Bioorganic & Medicinal Chemistry Letters 19 (2009) 1559–1563

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Biochemical basis for differences in metabolism-dependent genotoxicity by two diazinylpiperazine-based 5-HT$_{2C}$ receptor agonists

Amit S. Kalgutkar *, Jonathan N. Bauman, Kim F. McClure, Jiri Aubrecht, Santo R. Cortina, Janvi Paralkar

Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340, USA

ARTICLE INFO

Article history:
Received 16 January 2009
Revised 5 February 2009
Accepted 9 February 2009
Available online 12 February 2009

Keywords:
Salmonella
Reactive metabolite
P450
DNA
Covalent binding
Glutathione
Cyanide
Amine

ABSTRACT

The biochemical basis for S9-dependent mutagenic response of the 5-HT$_{2C}$ receptor agonist and diazinylpiperazine derivative **1** in the *Salmonella* Ames assay involves P450-mediated bioactivation to DNA-reactive quinone-methide, aldehyde and nitrone intermediates. Mechanistic information pertaining to the metabolism of **1** was used in the design of diazinylpiperazine **5** to eliminate the safety liability. While **5** was negative in the Ames assay, the compound retained the ability of **1** to form certain electrophilic intermediates. Plausible hypotheses that can collectively account for the differences in mutagenic response of the two piperazine analogs are discussed.

The *Salmonella* Ames assay is an integral part of drug safety evaluation and is required by regulatory agencies for drug approvals worldwide. Since positive findings in this assay have a good correlation with the outcome of rodent carcinogenicity studies, a positive result generally leads to the discontinuation of development of drugs particularly those intended for non-life threatening indications.[1,2] An example of such a situation was highlighted in our recent studies on the 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor agonist and potential anti-obesity agent **1** (Scheme 1).[3] Compound **1** demonstrated a serious genetic safety liability that manifested as a positive finding in the *Salmonella* reverse mutation assay in a S-9/NADPH-dependent fashion. Consequently, **1** was discontinued from preclinical development despite exhibiting attractive in vivo pharmacology in animal models of food intake and obesity.[3] The irreversible incorporation of radioactivity in calf thymus DNA following incubations with [^{14}C]-**1** in the presence of rat S-9/NADPH indicated that **1** was bioactivated to a reactive intermediate(s) that covalently bound to DNA.[3]

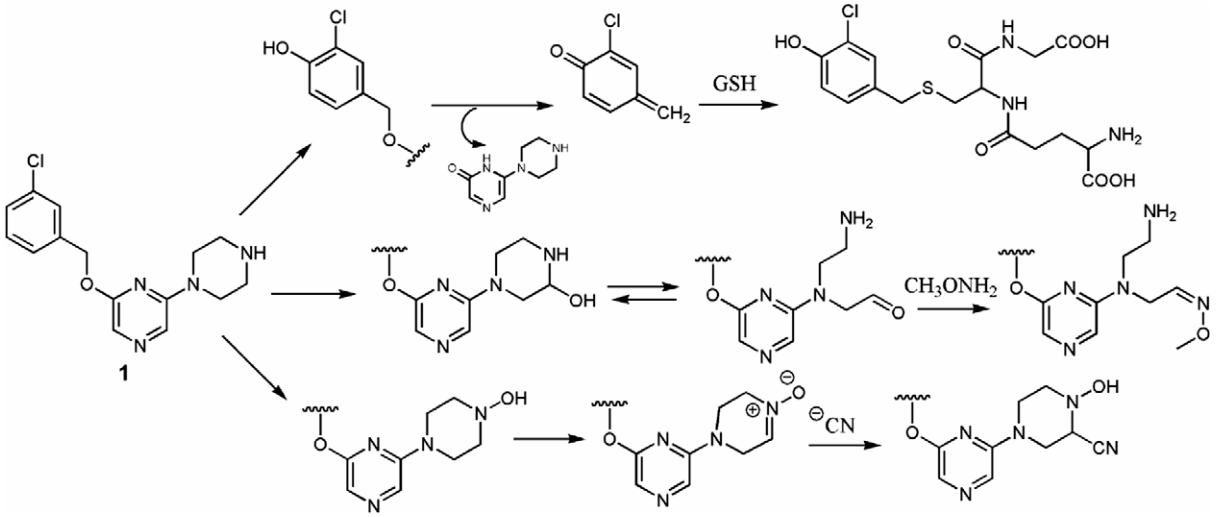
Reactive metabolite trapping studies in S-9/NADPH incubations led to the detection of glutathione (GSH), methoxylamine and cyanide conjugates of **1** and/or its downstream metabolites(s). Mass spectral characterization of the conjugates provided indirect information on the structures of the DNA-reactive intermediates and the oxidative pathways leading to their formation (Scheme 1).[3]

Elucidation of the structure of the GSH conjugate was consistent with a bioactivation pathway involving initial aromatic ring hydroxylation on the 3-chlorobenzyl motif in **1** followed by β-elimination to a quinone-methide that reacted with GSH. In contrast, structural characterization of the methoxylamine and cyanide conjugates indicated metabolism had occurred on the piperazine ring in **1** to yield reactive aldehyde and nitrone intermediates as shown in Scheme 1. The observation that methoxylamine and GSH reduced mutagenicity in the *Salmonella* assay suggested that the trapping agents competed with DNA towards reaction with electrophilic intermediates. Overall, this exercise provided a rationale on which to base a subsequent chemical intervention strategy to design 5-HT$_{2C}$ agonists devoid of the safety hurdles.

SAR studies for the diazinylpiperazine class of 5-HT$_{2C}$ agonists revealed that 3-chlorobenzyloxy region in **1** was liberal in terms of the substituents tolerated; consequently, quinone-methide formation could be prevented through the introduction of functional groups that would be incapable of undergoing β-elimination chemistry after potential phenyl ring hydroxylation. While standard tactics for blocking reactive metabolite formation usually involves removal of the "offending" functionality or incorporation of metabolic "soft-spots" in the vicinity of the bioactivation site,[4] we knew, early on, that SAR around the piperazine ring was fairly tight. For instance, incorporation of a piperidine ring instead of the pipera-

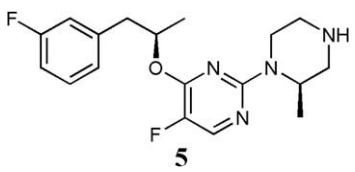
* Corresponding author.
E-mail address: amit.kalgutkar@pfizer.com (A.S. Kalgutkar).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.02.032



Scheme 1. Proposed P450-catalyzed bioactivation pathways of 5-HT_{2C} agonist **1** in NADPH-supplemented aroclor 1254-induced rat S9.

zine or insertion of methyl groups α to the secondary piperazine nitrogen in **1** resulted in pharmacologically inactive compounds. A breakthrough was achieved when the methyl group was incorporated α to the tertiary piperazine nitrogen. Not only were compounds containing the structural change devoid of metabolism-dependent mutagenicity but some (e.g., diazinylpiperazine **5**) also retained the primary pharmacology and pharmacokinetic attributes of **1**.[5,6] Interestingly, incorporation of an additional methyl group on the C-5 carbon in **5** and related compounds resulted in pharmacologically inactive compounds.

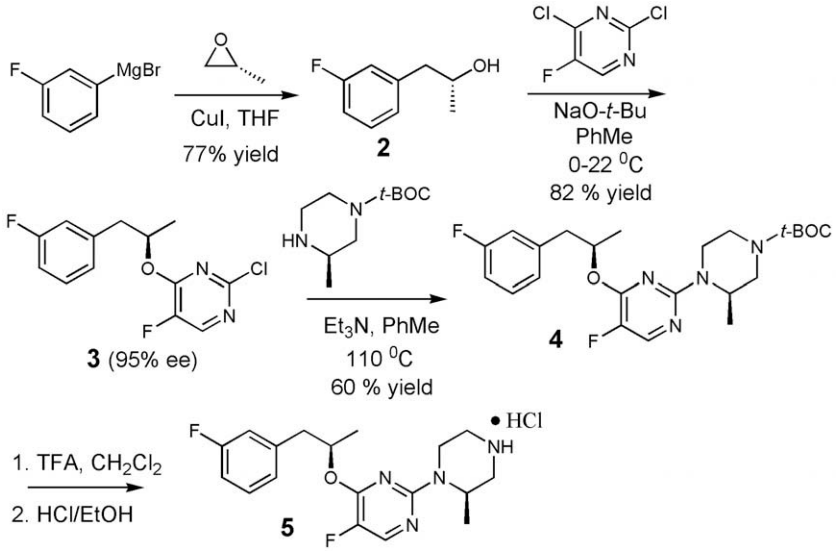


5

Of much interest were the findings that absence of S9/NADPH-dependent mutagenicity of **5** did not correlate with complete elimination of reactive metabolite formation. The results of the metabolism studies and plausible hypotheses for the disconnect is discussed herein.

The synthesis of compound **5** is shown in Scheme 2.[7] Grignard reaction of 3-fluorophenylmagnesium bromide with (*R*)-(+)-propylene oxide afforded the chiral primary alcohol derivative **2**, which was reacted with 2,4-dichloro-5-fluoropyrimidine in the presence of strong base yielding **3** in 95% ee. The *tert*-BOC-protected-piperazine **4** was prepared from **3** via base-catalyzed reaction with (*R*)-*tert*-butyl-3-methylpiperazine-1-carboxylate. Deprotection of the *tert*-BOC group in **4** with mild acid followed by treatment with ethanolic hydrochloric acid furnished the corresponding HCl salt of **5**.

The genetic safety of **5** was evaluated using the *Salmonella* reverse mutation assay.[8] To simulate the effect of drug metabolizing enzymes, the assay also included a segment where cells were exposed to aroclor 1254-induced rat liver S-9. To cover a broad spectrum of mutagenic mechanisms, a set of 4 commonly used tester strains TA98, TA100, TA1535 and TA1537 were utilized to evaluate mutagenicity of **5**.[9] The exposure of *Salmonella* cells with **5** in the absence or presence of S9 and NADPH co-factor did not result in a statistically significant increase of reverse mutations in any of



Scheme 2. Synthesis of the hydrochloride salt of **5**.

the strains examined (*a maximum 1.34-fold increase in reverse mutations in strain TA100 was discerned in the 5 mg/plate segment*). Under identical experimental conditions, **1** displayed statistically significant 3- to 6-fold increases in reverse mutations in strains TA100 and TA1537 at 0.63 mg/plate in a S9/NADPH-dependent fashion.[3]

Qualitative studies into the ability of **5** to form reactive metabolites were conducted.[10] Unlike **1**, **5** does not possess the 3-chlorobenzyloxy motif and therefore cannot form the reactive quinone-methide observed with **1**. Indeed, liquid-chromatography tandem mass spectrometry (LC–MS/MS) analysis of an incubation mixture comprising of NADPH- and GSH-supplemented aroclor 1254-induced rat S9 and **5** did not reveal the formation of GSH conjugates. In contrast, LC–MS/MS analysis of S9 incubations containing **5**, NADPH and methoxylamine or KCN led to the detection of a single conjugate (methoxylamine conjugate **6**: MH^+ = 394 Da; cyanide conjugate **7**: MH^+ = 390 Da) in each case (Figures 1 and 2, panel A). The formation of **6** and **7** was abolished when NADPH and/or trapping agents were omitted from the incubations.

The molecular weight of **6** was consistent with the addition of one molecule of amine to **5**, whereas, the molecular ion observed for **7** suggested addition of one molecule of cyanide to a monohydroxylated metabolite of **5**. The collision-induced dissociation (CID) spectra of **6** and **7** are shown in Figures 1 and 2, panel B. For purposes of comparison, the CID spectrum of **5** is depicted in Figure 3. The presence of the common fragment ion at *m/z* 109 in the mass spectra of **5**, **6** and **7** established that the structural integrity of the 3-fluorophenylpropyl group in the conjugates was maintained. Based on the additional fragment ions, a proposed structure[11] for **6** that is consistent with the observed mass spectrum is shown in Figure 1, panel A. The fragment ion in the CID spectrum of **7** (Figure 2, panel B) at *m/z* 254 suggested that hydroxylation and subsequent cyanide addition had occurred on the pyrimidinylpiperazine motif. A proposed structure for **7** that is consistent with the observed mass spectrum are shown in Figure 2, panel A.

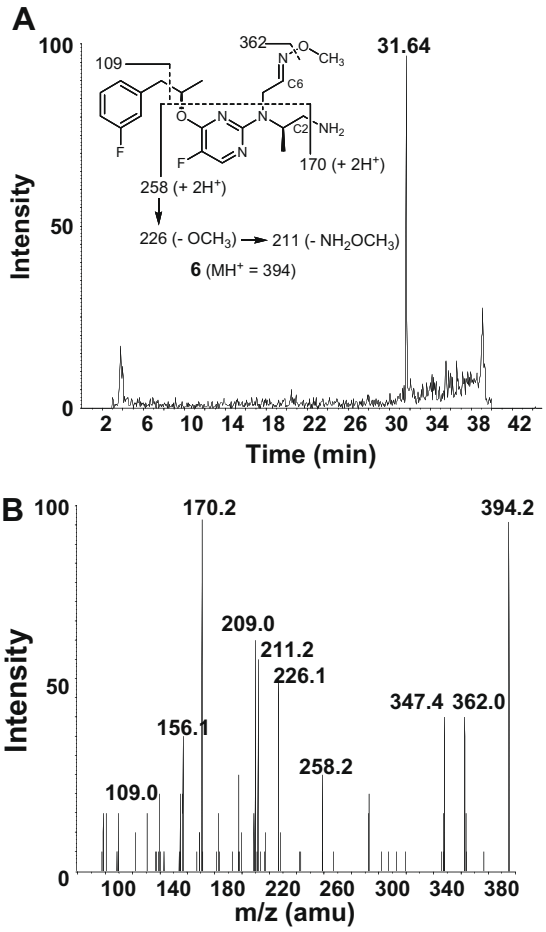


Figure 1. Extracted ion chromatogram of the methoxylamine conjugate **6** of **5** (panel A) following incubation with aroclor 1254-induced rat liver S-9, NADPH, **5** (20 μM), and methoxylamine (1 mM). Panel B shows the product ion spectra obtained by CID of the MH^+ ion (*m/z* 394) of **6**. The origins of the diagnostic ions are as indicated.

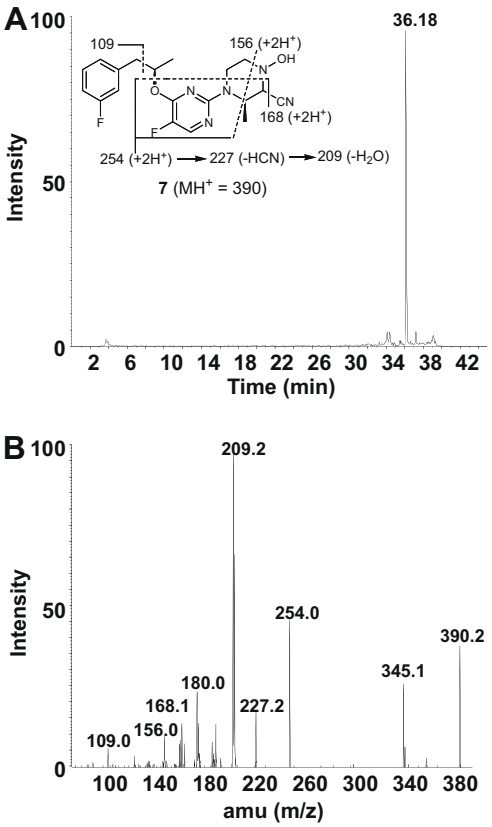


Figure 2. Extracted ion chromatogram of the cyanide conjugate **7** of **5** (panel A) following incubation with aroclor 1254-induced rat liver S-9, NADPH, **5** (20 μM), and KCN (1 mM). Panel B shows the product ion spectra obtained by CID of the MH^+ ion (*m/z* 390) of **7**. The origins of characteristic diagnostic ions are as indicated.

Overall, the amine and cyanide conjugates of **5** are analogous to the ones observed with **1** suggesting that the piperazine ring in the two compounds undergoes identical bioactivation pathways as shown in Scheme 1 with **1**. Thus, P450-mediated hydroxylation on a carbon α to a secondary piperazine nitrogen atom in **5** would afford the unstable carbinolamine intermediate (in equilibrium with the ring-opened aldehyde intermediate[12]), condensation of which, with methoxylamine would give rise to Schiff base **6**. For **7**, initial oxidation of the secondary piperazine nitrogen in **5** would furnish the hydroxylamine intermediate, which, upon a two-electron oxidation on the α-carbon-nitrogen bond would lead to the nitrone derivative capable of reacting with cyanide ion and generating cyanide conjugate **7** in a manner similar to that observed for related acyclic analogs.[13,14]

These studies present an interesting conundrum in that both **1** and **5** form DNA-reactive metabolites, but only **1** exhibits metabolism-based genotoxic response. The likelihood that lower aqueous solubility of **5** (compared with **1**) results in precipitation in the Ames assay leading to a false negative result can be excluded be-

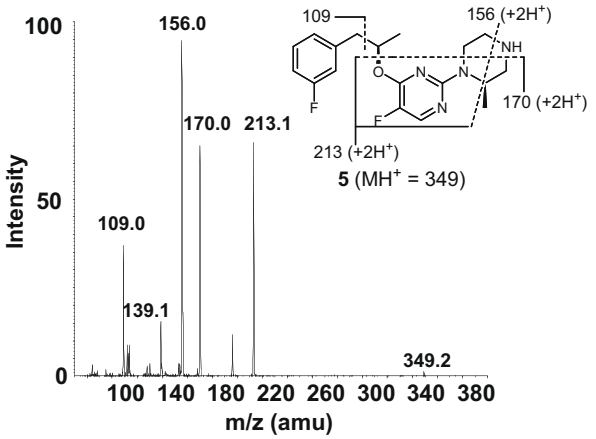


Figure 3. Product ion spectrum obtained by the CID of the MH^+ ion (*m/z* 349) of **5**.

cause the fumarate and HCl salt forms of **1** and **5**, respectively, used in genotoxicity testing, possessed comparable solubility.[15]

There are several other biochemical hypotheses which can individually or collectively account for the disconnect in mutagenicity between the two compounds. For instance, it is possible that statistically significant increases in reverse mutations via DNA adduction to electrophilic intermediates is dependent upon the formation of aldehyde, nitrone and quinone-methide reactive metabolites; a scenario only likely with **1**. While S9/NADPH-dependent covalent modification of DNA by [^{14}C-**1**] (*radiolabeled on the pyrazine carbons*) and the decrease in mutagenicity in the *Salmonella* assay upon co-incubation with methoxylamine strengthens the role of the aldehyde and nitrone as a DNA-reactive intermediates[3], the contribution of the liberated quinone-methide derivative in the mutagenic response of **1** cannot be ruled out, especially since addition of GSH in the *Salmonella* assay did result in attenuation of the mutagenic response of **1**.[3]

Apart from changes in bioactivation profile, other suitable explanations for mutagenicity differences of **1** and **5** include: (a) differences in rates of substrate consumption in S9 and/or (b) substrate inhibition of P450 enzymes responsible for reactive metabolite formation.[16,17] The likelihood that greater metabolic resistance of **5** (compared with **1**) in S9 significantly decreases reactive aldehyde and nitrone formation and therefore abrogates mutagenicity was explored by comparing half-lives of **1** and **5** in the metabolic activation system.[18] As shown in Figure 4, **5** was ~3-fold more resistant towards metabolism in NADPH-supplemented S9 when compared with **1** ($T_{1/2}$ for **5** ~13–19 min; $T_{1/2}$ for **1** ~4–7 min). Furthermore, LC–MS/MS analysis of the peak height ratios of methoxylamine conjugate / parent compound with both **1** and **5** revealed ~5-fold lower conversion of **5** to **6** when compared with methoxylamine conjugate formation with **1**.

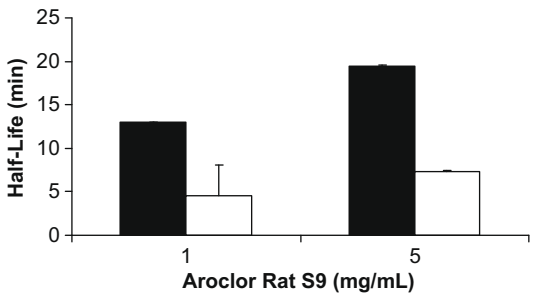


Figure 4. Differences in metabolic half-lives of **1** (open bars) and **5** (filled bars) in NADPH-supplemented aroclor 1254-induced rat S9.

Next, the possibility that lack of mutagenicity of **5** can occur via substrate inhibition of the cytochrome P450's responsible for reactive metabolite formation was examined. In human liver microsomes (HLM), the half-lives of **1** ($T_{1/2}$ = 8 min) and **5** ($T_{1/2}$ = 20 min) were only increased when incubations were conducted in the presence of the P4503A4 and P4501A2-selective inhibitors, ketoconazole and furafylline, respectively, ($T_{1/2}$ for **1** and **5** were >90 min and 40–45 min with ketoconazole and furafylline, respectively). This observation suggests that P4503A4 and P4501A2 are principally involved in the metabolism of **1** and **5**.[19] Based on this information, P4501A2 and 3A4 inhibition by **1** and **5** was evaluated in HLM.[20] Compound **5** inhibited P4501A2 and P4503A4 with IC_{50} values of 0.4 μM and 4.9 μM, respectively. In contrast, **1** was a weak inhibitor of P4503A4 (IC_{50} of 14 μM) and had virtually had no inhibitory effect on P4501A2 activity (IC_{50} > 50 μM). Considering that aroclor 1254 induction in the rat results in marked increases in activities of P450 isozymes (especially those responsible for metabolism/bioactivation of **1** and **5**: >35-fold induction of P4501A and >2-fold induction of P4503A)[17], it is quite possible that at the high substrate concentrations used for the Ames test **5** inhibits its own metabolism in the S9 segment resulting in reduction of reactive intermediates and consequently lack of mutagenicity.

In summary, insights into the mechanisms of reactive metabolite formation with the 5-HT_{2C} receptor agonist **1** assisted us in the design of follow-on compounds to eliminate genotoxic response. Diazinylpiperazine **5** retained the attractive primary pharmacology and pharmacokinetic properties of **1** and was devoid of in vitro mutagenicity in the *Salmonella* assay. Despite the negative result in the genotoxicity assay, the compound retained the ability of **1** in forming DNA-reactive metabolites derived from piperazine ring bioactivation. Lack of mutagenicity with **5** despite forming reactive metabolites may be rationalized on the basis of occurrence of several events in tandem such as: (a) absence of quinone methide formation (the formation of this metabolite may be essential along with formation of the aldehyde and nitrone intermediates for mutagenicity to occur), (b) slower rates of metabolism of **5** (when compared with **1**) and (c) self-catalyzed inhibition of its own metabolism. The latter two phenomenon could potentially reduce the "critical mass" of reactive metabolites required for DNA adduction leading to mutagenicity.

References and notes

1. Kim, B. S.; Margolin, B. H. *Environ. Mol. Mutagen.* **1999**, *34*, 297.
2. Zeiger, E. *Regul. Toxicol. Pharmacol.* **1998**, *28*, 85.
3. Kalgutkar, A. S.; Dalvie, D. K.; Aubrecht, J.; Smith, E. B.; Coffing, S. L.; Cheung, J. R.; Vage, C.; Lame, M. E.; Chiang, P.; McClure, K. F.; Maurer, T. S.; Coelho, R. V., Jr.; Soliman, V. F.; Schildknegt, K. *Drug Metab. Dispos.* **2007**, *35*, 848.
4. Kalgutkar, A. S.; Fate, G.; Didiuk, M. T.; Bauman, J. *Expert Rev. Clin. Pharmacol.* **2008**, *1*, 515.
5. (a) Siuciak, J. A.; Chapin, D. S.; McCarthy, S. A.; Guanowsky, V.; Brown, J.; Chiang, P.; Marala, R.; Patterson, T.; Seymour, P. A.; Swick, A.; Iredale, P. A. *Neuropharmacology* **2007**, *52*, 276; b In primary in vitro screens for the 5-HT_2 receptor subtypes, both **1** and **5** are potent, functional agonists at the 5-HT_{2C} receptor with EC_{50} values of 0.6 nM (96% maximal 5-HT activation) and 1.7 nM (88% maximal 5-HT activation), respectively. The EC_{50} for 5-HT_{2A} agonism by **1** and **5** was 381 nM (67% activation) and 4441 nM (62% activation), respectively. While **1** also displayed weak agonism of the 5-HT_{2B} receptor (EC_{50} = 201 nM, 26% activation), **5** was an antagonist of this receptor. In acute in vivo studies, oral administration of **1** and/or **5** to Wistar rats resulted in the dose-dependent inhibition of both spontaneous, nocturnal food intake and fasting-induced refeeding. Furthermore, oral administration of **1** and **5** at 30 mg/kg daily to Wistar rats for four days resulted in ~8–10% inhibition of cumulative food intake and ~25% reduction in body weight over the four-day period.
6. Predicted human blood clearance from liver microsomes for both 1 and 5 is ~4.0 mL/min/kg, respectively. Protocols for measuring half-lives in human liver microsomes and subsequent scaling to blood clearance have been published. See Obach, R. S. *Drug Metab. Dispos.* **1999**, *27*, 1350–1359.

7. Analytical data for **5**: Melting point = 230 °C, MH^+ = 349. ^1H NMR (400 MHz, methanol-d_4) δ 8.00 (d, J = 2.9 Hz, 1 H), 7.21–7.26 (m, 1H), 7.00–7.05 (m, 2 H), 6.87–6.98 (m, 1 H), 5.52–5.56 (m, 1 H), 4.99–5.02 (m, 1 H), 4.60 (dd, J = 14.9, 2.9 Hz, 1 H), 3.21–3.41 (m, 4 H), 2.94–3.09) (m, 3 H), 1.37 (d, J = 6.2 Hz, 3 H), 1.26 (d, J = 7.0 Hz, 3 H). ^{13}C NMR (100 MHz, dimethyl sulfoxide-d_6) δ 162.0 (J = 242 Hz), 157.1 (J = 10.5 Hz), 155.8, 143.8 (J = 19.6 Hz), 140.4 (J = 8.3 Hz), 139.6 (J = 246 Hz), 130.1 (J = 8.3 Hz), 125.5, 166.1 (J = 21.1 Hz), 113.2 (J = 21.1 Hz), 73.3, 45.9, 44.1, 42.2, 40.8, 35.4, 19.3, 13.9. Analysis calculated for $C_{18}H_{23}ClF_2N_4O$: C, 56.18; H, 6.02 N, 14.56. Found: C, 56.28; H, 5.84; N, 14.37. $[\alpha]_{22}^{D}$ −0.24 (c 0.12, methanol).
8. The *Salmonella* reverse mutation assay was performed using *Salmonella* typhimurium tester strains TA98, TA100, TA1535 and TA1537. Briefly, the *Salmonella* cells were treated in soft agar overlays with **5** dissolved in DMSO at concentrations ranging from 0.015 to 5 mg/plate or an appropriate amount of DMSO in presence or absence of aroclor 1254-induced rat liver S9 mixture and a NADPH-regenerating system. The number of visible revertant colonies present after 72 h incubation at 37 °C was recorded and fold of change over DMSO-treated control plates was calculated. At least three independent experiments with each *Salmonella* tester strain were performed. The analysis of the data included calculating average and SEM. The statistical significance was determined using the t-test. A 2-fold statistically significant ($p \leqslant 0.05$) increase of number of revertant colonies over the DMSO-treated controls was considered as a criterion for positive response in the *Salmonella* assay.
9. Maron, D. M.; Ames, B. N. *Mutat. Res.* **1984**, *113*, 173.
10. Stock solutions of **5** were prepared in DMSO. Incubations were carried out at 37 °C for 60 min. The incubation volume was 1 mL and consisted of the following: 0.1 M potassium phosphate buffer (pH 7.4) containing $MgCl_2$ (10 mM), aroclor 1254-induced rat liver S-9 fraction (final protein concentration = 1 mg/mL), NADPH (1.2 mM), **5** (20 μM), and methoxylamine (1 mM) or potassium cyanide (1 mM) or GSH (1 mM). Incubations that lacked either NADPH or trapping agents served as negative controls, and reactions were terminated by the addition of ice-cold acetonitrile (1 mL). The solutions were centrifuged (3,000*g*, 15 min) and the supernatants were dried under a steady nitrogen stream. The residue was reconstituted with mobile phase and analyzed for metabolite formation by LC–MS/MS using analytical conditions described in ref. 3.
11. The fragment ion at *m/z* 170 in the CID spectrum of **6** and parent compound **5** suggests that the site of amine addition is on the C6 carbon and not the C2 carbon. This may be due to steric hindrance by the adjacent C3 methyl group.
12. Castagnoli, N., Jr.; Rimoldi, J. M.; Bloomquist, J.; Castagnoli, K. P. *Chem. Res. Toxicol.* **1997**, *10*, 924.
13. Clark, N. G.; Carokill, E. *Tetrahedron Lett.* **1977**, *31*, 2717.
14. Kim, T. R.; Kim, Y. H.; Pyun, S. Y. *Bull. Korean Chem. Soc.* **1999**, *20*, 712.
15. Compound **1**. HCl salt: solubility in PBS (pH 6.6) = 0.2 mg/mL; solubility in unbuffered water (pH 5.0) = 1.5 mg/mL. Compound **5**. HCl salt: solubility in PBS (pH 6.1) = 0.6 mg/mL; solubility in unbuffered water (pH 5.5) = 5.0 mg/mL.
16. Obach, R. S.; Dobo, K. L. *Environ. Mol. Mutagen.* **2008**, *49*, 631–641.
17. Ku, W. W.; Bigger, A.; Brambilla, G.; Glatt, H.; Gocke, E.; Guzzie, P. J.; Hakura, A.; Honma, M.; Martus, H. J.; Obach, R. S.; Roberts, S. *Mutation Res.* **2007**, *627*, 59.
18. Half-life ($t_{1/2}$) were determined in triplicate after incubation of **1** and **5** (1 μM) in NADPH (1.2 mM) and aroclor 1254-induced rat S9 (1 and 5 mg/mL) in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C. Periodically, aliquots of the incubation mixture at 0, 5, 15, and 30 min (time period associated with reaction linearity) were monitored for percentage remaining substrate by LC–MS/MS.
19. The formation of conjugates (GSH, methoxylamine and cyanide conjugates for **1** and methoxylamine and cyanide conjugates for **5**) were observed in both recombinant P4503A4 and P4501A2 incubations.
20. Walsky, R. L.; Obach, R. S. *Drug Metab. Dispos.* **2004**, *32*, 647.