Biosynthesis of Fluorinated Analogs of Drugs Using Human Cytochrome P450 Enzymes Followed by Deoxyfluorination and Quantitative Nuclear Magnetic Resonance Spectroscopy to Improve Metabolic Stability

R. Scott Obach, Gregory S. Walker, and Michael A. Brodney

Pharmacokinetics, Pharmacodynamics, and Drug Metabolism (R.S.O., G.S.W.) and Worldwide Medicinal Chemistry (M.A.B.), Pfizer Inc., Groton, Connecticut and Cambridge, Massachusetts

Received January 5, 2016; accepted February 24, 2016

ABSTRACT

Replacement of hydrogen with fluorine is a useful drug design strategy when decreases in cytochrome P450 (P450) metabolic lability are needed. In this paper, a facile two-step method of inserting fluorine into metabolically labile sites of drug molecules is described that utilizes less than 1 mg of starting material and quantitative NMR spectroscopy to ascertain the structures and concentrations of products. In the first step, hydroxyl metabolites are biosynthesized using human P450 enzymes, and in the second step these metabolites are subjected to deoxyfluorination using diethylaminosulfur trifluoride (DAST). The method is demonstrated using midazolam, celecoxib, ramelteon, and risperidone as examples and CYP3A5, 2C9, 1A2, and 2D6 to catalyze the hydroxylations. The drugs and their fluoro analogs were tested for metabolic lability. 9-Fluoroderiperidone and 4'-fluoroceloxib were 16 and 4 times more metabolically stable than risperidone and celecoxib, respectively, and 2-fluororamelteon and ramelteon were metabolized at the same rate. 1'-Fluoromidazolam was metabolized at the same rate as midazolam by CYP3A4 but was more stable in CYP3A5 incubations. The P450-catalyzed sites of metabolism of the fluorine-containing analogs were determined. Some of the metabolites arose via metabolism at the fluorine-substituted carbon, wherein the fluorine was lost to yield aldehydes. In summary, this method offers an approach whereby fluorine can be substituted in metabolically labile sites, and the products can be tested to determine whether an enhancement in metabolic stability was obtained.

Introduction

Drug design can have many simultaneous challenges of optimizing target potency, selectivity, dispositional properties, and safety. Among dispositional properties, rapid metabolic intrinsic clearance is a commonly encountered problem that can result in unacceptable projected dosing regimens (required dose level is too high owing to high clearance and first-pass extraction, and/or dosing frequency is too high owing to short half-life), as well as a greater potential for pharmacologically active or toxic metabolites. Cytochrome P450-mediated metabolism is the most frequently encountered reason for excessively high intrinsic clearance. Common strategies to mitigate high metabolic turnover from P450-mediated clearance include optimizing physicochemical properties such as lipophilicity, molecular weight, and/or pKa of basic amines. Additional strategies to reduce metabolic clearance involve blocking or modifying a metabolic short-spot by the use of chemical isosters or groups that modify the electronics or sterics at a metabolic site. By introducing specific design elements proximal to or in place of a site of rapid P450-catalyzed metabolism, the intrinsic clearance can be attenuated by decreasing metabolic rate or changing the site of metabolism. Molecular properties and specific structural entities will impact binding of molecules to P450 enzymes, and the subsequent orientation and chemical reactivity of specific positions on these molecules dictates the observed metabolic products. Since the P450 catalytic mechanism for aliphatic hydroxylation involves abstraction of a hydrogen atom (Groves, 2005), replacing a rapidly abstracted hydrogen with a halogen atom such as fluorine is a commonly employed strategy to reduce clearance.

Incorporating a fluorine in drug design is a powerful strategy to improve compound properties (Purser, et al., 2008; Gillis, et al., 2015; Murphy and Sandford, 2015). The small size and electronegativity of fluorine can impart dramatic changes on compound conformation, permeability, pKa, potency, and metabolism. Specifically, fluorination at a site of P450 metabolism may result in either a decrease in metabolic rate or a change in the site of metabolism, thus fluorine substitution is a commonly used approach to modulate drug metabolism. Therefore, an ability to easily carry out fluorinations of lead structures at metabolic sites would be a valuable technique to rapidly assess whether such a substitution would have a beneficial impact in drug research projects. Reagents to carry out deoxyfluorination reactions have been described (Al-Maharik and O’Hagan, 2011), with one of the oldest and best described being diethylaminosulfur trifluoride (DAST; Hudlicky, 1988). DAST reactions can be done at ambient conditions in aprotic solvents and, as we show in this work, can be done in low volume at microgram scale. Although not proven, a proposed mechanism for deoxyfluorination reactions using DAST begins with reaction of an aliphatic alcohol group with the sulfur and displacement of fluoride as
HF. This intermediate can undergo either subsequent $S_N2$ displacement of the alkyl group by the released fluoride or a dissociative $S_N1$ mechanism and recombination with fluoride to yield the alkyl fluoride product (Baptista, et al., 2006). Overall, this approach would represent an alternative to classic total synthesis approaches.

In a recent report we described a method for generating nanomole quantities of drug metabolites (Walker, et al., 2014). Employment of cryomicroprobe $^1$H–nuclear magnetic resonance (NMR) spectroscopy to characterize the isolated metabolites in both a qualitative and quantitative fashion yielded solutions of drug metabolites of known concentration and structure, and these solutions could then be useful for biologic testing, or used as authentic standard stock solutions for high-performance liquid chromatography (HPLC)–mass spectroscopy (MS) bioanalysis methods, among other applications. While NMR has been historically viewed as a qualitative technique useful for structural characterization, it is also a valuable tool for quantitation. A significant advantage NMR holds over other analytical techniques (i.e., LC-MS, LC-UV) is its uniform response that is independent of structure. As an

![Reaction schemes for P450-catalyzed biosynthetic hydroxylation and deoxyfluorination of four drugs.](image)

**Fig. 1.** Reaction schemes for P450-catalyzed biosynthetic hydroxylation and deoxyfluorination of four drugs.
example, the response for a given hydrogen in a steroid from a 1 mM solution should be equal to the response for a given hydrogen in a glucuronide from a 1 mM solution. This uniform response allows an NMR instrument to be calibrated with a known compound and quantitate samples in the absence of authentic standards (Walker, et al., 2011).

In this report, we describe a facile method whereby drugs that are rapidly metabolized by P450 enzymes can be converted into analogs fluorinated at the site of enzymatic hydroxylation using a hybrid procedure of biosynthesis, chemical synthesis, and quantitative NMR spectroscopy (Fig. 1). A previous report by Rentmeister et al. (2009) coupled the use of a bacterially derived panel of P450 enzymes and subsequent deoxy fluorination to yield fluorinated derivatives on a scale of tens of milligrams. In the present work, synthesis is done at nanoscale, so that fluorinated analogs can be quickly prepared for the purpose of testing whether fluorine substitution yields an improvement in metabolic lability. Hydroxy metabolites are prepared by biosynthesis using the very P450 enzymes responsible for the high lability and then subjected to nanomole-scale deoxy fluorination using DAST. The fluorinated analogs are isolated and analyzed by quantitative NMR spectroscopy to determine the structure and concentration of the stock solution. The material can then be tested for biologic activity and metabolic stability. By using the same enzymes responsible for rapid metabolism, the likelihood that the fluorinated analog ultimately generated will have improved metabolic stability will be greater. The method is described herein using four well known drugs as examples.

Methods and Materials

Materials. Drugs were obtained from the following vendors: midazolam (Cerilliant, Round Rock, TX), celecoxib and ramelteon (Pfizer, Groton, CT), and risperidone (Sigma-Aldrich, St. Louis, MO). Heterologously expressed recombinant human P450 enzymes in microsomes from Sf9 cells were custom prepared under contract by Panvera (Madison, WI). Pooled human liver microsomes from 50 donors of both sexes were prepared under contract by BD Gentest (Woburn, MA). Hexadecuterated dimethylsulfoxide (DMSO) was from Cambridge Isotope Laboratories (Tewksbury, MA). NADPH and DAST (1 M concentration of the stock solution) or recombinant P450 enzymes (1 mM in CH3Cl) were from Sigma-Aldrich. Note that DAST is a caustic chemical and should be used with appropriate personal protective equipment in a laboratory chemical fume hood.

General Biosynthesis and Fluorination Procedure. Substrates (20 μM) were incubated with recombinant P450 enzymes (1–2 nmol), NADPH (1.3 mM), and MgCl2 (3.3 mM) in 40 mM potassium phosphate buffer (100 mM, pH 7.5) in a shaking water bath maintained at 37°C. Incubation times varied from 40 minutes to 2 hours. Incubations were terminated with addition of CH3CN (40 ml) and the precipitated material was removed by spinning the mixtures in a centrifuge for 5 minutes at 1700g. The supernatant was partially evaporated in a vacuum centrifuge (Genevac, Gardiner, NY) for 2 hours. To the remaining mixture was added 0.5 ml neat formic acid, 0.5 ml CH3CN, and water to a final volume of 50 ml. This mixture was spun in a centrifuge at 40,000g for 30 minutes. The clear supernatant was applied to an HPLC column (Polaris C18, 4.6 × 250 mm; 5-μm particle size) through a Jasco HPLC pump at a rate of 0.8 ml/min. After the entire

TABLE 1

Fluorinated analogs of four drugs generated by biosynthesis of hydroxyl metabolites and deoxy fluorination using DAST

<table>
<thead>
<tr>
<th>Parent Drug</th>
<th>Biosynthesis Enzyme</th>
<th>Fluorinated Product</th>
<th>Stock Concentration Obtained (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam</td>
<td>CYP3A5</td>
<td>1'-Fluoromidazolam</td>
<td>1.35</td>
</tr>
<tr>
<td>Ramelteon</td>
<td>CYP1A2</td>
<td>2'-Fluororamelteon</td>
<td>0.26</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>CYP2C9</td>
<td>4'-Fluorocelecoxib</td>
<td>2.32</td>
</tr>
<tr>
<td>Risperidone</td>
<td>CYP2D6</td>
<td>9'-Fluoroisperidone</td>
<td>0.96</td>
</tr>
</tbody>
</table>

sample was applied, an additional ~5 ml of mobile phase (0.1% formic acid containing 1% CH3CN) was pumped through the system. The column was moved to a ThermoFisher LTQ HPLC-MS system (Thermo Fisher Scientific, Sunnyvale, CA) containing a photodiode array detector, and a mobile phase gradient was applied to elute material of interest. The mobile phase comprised 0.1% formic acid in water and CH3CN and was run at a flow rate of 0.8 ml/min. The gradient began by using a dummy injection of water. The eluent passed through the photodiode array detector scanning from 200–400 nm and then to a splitter (ratio was approximately 15:1) with the larger portion going to a LEAP Technologies fraction collector (Leap Technologies, Carrboro, NC). Fractions were collected every 20 seconds, generally over a total run time of 60–80 minutes. The remainder was introduced into the mass spectrometer operated in the positive ion mode. MS3 and MS4 data were collected with source temperature and potential settings optimized for signal and fragmentation for each parent drug. Fractions containing hydroxyl metabolites of interest were analyzed on a ThermoFisher Orbitrap Elite HUPLC-UV-MS system containing an Acquity column (HSS T3 C18, 2.1 × 100 mm, 1.7-μm particle size) using the mobile phases described above at a flow rate of 0.4 ml/min and an injection volume of 5 μl. Fractions containing single peaks by UV and the desired protonated molecular ions were combined and evaporated by vacuum centrifugation in 15-ml conical polypropylene centrifuge tubes.

To the dried residues was added 0.15 ml of 150 mM DAST in CH3Cl. The tubes were capped and initially mixed, and then allowed to stand at room temperature for 2 hours. The reaction mixtures were processed by addition of 1 M Na2CO3 in water (0.5 ml) and extraction with ethyl acetate (2 × 2 ml). The solvent was removed by vacuum centrifugation and the residue was reconstituted in 20 μl CH3CN and 80 μl 0.1% HCOOH. This material was injected onto the aforementioned Polaris C18 column. ThermoFisher LTQ HPLC-UV-MS system with LEAP Technologies fraction collector. Fractions were collected every 20 seconds and those containing fluorinated analogs of interest were analyzed on the aforementioned ThermoFisher Orbitrap Elite HUPLC-UV-MS system. Fractions were pooled as appropriate and the solvent was removed by vacuum centrifugation. Specific details of reaction and HPLC-MS conditions for each drug are listed in the Supplemental Information.

Quantitative Nuclear Magnetic Resonance Analysis. Dried residues containing fluorinated analogs were dissolved in 0.04 ml of DMSO-d6 “100%” (Cambridge Isotope Laboratories, Andover, MA) and placed in a 1.7-mm NMR tube in a dry argon atmosphere. 1H and 13C spectra were referenced using residual DMSO-d6 (δH = 2.50 ppm relative to TMS, δ0 = 0.00, δ13C = 39.50 ppm relative to TMS, δ0 = 0.00). NMR spectra were recorded on an Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA) controlled by TopSpin v3.2 and equipped with either a 1.7-mm TCI CryoProbe or a 5-mm BBO CryoProbe (Bruker BioSpin). One-dimensional 1H spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of approximately 7 seconds. The one-dimensional 13C data were recorded using the standard pulse sequence (g=20,2) provided by Bruker. One-dimensional 13C spectra were recorded using an approximate sweep width of 11,000 Hz and a total recycle time of approximately 7.5 seconds. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. The two-dimensional data were recorded using the standard pulse sequences provided by Bruker. Postacquisition data processing was performed with either TopSpin v3.2 or MestReNova V8.1 (Mestrelab Research, Santiago de Compostela, Spain).

Metabolic Lability Experiments. Drugs and their fluorinated analogs at a substrate concentration of 0.2 μM were incubated with human liver microsomes (0.2–2.0 mg/ml depending on the test compound) or recombinant P450 enzymes (10–100 pmol/ml, depending on the test compound), NADPH (1.3 mM), and MgCl2 (3.3 mM) in an incubation volume of 1 ml potassium phosphate buffer (100 mM, pH 7.45) at 37°C. Incubations were commenced with the addition of NADPH, and aliquots of 0.1 ml were removed at 0, 2, 5, 10, 15, 20, and 60 minutes and added to 0.5 ml CH3CN containing clozapine (0.04 μM) as an internal standard. Terminated incubation mixtures were spun in a centrifuge for 5 minutes at 1700g, the supernatant was removed in a vacuum centrifuge, and residues were reconstituted in 0.2 ml 1% HCOOH in 15% CH3CN and analyzed by HPLC-MS. Ten microliters of supernatant were injected onto an Acquity column (HSS T3 C18, 2.1 × 100 mm, 1.7-μm particle size) coupled with a ThermoFisher Orbitrap Elite HUPLC-MS system. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 ml/min. Initial mobile phase

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conditions were at 10% B, increased linearly to 50% B at 2 minutes, then to 95% B at 3.2 minutes, held at this composition for 1.5 minutes, followed by re-equilibration at initial conditions for 0.8 minutes. The eluent was introduced into the ion source operated in the positive ion mode at a temperature of 350°C, capillary temperature of 300°C, and sheath gas and auxiliary gas settings at 25. Scanning was done from m/z 250 to 450 at a resolution setting of 30,000. Analyte peak areas were determined by integration of ion currents of protonated molecular ions to a range of 5 ppm. Analyte response was corrected by the internal standard response, and the peak area ratios were normalized to that measured at the t = 0 sampling point.

The decline in peak area ratio was plotted over the incubation time, and the first order rate constant was determined using the data that showed a log-linear relationship using GraphPad Prism (v6.03; GraphPad Software, San Diego, CA). Intrinsic clearance was calculated as:

\[
CL_{int} = -\frac{k}{[E]}
\]

where [E] is the concentration of microsomal protein or P450 used in the incubation and -k is the negative slope of the decline of natural log substrate concentration versus incubation time plot.

Fig. 2. High resolution MS^n, 1H-NMR, and 19F-NMR spectra for 1-fluoromidazolam.
Biotransformation of Fluorinated Analogs. Fluorinated analogs (10 μM) were incubated with recombinant P450 enzymes (10 pmol/ml CYP3A4 and 3A5 with 1'-fluoromidazolam, 25 pmol/ml CYP1A2 for 2-fluororamelteon, 50 pmol/ml CYP2C9 for 4'-fluorocelecoxib, 100 pmol/ml CYP2D6 for 9-fluorosperidone), NADPH (1.3 mM), and MgCl₂ (3.3 mM) in an incubation volume of 0.4 ml potassium phosphate buffer (100 mM, pH 7.45) at 37°C. The stock solutions of fluorinated analogs in DMSO-d₆ were evaporated in a Genevac to remove the solvent prior to metabolic incubations and the incubation buffer was added to the tube and sonicated for 10 minutes. Incubations were carried out for 30 minutes, terminated by addition of 2 ml CH₃CN, and spun in a centrifuge at 1700g for 5 minutes to remove precipitated protein. The supernatant was evaporated in a Genevac and reconstituted in 0.08 ml 1% HCOOH for analysis by UHPLC-UV-MS using the aforementioned column and system.

Results

Biosynthesis of Hydroxy Metabolites, Fluorination, and Structure Determination

Four drugs were successfully converted to their fluorine-containing analogs using human P450 enzymes and DAST-mediated deoxyfluorination (Table 1). A description of each preparation follows.

Fluoromidazolam. Incubation of midazolam with CYP3A5 yielded hydroxymidazolam, which was purified and subjected to deoxyfluorination by DAST. The resulting fluoro analog of midazolam was isolated by HPLC and analyzed by high resolution mass spectrometry and NMR spectroscopy. The protonated molecular
ion of m/z 344.0757 was consistent with an empirical formula of C18H13N3ClF4 (–1.0 ppm; Fig. 2). As midazolam is well known to be hydroxylated at the 1’- and 4-positions by CYP3A enzymes (Gorski, et al., 1994), the most likely sites of fluorine substitution are these two positions. However, the major fragment ions resulted from simple losses of chlorine, HF, and methylamine and thus were not informative for structure assignment. Analysis by NMR spectroscopy provided data to assign the site of fluorination on the 1’ position (Fig. 2). The 1H spectrum of the isolated fluoromidazolam contained no methyl resonance and contained a set of new resonances integrating to one hydrogen each at δ5.34 (dd, J = 50.1, 11.8 Hz) and δ5.70 (dd, J = 50.1, 11.8 Hz), Fig. 2. These new resonances are assigned as the methylene of the 1’ position of the fluoromidazolam. This structure is additionally supported by the 1H-13C heteronuclear single quantum coherence (HSQC) data (Supplemental Figs. 1 and 2). As with the 1H spectrum there is no evidence of a methyl. Furthermore the new resonances at δ5.34 and δ5.70 can be correlated to a carbon at δ76.5, which is consistent with a CH2F moiety. All of this data, as well as all other data collected, supports the proposed fluorination at the 1’-position.

**Fig. 4.** High resolution MSn, 1H-NMR, and 19F-NMR spectra for 4’-fluorocelecoxib.
Fluororamelton. Incubation of ramelteon with CYP1A2 generated three hydroxyl metabolites; however, after reaction with DAST only one fluorinated analog was obtained with a protonated molecular ion of m/z 278.1550, consistent with an empirical formula of C_{16}H_{21}NO_{2}F (~0.3 ppm, Fig. 3). Fragment ions showed that the site of fluorination was on the propanamide, which is consistent with a known major metabolite of ramelteon generated by CYP1A2 (Obach and Ryder, 2010). Analysis by NMR spectroscopy demonstrated that the site of fluorination is on the 2-position. In the ^1H spectrum of the isolated fluororamelton, the methyl group is assigned to a set of resonances integrating to three hydrogens at δ1.43 (dd, J = 24.5, 6.7 Hz), Fig. 3. In addition to these resonances, a methine resonance at δ4.99 (dq, J = 49.7, 7.3) can be correlated through the ^1H-^13C HSQC data to a carbon at δ88.8 Both the ^1H and ^13C shifts are consistent with a R-CHF-CH_{3} moiety (Supplemental Figs. 3 and 4). 2-Hydroxyramelteon is reported as the major metabolite of ramelteon and the stereochemistry at position 2 is reported as the (S)-isomer (Nishiyama, et al., 2014). From the one- and two-dimensional NMR spectral data it appears that there was a single 2-fluororamelton diastereoisomer, and HPLC analysis also shows a single peak for the material. Depending on the substrate, the DAST reaction can proceed through either an SN_{1} mechanism that would result in a mixture of stereoisomers or an SN_{2} reaction that would
result in a single isomer of inverted configuration from the hydroxyl precursor. Since there was a single isomer of 2-fluoromelateon made, it is proposed that it is the single stereoisomer of R-configuration at the 2-position.

**Fluorocelecoxib.** Celecoxib was incubated with CYP2C9, which is known to yield a hydroxyl metabolite on the tolyl methyl position (Tang, et al., 2000). Deoxyfluorination of the isolated hydroxycelecoxib metabolite yielded a fluorinated analog with a protonated molecular ion of \( m/z \) 400.0738, indicative of an empirical formula of \( C_{27}H_{27}NO_2F_2 \) (0.2 ppm). The mass spectral fragmentation pattern was not very informative regarding structure assignment as most fragments merely reflected sequential losses of HF (Fig. 4); however, as there is only one aliphatic site for oxidation and DAST does not deoxyfluorinate phenols, there is only one possible site for modification. This was confirmed with NMR spectroscopy, wherein the 1H spectrum of the isolated fluorocelecoxib contained no methyl resonance and contained a set of new resonances integrating to one hydrogen each at \( \delta 5.42 \) and \( \delta 5.50 \) (Fig. 4). These new resonances are assigned as the methylene of the 4 position of the fluorocelecoxib. This structure is additionally supported by the 1H-13C HSQC data (Supplemental Figs. 5 and 6). As with the 1H spectrum there is no evidence of a methyl. Furthermore, the new resonances at \( \delta 4.42 \) and \( \delta 5.50 \) can be correlated through the 1H-13C HSQC data to a carbon at 88.4, which is consistent with a CH2F moiety. All of this data, as well as all other data collected, supports the proposed fluorination at the 4 position.

**Fluororisperidone.** Risperidone was incubated with CYP2D6 and the isolated hydroxysperidone intermediate was subjected to deoxyfluorination with DAST. The subsequent fluororisperidone product had a protonated molecular ion of \( m/z \) 429.2094, consistent with an empirical formula of \( C_{30}H_{27}NO_2SF_2 \) (0.6 ppm). The fragment ion at \( m/z \) 209 and its subsequent loss of HF suggests that the site of fluorination is on the tetrahydropyridopyrimidone portion of risperidone (Fig. 5). Subsequent analysis by NMR showed that the site of fluorination is on the 9-position, which is consistent with the known site of hydroxylation of risperidone (Mannens, et al., 1993; Fang, et al., 1999). In the 1H spectrum of the isolated fluororisperidone the set of resonances at \( \delta 65.35 \) (dt, J = 48.2, 4.8 Hz) is assigned as the 9 position CHF. In addition to these resonances, the methine resonance at \( \delta 5.35 \) can be correlated through the 1H-13C HSQC data to a carbon at \( \delta 87.4 \) ppm. Both the 1H and 13C shifts are consistent with a R-CHF-CH2-R moiety. All of this data, as well as all other data collected (Supplemental Figs. 7 and 8), supports the proposed fluorination at the 9 position. CYP2D6 has been reported to generate only the R-hydroxy stereoisomer at position 9 (Yasui-Furukori, et al., 2001), while the DAST reaction has the potential to generate a mixture of fluoro stereoisomers (via Sn1 reaction) or a single stereoisomer with inverted structure (via Sn2 reaction). The NMR spectral and chromatographic data strongly suggest that a single enantiomer was generated, and on the basis of the mechanism of reaction, the S-isomer was proposed.

### Metabolic Lability

The four fluorinated analogs were incubated with the P450 enzymes largely responsible for metabolism of the respective nonfluorinated analogs, and the CL\(_{int}\) data obtained were compared to determine the impact, if any, of fluorine substitution at the metabolically labile site. Data are listed in Table 2. In two instances (midazolam versus 1'-fluoromidazolam with CYP3A4 and ramelteon versus 2-fluororametone with CYP1A2) there was minimal to no observed difference in metabolic lability, whereas in the other three there was a marked decrease in metabolism with fluorination. 9-Fluororisperidone showed the most marked decrease in lability compared with its nonfluoro counterpart with a 16-fold decrease in metabolism by CYP2D6. Fluorocelecoxib showed an almost 4-fold decrease in metabolic lability catalyzed by CYP2C9. Interestingly, 1'-fluoromidazolam was metabolized as quickly as midazolam by CYP3A4 but in CYP3A5 it was 3-fold more stable. Overall the data indicate that the impact of fluorine substitution at a site of P450-catalyzed hydroxylation is not predictable and may be governed by other physicochemical properties such as lipophilicity. The data also support using this approach to modify particular P450s responsible for metabolism as a strategy to attenuate drug-drug interactions or overreliance on an enzyme known to be subject to genetic polymorphism.

### Biotransformation of Fluorinated Analogs

The four fluorinated analogs were incubated with recombinant P450 enzymes to provide insight into how biotransformation reactions contribute to changes in metabolic lability. The quantities of metabolites generated were too low to yield NMR data, so structures of metabolites were proposed from MS data. Comparison was made to the known metabolite profiles for the nonfluorinated analogs. High resolution mass spectrometric data for the metabolites of the fluorinated analogs is in the Supplemental Information.

**Biotransformation of 1'-Fluoromidazolam by CYP3A4 and 3A5.** 1'-Fluoromidazolam was metabolized by both CYP3A4 and 3A5 to a similar array of metabolites with a proposed scheme in Fig. 6. Metabolism on the 1'-carbon yielded metabolites that lost the fluorine—presumably via generation of an initial unstable halohydrin that lost fluoride to yield the aldehyde metabolite (peak 7; \( m/z \) 340.0649) that was subsequently reduced to 1'-hydroxymidazolam (peak 1; \( m/z \) 342.0801) or oxidized to the carboxylic acid (peak 4; \( m/z \) 356.0597). There was also a
large peak in the CYP3A5 incubation, where one carbon atom was lost (peak 2; m/z 312.0697), and is hypothesized to arise via decarboxylation of peak 4. This is an unprecedented pathway and would require further exploration to confirm or refute this proposed pathway and mechanism. The other initial pathway yielded putative ring-opened metabolites that lost a nitrogen atom (peak 8; m/z 361.0545), similar to those seen for midazolam (Song and Abramson, 1993). These are proposed to arise via initial 4-hydroxylation that ring-opens to the imine/aldehyde, with the imine hydrolyzing to the ketone. Thus, the sites of metabolism of 1'-fluoromidazolam by CYP3A are largely the same as those for midazolam itself (Heizmann and Ziegler, 1981; Gorski, et al., 1994).

**Biotransformation of 2-Fluororamelteon by CYP1A2.** 2-Fluororamelteon was metabolized by CYP1A2 at the same rate as ramelteon. There were five peaks in the HPLC-UV chromatogram from the incubation extract (Fig. 7) of a similar pattern observed for ramelteon metabolism by CYP1A2 (Obach and Ryder, 2010). A pair of interconverting hydroxyl diastereomers (peak 1) were observed that have base peaks of a sodium adduct (m/z 316.1317) and in-source dehydration (m/z 276.1393), with fragmentation that suggests hydroxylation on the indenofuran system. The other major hydroxyl metabolite (peak 3; m/z 294.1499) has substitution on the aromatic ring suggested by the fragmentation pattern and small red shift in UV wavelength absorption maximum. The third metabolite (peak 4) had a protonated molecular ion of m/z 276.1592, indicating the replacement of fluorine with oxygen, and this metabolite had the same retention time and mass spectral fragmentation as 2-hydroxyramelteon. The fourth (peak 2) arose via hydroxylation and a loss of 2 H (m/z 292.1244) and no further data were gathered. The proposed metabolic pathways for fluororamelteon are in Fig. 7.

**Biotransformation of 4'-Fluoroclocecoxib by CYP2C9.** 4'-Fluoroclocecoxib was metabolized by CYP2C9 at the position of the fluoromethyl. Three metabolites were observed (Fig. 8) and are proposed to be the methyl alcohol (peak 1), aldehyde (peak 3), and carboxylic acid (peak 2). High resolution mass spectra yielded protonated molecular ions of m/z 398.0776, 396.0623, and 412.0577, all of which indicate the loss of one of the fluorines. Hydroxylation of the fluoromethyl group would result in elimination of the fluoride from the halohydrin to yield the aldehyde, which can undergo subsequent reduction or further oxidation to the alcohol and carboxylic acid, respectively. The proposed metabolism scheme is shown in Fig. 8.

**Biotransformation of 9-Fluororisperidone by CYP2D6.** 9-Fluororisperidone was metabolized by CYP2D6 to two metabolites (Fig. 9): a major one with a protonated molecular ion of m/z 445.2044 consistent with the addition of an oxygen atom (peak 2) and a minor one at m/z 427.2142 indicating the replacement of a fluoride with a hydroxyl (peak 1). The exact site of hydroxylation in peak 2 cannot be determined from MS data alone; however, the fragmentation pattern is consistent with the hydroxyl being introduced to one of the three aliphatic carbons.

![Fig. 6. HPLC-UV traces for CYP3A4 and 3A5 1'-fluoromidazolam incubation extracts and proposed metabolic pathways. Peak P refers to unchanged parent. Peak 1 matched 1'-hydroxymidazolam. High resolution mass spectral data for these metabolites are included in Supplemental Figs. 9–16.](image-url)
on the terahydropyridopyrimidone portion. The other metabolite was a match with 9-hydroxyrisperidone. The proposed metabolic scheme for 9-fluororisperidone is shown in Fig. 9.

Discussion

Fluorine is an important atom in drug design, and in this report we have described a facile method to introduce fluorine into drug molecules at sites that are prone to hydroxylation by human P450 enzymes. The quantities of fluorinated products generated are small (nanomoles); however, the use of quantitative NMR spectroscopy reduces the material required to verify the structure and permits a concentrated stock solution to be obtained that can be used for in vitro biologic testing and drug metabolism experiments. Four drugs well known to undergo metabolism by different human P450 enzymes were selected to demonstrate this method, and the impact of fluorine substitution was evaluated in follow up metabolic lability and metabolite identification experiments.

For the four drugs tested, fluorination had different impacts on metabolic lability. 9-Fluororisperidone and 4-9-fluorocelecoxib were considerably more stable in CYP2D6 and CYP2C9 incubations, respectively, than their nonfluorinated counterparts, whereas 2-fluororametelone was metabolized at a comparable rate as rametelone by CYP1A2. It is interesting to note that for risperidone and celecoxib, metabolism by CYP2D6 and CYP2C9 primarily occurs at a single site and when this site possesses fluorine, the rate of metabolism is reduced. The CYP2D6 mediated hydroxylation that did occur for 9-fluororisperidone occurred at a nearby site; also, a small amount of oxidative defluorination of the 9-position was observed. The metabolism of 4'-fluorocelecoxib by CYP2C9 still occurred at the same carbon as in celecoxib (albeit at one-fourth the rate), and the resulting metabolites lost the fluorine. It is most likely that one of the remaining two H-atoms on the methyl is abstracted and the hydroxy intermediate that is generated spontaneously loses fluoride to yield the observed aldehyde. The case of 2-fluororametelone is different in that CYP1A2 catalyzes hydroxylation at multiple sites on rametelone (Obach and Ryder, 2010) and thus blocking only one of these with a fluorine atom does not yield a decrease in metabolic lability. The CYP1A2-generated metabolite profile of 2-fluororametelone was similar to that of rametelone itself, albeit metabolism at the 2-position was less. There was a small amount of oxidative defluorination of 2-fluororametelone. Overall, this suggests that this approach for reducing metabolism would work best for compounds that are metabolized rapidly, but at a single site. For compounds already metabolized at multiple sites, fluorine substitution may only result in “metabolic switching,” wherein blocking a site of metabolism only results in a change to an alternate position but no
decrease in overall rate of metabolism. However, these same effects were not necessarily noted in human liver microsomes. 4′-Fluorocelecoxib was metabolized more rapidly than celecoxib, and the marked difference between 9-fluororisperidone and risperidone with CYP2D6 was substantially lower in liver microsomes. It is probable that other P450 enzymes contribute to the metabolism of the fluoro analogs, increasing the rate. Preliminary investigation has shown that 4′-fluorocelecoxib is metabolized by CYP2D6 and CYP2C19, whereas fluororisperidone is metabolized by CYP3A4 by N-dealkylation at the piperidine (unpublished observations). Ramelteon is metabolized by CYP1A2, 2C19, and 3A4, and it is possible that the fluorinated analog is a better substrate for CYP2C19 and/or 3A4. Thus it cannot be assumed that fluorination will always result in an overall decrease in metabolic lability.

The case of 1′-fluoromidazolam was interesting in that fluorination yielded a different impact on CYP3A4 versus 3A5-mediated metabolism. The rate by the former was largely unaffected, but for the latter there was a 3-fold decrease. The sites of metabolism for both enzymes (i.e., 1′- and 4-positions) appeared to be the same, and similar to those for midazolam itself. Like the other fluoro analogs, there was some oxidative defluorination observed for 1′-fluoromidazolam. Also, 1′-fluoromidazolam was shown to lose the 1′-CH₂F group entirely and this may occur via an initial oxidative defluorination and further oxidation at this position that can result in overall C–C bond cleavage. We have proposed that this occurs via the carboxy metabolite (which is not formed in the absence of fluorine at that position), but more in-depth work would be needed to support or refute this proposal.

The procedure described can yield fluorinated analogs of drugs within a couple of days, which will almost always be faster than redesigning a synthetic scheme for preparing fluorinated analogs of a lead compound. Also, the method has the potential to yield multiply fluorinated analogs if the P450-generated metabolite is either multiply hydroxylated and/or a ketone/aldehyde, and this has been observed for other compounds to which this method has applied (data on file). However, there are practical limitations to the approach, notwithstanding the very low quantities generated and the need for relatively expensive cryomicroprobe NMR instrumentation. Hydroxy compounds generated enzymatically need to be able to withstand the DAST reaction, which can cause dehydration of some alcohols instead of deoxyfluorination. Also, very importantly, DAST works best on aliphatic alcohols, especially benzylic alcohols like three of the four examples used in this paper, and it does not deoxyfluorinate phenols. Future efforts are

![Fig. 8. HPLC-UV trace for the CYP2C9 4′-fluorocelecoxib incubation extract and proposed metabolic pathways. Peak P refers to unchanged parent and the peak marked X designates nondrug related material. Peak 1 matched 4′-hydroxycelecoxib by retention time and mass spectrum. High resolution mass spectral data for these metabolites are included in Supplemental Figures 20–22.](image)
aimed toward expanding the range of metabolites that can be converted to their fluoro substituted analogs, exploring other fluorination reagents for this purpose (Al-Maharik and O’Hagan, 2011; Tang et al., 2011; Fujimoto and Ritter, 2015; Nielsen et al., 2015), investigating the utility of other sources of P450 activity (e.g., liver microsomes) for the enzymatic portion of the procedure, and working toward generating multiply fluorinated analogs from secondary metabolites possessing more than one hydroxyl group. Furthermore, the methodology described in the paper has utility in diversification of lead molecules in order to impact other properties critical in drug discovery, such as potency, selectivity, DDI, and distribution properties. Application of these methods to a contemporary medicinal chemistry program is underway and will be reported in due course.

Acknowledgments

The authors acknowledge Raman Sharma for NMR analysis of other unpublished fluorination products made previously using this approach.

Authorship Contributions

Participated in research design: Obach, Walker, Brodney.
Conducted experiments: Obach, Walker.
Performed data analysis: Obach, Walker.

Wrote or contributed to the writing of the manuscript: Obach, Walker, Brodney.

References


Address correspondence to: Dr. R. Scott Obach, Pfizer Inc., Eastern Point Road, Groton, CT 06340. r.scott.obach@pfizer.com
BIOSYNTHESIS OF FLUORINATED ANALOGUES OF DRUGS USING HUMAN CYTOCHROME P450 ENZYMES FOLLOWED BY DEOXYPFLUORINATION AND QUANTITATIVE NMR SPECTROSCOPY TO IMPROVE METABOLIC STABILITY

Supplemental Figures

R. Scott Obach, Gregory S. Walker, and Michael A. Brodney
Supplemental Figure 1

$^{1}$H-$^{1}$H COSY Fluoromidazolam
$^{1}H-^{13}C$ HSQC Fluoromidazolam

Supplemental Figure 2
$^{1}H-^{1}H$ COSY Fluororamelteon

Supplemental Figure 3
$^1$H-$^{13}$C HSQC Fluororamelteon

Supplemental Figure 4
Supplemental Figure 5

$^1\text{H}-^1\text{H}$ COSY Fluorocelecoxib
Supplemental Figure 6
$^{1}$H-$^{1}$H COSY Fluororisperidone

Supplemental Figure 7
$^{1}H-^{13}C$ HSQC Fluororisperidone

Supplemental Figure 8
High Resolution Mass Spectra for Metabolites of 1’-Fluoromidazolam
m/z 342 (Peak 1; 1’-Hydroxymidazolam)

Supplemental Figure 9
Supplemental Figure 10
m/z 359 (Peak 3)

Supplemental Figure 11
**m/z 356 (Peak 4)**

**Supplemental Figure 12**

**MS\(^1\)**

**MS\(^2\) of m/z 356**

**MS\(^3\) of m/z 338**

Chemical structure and mass spectra of m/z 356 and m/z 338. The figure shows the mass spectra for MS\(^1\), MS\(^2\), and MS\(^3\) with peaks at various m/z values. The peaks represent the relative abundance of ions at different m/z ratios.
m/z 377 (Peak 5)

Supplemental Figure 13
m/z 329 (Peak 6)

Supplemental Figure 14
m/z 340 (Peak 7)

Supplemental Figure 15
m/z 361 (Peak 8)

Supplemental Figure 16
High Resolution Mass Spectra for Metabolites of 2-Fluororamelteon
m/z 294 (m/z 276=H$_2$O; m/z 318=Na$^+$ adduct) (Peak 1)

Supplemental Figure 17
m/z 294 (Peak 3)

Supplemental Figure 18
m/z 276 (Peak 4)

Supplemental Figure 19
High Resolution Mass Spectra for Metabolites of 4’-Fluorocelecoxib
m/z 398 (Peak 1)

Supplemental Figure 20
m/z 412 (Peak 2)

Supplemental Figure 21
m/z 396 (Peak 3)

Supplemental Figure 22
High Resolution Mass Spectra for Metabolites of 9-Fluororisperidone
m/z 427 (Peak 1)

补充分析图23
Supplemental Figure 24