Application of a Deuterium Replacement Strategy to Modulate the Pharmacokinetics of 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylpyrazolo[5,1-b]oxazole, a Novel CRF₁ Antagonist

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ABSTRACT

Deuterium isotope effects were evaluated as a strategy to optimize the pharmacokinetics of 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylpyrazolo[5,1-b]oxazole (NVS-CRF38), a novel corticotropin-releasing factor receptor 1 (CRF₁) antagonist. In an attempt to suppress O-demethylation of NVS-CRF38 without losing activity against the CRF₁ receptor, the protons at the site of metabolism were replaced with deuterium. For in vitro and in vivo studies, intrinsic primary isotope effects (Kᵢ/K₀) were determined by the ratio of intrinsic clearance (CLᵢn) obtained for NVS-CRF38 and deuterated NVS-CRF38. In vitro kinetic isotope effects (Kᵢ/K₀) were more pronounced when CLᵢnt values were calculated based on the rate of formation of the O-desmethyl metabolite (Kᵢ/K₀ ∼7) compared with the substrate depletion method (Kᵢ/K₀ ∼2). In vivo isotope effects were measured in rats after intravenous (1 mg/kg) and oral (10 mg/kg) administration. For both administration routes, isotope effects calculated from in vivo CLᵢnt corresponding to all biotransformation pathways were lower (Kᵢ/K₀ ∼2) compared with CLᵢnt values calculated from the O-demethylation reaction alone (Kᵢ/K₀ ∼7). Comparative metabolite identification studies were undertaken using rat and human microsomes to explore the potential for metabolic switching. As expected, a marked reduction of the O-demethylated metabolite was observed for NVS-CRF38; however, levels of NVS-CRF38’s other metabolites increased, compensating to some extent for the isotope effect.

Introduction

For many years, scientists have used the kinetic deuterium isotope effect, whereby C-H bonds are substituted for more stable C-D bonds, to investigate drug metabolism reactions (Parli and McMahon, 1973; Obach, 2001). Potentially, this approach offers an opportunity to modulate the in vivo disposition and pharmacokinetics of molecules while maintaining selectivity and potency for a given target.

Deuterium replacement strategies have been applied to enzyme systems, including cytochrome P450 enzymes (Krauser and Guengerich, 2005; Kim et al., 2006), monoamine oxidase (Belbeu et al., 1961; Ottoboni et al., 1989), aldehyde oxidase (Sharma et al., 2012), and alcohol/alddehyde dehydrogenase (Ghanayem et al., 1987). The goal of such strategies includes extension of elimination half-life, optimization of dose and dosing regimen, and mitigation of risks associated with drug-drug interactions (Tanabe et al., 1969; Shao et al., 2006). This strategy has attracted significant commercial interest and has been the subject of recent reviews (Shao and Hewitt, 2010; Guengerich, 2013).

Deuterium isotope strategies will not solve pharmacokinetic issues for every compound; the success of this approach depends on several factors, including the enzymology of drug metabolism and elimination mechanisms for a given compound. For some drug metabolism reactions, C-H bond cleavage may not be the rate-limiting step; rate-limited product release has been proposed to account for the lack of isotope effects for cytochrome P450 2E1 catalyzed ethanol oxidation (Bell and Guengerich, 1997). Contribution to drug metabolism by enzyme systems which are not amenable to deuterium isotope effects may confound this approach. This limitation has been exemplified by in vitro and in vivo studies on the aldehyde oxidase substrates carbazeran and zoniporide (Sharma et al., 2012). For these compounds, marked isotope effects were observed in vitro using liver cytosol; however, in hepatocytes and in vivo, these effects were attenuated by the involvement of other enzyme systems or elimination mechanisms. In vivo deuterium replacement strategies may be confounded by metabolic switching. This term describes the situation whereby slowing a specific drug metabolism reaction with deuterium results in metabolism being diverted to other (albeit previously minor) pathways. The challenges associated with metabolic switching are nicely exemplified with in vitro microsomal studies for the antiviral drug boceprevir; for this compound,

ABBREVIATIONS: AUC₀→∞, area under the plasma concentration-time curve calculated by the trapezoidal rule and extrapolated to infinity; CL₀, total intravenous blood clearance; CLᵢn, intrinsic clearance; CRF₁, corticotropin-releasing factor receptor 1; DMSO, dimethylsulfoxide; d-NVS-CRF38, [¹³CD₃] NVS-CRF38; fm, fraction of drug systemically available that is converted to a metabolite; Kᵢ/K₀, intrinsic primary isotope effect; LC-MS/MS, liquid chromatography–tandem mass spectrometry; O-desmethyl NVS-CRF38, 4-(7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-2,6-dimethylpyrazolo[5,1-b]oxazole-3-yl)-3-methylphenol; P450, cytochrome P450; Vₛₛₚ, steady-state volume of distribution.
simultaneous deuteration at five different positions was required to achieve a modest decrease in rat liver microsome intrinsic clearance (Morgan et al., 2011). Isotope replacement strategies have also been used as an approach to minimize the exposure of undesirable metabolites; several examples are provided in the review by Nelson and Trager (2003).

For deuterated drugs, unpredictable translation of isotope effects from in vitro drug metabolism systems to the in vivo situation is a key challenge; often, little change is observed in terms of in vivo clearance. We recently evaluated the preclinical pharmacokinetics and metabolism of 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylpyrazolo[5,1-b]oxazole (NVS-CRF38), a novel corticotropin-releasing factor receptor 1 (CRF1) antagonist (Fig. 1). The aim of this study was to evaluate the impact of deuteration as a strategy to modulate the pharmacokinetics and disposition of this compound. Previous metabolite identification studies for NVS-CRF38 (R. A. Stringer et al., in press) indicate that O-demethylation is a major biotransformation pathway. Therefore, in an attempt to attenuate this reaction, we synthesized and measured the in vitro and in vivo pharmacokinetic parameters for both the O-CH3 and O-CD3 analogs for this compound. Deuterium isotope effects are explored in terms of both the disposition of the parent compounds and the formation rate of the O-demethylated metabolite common to both compounds. Comparative pharmacokinetic data are complemented with metabolite identification studies to explore the potential for compensatory metabolic pathways.

Materials and Methods

Chemicals. NVS-CRF38 and 4-(7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-2,6-dimethylpyrazolo[5,1-b]oxazole-3-yl)-3-methylphenol (O-desmethyl NVS-CRF38) were synthesized at the Novartis Institutes for Biomedical Research (Horsham, UK). [13CD3] NVS-CRF38 (d-NVS-CRF38) was synthesized at the Novartis Institutes for Biomedical Research (Basel, Switzerland). All other solvents and chemicals were of analytical grade or better.

Metabolite Identification. Aliquots of microsomes (0.5 ml) were rapidly thawed in a water bath at 37°C and diluted with an appropriate amount of 100 mM phosphate buffer (pH 7.4) to achieve a protein concentration of 1.6 mg/ml. An appropriate amount of NVS-CRF38 or d-NVS-CRF38 was dissolved into dimethyl sulfoxide (DMSO) for a 100 mM stock solution; this was diluted to 1200 µM in 30:70 (v/v) acetonitrile/water and the further diluted 1:4 (v/v) with 100 mM (pH 7.4) potassium phosphate buffer. Three hundred microliters of the microsome preparation was added to a 4-ml glass vial containing 300 µl of reduced NADPH (2 mM) dissolved in 100 mM (pH 7.4) potassium phosphate buffer. The vial was mixed and immersed in a water bath at 37°C for 10 minutes. Reactions were initiated by the addition of 20 µl of NVS-CRF38 (300 µM drug solution). The final assay solvent concentrations were 0.01% DMSO and 0.25% acetonitrile. For the control sample, 1.8 ml of ice-cold acetonitrile and frozen at −80°C. The final microsomal protein concentration was 0.8 mg/ml and the substrate concentration was 10 µM. On the day of analysis, the frozen precipitated samples were allowed to thaw, and an aliquot of sample (2 µl) was evaporated to dryness, reconstituted with 100 µl of acetonitrile, mixed, and diluted with an equal volume of water. Samples were centrifuged at 2000 × g for 15 minutes prior to analysis.

Sample analysis was performed using an LTQ Orbitrap mass spectrometer and Accela LC system (Thermo Scientific, Hemel, UK). NVS-CRF38 was analyzed using an Acquity C18 BEH analytical column (1.7 µm particle size, 150 × 2.1 mm; Waters, Manchester, UK) maintained at 45°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). Initially, the gradient profile was maintained at 5% B for 1 minute, and then a linear gradient was programmed from 5% to 70% B in 90 minutes and maintained at 70% for 10 minutes. To ensure efficient washing of the column, solvent B was increased to 90% in a linear fashion over 10 minutes, maintained for 5 minutes, and then returned to the initial conditions. The flow rate was 0.3 ml/min into the electrospray source. Samples were detected by scanning between 100 and 1300 m/z with ionization in positive mode and normalized collision energy of 35%. Ten microliters of sample was injected into the LC-MS/MS system.

Microsomal Intrinsic Clearance Determination. Compound stock solutions (10 mM) were prepared by dissolving an appropriate amount of either NVS-CRF38 or d-NVS-CRF38 in DMSO. This standard solution was diluted to 120 µM with 30% acetonitrile in water and further diluted to 6 µM with 100 mM phosphate buffer at pH 7.4. Five microliters of this solution was added to a total volume of 300 µl of microsomal incubate for a substrate concentration of 100 nM. Aliquots of microsomes (0.5 ml) were rapidly thawed in a water bath at 37°C and diluted with an appropriate amount of 100 mM phosphate buffer (pH 7.4) to achieve a protein concentration of 1.6 mg/ml. One hundred fifty microliters of this microsomal preparation was added to a 96-well plate containing 5 µl of 6 µM drug solution. The plate was mixed and immersed in a shaking water bath at 37°C for 10 minutes. At time point 0 minutes, 25 µl of incubate was quenched with 150 µl of ice-cold acetonitrile and diluted with 25 µl of cofactor solution containing potassium phosphate buffer and NADPH (2 mM). Reactions were initiated by addition of 125 µl of cofactor solution and mixed thoroughly. Final concentrations of the assay components were phosphate buffer (100 mM) and NADPH (1 mM). The final assay solvent concentrations were 0.003% DMSO and 0.08% acetonitrile. At predetermined time points of 5, 15, 30, 45, and 60 minutes, 50 µl of incubate was dispensed into 150 µl of ice-cold acetonitrile. Calibration curves for O-desmethyl NVS-CRF38 were prepared in rat and human microsomes. Twelve microliters of a 10 mM O-desmethyl NVS-CRF38 DMSO stock solution was added to 998 µl of 30% acetonitrile and mixed to form a 120 µM solution, which was diluted 1:4 (v/v) with 100 mM phosphate buffer and mixed. Five microliters was added to 150 µl of either human or rat microsomes (0.8 mg/ml) omitting the cofactor NADPH. After mixing this, 1 µM calibrant was serially diluted 1:3 seven times with microsomal matrix; the calibration range for O-desmethyl NVS-CRF38 in microsomes was 1.4–1000 nM. Fifty-microliter aliquots of calibration standard were dispensed into 150 µl of ice-cold acetonitrile. Prior to LC-MS/MS analysis, samples were centrifuged for 5 minutes at 2000 × g, and 150 µl of supernatant was removed and diluted with 150 µl of water and 30 µl of internal standard (gliburide 2 µM, in 10% acetonitrile in water).

For calculations of intrinsic clearance (CLint), the analyte/external standard peak area ratios were converted to concentration drug remaining, using T = 0 response as 100%. All data were fitted with a monoeXponential decay using eq. 1:

\[ \text{R}_0 = R_s \cdot e^{-k_t} \]

where \( R \) is the peak area ratio, \( R_0 \) is the peak area ratio at zero time, and \( k_t \) is the elimination rate constant (min⁻¹). The elimination rate constant was used to

![NVS-CRF38](image_url)
calculate the in vitro CL_{int} (units: microliter per minute per milligram of microsomal protein) according to eq. 2:

\[ \text{In vitro CL}_{\text{int}} = (k \cdot 1/P \cdot 1000) \]

where \( k \) is the elimination rate constant and \( P \) is the microsomal protein concentration (milligrams per milliliter).

Scaled in vivo CL_{int} (units: milliliter per minute per kilogram) was calculated using eq. 3:

\[ \text{In vivo CL}_{\text{int}} = \text{In vitro CL}_{\text{int}} \cdot \text{MPPGL} \cdot \text{LW}/1000 \]

where MPPGL is the microsomal recovery factor (45 mg of microsomal protein per gram of liver) and LW is liver weight (40 g/kg).

To estimate CL_{int} values for formation of the metabolite, a pseudo drug depletion curve was determined by subtracting the concentration of O-desmethyl NVS-CRF38 from each time point from the initial substrate concentration. This simulated depletion curve was treated as described earlier.

**Enzyme Kinetics in Liver Microsomes.** Saturated solutions of NVS-CRF38 and d-NVS-CRF38 were prepared by mixing 5 mg of compound overnight in 100 mM potassium phosphate buffer at 37°C. The following morning, the samples were filtered using a 0.22-μm syringe filter. The filtrate was analyzed for dissolved drug substance by high-performance liquid chromatography and UV light detection. Saturated solutions were achieved in buffer at 682 μM for NVS-CRF38 and 522 μM for d-NVS-CRF38. Saturated drug solutions were combined with liver microsomes (20 mg/ml, same batches as described previously) and blank 100 mM potassium phosphate buffer (pH 7.4) for a substrate concentration of 210 μM and microsomal protein concentration of 0.84 mg/ml. Drug-free microsomes at 0.84 mg/ml were prepared by dilution in phosphate buffer. In a 96-well plate, the drug/microsome solution was serially diluted (by 50% each time) with drug-free microsomes (rat and human in duplicate), yielding 12 samples in total ranging from 210 μM to 100 nM. Reactions were initiated by the addition of 5 μl of NADPH (21 mM dissolved in 100 mM phosphate buffer). After cofactor addition, the plates were thoroughly mixed and left in a shaking incubator at 37°C for 20 minutes. Reactions were terminated by the addition of 300 μl of ice-cold acetonitrile and diluted with 300 μl of water. Sixty microliters of the internal standard glyburide (2 μM in 10% methanol:90% water) was added to each sample. Samples were mixed and centrifuged at 2000 (x g) for 15 minutes, and 250 μl of supernatant was transferred to a sample plate for analysis. Five microliters of sample was injected into the LC-MS/MS system.

**Pharmacokinetic Studies.** Animal procedures were conducted in accordance with the British Home Office regulations (Scientific Procedures) Act of 1986, UK. Pharmacokinetic studies were conducted using male Sprague-Dawley rats with an average weight of 257 g (range 245–269 g). For intravenous dosing, NVS-CRF38 and d-NVS-CRF38 were formulated as a solution in 1:1 (v/v) poly(ethylene glycol) 200 (PEG200):saline (final drug concentration 1 mg/ml). The compounds dissolved completely with vigorous shaking and sonication. Rat (concentration 1 mg/ml). The compounds dissolved completely with vigorous shaking and sonication. Rats (n = 4 per group) with surgically implanted lateral tail vein cannulae were administered either NVS-CRF38 or d-NVS-CRF38 by oral gavage; the dose volume was 5 ml/kg.

Serial blood samples (0.15 ml) were taken via the implanted catheter at defined time points to 72 hours. Intravenous blood samples were taken at 0.05, 0.1, 0.25, 0.75, 2, 4, 6, 24, 30, 48, 54, and 72 hours. For the oral studies, blood samples were taken at 0.25, 0.75, 1.5, 3, 6, 24, 30, 48, 54, and 72 hours. Following removal of each blood sample, an equal volume of heparinized saline was injected into the animal via the sampling catheter. Blood samples (50 μl) were transferred to a microtiter plate, and the plate was frozen (–80°C) until analysis. Pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin (Certara, St. Louis MO) version 6.1 (build 6.1.0.173). The C_{max} and time to reach C_{max} were used as the observed data. Total intravenous blood clearance (CL_{int}) was calculated as dose/AUC_{int, i.v.}, with AUC_{int, i.v} as the area under the blood concentration-time curve calculated by the trapezoidal rule and extrapolated to infinity using the apparent terminal disposition rate constant (\( \lambda_d \)), determined by regression analysis of the linear terminal portion of the log blood concentration-time curve. The apparent terminal half-life was estimated from the terminal rate constant with half-life = ln(2)/\( \lambda_d \). For intravenous dosing, the steady-state volume of distribution (V_{ss}) was calculated as V_{ss} = MRT × CL_{int}, with the mean residence time (MRT) being defined as the area under the first moment curve AUMC_{int, i.v.} divided by AUC_{int, i.v.}. Oral bioavailability (F) was calculated by the ratios of dose-normalized AUC_{int, i.v.} after oral and intravenous dosing.

In vivo intrinsic clearance values after intravenous dosing were calculated using the well-stirred liver model (eq. 4):

\[ \text{In vivo CL}_{\text{int}} = Q_H \cdot \text{CL}_{\text{int}}/[f_u \cdot (Q_H - \text{CL}_{\text{int}})] \]

Where \( Q_H \) is hepatic blood flow and \( f_u \) is the ratio of unbound and whole drug concentrations available for binding. After oral drug administration, in vivo intrinsic clearance was calculated using eq. 5:

\[ \text{In vivo CL}_{\text{int}} = \text{Dose}/(\text{AUC} \cdot f_u) \]

where in vivo fraction of drug systemically available that is converted to a metabolite (fm) was calculated using eq. 7:

\[ \text{fm} = \text{AUC}_{\text{int}} \text{ after i.v. drug}/(\text{AUC}_{\text{int}} \text{ after i.v. metabolite}) \]

where \( \text{AUC}_{\text{int}} \) is the area under the curve for the O-desmethyl NVS-CRF38. Kinetic deuterium isotopes effects (KH/KD) based on either total in vivo CL_{int} or CL_{int} for NVS-CRF38 O-demethylation alone were calculated by eq. 8:

\[ \text{In vivo KH}/\text{KD} = \text{NVS-CRF38 in vivo CL}_{\text{int}}/\text{d-NVS-CRF38 in vivo CL}_{\text{int}} \]

Bioanalysis of plasma samples was undertaken using a solvent precipitation approach followed by LC-MS/MS. Calibration curve concentration ranges for

**TABLE 1**

Intrinsic clearance values for NVS-CRF38 and [13C5]NVS-CRF38 in liver microsomes

Intrinsic clearance values were determined by either substrate depletion or metabolite formation in mixed-gender human or male Sprague-Dawley rat liver microsomes. The initial substrate concentration was 100 nM and the microsomal protein concentration was 0.8 mg/ml. The cofactor NADPH was included to activate cytochrome P450 enzymes. Data are the mean and S.D. of three determinations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat</th>
<th>Human</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/min/kg microsomal protein</td>
<td>ml/min/kg microsomal protein</td>
<td>ml/min/kg microsomal protein</td>
<td>ml/min/kg microsomal protein</td>
</tr>
<tr>
<td>NVS-CRF38</td>
<td>32.9 ± 3.6</td>
<td>17.2 ± 2.8</td>
<td>18.2 ± 6.9</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>[13C5]NVS-CRF38</td>
<td>15.8 ± 4.7</td>
<td>10.7 ± 2.8</td>
<td>2.5 ± 0.4</td>
<td>0.85 ± 0.34</td>
</tr>
<tr>
<td>( K_d/K_c )</td>
<td>2.2 ± 0.8</td>
<td>1.7 ± 0.5</td>
<td>7.1 ± 2.1</td>
<td>5.4 ± 0.9</td>
</tr>
</tbody>
</table>
NVS-CRF38 and d-NVS-CRF38 ranged from 7.6 to 16,668 nM (8-point calibration curve with a 1:3 dilution per point). For O-desmethyl NVS-CRF38, the calibration curve concentration ranged between 6.9 and 556 nM (5-point calibration curve with a 1:3 dilution per point). For each analyte, the calibration curve was fitted with a quadratic function weighted to 1/x. Calibration samples were extracted alongside study samples. Fifty microliters of either calibrant or study blood sample was precipitated with 150 μl of acetonitrile and mixed for 5 minutes. After centrifugation at 2000 (× g) for 10 minutes, 120 μl of the supernatant was removed and diluted with 120 μl of water, then 30 μl of internal standard was added (glyburide at 2 μM in 10% methanol dissolved in water). LC-MS/MS analysis was performed using a Quattro Premier mass spectrometer and Acquity UPLC system (Waters). Analytes and the internal standard glyburide were analyzed using an Acquity UPLC BEH C18 analytical column (1.7-μm particle size, 50 × 2.1 mm; Waters). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). A linear gradient was programmed from 5% to 95% B in 4.5 minutes; the mobile phase was maintained at 95% B for 1 minute and then returned to the initial conditions. The flow rate was 0.5 ml/min into the electrospray source. Samples were detected using multiple reaction monitoring, and the parent-to-daughter transitions were 352.19→255.08 m/z (cone voltage 45 V and collision energy 25 eV) for NVS-CRF38, 356.29→259.17 m/z (cone voltage 40 V and collision energy 25 eV) for d-NVS-CRF38, 338.19→241.08 m/z (cone voltage 45 V and collision energy 25 eV) for O-desmethyl NVS-CRF38, and 494.16→369.09 m/z (cone voltage 25 V and collision energy 15 V) for the internal standard glyburide. Five microliters of sample was injected into the LC-MS/MS system.

**Results**

The metabolic stability of NVS-CRF38 and d-NVS-CRF38 was measured in rat and human liver microsomes (Table 1); CLint estimates were based on both depletion of the parent drug and formation of the O-desmethyl metabolite. Using the drug depletion method, CLint values for d-NVS-CRF38 were reduced by 40%–50% in both rat and human liver microsomes when compared with unlabeled compound. This reduction in microsomal CLint using the drug depletion method corresponded to a KH/KD value of ~2. When CLint values were calculated based on formation of the O-desmethyl metabolite, the isotope effect was more pronounced. Formation of this specific metabolite was reduced by 86% in rat microsomes and 81% in human microsomes, thus KH/KD values for microsomal CLint based on metabolite formation were in the range of 5.4–7.1.

![Fig. 2. Hanes-Woolf plots for NVS-CRF38 and [13CD3]NVS-CRF38 O-demethylation in rat (A) and human (B) liver microsomes. The microsomal protein concentration is 0.8 mg/ml and incubation time is 20 minutes. Data are the mean and S.D. of three determinations.](image)

![Fig. 3. Plasma concentration-time profiles for NVS-CRF38 (A) and [13CD3]NVS-CRF38 (B) after intravenous administration to male Sprague-Dawley rats. Compounds were formulated as a solution in 1:1 (v/v) poly(ethylene glycol) 200: saline; the dose volume was 1 ml/kg. Data are the mean and S.D. of four rats.](image)
Transformed substrate saturation curves comparing rates of NVS-CRF38 O-demethylation in rat and human microsomes are provided in Fig. 2. Substrate saturation curves were transformed using the Hanes-Woolf method. For rats (Fig. 2A), deuteration had no marked effect on the K_m for the O-demethylation reaction; however, a 5-fold reduction in V_max (~5-fold) was observed. In human liver microsomes, an isotope was apparent by visual inspection of the data (Fig. 2B); determination of reliable kinetic parameters was not possible using models describing the involvement of either one or two enzymes due to the multiphasic nature of the Hanes-Woolf plot.

After intravenous administration to male rats, blood concentrations for both NVS-CRF38 and d-NVS-CRF38 declined in a biexponential manner (Fig. 3A). Pharmacokinetic parameters are presented in Table 2; both compounds were rapidly distributed to tissues, and volume of distribution values were comparable (V_ss = 3 to 4 l/kg). Blood clearance values were 2-fold higher for unlabeled NVS-CRF38 (CLb = 21.2 ± 0.17 ml/min/kg) compared with d-NVS-CRF38 (CLb = 9.7 ± 0.04 ml/min/kg). Consequently, a shorter elimination half-life was observed for the unlabeled compound. For both compounds, the O-desmethyl metabolite was detected in blood samples over the entire intravenous study time course (Fig. 3B); maximum concentrations in blood occurred 0.8 hours after administration of NVS-CRF38 and 0.08 hours after administration of d-NVS-CRF38. After incubation for 1 hour, the initial peak of the O-demethylated metabolite (M7) was observed.

Exposed to the i.v. pharmacokinetic parameters for this metabolite ([13CD3] NVS-CRF38) was generated from oral exposure of parent drug (accounting for all biotransformation routes) after i.v. dosing was estimated at 20.3 ± 31.0 ml/min/kg; for d-NVS-CRF38 this value decreased to 84.1 ± 7.2 ml/min/kg, yielding an in vivo kinetic isotope effect corresponding to all metabolic pathways of 2.4 ± 0.5. In vivo CLint values for the O-desmethyl metabolite were calculated by multiplying the total in vivo CLint by f_m; using this approach, the CLint after administration of NVS-CRF38 was estimated at 42.3 ± 6.5 and was reduced to 5.7 ± 0.5 ml/min/kg after i.v. administration of d-NVS-CRF38. Therefore, when the O-demethylation reaction is considered in isolation, a much higher in vivo kinetic isotope effect is observed (Kf/KD = 7.6 ± 1.6). After oral administration, both NVS-CRF38 and d-NVS-CRF38 were rapidly absorbed, reaching maximum concentrations ~1 hour post dose; oral bioavailability was complete for both compounds (Fig. 4A; Table 2). Exposure of the O-demethylated metabolite (Fig. 4B) was 2.5-fold lower after administration of d-NVS-CRF38. In vivo CLint estimates generated from oral exposure of parent drug (accounting for all biotransformation routes) indicate a modest kinetic isotope effect (Kf/KD = 2.1 ± 0.4). The isotope effect was more marked when CLint estimates were based on the O-demethylation reaction alone (Kf/KD = 6.5 ± 0.3). A summary of Kf/KD values for d-NVS-CRF38 in rats is provided in Table 3.

Metabolite identification studies were undertaken using rat and human liver microsomes to compare the metabolic pattern for NVS-CRF38 and d-NVS-CRF38. After incubation for 1 hour, the initial peak areas for NVS-CRF38 (initial substrate concentration 10 μM) decreased by ~30% in both rat and human microsomes. The metabolic patterns for these compounds are presented in Fig. 5. In rat microsomes, six metabolites were detected; these included three aromatic oxygen adducts located on the alkoxyaryl ring (M1, M4, M5), one aliphatic oxygen adduct on the same ring system (M3), and an additional aliphatic oxygen adduct (M6) which was located on either the pyrazolo-oxazole core or the 1,3,4-triazole ring. In addition, the demethylated metabolite (M7) was observed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NVS-CRF38</th>
<th>[13CD3] NVS-CRF38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cmax (nM)</td>
<td>74 ± 20</td>
<td>9 ± 1**</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>AUCinf (nM·h)</td>
<td>2255 ± 211</td>
<td>4853 ± 327**</td>
</tr>
<tr>
<td>CLb (ml/min/kg)</td>
<td>21.2 ± 2.0</td>
<td>9.7 ± 0.7**</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>0.8 ± 0.7</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>6.9 ± 1.8</td>
<td>5.2 ± 1.4</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.6 ± 0.3</td>
<td>6.5 ± 1.5*</td>
</tr>
<tr>
<td>fm</td>
<td>0.21 ± 0.08</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Oral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
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<td>10</td>
</tr>
<tr>
<td>Cmax (nM)</td>
<td>4111 ± 265</td>
<td>6148 ± 1506</td>
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<tr>
<td>Tmax (h)</td>
<td>0.8 ± 0.0</td>
<td>1.3 ± 1.0</td>
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<tr>
<td>AUCinf (nM·h)</td>
<td>23,391 ± 1475</td>
<td>60,218 ± 14,726*</td>
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<tr>
<td>Oral half-life (h)</td>
<td>3.5 ± 0.6</td>
<td>5.6 ± 1.1*</td>
</tr>
<tr>
<td>F (%)</td>
<td>104 ± 7</td>
<td>124 ± 30</td>
</tr>
<tr>
<td>Blood distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fo</td>
<td>0.17 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>BPR</td>
<td>0.83 ± 0.07</td>
<td>0.86 ± 0.05</td>
</tr>
</tbody>
</table>

BPR, blood-to-plasma ratio; MRT, mean residence time; Tmax, time to reach Cmax.

*Significant differences (Student’s t test) versus values for NVS-CRF38 at the P < 0.05 level.

**Significant differences (Student’s t test) versus values for NVS-CRF38 at the P < 0.01 level.
Peak areas for each metabolite were compared using either NVS-CRF38 or d-NVS-CRF38 as a substrate (Fig. 6). Comparatively, levels of the O-desmethyl metabolite M7 were markedly lower for incubations with d-NVS-CRF38 by 7.5-fold in rat liver microsomes and 3-fold in human liver microsomes. Reduction of M7 formation with d-NVS-CRF38 was associated with a relative increase in formation of the other metabolites (M1, M3, M5, and M6) in rat liver microsomes. In human liver microsomes, metabolic switching was particularly marked with clear increases in peak areas for M1, M3, M4, M5, and M6. In addition, two further metabolites were observed (M10 and M11) which were not detected with the unlabeled compound. Metabolites for NVS-CRF38 which have been previously observed after incubation with cryopreserved hepatocytes (R. A. Stringer et al., in press), including the carboxylic acid (M2) and conjugated metabolites of M7 including the sulfate (M8) and glucuronide (M9), were not observed in this in vitro study with hepatic microsomes.

**Discussion**

Deuterium-for-hydrogen substitution is an attractive way to increase drug exposure by slowing down oxidative metabolism. Often deuteration decreases the rate of biotransformation reactions in vitro; translation between in vitro observations and in vivo isotope effects are somewhat uncertain. Many factors may confound this approach, including the contribution to in vivo metabolism by enzymes which do not rely on C-H bond breakage. No isotope effect was observed after administration of deuterated propofol to mice (Helfenbein et al., 2002); this may be attributed to the fact that glucuronidation accounts for a significant proportion of propofol metabolism (Simons et al., 1988). Contribution to in vivo clearance by nonmetabolic elimination mechanisms (urinary and/or biliary excretion of unchanged drug) may also attenuate in vivo deuterium isotope effects; biliary excretion of zoniporide (~40% of dose) is likely to contribute to the lack of isotope effects observed for this compound in rats (Sharma et al., 2012). The success of the deuterium replacement strategy will also be dependent on physiological factors such as hepatic blood flow; for compounds with CL$_{int}$ values well in excess of hepatic blood flow, isotope effects observed in vitro are unlikely to translate to comparable differences for in vivo clearance (Sharma et al., 2012).

When considering NVS-CRF38 for a deuteration replacement strategy, we carefully considered these potential pitfalls. Previous drug metabolism studies conducted using cryopreserved hepatocytes from rats, dogs, monkeys, and humans indicate that primary metabolic routes are inhibited by the nonselective cytochrome P450 (P450) inhibitor 1-aminobenzotriazole (R. A. Stringer et al., in press), implicating P450 enzymes in primary biotransformation reactions for this compound. We also considered the involvement of other elimination mechanisms which could potentially negate the impact of pharmacokinetic changes mediated by deuterium replacement. Excretion studies in rats indicate that elimination of NVS-CRF38 (at least in the rat) was virtually entirely hepatic metabolic, with very little contribution from either renal or biliary excretion of unchanged drug.

For NVS-CRF38, scaled microsomal intrinsic clearance values were either close to or below hepatic blood flow, suggesting that changes in the CL$_{int}$ of this compound are likely to translate into observable changes in hepatic clearance. Since the extrapolation of in vitro CL$_{int}$ to the in vivo situation also requires consideration of the free fraction in blood, we also considered potential differences in blood distribution.

**Table 3**

In vivo kinetic isotope effects in male Sprague-Dawley rats after intravenous and oral administration.

<table>
<thead>
<tr>
<th>Biotransformation Pathway(s)</th>
<th>Route</th>
<th>In Vivo CL$_{int}$ NVS-CRF38</th>
<th>[13CD3] NVS-CRF38</th>
<th>K$_D$/K$_O$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mI/min/kg</td>
<td>mI/min/kg</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>i.v.</td>
<td>203.6 ± 31.0</td>
<td>84.1 ± 7.2</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>O-demethylation</td>
<td>i.v.</td>
<td>42.3 ± 6.5</td>
<td>5.7 ± 0.5</td>
<td>7.6 ± 1.6</td>
</tr>
<tr>
<td>Total</td>
<td>PO</td>
<td>119.7 ± 7.7</td>
<td>58.4 ± 12.5</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>O-demethylation</td>
<td>PO</td>
<td>24.9 ± 1.6</td>
<td>3.9 ± 0.8</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

PO, by mouth.
for the two compounds. No marked differences were observed between the proto and deutero form in terms of either plasma-protein binding or partition into red blood cells.

NVS-CRF38 has several biotransformation pathways in addition to the O-demethylation reaction; it is quite conceivable that blocking the O-demethylation pathway will simply divert the compound through other biotransformation routes. The success of deuterium replacement (in terms of modulating disposition of the parent compound) is dependent on the relative capacity and catalytic efficiency of these reactions versus O-demethylation.

In terms of primary routes of metabolism, the metabolic pattern for NVS-CRF38 generated in this study was in good agreement with previously published studies using cryopreserved hepatocytes (R. A. Stringer et al., in press). Several secondary metabolites previously observed in hepatocytes, including the carboxylic acid (M2) and conjugated metabolites of M7 (sulfate and glucuronide), were not detected using microsomes. For this study, additional changes were made to the metabolite identification protocol to enable a more accurate assignment of drug metabolites. Specifically, an evaporation/reconstitution step was incorporated to enrich the concentration of metabolites in the final in vitro sample, thus enabling more precise metabolite identification. In addition, the chromatographic run time was increased to ensure complete separation of drug metabolites. As a result, we were able to identify that, in addition to O-demethylation, NVS-CRF38 undergoes aromatic ring hydroxylation at the three available positions located to the alkoxyaryl ring, aliphatic hydroxylation of the core, and aliphatic hydroxylation on the triazole ring.

For NVS-CRF38, the disparity between $K_{\text{H}}/K_{\text{D}}$ determined based on either all metabolic pathways or O-demethylation alone and the multitude of biotransformation routes for NVS-CRF38 strongly suggest that metabolic switching occurs in vivo. These observations are supported by comparative in vitro metabolite identification studies which demonstrate that, for d-NVS-CRF38 (compared with NVS-CRF38), a reduction in formation of O-desmethyl NVS-CRF38 was associated with an increase in peak areas for the other metabolites. It is important to acknowledge that these metabolite identification studies

![Fig. 5. Metabolic patterns for NVS-CRF38 and [13CD3] NVS-CRF38 after incubation in human and rat liver microsomes for 20 minutes. The substrate concentration was 5 $\mu$M and microsomal protein concentration was 0.8 mg/ml.](image)
were performed using nonradiolabeled compound and are not quantitative in terms of the amounts of metabolites formed. However, we considered this approach suitable for comparing the efficiency of O-demethylation between these two compounds.

Enzyme kinetic studies were undertaken to explore the mechanism behind NVS-CRF38 isotope effects and determine whether isotope effects were attributed to differences in substrate binding to P450 or catalysis. Rat microsomal incubations clearly indicate that the binding affinity ($K_m$) is unchanged for the two compounds; however, a marked reduction in $V_{max}$ is apparent for d-NVS-CRF38, supporting C-H bond breaking as the rate-limiting step for NVS-CRF38 O-demethylation. For the rat, $V_{max}/K_m$ ratios are in good agreement with O-demethylation $Cl_{int}$ values determined from substrate depletion studies at a single substrate concentration. The situation with enzyme kinetic studies in human liver microsomes is not so clear due to the curved nature of the transformed kinetic profiles. We speculate that multiple human P450 isoforms contribute to the metabolism of NVS-CRF38, and that each of these isoforms has different enzyme kinetics. We did attempt to fit the human data with models describing the involvement of two enzymes; however, the fit was poor.

For our study, good correlation was observed between isotope effects observed with in vitro drug metabolism systems and those observed in vivo; clearly, that is not always the case, and for many compounds, marked in vitro isotope effects do not translate into in vivo effects of comparable magnitude. A detailed understanding of drug disposition in terms of mechanisms of elimination and enzymology of metabolism is important to identify drug candidates which are most likely to benefit from deuterium substitution. In terms of quantitative translation of in vitro metabolism results, P450 substrates are particularly attractive due to the existence of well characterized scaling procedures that have been developed to scale from in vitro clearance to the in vivo situation (Houston, 1994).

 Compared with drug development activities for nonlabeled small-molecule drugs, drug development activities for deuterium-substituted analogs are not foreseen to be significantly different from their nonlabeled counterpart. In a regulatory context, even a deuterium-labeled version of a marketed drug is considered a new molecular entity. This "new molecular entity" approach for deuterium-labeled drugs is consistent with current practices and strategies that leverage on the "uniqueness" of deuterium-labeled drugs. The precedence for intellectual property protection and patents is well established and used for deuterium-labeled drug candidates. Importantly, a deuterium-labeled analog should offer a unique benefit or property for the patient which is an improvement in comparison with the nonlabeled counterpart, or conventional therapies.

Although health authority submissions and subsequent approval would be necessary for deuterium-labeled drugs, the information and knowledge available from the nonlabeled drug might be helpful and beneficial. The available information and data from the nonlabeled drug could help streamline, focus, and shape the drug development process, which could result in a more efficient and shorter path to market.

Cost of goods is also an important consideration for deuterium-labeled drugs, and this would be dependent on the individual characteristics of a given molecule. The broad range of costs and synthetic complexities for deuterium-labeled drugs would be analogous to the marketed nonlabeled drugs. Although cost of goods for a deuterium-labeled version might be somewhat more expensive than a nonlabeled analog, this factor will likely not be prohibitive for every compound. The synthesis of a final deuterium-labeled drug product would be optimized for efficiency and cost in an analogous manner to that of nonlabeled drugs.

One potential consequence of in vivo metabolic switching is that, for deuterated drugs, previously undetected metabolites may be observed. The application of deuteration as a method to minimize the formation of toxic metabolites has been demonstrated in mice with the hepatotoxin N-methylformamide (Threadgill et al., 1987). In the 1970s, Merck successfully adopted a deuteration strategy to mitigate the toxicity of 3-fluoropyruvate, a metabolite of fluoro-d-alanine catalyzed by d-amino acid oxidase. This compound, intended as part of a combination therapy for chronic obstructive pulmonary disease, did not reach the market due to non-deuterium-related toxicity (Darland et al., 1986).

Based on our experience with NVS-CRF38, the effectiveness of a deuterium replacement strategy will be highly dependent on the aims. If one wishes to change the pharmacokinetic properties of a drug molecule (for instance, increase elimination half-life, reduce dose and dosing frequency), then in vivo isotope effects are likely to be relatively minor due to compensation by other metabolic pathways. The application of deuteration to minimize specific metabolites is attractive in that it enables one to harness the full magnitude of isotope effects and is not impacted by metabolic switching in vivo. The process, which could result in a more efficient and shorter path to market.

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knowledge of metabolic patterns for both deuterated and unsubstituted drug candidates and efficacy/safety data pertaining to each metabolite is important to enable an informed decision regarding the suitability of this approach.

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Authorship Contributions

Participated in research design: Stringer, Williams, Picard, Krauser, Sohal, Kretz, McKenna.

Conducted experiments: Stringer, Williams, Krauser.

Performed data analysis: Stringer, Williams, Krauser.

Wrote or contributed to the writing of the manuscript: Stringer, Krauser, Sohal.

References


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