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Dexamethasone prodrugs as potent suppressors of the immunostimulatory effects of lipid nanoparticle formulations of nucleic acids

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ABSTRACT

Lipid nanoparticles (LNPs) are playing a leading role in enabling clinical applications of gene therapies based on DNA or RNA polymers. One factor impeding clinical acceptance of LNP therapeutics is that LNP formulations of nucleic acid polymers can be immunostimulatory, necessitating co-administration of potent corticosteroid immunosuppressive agents. Here, we describe the development of hydrophobic prodrugs of a potent corticosteroid, dexamethasone, that can be readily incorporated into LNP systems. We show that the presence of the dexamethasone prodrug LD003 effectively suppresses production of cytokines such as KC-GRO, TNFα, IL-1β and IL-6 following intravenous administration of LNP loaded with immune stimulatory oligodeoxynucleotides containing cytosine-guanine dinucleotide motifs. Remarkably, LD003 dose levels corresponding to 0.5 mg/kg dexamethasone achieve a greater immunosuppressive effect than doses of 20 mg/kg of free dexamethasone. Similar immunosuppressive effects are observed for subcutaneously administered LNP-siRNA. Further, the incorporation of low levels of LD003 in LNP containing unmodified mRNA or plasmid DNA significantly reduced pro-inflammatory cytokine levels following intravenous administration. Our results suggest that incorporation of hydrophobic prodrugs such as LD003 into LNP systems could provide a convenient method for avoiding the immunostimulatory consequences of systemic administration of genetic drug formulations.

1. Introduction

Nucleic acid-based therapeutics such as antisense oligodeoxynucleotides (ASO) or short-interfering RNA (siRNA) for gene silencing, and messenger RNA (mRNA) or plasmid DNA (pDNA) for gene expression can be potent inducers of the innate immune response in vertebrates [1, 2]. Therapeutic use of nucleic acid-based macromolecules often requires sophisticated delivery vehicles, which can further exacerbate this response [3–5]. Lipid nanoparticles (LNP) that contain ionizable amino-lipids are the most clinically advanced delivery system for nucleic acid therapeutics [6]. LNP systems can give rise to “flu-like” symptoms and hypotension from the activation of toll-like receptors and increases in serum cytokine levels even when they contain a payload (e.g. siRNA) that has been engineered to minimize immunostimulatory potential [7–9]. Prophylactic administration of corticosteroids reduces LNP-siRNA mediated immune stimulation [8], but breakthroughs can occur for a variety of nanoparticle formulations [10, 11]. Furthermore, while siRNA can be designed and synthesized *in silico* with modifications that reduce immune stimulation, larger nucleic acids such as mRNA or pDNA are more difficult to modify chemically. Immune responses can also result in the rapid clearance of subsequent administrations of LNP [12–17], greatly limiting the utility of such formulations. Thus, the potential immunostimulatory properties of LNP and other formulations of genetic drugs are a major challenge for clinical advancement of gene therapies in general.

Dexamethasone is a potent synthetic corticosteroid used for the treatment of a number of inflammatory and autoimmune conditions such as Crohn's disease, asthma, ulcerative colitis, rheumatoid arthritis and immune thrombocytopenia. It has also been used for certain hematological malignancies [18, 19], and prophylactically to reduce inflammatory responses to therapeutic treatments such as antibiotics and some chemotherapeutics [20, 21]. It has been shown previously that co-administration of dexamethasone with LNP greatly reduces immune stimulation [8, 22]. Here, we demonstrate that the incorporation of a dexamethasone prodrug directly into LNP containing various types of nucleic acid cargos can greatly reduce the level of pro-inflammatory

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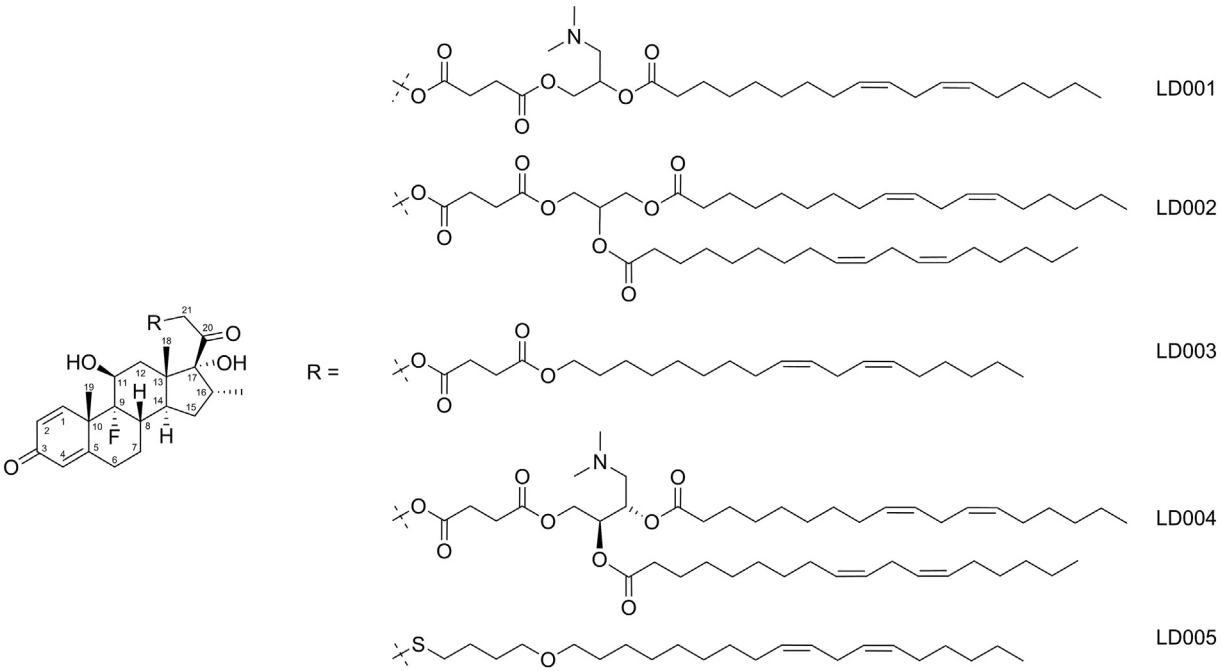

Fig. 1. Structures of lipophilic dexamethasone prodrugs. Dexamethasone prodrugs of varying hydrophobicity were synthesized. LD001-LD004 contain a succinate linker between dexamethasone and either one or two C18 hydrocarbon moieties. LD001 and LD004 also contain a tertiary amine group. LD005 is composed of dexamethasone conjugated with a single C18 hydrocarbon chain via an ether linkage.

Table 1
Prodrug and lipid nanoparticle parameters.

Prodrug	Predicted LogP (or LogD)[a]	Particle diameter (nm)	PdI	% CpG ASO entrapment	% Prodrug entrapment
LD001	5.1/7.6	46	0.08	100%	~ 40
LD002	15.0	46	0.09	100%	> 95
LD003	8.9	49	0.06	99%	> 95
LD004	11.6/14.0	48	0.04	98%	~ 60
LD005	10.4	50	0.06	99%	> 95

[a] LogD predicted at two pH values (pH 4/pH 7.4).

cytokines induced by the LNP. In order to incorporate dexamethasone directly into the particle, lipophilic acyl/alkyl moieties were chemically conjugated to dexamethasone via biodegradable linkers. Various dexamethasone prodrugs were synthesized and tested to better understand the requirements for effective incorporation within the LNP. Finally, we show these prodrugs ameliorate pro-inflammatory cytokine induction using LNP that contain ASO, siRNA, mRNA or plasmid DNA.

2. Material and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). Amino-lipids 3-(dimethylamino) propyl(12Z,15Z)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl]henicosa-12,15-dienoate (DMAP-BLP), (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate (DLin-MC3-DMA) and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) were synthesized by BioFine International (Vancouver, BC) [23, 24]. (*R*)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate (PEG-DMG) and (*R*)-2,3-bis(stearyloxy)propyl-1-(methoxy poly(ethylene glycol)2000 carbamate (PEG-DSG) were synthesized as previously described [25]. Phosphorothioated unmethylated cytosine-guanine containing oligodeoxynucleotide (CpG) complementary to the initiation codon region of the human/mouse c-myc proto-oncogene (5'-AACGTTGAGGGGCAT-3′) was purchased from TriLink Biotechnologies (San Diego, CA). This sequence has been previously identified as highly immunostimulatory when administered to mice due to the presence of the CpG dinucleotide motif [5, 15, 26, 27]. The sense and antisense sequences of siRNA targeting coagulation factor VII (siFVII) are 5′-GGAucAucucAAGucuuAcT*T-3′ and 5′-GuAAGAcuuGAGAuGAuccT*T-3, respectively with 2'fluoro-modified nucleotides represented in lower case and phosphorothioate linkages by asterisks. siFVII was synthesized by Integrated DNA Technologies (Coralville, IA) [25, 28]. *In vitro* transcription of firefly luciferase mRNA was carried out as described previously [29]. pGL4.51 plasmid DNA (Promega Madison, WI) was prepared using a Qiagen EndoFree Plasmid Giga Kit as per manufacturer's instructions (Germantown, MD).

2.2. Synthesis of lipophilic-dexamethasone prodrugs

Detailed description of the synthesis of dexamethasone prodrugs LD001-LD005 can be found in Supplemental Methods.

2.3. Preparation of lipid nanoparticles

LNP containing various types of nucleic acids (CpG, siRNA, mRNA, pDNA) were prepared by rapid mixing through a T-junction mixer as previously described [30, 31]. Briefly, amino-lipid, DSPC, cholesterol and PEG-DMG were dissolved in ethanol at the mole ratio of 50, 10, 38.5 and 1.5 respectively. Dexamethasone prodrugs were incorporated at 1, 4 or 10 mol% at the expense of amino-lipid. Amino-lipids were chosen based on previous work. For CpG and siRNA, DMAP-BLP [23, 28, 32] was used while DLin-MC3-DMA [24] and DLin-KC2-DMA [33] were used for the delivery or mRNA and pDNA, respectively. Nucleic acids were dissolved in 25 mM acetate buffer at pH 4.0 such that the final mixture would have a defined nucleic acid to lipid weight/μmol ratios (0.056 for CpG and siRNA, 0.028 for mRNA and pDNA). Ethanolic and aqueous mixtures were mixed together at a 1:3 volume and flow rate ratio with final flow rates > 10 mL/min at room temperature. Resulting mixtures containing 25% ethanol were dialyzed against