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In vitro cytochrome P450- and transporter-mediated drug interaction potential of 6β-hydroxy-21-desacetyl deflazacort—A major human metabolite of deflazacort

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Abstract

6β-Hydroxy-21-desacetyl deflazacort (6β-OH-21-desDFZ) is a major circulating but not biologically active metabolite of deflazacort (DFZ). In vitro studies were performed to evaluate cytochrome P450 (CYP)- and transporter-mediated drug interaction potentials of 6β-OH-21-desDFZ. Up to 50 µM, the highest soluble concentration in the test system, 6β-OH-21-desDFZ weakly inhibited (IC$_{50}$ > 50 µM) the enzyme activity of CYPs 1A2, 2B6, 2C8, 2C9, and 2D6, while moderately inhibiting CYP2C19 and CYP3A4 with IC$_{50}$ values of approximately 50 and 35 µM, respectively. The inhibition was neither time-dependent nor metabolism-based. Incubation of up to 50 µM 6β-OH-21-desDFZ with plated cryopreserved human hepatocytes for 48 h resulted in no meaningful concentration-dependent induction of either mRNA levels or enzyme activity of CYP1A2, CYP2B6, or CYP3A4. In transporter inhibition assays, 6β-OH-21-desDFZ, up to 50 µM, did not show interaction with human OAT1, OAT3, and OCT2 transporters. It weakly inhibited (IC$_{50}$ > 50 µM) human MATE1, MATE2-K, and OCT1 transporter activity, and moderately inhibited human MDR1, OATP1B1, and OATP1B3 transporter activity with IC$_{50}$ values of 19.81 µM, 37.62 µM, and 42.22 µM, respectively. ^{14}C-6β-OH-21-desDFZ was biosynthesized using bacterial biotransformation and the subsequent study showed that 6β-OH-21-desDFZ was not a substrate for human BCRP, MDR1, MATE1, MATE2-K, OAT1, OATP1B1, OATP1B3, and OCT2 transporters, but appeared to be an in vitro substrate for the human OAT3 uptake transporter. At plasma concentrations of 6β-OH-21-desDFZ seen in the clinic, CYP- and transporter-mediated drug–drug interactions are not expected following administration of a therapeutic dose of DFZ in Duchenne muscular dystrophy (DMD) patients.

Abbreviations: A-B, apical to basolatera; B-A, basolateral to apical; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CYP450, cytochrome P450; DMD, Duchenne muscular dystrophy; DMSO, dimethyl sulfoxide; ER, efflux ratio; FDA, Food and Drug Administration; HBSS, Hank's buffered salt solution; HEK293, human embryonic kidney 293 cells; HLMs, human liver microsomes; IC$_{50}$, concentration of an inhibitor that causes a 50% decrease in enzyme or transporter activity; KH, Krebs–Henseleit; LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LSC, liquid scintillation counting; MATE, multidrug and toxin extrusion transporter; MDCKII cells, Madin–Darby canine kidney cells; MDR, multidrug resistance; MDR1, multidrug resistance protein 1; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; P$_{app}$, apparent permeability coefficient; P-gp, P-glycoprotein; UPT, uptake transporter (assay).

1 | INTRODUCTION

Deflazacort (DFZ), a glucocorticoid with anti-inflammatory and immunosuppressive effects, is used to treat a variety of diseases, including Duchenne muscular dystrophy (DMD). DFZ was approved by the U.S. Food and Drug Administration (FDA) as a treatment for DMD patients aged ≥2 years (https://www.emflaza.com/). Currently, DFZ is the only small molecule drug approved in the United States for the treatment of DMD. DMD is a muscle disease caused by X-linked mutations to the gene encoding dystrophin protein leading to recurrent muscle fiber damage during contraction. The inflammatory response to fiber damage is a compelling mechanism for disease exacerbation.[1] The incidence of DMD is approximately 1 in 3500 live male births.[2,3] Corticosteroids may be prescribed to improve muscle strength and slow disease progression by suppressing muscle inflammation.[4]

DFZ is an inactive pro-drug which is rapidly converted by esterases to the active metabolite 21-desacetyl deflazacort (21-desDFZ) after oral administration. 21-desDFZ is further metabolized mainly by CP3A4 to several other inactive metabolites.[5] 21-desDFZ and 6β-hydroxy-21-desacetyl deflazacort (6β-OH-21-desDFZ) are the major renally excreted metabolites together accounting for 59%, 66%, and 52% of the urinary radioactivity in rats, dogs, and humans, respectively.[6] 6β-OH-21-desDFZ was also reported to be a minor circulating metabolite in rats and dogs, but a major plasma metabolite in monkeys and humans following a single DFZ oral dose.[7] Along with other minor metabolites, 6β-OH-21-desDFZ and 21-desDFZ were confirmed to be the major circulating metabolites, accounting for 25% and 33% of 0–24 h plasma levels of total radioactivity in healthy male subjects following a single oral dose of 60 mg ^{14}C-DFZ.[8]

In transporter and metabolism study assays, radiolabeled substrates are commonly used and are preferred if available.[9,10] However, chemical synthesis of [14]C-6β-OH-21-desDFZ is challenging since multiple steps as well as stereospecific reactions are required and which are time-consuming and costly. Therefore, an alternative approach, microbial biotransformation, was used to generate [14]C-6β-OH-21-desDFZ. Due to the capability of a wide range of expression of drug-metabolizing enzymes, bacteria and fungi have been successfully used in generating human metabolites of interest that are challenging for chemical synthesis.[11]

6β-OH-21-desDFZ was demonstrated to be a major circulating metabolite in healthy male subjects following a single oral dose of DFZ.[6,8] Its pharmacokinetic drug interaction potential was not fully characterized and was subsequently required by the U.S. FDA post DFZ approval in 2017. The objective of this paper was to evaluate the in vitro CYP- and transporter-mediated drug interaction potentials of 6β-OH-21-desDFZ per the FDA guidance at the time of the study.[12] A novel approach to preparing radioactively labeled [14]C-6β-OH-21-desDFZ using microbial biotransformation for transporter substrate assay is also presented.

2 | MATERIALS AND METHODS

2.1 | Materials

Non-labeled 6β-OH-21-desDFZ was obtained from Toronto Research Chemicals and Santa Cruz Biotechnology. [14]C-6β-OH-21-desDFZ was biosynthesized using microbial biotransformation (details are provided in the Supporting Information). Human liver microsomes (HLMs) were purchased from BioIVT (pool of 25 donors). CYP and transporter inhibitors, substrates, internal standards, and specialty chemicals were purchased from Corning Gentest, Sigma, Toronto Chemical Research, and Santa Cruz Biotechnology. General reagents were purchased from Thermo Fisher Scientific or VWR. Plateable cryopreserved hepatocytes from three different human donors and incubation medium were provided by BioIVT. Inside-out membrane vesicles prepared from HEK293 cells overexpressing human ABC transporters, MDCKII or HEK293 cells stably expressing the respective uptake transporters, and control and BCRP- or MDR1-transfected MDCKII cells were obtained from SOLVO Biotechnology.

2.2 | Methods

2.2.1 | Biosynthesis of [14]C-6β-OH-21-desDFZ

The experimental details are summarized in the Supporting Information. Briefly, a set of 19 bacterial strains and 18 fungal strains were screened for production of 6β-OH-21-desDFZ using DFZ as substrate. The highest yielding bacterial strain ("Sp1", a proprietary bacterial strain of the Order *Actinomycetales*) was selected for progression to scale-up production which led to the isolation and purification of 7.9 mg of >95% pure metabolite. The identity of the isolated metabolite was confirmed by LC–MS/MS and nuclear magnetic resonance (NMR) analysis, and comparison to authentic commercial standards of both 6α-OH-21-desDFZ and 6β-OH-21-desDFZ isomers to confirm unequivocally the microbial biotransformation product as 6β-OH-21-desDFZ. Production of [14]C-6β-OH-21-desDFZ was achieved by performing the same fermentation steps using ^{14}C-DFZ as starting material. Iterative preparative LC isolation and purification resulted in the production of 62.1 μCi [14]C-6β-OH-21-desDFZ, with specific activity of 57.8 mCi/mmol, radiochemical purity of 99.2%, and chemical purity of 98.7%.

2.2.2 | CYP inhibition and induction

CYP inhibition

6β-OH-21-desDFZ was separately incubated with CYP probe substrates, phenacetin (CYP1A2), bupropion (CYP2B6), paclitaxel (CYP2C8), diclofenac (CYP2C9), S-mephenytoin (CYP2C19), dextromethorphan (CYP2D6), midazolam (CYP3A4), and testosterone (CYP3A4) in pooled HLMs with and without preincubation in the presence and absence of NADPH for 30 min at 37°C. For the direct inhibition assays, the reactions were initiated by adding the substrate and NADPH solution and incubating at 37°C for 5 ± 2 min with no preincubation. For the time-dependent and mechanism-based inhibition assays, 6β-OH-21-desDFZ was incubated with HLMs (0.5 mg/ml, 0.25 mg/ml for midazolam) in the presence of phosphate-buffered saline, pH 7.4, or NADPH at 37°C for 30 min. After the preincubation, the incubation mixtures were diluted 10-fold by adding the substrate/NADPH solution and incubating at 37°C for 5 ± 2 min. The final incubation concentrations were 0.02, 0.07, 0.2, 0.6, 1.9, 5.6, 16.7, and 50.0 μM for 6β-OH-21-desDFZ; 0.05 mg/ml (0.0250 mg/ml for midazolam) protein concentrations for HLMs in phosphate-buffered saline, pH 7.4, and the total organic solvent (acetonitrile, methanol, or ethanol) was <0.25% (v/v). At the completion of incubation, the reactions were terminated by the addition of acetonitrile with internal standard and the metabolite of each specific CYP substrate was quantified using a validated UPLC-MS/MS method. The UPLC-MS/MS system consisted of an Acquity UPLC coupled with a Xevo TQ-S Mass Spectrometer. An ACE Excel 5 C18 PFP (100 × 2.1 mm, 5.0 μm) column (MAC-MOD Analytical, Inc.) was maintained at 40°C. The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in methanol. The linear gradients were: 20% B for 0.2 min and increased to 84% B in 9.8 min at a flow rate of 0.3 ml/min; 84% to 95% B in 0.1 min and maintained at 95% B for 1 min at a flow rate of 0.6 ml/min; 95% to 20% B in 0.1 min and maintained at 20% for 0.6 min at a flow rate of 0.6 ml/min; the flow rate was decreased to 0.3 ml/min and held at 20% B for 0.1 min. For the mass spectrometer, the source and desolvation temperatures were set at 150°C and 650°C, respectively. The IC_{50} values were calculated using the four-parameter fitting routine in GraFit 7.0 (Erithacus Software).

CYP induction

Plated cryopreserved human hepatocytes from three human donors were incubated with 0.002, 0.010, 0.050, 0.250, 1.25, 5.00, 10.0, and 20.0 μM of 6β-OH-21-desDFZ in hepatocyte incubation medium for 48 h. Omeprazole, phenobarbital, and rifampin were used as positive induction controls for CYP1A2, CYP2B6, and CYP3A4, respectively, and gatifloxacin was included as a negative induction control. Briefly, on Day 1, plateable cryopreserved hepatocytes were suspended in hepatocyte plating medium and were added to individual wells of a 96-well collagen-coated plate at a concentration of 0.5 × 10⁶ viable cells/ml. On Day 2, after 24 h incubation at 37°C, 95% humidity, and 5% CO_2, the medium was removed from each well, and 100 μl of the ECM gel solution at 0.25 mg/ml was added.

The covered plates were incubated at 37°C, 95% humidity, and 5% CO_2. On Days 3 and 4, the medium was removed from each well, and 100 μl of the test article working solutions, the positive control solutions, vehicle controls, and negative control solutions were added in triplicate. The covered plates were incubated at 37°C, 95% humidity, and 5% CO_2. On Day 5, the medium was removed from each well, and 100 μl of the probe substrate solutions was added in triplicate to the appropriate plates. The covered plates were incubated at 37°C, 95% humidity, and 5% CO_2. After 20 min incubation, samples of the medium were removed and analyzed for the metabolites of the probe substrates using UPLC-MS/MS as described in the CYP inhibition section. Total mRNA from each well was isolated using the Agencourt RNAdvance Cell v2 kit (Beckman Coulter). The mRNA was reverse-transcribed into cDNA using the High Capacity RNA-to-cDNA kit from Applied Biosystems. PCR quantification of the cDNA was performed using the ViiA7TM Real-Time PCR system from Applied Biosystems. CYP1A2, CYP2B6, and CYP3A4 mRNA were quantified using TaqMan® Gene Expression Assays with the TaqMan® Fast Advanced Master Mix (Applied Biosystems). GAPDH was used as the endogenous control (Applied Biosystems). The ViiA7 software was used to normalize the cycle thresholds for the CYP mRNA in each well and quantify the amount of the CYP mRNA in the wells with compounds relative to the wells with vehicle alone.

2.2.3 | Transporter interaction studies

Transporter inhibition assays

6β-OH-21-desDFZ and the probe substrate were incubated in triplicate with inside-out membrane vesicles prepared from HEK293 cells overexpressing human BCRP, BSEP, or MDR1 transporter (total protein: 50 μg/well or 25 μg/well for BCRP). Incubations were carried out in the presence of 4 mM ATP or AMP to distinguish between transporter-mediated uptake and passive diffusion into the vesicles. 6β-OH-21-desDFZ at 0.07, 0.2, 0.6, 1.9, 5.6, 16.7, and 50 μM was added to the reaction mixture in DMSO (1% of the final incubation volume). Reaction mixtures were preincubated for 15 min at 37 ± 1°C (or 32 ± 1°C for BCRP and MDR1). Reactions were initiated by the addition of 25 μl of 12 mM ATP or 12 mM AMP and were quenched by the addition of 200 μl of ice-cold washing buffer and immediate filtration via glass fiber filters mounted to a 96-well filter plate. The filters were washed (5 × 200 μl of ice-cold washing buffer), dried, and the amount of substrate inside the filtered vesicles was determined by liquid scintillation counting (LSC). Probe substrates used were 1 μM ³H-estrone-3-sulfate for BCRP, 0.2 μM ³H-taurocholic acid for BSEP, and 1 μM ³H-N-methyl-quinidine for MDR1.

Uptake experiments were performed using MDCKII or HEK293 cells stably expressing the respective human uptake transporters, that is, OAT1, OAT3, OCT1, OCT2, OATP1B1, OATP1B3, MATE1, and MATE2-K. Cells were cultured at 37 ± 1°C in an atmosphere of 95:5 air:CO_2 and were plated onto standard 96-well tissue culture plates at 1 × 10⁵ cells/ml. Prior to the experiment, the medium was removed, and the cells were washed twice with 100 μl of assay

buffer (KH pH 7.4 or pH 8.0, or HBSS pH 7.4). Uptake experiments were carried out at 37 ± 1°C in 50 µl in the respective buffer containing the probe substrate and 6β-OH-21-desDFZ or solvent. The organic solvent concentration was equal in all wells and did not exceed 1.5% (v/v). The final concentrations of 6β-OH-21-desDFZ were at 0.07, 0.2, 0.6, 1.9, 5.6, 16.7, and 50 µM. Substrates used were: tenofovir for OAT1, estrone-3-sulfate for OAT3, metformin for OCT1 and OCT2, β-estradiol 17-β-D-glucuronide for OATP1B1, cholecystokinine-8 for OATP1B3, and metformin for MATE1 and MATE2-K. After the experiment, the cells were washed twice with 100 µl of cold appropriate buffer and lysed with 50 µl of 0.1 M sodium hydroxide. The extent of radiolabeled probe substrate transport was determined by measuring an aliquot from each well for LSC.

Transporter substrate assays

MDCKII monolayer assays. Control, MDCKII-BCRP, and MDCKII-MDR1 cells were cultured in Dulbecco's Modified Eagle Medium with 4.5 g/l glucose supplemented with 10% (v/v) fetal bovine serum at 37 ± 1°C in an atmosphere of 95:5 air:CO_2 in cell culture flasks prior to seeding into 24-transwell inserts. Cells were cultured on the inserts with 400 µl medium per well on the apical side and 25 ml in a single-well receiver tray for all 24 wells on the basolateral side for 96 h. Medium was changed 24 h before the experiment. Transepithelial electric resistance of each well was measured to confirm the confluency of the monolayers after the experiments. Permeability incubations were carried out in Hank's Buffered Salt Solution (HBSS) at 37 ± 1°C. Bidirectional transport of ^{14}C-6β-OH-21-desDFZ was determined through control, MDCKII-BCRP, and MDCKII-MDR1 cell monolayers. Cells were preincubated in assay buffer for 10 min to allow cells to adjust to the medium. Assay buffer containing ^{14}C-6β-OH-21-desDFZ at four concentrations (0.5, 1, 2.5, and 5 µM) was then added to the appropriate apical (400 µl) or basolateral chamber (800 µl). The final concentration of DMSO in the incubations did not exceed 0.5% (v/v). The prazosin or digoxin efflux ratio was determined as a positive control for BCRP or MDR1 function. After incubation at 37 ± 1°C, aliquots (100 µl) were taken from the receiver chambers to determine the amount of translocated ^{14}C-6β-OH-21-desDFZ and controls. Bidirectional transport of ^{14}C-6β-OH-21-desDFZ in control, MDCKII-BCRP, and MDCKII-MDR1 cells was determined by LSC. The relative activity was calculated as:

$$\text{Relative activity \%} = \frac{A - B}{C - D} \times 100$$

where for vesicular transport inhibition assays, A is the amount of translocated substrate in the presence of test compound and ATP, B is the amount of translocated substrate in the presence of test compound and AMP, C is the amount of translocated substrate in the presence of solvent and ATP, and D is the amount of translocated substrate in the presence of solvent and AMP. While for uptake transporter inhibition assays, A is the amount of translocated substrate in the presence of test compound in transfected cells, B is the amount of translocated substrate in the presence of test compound in control cells, C is the

amount of translocated substrate in the presence of solvent in transfected cells, and D is the amount of translocated substrate in the presence of solvent in control cells. IC_{50} values were derived from a four-parametric logistic equation as shown below:

$$V = Bottom + \frac{Top - Bottom}{\left([I]/IC_{50}\right)^n}$$

where V is the velocity of transporter activity, *Top* is the maximal response value, *Bottom* is the maximally inhibited response value, $[I]$ is the inhibitor concentration, IC_{50} represents the value at which transporter activity is inhibited by 50%, and n is a Hill coefficient.

The curve was fitted to the relative activity versus test compound concentration plot using non-linear regression.

Uptake transporter substrate assays. Uptake experiments were performed using MDCKII or HEK293 cells stably expressing the respective human uptake transporters, that is, MATE1, MATE2-K, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2. Cells were cultured at 37 ± 1°C in an atmosphere of 95:5 (air:CO_2) and were plated onto standard 24-well tissue culture plates at a density 5 × 10^5 cells/well. The uptake of the ^{14}C-6β-OH-21-desDFZ was determined at two incubation time points (2 and 20 min) and at four concentrations (0.5, 1, 1.25, and 5 µM) of ^{14}C-6β-OH-21-desDFZ. In order to confirm the interaction, where applicable, the transporter-specific uptake of ^{14}C-6β-OH-21-desDFZ was determined in the presence of a known inhibitor of the respective transporter. Before the experiment, the medium was removed, and the cells were washed twice with 300 µl of KH (pH 7.4 or 8) or HBSS buffer (pH 7.4). Cellular uptake of ^{14}C-6β-OH-21-desDFZ into the cells was measured by adding 300 µl of assay buffer containing ^{14}C-6β-OH-21-desDFZ and incubating them at 37 ± 1°C. Reactions were quenched by removing the assay buffer and the cells were washed twice with 300 µl of assay buffer. Cells were lysed by adding 300 µl of 0.1 M sodium hydroxide and incubated for 10 min at 37 ± 1°C. The amount of ^{14}C-6β-OH-21-desDFZ in the cell lysate was determined by LSC. The amount of protein in each well was quantified using the BCA kit for protein determination (Sigma-Aldrich, St Louis, MO, USA). The fold accumulation value for uptake transporter substrate assays was defined as the ratio of uptake of ^{14}C-6β-OH-21-desDFZ or probe substrate into transfected and control cells. The following equation was used to calculate the apparent permeability coefficient (P_{app}):

$$P_{app} = \frac{dQ}{dT} \times \frac{1}{A \times C_0}$$

where dQ is the amount of transported test compound, dT is the incubation time, A is the surface of porous membrane in cm^2, and C_0 is the initial concentration of the compound in the donor compartment.

The efflux ratio (ER) is given as:

$$ER = \frac{P_{app} B - A}{P_{app} A - B}$$

For MDCKII-MDR1/BCRP cells, net efflux ratios were calculated as:

$$\text{net ER} = \text{ER}_T - \text{ER}_P$$

where ER_T is the efflux ratios in the transfected cells and ER_P is the efflux ratios in the parental cells.

3 | RESULTS

3.1 | CYP inhibition

CYP inhibition potential of 6β-OH-21-desDFZ (up to 50 μM, the highest soluble concentration in the test system) was investigated in pooled HLMs with and without preincubation in the presence and absence of NADPH for 30 min at 37°C (Table 1). 6β-OH-21-desDFZ exhibited modest inhibition for 2C19 and 3A4 (with midazolam as probe substrate) with IC_{50} values of approximately 50 and 35 μM, respectively, and weak inhibition for 1A2, 2B6, 2C8, 2C9, 2D6, and 3A4 (with testosterone as probe substrate) with estimated IC_{50} values greater than the highest tested concentration of 50.0 μM. In the assessment of preincubation of 6β-OH-21-desDFZ in the absence and presence of NADPH, no shift in the IC_{50} values was noted for any of the tested isoforms. Thus, 6β-OH-21-desDFZ did not appear to be a time-dependent or mechanism-based inhibitor of the tested CYP isoforms.

3.2 | CYP induction

Hepatocytes from all three human donors used in the study responded to exposure of all the prototypical inducers at both the level of mRNA and enzyme activity. 6β-OH-21-desDFZ at all concentrations tested (0.002, 0.01, 0.05, 0.25, 1.25, 5.00, 10.0, and 20.0 μM) did not show concentration-dependent induction response for CYP1A2, CYP2B6, and CYP3A4 at the level of mRNA or enzyme

activity (Table 2). Thus, 6β-OH-21-desDFZ did not appear to be an inducer of CYP1A2, CYP2B6, and CYP3A4 in vitro.

3.3 | Transporter inhibition assays

6β-OH-21-desDFZ up to 50 μM (the highest soluble concentration in the test system) was evaluated for its potential to inhibit transporter activity using inside-out membrane vesicles prepared from HEK293 cells overexpressing human BCRP, BSEP, or MDR1 transporter and using MDCKII or HEK293 cells stably expressing the respective human uptake transporters, that is, OAT1, OAT3, OCT1, OCT2, OATP1B1, OATP1B3, MATE1, and MATE2-K. 6β-OH-21-desDFZ did not show interaction with most of the transporters investigated with the exception of the slight inhibition of MDR1, OATP1B1, and OATP1B3 transporter activity (Table 3).

3.4 | Transporter substrate assays

Bidirectional permeability of [14]C-6β-OH-21-desDFZ was evaluated using control (mock-transfected cells for BCRP and parental cells for MDR1) and BCRP- or MDR1-transfected MDCKII cell monolayers (Table 4). [14]C-6β-OH-21-desDFZ did not show higher permeability in the B-A direction than in the A-B direction, indicating that there was no active transport of this compound in the MDCKII-BCRP or MDCKII-MDR1 cells. The highest observed net efflux ratio was 0.17 at 2.5 μM in the case of BCRP and 0.59 at 1 and 5 μM in the case of MDR1.

Substrate potential of [14]C-6β-OH-21-desDFZ for uptake transporters was evaluated using MDCKII or HEK293 cells stably expressing the respective human uptake transporters, that is, MATE1, MATE2-K, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2. Transporter-specific accumulation of [14]C-6β-OH-21-desDFZ, 2.71-fold at 5 μM and 20 min, was observed in OAT3-expressing cells. The OAT3 inhibitor, probenecid, decreased the transporter-specific accumulation of [14]C-6β-OH-21-desDFZ to 1.08-fold, indicating that [14]C-6β-OH-21-desDFZ is an OAT3 substrate in vitro. In contrast, accumulation of [14]C-6β-OH-21-desDFZ

TABLE 1 Inhibition of CYP activities by 6β-hydroxy-21-desacetyl deflazacort (n = 3)

CYP	IC$_{50}$ (μM)		
	No preincubation	Preincubation/PBS	Preincubation/NADPH
CYP1A2	>50	>50	>50
CYP2B6	>50	>50	>50
CYP2C8	>50	>50	>50
CYP2C9	>50	>50	>50
CYP2C19	~50	48.0	>50
CYP2D6	>50	>50	>50
CYP3A4 (Midazolam)	35	>50	>50
CYP3A4 (Testosterone)	>50	>50	>50

Abbreviations: CYP, cytochrome P450; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; PBS, phosphate-buffered saline.

TABLE 2 CYP induction potential of 6β-hydroxy-21-desacetyl deflazacort using mRNA and enzyme activity as the endpoints

| | CYPs | 1A2 | 2B6 | 3A4 | | | | | | |
| | Individual subjects | AKB | GQJ | LYZ | AKB | GQJ | LYZ | AKB | GQJ | LYZ |
Test compound	Concentration (μM)	mRNA fold induction (n = 3)								
6β-OH-21-desDFZ	0.002	0.978	0.657	0.665	0.643	0.680	0.704	0.739	0.450	0.562
	0.01	0.871	0.735	0.744	0.522	0.750	0.745	0.714	0.583	0.484
	0.05	0.928	0.735	0.701	0.571	0.736	0.721	0.739	0.507	0.450
	0.25	0.979	0.667	0.610	0.622	0.794	0.726	0.817	0.681	0.684
	1.25	0.856	0.641	0.612	0.482	0.613	0.706	0.622	0.510	0.502
	5	0.786	0.628	0.635	0.515	0.616	0.791	0.856	0.647	0.615
	10	0.840	0.619	0.647	0.596	0.663	0.806	1.02	0.890	0.764
	20	0.886	0.741	0.723	0.809	0.756	0.840	1.95	1.74	1.33
Omeprazole	100	34.8	39.6	40.5	NA	NA	NA	NA	NA	NA
Phenobarbital	750	NA	NA	NA	12.9	11.2	10.2	NA	NA	NA
Rifampin	10	NA	NA	NA	NA	NA	NA	20.9	39.0	27.9
Gatifloxacin	10	1.12	0.941	0.898	0.685	0.965	0.989	0.591	0.612	1.18
		Enzyme activity fold induction (n = 3)								
6β-OH-21-desDFZ	0.002	0.913	0.783	1.01	0.960	0.921	0.836	0.601	0.600	0.626
	0.01	0.880	0.671	0.892	0.895	0.826	0.829	0.635	0.595	0.543
	0.05	0.838	0.649	0.812	0.944	0.767	0.829	0.657	0.604	0.545
	0.25	0.832	0.753	0.747	0.963	0.752	0.810	0.769	0.685	0.668
	1.25	1.27	0.752	0.909	1.02	0.705	0.883	0.593	0.547	0.524
	5	1.18	0.770	0.870	1.22	0.832	1.01	0.690	0.451	0.542
	10	1.07	0.772	0.954	1.20	0.814	1.13	0.637	0.446	0.563
	20	1.18	0.699	0.955	1.40	0.896	1.08	0.856	0.561	0.637
Omeprazole	100	5.48	3.55	8.36	NA	NA	NA	NA	NA	NA
Phenobarbital	750	NA	NA	NA	9.73	11.7	5.58	NA	NA	NA
Rifampin	10	NA	NA	NA	NA	NA	NA	12.5	22.0	19.4
Gatifloxacin	10	0.805	0.744	0.996	1.05	0.719	0.875	0.861	0.881	1.44

Abbreviations: 6β-OH-21-desDFZ, 6β-hydroxy-21-desacetyl deflazacort; CYP, cytochrome P450; mRNA, messenger ribonucleic acid; NA, not applicable.

was similar in the MATE1-, MATE2-K-, OAT1-, OATP1B1-, OATP1B3- and OCT2-expressing cells and in the control cells (transporter-specific fold accumulations were <2), indicating no active accumulation of [14]C-6β-OH-21-desDFZ under the conditions tested.

4 | DISCUSSION AND CONCLUSION

6β-OH-21-desDFZ was reported to be a prominent but biologically inactive metabolite of DFZ in rats, dogs, monkeys, and humans following a single DFZ oral dose.[7] Following a single oral dose of 60 mg ^{14}C-DFZ in healthy male subjects, 6β-OH-21-desDFZ accounted for 25% of 0-24 h plasma total radioactivity.[8] As a major circulating metabolite in humans, the in vitro CYP- and transporter-mediated drug interaction potentials of 6β-OH-21-desDFZ were systematically evaluated per the FDA guidance at the time of the study.[12]

Both direct and time-dependent and mechanism-based inhibition potentials of CYPs were evaluated in HLMs. Of the CYP enzymes tested, 6β-OH-21-desDFZ exhibited modest inhibition of 2C19 and 3A4 (with midazolam as probe substrate) with IC_{50} values of approximately 50 and 35 μM, respectively, and weak inhibition of 1A2, 2B6, 2C8, 2C9, 2D6, and 3A4 (with testosterone as probe substrate) with estimated IC_{50} values greater than the highest tested concentration of 50.0 μM. 6β-OH-21-desDFZ did not appear to be a time-dependent or mechanism-based inhibitor of the CYP isoforms tested since no IC_{50} shift was noted after preincubation of 6β-OH-21-desDFZ in the absence and presence of NADPH (Table 1). In human hepatocytes, up to 20.0 μM 6β-OH-21-desDFZ showed no concentration-dependent induction of CYP1A2, CYP2B6, or CYP3A4 at levels of mRNA or enzyme activity (Table 2). In transporter inhibition studies, up to 50 μM 6β-OH-21-desDFZ showed no inhibitory interaction with human efflux (BCRP and BSEP) and

uptake (OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K) transporters. However, 6β-OH-21-desDFZ modestly inhibited MDR1, OATP1B1, and OATP1B3 transporter activity with estimated IC_{50} values of 19.8, 36.7, and 42.2 μM, respectively (Table 3).

For high specificity of transporter-mediated substrate accumulation, ^{14}C-6β-OH-21-desDFZ was biosynthesized using a proprietary "SP1" bacterial strain of the Order *Actinomycetales* and was applied in the subsequent transporter substrate evaluation. In bidirectional permeability assay using BCRP- or MDR1-transfected MDCKII cell monolayers, ^{14}C-6β-OH-21-desDFZ did not show higher permeability in the B-A direction than in the A-B direction, indicating that

TABLE 3 Inhibition of transporter activities by 6β-hydroxy-21-desacetyl deflazacort (*n* = 3)

Transporter and assay type	Maximum inhibition at 50 μM (% of control)	IC_{50} (μM)
BCRP VT	<20	NA
BSEP VT	27	NA
MDR1 VT	78	19.8 (15.9–24.7)[a]
MATE1 UPT	26	NA
MATE2-K UPT	26	NA
OAT1 UPT	<20	NA
OAT3 UPT	<20	NA
OATP1B1 UPT	67	36.7 (12.2–111)[a]
OATP1B3 UPT	57	42.2 (33.9–52.6)[a]
OCT1 UPT	23	NA
OCT2 UPT	<20	NA

Abbreviations: BCRP, breast cancer resistance protein; BSEP, bile salt export pump; IC_{50}, concentration of an inhibitor that causes a 50% decrease in enzyme or transporter activity; MATE, Multidrug and toxin extrusion transporter; MDR1, multidrug resistance protein 1; NA, not applicable; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; UPT, uptake assay; VT, vesicular transport assay.
[a]95% confidence interval.

there was no BCRP- or MDR1-mediated active transport of ^{14}C-6β-OH-21-desDFZ. In MDCKII or HEK293 cells stably expressing the respective human uptake transporters, transporter-specific accumulation of ^{14}C-6β-OH-21-desDFZ was only observed in OAT3-expressing cells, but not in MATE1-, MATE2-K, OAT1-, OATP1B1-, OATP1B3-, and OCT2-expressing cells in which similar levels of ^{14}C-6β-OH-21-desDFZ accumulation were observed in transporter-expressing cells as in the control cells indicating no active transport of ^{14}C-6β-OH-21-desDFZ by these transporters (Table 4). Although 6β-OH-21-desDFZ showed substrate potential for OAT3 in vitro, given the lower administered dose (0.9 mg/kg), route of elimination, and large therapeutic window of DFZ, clinical DDI due to OAT3 inhibition is not meaningful.

Since a very low concentration of liver microsomal protein (0.05 mg/ml [0.0250 mg/ml for midazolam]) was used for IC_{50} determinations in the CYP inhibition assays, 6β-OH-21-desDFZ at the estimated IC_{50} concentration or at the highest soluble concentration used in the assay is considered to reflect a free concentration. The same is assumed to be true for transporter interaction assay in which 6β-OH-21-desDFZ up to its highest soluble concentration in the buffer was used for assay of transporter activity.

Following repeat daily administration of DFZ at a dose at 0.9 mg/kg/day in DMD patients (children 4 to 11 years old and adolescents 12 to 16 years old), the plasma C_{max} of 6β-OH-21-desDFZ at steady state was 125 ng/ml and 159 ng/ml in children and adolescents, respectively, with a mean value of 142 ng/ml (0.34 μM) in combined patient populations (PTC internal data). Although 6β-OH-21-desDFZ showed weak inhibition of CYP2C19, CYP3A4, MDR1, OATP1B1, and OATP1B3 activities in vitro with IC_{50} values of 50, 35, 19.8, 37.6, and 42.2 μM, respectively, the corresponding estimated plasma C_{max}/IC_{50} ratios were 0.007, 0.01, 0.017, 0.009, and 0.008 for CYP2C19, CYP3A4, MDR1, OATP1B1, and OATP1B3, respectively. To be conservative, assuming 6β-OH-21-desDFZ in human plasma is 100% free (as a reference, the protein binding of 21-desDFZ in human plasma is

TABLE 4 Substrate potential of 6β-hydroxy-21-desacetyl deflazacort for human transporters (*n* = 3)

Transporter and assay type	Maximum net ER or fold accumulation over control	Substrate potential
BCRP ML	0.17 at 2.5 μM	No
MDR1 ML	0.59 at 1 and 5 μM	No
MATE1 UPT	1.18 at 1 μM and 2 min	No
MATE2-K UPT	1.08 at 2.5 μM and 20 min	No
OAT1 UPT	1.15 at 2.5 μM and 20 min	No
OAT3 UPT	2.71 at 5 μM and 20 min. Inhibitor-reduced accumulation from 1.75 to 1.08	Yes
OATP1B1 UPT	1.02 at 2.5 μM and 20 min	No
OATP1B3 UPT	1.47 at 1 μM and 20 min	No
OCT2 UPT	1.32 at 1 μM and 20 min	No

Abbreviations: BCRP, breast cancer resistance protein; MATE, Multidrug and toxin extrusion transporter; MDR1, multidrug resistance protein 1; ML, monolayer assay; net ER, net efflux ratio; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; UPT, uptake assay.

about 40%).[5] These C_{max}/IC_{50} ratios are all lower than the corresponding cut-off values (0.02 for CYP2C19 and CYP3A4, and 0.1 for MDR1, OATP1B1, and OATP1B3) set for these CYP enzymes and transporters as a static model per the most recent FDA guidance.[10] Therefore, at the therapeutic dose of DFZ, inhibition of CYP2C19, CYP3A4, MDR1, OATP1B1, and OATP1B3 activities by 6β-OH-21-desDFZ metabolite is not expected in the clinic.

There is extensive clinical experience for DFZ in DMD patients and in patients with other inflammatory conditions, and its exposure levels are well studied. The side effects of DFZ in humans are well characterized and consistent with the glucocorticoid class, but DFZ is associated with better efficacy and less weight gain compared to that seen with prednisone.[4,13,14]

In summary, 6β-OH-21-desDFZ as a major circulating metabolite in humans was shown to be an in vitro substrate for human OAT3. 6β-OH-21-desDFZ did not induce mRNA expression or enzyme activity of CYP1A2, CYP2B6, and CYP3A4. Furthermore, 6β-OH-21-desDFZ did not show meaningful inhibitory interactions with most of the CYPs and transporters investigated. 6β-OH-21-desDFZ modestly inhibited CYP2C19, CYP3A4, MDR1, OATP1B1, and OATP1B3 activities ($IC_{50} \geq 20$ μM). On the basis of the low plasma exposure of 6β-OH-21-desDFZ (142 ng/ml or 0.34 μM) at the therapeutic dose of DFZ and low potency of inhibition observed in vitro, clinically relevant drug–drug interactions of 6β-OH-21-desDFZ mediated by CYPs and transporters are not expected.

ETHICS STATEMENT

All studies were conducted in accordance with all applicable ethical requirements.

DISCLOSURES

The study was sponsored by PTC Therapeutics, Inc. RK, BB, and JM are current employees of PTC Therapeutics. JMC was formerly employed by PTC and is currently a consultant for PTC.

AUTHOR CONTRIBUTIONS

JM and RK participated in research design; JM and RK conducted experiments; RM and SR synthesized ^{14}C-6β-OH-21-desDFZ; JM and RK performed data analysis, and JM, BB, RM, SR, JMC, and RK wrote or contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.