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RESEARCH ARTICLE

Deuterium isotope effects in drug pharmacokinetics II: Substrate-dependence of the reaction mechanism influences outcome for cytochrome P450 cleared drugs

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Abstract

Two chemotypes were examined in vitro with CYPs 3A4 and 2C19 by molecular docking, metabolic profiles, and intrinsic clearance deuterium isotope effects with specifically deuterated form to assess the potential for enhancement of pharmacokinetic parameters. The results show the complexity of deuteration as an approach for pharmacokinetic enhancement when CYP enzymes are involved in metabolic clearance. With CYP3A4 the rate limiting step was chemotype-dependent. With one chemotype no intrinsic clearance deuterium isotope effect was observed with any deuterated form, whereas with the other chemotype the rate limiting step was isotopically sensitive, and the magnitude of the intrinsic clearance isotope effect was dependent on the position(s) and extent of deuteration. Molecular docking and metabolic profiles aided in identifying sites for deuteration and predicted the possibility for metabolic switching. However, the potential for an isotope effect on the intrinsic clearance cannot be predicted and must be established by examining select deuterated versions of the chemotypes. The results show how in a deuteration strategy molecular docking, in-vitro metabolic profiles, and intrinsic clearance assessments with select deuterated versions of new chemical entities can be applied to determine the potential for pharmacokinetic enhancement in a discovery setting. They also help explain the substantial failures reported in the literature of deuterated versions of drugs to elicit a systemic enhancement on pharmacokinetic parameters.

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Introduction

Because of the potential to enhance pharmacokinetic properties or decrease toxicity by virtue of a kinetic deuterium isotope effect, the replacement of hydrogen by deuterium at non-exchangeable carbon-hydrogen bonds of drug molecules has received extensive attention as indicated by an exponential increase over the past decade in patent applications for deuterated versions of existing pharmaceuticals and new chemical entities [1,2]. As reported previously

these authors are articulated in the ‘author contributions’ section.

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by us for aldehyde oxidase-cleared drugs, successful application of a deuteration strategy requires a clear understanding of the metabolic and systemic clearance mechanisms, and species differences in metabolic pathways [3].

Cytochrome P450 enzymes (CYP) are responsible for over 90% of all metabolic clearance of drugs and xenobiotics, and three quarters of these reactions are attributable to five CYP isoforms (1A2, 2C9, 2C19, 2D6, and 3A4) with CYP3A4 contributing approximately 27% to the metabolism of all marketed drugs [4]. Thus, any deuteration strategy must consider the complex reaction mechanisms of these enzymes that can confound a deuteration strategy leading to a failure to achieve significant systemic pharmacokinetic gain even when metabolism by these enzymes may be rate-limiting in systemic clearance [5–8]. Examples of such mechanistic complexity include: a) Differences in reaction mechanisms of C-H bond cleavages such as the N- and O- dealkylation reactions, where single electron transfer and hydrogen atom abstraction mechanisms can have substantial differences in the magnitude of their intrinsic deuterium isotope effect [9,10]; b) Deuterium-induced metabolic switching to proximal or distal non-deuterated sites [11,12] which is possibly due to multiple binding orientations of a molecule within an active site, or freedom for a bound molecule to “tumble” within an active site because of the large active site cavity of some CYP enzymes, such that oxidation at a non-deuterated site compensates for decreased metabolism at the deuterated site resulting in loss of an isotope effect on the intrinsic clearance and a redistribution of the relative abundance of metabolites; and c) A rate limiting release of product resulting in masking of the intrinsic deuterium isotope effect ($^{H}k/^{D}k$) on the intrinsic clearance ($^{H}V_m/K_m\,/\,^{D}V_m/K_m$) [13,14].

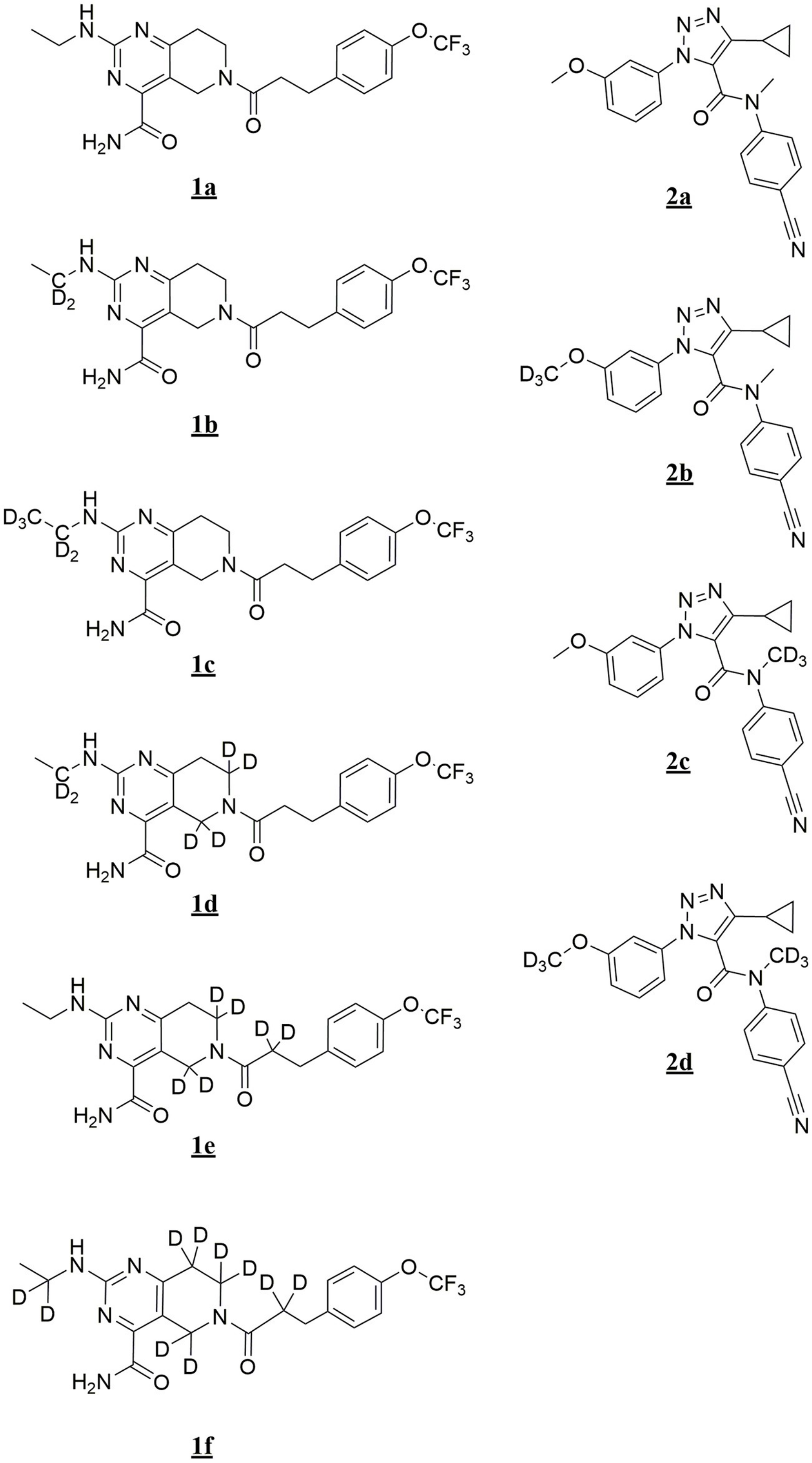
In this study we examined two structurally distinct chemo-types (Fig 1, **1a** and **2a**) where in-vitro clearance predictions with hepatic microsomes and hepatocytes suggested a blood flow-limited, CYP-mediated oxidative metabolic clearance. Using virtual molecular docking with CYPs 3A4 and 2C19, metabolic profiles and intrinsic clearance isotope effects with human liver microsomes and recombinant CYPs 3A4 and 2C19 with deuterated versions of **1a** and **2a**, we demonstrate the mechanistic complexities of CYP-catalyzed reactions where the rate limiting step may be determined by the substrate under consideration. The two chemotypes examined also provide an understanding of how to address a deuteration strategy for new chemical entities, and helps explain the numerous reports where deuteration has been largely ineffective in substantially altering the in-vivo pharmacokinetics of some CYP-cleared compounds [5–8].

Materials and methods

The synthesis and characterization of chemotypes **1a** and **2a** have been previously reported [15,16]. Synthesis procedures for **1a** and analytical data for deuterated analogs of **1a** and **2a** are presented in Supporting information (S1 File). The identities of primary metabolites from **1a** and **2a** were established from their mass spectral fragment patterns and are presented in Supporting information (S2 File).

Molecular docking

Structures of **1a** and **2a** were constructed using ChemBioOffice (PerkinElmer Inc. Waltham, MA) and stored in SD format. These structures were then modified with a customized script written in Python: the explicit hydrogen atoms were added, formal charge was calculated, and the structures were transformed into PDB format, with the integration of the OEChem Toolkit (OpenEye Scientific Software Inc., Santa Fe, NM). The derived molecular structures were further optimized with a DFT/B3LYP (Becke three-parameter Lee-Yang-Parr) approach using a 6-31G** basis set in Gaussian 09 (Gaussian, Inc., Wallingford, CT). The energetically



1a **1b** **1c** **1d** **1e** **1f**

2a **2b** **2c** **2d**

Fig 1. Structures of chemotypes 1a and 2a and their respective deuterated forms examined in this study.

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minimized structures of **1a** and **2a** were then modified by AutoDockTools (The Scripps Research Institute, La Jolla, CA) with flexible torsions defined and Gasteiger atomic charges assigned, as the final ligand input files for docking. To prepare the protein templates, three-dimensional coordinates of CYP3A4 and CYP2C19 structures were collected from both Protein Data Bank and Pfizer's Protein Structure Database. The protein templates were selected and customized specifically for **1a** and **2a** with previous findings [17]. Specifically, for CYP3A4, the core template was 3NXU, a crystal structure determined at resolution of 2.0 Å with the inhibitor ritonavir bound [18]; for CYP2C19, 4GQS, a crystal structure determined at resolution of 2.9 Å complexed with the inhibitor (2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl)methanone was used as the template [19]. These templates were further modified by AutoDockTools to add polar hydrogen atoms, Kollman partial charges, and solvation parameters. The partial charge of the iron (Fe) was assigned as 0.262 and the proximal oxygen (O) as -0.342, with the compound I Fe-O length assigned as 1.6 Å, according to previously quantum mechanically derived heme parameters [20]. The active site space of CYP3A4 and CYP2C19 was defined by AutoGrid 4.0 (The Scripps Research Institute), which pre-calculates the van der Waals, hydrogen bonding, electrostatics, torsional, and solvation interactions between protein and studied compounds. Docking procedures were accomplished with AutoDock 4.0 (The Scripps Research Institute) on Pfizer's high performance computing Linux clusters. The globally optimized conformation and orientation of compound **1a** and **2a** were searched using a Lamarckian generic algorithm, a hybrid of generic algorithms and an adaptive local search method. The derived 100 docking poses for each compound were clustered according to RMSD (root-mean-square deviation). The binding poses with the lowest binding energies and within 5Å to the heme iron-oxo were automatically chosen by customized scripts for further analysis, and visualized using PyMOL (Schrödinger, LLC, New York, NY).

Reactions with human liver microsomes and recombinant CYP enzymes for the assessment of metabolic profiles, relative CYP isoform activities, and intrinsic clearance isotope effects

Microsomal and recombinant CYP reactions were conducted at 37 ˚C in final volumes of 1.0 mL (for first order substrate depletion rate constant assessment at substrate concentrations of 1.0 μM), and 2.0 mL (for metabolic profiles at substrate concentrations of 10 μM). Each reaction contained 100 mM potassium phosphate buffer pH 7.4, either 0.5 mg/mL human liver microsomal protein or 10 pmol/mL rCYP isoform co-expressed with cytochrome P450 oxido-reductase in insect cell membranes. Reactions were initiated by the addition of 3.0 mM NADPH or an NADPH regenerating system (0.3mM $NADP^+$, 1 mM isocitrate, 0.5 mM $MgCl_2$ and 1.0 unit isocitrate dehydrogenase).

For metabolic profiles, reactions were incubated for 30 minutes at 37 ˚C then quenched by adding 5.0 mL of acetonitrile. After mixing, the samples were centrifuged at 1800 x g for 20 minutes and the supernatants were decanted and dried at room temperature under reduced pressure in a vacuum centrifuge. The residues were re-suspended in 200 μL of acetonitrile: DMSO:water (5:20:75), centrifuged as above to remove insoluble material and a 50 to 100 μL aliquot of the supernatant was analyzed by LC/MS as described below.

For relative activities of CYP isoforms and intrinsic clearance, substrate depletion at 1.0 μM was the method of choice for assessment of the depletion rate constants as each substrate produced multiple metabolites, and an estimation of Km for substrates (**1a** and **2a**) by the substrate depletion method showed that their respective Km's were greater than 5 μM [21,22]. Relative CYP activities were determined from the ratio of the depletion rate constant for each isoform relative to that for CYP3A4. Intrinsic clearance isotope effects were determined from competitive reactions using 1:1 mixtures of the protio and appropriate deuterio form of the substrate at 0.5 μM each. Eight 100 μL aliquots each were removed over a period of 70 to 90 minutes and added to 100 μL of a 0.1 μM solution of an internal standard in methanol to quench the enzymatic reaction. The samples were filtered through a high protein-binding capacity filter membrane in a 96-well format. The filtrates were evaporated in a vacuum centrifuge to near dryness, diluted with 125 μL of water, and a 99 μL aliquot was analyzed by reversed phase chromatography/mass spectrometry using selected reaction monitoring. Rate constants were determined from the semi-logarithmic plots of the time versus ratio of the area under the peak for specific transitions of the various substrates and the internal standard. The intrinsic clearance isotope effect was determined from the ratio of the rate constants for the protio- and respective deuterio- forms.

LC-MS methods

An integrated Thermo-Finnigan LC/MS system consisting of a Surveyor Autosampler, LC pump, diode array detector and either an Orbitrap or LCQ mass spectrometer auto-tuned with the protio-form of the compound of interest were used in all analytical work. Two chromatographic conditions were used for analysis. For rate measurements, a steep linear gradient from 30 to 95% acetonitrile was used with a Phenomenex Luna C18, 5 μm 2 x 50 mm column. For the identification of metabolites, a shallow gradient from 5 to 95% acetonitrile at a linear rate of 2.25% per minute was used with a Phenomenex Luna C-18, 3 μm, 4.6 x 150 mm column. The gradients used are shown in Tables 1 and 2.

Metabolites were identified by standard techniques that include: extraction of ion masses from the total ion current corresponding to known metabolic transformations; identifying drug-derived substances by extracting ion masses from the MS2 and MS3 ion chromatograms that are common to the parents in their MS2 and MS3 spectra; and, examining fragment patterns of ions in the total ion current spectrum to determine if they are drug-related, in regions where UV (250–400 nm) absorbing peaks occurred.

Results

Molecular modeling

Molecular docking studies of compound **1a** with CYP3A4 showed two energetically favored binding clusters (clustered at RMSD 2.0 Å) where either the pyrimidino-piperidine ring (Fig 2 Panel A) or the terminal *N*-ethyl moiety (Fig 2 Panel B) of compound **1a** are in proximity to the heme iron of CYP3A4 for aliphatic hydroxylations at the pyrimidino-piperidine ring and *N*-deethylation reactions, respectively. Other binding clusters that were also energetically favored (1–2 kcal/mol within the lowest-energy binding cluster) but not in an orientation for a typical P450-catalyzed reaction, were not included for analysis, an approach we previously reported [23–27]. For example, the binding pose with the trifluoromethyl moiety of **1a** closest

Table 1. Steep gradient for LC/MS analysis.

Time (min)	0.1% formic acid in water	Acetonitrile	Flow rate (μL/min)
0	70	30	500
2.0	70	30	500
5.0	5	95	500
6.0	5	95	500
6.5	95	5	500
8.0	95	5	500

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