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High potency of lipid conjugated TLR7 agonist requires nanoparticulate or liposomal formulation

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ABSTRACT

Conjugation of small molecule agonists of Toll-like receptor 7 (TLR7) to proteins, lipids, or polymers is known to modulate potency, and the physical form or formulation of these conjugates is likely to have a major effect on their immunostimulatory activity. Here, we studied the effect of formulation on potency of a 1,2-di-(9Z-octadecenoyl)-*sn-glycero*-3-phosphoethanolamine (DOPE) conjugated TLR7 agonist (DOPE-TLR7a) alongside assessing physical form using Dynamic Light Scattering (DLS), Nanosight Particle Tracking (NTA) analysis and Small Angle X-ray Scattering (SAXS). A very high potency of DOPE-TLR7a conjugate (EC_{50} around 9 nM) was observed either when prepared by direct dilution from DMSO or when formulated into 400–700 nm large multilamella liposomes containing dimethyldioctadecylammonium bromide salt (DDA) and DOPE. When prepared by dissolution in DMSO followed by dilution in aqueous culture medium, 93 ± 5 nm nanoparticles were formed. Without dilution from solution in DMSO, no nanoparticles were observed and no immunostimulatory activity could be detected without this formulation step. SAXS analysis of the conjugate after DMSO dissolution/water dilution revealed a lamellar order with a layer spacing of 68.7 Å, which correlates with arrangement in groups of 3 bilayers. The addition of another immunostimulatory glycolipid, trehalose-6,6-di-behenate (TDB), to DOPE:DDA liposomes gave no further increase in immunostimulatory activity beyond that provided by incorporating DOPE-TLR7a. Given the importance of nanoparticle or liposomal formulation for activity, we conclude that the major mechanism for increased potency when TLR7 agonists are conjugated to macromolecules is through alteration of physical form.

1. Introduction

Recently the molecular pathways of immune activation both by pathogen associated molecular patterns (PAMP) recognition *via* pattern recognition receptors (PRR) such as TLR (Medzhitov and Janeway, 1998; Medzhitov et al., 1997; Takeda and Akira, 2004) and also by particulate adjuvants for example by nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3)/inflammasome mediated pathways (Eisenbarth et al., 2008) have been elucidated. These advances include insight into the immunomodulatory properties of alum, which was thought to rely on a deposition effect to enhance the persistence of presented antigens to antigen presenting cells (APC) (Verdier et al., 2005). However this paradigm has been challenged by studying the rapid release of ^{14}C labelled antigens administered with alum (Gupta et al., 1996) and the excision of the injection site shortly after administration does not significantly impact on the immune response to the antigen (Lindblad, 2004). The increased immunogenicity of alum adsorbed antigen may therefore lie in the efficiency of APC to phagocytise particulate antigens compared to the macropinocytosis of antigen alone despite the lack of PRR agonist (Morefield et al., 2005). The physical form of the antigen is therefore clearly of equal importance to associated immunostimulatory activity through PRR. Recent attempts at rational vaccine design therefore manipulate both the innate molecular recognition pathways and the physical form of the antigen. For example, when a small molecule TLR7/8 agonist was conjugated to a temperature responsive polymer support to form nanoparticles, the most significant increase in immunogenicity was seen from the formation of particles as opposed to

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modulation of TLR7/8 agonist loading (Lynn et al., 2015).

This detailed molecular and cellular insight confirms multiple previous observations highlighting the importance of formulation and physicochemical form of vaccine adjuvants for maximising immunostimulatory activity. For example, an increase in potency following liposomal formulation on muramyl dipeptide was observed as far back as 1984 (Sone et al., 1984) the immunostimulatory activity of which has been more recently attributed to nucleotide-binding oligomerization domain-like receptors (NOD) activation. Likewise, the *in vivo* adjuvant activity of TLR2 stimulatory lipopeptides was found to be strongly dependent on physical form, with particulate components primarily responsible for vaccine activity *in vivo* (Tsunoda et al., 1999). The physical form and self-assembly properties of TLR2 stimulatory lipopeptides have recently been studied in much greater detail, revealing that aggregation into micellular or fibrillar structures, has a profound effect on biological activity (Castelletto et al., 2016; Hamley et al., 2014).

The most intensely studied synthetic immunostimulatory small molecule PRR agonists target the sensors of viral RNA TLR7 and TLR8 (Heil et al., 2003; Hemmi et al., 2002; Takeda and Akira, 2004). Indeed, the imidazoquinoline Imiquimod was clinically approved in 1997, 5 years before its ability to trigger TLR7 was identified (Hemmi et al., 2002). Many studies have reported the relative potency of different families of small molecule agonists for TLR7 and TLR8, and while different cellular assays and endpoints are used, it is still informative to compare their potency which varies widely. To date, the most potent reported were in a library of 2-substituted 8-hydroxyadenines some of which had minimum effective concentrations (MEC) as low as 1–10 nM (Hirota et al., 2002). The majority of compounds in contrast have reported activity in the 0.1–10 μM range including imidazoquinolines with a ED_{50} or MEC in μM level (Kurimoto et al., 2004). Several structure-activity relationship (SAR) studies have focused on identifying a suitable location for conjugation to protein antigens or macromolecules without reducing potency following conjugation (Chan et al., 2009; Wille-Reece et al., 2005a; Wu et al., 2007a). Interestingly, one of the most studied examples, UC-1V150, is by no means the most potent TLR7 molecule in its unconjugated form, with EC_{50} of around 500 nM (Wu et al., 2007a). This contrasts with structurally similar 8-oxoadenine derivatives reported with EC50 as low as 5 nM (Czarniecki, 2008) and rationally designed compounds based on pharmacophore molecular docking studies as low as 0.5 nM have been reported (Yu et al., 2013).

The potency of UC-1V150 is significantly increased following conjugation to proteins such as serum albumin (Wu et al., 2007b) even taking into account that the conjugates typically have a drug substitution ratio of up to 5:1 meaning that stated molar concentrations underestimate the total quantity of TLR7-agonist small molecule drug by up to 5-fold. Similarly conjugation of UC1V150 to the anti-CD20 antibody rituximab demonstrates a similar increase in potency compared to the free unconjugated TLR7 agonist (Gadd et al., 2015). Imidazoquinoline compounds such as 3M-012 conjugated to HIV Gag P41 protein show a similar increase in potency over the unconjugated and the protein mixed with TLR7/8 agonist (Wille-Reece et al., 2005b). As well as protein/peptide conjugation of TLR agonists, other published examples of macromolecular conjugates include polymers such as PEG (Chan et al., 2011), phospholipids (Chan et al., 2009) and glycoconjugates (Donadei et al., 2016; Shinchi et al., 2015). These examples typically note an increase in potency compared to the unconjugated agonist or agonist mixed with the antigen/scaffold. Other TLR agonists have likewise been conjugated to macromolecules with modulation of activity (Yu et al., 2017). Furthermore, conjugation alters *in vivo* responses including altered duration and location of cytokine induction, and when humoral response was studied modification of the antibody titre and ratio of immunoglobulin G (IgG) subtypes (Donadei et al., 2016; Shinchi et al., 2015; Smirnov et al., 2011). Further benefits of conjugation were also observed beyond simply increasing potency; for example conjugated or unconjugated TLR7 agonist were both able to induce dendritic cell (DC) maturation, but cross presentation was increased when TLR7 agonist was conjugated directly to antigen (Oh et al., 2011; Oh and Kedl, 2010).

A lipid modified member of the imidazoquinoline family TLR7 agonist was shown to be a potent vaccine adjuvant *in vivo*, but the *in vitro* immunostimulatory activity of this compound was not reported (Smirnov et al., 2011). However, the *in vitro* activity of the unconjugated agonist was reported in the low μM range similar for the structurally related R848 compound. In contrast to the highly potent UC-1V150 conjugated to the phospholipid DOPE that had EC_{50} of approximately 50 nM *in vitro* when analysing IL12p40 concentrations, compared to the unconjugated agonist of aproximatly 1 μM (Chan et al., 2009). Separately, immunostimulatory liposomal formulations have been studied extensively for vaccine formulation as an antigen delivery and/or depot system. While the majority of liposome-forming lipids lack intrinsic immunostimulatory activity, addition of the glycolipid trehalose-6,6-dibehenate (TDB) provides direct immunostimulatory activity to liposomes by triggering the PRR minkle *via* Syk/CARD9 (Matsunaga and Moody, 2009; Vangala et al., 2006; Yamasaki et al., 2008).

Here, we studied the influence of the physical form of lipid conjugated TLR7 agonist on immunostimulatory potency. We specifically focussed on phospholipid-conjugated forms of 2-substituted 8-hydroxyadenines which are potent TLR7 agonists (Chan et al., 2009). The physical form of this conjugate and its influence on biological activity has not previously been reported, and we therefore describe here how either nanoparticles formed by dilution from solvent, or a liposomal formulation, are required for this conjugate to be active *in vitro* and provide further physical characterisation by SAXS.

2. Material and methods

2.1. Materials

RPMI 1640 supplemented with Glutamax & HEPES & Phenol Red, performance plus heat inactivated foetal bovine serum, phosphate buffered saline and 2-mercaptoethanol were from Life Technologies (Paisley, UK). ExtrAvidin alkaline phosphatase conjugate, Sigmafast pNPP, PBS tablets, sodium azide and EDTA (0.5 M) were from Sigma Aldrich (Gillingham, UK). The IL-12p40 capture antibody C 15.6 and biotinylated IL-12p40/70 detection antibody C17.8 were from eBioscience (Hatfield, UK). Nunc® Maxisorb 96 well ELISA plates were from Thermo Fisher scientific (Paisley, UK). Reagents for chemical synthesis were purchased from Sigma Aldrich (Gliiingham, UK) or Fluorochem (Hadfield, UK) and used without further purification.

2.2. Synthesis

TLR7 agonist with carboxyl site on the para-benzyl group for conjugation (**7**, Fig. 1) and DOPE-TLR7 agonist conjugate (**8**, Fig. 1) were synthesised according to previous methods (Wu et al., 2007b) and obtained in good yields as white crystalline powders. A similar TLR7 agonist without the carboxyl conjugation site on the para-benzyl group (**9**, Fig. 1) was also synthesised for activity comparison. Full synthetic methodology and comprehensive characterisation data is provided in Supplementary information, including a crystal structure for **9** (Supplementary information).

4-{[6-Amino-2-(2-methoxyethoxy)-8-oxo-7*H*-purin-9(8*H*)-yl]methyl}benzoic Acid (**7**, Fig. 1)

^{1}H NMR (DMSO-d_6) δ 12.94 (1H, s, ArC(O)OH), 10.03 (1H, s, NHC(O)N), 7.89 (2H, d J = 8.2, 2x *o*-ArH), 7.39 (2H, d, J = 8.2, 2x *m*-ArH), 6.51 (2H, s, NH_2), 4.94 (2H, s, $ArCH_2N$), 4.25 (2H, t, J = 4.2, $OCH_2CH_2OCH_3$), 3.57 (2H, t, J = 4.2, $OCH_2CH_2OCH_3$), 3.26 (3H, s, $OCH_2CH_2OCH_3$). ^{13}C NMR (DMSO-d_6) δ167.00, 159.83, 152.17, 149.08, 147.75, 141.98, 129.83, 129.55, 127.39, 98.34, 70.15, 65.26,

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