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Targeted Delivery of LXR Agonist Using a Site-Specific Antibody-Drug Conjugate

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Abstract

Liver X receptor (LXR) agonists have been explored as potential treatments for atherosclerosis and other diseases based on their ability to induce reverse cholesterol transport and suppress inflammation. However, this therapeutic potential has been hindered by on-target adverse effects in the liver mediated by excessive lipogenesis. Herein, we report a novel site-specific antibody-drug conjugate (ADC) that selectively delivers a LXR agonist to monocytes/macrophages while sparing hepatocytes. The unnatural amino acid *para*-acetylphenylalanine (pAcF) was site-specifically incorporated into anti-CD11a IgG, which binds the α -chain component of the lymphocyte function-associated antigen 1 (LFA-1) expressed on nearly all monocytes and macrophages. An aminooxy-modified LXR agonist was conjugated to anti-CD11a IgG through a stable, cathepsin B cleavable oxime linkage to afford a chemically-defined ADC. The anti-CD11a IgG-LXR agonist ADC induced LXR activation specifically in human THP-1 monocyte/macrophage cells in vitro (EC₅₀ ~ 27 nM), but had no significant effect in hepatocytes, indicating that payload delivery is CD11a-mediated. Moreover, the ADC exhibited higher fold activation compared to a conventional synthetic LXR agonist T0901317 (Tularik) (~3 fold). This novel ADC represents a fundamentally different strategy that uses tissue targeting to overcome the limitations of LXR agonists for potential use in treating atherosclerosis.

INTRODUCTION

With the recent FDA approval of two antibody-drug conjugates (ADCs), namely, Adcetris (brentuximab vedotin for Hodgkin's lymphoma) and Kadcyla (ado-trastuzumab emtansine

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Supporting Information. Additional experimental details and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

for Her2 positive breast cancer), along with the increasing number of such agents currently in clinical trials, ADCs have garnered significant interest in oncology.^{1,2} ADCs can deliver cytotoxic agents selectively to tissues by binding to antigens highly expressed on specific cell types, followed by internalization and subsequent intracellular drug release.³ The net result is that the small-molecule payloads are delivered directly to the cell type of interest, reducing unwanted side effects resulting from off-target and/or off-tissue toxicity. Despite significant progress in the development of ADCs, few indications outside oncology have been explored.⁴ Recently, we reported an immunosuppressive anti-CXCR4 ADC that can selectively deliver a kinase inhibitor to human T lymphocytes and suppress T-cell receptor (TCR)-mediated T-cell activation and cytokine expression *in vitro*.⁵ We now describe the use of an ADC to deliver an LXR nuclear receptor agonist selectively to monocyte/macrophage cells, thereby avoiding unwanted lipogenic effects in hepatocytes.

Atherosclerosis is a chronic progressive disease that accounts for approximately 35% of all deaths in the United States.⁶ One of the underlying processes that drives atherosclerosis is the formation of oxidized low-density lipoproteins (oxLDL) and a subsequent inflammatory response.⁷ Macrophages recruited as part of this response engulf, but fail to adequately process, oxLDL particles and transform into lipid-laden foam cells whose accumulation in the intima leads to the formation of atherosclerotic lesions. When left untreated, these lesions will cause the vessel to rupture leading to fatal thrombosis. Under non-pathogenic conditions, normal macrophages exert atheroprotective effects by promoting the efflux of cholesterol to the liver for bile secretion via reverse cholesterol transport (RCT).⁸ RCT is triggered upon activation of the liver X receptors (LXR- α and LXR- β), sterol-responsive nuclear receptors that control the transcription of several genes directly involved in cholesterol homeostasis^{9,10} and lipid metabolism, such as ATP-binding cassette transporters *ABCG1* and *ABCA1*, lipoprotein lipase (*LPL*), and apolipoprotein E (*ApoE*).^{11–13} Additionally, LXR activation suppresses an array of inflammatory genes, namely, iNOS, COX-2, IL-6, and MCP-1/3 and results in inhibition of macrophage proliferation and foam cell formation.^{14,15}

While both LXR- α and LXR- β can be found in monocytes and macrophages as well as other tissues (intestine, kidney, adipose), LXR- α is also highly expressed in the liver.^{11,16} In contrast to LXR activation in macrophages, activation of LXR in the liver increases hepatic triglyceride levels, which is an independent risk factor for the development of atherosclerosis. Indeed, although numerous studies demonstrated significant reduction in atherosclerotic lesions in murine models upon treatment with synthetic LXR agonists GW3965 and T0901317,¹⁵ a significant increase in hepatic lipogenesis and plasma triglyceride levels was also observed.^{15,17} This effect on triglyceride levels was notably absent in LXR- α knockout mice treated with synthetic LXR agonist,¹⁷ giving rise to drug discovery efforts aimed at generating LXR- β -selective agonists.^{18,19} However, to date these efforts have not delivered compounds that have progressed into patients, likely due to an inability to obtain sufficient selectivity.^{20–22} Targeted delivery of an LXR agonist to macrophages by an ADC represents a fundamentally new approach to selectively engage the LXR pathway in the treatment of atherosclerosis.

RESULTS AND DISCUSSION

Design and Synthesis of the LXR agonist

GW3965²³ (**1**) and T0901317⁸ (**2**) are two widely used LXR agonists to study cardiovascular diseases. However, based on the reported structure-activity relationships (SAR), these two agonists fit tightly within the LXR receptor binding site and, as such, would be difficult to derivatize with a linker for antibody conjugation while retaining potency (Figure 1).^{24–26} Recently, Wyeth reported a series of highly potent LXR agonists that tolerate various modifications at the sulfonamide group,^{20–22,27} suggesting that this core structure should be useful for generating a highly potent, antibody-linked LXR agonist. (Figure 1, 3). To test this notion, compound **3** with an aminoethyl sulfonamide substituent was synthesized (Scheme 1 and Scheme S3) and LXR binding was tested in comparison with **1** and **2** (LXR- α /LXR- β LanthaScreen, Life Technologies). Indeed, the modification was well-tolerated and **3** maintained similar potency to GW3965 and T0901317 (Figure 1 and Figure S1).

Next, we designed and synthesized the linker-derivative of the LXR agonist to be used for antibody conjugation. We considered several different linker release strategies, including disulfides, acid-labile hydrazones, and protease cleavable linkers.^{5,28,29} Among these, we chose a protease cleavable phenylalanine-lysine (Phe-Lys) dipeptide, a stable linker that is rapidly hydrolyzed by the lysosomal enzyme Cathepsin B (CatB),³ resulting in the release of the free LXR agonist **3** inside the cell. A terminal aminooxy moiety was also incorporated to allow for site-specific conjugation to the antibody. To synthesize aminooxy-modified CatB-LXR agonist **10**, 3-bromobenzenesulphonyl chloride was reacted with 2-(methylamino)ethanol to afford 3-bromobenzenesulfonamide **6** in 95% yield (Scheme 1). Next, **6** was coupled to a commercially-available quinolone **7** in the presence of dimethylglycine hydrochloride in a cesium carbonate/copper(I) iodide/dioxane solution to afford compound **8** in moderate yield. The alcohol group of **8** was then converted to the methanesulfonate, which in turn was converted to amine **3** in high yield using ammonia in methanol. Coupling of **3** with the pre-formed tosylated-PEGylated dipeptide **9** (Scheme S1 and Scheme S2) was carried out using EDCI/HOBt in 51% yield. The resulting product was reacted with N-hydroxyphthalimide to form the alkoxyamine. Sequential deprotection of the Boc and phthalimide groups provided the final product, aminooxy-CatB-LXR agonist **10** in an overall 27% yield (Scheme 1).

We next evaluated the stability of aminooxy-CatB-LXR agonist in cell culture media. Compound **10** was incubated in growth media (RPMI, 10% FBS, 0.1% β -mercaptoethanol, 1 mM sodium pyruvate and 100 U/ml penicillin-streptomycin) at 37 °C. Samples were extracted at different time points and release of parent compound was analyzed by LC-MS. The results indicate the aminooxy-CatB-LXR agonist is completely stable after 24 hours. (Figure S2A). We also analyzed cleavage of the Phe-Lys dipeptide by incubating **10** with purified CatB enzyme (EMD Millipore). Notably, after 2 hours of incubation with CatB, formation of a new peak is observed that corresponds to the mass of the desired cleavage product (Figure S2B). Taken together, these results indicate the aminooxy-CatB-LXR agonist is stable, but can be efficiently released upon enzymatic activation.

Design and Synthesis of anti-CD11a IgGX-LXR Agonist ADC

With our linker-derivatized LXR agonist in hand, we proceeded with the synthesis of the corresponding ADC. To selectively deliver a LXR agonist to macrophages, we used CD11a as the target antigen. CD11a is the α -chain component of the lymphocyte function-associated antigen 1 (LFA-1). Although CD11a is expressed on most leukocytes, including lymphocytes and granulocytes, expression is abundant on monocytes and macrophages, and importantly, CD11a is not expressed on hepatocytes.^{30–32} Moreover, an increase in the expression of CD11a on monocytes is correlated with atherosclerotic coronary stenosis.³³ CD11a receptors also internalize rapidly, and there are high affinity antibodies readily available ($K_d \sim 2.2$ nM) making it an attractive choice for an ADC.^{31,32,34}

Compared to non-specific conjugation that utilizes surface-exposed lysines on the antibody, site-specific conjugation strategies have been shown to improve stability, pharmacokinetics, and the drug safety profile of the resulting ADCs.^{35–38} In this study, we utilized unnatural amino acid (UAA) technology to incorporate a bio-orthogonal moiety (*para*-acetylphenylalanine, pAcF) site-specifically into anti-CD11a IgG for reaction with the terminal aminoxy group on our LXR agonist. Accordingly, we cloned the variable light and heavy regions of Efalizumab (humanized monoclonal anti-CD11a antibody)³⁹ into an expression vector containing the orthogonal aaRS/tRNA pair necessary for UAA incorporation in mammalian cells.⁴⁰ We incorporated pAcF at position A122 in the constant region of the heavy chain, as this site has previously shown excellent coupling efficiency (>95% for oxime ligation) and good drug-linker stability.^{35,41} We then transiently transfected Chinese Hamster Ovary (CHO) suspension cells with the expression vector, and the cells were grown for 7–10 days in pAcF-supplemented media to afford anti-CD11a A122pAcF IgG (referred to herein as anti-CD11a IgGX) in > 4 mg/L yield (purified by Protein A chromatography). The identity and purity of the antibody was confirmed by denaturing SDS-PAGE gel electrophoresis (Figure 2B) and electrospray-ionization mass spectrometry (ESI-MS) (Figure S3). Additionally, anti-Her2 A121pAcF IgG (anti-Her2 IgGX) and anti-Her2 K169pAcF Fab (anti-Her2 FabX) were expressed in CHO cells³⁵ and *E. coli*,⁴² respectively, (See Materials and Methods, Supporting Information) and were used as negative controls in subsequent assays (Figure S3).

Next, to covalently link the aminoxy-CatB-LXR agonist (**10**) to anti-CD11a IgGX, we treated the mutant antibody (67–80 μ M) with 40 equivalents of **10** in sodium acetate buffer (pH 4.5) in the presence of 10 mM acetic hydrazide (catalyst) at 37 °C for 48 hours (Figure 2A). Excess LXR agonist was removed by buffer exchange with an Amicon concentrator (30 kDa MWCO). Denaturing SDS-PAGE analysis of anti-CD11a IgGX-CatB-LXR agonist indicated that upon treatment with dithiothreitol (DTT), the conjugate gave rise to two bands with molecular weights corresponding to the heavy (~50 kDa) and light chains (~25 kDa) (Figure 2B). As assessed by ESI-MS, the heavy chain peak had an increase in MW of ~1030 Da (compared to unconjugated anti-CD11a IgGX, Figure S3) corresponding to the LXR agonist-modified product; >95% of anti-CD11a IgGX was successfully coupled to the LXR agonist (Figure 2C). Taken together, these results demonstrate the ability to site-specifically modify anti-CD11a IgGX with an LXR agonist, resulting in a chemically-defined, homogeneous product with a defined drug to antibody ratio (DAR) of 2.

Binding and Internalization of anti-CD11a IgGX

We next determined the affinity, specificity and internalization of the mutant CD11a antibody. Anti-CD11a IgGX and negative controls (anti-Her2 IgGX and anti-Her2 FabX) were site-specifically conjugated to aminooxy-Alexa Fluor 488 (AF488, Life Technologies) in a similar manner as described above (Figure S4). To assess the binding affinity of anti-CD11a IgGX and confirm it does not bind hepatocytes, we incubated human THP-1 monocyte/macrophage cells and human HepG2 hepatoma cells with anti-CD11a IgGX-AF488, anti-Her2 IgGX-AF488 and anti-Her2 FabX-AF488 and analyzed the results by flow cytometry. Because monocytes/macrophages have Fc γ receptors,⁴³ we also examined the binding in the presence of an Fc blocking reagent (BD biosciences). As depicted in Figure 3A, anti-CD11a IgGX-AF488 binds in the absence of Fc block, but still retains excellent binding affinity ($K_d \sim 0.5$ nM) once the Fc receptors are blocked, indicating the binding is CD11a-mediated (Figure 3A and Figure S5). Conversely, anti-Her2 IgGX-AF488 also binds to THP-1 cells in the absence of Fc block, but does not bind once the Fc receptors are blocked, indicating that the binding of anti-Her2 IgG is Fc-mediated. To further verify this result, we also tested a fragment of Her2 (anti-Her2 FabX-AF488), which does not contain the Fc region. As expected, the anti-Her2 FabX-AF488 does not bind to THP-1 cells. Furthermore, incubation of anti-CD11a IgGX-AF488 with HepG2 cells did not result in any peak shift by flow cytometry, indicating anti-CD11a IgGX binds CD11a selectively and does not have an affinity towards hepatocytes (Figure 3A and Figure S6).

Given the high selectivity and affinity of anti-CD11a IgGX, we next examined its internalization by THP-1 cells, which is required for successful delivery of the LXR agonist. Confocal microscopy was used to determine the internalization efficiency of the anti-CD11a antibody into THP-1 cells in the presence or absence of Fc block (Figure 3B and Figure S7). As depicted in Figure 3B, anti-CD11a IgGX-AF488 was observed in the cytoplasm (green spots) within 2 hours at 37 °C, which indicates efficient endocytosis. This effect was inhibited at 4 °C or in the presence of 20-fold excess of unconjugated anti-CD11a IgGX, which further confirms the internalization is CD11a-mediated. Anti-Her2 IgGX-AF488 was used as a negative control; no internalization was observed.

In Vitro LXR Transactivation Assay

Next, we evaluated the *in vitro* activity of the anti-CD11a IgGX-CatB-LXR agonist using an LXR-driven luciferase stably incorporated into THP-1 and HepG2 cells. This vector encodes the firefly luciferase reporter gene under the control of a CMV promoter and tandem repeats of the LXR- α transcriptional response element. Cells were treated with anti-CD11a IgGX-CatB-LXR agonist, T0901317 (**2**, positive control) or aminooxy-CatB-LXR agonist (**10**) for 24 hours in the presence or absence of 10% human AB serum to block Fc γ receptors. Cells were then treated with ONE-Glo and luminescence was read on an Envision plate reader. Notably, LXR activation by the anti-CD11a IgGX-CatB-LXR agonist was selectively induced in THP-1 cells independent of Fc blockade (Figure 4). The ADC exhibits strong agonist activity with an $EC_{50} = 26.7 \pm 1.3$ nM compared to T0901317 ($EC_{50} = 79.0 \pm 1.1$ nM) in THP-1 cells. In HepG2 cells, anti-CD11a IgGX-CatB-LXR agonist did not induce significant activation, while T0901317 was equipotent in these cells relative to THP-1. Furthermore, no activity was detected in either cell type treated with compound **10**, most

likely due to a lack of cell permeability. These results indicate that the anti-CD11a ADC can selectively and effectively deliver a LXR agonist to THP-1 cells.

CONCLUSIONS

In summary, we generated a site-specific anti-CD11a IgG-LXR agonist ADC with a DAR of 2 that selectively activates LXR in THP-1 monocyte/macrophage cells (versus HepG2 hepatocytes) with 3-fold greater potency than a known small molecule LXR agonist. This ADC represents a new approach to selectively eliciting the beneficial effects of LXR agonists on reverse cholesterol transport and suppression of inflammation in macrophages while avoiding detrimental lipogenic effects in liver cells. In order to translate this approach to *in vivo* mouse models, we are currently generating a mouse cross-reactive ADC based on the reported Efalizumab surrogate muM17, a chimeric antibody based on a rat IgG2a anti-mouse CD11a monoclonal antibody.³⁰ In addition, we are constructing an Fc-null anti-CD11a IgG with several mutations in the Fc region to prevent binding and internalization through Fc γ receptors and further improve selectivity and suitability for use *in vivo*.⁴⁴ Pharmacokinetics, tissue distribution and clearance of the ADC will also be evaluated. These experiments may lead to a novel therapeutic modality for the treatment of atherosclerosis. Finally, we continue to explore other tissue-targeted pharmacology outside of cancer in an effort to expand the utility of antibody drug conjugates toward safer therapies.

EXPERIMENTAL SECTION

LXR Agonist Conjugation to Antibodies via Oxime Ligation

A solution of the anti-CD11a IgGX (1–1.5 mg, 6.7–10 nmol) in 100 mM sodium acetate buffer, pH 4.5 (final concentration of antibody ranges from 10–12 mg/mL) was reacted with 40–60 equiv. of the amonnoxy-CatB-LXR agonist (**10**) in the presence of 10 mM acetic hydrazide. The mixture was left to react at 37°C for 2 days. Afterwards, the anti-CD11a IgGX-CatB-LXR agonist ADC was isolated from excess small molecule using a Zeba spin desalting column (40 kDa MWCO, Thermo Scientific) and Amicon filter (30 kDa MWCO, EMD Millipore).

Flow Cytometry Analysis

An appropriate number of THP-1 or HepG2 cells (~300k/well) were plated in a 96-well plate. Before treatment with the Alexa Fluor 488-conjugated antibodies, cells were first incubated with or without the human Fc block (100 μ L with final concentration of 0.0125 mg/mL, BD Biosciences) in FACS buffer (2% BSA, 1 mM EDTA, in 1X PBS) for 15 min at room temperature. Afterwards, the cells were spun down at 1000 rpm for 30 s and cells were treated with 100 μ L of 2 nM each of the IgG- or Fab-Alexa Fluor 488 conjugates (except for the control cells) in FACS buffer at 4 °C in the dark, with gentle rocking (for the binding affinity measurement, cells were treated with different concentrations of the dye-conjugated antibodies ranging from 0 to 50 nM). After 1 h, cells were washed 3x (200 μ L of cold buffer, with repeated centrifugation and cell resuspension). Finally, cells were resuspended in 150

μL of the same buffer for subsequent FACS analysis (BD Accuri). All the results were processed using FlowJo software.

In Vitro LXR Transactivation Assay

THP-1 and HepG2 cells were purchased from ATCC. THP-1 cells were cultured in RPMI (1 mM glucose) with 10% FBS, 0.1% β-mercaptoethanol, 1 mM sodium pyruvate and 100 U/ml penicillin-streptomycin. HepG2 cells were cultured in MEM:F12 (v/v = 1:1) with 10% FBS, 1 mM sodium pyruvate and 100 U/ml penicillin-streptomycin. Signal lenti LXR-α luciferase reporter (cat no: CLS-7041L) was purchased from Qiagen. Cells were transfected with lenti LXR-α luciferase reporter according to manufacturer's instruction, and positively transfected cells were selected by adding 1 μg/ml puromycin. For ADC activity experiment, 10,000 cells/well were plated in 384-well plate with or without 10% human AB serum (cat no: H4522, Sigma Aldrich) and were incubated with T0901317 (**2**), anti-CD11a IgGX-CatB-LXR agonist or aminooxy-CatB-LXR agonist (**10**) at suggested concentrations in a total volume of 25 μL. After incubation for 24 hours, 5 μL ONE-Glo luciferase assay reagent (Promega) was added into the cell culture according to manufacturer's instruction, and the reporter gene activity signal was read by a PerkinElmer EnVision Multilabel Reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Synthetic LXR agonists and their LXR-α and LXR-β binding affinities, shown in a simplified binding model.

Figure 2.

Construction and characterization of the ADC, anti-CD11a IgGX-CatB-LXR agonist. A) Site-specific conjugation of the CatB-LXR agonist to anti-CD11a IgGX via a ketone-hydroxylamine condensation reaction in acetate buffer at 37 °C to form anti-CD11a IgGX-CatB-LXR agonist with a stable oxime linkage. B) SDS-PAGE analysis of the antibodies with and without DTT treatment; Lanes 2 and 5 = anti-CD11a IgGX, Lanes 3 and 6 = ADC. C) ESI-MS data showing both the light chain and CatB-LXR agonist-modified heavy chain of the ADC after treatment with PNGase F (Promega, 12 h at 37 °C in PBS pH 7.4) and subsequent reduction of the disulfide bonds with 10 mM DTT: Calc. MW = 50,699 Da, Observed MW = 50,697 Da.

Figure 3.

Assessment of binding, internalization, and *in vitro* agonist activity of the ADC. A) Binding of AF488-conjugated antibodies on THP-1 and HepG2 cells as analyzed by flow cytometry. Cells were incubated either with 2 nM of the dye-conjugated antibodies or with increasing concentrations of antibody (0–50 nM, right) for 1 h at 4 °C in the presence or absence of Fc block (Human Fc block, BD Biosciences). B) Confocal microscopy analysis of anti-CD11a IgGX internalization in the presence of Fc block. THP-1 cells were incubated with 50 nM anti-CD11a IgGX-AF488 for 2 h at i) 37 °C, ii) 4 °C, or iii) in the presence of 1 μ M (20-fold) anti-CD11a IgGX at 37 °C. Anti-Her2 IgGX-AF488 (iv) was used as a negative control. Cells were then fixed, stained with Hoechst (blue, nucleus), Alexa Fluor 594-conjugated wheat germ agglutinin (red, membrane) and imaged with a Zeiss 710 confocal microscope. Bar = 10 μ m.

Figure 4.

Evaluation of agonist activity of anti-CD11a IgGX-CatB-LXR agonist, T0901317 small molecule agonist (2), and aminoxy-CatB-LXR agonist free drug (10) in the presence and absence of 10% human AB serum. Data were normalized to the highest concentration of T0901317 (set at 100%).

Scheme 1.
Synthesis of aminoxy-modified Cathepsin B-LXR agonist.