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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BPR, blood-to-plasma ratio, fm, fraction of drug systemically available that is converted to a metabolite, $K_{H}/K_{D}$, intrinsic primary isotope effect, LC-MS/MS, liquid chromatography tandem mass spectrometry, P450, cytochrome P450, MPPGL, microsomal recovery factor
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 CRF₁ receptor 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/Kᵦ) were determined by the ratio of intrinsic clearance (CL⁻¹) obtained for NVS-CRF38 and deuterated NVS-CRF38. In vitro kinetic isotope effects (Kᵢ/Kᵦ) were more pronounced when CL⁻¹ values were calculated based on the rate of formation of the O-desmethyl metabolite (Kᵢ/Kᵦ ~7), compared to the substrate depletion method (Kᵢ/Kᵦ ~2). In vivo isotope effects were measured in the rat after intravenous (1 mg/kg) and oral (10 mg/kg) administration. For both administration routes isotope effects calculated from in vivo CL⁻¹ corresponding to all biotransformation pathways were lower (Kᵢ/Kᵦ ~2) compared to CL⁻¹ values calculated from the O-demethylation reaction alone (Kᵢ/Kᵦ ~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 the 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 whilst maintaining selectivity and potency for a given target.

Deuterium replacement strategies have been applied to enzymes systems including cytochrome P450 enzymes (Krauser and Guengerich, 2005; Kim, et al., 2006), monoamine oxidase (Belleau, et al., 1961; Ottoboni, et al., 1989), aldehyde oxidase (Sharma, et al., 2012) and alcohol/aldehyde dehydrogenase (Ghanayem, et al., 1987). The goal of such strategies includes extension of elimination half-life, optimization of dose and dosing regimen and mitigating risks associated with drug-drug interactions (Shao, et al., 2006; Tanabe, et al., 1969). This strategy has attracted significant commercial interest and has been the subject of a 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 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 zonipiride (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 simultaneous deuteration at five different positions were 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 Nelson’s review (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 CRF1 antagonist (Figure 1). The aims of this study were 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 (Stringer et al., submitted for publication) 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 analogues for this compound. Deuterium isotope effects are explored both in terms of 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.** 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) and 4-(7-3,5-dimethyl-1H-1,2,4-triazol-1-yl-2,6-dimethylpyrazolo[5,1-b]oxazol-3-yl-3-methylphenol (O-desmethyl NVS-CRF38) were synthesized at the Novartis Institute for Biomedical Research (Horsham, UK). [\(^{13}\)CD\(_3\)] NVS-CRF38 (d-NVS-CRF38) was synthesized at the Novartis Institute for Biomedical Research (Basel, Switzerland). All other solvents and chemicals were of analytical grade or better.

**Metabolite identification.** 0.5 mL aliquots of microsomes 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 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 pH 7.4 100 mM potassium phosphate buffer. 300 µL of the microsome preparation was added to a 4 mL glass vial containing 300 µL of reduced NADPH (2 mM) dissolved in pH 7.4 100 mM 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 0.003% DMSO and 0.25% acetonitrile. For the control sample 1.8 mL of ice-cold acetonitrile was present in the sample prior to reaction initiation by addition of compound. A blank sample was prepared containing all assay reagents to check for endogenous background peaks. After incubation for 20 minutes microsome samples were precipitated with 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 LC-MS/MS analysis the frozen precipitated samples were allowed to thaw, an aliquot of sample (2 mL) was evaporated to dryness, reconstituted with 100 µL of acetonitrile, mixed and diluted with an equal volume of water. Samples were centrifuged at 2000 (xg) for 15 minutes and loaded onto the LC-MS/MS system.
LC-MS analysis was performed using an LTQ Orbitrap mass spectrometer and Accela LC system (Thermo Finnigan, UK). NVS-CRF38 was analyzed using an Acquity C18 BEH analytical column (1.7 μm particle size; 150 x 2.1 mm; Waters, 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 to 1300 m/z with ionization in positive mode and normalized collision energy of 35%. 10 μL of sample was injected into the LC-MS/MS system.

**Microsomal intrinsic clearance determination.** 10 mM compound stock solutions 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. 5 μL of this solution was added to a total volume of 300 μL of microsomal incubate for a substrate concentration of 100 nM. 0.5 mL aliquots of microsomes were rapidly thawed in a water bath at 37°C and dilution with an appropriate amount of 100 mM phosphate buffer (pH 7.4) to achieve a proteins concentration of 1.6 mg/mL. 150 μL 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. For a 0 minute time point 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. 12 µL of a 10 mM O-desmethyl NVS-CRF38 DMSO stock solution was added to 998 µL of 30% acetonitrile and mixed for a 120 µM solution, this was diluted 1:4 v/v with 100 mM phosphate buffer and mixed. 5 µL was added to 150 µL 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 to 1000 nM. 50 µL 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 (xg), 150 µL of supernatant was removed diluted with 150 µL of hplc grade water and 30 µL of internal standard (glyburide 2 µM, in 10% acetonitrile in water).

For calculations of intrinsic clearance (CL_{int}) the analyte/internal 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 equation 1. The elimination rate constant (k) was used to calculate in vitro CL_{int} (equation 1).

\[
R(t) = R_0 e^{-kt}
\]  

Where \( R \) is the peak area ratio, \( R_0 \) is the peak area ratio at zero time and k is the elimination rate constant (min^{-1}). The elimination rate constant was used to calculate the in vitro CL_{int} (units µL per minute per mg of microsomal protein) according to equation 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 (mg/mL).

Scaled in vivo CL_{int} (units, mL/min/kg) was calculated using equation 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 formed at each
time-point from the initial substrate concentration. This simulated depletion curve was treated as described above.

**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 ultraviolet 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 pH 7.4 100 mM potassium phosphate buffer 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 the diluted with 300 µL of ultrapure water. 60 µL of the internal standard glyburide (2 µM in 10% methanol:90% water) was added to each sample. Samples were mixed at centrifuged at 2000 (xg) for 15 minutes, 250 µL of supernatant was transferred to a sample plate for analysis. 5 µL 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 257g (range 245 to 269g). For intravenous dosing NVS-CRF38 and d-NVS-CRF38 were formulated as a solution in 1:1 v/v PEG200:saline, final drug concentration 1 mg/mL. The compounds dissolved completely with vigorous shaking and sonication. Rats (n=4 per group) with a
surgically implanted lateral tail vein cannula were administered with either compound by bolus injection into the contra lateral tail vein, the dose volume was 1 mL/kg. For oral dosing compounds were formulated as a suspension in 0.5% methylcellulose and 0.5% Tween 80 for a final dose of 10 mg/kg. 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 heparinised saline was injected into the animal via the sampling catheter. Blood samples (50 µL) were transferred to a microtitre plate and the plate frozen (-80°C) until analysis. Pharmacokinetic parameters were calculated using non-compartmental analysis using WinNonlin version 6.1 (build 6.1.0.173). The C<sub>max</sub> and time to reach C<sub>max</sub> (T<sub>max</sub>) were used as the observed data. Total intravenous blood clearance (CL<sub>b</sub>) was calculated as dose/AUC<sub>0-∞</sub> with AUC<sub>0-∞</sub> as the area under the plasma concentration-time curve calculated by the trapezoidal rule and extrapolated to infinity using the apparent terminal disposition rate constant (λ<sub>z</sub>), determined by regression analysis of the linear terminal portion of the log plasma concentration-time curve. The apparent terminal half-life was estimated from the terminal rate constant with half-life = ln 2/λ<sub>z</sub>. For intravenous dosing, the steady state volume of distribution (V<sub>ss</sub>) was calculated as V<sub>ss</sub> = MRT · CL<sub>b</sub> with the mean residence time (MRT) being defined as the area under the first moment curve AUMC<sub>0-∞</sub> divided by AUC<sub>inf</sub> · T<sub>max</sub> and C<sub>max</sub> were determined as the time at which maximum drug concentrations were observed in blood after oral dosing.

Oral bioavailability (F) was calculated by the ratios of dose-normalized AUC<sub>0-∞</sub> after oral and intravenous dosing.

In vivo intrinsic clearance values after intravenous dosing were calculated using the well-stirred liver model equation 4.
In vivo CL\textsubscript{int} = Q_H \cdot CL_b / (f_{ub} (Q_H - CL_b)) \hspace{1cm} (4)

After oral drug administration in vivo intrinsic clearance was calculated using equation 5

In vivo CL\textsubscript{int} = Dose / (AUC \cdot f_{ub}) \hspace{1cm} (5)

In vivo CL\textsubscript{int} corresponding to NVS-CRF38 O-demethylation after either intravenous or oral administration was calculated by equation 6

In vivo CL\textsubscript{int}(NVS-CRF38 O-demethylation) = total in vivo CL\textsubscript{int} \cdot fm \hspace{1cm} (6)

Where in vivo fm was calculated using equation 7.

fm = AUC\textsubscript{(m)} after i.v. drug / AUC\textsubscript{(m)} after i.v. metabolite \hspace{1cm} (7)

Where AUC\textsubscript{(m)} is the area under the curve for the O-desmethyl NVS-CRF38. Kinetic deuterium isotopes effects (K_H/K_D) based on either total in vivo CL\textsubscript{int} or CL\textsubscript{int} for NVS-CRF38 O-demethylation alone were calculated by equation 8.

In vivo K_H/K_D = NVS-CRF38 in vivo CL\textsubscript{int} / d-NVS-CRF38 in vivo CL\textsubscript{int} \hspace{1cm} (8)

Bioanalysis of plasma samples was undertaken using a solvent precipitation approach followed by LC-MS/MS. Calibration curve concentration ranges for NVS-CRF38 and d-NVS-CRF38 ranged 7.6 nM 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. 50 \mu L of either calibrant or study blood sample was precipitated with 150 \mu L of acetonitrile and mixed for 5 minutes. After centrifugation at 2000 (xg) for 10 minutes, 120 \mu L of the supernatant was removed, diluted with 120 \mu L of water then 30 \mu L of internal standard was added (glyburide at 2 \mu M in 10% methanol dissolved in water). LC-MS/MS analysis was performed using a Quattro Premier mass spectrometer and Acquity UPLC system (Waters, UK). Analytes and the internal standard glyburide were analyzed using an Acquity UPLC BEH C18 analytical column (1.7 \mu m particle size; 50 x 2.1
DMD#57265

mm; Waters, UK). 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, 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 eV) for the internal standard glyburide. 5 μL of sample was injected into the LC-MS/MS system.
Results

The metabolic stability of NVS-CRF38 and d-NVS-CRF38 were measured in rat and human liver microsomes (Table 1), CL_{int} estimates were based on both depletion of the parent drug and formation of the O-desmethyl metabolite. Using the drug depletion method CL_{int} values for d-NVS-CRF38 were reduced by 40 to 50% in both rat and human liver microsomes when compared to unlabelled compound. This reduction in microsomal CL_{int} using the drug depletion method corresponded to a K_H/K_D value of 2. When CL_{int} 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 K_H/K_D values for microsomal CL_{int} based on metabolite formation were in the range 5.4 to 7.1.

Transformed substrate saturation curves comparing rates of NVS-CRF38 O-demethylation in rat and human microsomes are provided in Figure 2. Substrate saturation curves were transformed using the Hanes-Woolf method. For the rat (Figure 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 (Figure 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 bi-exponential manner (Figure 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 (CL_b = 21.2±2.0 mL/min/kg) compared to the d-NVS-CRF38 (CL_b = 9.7±0.7 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 (Figure 3b),
maximum concentrations in blood occurred after ~40 minutes. The AUC\(_{0\rightarrow\infty}\) for the O-desmethyl metabolite was 68% lower after administration of d-NVS-CRF38 compared to unlabeled compound (130±69 nM*hr versus 402±157 nM*hr respectively). The fraction of dose metabolized to this metabolite was calculated with reference to the i.v. pharmacokinetic parameters for this metabolite (AUC\(_{0\rightarrow\infty}\) = 1948±179 nM*hr, CL\(_b\) = 25±2 mL/min/kg, V\(_{ss}\) = 9.7±3.6 L/kg). In vivo fm for the O-desmethyl metabolite common to both compounds was 0.21±0.08 for NVS-CRF38 and 0.07±0.04 for d-NVS-CRF38. In vivo CL\(_{int}\) was calculated using the well stirred liver model, both NVS-CRF38 and d-NVS-CRF38 were weakly bound to blood constituents (f\(_{ub}\) = 0.17±0.02 versus 0.14±0.02 respectively). The total CL\(_{int}\) for NVS-CRF38 (corresponding to all biotransformation pathways) after i.v. dosing was estimated at 203.6±31.0 mL/min/kg for d-NVS-CRF38, this value decreased to 84.1±7.2 mL/min/kg yielding a in vivo kinetic isotope effect, corresponding to all metabolic pathways of 2.4±0.5. In vivo CL\(_{int}\) values for the O-desmethyl metabolite were calculated by multiplying the total in vivo CL\(_{int}\) by fm, using this approach the CL\(_{int}\) after administration of NVS-CRF38 was estimated at 42.3±6.5 was reduced to 5.7±0.5 mL/min/kg after i.v. administration of the d-NVS-CRF38. Therefore when the O-demethylation reaction is considered in isolation, a much higher in vivo kinetic isotope effect is observed (K\(_H\)/K\(_D\) = 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 (Figure 4a, Table 2). Exposure of the O-demethylated metabolite (Figure 4b) was 2.5-fold lower after administration of d-NVS-CRF38. In vivo CL\(_{int}\) estimates generated from oral exposure of parent drug (accounting for all biotransformation routes) indicate a modest kinetic isotope effect (K\(_H\)/K\(_D\) = 2.1±0.4). The isotope effect was more marked when CL\(_{int}\) estimates were based on the O-demethylation reaction alone (K\(_H\)/K\(_D\) = 6.5±0.3). A summary of K\(_H\)/K\(_D\) values for d-NVS-CRF38 in rats are 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 5 µM) decreased by ~30% in both rat and human microsomes. The metabolic pattern for these compounds are presented in figure 5, in rat microsomes six metabolites were detected, these included three aromatic oxygen adducts located to the alklyoxyaryl ring (M1, M4, M5), one aliphatic oxygen adduct on the same ring system (M3) and an additional aliphatic oxygen adduct (M6) which was located to either the pyrazolo-oxazole core or 1, 3, 4-triazole ring. In addition the demethylated metabolite (M7) was observed.

Peak areas for each metabolite were compared using either NVS-CRF38 or d-NVS-CRF38 as a substrate (Figure 6). Comparatively levels of the O-desmethyl metabolite M7 where 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 for 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, M11) which were not detected with the unlabeled compound. Metabolites for NVS-CRF38 which have been previously observed in after incubation with cryopreserved hepatocytes (Stringer et al., submitted for publication), 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 \textit{in vitro}, translation between \textit{in vitro} observations and \textit{in vivo} isotope effects are somewhat uncertain. Many factors may confound this approach including the contribution to \textit{in vivo} metabolism by enzymes which do not rely on C-H bond breakage.

No isotope effect was observed after administration of deuterated propofol (Helfenbein, et al., 2002), this may be attributed to the fact that glucuronidation accounts for significant proportion of propofol metabolism (Simons, et al., 1988). Contribution to \textit{in vivo} clearance by non-metabolic elimination mechanisms (urinary and/or biliary excretion of unchanged drug) may also attenuate \textit{in vivo} deuterium isotope effects, biliary excretion of zonipiride (~40% of dose) is likely to contribute to the lack of isotope effects observed for this compound in the rat (Sharma, et al., 2012). The success of deuterium replacement strategy will be also dependent on physiological factors such as hepatic blood flow; for compounds with CL\textsubscript{int} values well in excess of hepatic blood flow isotope effects observed \textit{in vitro} are unlikely to translate to comparable differences for \textit{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 rat, dog, monkey and humans indicate that primary metabolic routes are inhibited by the non-selective P450 inhibitor 1-aminobenzotriazole (Stringer et al., submitted for publication), implicating P450 enzymes in primary biotransformation reactions for this compound. We also considered the involvement of other elimination mechanisms which could potential 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\textsubscript{int} of this compound are likely to
translate into observable changes in hepatic clearance. Since the extrapolation of \textit{in vitro} \( \text{CL}_{\text{int}} \)
to the \textit{in vivo} situation also requires consideration of the free fraction in blood, we also
considered potential differences in blood distribution for the two compounds. No marked
differences were observed between the proto and deutero form either in terms of 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 (Stringer et al., submitted for publication). Several secondary metabolites
previously observed in hepatocytes, including the carboxylic acid (M2) and conjugated
metabolites of M7 (sulphate 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 \textit{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 consequence 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 alk oxyaryl ring, aliphatic
hydroxylation of the core and aliphatic hydroxylation of the triazole ring.

For NVS-CRF38 the disparity between \( K_D / K_H \) determined based on either all metabolic
pathways or O-demethylation alone and multitude of biotransformation routes for NVS-
CRF38 strongly suggest that metabolic switching occurs \textit{in vivo}. These observations are
supported by comparative \textit{in vitro} metabolite identification studies which demonstrate that for
d-NVS-CRF38 (compared to 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 were performed using non-radiolabelled 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 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 the 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 in vivo, clearly that is not always the case and for many compounds marked in vitro isotope effects to 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, cytochrome 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 to drug development activities for non-labeled small molecule drugs, drug development activities for deuterium substituted analogues are not foreseen to be significantly
different from their non-labeled counterpart. From 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 utilized for deuterium labeled drug candidates. Importantly, a deuterium labeled analogue should offer a unique benefit or property for the patient which is an improvement in comparison with the non-labeled counterpart, or conventional therapies.

Although health authority submissions and subsequent approval would be necessary for deuterium labeled drugs, the information and learnings available from the non-labeled drug might be helpful and beneficial. The available information and data from the non-labeled 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 non-labeled drugs. Although cost of goods for a deuterium labeled version might be somewhat more expensive than a non-labeled analogue, 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 costs in an analogous manner to that of non-labeled 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. Thorough knowledge of metabolic pattern 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.
Acknowledgements

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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


System-dependent effects of specific deuteration with aldehyde oxidase cleared drugs. *Drug Metab Dispos* **40**:625-634.


Legends for Figures

FIG. 1. Structure for NVS-CRF38 and [^{13}CD_{3}] NVS-CRF38

FIG. 2. Hanes-Woolf plots for NVS-CRF38 and [^{13}CD_{3}] NVS-CRF38 O-demethylation in A) rat and B) human liver microsomes. The microsomal protein concentration is 0.8 mg/mL and incubation time 20 minutes. Data is the mean and standard deviation of three determinations.

FIG. 3. Plasma concentration time profiles for A) NVS-CRF38 and B) [^{13}CD_{3}] NVS-CRF38 after intravenous administration to male Sprague-Dawley rats. Compounds were formulated as a solution in 1:1 v/v PEG200:saline, the dose volume was 1 mL/kg. Data is the mean and standard deviation of four rats.

FIG. 4. Plasma concentration time profiles for A) NVS-CRF38 and B) [^{13}CD_{3}] NVS-CRF38 after oral administration to male Sprague-Dawley. Compounds were formulated as a suspension in 0.5% methylcellulose and 0.5% Tween 80, the dose volume was 5 mL/kg. Data is the mean and standard deviation of four rats.

FIG. 5. A) Metabolic pattern for NVS-CRF38 and [^{13}CD_{3}] NVS-CRF38 after incubation in human and rat liver microsomes for 20 minutes. The substrate concentration was 5 µM and microsomal protein concentration was 0.8 mg/mL.

FIG. 6. Uncorrected peak areas for each of the parent compounds and associated metabolites are provided expressed as % of total peak area for A) male Sprague-Dawley rat microsomes and B) pooled human liver microsomes for 20 minutes.
### Table 1

**Intrinsic clearance values for NVS-CRF38 and [13CD3]NVS-CRF38 in liver microsomes**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Intrinsic clearance (mL/min/kg microsomal protein)</th>
<th>Rat (depletion)</th>
<th>Human (depletion)</th>
<th>Rat (formation)</th>
<th>Human (formation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVS-CRF38</td>
<td></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>[13CD3] NVS-CRF38</td>
<td></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_i/K_D</td>
<td></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>

Intrinsic clearances 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 0.8 mg/mL. The cofactor NADPH was included to activate cytochrome P450 enzymes. Data is the mean and standard deviation of three determinations.
Table 2

Pharmacokinetic parameters for NVS-CRF38 and \[^{13}CD_3\] NVS-CRF38 in male Sprague-Dawley rats after intravenous and oral administration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NVS-CRF38 Parent</th>
<th>NVS-CRF38 Metabolite</th>
<th>[^{13}CD_3] NVS-CRF38 Parent</th>
<th>[^{13}CD_3] NVS-CRF38 Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intravenous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>C(_{\text{max}}) (nM)</td>
<td>–</td>
<td>74±20</td>
<td>–</td>
<td>(^{+})9±1</td>
</tr>
<tr>
<td>T(_{\text{max}}) (hr)</td>
<td>–</td>
<td>0.8±0.0</td>
<td>–</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow\infty}) (nM*hr)</td>
<td>2255±211</td>
<td>402±157</td>
<td>(^{+})4853±327</td>
<td>130±69</td>
</tr>
<tr>
<td>CL(_{b}) (mL/min/kg)</td>
<td>21.2±2.0</td>
<td>–</td>
<td>(^{+})9.7±0.7</td>
<td>–</td>
</tr>
<tr>
<td>V(_{ss}) (L/kg)</td>
<td>3.3±0.7</td>
<td>–</td>
<td>3.8±0.6</td>
<td>–</td>
</tr>
<tr>
<td>Half-life (hr)</td>
<td>2.7±0.7</td>
<td>6.9±1.8</td>
<td>5.2±1.4</td>
<td>12.8±7.5</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>2.6±0.3</td>
<td>–</td>
<td>(^{+})6.5±1.5</td>
<td>–</td>
</tr>
<tr>
<td>fm</td>
<td>0.21±0.08</td>
<td>–</td>
<td>0.07±0.04</td>
<td>–</td>
</tr>
<tr>
<td><strong>Oral</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>10</td>
<td>–</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>C(_{\text{max}}) (nM)</td>
<td>4111±265</td>
<td>674±171</td>
<td>6148±1506</td>
<td>113±20</td>
</tr>
<tr>
<td>T(_{\text{max}}) (hr)</td>
<td>0.8±0.0</td>
<td>0.6±0.3</td>
<td>1.3±1.0</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow\infty}) (nM*hr)</td>
<td>23391±1475</td>
<td>4554±1510</td>
<td>(^{+})60218±14726</td>
<td>(^{+})1812±631</td>
</tr>
<tr>
<td>Oral half-life (hr)</td>
<td>3.5±0.6</td>
<td>9.3±2.2</td>
<td>(^{+})5.6±1.1</td>
<td>14.6±6.0</td>
</tr>
<tr>
<td>F (%)</td>
<td>104±7</td>
<td>–</td>
<td>124±30</td>
<td>–</td>
</tr>
<tr>
<td>Blood distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f(_{\text{ub}})</td>
<td>0.17±0.02</td>
<td>–</td>
<td>0.14±0.02</td>
<td>–</td>
</tr>
<tr>
<td>BPR</td>
<td>0.83±0.07</td>
<td>–</td>
<td>0.86±0.05</td>
<td>–</td>
</tr>
</tbody>
</table>

For intravenous administration the compounds were formulated in 50:50 v/v PEG200:saline, the dose volume was 1 mL/kg. For oral administration compounds were formulated as a...
suspension in 0.5% methylcellulose containing 0.5% Tween 80 and dosed at 5 mL/kg. Data is the mean and standard deviation of four rats. †, †† Denote significant differences (students t-test) versus values for NVS-CRF38 at the P<0.05 and P<0.01 levels respectively.
### 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} (mL/min/kg)</th>
<th>K_{H}/K_{D}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NVS-CRF38 [^{13}CD_{3}] NVS-CRF38</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>i.v.</td>
<td>203.6±31.0</td>
<td>84.1±7.2</td>
</tr>
<tr>
<td>O-demethylation</td>
<td>i.v.</td>
<td>42.3±6.5</td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>Total</td>
<td>p.o.</td>
<td>119.7±7.7</td>
<td>58.4±12.5</td>
</tr>
<tr>
<td>O-demethylation</td>
<td>p.o.</td>
<td>24.9±1.6</td>
<td>3.9±0.8</td>
</tr>
</tbody>
</table>
Figure 1

NVS-CRF38

$^{13}[CD_3]$ NVS-CRF38
Figure 3

(A) Concentration (nM) vs. Time (hr)

(B) Concentration (nM) vs. Time (hr)
Figure 4

A

B

Concentration (nM)

Time (hr)

Concentration (nM)

Time (hr)
Figure 5

\[ \text{Diagram showing molecular structures involving compounds M10, M11, M7, M6, M3, M1, M4, M5 with variables R = CH}_3 \text{ or CD}_3 \]