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Short Communication

Indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors activate the aryl hydrocarbon receptor

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ABSTRACT

Indoleamine 2,3-dioxygenase 1 (IDO1) plays a key role in the immune system by regulating tryptophan levels and T cell differentiation. Several tumor types overexpress IDO1 to avoid immune surveillance making IDO1 of interest as a target for therapeutic intervention. As a result, several IDO1 inhibitors are currently being tested in clinical trials for cancer treatment as well as several other diseases. Many of the IDO1 inhibitors in clinical trials naturally bear structural similarities to the IDO1 substrate tryptophan, as such, they fulfill many of the structural and functional criteria as potential AHR ligands. Using mouse and human cell-based luciferase gene reporter assays, qPCR confirmation experiments, and CYP1A1 enzyme activity assays, we report that some of the promising clinical IDO1 inhibitors also act as agonists for the aryl hydrocarbon receptor (AHR), best known for its roles in xenobiotic metabolism and as another key regulator of the immune response. The dual role as IDO antagonist and AHR agonist for many of these IDO target drugs should be considered for full interrogation of their biological mechanisms and clinical outcomes.

1. Introduction

The essential amino acid L-Tryptophan (Trp) is metabolized in a tissue-specific manner by the rate-limiting enzymes tryptophan 2,3-dioxygenase 2 (TDO2) and indoleamine 2,3-dioxygenase (IDO1 and IDO2) to produce L-Kynurenine (Kyn) (Austin and Rendina, 2015). IDO1 has garnered the most attention due to its key roles in inflammation, as Trp metabolites, particularly Kyn, act as regulators of immune cell differentiation and proliferation. As an example, IDO1 expression directs T cell polarization and decreases T cell proliferation (Taylor and Feng, 1991; Hwu et al., 2000; Jaronen and Quintana, 2014).

The Aryl Hydrocarbon Receptor (AHR) is a basic helix-loop-helix transcription factor that binds and responds to xenobiotics (Denison et al., 2002). The AHR is notable for its capacity to bind hundreds of identified ligands, the majority of which are exogenous environmental toxicants. Following ligand binding, the AHR translocates to the nucleus where it regulates the transcription of thousands of target genes in a ligand-specific manner (Karyala et al., 2004), the best known of which are a gene battery encoding proteins involved in xenobiotic metabolism.

T-cell polarization also involves the AHR (Mezrich et al., 2010; Quintana and Sherr, 2013). Exposure to some AHR agonists, including the potent environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), induces polarization of naïve T cells to an anti-inflammatory Treg status contributing to an immunosuppressive condition (Faith and Luster, 1979; Mohinta et al., 2015). The AHR responds to other ligands by promoting polarization of naïve T-cells to the pro-inflammatory Th17 cell phenotype (Veldhoen et al., 2008) demonstrating that the roles of the AHR in the immune response are complex and ligand-specific.

IDO1 gene expression is regulated by two pathways: an interferon gamma (IFNγ)-independent and an IFNγ-dependent pathway (Brandacher et al., 2007; Sarkar et al., 2007; Chen, 2011; Bessede et al., 2014). The AHR carries out its role in the IFNγ-independent pathway, in which the AHR binds the IDO1 promoter to directly regulate IDO1 transcription (Vogel et al., 2008; Mezrich et al., 2010), and second, by induction of the AHR-regulated miR-132/212 cluster, which also plays a

Abbreviations: 1MDT, 1-Methy-D-Tryptophan; 1MLT, 1-Methyl-L-Tryptophan; AHR, Aryl Hydrocarbon Receptor; DMSO, dimethylsulfoxide; DPBS, Dulbecco's Phosphate Buffered Saline; IDO, indoleamine 2,3-dioxygenase; INCB, INCB024360; Kyn, kynurenine; KA, kynurenic acid; NLG, NLG919; NORH, norharmane; QA, quinolinic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Trp, tryptophan.

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role in T-cell polarization (Nguyen et al., 2010; Nguyen et al., 2013). Additionally, the major IDO1 metabolite Kyn is itself an AHR ligand (Mezrich et al., 2010) producing a likely positive regulatory feedback loop between the AHR and IDO1 (Julliard et al., 2014).

IDO1 is frequently overexpressed in cancer cells causing a depletion of Trp in the microenvironment and leading to the suppression of immune-surveillance (Friberg et al., 2002; Brandacher et al., 2006; Liu et al., 2009; Ferdinande et al., 2012). Thus, IDO1 is a popular target for chemotherapeutic intervention, with a number of IDO1 inhibitors currently being tested in cancer clinical trials (Vacchelli et al., 2014; Austin and Rendina, 2015; Rohrig et al., 2015). For example, INCB024360 (INCB) is involved in at least eight clinical trials, either as a single agent or in combination with other drugs for treatment of melanomas, reproductive tract cancers, and other solid tumors; while NLG919 (NLG) is in a phase I clinical trial for treatment of solid tumors. It should be noted that the inhibition of IDO1 is also the goal in clinical trials for Huntington's disease (Mazarei and Leavitt, 2015), neurological disorders (Fujigaki et al., 2017), and autoimmunity and other diseases (Yeung et al., 2015).

Most IDO1 inhibitors are structurally similar to Trp by possessing a planar, polycyclic molecular configuration that is favorable for binding to the promiscuous AHR (Murray et al., 2014). In this report, we show that some IDO1 inhibitors currently in testing in clinical trials can also act as AHR agonists. The prospect that a compound that inhibits IDO1 activity and reduces immune tolerance may also trigger a wide range of AHR-mediated effects, including altered immune cell differentiation and polarization and an upregulation of IDO1 expression, poses a significant clinical challenge. The question arises whether the observed clinical effects of an IDO-inhibiting drug are due to the inhibition of IDO activity and/or to the activation of AHR signaling.

2. Materials and methods

2.1. Materials

INCB024360 (INCB), NLG919 (NLG) (MCE, Monmouth Junction, NJ), Norharmane (Norh) (Santa Cruz Biotechnology, Dallas, TX), Kynurenine (Kyn), Kynurenic acid (KA), Quinolinic acid (QA), 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), 1-Methyl-L-Tryptophan (1MLT), and 1-Methyl-D-Tryptophan (1MDT) (Sigma Aldrich, St. Louis MO) were dissolved in dimethylsulfoxide (DMSO) and used at the indicated concentrations. For the HepG2 (40/6) treatments, 1MLT and 1MDT were dissolved in 50% DMSO. The P450-Glo™ Assay kit specific for CYP1A1 (catalog# V8752) was purchased from Promega (Madison, WI).

2.2. Cell culture

Murine Hepa-1c1c7 (Hankinson et al., 1991) and H1L7.5c3 hepatocyte cell lines, in which the latter is derived from Hepa-1c1c7 cells and has a stably transfected luciferase reporter gene regulated by a promoter with multiple AHR response elements (He et al., 2011) (courtesy of Dr. Michael Denison, University of California, Davis, CA), were cultured in alpha Minimum Essential Medium (Corning, Manassas, VA), supplemented with 10% FBS (Hyclone Laboratory, Logan, UT), 2 mM L-glutamine, 0.2% penicillin/streptomycin, and 2.2 g/l sodium bicarbonate (Sigma Aldrich). The HepG2 (40/6) human hepatoma stable cell line (Long et al., 1998) containing the stably integrated pGudluc 6.1 luciferase reporter construct under the control of the *CYP1A1* enhancer were cultured in α-modified essential media (Sigma-Aldrich) supplemented with 8% fetal bovine serum (Hyclone Laboratories), 100 IU/ml penicillin/100 μg/ml streptomycin (Sigma-Aldrich). The Hepa-1c1c7, H1L7.5c3, and HepG2 (40/6) cells were maintained at 37 °C and 5% CO_2. H1L7.5c3 cells were seeded in white-walled, white-bottomed 96-well plates (Corning, Manassas, VA) at 4000 cells/well and incubated for 24 h in culture medium. After the 24-h incubation, the medium was removed, and the cells were washed once with Dulbecco's Phosphate Buffered Saline (DPBS) (Corning). The Hepa-1c1c7 and H1L7.5c3 cells were treated for an additional 24 h with the reagents at the indicated concentrations. HepG2 (40/6) cells were seeded in 12-well plates and cultured to ~80% confluence before treatment for an additional 4 h with the reagents at the indicated concentrations. DMSO did not exceed 0.1% concentration in the culture medium.

2.3. Luciferase assays

Luciferase assays were carried out using the H1L7.5c3 and HepG2 (40/6) cells. At the conclusion of the indicated exposures, H1L7.5c3 cells were removed from incubation and allowed to equilibrate to room temperature for 15 min. After equilibration, the medium was removed and the cells were washed twice with at room temperature with DPBS. The cells were lysed with 20 μl/well 1 × Passive Lysis Buffer (Promega, Madison, WI) and shaken for 20 min at room temperature. Luciferase activity was recorded using an LMax Luminometer Microplate Reader (Molecular Devices, Sunnyvale, CA) programmed to inject 50 μl of Luciferase Assay Reagent (Promega, Madison, WI) per well with a 10 s integration of emitted luminescence. For the HepG2 (40/6) luciferase assays (Murray et al., 2010), cells were removed from incubation and lysed in 400 μl of 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% (v/v) glycerol, and 1% (v/v) TritonX-100]. Lysate (20 μl) was combined with 80 μl of Luciferase Reporter Substrate (Promega, Madison, WI), and luciferase activity was measured with a TD-20e Luminometer (Turner Designs, Sunnyvale, CA).

2.4. Quantitative real-time polymerase chain reaction assays

To verify the microarray results, qPCR analysis was carried out using primers designed for mouse AHR target *Cyp1a1* mRNA (Mm00487218_m1) and mouse reference *Gapdh* mRNA (Mm99999915_g1) purchased from ThermoFisher Scientific, Inc. (Waltham, MA). Approximately 5 μg of total RNA from each H1L7.5c3 cell culture (three biological replicates per treatment) served as template for the cDNA synthesis. The cDNA was synthesized using TaqMan® assay kits with the Superscript III First-Strand Synthesis System (ThermoFisher Scientific, Inc.). The qPCR reactions were performed using the Fast Advanced Master Mix (ThermoFisher Scientific, Inc.) on a BioRad CFX96 System using version 3.1 software (BioRad, Hercules, CA) set at 40 cycles. Assays to determine levels of DNA contamination were carried out by omitting reverse transcriptase and mRNA template from the reactions.

For the HepG2 (40/6) cells, primers (Integrated DNA Technologies, Coralville, IA) for qPCR analysis (Murray et al., 2010) were selected to detect human *CYP1A1* mRNA and ribosomal protein L13a mRNA as a reference (see Table 1 in Murray et al., 2010). PCR was performed on a MyiQ (Bio-Rad Laboratories, Hercules, CA) system using PerfeCTa SYBR Green reagent (Quanta Biosciences, Gaithersburg, MD). In all cases, melting point analysis revealed amplification of a single product. Data acquisition and analysis were carried out using MyIQ software (Bio-Rad Laboratories).

2.5. CYP1A1 enzyme activity assays

Hepa-1c1c7 cells were plated in 24-well plates and incubated overnight as described above for the H1L7.5c3 hepatocytes to a growth confluency of 75-90%. All treatments were carried out in quadruplicate. The cells were transferred to a new 24-well plate and treated with DMSO vehicle, 10 nM TCDD, and 10 μM of the different IDO1 inhibitors for 12 h. The cells in the vehicle and TCDD-treated wells were treated again with DMSO and the test cells treated again with 10 μM of the different IDO1 inhibitors for an additional 12 h. After the 24 hr treatments, the cells were washed and then incubated at 37 °C for 3 h in culture