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Synthesis and immunoregulatory activities of conjugates of a Toll-like receptor 7 inert ligand

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ABSTRACT

In the synthesis and modification of the analogs of an adenine type of Toll-like receptor (TLR) 7 agonists, we found a special compound, 9-propionyloxy-8-hydroxy-2-(2-methoxyethoxy)-adenine (**6**). It is a synthesized TLR7 inert ligand, which does not respond to TLR7 itself. However, it can be coupled with protein or peptide antigens via propionyloxy functional group to promote their immunogenicity significantly. The compound was covalently coupled to protein and peptide to get the conjugates. The inductivity of cytokine production by the conjugates was 872.4-fold compared with the unconjugated antigens in vitro by mouse splenocyte. These data show that the immunostimulatory activity of inert TLR7 ligand can be endowed, and the activity of antigens can be amplified by conjugation with various proteins and peptides, thus broadening the potential therapeutic application and reducing the risk of TLR7 agonists' side effects.

The Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR) that recognize highly conserved components of diverse pathogens and induce host innate and adaptive immune responses.[1–3] These specific molecular patterns are broadly shared by pathogen molecules but are structurally distinct from host molecules,[4,5] such as cell wall structures (recognized by TLR1, -2, -4, -5, and -6 that respond to extracellular stimuli) and nucleic acids (recognized by TLR3, -7, -8, and -9 that respond to intracellular stimuli).[6,7] Many cancer researchers show their interest in the profound antitumoral activity of the agonists[8] because the small-molecule agonists could activate certain TLR pathways. The natural ligand for TLR7 was identified as G and U-rich ssRNA.[9] In addition, several synthesized low molecular weight agonists of TLR7 were found, including imidazoquinolines,[9] and purine-like molecules.[10–12]

Synthetic small molecules, such as 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320)[13] and 4-[6-amino-8-hydroxy-2-(2-methoxyethoxy)purin-9-ylm-ethyl]benzaldehyde (UC-1V150) have been shown to be potent and specific TLR7 agonists (Fig. 1).[8,14] The certain derivatives of adenine could activate immune cells via TLR7 by triggering antigen-presenting cell maturation program.[15] Because the systemic TLR activation could induce a rapid and potentially toxic cytokine syndrome, the immunotherapeutic activities of these agonists in vitro were difficult to translate to in vivo settings.[8,16,17] Accordingly, the major applications of TLR7 ligands in vivo were agents or immune adjuvants injected in small quantities.[8,9,18] A potential effective strategy is to conjugate the ligand to a macromolecule,[8,14,19,20] or linking itself to multimeric,[21,22] offering the possibility of drastically reducing systemic exposure of the adjuvant, yet maintaining relatively high local concentrations at the site of vaccination.[20,23,24]

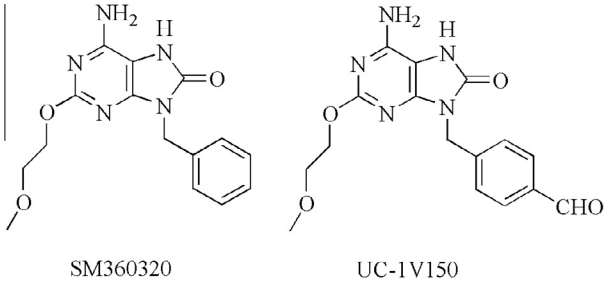

Figure 1. The molecular structures of purine class of potent TLR7 agonists.

As the majority of purine-like potent agonists were mostly dependent on 9-benzyl derivatives,[8,10–14] the effect of 9-benzyl

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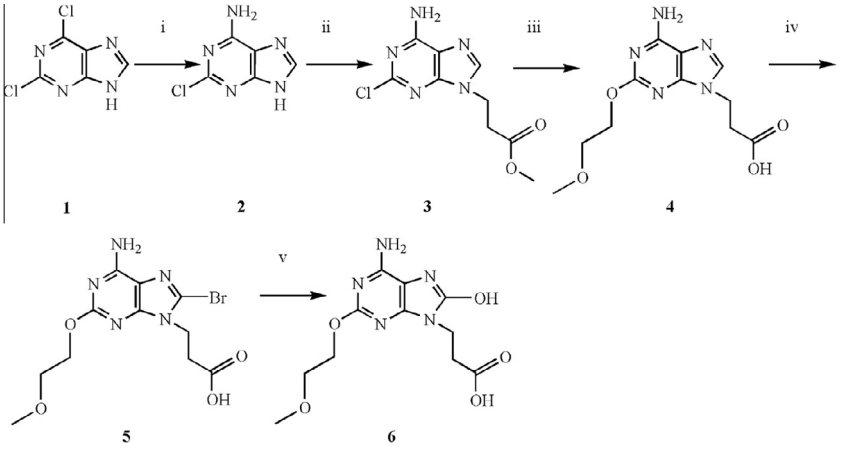
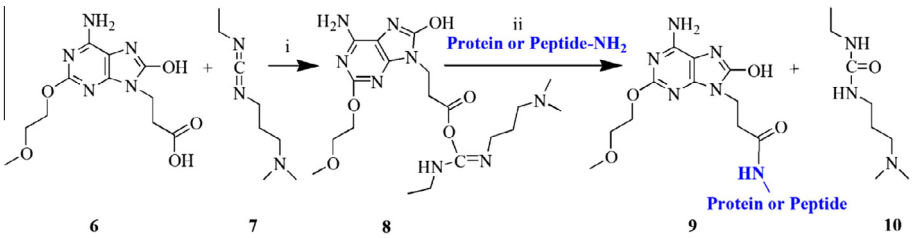


Scheme 1. General synthetic route for compound **6**. Reagents and conditions: (i) NH_3–MeOH, 80 °C; (ii) methyl 3-bromopropionate, K_2CO_3/DMF/KI, 25 °C; (iii) $CH_3OCH_2CH_2CH_2ONa/CH_3OCH_2CH_2CH_2OH$, 80 °C, reflux; (iv) Br_2/CH_2Cl_2, 25 °C; (v) CH_3ONa/CH_3OH/80 °C, reflux; HCl, 25 °C.



Scheme 2. Conjugation of compound **6** to protein or peptide. Reagents and conditions: (i) 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI), NHS, DMSO, 25 °C; (ii) DMSO or PBS, 4 °C.

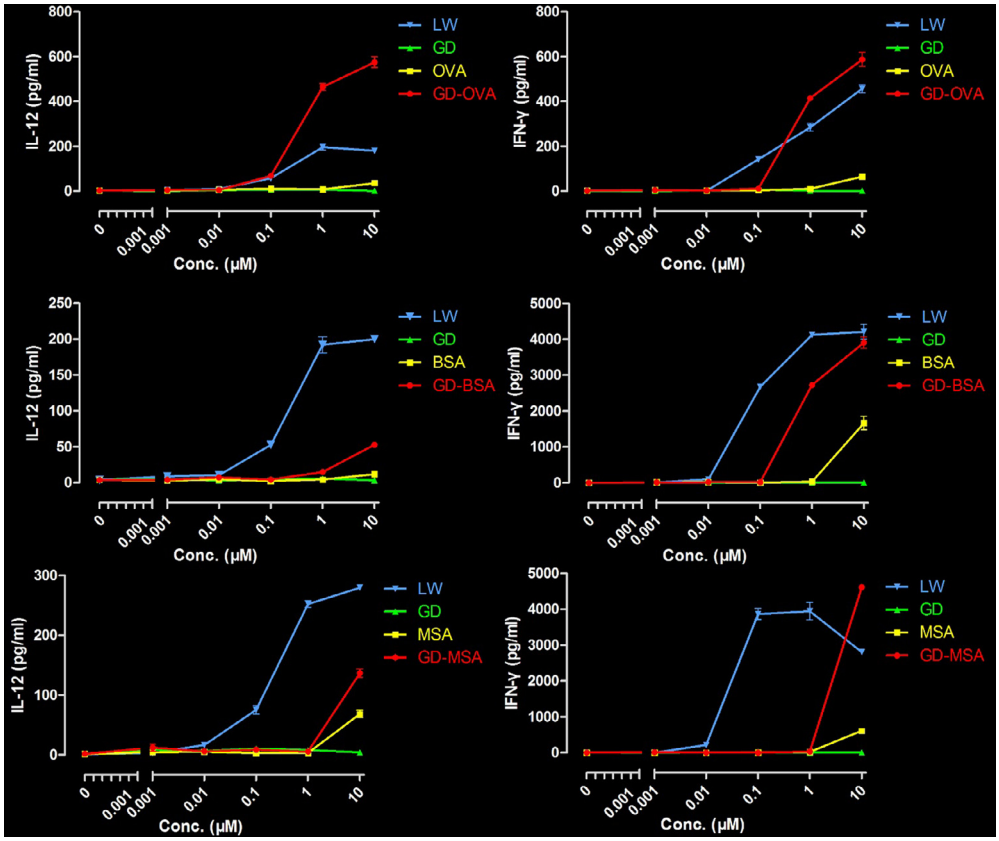


Figure 2. In vitro cytokine release enhancement in response to protein conjugates. Mouse splenocyte were treated with GD or GD-OVA, -BSA, -MSA conjugates at various concentrations as indicated. Culture supernatants were harvested 24 h later, and cytokine levels were measured by ELISA. LW is UC-1V150[8] as a control. Data are means ± SEM and a representative experiment of three independent experiments in triplicate per treatment is shown.

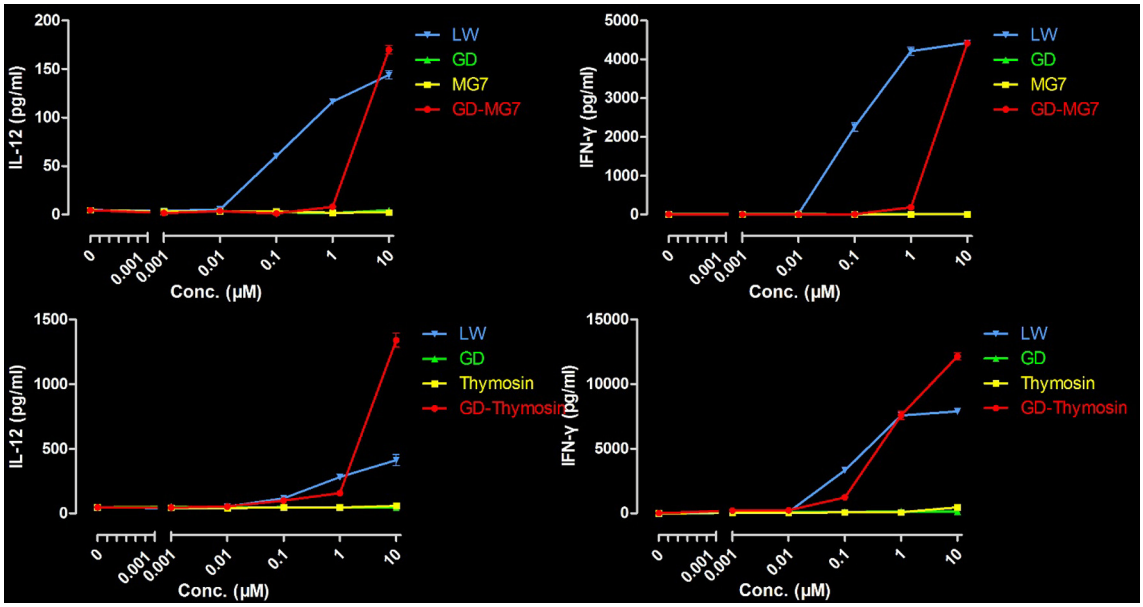


Figure 3. In vitro cytokine release enhancement in response to peptide conjugates, as described of Figure 2.

Table 1
Cytokine ratio in vitro treated with conjugated versus unconjugated antigens[a]

Drug (μM)	GD-OVA		GD-BSA		GD-MSA		GD-MG7		GD-thymosin	
	IL-12	IFN-γ	IL-12	IFN-γ	IL-12	IFN-γ	IL-12	IFN-γ	IL-12	IFN-γ
0.001	1.50	1.00	1.31	0.85	2.76	2.40	0.50	0.07	1.01	0.79
0.01	0.79	1.11	1.72	0.64	1.16	0.67	1.08	0.21	1.30	0.55
0.1	6.08	2.64	1.64	2.00	2.75	0.17	0.38	1.83	1.72	9.46
1	44.34	31.40	3.56	89.37	1.75	1.57	4.00	36.70	3.33	91.80
10	14.47	9.00	4.43	2.35	2.00	7.51	74.44	872.40	20.28	5.70

[a] 1×10^6/mL cells were incubated with serially diluted TLR7 conjugates or un-conjugates for 24 h. The levels of IL-12 and IFN-γ in the culture supernatants were measured by ELISA. Data are ratio of cytokine production in vitro mouse splenocyte treated with conjugated versus unconjugated antigens.

on adenine needs to be examined. We designed Scheme 1 to synthesize 9-propionyloxy-8-hydroxy-2-(2-methoxyethoxy)-adenine (**6**), an inert ligand of TLR7. Compound **6** was synthesized in five steps from 2,6-dichloropurine (Scheme 1), amount to C replace phenyl of substituent group at the C9 of UC-1V150. The structure of **2**–**6** were determined by NMR and MS.

The 9-propionyloxy of compound **6** enabled us to couple it with many different macromolecules, including proteins and peptides through EDCI.[25] It was covalently coupled to ovalbumin (OVA), bovine serum albumin (BSA), mouse serum albumin (MSA), monoclonal gastric cancer7 antigen (MG7) and Thymosin (Scheme 2). The coupling ratios of ligand to OVA, BSA, MSA, MG7 and Thymosin were 5, 6, 5, 1, and 4, respectively (detailed results were reported in Supplementary data).

All conjugates were then tested employing in vitro mouse splenocyte. Incubation of the mouse splenocyte with conjugates alone stimulated cytokine release. The free compound **6** did not stimulate cytokine release, and lost TLR7-agonistic properties (Figs. 2 and 3). After conjugated to macromolecules, such as proteins (OVA, BSA and MSA) (Fig. 2) and peptides (MG7 and Thymosin) (Fig. 3), comparison of the cytokine production profiles (IL-12 and IFN-γ) between original antigens vs. conjugates at various dosages (0.001–10 μM) demonstrated that the conjugates enhanced the potency 872.4 times most (Table 1). We also tested the effect of different substitution level on the immunostimulatory response. There was no significant difference statistically in production of IL-12 among 3 different coupling ratios (detailed results were reported in Supplementary data).

We sequentially prolongated the C9-carboxylic acid chain length and gained the C9-(4-carboxybutyl) and C9-(5-carboxyamyl) derivatives. The two derivatives have high activity self, but their conjugates have low activity compared with compound **6** in stimulating cytokines production (data not shown).

Overall, we synthesized inert purine-like ligands through minor modifications of the 9H-Purine scaffold resulting in compound **6**, amounted $-(CH_2)_3-$ tether to replace the phenyl group of UC-1V150. This compound did not stimulate cytokine release in vitro, which means it has no agonistic effect. However its properties were totally reconverted once it was conjugated to proteins or peptides. To be more specifically, the immunostimulatory activity of such inactive TLR7 ligand can be endowed, and the activity of low immunogenicity antigens can be amplified through such conjugations. This coupling method can effectively avoid hypersensitivity reactions caused by agonist in vivo. We hypothesized the mechanism of this phenomenon lies in that the dendritic cells and macrophages in the splenetic cultures proteolyzing the conjugates, and leaving behind the pharmacore attached to a few epitope peptides. One of the metabolites may have potent TLR7 activity compared to the starting material. By analogy, antibody-drug conjugates are degraded in target cells, leaving behind a tubulin inhibitor attached to a small peptide, that mediate effects. The novelty of our results is that we found a type of prodrug that is inert until activated within the TLR7 expressing target cells. Our results will hopefully broaden the potential therapeutic application of TLR7 ligands by reducing the risk of unexpected side effects and increasing sustained release time of adjuvant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.034. These data include MOL files and InChiKeys of the most important compounds described in this article.

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