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In vitro and in vivo pharmacology of NXT629, a novel and selective PPARα antagonist☆

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ABSTRACT

Peroxisome-proliferator activated receptors (PPAR) are members of the nuclear hormone receptor superfamily which regulate gene transcription. PPARα is a key regulator of lipid homeostasis and a negative regulator of inflammation. Under conditions of metabolic stress such as fasting or glucose deprivation, PPARα is upregulated in order to control gene expression necessary for processing alternate fuel sources (e.g. fatty acid oxidation) and thereby promote maintenance of cell viability. Clinically, PPARα expression is upregulated in diseased tissues such as melanoma, chronic lymphocytic leukemia, ovarian and prostate cancer. This may allow for cellular proliferation and metastasis. Importantly, genetic knockouts of PPARα have been shown to be protected against tumor growth in a variety of syngeneic tumors models. We hypothesized that a potent and selective PPARα antagonist could represent a novel cancer therapy. Early in our discovery research, we identified NXT629 (Bravo et al., 2014). Herein we describe the pharmacology of NXT629 and demonstrate that it is a potent and selective PPARα antagonist. We identify NXT629 as a valuable tool for use in in vivo assessment of PPARα due to its good systemic exposure following intraperitoneal injection. We explore the in vivo pharmacology of NXT629 and demonstrate that it is efficacious in pharmacodynamic models that are driven by PPARα. Finally, we probe the efficacy of NXT629 in disease models where PPARα knockouts have shown to be protected. We believe that PPARα antagonists will be beneficial in diseases such as ovarian cancer and melanoma where PPARα and fatty acid oxidation may be involved.

1. Introduction

Peroxisome proliferator activated receptor alpha (PPARα), a member of the nuclear hormone receptor superfamily, exists as a heterodimer with retinoid X receptor (RXR). Upon ligand binding and recruitment of the co-activator protein, the resulting ternary complex binds to the peroxisomal proliferator response element (PPRE) on the promoter region of the target genes and drives gene transcription. Endogenous ligands of PPARα include oleoylethanolamide (OEA) (Fu et al., 2003), which is reported to bind with nanomolar potency (Berger and Moller, 2002), as well as long chain fatty acids and leukotriene B_4 (LTB_4) which have micromolar potencies (Devchand et al., 1996). Numerous synthetic pharmacological agonists for PPARα have been described including fibrates, WY-14,643 and GW7647 (Willson et al., 2000).

PPARα, a key regulator of lipid homeostasis and a negative regulator of inflammation, is widely expressed in normal tissue with predominance in the liver, kidney, brown adipose tissue, skeletal muscle and heart. Expression is upregulated under conditions of metabolic stress such as fasting or glucose deprivation. This promotes maintenance of cell viability by increasing the expression of genes required for fatty acid oxidation in order to facilitate the use of alternate fuel sources (i.e. lipids rather than glucose). From a cancer perspective, expression of PPARα or its target genes is increased in diseased tissues such as melanoma (Eastham et al., 2008), chronic lymphocytic leukemia (Spaner et al., 2013), ovarian (Nieman et al., 2011) and prostate cancer (Collett et al., 2000). In patients with colorectal liver metastases, PPARα staining is associated with worse overall survival (Pang et al., 2015).

In PPARα knockout mice, transcription of genes involved in fatty acid oxidation (eg. *Cpt1a*, *Cact*) is impaired. Furthermore, genetic knockouts of PPARα have been shown to be protected against tumor

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growth in a variety of syngeneic tumors models (Kaipainen et al., 2007). Thus, we hypothesized that a potent and selective PPARα antagonist could represent a novel cancer therapy. To date, a limited number of PPARα antagonists have been disclosed, with GW6471 (Xu et al., 2002) and MK886 (Kehrer et al., 2001) being the most referenced. While GW6471 has been extensively utilized in vitro, it has been used only sparingly in vivo. MK886 is a potent inhibitor of 5-lipoxygenase activating protein (FLAP) and FLAP has previously been implicated in some oncogenic pathways. However in our luciferase-based reporter assay system, MK886 was cytotoxic at the concentrations at which inhibition of PPARα occurred (data not shown), complicating interpretation of results. Eli Lilly has described an acylsulfonamide series of PPARα antagonists (Etgen and Mantlo, 2003) however, these lack stability in murine plasma and are therefore not amenable for use in vivo.

Although our ultimate goal was to identify a molecule with oral bioavailability, we initially identified a compound with good systemic exposure following intraperitoneal injection, as valuable for use in vivo (Bravo et al., 2014). In the studies described herein, NXT629, a potent and selective PPARα antagonist, was profiled in a panel of in vitro and in vivo pharmacological models.

2. Material and methods

2.1. Chemicals

NXT629 (Fig. 1A), NXT969 (Fig. 1B) and GW590735 were synthesized at Inception Sciences. WY-16,643 was purchased from Sigma-Aldrich (St. Louis, MO). For all in vitro experiments, a 30 mM stock solution in DMSO was diluted to the appropriate concentration in assay buffer. In vivo dosing solutions were prepared in sterile saline (NXT629), sterile saline containing 2% Tween 20 (NXT969), or in a 1:1 mixture of DMSO and sesame oil (GW590735 and WY-16,643).

2.2. In vitro pharmacology

2.2.1. Functional reporter assay

Antagonist activity of test compound against human or mouse PPARα receptors was analyzed using commercial kits (Human or Mouse PPARα Reporter Assay System, Indigo Biosciences, State College, PA). PPARα reporter cells are dispensed into 96-well assay plates followed by immediate addition of test compounds in the presence or absence of the PPARα agonist GW7647 (20 nM). Following an overnight incubation, the treatment media is discarded and Luciferase Detection Reagent was added. The luminescence intensity of light emission from the ensuing luciferase reaction is directly proportional to the relative level of PPARα activation in the reporter cells. Luminescence was read using a Molecular Devices FlexStation 3 (Sunnyvale, CA).

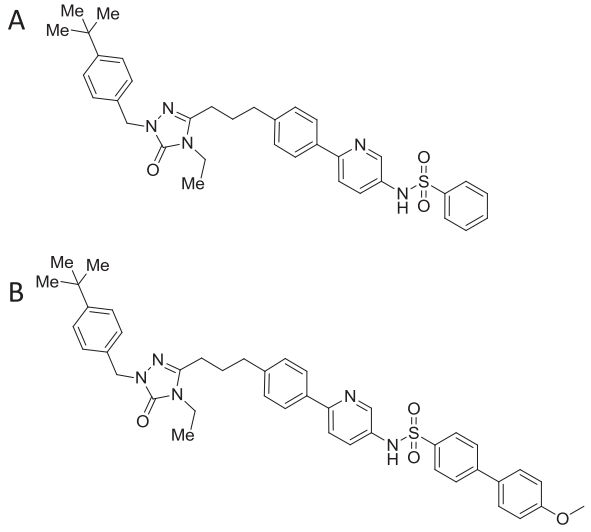

Fig. 1. Chemical structure of NXT629 (A) and NXT969 (B).

To determine whether NXT629 was a competitive antagonist of PPARα, the concentration of both NXT629 and GW7647 were varied in the PPARα reporter assay. Inhibitory concentration response curves were generated for NXT629 at 0, 30, 100 and 300 nM against a variable concentration of GW7647 (0.1 nM to 1 μM). We further explored whether potency of NXT629 shifts with increasing concentrations of agonist. This was accomplished by varying the concentration of NXT629 (1 nM to 10 μM) while maintaining a constant agonist concentration of either 100 nM or 1 μM.

2.2.2. Counterscreens

To determine selectivity of NXT629 for PPARα, antagonist activity against human PPARδ, PPARγ, estrogen receptor β, glucocorticoid receptor and thyroid receptor β was evaluated. NXT629 was also counterscreened against mouse PPARδ and PPARγ⊡ Counterscreens were performed using commercially available reporter assay kits (Indigo Biosciences, State College, PA) according to the manufacturer's instructions.

2.2.3. Real time-PCR of normal human skin and melanoma

In order to examine expression of PPARα, normal healthy or melanoma skin biopsy samples (n=2 donors each) were obtained from ConversantBio (Hunstville, Alabama). Samples were collected into RNALater (Qiagen, Venlo, The Netherlands). Normal skin and melanoma tumor tissues were weighed then coated and minced while in a solution of Trizol. For every 100 mg of tissue, 1 ml of TRIzol reagent was applied and subjected to homogenization with a Polytron handheld homogenizer. Total RNA was purified according to the manufacturer's TRIzol extraction protocols (Life Technologies, Carlsbad, CA). The resulting RNA was treated with (1U:25 μl) DNase I (Life Technologies, Carlsbad, CA). Reverse transcription of cDNA was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with 1 μg of RNA. The synthesized cDNA was mixed with primers (Table 3) and SsoFast EvaGreen Supermix With Low ROX (Bio-Rad, Hercules, CA). Real-Time PCR detection on the mix was performed in the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) following manufacturers suggested protocols and using an annealing temperature of 60 °C for 5 s.

2.3. In vivo pharmacology

2.3.1. Animals

Mice (CD-1, C57BL/6 N or Fox1nu females) and jugular vein cannulated Sprague-Dawley rats (male) were obtained from Harlan (Livermore, CA). Animals were given food and water ad libitum and allowed to acclimate for at least 5 days prior to initiation of experiments. All protocols were approved by the Inception Sciences Institutional Animal Care and Use Committee.

2.3.2. Pharmacokinetics

Rats were fasted overnight and dosed with NXT629, either *per os* (p.o.) (10 mg/kg) or intravenously (1 mg/kg). Blood was sampled via the jugular cannula at various time points after drug administration. Plasma was isolated and stored at −80 °C until analysis for NXT629 by liquid chromatography-mass spectrometry (LC/MS/MS).

CD-1 mice received NXT629 PO (10 mg/kg in 0.5% methyl cellulose) or intraperitoneally (i.p) (10 mg/kg in saline). A pharmacokinetics assessment was performed on the less potent analog NXT969, which served as a negative control. CD-1 mice received NXT969 p.o. (10 mg/kg in 0.5% methyl cellulose) or i.p (30 mg/kg in 2% Tween 20/98% saline). Mice were euthanized at various time points after dosing

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