, 24 base pairs, 26 base pairs, 28 base pairs, 30 base pairs, more than 30 base pairs, or any combination, sub-combination, range, or sub-range thereof. In another embodiment, the single-stranded nucleic acid duplex includes, but is not limited to, a stem-loop or hairpin loop RNA. In a further embodiment, the stem-loop RNA includes an RNA tetraloop opposite the single terminus. This RNA tetraloop stabilizes the stem-loop RNA molecule and/or blocks protein binding. Although discussed above in terms of an RNA molecule, as will be appreciated by those skilled in the art, these molecules are not limited solely to RNA bases and may include at least one DNA base.

[0038] Although discussed herein primarily with regard to phosphorylated nucleic acid duplexes, as will be appreciated by those skilled in the art, the disclosure is not so limited and may include any other suitable nucleic acid agonist for the PRR of interest. In some embodiments, the nucleic acid agonist includes a specific nucleic acid ligand for the PRR

and/or an immunostimulatory oligonucleotide. For example, the nucleic acid agonist may include a single stranded oligonucleotide, such as CpG ODN (TLR-9 ligand); double-stranded DNA, such as a cGAS ligand; single stranded RNA, such as TLR-7/8 ligands; any other suitable nucleic acid agonist; or a combination thereof.

[0039] Turning to the at least one macromolecule conjugated to each of the nucleic acid agonists disclosed herein, in some embodiments, the macromolecule includes any molecule having a molecular weight of at least 300 Da, at least 350 Da, at least 400 Da, at least 450 Da, at least 500 Da, at least 550 Da, between about 300 Da and about 10,000 Da, between about 300 Da and about 7,500 Da, between about 300 Da and about 5,000 Da, between about 300 Da and about 4,000 Da, between about 300 Da and about 3,000 Da, between about 300 Da and about 2,000 Da, between about 300 Da and about 1000 Da, between about 400 Da and about 1000 Da, between about 500 Da and about 1000 Da, between about 550 and about 1000 Da, greater than 1000 Da, or any combination, sub-combination, range, or sub-range thereof. In some embodiments, the macromolecule includes any covalently attached molecule that inhibits PRR binding or activity by at least 75% through steric hindrance. In some embodiments, the macromolecule includes a polymer, a peptide, a lipid, a nucleic acid molecule, a carbohydrate, any other suitable macromolecule, or a combination thereof. Suitable polymer macromolecules include, but are not limited to, poly(ethylene glycol), polyethers, polyesters, polycarbonates, polyvinyls, polyamino acids, polysulfobetaines, carboxybetaines, or combinations thereof. Additionally or alternatively, the polymer macromolecules may include pH-responsive and/or endosome destabilizing polymers.

[0040] In some embodiments, at least one of the macromolecules disclosed herein is conjugated to a 3' end of the nucleic acid duplex at the same terminus as the phosphorylated 5' end. For example, in one embodiment, the 5' end of one strand of a double stranded RNA duplex is phosphorylated and a macromolecule is conjugated to the 3' end of the complementary strand, such that the phosphate groups and the macromolecule are on separate strands at the same terminus. In another embodiment, the double stranded RNA duplex includes at least two phosphate groups at each 5' end and a macromolecule conjugated to at least one complementary 3' end. In a further embodiment, each strand of the double stranded RNA duplex includes at least two phosphate groups at the 5' end and a macromolecule conjugated to the complementary 3' end. Turning to embodiments where there is only one terminus, such as the single-stranded duplex, the 5' end of the single terminus is phosphorylated and the macromolecule is conjugated to the 3' end thereof. As used herein,

the phrase “conjugated to a 3’ end” refers to a macromolecule conjugated to the nucleic acid duplex at any nucleotide within 5 nucleotides of the 3’ end thereof.

[0041] Additionally or alternatively, the macromolecule may be conjugated to the gamma phosphate of the phosphate group at the 5’ end of the nucleic acid duplex. In one embodiment, the conjugation of the macromolecule to the gamma phosphate includes formation of a P^{III} - P^V cyclic anhydride, followed by oxidation with *t*BuOOH and washing with anhydrous acetonitrile to form a solid-phase-bound RNA cyclotriphosphate, and then reaction with a macromolecule solution in acetonitrile. Although discussed above primarily with regard to conjugation of the macromolecule to a 3’ end of a nucleic acid duplex, as will be appreciated by those skilled in the art, the disclosure is not so limited and includes the macromolecule conjugated to any other nucleic acid agonist disclosed herein in any position suitable for inhibiting binding to and/or activation of the associated PRR.

[0042] According to one or more of the embodiments disclosed herein, the macromolecule may be conjugated to the nucleic acid agonist through any suitable linking agent. In some embodiments, the linking agent is a cleavable and/or environmentally reactive linking agent, such as, but not limited to, reactive oxygen species (ROS) cleavable linking agents, reactive nitrogen species cleavable linking agents, pH cleavable linking agents, redox cleavable linking agents, enzymatically cleavable linking agents, light sensitive linking agents, temperature responsive linking agents, oxygen sensitive linking agents, reductase sensitive linking agents, or a combination thereof. For example, in one embodiment, redox sensitive linking agents include, but are not limited to, glutathione sensitive linkers (e.g., disulfide linkers), nitroreductase/NADH sensitive linkers, or a combination thereof. In another embodiment, the pH sensitive linking agent includes, but is not limited to, hydrazone, silyl ethers, other low pH sensitive linking agents, or a combination thereof. In a further embodiment, the enzymatically cleavable linking agents include, but are not limited to, matrix metalloproteinases, dipeptide/*p*-aminobenzyl alcohol systems (which are tunable for half-lives between 9 and 240 h), lysosomal, beta-glucuronidase, intracellular esterases, or a combination thereof.

[0043] Without wishing to be bound by theory, it is believed that conjugation of the macromolecule to the nucleic acid agonist inhibits binding of the agonist to the PRR through steric hindrance. By providing selective removal of at least a portion of the macromolecule, the nucleic acid agonists disclosed herein provide selective activation of the associated receptors, thus avoiding unwanted systemic activation. Accordingly, in some embodiments,

such as where the macromolecule is conjugated through an environmentally sensitive linking agent, the nucleic acid agonists disclosed herein form a new class of nucleic acid prodrugs. For example, in one embodiment, upon removal of at least a portion of the macromolecule conjugated to a phosphorylated nucleic acid duplex, the RIG-I receptor recognizes the 5' phosphorylated duplex, regardless of nucleotide sequence, and triggers a response. While the portion of the macromolecule removed depends upon the specific macromolecule, nucleic acid agonist, and PRR, as used herein, the term "at least a portion of the macromolecule" refers to any portion which removal thereof restores at least 25% of the agonist activity. In some embodiments, this includes any portion that leaves behind a residual macromolecule and/or linker of less than 1000 Da. In some embodiments, this includes any portion that leaves behind a residual macromolecule and/or linker of less than 900 Da, less than 800 Da, less than 700 Da, less than 600 Da, less than 500 Da, less than 400 Da, less than 300 Da, less than 200 Da, or any combination, sub-combination, range, or sub-range thereof.

[0044] In some embodiments, these prodrugs are capable of activating the PRR pathway, such as the RIG-I pathway or any other suitable PRR pathway, in response to environmental stimuli that cleave at least a portion of the macromolecule from the nucleic acid agonist. In one embodiment, the environmental stimuli include any stimuli corresponding to the environmentally sensitive linker. As will be appreciated by those skilled in the art, the specific environmental stimuli will depend upon the specific linking agent attaching the macromolecule to the nucleic acid agonist, and the linker may be passively or actively exposed to the environmental stimuli. For example, the pH cleavable linking agents are cleaved when exposed to specific pH, which may be present in some environments but not in other. In another example, the light sensitive linking agents are cleaved when exposed to specific light waves, which may be applied to selected areas of a subject. Through the use of different linker chemistries and conjugation sites, the PRR agonists disclosed herein may form various bioconjugates and prodrugs based on the nucleic acid agonists disclosed herein, such as, but not limited to, agonist-antibody conjugates for tumor targeting and/or agonist-antigen conjugates for vaccination. Accordingly, in some embodiments, the prodrugs may be used as vaccine adjuvants, cancer immunotherapies, anti-viral agents, or any other application where selective activation of the RIG-I pathway or any other suitable pathway is desired.

[0045] Also provided herein, in some embodiments, is a composition including the PRR agonist and a carrier system. The carrier system includes any suitable compound that improves physiochemical and/or pharmacokinetic properties of the PRR agonist when

attached and/or linked to the nucleic acid agonist thereof. In some embodiments, the macromolecule forms the carrier system. Alternatively, in some embodiments, the carrier system is attached and/or linked to the nucleic acid agonist in addition to the macromolecule. Suitable carrier systems include, but are not limited to, polymeric compounds, liposomes, micelles, microspheres, nanoparticles, or a combination thereof. For example, suitable carrier systems include, but are not limited to, linear and branched cationic polymers, including both synthetic and naturally occurring polymers (e.g., chitosan, poly(L-lysine), polyethylenimine (PEI), (dimethylamino)ethyl methacrylate (DMAEMA), poly(beta-amino esters, and cell penetrating peptides); dendrimers, including cationic dendrimers for electrostatic complexation of RNA and dendrimer-RNA conjugates; DMAEMA-block-(DMAEMA-co-BMA-co-PAA) where BMA=butyl methacrylate and PAA=propyl acrylic acid; antibodies for generating antibody-duplex conjugates; poly(lactic-co-glycolic acid) (PLGA) micro- and nanoparticles; lipid and lipidoid nanoparticles; or combinations thereof.

[0046] In one embodiment, the carrier system is attached and/or linked to the PRR agonist through a labile bridge. In another embodiment, the carrier system encapsulates the PRR agonist. In a further embodiment, the PRR agonist is electrostatically complexed with the carrier system. As will be appreciated by those skilled in the art, suitable carrier systems will depend upon the target site for nucleic acid agonist delivery/PRR activation.

[0047] The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the presently-disclosed subject matter.

EXAMPLES

[0048] Example 1

[0049] This Example describes the generation of a “synthetic overhang” by conjugating macromolecules to the 3’ end of the complement strand to a 5’ triphosphorylated RNA to block the immunostimulatory properties of the nucleic acid and overcome the risk of increased toxicity resulting from induction of systemic anti-viral innate immunity through systemic administration of RIG-I agonists. This Example also shows restoration of agonist activity in response to specific environmental stimuli when the synthetic overhang is linked to the RNA *via* a cleavable linker (**FIG. 1**).

[0050] To generate the synthetic overhang, monomethyl poly(ethylene glycol) (PEG) was conjugated to pppRNA. PEG was chosen as a synthetic overhang due to its aqueous solubility, biocompatibility, and widespread use in clinically approved biomacromolecular therapeutics. Investigation of the effect of PEG molecular weight, conjugation site, and linker cleavability on the ability of PEG-pppRNA conjugates to activate the RIG-I pathway *in vitro* is also discussed below.

[0051] MATERIALS AND METHODS

[0052] *Materials*

[0053] Methoxy-poly(ethylene glycol) functionalized with ortho-pyridinyl disulfide (PEG-OPSS, M_n 5000), and methoxy-poly(ethylene glycol) functionalized with maleimide at varying molecular weights (PEG-MAL, M_n 550, 1000, 2000, and 5000) were obtained from Creative PEGWorks (Chapel Hill, NC). Oligonucleotide synthesis reagents were purchased from BioAutomation (Irving, TX). Dithiothreitol (DTT), reduced glutathione (GSH), chromatography-grade methanol, and oligonucleotide triphosphorylation and deprotection reagents were obtained from Sigma-Aldrich (St. Louis, MO). Heat-inactivated fetal bovine serum (HI-FBS), phosphate-buffered saline (PBS), penicillin/streptomycin solution, and Roswell Park Memorial Institute 1640 medium (RPMI) and Dulbecco's Modified Eagle Medium (DMEM) were procured from Gibco (Grand Island, NY). 0.5 M disodium N,N,N',N'-ethylenediamine tetraacetic acid (EDTA) solution was purchased from Corning (Corning, NY). Lipofectamine 2000 was acquired from Invitrogen (Carlsbad, CA). Chromatography-grade triethylamine and glacial acetic acid were obtained from Fisher Scientific (Hampton, NH). All other reagents were analytical grade.

[0054] *Chemically Modified RNAs*

[0055] 5'-triphosphorylated sense strand RNA (sequence: 5' – CGU UAA UCG CGU AUA CGC CUA U – 3') (SEQ ID NO: 1) was synthesized on a MerMade 12 automated RNA-DNA synthesizer (BioAutomation) as described previously. Base deprotection was carried out using ammonium hydroxide – methylamine solution (AMA) as described previously with minor modifications. The oligonucleotide on polymer support was transferred into a glass vial with 4 mL of AMA solution (1:1 (v/w) mixture of 30% ammonium hydroxide and 40% methylamine) before incubation at 65°C for 10 min to remove the oligonucleotide from the support and to remove protecting groups from bases and phosphates. After cooling on ice for 10 min, the supernatant was transferred to a clean vial.

The support was washed with 500 μ L of acetonitrile:water:ethanol mixture (1:1:3 v/w) and the wash was combined with the supernatant. The resulting mixture was evaporated to dryness using a SpeedVac to yield a pellet, which was rewetted with 500 μ L of ethanol and evaporated to dryness again.

[0056] Deprotection of the 2'-OH groups was carried out as described previously with minor modifications. The pellet was dissolved in 500 μ L of a 1M solution of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran and incubated at room temperature for at least 24 h with gentle shaking. 500 μ L of sodium acetate (pH 6.0) was then added and the mixture before being evaporated to a volume of $\sim$ 500 μ L. The resulting mixture was extracted 3 times with ethyl acetate to remove TBAF, and the deprotected oligonucleotide was precipitated with 1.6 mL of ethanol and purified on a 16% denaturing polyacrylamide gel. The oligonucleotide was visualized by UV-shadowing, excised from the gel and eluted by incubation of gel slices in a solution containing 10 mM MOPS (pH 6.0), 1 mM EDTA, and 300 mM NaCl at 4 $^{\circ}$ C overnight with gentle shaking.

[0057] 3'- and 5'- disulfide-modified antisense strand RNA (sequence: 5' – AUA GGC GUA UUA UAC GCG AUU AAC G – 3') (SEQ ID NO: 2), as well as 5'-non-phosphorylated sense strand and unmodified antisense strand RNAs were purchased from Integrated DNA Technologies (Coralville, IA). 3' or 5'-disulfide modified antisense RNA was annealed to 5'-triphosphorylated sense RNA or 5'-unphosphorylated control sense RNA to yield double-stranded RNA (pppRNA and cRNA, respectively) by incubating equimolar concentrations of each strand in 100 mM NaCl at 90 $^{\circ}$ C and slowly reducing the temperature to room temperature over 45 min.

[0058] *Synthesis of PEG-RNA Conjugates*

[0059] To synthesize PEG-RNA conjugates, thiolated double-stranded RNA in the form of a protected disulfide was reduced by adding 1.2 mg of dithiothreitol (DTT, 80 μ mol) to a solution containing 125 μ g of RNA (pppRNA or RNA, 7.75 nmol) suspended in water. After incubating for 30 min at room temperature, the excess DTT and other small-molecule reaction byproducts were removed by buffer exchange into a solution containing PBS, 10 mM EDTA, and 0.02% sodium azide (w/v) with a size exclusion chromatography column (ZebaTM Spin Desalting Columns, Thermo Scientific, Waltham, MA). 15 μ g of the desalted, thiolated RNA (950 pmol) was then combined with 1.2 mg of functionalized mPEG (mPEG-OPSS or mPEG-MAL, 240 nmol) and was allowed to react overnight at room temperature.

Aliquots of the resulting products were then taken for agarose gel analysis, and the remainder was buffer exchanged into 50 mM triethylammonium acetate, pH 7.5 by centrifugal diafiltration (Amicon Ultra centrifugal filters, 10 kDa MWCO, EMD Millipore, Burlington, MA) and analyzed by ion-paired reverse-phase HPLC on a Clarity C18 column (50 mm × 4.6 mm, 5 μm, Phenomenex, Torrance, CA) equipped with a dual-channel UV detector at 260 and 280 nm (Waters, Milford, MA) and utilizing the following method: buffer A, 50 mM triethylammonium acetate, pH 7.5; buffer B, methanol; gradient, 5% to 100% buffer B in 50 min after 5 min dwell time; flow rate 1 mL/min; ambient column temperature. The appropriate HPLC fractions were pooled (**Table 1**), then concentrated and buffer exchanged into ultrapure water by centrifugal diafiltration (3 kDa MWCO).

Table 1: Times during which elute was collected during HPLC for each sample.

Sample	Collection Begin (min)	Collection End (min)
unconjugated pppRNA	15.4	19.8
pppRNA + 550-mal	19	25
pppRNA + 1k-mal	22	26
pppRNA + 2k-mal	25	31
pppRNA + 5k-mal	33	37
pppRNA + 5k-ss	33	37
pppRNA + 5k-mal (opposite side)	33	37

[0060] *Characterization of PEG-RNA Conjugates*

[0061] To verify PEG conjugation and product purity, the HPLC purified products were imaged after agarose gel electrophoresis in Tris-borate-EDTA buffer with 2% agarose at 148 V for 30 or 75 min. Area-under-the-curve absorbance ratios were evaluated during HPLC analysis to estimate conjugation efficiency. This analysis was performed using absorbance values at 280 nm, as disulfide bonds absorb weakly between 250 and 270 nm. To verify the cleavability and non-cleavability of the disulfide and maleimide linkages (respectively), the reaction products were incubated in 10 mM GSH at 37 °C for 24 h before visualization via agarose gel electrophoresis and analysis as previously described.

[0062] *Cell Culture*

[0063] All cell culture assays were performed using A549 Dual Reporter cells (Invivogen). A549s were cultured in DMEM supplemented with 4.5 g/L glucose, 10% HI-FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37° C in a humidified atmosphere containing 5% CO₂. 5,000 cells were plated into 96-well plates and transfected in quadruplicate with Lipofectamine 2000 transfection reagent according to the manufacturer's instructions with 20 nM concentrations of conjugates. Dose sweeps started at 20 nM, followed by serial 2-fold dilutions down to 156.25 pM. IFN-β concentrations in cell supernatant were determined using a Lumikine hIFN-β kit (Invivogen) according to the manufacturer's instructions. Luminescent reporter assays were performed using QUANTI-Luc (Invivogen) according to the manufacturer's instructions. Measurements were taken using a Synergy H1 microplate reader (BioTek, Winooski, VT). All measurements were normalized after baselining to the average value of the PBS-treated negative control group.

[0064] RESULTS AND DISCUSSION

[0065] The synthesis scheme used to conjugate monofunctionalized PEG to pppRNA is depicted in **FIG. 2**. In summary, the *n*-hydroxypropyl disulfide protecting group was removed by reduction with excess dithiothreitol (DTT) to yield double stranded oligonucleotides with a free thiol group at the 3' end of strand complementary to 5' ppp-containing strand. Thiolated pppRNAs were subsequently reacted with *ortho*-pyridyl disulfide- or maleimide-functionalized PEG of various molecular weight (MW) to yield pppRNA conjugates containing either a reducible disulfide linkage (PEG_{MW}-SS-pppRNA) or a stable thioether linkage (PEG_{MW}-mal-pppRNA).

[0066] Conjugation efficiency was determined by area-under-the-curve integration during HPLC purification (**Table 2**). Agarose gel electrophoresis depicted shifts in RNA migration distances that were consistent with the molecular weight of the conjugated PEG (**FIG. 3A**). Additional gel electrophoresis was performed to further confirm the absence of unconjugated pppRNA in the HPLC-purified PEG₅₅₀-mal-pppRNA conjugate (**FIG. 3B**). These procedures were repeated with control RNA lacking the 5' ppp moiety (cRNA) to produce negative control conjugates (**Table 3**). To evaluate whether synthetic overhangs could be removed when linked *via* a reducible disulfide bond but not a thioester linkage, cRNA conjugates synthesized with 5kDa PEG were incubated with cytosolic levels (10 mM) of glutathione prior to electrophoresis. cRNA was completely liberated from disulfide-linked overhangs,

whereas conjugates with stable maleimide linkers remained intact (**FIG. 3C**), demonstrating that cleavable linkers can be used for stimuli-responsive removal of synthetic overhangs.

Table 2: Conjugation efficiencies of PEG-pppRNA conjugates used in this study.

Sample	% Conjugation
pppRNA + 550-mal	86.6
pppRNA + 1k-mal	37.3
pppRNA + 2k-mal	≥98
pppRNA + 5k-mal	≥98
pppRNA + 5k-ss	63.1
pppRNA + 5k-mal (opposite side)	95.3

Table 3: Conjugation efficiencies of PEG-cRNA conjugates used in this study.

Sample	% Conjugation
cRNA + 5k-mal	≥98
cRNA + 5k-ss	59.5

[0067] To evaluate the effect of synthetic overhangs on RIG-I activation, PEG-pppRNA conjugates of indicated overhang molecular weight and linker chemistry were complexed with a lipid-based transfection reagent (Lipofectamine 2000) to mediate cytosolic delivery, and incubated with human lung carcinoma cells with an interferon regulatory factor (IRF) pathway reporter gene (A549-Dual). The effect of introducing a propanthiol group on the 3' end of the complement strand was first evaluated by comparing IRF pathway activation to unmodified pppRNA. A significant effect on ligand activity was not observed (**FIG. 4**). The effect of PEG overhang molecular weight was then examined using maleimide-linked PEG overhangs of 550 Da, 1 kDa, 2 kDa, and 5 kDa. Interestingly, conjugation of 550 Da PEG had minimal impact on ligand activity, whereas all larger molecular weights (1, 2, 5 kDa) dramatically inhibited ligand activity to approximately the same extent, suggesting a minimum molecular weight threshold for successful ablation of RIG-I activation (**FIG. 5A**). This is conceptually consistent with the ability of RIG-I to recognize pppRNA with short (*i.e.*, 1-3 nucleotides) 3' RNA overhangs, albeit at the expense of activity. Additionally,

PEG_{5k}-cRNA conjugates were delivered to cells to evaluate the degree of immunogenicity present solely due to the presence of the synthetic overhang and linker chemistry. As expected, control RNA without the 5'ppp moiety (cRNA) lacked immunostimulatory activity, which was not effected by conjugation of 5kDa PEG (**FIG. 6**).

[0068] Next, to demonstrate the importance of conjugation site specificity in inhibiting pppRNA activity, 5kDa PEG was conjugated *via* a thioester linkage at the 5' end of the strand complementary to the 5'ppp-containing strand such that the PEG was conjugated at the opposite end of the 5'ppp group (**FIG. 5B**). Equivalent activity was observed between unconjugated pppRNA and pppRNA conjugated with PEG_{5k} on the opposite end, demonstrating that blockade of RIG-I activity with PEG synthetic overhangs requires conjugation on the same end as the 5'ppp group on the dsRNA.

[0069] To establish that RIG-I activation could be restored via removal of the overhang, the activity of pppRNA conjugated to PEG_{5k} overhangs via a stable thioester bond or disulfide bridge, which would be anticipated to be cleaved in the reducing environment of the cytosol enabling recognition of pppRNA by RIG-I, was further compared. Indeed, the pppRNA conjugated to PEG via a disulfide was significantly more active than its thioether counterpart (**FIG. 5C**), though slightly less active than the conjugated pppRNA, which is believed to be a consequence of partial disulfide bond reduction and incomplete liberation of pppRNA from the overhang. To further quantify the effect of molecular weight, conjugation site, and linker chemistry on pppRNA activity, the estimated median effective concentrations (EC₅₀) of the conjugates and relevant controls described above are shown in **FIG. 5D**.

[0070] Finally, as a proof-of-concept demonstration of environmentally-triggerable activation of innate immunity, secretion of human interferon beta (hIFN- β 1), a critical mediator of anti-tumor immune responses, by A549 lung cancer cells treated with pppRNA bearing reduction-responsive (disulfide) or non-responsive (maleimide) 5kD PEG overhangs was quantified. As shown in **FIG. 7**, only conjugates synthesized with the cleavable disulfide linker were capable of stimulating production of hIFN- β 1. While in such an *in vitro* system removal of the overhang is likely mediated primarily by intracellular glutathione, which has been shown to be elevated in cancer cells, the increased extracellular levels of glutathione associated with tumors might also be exploited to increase ligand activity in the tumor microenvironment prior to cellular entry.

[0071] CONCLUSION

[0072] This Example demonstrates that synthetic polymer overhangs can be designed to inhibit activation of RIG-I by pppRNA, and that conjugation of synthetic overhangs using environmentally cleavable linkers provides a mechanism for their removal and restoration of pppRNA immunostimulatory properties. This Example also shows that abrogation of activity via synthetic overhangs is at least partially dependent on PEG molecular weight, with a molecular weight of between 550 and 1000 Da being inhibiting RIG-I activity. Furthermore, this Example shows that blockade of RIG-I activity is conjugation site-dependent, as ligation of PEG to the opposite end of the 5' ppp group did not influence ligand activity.

[0073] Collectively, this work demonstrates that conjugation of macromolecule overhangs to pppRNA through cleavable linkers is a viable strategy for the development of environmentally triggerable RIG-I-targeting prodrugs. These findings also support the potential to use a diversity of cleavable linker chemistries with sensitivities to different stimuli to create overhangs that can be removed under specific environmental conditions (**FIG. 8**). For example, in addition to the reduction-sensitive linkage described in this Example, pH labile, reactive oxygen species (ROS) responsive, and/or matrix metalloproteinase cleavable linkers may be employed to synthesize pppRNA-based prodrugs that enrich RIG-I activation in tumors. Moreover, elevated intracellular levels of glutathione, ROS, or enzymatic activity in specific cell populations may be exploited for triggerable RIG-I activation.

[0074] Further still, these findings indicate that short PEG overhangs (550 Da) appear to minimally effect RIG-I activation (**FIG. 5A**), which implies that there is a tolerance for residual overhang material that may remain following linker cleavage. While this phenomenon may be dependent on nature of the linker and overhang chemistry, the data suggest a degree of wiggle room for molecular design of environmentally responsive linkers. Additionally, the dependence of linker chemistry and conjugation site on RIG-I activation have important implications for design of other bioconjugates and prodrugs based on pppRNA, including pppRNA-antibody conjugates for tumor targeting and pppRNA-antigen conjugates for vaccination. Overall, the design of synthetic polymer overhangs for RNA ligands of the RIG-I pathway represents an important development in expanding the utility of this potent and emerging class of PRR agonist. Most importantly, the ability to block RIG-I activation using conjugated synthetic overhangs, but restore activity in an environmentally responsive manner, offers safe and therapeutically relevant prodrugs for localized induction of innate immunity.

[0075] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference, including the references set forth in the following list:

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[0076] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and are herein described below in detail. It should be understood, however, that the description of specific embodiments is not intended to limit the disclosure to cover all modifications, equivalents and alternatives falling within the spirit and scope of the disclosure as defined by the appended claims.

CLAIMS

What is claimed is:

1. A selective pattern recognition receptor (PRR) agonist, comprising:
a nucleic acid agonist; and
a macromolecule conjugated to the nucleic acid agonist.
2. The selective PRR agonist of claim 1, wherein the nucleic acid agonist comprises a nucleic acid duplex having at least two phosphoryl groups attached to a 5' end thereof.
3. The selective PRR agonist of claim 2, wherein the nucleic acid duplex comprises a double stranded nucleic acid molecule.
4. The selective PRR agonist of claim 2, wherein the nucleic acid duplex comprises a single-stranded nucleic acid molecule.
5. The selective PRR agonist of claim 4, wherein the single-stranded nucleic acid molecule comprises a hairpin loop RNA molecule.
6. The selective PRR agonist of any of claims 2-5, wherein the at least two phosphoryl groups are selected from the group consisting of a diphosphate group and a triphosphate group.
7. The selective PRR agonist of any of claims 1-5, wherein the macromolecule has a molecular weight of at least 550 Da.

8. The selective PRR agonist of any of claims 1-5, further comprising an environmentally selective linker conjugating the macromolecule to the nucleic acid agonist.
9. The selective PRR agonist of claim 8, wherein the environmentally selective linker is selected from the group consisting of reactive oxygen species (ROS) sensitive linking agents, pH sensitive linking agents, redox sensitive linking agents, enzymatically cleavable linking agents, light sensitive linking agents, and combinations thereof.
10. The selective PRR agonist of claim 9, wherein the redox sensitive linking agents are selected from the group consisting of glutathione sensitive linkers, nitroreductase/NADH sensitive linkers, and combinations thereof.
11. The selective PRR agonist of claim 9, wherein the pH sensitive linking agents are selected from the group consisting of hydrazone, silyl ethers, other low pH sensitive linking agents, and combinations thereof.
12. The selective PRR agonist of claim 9, wherein the enzymatically cleavable linking agents are selected from the group consisting of matrix metalloproteinases, dipeptide/*p*-aminobenzyl alcohol systems, lysosomal, beta-glucuronidase, intracellular esterases, and combinations thereof.
13. The selective PRR agonist of any of claims 2-5, wherein the macromolecule is conjugated to a 3' end of the nucleic acid duplex, the 3' end that the macromolecule is conjugated to and the 5' end that the at least two phosphoryl groups are attached to being at a single terminus of the nucleic acid duplex.
14. The selective PRR agonist of claim 13, wherein the agonist is a retinoic acid-inducible gene I (RIG-I) agonist.

15. The selective PRR agonist of any of claims 2-5, wherein the macromolecule is conjugated to a phosphoryl group attached to the 5' end of the nucleic acid duplex.
16. The selective PRR agonist of any of claims 2-5, wherein removal of at least a portion of the macromolecule permits binding of the agonist to a PRR.
17. A method of selectively activating a pattern recognition receptor (PRR), the method comprising:
 - administering the selective PRR agonist of claim 1 to a subject; and
 - cleaving at least a portion of the macromolecule conjugated to the nucleic acid agonist;
 - wherein the cleaving of at least a portion of the macromolecule permits the agonist to bind a PRR.
18. The method of claim 17, further comprising an environmentally selective linker conjugating the macromolecule to the nucleic acid agonist.
19. The method of claim 18, wherein the cleaving step comprises passively or actively subjecting the agonist to an environmental stimulus corresponding to the environmentally selective linker.
20. The method of claim 17, further comprising a carrier system attached to the agonist.

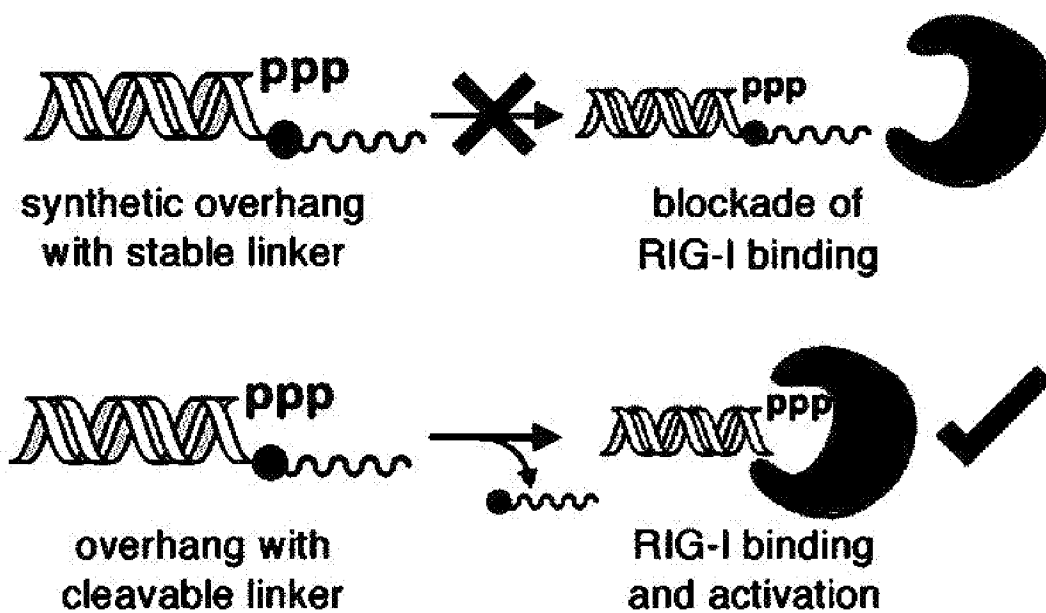


FIG. 1

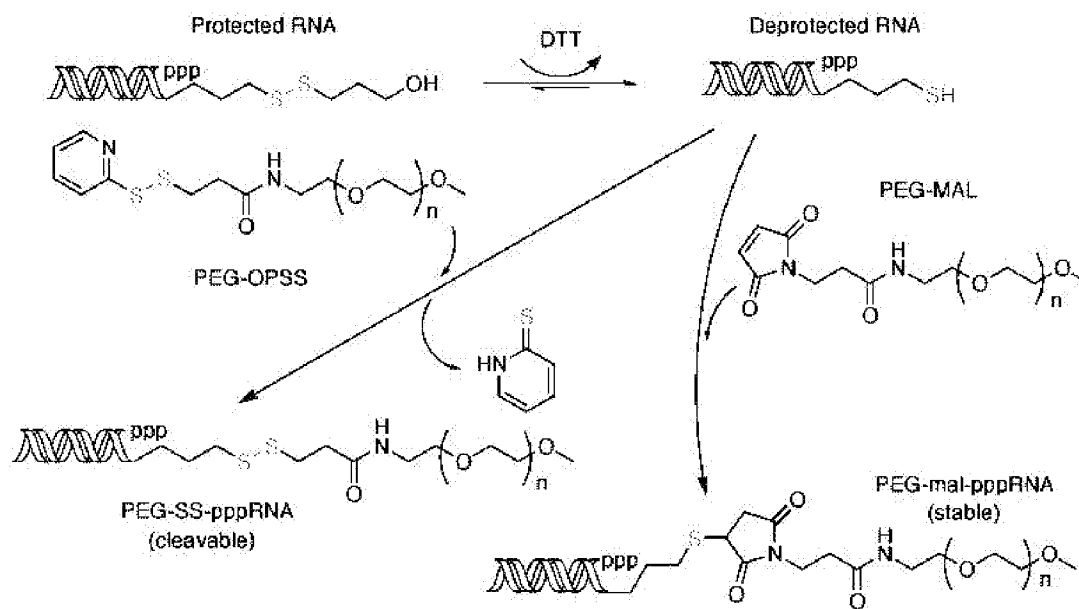


FIG. 2

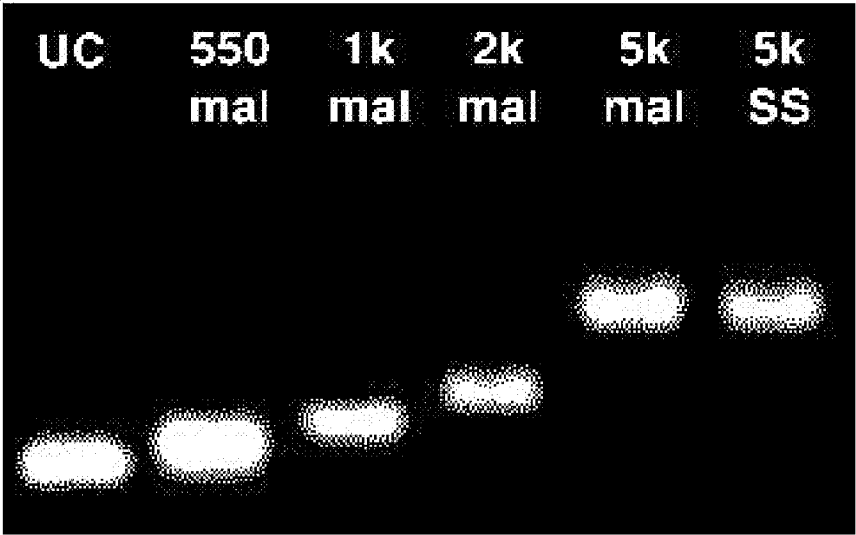


FIG. 3A

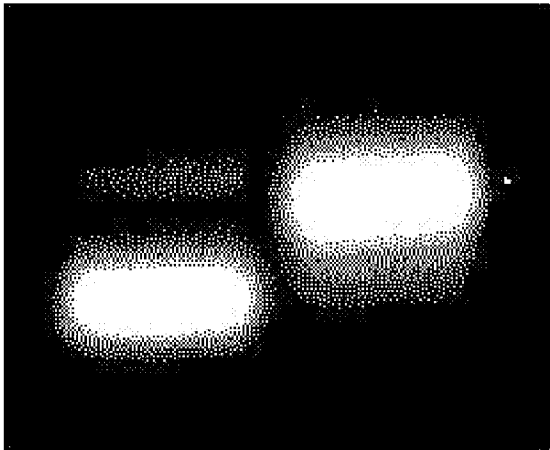


FIG. 3B

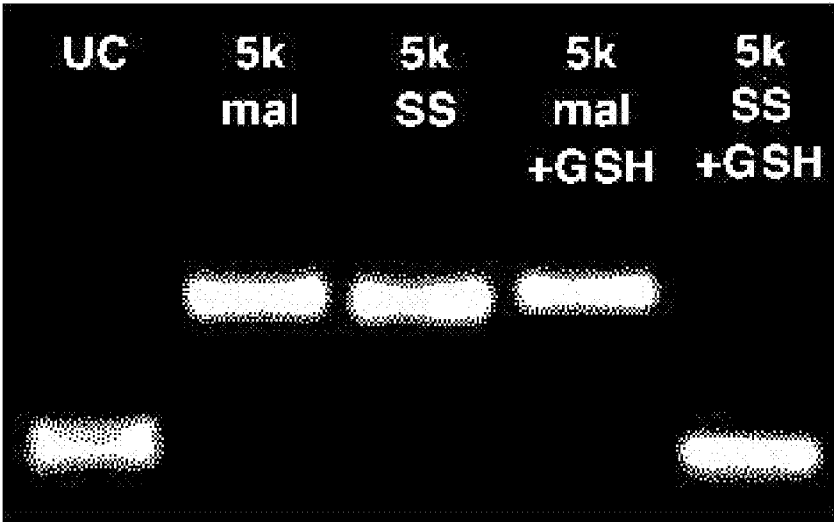


FIG. 3C

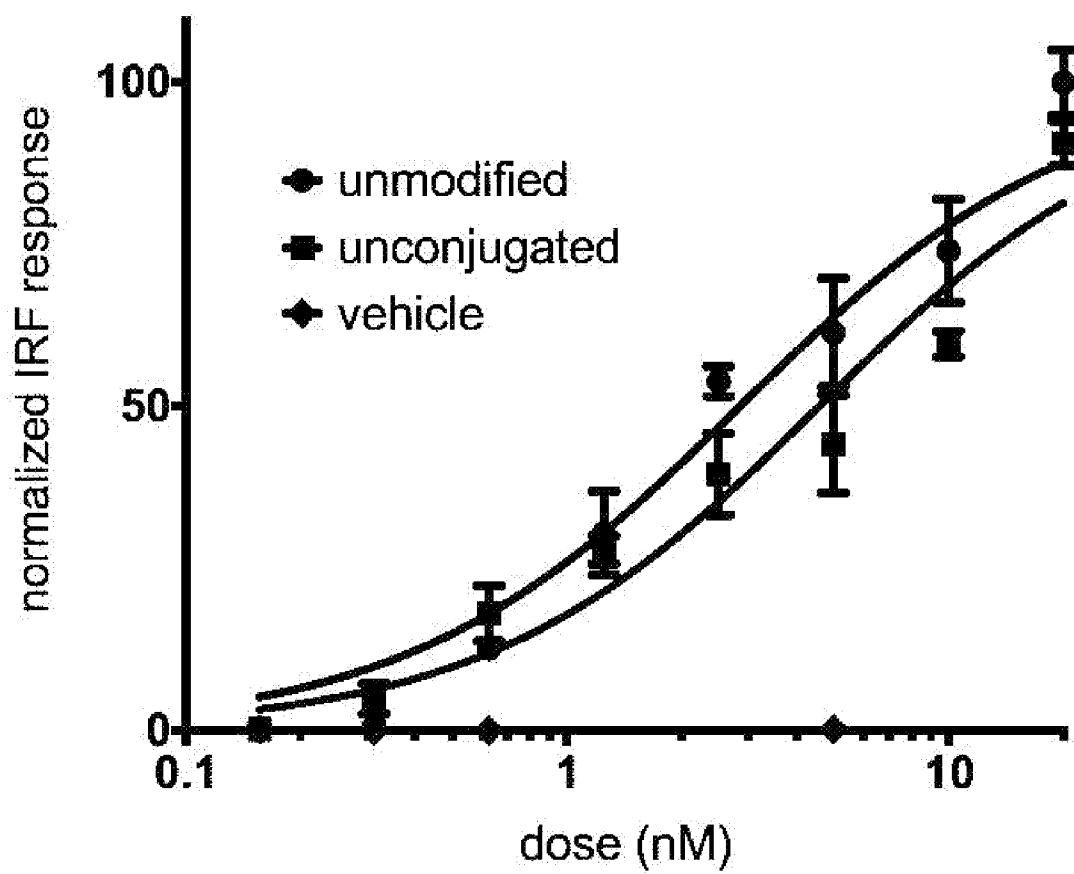


FIG. 4

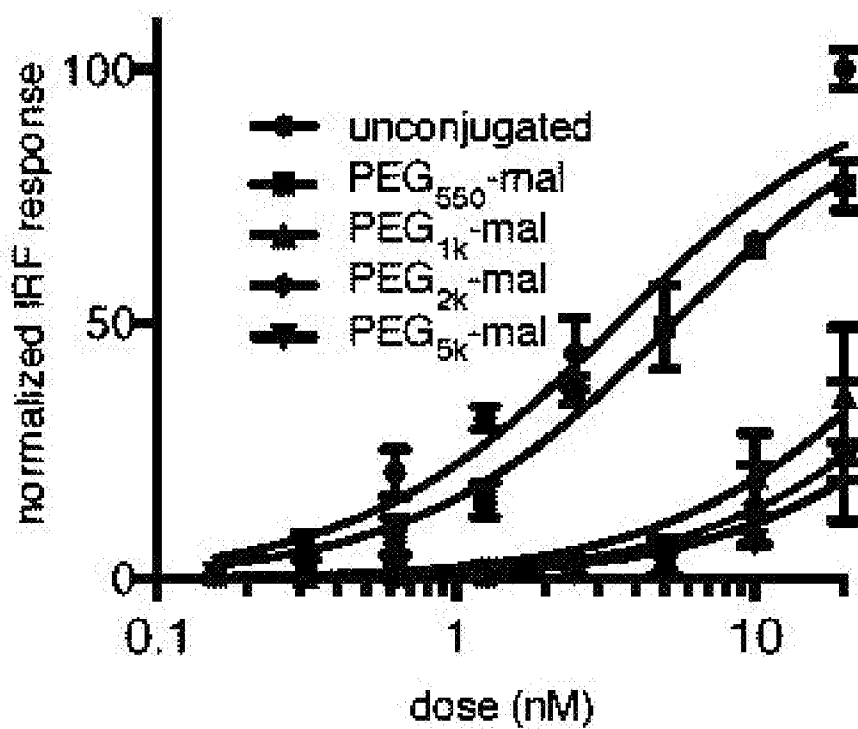


FIG. 5A

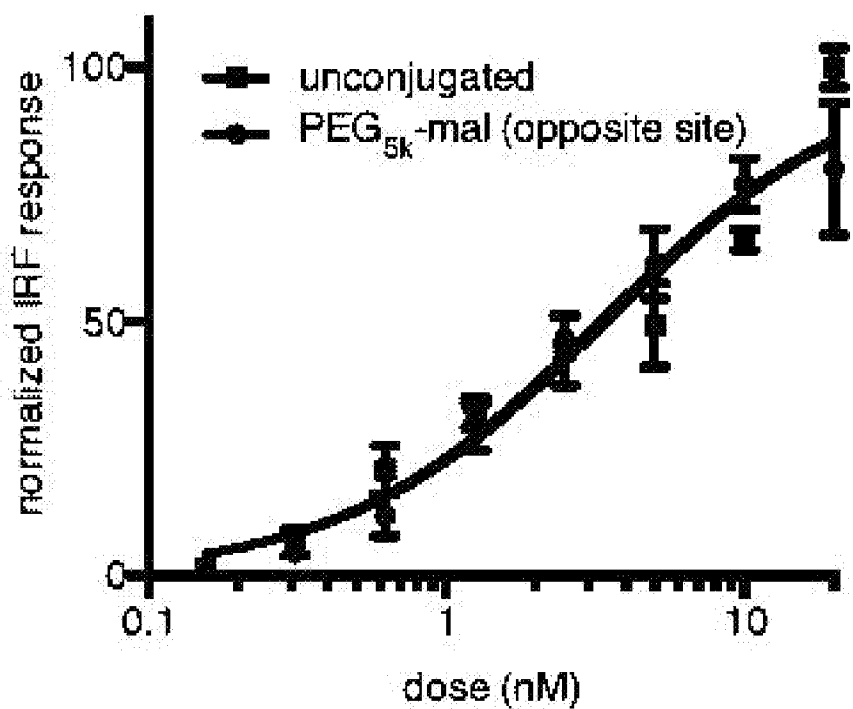


FIG. 5B

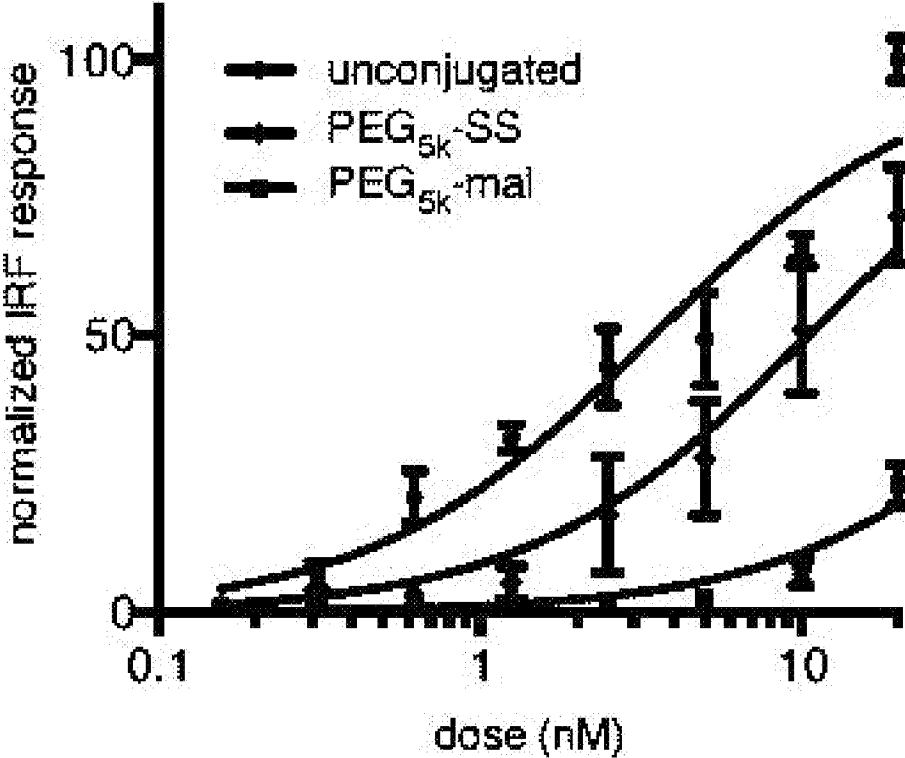


FIG. 5C

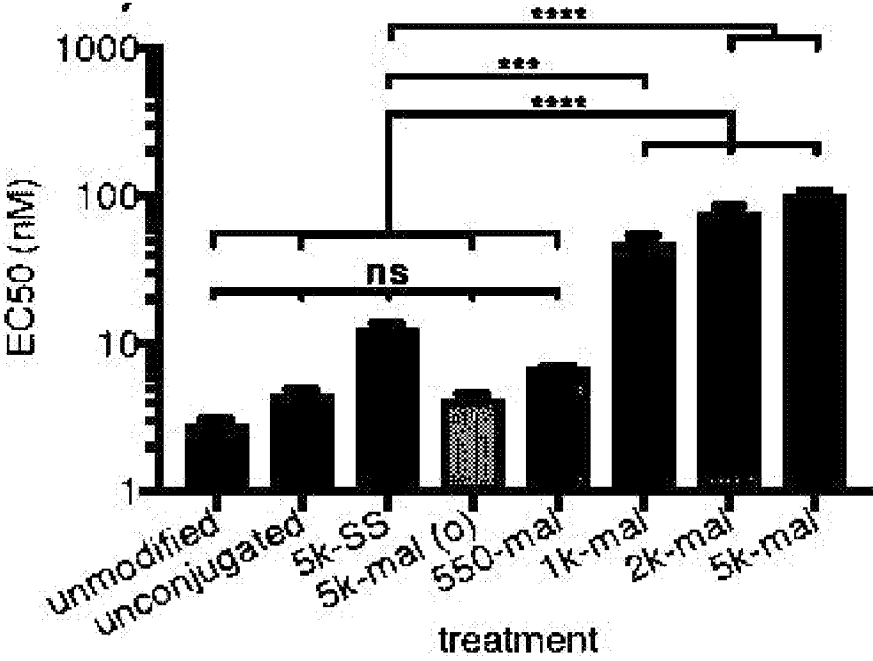


FIG. 5D

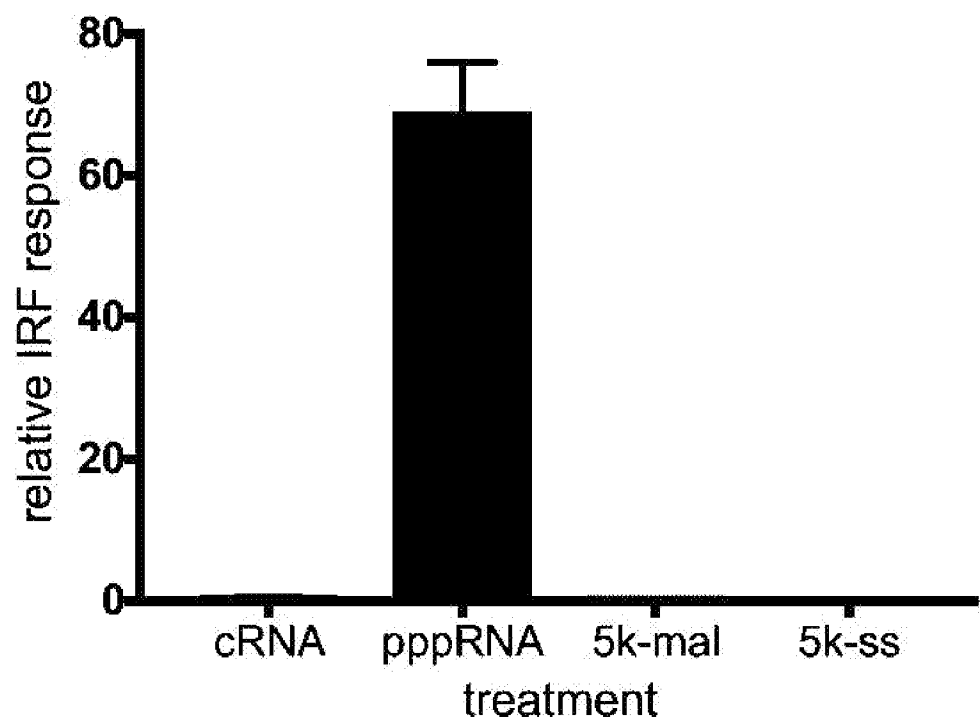


FIG. 6

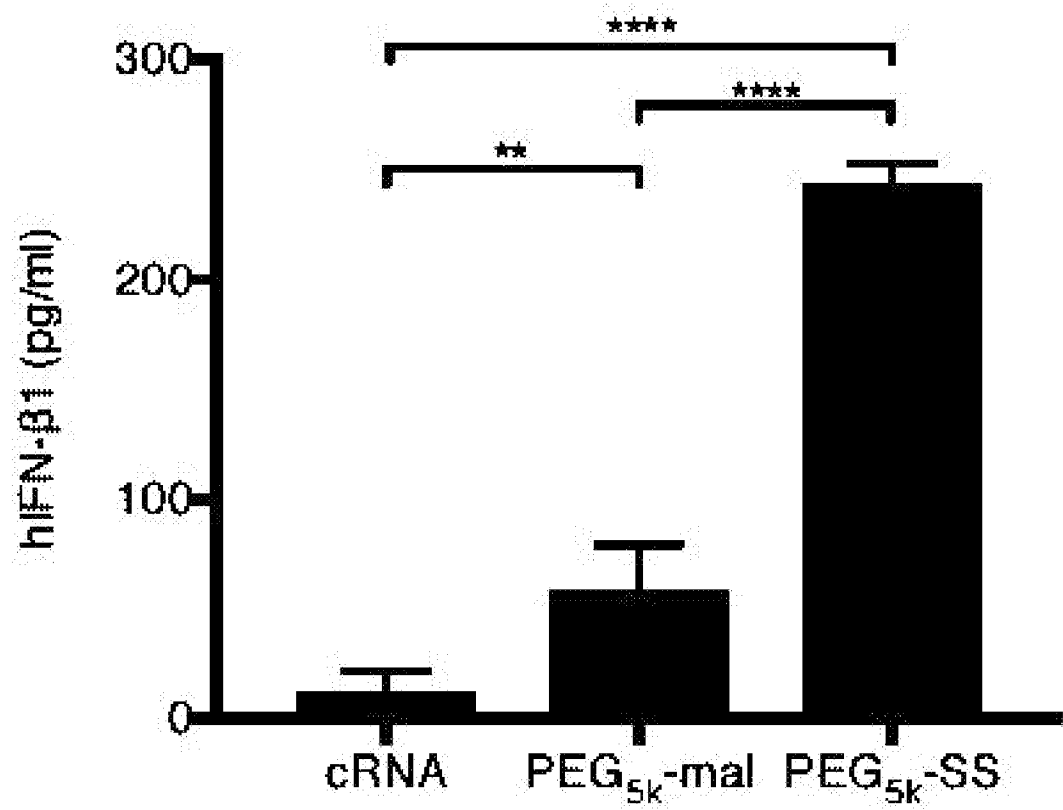


FIG. 7

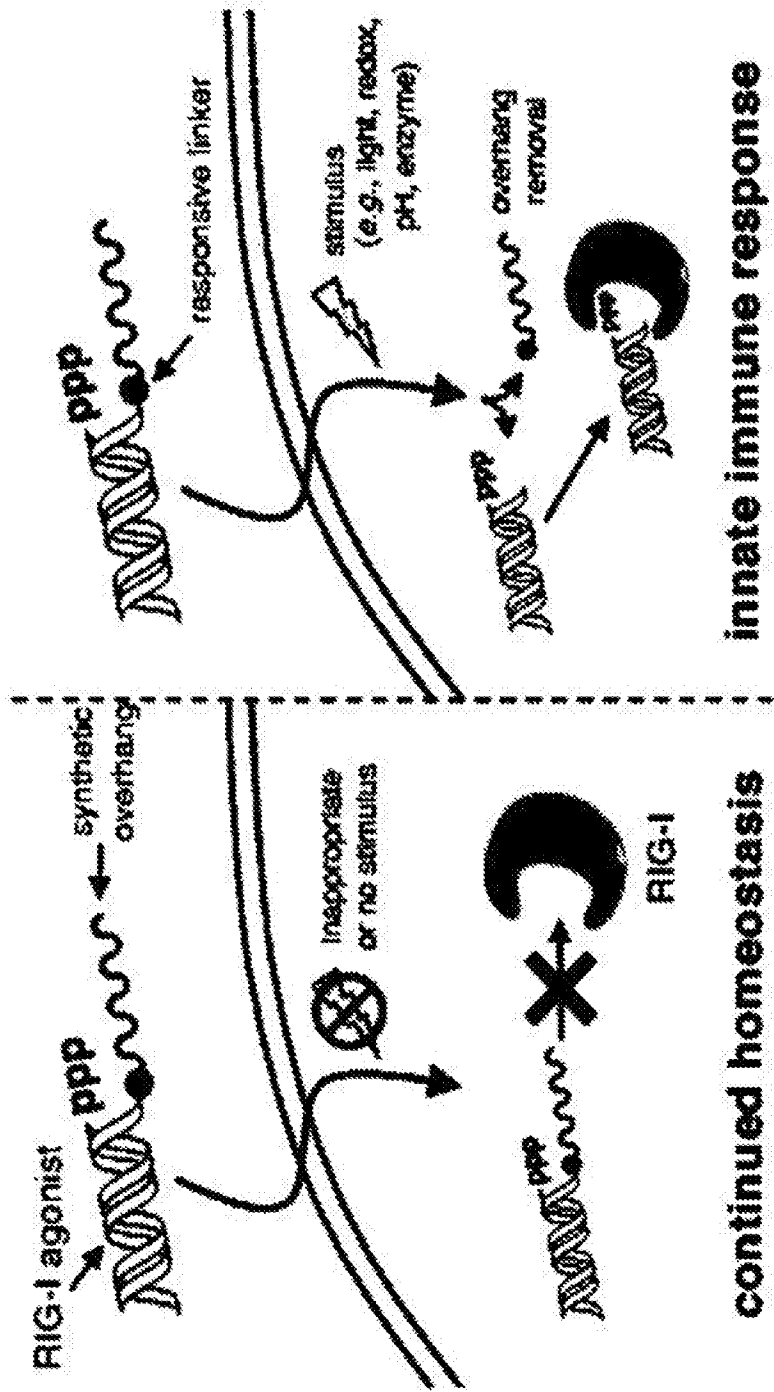


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/12929

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

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2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/12929

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/00, A61K 39/39 (2019.01)
CPC - A61K 39/0011, A61K 39/39, A61K 2039/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0287023 A1 (MCGILL UNIVERSITY et al.) 25 Sept 2014 (25.09.2014) para [0007], [0009], [0154], [0158], [0162], [0163], [0182], [0234]; Fig. 1A	1-20
Y	MISHRA et al., "PEGylation in anti-cancer therapy: An overview," Asian Journal of Pharmaceutical Science, 14 Sept 2015, Vol. 11, No. 3, pg. 337-348. Especially Abstract; pg 337, col 2, last para; pg 338, col 2, para 1; pg 339, col 1, para 2; Fig. 3, 5	1-20
Y	HEIDEL et al., "Cyclodextrin-Containing Polymers: Versatile Platforms of Drug Delivery Materials," Journal of Drug Delivery, 2012, Vol. 2012, Article ID 262731, pg. 1-17. Especially pg 3, col 1, para 2; Figure 1	20

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

5 March 2019

Date of mailing of the international search report

24 APR 2019

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