Strategies toward optimization of the metabolism of a series of serotonin-4 partial agonists: investigation of azetidines as piperidine isosteres

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Strategies toward optimization of the metabolism of a series of serotonin-4 partial agonists: investigation of azetidines as piperidine isosteres

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Abstract

1. The first generation 5HT-4 partial agonist, 4-\{4-\{4-Tetrahydrofuran-3-yloxy\}-benzo[d]isoxazol-3-yl\}oxy\}-piperidin-1-ylmethyl\}-tetrahydropyran-4-ol, PF-4995274 (TBPT), was metabolized to N-dealkylated (\textsuperscript{M1}) and an unusual, cyclized oxazolidine (\textsuperscript{M2}) metabolites. \textsuperscript{M1} and \textsuperscript{M2} demonstrated pharmacological activity at 5HT receptor subtypes warranting further investigation into their dispositional properties in humans; \textsuperscript{M2} was a minor component in vitro but was the pre-dominant metabolite identified in human plasma.

2. To shift metabolism away from the piperidine ring of \textsuperscript{TBPT}, a series of heterocyclic replacements were designed, synthesized, and profiled. Groups including azetidines, pyrrolidines, as well as functionalized piperidines were evaluated with the goal of identifying an alternative group that maintained the desired potency, functional activity, and reduced turnover in human hepatocytes.

3. Activities of 4-substituted piperidines or pyrrolidine analogs at the pharmacological target were not significantly altered, but the same metabolic pathways of N-dealkylation and oxazolidine formation were still observed. Altering these to bridged ring systems lowered oxazolidine metabolite formation, but not N-dealkylation.

4. The effort concluded with identification of azetidines as second-generation 5HT\textsubscript{4} partial agonists. These were neither metabolized via N-dealkylation nor converted to cyclized oxazolidine metabolites rather oxidized on the isoxazole ring. The use of azetidine as a replacement for aliphatic aza-heterocyclic rings in drug design to alter drug metabolism and pharmacology is discussed.

Keywords

Alicyclic amine, azetidine, metabolism, piperidine

Introduction

As described previously (Johnson et al., 2012), 5-HT\textsubscript{4} receptor agonists reportedly stimulate brain acetylcholine (ACh) release and have emerged as potential therapeutics for the treatment of Alzheimer’s disease. The piperidine-tetrahydropyran (THP) series identified previously (Brodney et al., 2012) was active at 5HT\textsubscript{4d} receptors with an advantage of incorporating varying degrees of partial agonism at these receptors. Optimization of the piperidine THP analogs for binding affinity, intrinsic partial agonism, and acceptable predicted dosing regimen in humans (i.e. <50 mg per os, once daily) resulted in progression of compound \textsuperscript{1} (PF-04995274; TBPT) to early clinical development (Brodney et al., 2012).

In humans, a preliminary investigation of metabolite profile of compound \textsuperscript{1} in pooled plasma (compound \textsuperscript{1} dosed P.O., 40 mg, QD) revealed that metabolite \textsuperscript{M2}, a cyclized oxazolidine, was present in plasma in greater concentrations than the parent drug. Extensive characterization (Sawant-Basak et al., 2013) of \textsuperscript{M2} suggested that it was formed via oxidation of the piperidine ring to a putative iminium metabolite, which cyclized to the oxazolidine by intramolecular –OH attack on the iminium ion (Figure 1). We demonstrated that \textsuperscript{M2} was active as a partial agonist at 5-HT\textsubscript{4d} receptors, confounding our quantitative understanding of target pharmacology. In addition, profiling \textsuperscript{M2} across several receptors showed that this metabolite possessed activity as a 5HT\textsubscript{2b} receptor agonist, which is associated with an increase in cardiovascular safety risks (Fitzgerald et al., 2000; Greffe et al., 2007). In addition to \textsuperscript{M2}, compound \textsuperscript{1} also was metabolized to a minor metabolite \textsuperscript{M1}, an N-desalkyl piperidine metabolite, which was a 5-HT\textsubscript{3} antagonist.

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and a selective CYP1A2 reversible inhibitor (internal data). While the latter could be further understood using clinical drug–drug interaction trials and physiologically based pharmacokinetic modeling, occurrence of the major metabolite M2 was a potential concern during development of compound 1. Hence research efforts were triggered to identify analogs of compound 1 with an aim to reduce or eliminate the generation of M2-like structures as metabolites, as well as reduce the generation of M1. Elimination of the two pathways required understanding the mechanism of piperidine ring oxidation and deriving a qualitative structure–metabolism relationship using in vitro human hepatic systems. In this report, we discuss the structure–metabolism relationships observed and introduce novel analogs with improved profile, which eventually yielded the azetidine analog (compound 7).

**Materials and methods**

**Materials**

Compounds 1–10 (Table 1) were prepared at Pfizer Inc. (Groton, MA). Human liver microsomes pooled from 50 donors (a mixture of males and females) were prepared by BD Gentest (Corning, NY) under contract to Pfizer. NADPH was obtained from Sigma-Aldrich (St. Louis, MO). Other reagents and solvents were from common laboratory suppliers.

**Synthesis: general procedure for compounds 1–3 and 5–10**

To the required Boc-amino alcohol (0.20 mmol) in a 2 dram vial was added 4-fluorobenz[d]isoxazol-3-ol (30.6 mg, 0.20 mmol) as a suspension in anhydrous toluene (1 mL). A solution of cyanomethylene tri-butylphosphorane (67.6 mg, 0.28 mmol) in anhydrous toluene (0.5 mL) was then added and the vial was capped and heated at 90 °C for 18 h. The solvent was then evaporated to dryness and to the residue was added a solution of (R)-tetrahydrofuran-3-ol (70.5 mg, 0.8 mmol) in anhydrous tetrahydrofuran (0.5 mL) followed by a solution of sodium hexamethyldisilazide (0.8 mL, 0.8 mmol, 1 N in tetrahydrofuran) was added. The resulting mixture was then heated at 60 °C for 20 h. The solvent was evaporated, and, to the residue, was added a mixture of trifluoroacetic acid (1 mL) and dichloroethane (1 mL). The reaction mixture was then stirred at room temperature for 2.5 h and the solvent was removed under reduced pressure. The residue was partitioned between saturated sodium bicarbonate (1.5 mL) and ethyl acetate (2.5 mL). The biphasic mixture was separated and the aqueous portion was extracted three additional times with ethyl acetate (2.5 mL). The combined organics were dried with anhydrous sodium sulfate, it was filtered and the filtrate was concentrated under reduced pressure. The remaining residue was suspended in a 3:1 mixture of isopropanol/water (1 mL) and triethyl amine (0.056 mL, 0.4 mmol) was added followed by a solution of 1,6-dioxaspiro[2.5]octane (25 mg, 0.22 mmol) dissolved in 2-propanol (0.1 mL). The vial was capped and heated at 80 °C for 4 h. The solvent was evaporated and the crude residue was purified via HPLC purification. Purification conditions and analytical data of the 5HT4 analogs are listed in Supplementary Table 1 and Figure 2.

**Synthesis of compound 4**

To compound 1 (100 mg, 0.213 mmol) in 5:1 dimethylformamide and tetrahydrofuran (6 mL) was added sodium hydride

![Figure 1. General synthetic scheme of compounds 1–10.](image-url)
Table 1. Estimates of percent conversion of compound 1 (TBPT) and analogs to N-dealkylated (M1) and cyclized oxazolidine (M2) metabolites.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure type</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (TBPT)</td>
<td>Piperidine</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Pyrrolidine</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>3-Azabicyclo[3.1.0]hexane</td>
<td>58</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Piperidine with methoxy</td>
<td>69</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>4-Methylpiperidine</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>4-Fluoropiperidine</td>
<td>93</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 2. Proposed metabolism of the piperidine nitrogen to N-dealkylation (M1) and formation of an oxazolidine metabolite (M2) via an iminium ion intermediate.

(continued)
(60% dispersion in mineral oil, 25.6 mg, 0.639 mmol) and the reaction mixture was stirred at room temperature for 30 min. Methyl iodide (242 mg, 1.704 mmol) was added drop-wise and the reaction was stirred at room temperature for 16 h. Saturated aqueous ammonium chloride (20 mL) was added and the aqueous phase was washed with ethyl acetate (3 × 50 mL). Organic extracts were combined, dried over anhydrous sodium sulfate, filtered, and concentrated. The crude residue was purified by reverse phase HPLC. Purification conditions and analytical data of the 5HT₄ analogs are listed in Supplementary Table 1 and Figure 2.

### Incubation procedure for metabolite identification

Test compounds 1–10 were incubated with pooled human liver microsomes (pool of 50 livers purchased from BD Gentest (Woburn, MA) (2 mg of total microsomal protein/mL) and NADPH (1.3 mM) in 1 mL of 0.1 M potassium phosphate buffer, pH 7.45, containing MgCl₂ (3.3 mM) at 37 °C for 30 min in a shaking water bath. Incubations were terminated with addition of CH₃CN (5 mL) to precipitate the protein, spun in a centrifuge for 5 min at 1700 g, and the supernatants were evaporated in a vacuum centrifuge. Residues were reconstituted in 0.2 mL 1% HCOOH for analysis by HPLC-MS.

### HPLC-MS analysis for metabolite identification

Reconstituted incubation extracts were analyzed on a Thermo HPLC-MS system (Thermo Fisher Scientific, Inc., Waltham, MA) composed of a Surveyor HPLC system and photodiode array (PDA) UV/VIS detector in line with an LTQ mass spectrometer operated in the positive mode with data-dependent scanning. The column was a Polaris (Thermo Fisher

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure type</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azetidine; n = 1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Azetidine; n = 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>3-Oxa-9-azabicyclo[3.3.1] nonane</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>Tropane</td>
<td>47</td>
<td>ND</td>
</tr>
</tbody>
</table>

*aPercentage for M1 include M1 itself and downstream metabolites.

bND, not detected.
Scientific, Inc., Waltham, MA) C_{18} (250 × 4.6 mm; 5 μm particle size) with mobile phase at a flow rate of 0.8 mL/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The composition was held at 10% B for 5 min, followed by a linear gradient to 50% B at 50 min and subsequent wash and re-equilibration steps. The effluent passed through the PDA detector scanning from 200 to 400 nm, the flow split with approximately 10% going to the ion trap mass spectrometer. Source parameters and potentials were adjusted to optimize the signal and fragmentation for compound 1. Percentages comprised for each metabolic pathway were estimated by integrating the UV peak areas for each metabolite and parent compound using the combined trace at 280–320 nm. Peak areas for all metabolites were summed, and the percentage that each metabolite comprised was calculated relative to this sum. This assumes that extinction coefficients for metabolites were similar to each other in this wavelength range.

**Determination of in vitro intrinsic clearance in human liver microsomes**

Compounds 1–10 were prepared as solutions in methanol. The final concentration of methanol in the incubation media was 0.2% (v/v). In vitro t_{1/2} of compounds 1–10 was determined in triplicate in an incubation containing substrate (2 μM) within human liver microsomes (P450 concentration, 0.25 μM) in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. The total incubation volume was 1 ml. The reaction mixture was pre-warmed at 37°C for 2 min before adding NADPH (1.2 mM). Aliquots (75 μl) of the reaction mixture at 0, 5, 10, 15, and 30 min were added to acetonitrile (200 μl) containing internal standard (PF-01352968) (0.05 μg/ml), and the samples were centrifuged at 2500 g for 5 min prior to liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis of compounds 1–10 using multiple reaction monitoring (MRM). For control experiments, NADPH was omitted from these incubations.

The microsomal half-life (t_{1/2}) was obtained from a log-linear plot of the substrate depletion versus the incubation time and was scaled to hepatic intrinsic clearance (CL_{int}) using the following equation:

\[
CL_{int, app, scaled} = 0.693 \cdot \frac{1}{T_{1/2}} \cdot \frac{g \text{ liver weight}}{kg \text{ body weight}} \cdot \frac{g \text{ liver}}{mg \text{ microsomal protein}} \cdot \frac{45 mg \text{ microsomal protein}}{ml \text{ incubation}}
\]

in which, the term T_{1/2} refers to the in vitro half-life.

**Agonist-induced cAMP elevation in human 5-HT_{4d} transfectend HEK293 cells**

Human 5-HT_{4d} transfected HEK293 cells (DNA 2.0, Menlo Park, CA) were grown at 37°C and in 5% CO_{2} in DMEM (without sodium pyruvate) supplemented with 10% FBS, 20 mM HEPES (pH 7.4) and 200 μg/mL hygromycin B (Gibco, Waltham, MA). The cells were grown to 60–80% confluence and frozen in liquid nitrogen vapor phase as assay-ready vials. On the day of the experiment, compound dissolved in DMSO was diluted in assay buffer containing PBS, 5 μM HEPES, and 500 μM IBMX (final concentrations). The cells were rapidly thawed in a 37°C water bath and centrifuged to remove DMSO. The cell pellet was then re-suspended in PBS and the cells were counted and diluted appropriately. The reaction was initiated by addition of the cells into 384 well plates containing compound; the final number of cells used in the assay was 5000 cells per well. After incubation for 30 minutes at 37°C, Cisbio (Medford, MA) cAMP Dynamic 2 screening kit reagents (cat# 62AM4PEB) were added to the plate to stop the reaction. Homogenous time-resolved fluorescence-based cAMP (Schering, Merck & Co., Inc., Kenilworth, NJ) detection was determined according to the instruction of the manufacturer. A Wallac Envision was used to measure homogeneous time resolved fluorescence (HTRF) (excitation 320 nm, emission 665 nm/620 nm, delay time 50 μs, window time 400 μs). Data were analyzed based on the ratio of fluorescence intensity of each well at 620 nm and 665 nm followed by cAMP quantification using a cAMP standard curve. Enhancement of cAMP production elicited by each compound was normalized to the amount of cAMP produced by 1 μM serotonin (Sigma, St. Louis, MO).

**Human MDR1-MDCK and RRCK transwell assays**

Human MDR1 transfected MDCK cell line (source: Dr. Piet Borst, Netherlands Cancer Institute, Amsterdam, The Netherlands) and RRCK cell line (generated in house at Pfizer Inc., Groton, MA) assays were employed to measure the apparent permeability of test compound. Apparent permeability (P_{app}) of compound was determined in apical to basolateral (A→B) and basolateral to apical (B→A) directions in triplicates by incubation with compounds 1–10 for 2 h at 37°C. Samples of medium (20 μl) from donor and acceptor compartments were analyzed by tandem liquid chromatography and mass spectrometry (LC/MS-MS). Efflux ratio was calculated as (ER = BA/AB) Detailed protocols for these assays have been described and reported previously (Feng et al., 2009).

**Molecular modeling**

The hydrogen-atom abstraction workflow described in this manuscript was implemented using Discovery Studio and Materials Studio components in Pipeline Pilot 9.1 (Accelrys, Inc, San Diego, CA). For compounds 1, 2, and 7, the workflow was performed stepwise as follows:

- Find the lowest energy conformation of the parent structure (Catalyst; method = BEST).
- Compute semi-empirical QM energy of the lowest energy parent conformer, \( E_{parent} \) (VAMP; Hamiltonian = AM1, maximum step size = 0.1).
- Generate a radical by abstracting a hydrogen atom from the parent structure (materials script or manually drawn).
- Find the lowest energy conformation of the radical structure (Catalyst; method = BEST).
- Compute semi-empirical QM energy of the lowest energy radical conformer, \( E_{radical} \) (Catalyst; method = BEST).
- Repeat steps 3–5 for every hydrogen atom in the structure.
- Compute the relative free energies of all radical structures ($E_{\text{radical}} - E_{\text{radical,min}}$) and the free energies of each radical structure relative to the parent structure ($E_{\text{radical}} - E_{\text{parent}}$).

**Results**

**Synthesis of compounds 1–10**

Identification of the pharmacologically active M2 metabolite of 1 (TBPT) prompted us to probe various structural modifications to limit metabolite formation. We altered the amine ring size, the amine $pK_a$, and the adjacent functional groups around the metabolically labile site, as we postulated that these changes could impact the metabolic profile. To facilitate rapid exploration of these modifications, we developed a parallel chemistry protocol to rapidly synthesize fully elaborated 5HT$_4$ analogs from a common (4-fluorobenzo[d]isoxazol-3-ol) (11) intermediate. A series of N-Boc protected heterocyclic alcohols (12) were coupled with the (4-4-fluorobenzo[d]isoxazol-3-ol) to generate the isoxazole analogs (13). The use of cyanomethylene tri-$N$-butylphosphorane (CMBP) was critical for the Mitsunobu reaction as the byproducts formed (acetonic tetrahydrofuran-3-ol) convert cleanly to the aryl ether (14). Subsequent removal of the Boc protecting group with trifluoroacetic acid followed by epoxide opening provides crude fully elaborated 5HT$_4$ analogs (15) which are purified via HPLC (Supplementary Table 1 and Figure 2).

**Metabolism of compound 1 and analogs in human liver microsomes**

The HPLC-UV metabolic profiles of TBPT (compound 1) and its nine analogs are shown in Figures 3 and 4. Metabolites arising via N-dealkylation of the aliphatic aza-heterocyclic moiety (piperidine, or other rings) are designated as M1, with downstream metabolites designated as M1a or M1b; metabolites wherein an iminium ion is initially formed followed by cyclization to the oxazolidine are designated as M2, and all other pathways are named M3, M4, etc. Compound 1 was metabolized to five products, three initially arising from N-dealkylation (M1, M1a, and M1b), one cyclized oxazolidine (M2), and one via hydroxylation of the benzisoxazole (M3). This is consistent with previous observations (Sawant-Basak et al., 2013). Estimation of the percentage of metabolism proceeding through the N-dealkylation and oxazolidine pathways yielded values of 70% and 5%, respectively (Table 1).

A similar analysis was done for the other nine compounds (Figures 3 and 4), and estimates of N-dealkylation (M1 plus downstream metabolites) and iminium ion formation that leads to oxazolidine formation (M2) were made (Table 1). Replacement of the piperidine ring of compound 1 with a pyrrolidine (compound 2) caused an increase in the pathway that yields the oxazolidine product. Others that yielded detectable levels of the oxazolidine metabolites contained piperidines or 3-azabicyclo[3.1.0]hexane (compounds 3, 4, and 5). Interestingly, 4-fluoro substitution of the piperidine (compound 6) did not yield any oxazolidine metabolite, but the generation of N-dealkylation products was extensive. Bridged ring systems (compounds 9 and 10) did not yield any oxazolidine metabolites but N-dealkylation occurred. The most important finding regarding metabolism to N-dealkyl and oxazolidine metabolites was observed for azetidine-containing compounds 7 and 8: neither of the reactions were observed, and the major metabolites observed for these (designated as M3 in Figure 4) arose via hydroxylation of the benzisoxazole ring. This indicates that replacement of 5- and 6-membered aliphatic aza-heterocyclic rings with the

![Figure 3. UV–VIS chromatograms of human liver microsomal incubations of compounds 1–5. Compound numbers are designated in parentheses. Metabolites designated as M1, M1a, and M1b arise via N-dealkylation. M2 represents oxazolidine metabolites and those designated as M3, M4 and M5 refer to other metabolites.](image-url)
4-membered azetidine had a profound effect on the oxidation of the nitrogen (or the alpha carbon). The estimates of percentages of metabolism proceeding through the N-dealkylation and iminium ion routes are listed in Table 1.

**Comparison of 5HT4 partial agonism, intrinsic clearance, and permeability data for compounds 1–10**

Table 2 summarizes the target pharmacology, intrinsic clearance, and permeability data of compounds 1–10. Brodney et al. (2012) have demonstrated that the substituents at the piperidine and benzisoxaole groups attributed to optimization of intrinsic activity at 5HT4 and in vitro properties of absorption, distribution, and clearance. As observed in Table 2, compounds 5, 8, and 9 resulted in loss of functional potency at the target pharmacology. Similarly, changes in the piperidine ring size resulted in similar trends in human liver microsomal intrinsic clearance for compounds 1–8 and 10. The significantly increased intrinsic clearance of compound 9 in human liver microsomes could be attributed to the high (measured) eLogD of compound 9. While we did not observe loss of intrinsic permeability of any of the piperidine analogs, in vitro data suggested that the azetidine analogs 7 and 8 were potentially effluxed in human MDR1-transfected MDCK cell lines.

**Discussion**

The first generation 5HT4 partial agonist, TBPT (compound 1), was metabolized via N-dealkylation to M1 and to a putative iminium metabolite (M2): both metabolites arising from oxidation of the piperidine ring. M1 demonstrated antagonism at 5HT3 and was also observed to be a reversible inhibitor of CYP1A2. In addition, metabolism of the piperidine core of 1 to a cyclic quaternary piperidinium ion metabolic intermediate was further complicated by the presence of the hydroxyl on the carbon β to the iminium nitrogen. This hydroxyl group serves as a nucleophile that can then undergo an intramolecular cyclization reaction to yield the previously described oxazolidine metabolite M2 (Sawant-Basak et al., 2013). As M1 and M2 were deemed to be a problem in the further clinical development of compound 1 due to their off-target pharmacology at the 5HT4 and 5HT2b receptors, respectively, and due to their high abundance in human plasma, efforts were undertaken to alter compound 1 to prevent this metabolic route, while maintaining the essential β-hydroxy group and other key structural elements of compound 1.

In the case of N-substituted alicyclic amines, metabolism of a piperidine group can result in N-dealkylation of pendant alkyl groups or the formation of cyclic iminium ions. For the latter, the stability of the iminium ion versus its tautomers (carbinolamine, enamine, and open chain amino aldehyde) is dictated by the electronics of the molecule, favorability of the ring structure (especially for 5- and 6-membered rings), solvent and pH environment (Cervinka, 1987).

The oxidative metabolism of tertiary amines is known to be catalyzed by CYP450, peroxidases, FMOs, and MAOs (Kedderis & Hollenberg, 1983; Shaffer et al., 2001) either by sequential electron transfer (SET) of nitrogen oxidation or by hydrogen atom abstraction (HAT) from the α-carbon. Evidence of small kinetic isotope effects (<2) (Guengerich, 2002; Guengerich et al., 1996; Okazaki & Guengerich, 1993) have resulted in favoring the oxidation of alkyl amines via the sequential electron transfer. This sequential electron transfer pathway involves initial single electron transfer from the nitrogen resulting in an intermediate iminium cation radical.
followed by deprotonation at one of the $\pi$-carbons to form the iminium ion (Guengerich et al., 1996). Hydrolysis of non-cyclic iminium ions generally results in the N-desalkylation metabolite and the corresponding carbonyl compound, whereas cyclic iminium ions can be stable. Alternatively, others have proposed that the reaction begins via oxidation of the $\pi$-carbon via hydrogen atom abstraction followed by recombination of the hydroxyl and carbon radicals to yield the carbinaloline (Carlson et al., 1995). The carbinaloline can tautomerize to the cyclic iminium ion or undergo N–C bond scission to generate the N-dealkylation products. In either case, it was evident that elimination of metabolism at the piperidine nitrogen of compound 1 that leads to M1 and M2 would require altering the piperidine ring.

When the piperidine ring was replaced with pyrrolidine to give analog 2, target functional agonist potencies and scaled $C_{\text{int}}$ were not significantly altered; however, both, the N-dealkylation and oxazolidine metabolites, were still observed. Metabolism of pyrrolidines to M1 and M2 was expected based on well understood metabolism of nicotine and related molecules (Carlson et al., 1995). Similarly, altering the pyrrolidine to the [3.1.0] fused ring analog 3 reduced the formation of M2 but left the formation of M1 largely unchanged (Figure 3). This structure–metabolism relationship suggested that steric congestion around the piperidine ring had the potential to shift metabolism. To this end, the methyl group in the 4-position of the piperidine (5) failed to shift metabolism away from the N-dealkylation pathway. Modulation of the piperidine $pK_a$ was an additional strategy to impact metabolism of the piperidine nitrogen. Introducing a fluoro group at the 4-position of piperidine (6) resulted in a 1 unit drop in $pK_a$ (compound 1, $pK_a = 8.4$; compound 6, $pK_a = 7.3$) which eliminated the M2 pathway. Unfortunately, this did not impact the reactivity of the piperidine nitrogen as observed by formation of the N-dealkylated metabolite. Unfortunately, the N-dealkylated metabolite from the M1 pathway was still observed. As an additional blocking strategy, we tested the impact of capping the free $\beta$-hydroxyl of the tetraydropyrany ring (compound 4); but M2 formation still occurred presumably due to O-demethylation of the methoxy along with the same piperidine oxidation that occurred with TBPT.

Replacing the piperidine with bulkier bridged-bicyclic linkers (analogs 9 and 10) resulted in complete elimination of the M2 pathway, but not M1. This observation is supported by recent work on 4-aminopiperidines (Sun & Scott, 2011), where steric bulk on the piperidine nitrogen (e.g. indoramine, lorcaimide, sabeluzole, and astemizole) resulted in unfavorable, high-energy conformations at the enzyme active site, ultimately reducing the N-dealkylation pathways. In the case of analog 9, the ether bridgehead also had a dramatic impact on $pK_a$, which presumably contributed to the overall lack of 5HT4 potency.

In contrast to the design strategies described so far, further reducing the ring size of the piperidine to an azetidine ring provided an opportunity to investigate the impact of a more strained ring system on the metabolism. Interestingly, azetidine analogs 7 and 8 showed no detectable formation of the M1 or M2 metabolites. Instead, as observed in Figure 4, both the azetidine analogs (compounds 7 and 8) resulted in complete metabolic switching of alicyclic amine to predominant hydroxylation of benzisoxazole. This is only partially explained with the marginally lower basicity of the azetidine analogs (Table 2). A potential reduction in $pK_a$ has been attributed by (Ohwada et al., 2004) to the orientation/hybridization of the lone pair and not due to ring strain. However, we observed no trends between $pK_a$ of the piperidine nitrogen of 5HT4 analogs (1–10) and elimination of metabolism of the piperidine group. With no clear physicochemical property explanation for the superior metabolism profile of the azetidine analogs, we considered more rigorous molecular modeling approaches. The limited role of $pK_a$ would suggest that these analogs are metabolized through a hydrogen atom extraction pathway. Under this assumption, the rate-limiting, radical formation would commence through abstraction of hydrogen on the carbon alpha (C2) to the cyclic amine. To determine if this initial step leads to the observed differences in metabolism, we employed a workflow to compute the free-energy of hydrogen atom abstraction for azetidine 7 and its partners, piperidine 1 and pyrrolidine 2.
The results of these calculations, shown in Figure 5, are in excellent agreement with the experimental results. The calculations show, for the piperidine and pyrrolidine, that the hydrogens on C2 have the lowest free-energy of hydrogen-atom abstraction. In contrast, the lowest free-energy of abstraction for the azetidine analog was on the methylene linker adjacent to the cyclic amine. Interestingly, no metabolism has been observed at this position in the azetidine series. The relative free energy of abstraction for the azetidine C2 hydrogens is 8.7 kcal/mol, which would be a significant energy barrier to overcome. To put the radical energies into perspective and allow a reasonable comparison of the three molecules, one should also consider the inherent strain energy of the parent structures and compute the radical free-energies relative to the parent structure energy. These calculations are also in alignment with the experimental findings, showing that hydrogen atom abstraction from azetidine 7 is 11.8 kcal/mol higher in energy relative to piperidine 1 ($E_{rel} = 0.0$) and pyrrolidine 2 ($E_{rel} = 1.0$ kcal/mol). Taken in totality, the results of this modeling work suggest that the differences observed in metabolism between the azetidine analogs and other cyclic amines can be attributed, at least in part, to a higher energy of hydrogen-atom abstraction.

While β-azetidinones are commonly observed in classical antibiotic therapies, intact azetidines have received limited attention but offered promising avenues in discovery of small molecules. Hubbs et al. (2015) has demonstrated that substitution of a morpholine with azetidine disrupted the interactions of morpholine with heme porphyrin ring system, and was successfully applied as a strategy to reduce CYP450 inhibition of the unsaturated morpholine. Packiarajan et al. (2012) have reported a series of azetidinyl oxadiazoles as novel mGluR5 positive allosteric modulators (PAMs) where the azetidine carboxamides due to their low molecular weight (<350) and optimal eLog P (<3) showed improved physicochemical and pharmacokinetic properties in comparison to N-aryl pyrrolidinonyl oxadiazole leads. Similarly, Zhang et al. (2012) have reported the discovery of cyclohexyl azetidinyl functionality as a requisite of potent CCR2 antagonists. However, all such reports lack a methodical evaluation of azetidine as isosteric replacement for the optimization of metabolic clearance of cyclic amines. Here we propose azetidine as a prospective metabolism-guided isostere of cyclic amines to restrict or eliminate the oxidative metabolism associated with other cyclic amine ring systems (pyrrolidine, piperidine, etc.). This metabolic isostere offered several advantages in that it decreased the intrinsic CYP450 mediated turnover and also showed the potential to conserve the basicity of alicyclic nitrogen causing minimal hindrance to pharmacophoric attributes of $(\mathrm{CH}_2)_nNR$ as seen in the case of compound 7.

While the chemical and metabolic attributes of 7 and 8 were similar, compound 7 retained functional potency similar to that of TBPT and hence was identified as the most appropriate lead compound as a follow on to TBPT. In conclusion, we have demonstrated azetidine 7 as a successful replacement of the metabolically labile piperidine ring system of 1.

**Declaration of interest**

The authors have no declarations of interest to report.

**References**


Supplementary materials are available online