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Environmentally Triggerable RIG-I Agonists Using Synthetic Polymer Overhangs

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Abstract

Retinoic acid-inducible gene I (RIG-I) is a cytosolic pattern recognition receptor (PRR) that potently activates antiviral innate immunity upon recognition of 5' triphosphorylated double-stranded RNA (pppRNA). Accordingly, RNA ligands of the RIG-I pathway have recently emerged as promising antiviral agents, vaccine adjuvants, and cancer immunotherapeutics. However, RIG-I is expressed constitutively in virtually all cell types, and therefore administration of RIG-I agonists causes risk for systemic inflammation and possible dose-limiting toxicities. Here, we establish proof-of-concept and initial design criteria for pppRNA prodrugs capable of activating the RIG-I pathway in response to specific environmental stimuli. We show that covalent conjugation of poly(ethylene glycol) (PEG) to the 3' end of the complementary strand, *i.e.*, on the same side but opposite strand as the 5' triphosphate group, can generate a synthetic overhang that abrogates RIG-I activation. Additionally, conjugation of PEG through a cleavable linker - here, a reducible disulfide bond - allows for removal of the synthetic overhang and restoration of immunostimulatory activity. Furthermore, we demonstrate that blockade of RIG-I activation via synthetic overhangs is dependent on PEG molecular weight, with a critical molecular weight between 550 and 1000 Da required to inhibit activity. Additionally, we demonstrate that blockade of RIG-I activity is conjugation site-dependent as ligation of PEG to the opposite end of the RNA did not influence ligand activity. Collectively, this work demonstrates that conjugation of synthetic

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

Supporting Information

Materials and methods, supporting tables S1–S3, supporting figures S1–S3, and supporting references.

polymer overhangs to pppRNA through cleavable linkers is a viable strategy for the development of environmentally triggerable RIG-I-targeting prodrugs.

Graphical Abstract

Introduction

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.⁽¹⁾ 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 bolster anti-tumor immunity or improve the efficacy of vaccines.^(2–7) 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.^(8–11) 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).^(12–16) This challenge has spawned the development of scaffolds and controlled release depots that localize inflammatory cues,^(17, 18) nanoparticulate carriers and bioconjugates that enhance accumulation of immune activators in target tissues,^(19, 20) immunostimulatory antibody-drug conjugates,⁽²¹⁾ and, more recently, stimuli-responsive immunomodulators.⁽²²⁾

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.^(1, 11, 23–25) 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.^(2–5, 19, 26–30) An important nucleic acid sensor 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, doublestranded RNA that contains a triphosphate group at the 5' end (pppRNA).^(31–35) 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).^(34, 36–38) Additionally, activation of

RIG-I in cancer cells has been shown to increase their immunogenicity as well as induce immunogenic cell death.(39–44) Hence, agonists of the RIG-I pathway have recently emerged as a promising class of cancer immunotherapeutic and are currently being evaluated in a clinical trial (NCT03065023) via an intratumoral administration route.(39, 41, 45) Unlike many other PRRs, which are expressed primarily in hematopoietic cells and often restricted to specific subsets of immune cells,(46) RIG-I is present in the cytosol of virtually all cell types.(6, 31) This characteristic of RIG-I is a double-edged sword: on one hand, the ubiquity of RIG-I expression could allow for the design of a universal innate immune activator for cancer immunotherapy that is independent of the presence of specific cell populations within tumors; on the other hand, systemic administration of RIG-I agonists risks induction of systemic anti-viral innate immunity with increased potential for toxicity.

Towards overcoming this challenge, we looked to naturally occurring viral host evasion tactics for inspiration. Some viruses are capable of evading detection by RIG-I by leaving unpaired bases at the 5' triphosphate-containing end, resulting in an overhang that prevents interaction of the RNA with RIG-I.(47, 48) With this in mind, we hypothesized that we could conjugate polymers to the 3' end of the complement strand of a 5' triphosphorylated RNA to generate a “synthetic overhang” that would block the immunostimulatory properties of the nucleic acid. Importantly, we also hypothesized that agonist activity can be restored in response to specific environmental stimuli if the synthetic overhang was linked to the RNA via a cleavable linker (Scheme 1). To test these hypotheses, we conjugated monomethyl poly(ethylene glycol) (PEG) to pppRNA. PEG was chosen as a synthetic overhang due to its aqueous solubility, biocompatibility, and widespread use in clinically approved biomacromolecular therapeutics.(49) Additionally, PEG-RNA conjugates have been thoroughly explored in the context of small interfering RNA (siRNA), which are structurally and compositionally similar to our RIG-I agonist.(50, 51) Here, we investigated 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*.

Results and Discussion

The synthesis scheme used to conjugate monofunctionalized PEG to pppRNA is depicted in Scheme 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}-malpppRNA).

Conjugation efficiency was determined by area-under-the-curve integration during HPLC purification (Table S1). Agarose gel electrophoresis depicted shifts in RNA migration distances that were consistent with the molecular weight of the conjugated PEG (Figure 1a). Additional gel electrophoresis was performed to further confirm the absence of unconjugated pppRNA in the HPLC-purified PEG550-mal-pppRNA conjugate (Figure S1). These procedures were repeated with control RNA lacking the 5'ppp moiety (cRNA) to

produce negative control conjugates (Table S2). These data collectively demonstrate the purity of the synthesized materials used in the following experiments. To evaluate whether synthetic overhangs could be removed when linked *via* a reducible disulfide bond but not a thioether linkage, cRNA conjugates synthesized with 5kDa PEG were incubated with cytosolic levels (10 mM) of glutathione prior to electrophoresis.⁽⁵²⁾ cRNA was completely liberated from disulfide-linked overhangs, whereas conjugates with stable maleimide linkers remained intact (Figure 1b), demonstrating that cleavable linkers can be used for stimuli-responsive removal of synthetic overhangs.

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). We first evaluated the effect of introducing the propanthiol reactive handle on the 3' end of the complement strand by comparing IRF pathway activation to unmodified pppRNA, and did not observe a significant effect on ligand activity (Fig S2; $EC_{50_{unmodified}}=2.9\pm0.2$ nM; $EC_{50_{modified}}=4.6\pm0.4$ nM). Next, we examined the effect of PEG overhang molecular weight 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 IRF pathway activation ($EC_{50_{550}}=6.8\pm0.3$ nM), whereas all larger molecular weights (1, 2, and 5 kDa) dramatically inhibited ligand activity to approximately the same extent ($EC_{50}\cong 50$ –100 nM), suggesting a minimum molecular weight threshold for successful ablation of RIG-I activation (Figure 2a). 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.^(31, 53) Additionally, cells were treated with PEG_{5k}-cRNA conjugates to evaluate any changes in immunostimulatory activity due to the presence of the synthetic overhang or linker chemistry. As expected, control RNA without the 5'ppp moiety (cRNA) lacked immunostimulatory activity, and this was not influenced by conjugation of 5kDa PEG (Fig S3).

We next sought to demonstrate the importance of conjugation site specificity in inhibiting pppRNA activity. To do this, we conjugated 5kDa PEG *via* a thioether 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 (Figure 2b). Equivalent activity was observed between unconjugated pppRNA and pppRNA conjugated with PEG_{5k} on the opposite end ($EC_{50}=4.3\pm0.3$ nM), demonstrating that blockade of RIG-I activity with PEG syntheticoverhangs requires conjugation on the same end as the 5'ppp group on the dsRNA.

To establish that RIG-I activation could be restored via removal of the overhang, we further compared the activity of pppRNA conjugated to PEG_{5k} overhangs via a stable thioether bond or disulfide bridge, which would be anticipated to be cleaved in the reducing environment of the cytosol thereby enabling recognition of pppRNA by RIG-I.⁽⁵²⁾ Indeed, the pppRNA conjugated to PEG via a disulfide was significantly more active than its thioether counterpart (Figure 2c; $EC_{50_{5k-SS}}=12.9\pm1$ nM, $EC_{50_{5k-mal}}=100\pm8$ nM) though slightly less active than the unconjugated pppRNA, which we postulate is a consequence of partial disulfide bond reduction and incomplete liberation of pppRNA from the overhang.

Additionally, the reduced activity of SS-linked PEG-pppRNA conjugates could be at least partially due to reduced complexation with Lipofectamine.(54) We also suspect that the minor activity observed after high doses of maleimide-linked PEG overhangs of molecular weight greater than 1 kDa is due to extremely slow, but nonetheless still present, degradation kinetics of maleimide-thiol adducts in reducing environments.(55, 56) To summarize 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 Figure 2d.

Finally, as a proof-of-concept demonstration of environmentally-triggerable activation of innate immunity, we quantified secretion of human interferon beta (hIFN- β 1), a critical mediator of anti-tumor immune responses,(36, 57) by A549 lung cancer cells treated with pppRNA bearing reduction-responsive (disulfide) or non-responsive (maleimide) 5kD PEG overhangs. As shown in Figure 3, the conjugate synthesized with the cleavable disulfide linker stimulated significantly more hIFN- β 1 production than that with the more stable thioether linker. 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. (55, 59)

Conclusion

In this study, we have demonstrated 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. Additionally, we show that a minimum PEG molecular weight is required to abrogate activity and that conjugation of PEG to the same end as the 5'ppp group is critical for ablation of RIG-I activation. Our findings 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. For example, in addition to the reduction-sensitive linkage described herein, pH labile, reactive oxygen species (ROS) responsive, and/or matrix metalloproteinase cleavable linkers could potentially be employed to synthesize pppRNA-based prodrugs that enrich RIG-I activation in tumors.(60, 61) Moreover, elevated intracellular levels of glutathione, ROS, or enzymatic activity in specific cell populations may be exploited for triggerable RIG-I activation. Importantly, our findings indicate that short PEG overhangs (550 Da) appear to minimally effect RIG-I activation (Figure 3b), 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 and must be evaluated accordingly, our data suggest a degree of flexibility 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 many opportunities to develop safe and therapeutically relevant prodrugs for localized induction of innate immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of PEG-pppRNA conjugates containing stable (mal) and reducible (SS) linker chemistries by gel electrophoresis. **a)** Representative agarose gel demonstrating reduced migration distance with increased PEG overhang molecular weight. UC = unconjugated pppRNA. PEG molecular weight and linker chemistry of PEG-pppRNA conjugates is indicated above each lane. **b)** Agarose gel electrophoresis of unconjugated cRNA (UC) and cRNA conjugates with PEG_{5k} overhangs and indicated linker chemistry. Conjugates incubated with 10 mM glutathione (+GSH) are indicated.

Figure 2.

Effect of PEG synthetic overhang molecular weight, linker cleavability, and conjugation site on pppRNA mediated activation of interferon regulatory factor (IRF) pathway. **a)** Dose-response curve comparing the response to unconjugated pppRNA and pppRNA conjugated with to PEG of indicated molecular weight via a maleimide (mal) group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by student's t -tests comparing PEG₅₅₀-mal and PEG_{1k}-mal, correcting for multiple comparisons using the Holm-Sidak method. Each dose was analyzed individually without assuming a constant standard deviation. **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 IRF response stimulated by unconjugated pppRNA to that generated by pppRNA conjugated to PEG_{5k} overhangs via indicated linker chemistry. Statistical analysis compares PEG_{5k}-mal and PEG_{5k}-SS and is otherwise identical to that performed in Fig. 2a. **d)** Comparison of calculated EC₅₀ for 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.

Figure 3:
Concentration of hIFN-β1 secreted by A549-Dual cells treated with PEG_{5k}-pppRNA conjugates with indicated linker chemistry or control RNA (cRNA) at 20 nM RNA ** $p < 0.01$ **** $p < 0.0001$ one-way ANOVA with Tukey's test. Lipofectamine was used for 3pRNA transfection according to the manufacturer's instructions.

Scheme 1.

Illustration depicting the hypothesis that synthetic polymer overhangs can be used 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.

Scheme 2:
Synthesis of PEG-pppRNA conjugates used in this study.