ACS Medicinal Chemistry Letters

Letter

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Synthesis and Evaluation of Conjugates of Novel TLR7 Inert Ligands as Self-Adjuvanting Immunopotentiators

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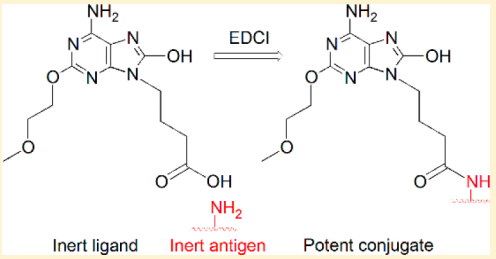
Supporting Information

ABSTRACT: During the design and synthesis of a series of 8-hydroxy-2-(2-methoxyethoxy)-adenine derivatives bearing various substituted −RCOOH groups at the 9-position, we identified a TLR7-inert ligand, which does not activate TLR7 signaling pathway. Of interest, the coupling of weakly immunogenic antigens via the −RCOOH group was able to significantly enhance the immunogenicity of the antigens. Herein, an inert ligand, 9-(3-carboxypropyl)-8-hydroxy-2-(2-methoxyethoxy)-adenine (**5**, GD2), was synthesized and conjugated to 5 different weakly immunogenic antigens (BSA, OVA, MSA, MG7, and thymosin). Compared with the GD2 and the potent agonist UC-1 V150, all conjugates demonstrated potent immunogenicity *in vitro* and *in vivo*. All conjugates induced prolonged increases, while UC-1 V150 showed a rapid decline in the levels of proinflammatory cytokines following initial increases. These data indicate that the immunostimulatory activity of TLR7-inert ligands could be amplified and prolonged by conjugation to antigens, thus broadening the potential therapeutic application of these agents.



KEYWORDS: *Toll-like receptor agonist, adenine derivative, immunostimulatory activity*

Early in the mid-1980s, a subset of synthetic derivatives and/or analogues of guanosine were found to activate the innate immune response. Structure−activity studies by many chemists revealed that compounds with purine,[1] 7-deazapurine,[2] and 9-deazapurine[3] ring systems were active ligands (Figure 1). Some purine analogues were reported to be potent Toll-like receptor 7 (TLR7) agonists, with the ability to induce the rapid production of cytokines. Among these synthetic analogues, 7-thia-8-oxoguanosine (TOG),[4] 7-allyl-8-oxoguanosine (loxoribine),[5] 9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine (SM-360320),[6] methyl 2-(3-{[6-amino-2-butoxy-8-oxo-7*H*-purin-9(8*H*)-yl]-methyl}phenyl)acetate (SM-324405),[7,8] and 4-[6-amino-8-hydroxy-2-(2-methoxyethoxy)purin-9-ylmethyl]benzaldehyde (UC-1 V150)[9,10] have been observed to be potent TLR7 agonists *in vitro* (Figure 1).[9−11] However, the *in vitro* immunotherapeutic activities of TLR7 ligands have been difficult to correlate with *in vivo* tests due to differences in pharmacokinetics or pharmacodynamics.[11−13] TLR7 are active when proteolytically processed and loaded into endosomes.[14−16] The conjugation of various chemical entities to a protein or peptide is known to facilitate endocytosis, enhance immunostimulatory activity, and reduce the risk of toxic cytokine syndrome.[17−22] Therefore, we hypothesized that the conjugation of a TLR7 inert ligand with various weakly immunogenic antigens could enhance innate immune system activation. Therefore, we aimed to synthesize a TLR7 inert ligand (or weak agonist) and to determine whether its conjugation to weakly immunogenic antigens could alter its pharmacodynamics. This strategy may broaden the potential therapeutic application of TLR7 ligands by not only reducing the risk of toxic cytokine syndrome but also increasing sustained release times.

In the present study, we designed and synthesized an inert ligand of TLR7 (Scheme 1), 9-(3-carboxypropyl)-8-hydroxy-2-(2-methoxyethoxy)-adenine (compound **5**, designated here as GD2). GD2 was synthesized in four steps from 2-chloroadenine (**1**). The structure of compounds **2** to **5** were determined by NMR and MS. The 3-carboxypropyl group at C9 enabled us to couple the compound with many different macromolecules using EDCI.[23] GD2 was covalently coupled to three protein and two peptide antigens. The coupling ratios of GD2 to BSA, OVA, MSA, MG7, and thymosin were about 6, 5, 5, 1, and 4, respectively (Supporting Information). Subsequently, we confirmed that all of the conjugates stimulated mouse BMDM exclusively via TLR7 activation (Figure 2).

The potency of all of the conjugates was evaluated using a mouse splenocyte cytokine production assay *in vitro* (Figure 3).

Received: September 7, 2014
Accepted: January 30, 2015
Published: January 30, 2015

DOI: 10.1021/ml5003647
ACS Med. Chem. Lett. 2015, 6, 249−253

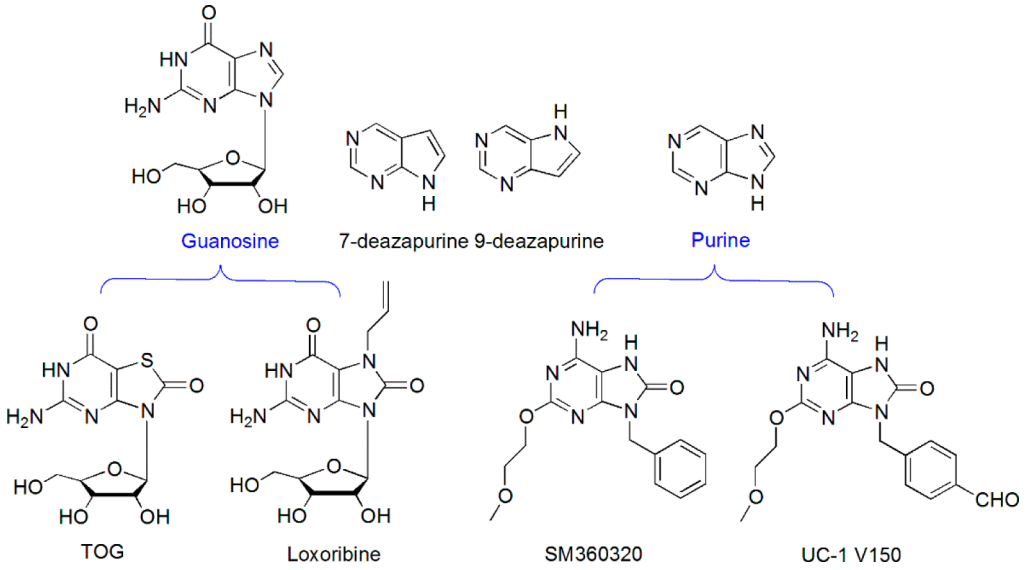
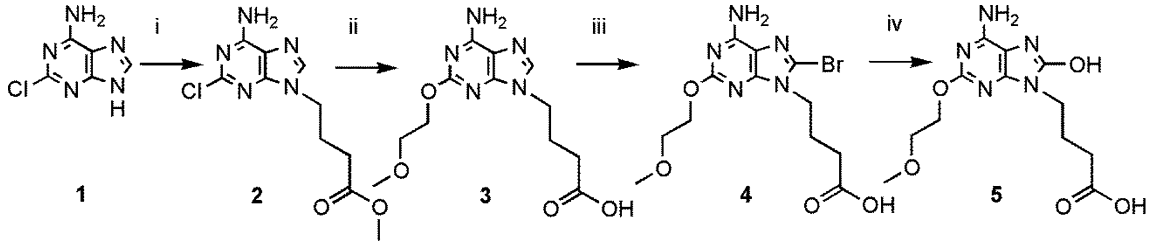


Figure 1. Ring systems and molecular structures of the purine class of key agonists.

Scheme 1. Synthesis of Compound 5[a]



[a]Reagents and conditions: (i) methyl 4-bromobutyrate, K_2CO_3/dimethylformamide/KI, 30 °C; (ii) $CH_3OCH_2CH_2CH_2ONa$/$CH_3OCH_2CH_2CH_2OH$, reflux; (iii) Br_2/CH_2Cl_2, 30 °C; (iv) MeOH/10 M NaOH, 1:1, reflux.

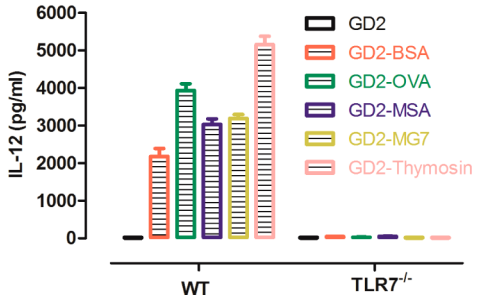


Figure 2. Confirmation of conjugate-mediated stimulation via TLR7 activation. BMDM (1×10^6/mL) derived from wild-type or TLR7$^{-/-}$ mice was incubated with 10 μM GD2 or 2 μM conjugates for 24 h. The levels of IL-12 in the culture supernatants were measured by ELISA. The data are shown as the mean ± SEM of triplicates and are representative of three independent experiments.

The cells were incubated with serially diluted conjugates for 24 h, and the levels of cytokines released in the media were subsequently determined by ELISA. The results indicated that UC-1 V150 was more potent as a cytokine inducer, compared with GD2 (524-fold for IFN-γ and 61-fold for IL-12), which indicated that GD2 is a TLR7-inert ligand (Figure 3A,B). The conjugates were more potent cytokine inducers, compared with unconjugated GD2 ($P < 0.0001$). The cytokine IFN-γ production levels were 488-, 73-, 575-, 550-, and 1511-fold higher for the BSA, OVA, MSA, MG7, and thymosin conjugates, respectively, compared to GD2 and as normalized to the 10 μM level of each treatment (Figure 3A). The IL-12 production levels for these molecules were 16-, 175-, 42-, 52-, and 409-fold higher than that of GD2, respectively (Figure 3B). The conjugates were equally potent as or within one-digit-fold potency of free UC-1 V150. Equivalent concentrations of free antigens induced minimal or undetectable cytokine levels (Figure 3A,B).

To determine the kinetics of proinflammatory cytokines induced by the different conjugates and free drugs, C57BL/6 mice were injected intravenously with the agents, and then the cytokine levels in the serum were determined at various time intervals (Figure 4A,B) and compared with that induced by the control group. The results indicated that the maximum induction of IFN-γ and IL-12 was observed 2 h after injection for all of the drugs. The cytokine levels induced by the conjugates were sustained for up to 6 h. In contrast, the cytokine levels induced by UC-1 V150 declined rapidly after 2 h. Any induction of cytokine levels by free GD2 was undetectable. Sera from control mice that received saline or free antigens revealed extremely low or undetectable cytokine levels.

To determine the kinetics of adaptive immune responses, mice were immunized with GD2, BSA, OVA, GD2-BSA, or GD2-OVA, and the levels of antigen-specific IgG in the serum were assayed at various time intervals (Figure 5A,B). The induction of IgG was observed after 14 days in mice immunized with GD2-BSA or GD2-OVA. The levels of antigen-specific IgG continuously increased during the 30-day testing interval.

DOI: 10.1021/ml5003647
ACS Med. Chem. Lett. 2015, 6, 249−253

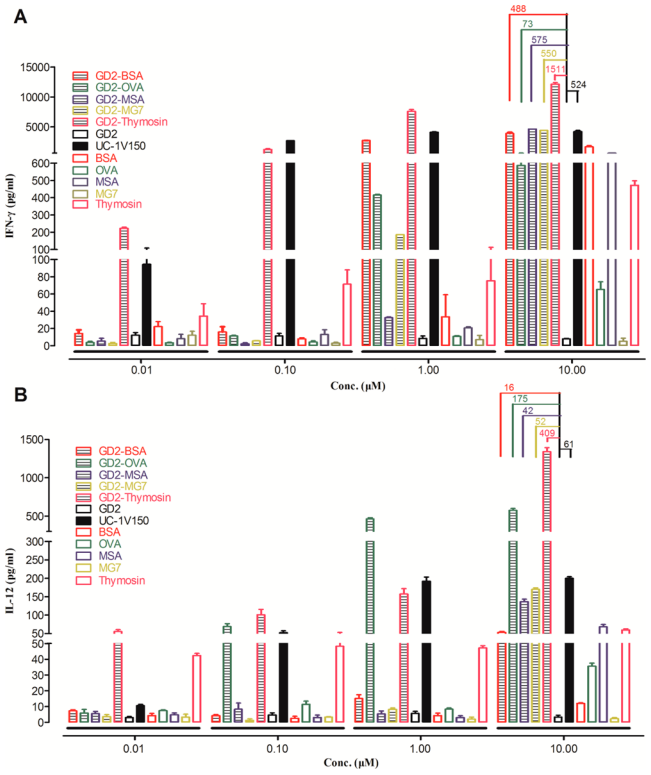


Figure 3. *In vitro* cytokine release in response to free drugs, antigens, or conjugates. One × 10^6/mL mouse splenocyte cells were incubated with serially diluted free GD2 or UC-1 V150 as indicated at the *x*-axis, respectively. Considering that the conjugation ratios are different in each antigen, the concentrations of BSA, OVA, MSA, MG7, thymosin, and their conjugates were used with one-sixth, -fifth, -fifth, equal, or a quarter of the number as indicated at the *x*-axis, respectively. The levels of IFN-γ (A) and IL-12 (B) in the culture supernatants were measured by ELISA. The data are shown as the mean ± SEM of triplicates and are representative of three independent experiments. The colored numbers indicate the cytokine concentration ratios for conjugated vs free GD2.

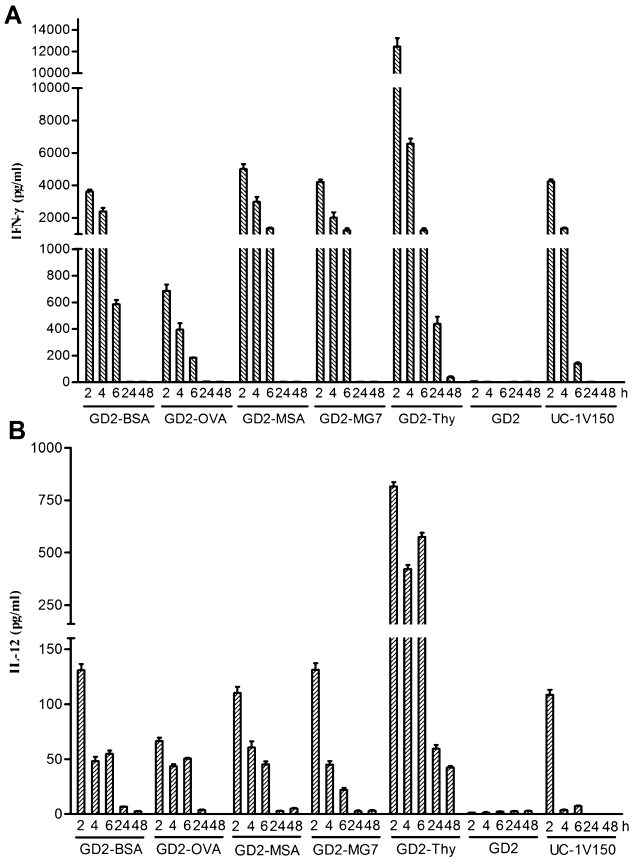


Figure 4. Kinetics of proinflammatory cytokine induction by the different conjugates and free drugs *in vivo*. C57BL/6 mice (n = 5 per group) were intravenously injected with 0.18 μmol of UC-1 V150, GD2, or conjugates. Serum samples were collected at the indicated times after injection. The cytokine levels were measured by Luminex assay. The data are shown as the mean ± SEM of two independent experiments.

Meanwhile, sera from control mice that received PBS, BSA, OVA, or free GD2 revealed extremely low or undetectable levels of IgG.

To avoid the effects of endotoxin contamination, only conjugates or drugs with an endotoxin concentration of less than 5 EU/mg were used throughout the experiments. We evaluated potential endotoxin contamination using BMDM from TLR4$^{-/-}$ and wild-type mice. The levels of cytokine production were similar between TLR4$^{-/-}$ and wild-type samples, indicating that endotoxin contamination was negligible (Figure S1, Supporting Information).

One potential problem that has been observed in agonist drug development is the potential induction of the toxic cytokine syndrome by agonists.[11,18,24] Some strategies to overcome this challenge include the conjugation of the ligand drug to a macromolecule[17−22] or link itself to multimeric,[20,25] thus facilitating the possibility of drastically reducing the systemic exposure to the adjuvant while maintaining relatively high local concentrations at the site of vaccination.[20,22,26] We synthesized an inert TLR7 ligand and revealed its self-adjuvanting immunoregulatory activity when bound to model protein and peptide antigens. GD2 is an inert and versatile synthetic small-molecule TLR7 ligand. The compound could be coupled to a variety of macromolecules for the enhancement of activity and

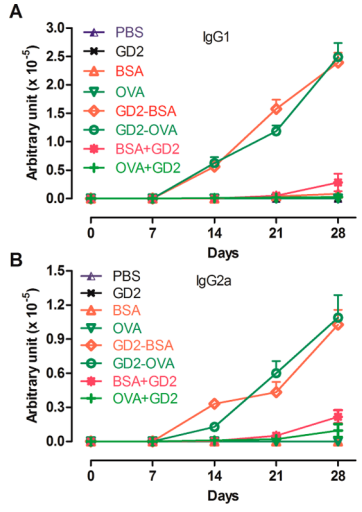


Figure 5. Kinetics of IgG induction by GD2-BSA or GD2-OVA *in vivo*. C57BL/6 mice (n = 6 per group) were immunized subcutaneously at the base of the tail on days 0 and 7 with 5 nmol of GD2, 0.83 nmol of BSA, 0.83 nmol of GD2-BSA, (0.83 nmol of BSA + 5 nmol of GD2), 1 nmol of OVA, 1 nmol of GD2-OVA, or (1 nmol of OVA + 5 nmol of GD2) per mouse. Sera samples were collected at 7-day intervals from days 0 to 30. BSA- or OVA-specific IgG1 (A) and IgG2a (B) were measured by ELISA. The data are shown as mean ± SEM of triplicate in the representative data of three independent experiments.

DOI: 10.1021/ml5003647
ACS Med. Chem. Lett. 2015, 6, 249−253

a longer duration of action, compared with the free monomeric drug. This delicate design would effectively reduce the cytokine syndrome caused by agonists *in vivo* and broaden the potential therapeutic application of these agents.[10]

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental procedures and supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by grants from the Basic Research Program of Shenzhen (Grant No. JCYJ20130326110139687, 20120613113228732, and JC201005250060A), Shenzhen Development and Reform Commission (Grant No. 2012553 and 2012886), China Postdoctoral Science Foundation (Grant No. 2014 M552234), and National Natural Science Foundation of China (Grant No. 81273374 and 81202396).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The author thank Wang Li, Xiaoli Zhang, Jiaxing Song and Bo Li for technical assistance.

■ ABBREVIATIONS

BMDM, bone marrow derived macrophages; BSA, bovine serum albumin; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO-d_6, dimethyl sulfoxide-d_6; EDCI, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride; ELISA, enzyme linked immunosorbent assay; ESI-TOF, electrospray ionization-time-of-flight; IFN, interferon; IgG, Immunoglobulin G; LAL, Limulus Amoebocyte Lysate; IL-12, Interleukin 12; MG7, monoclonal gastric cancer7 antigen; MS, mass spectrometry; MSA, mouse serum albumin; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, phosphate buffered saline; TLR, Toll-like receptor

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