

## Characterization of Non-Nitrocatechol Pan and Isoform Specific Catechol-O-methyltransferase Inhibitors and Substrates

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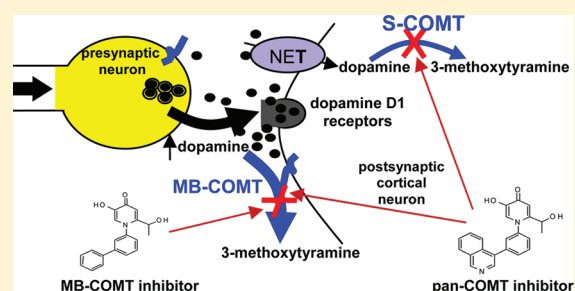
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**ABSTRACT:** Reduced dopamine neurotransmission in the prefrontal cortex has been implicated as causal for the negative symptoms and cognitive deficit associated with schizophrenia; thus, a compound which selectively enhances dopamine neurotransmission in the prefrontal cortex may have therapeutic potential. Inhibition of catechol-O-methyltransferase (COMT, EC 2.1.1.6) offers a unique advantage, since this enzyme is the primary mechanism for the elimination of dopamine in cortical areas. Since membrane bound COMT (MB-COMT) is the predominant isoform in human brain, a high throughput screen (HTS) to identify novel MB-COMT specific inhibitors was completed. Subsequent optimization led to the identification of novel, non-nitrocatechol COMT inhibitors, some of which interact specifically with MB-COMT. Compounds were characterized for in vitro efficacy versus human and rat MB and soluble (S)-COMT. Select compounds were administered to male Wistar rats, and ex vivo COMT activity, compound levels in plasma and cerebrospinal fluid (CSF), and CSF dopamine metabolite levels were determined as measures of preclinical efficacy. Finally, novel non-nitrocatechol COMT inhibitors displayed less potent uncoupling of the mitochondrial membrane potential (MMP) compared to tolcapone as well as nonhepatotoxic entacapone, thus mitigating the risk of hepatotoxicity.

**KEYWORDS:** Catechol-O-methyltransferase, high throughput screen, dihydroxyphenylacetic acid, homovanillic acid, fluorescence polarization and hepatotoxicity



Schizophrenia is a chronic, debilitating disease estimated to occur in 1% of the population.<sup>1</sup> Effects of the disease are manifested in patients as any one or combination of three symptom domains; positive symptoms, negative symptoms and cognitive deficit.<sup>2</sup>

The negative symptoms and cognitive impairment present in individuals with schizophrenia represent an unmet medical need.<sup>3,4</sup> Reduced dopamine neurotransmission in the prefrontal cortex (PFC) has been implicated as causal for these symptoms.<sup>5–7</sup> Catechol-O-methyltransferase (COMT) is the preferential mechanism for catabolism of dopamine in cortical areas.<sup>8–11</sup> The enzyme exists as two major isoforms where the membrane (MB) form is more predominant in human brain and the soluble (S) form is more predominant in peripheral tissues.<sup>12–15</sup> Preferential expression of MB versus S-COMT in

human brain is an evolutionary change and in contrast to rat; where S-COMT predominates.<sup>16</sup> The only difference between human MB and S-COMT is the inclusion of an extra 50 hydrophobic amino acids (43 in rat) in MB-COMT. As a critical player in the manipulation of cortical dopamine levels, inhibition of COMT may serve as a promising adjunct therapy for schizophrenia.<sup>9,17</sup>

There are numerous functional single nucleotide polymorphisms (SNPs) in COMT. The majority of human studies have focused on a SNP common in the COMT gene which leads to the replacement of valine (Val) with methionine (Met)

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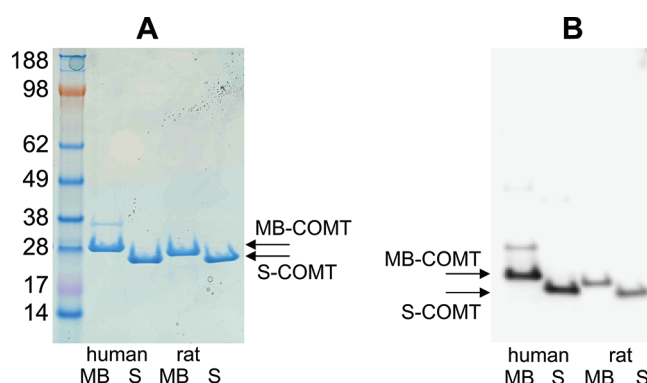
at codon 108 of S-COMT and codon 158 of MB-COMT.<sup>18</sup> Val-COMT isolated from human PFC is ~40% more catalytically active in vitro than enzyme containing the Met allele.<sup>19</sup> Various investigations indicate that healthy controls with the Met allele perform better on certain working memory tasks,<sup>20–22</sup> but not always.<sup>23</sup> Improvements on working memory tasks have been reported in Val 158 homozygote healthy subjects treated with the COMT inhibitor tolcapone.<sup>24,25</sup> However, based on a number of haplotypes that influence COMT expression and activity, it seems likely that associated phenotypes result from a multifaceted set of genetic variations.<sup>26</sup>

The nitrocatechol COMT inhibitors tolcapone and entacapone are successfully utilized as an adjunct therapy for Parkinson's disease, where inhibition of COMT in the periphery improves the bioavailability of L-dopa in brain. Despite successful clinical use, idiosyncratic toxicity is associated with tolcapone<sup>27</sup> and although entacapone is not associated with hepatotoxicity; like tolcapone, its use is sometimes limited by severe diarrhea due to peripheral inhibition.<sup>28</sup> Inhibition of S-COMT in the periphery has been proposed to contribute to tolcapone linked hepatotoxicity,<sup>17</sup> thus highlighting an advantage of identifying and characterizing MB-COMT specific inhibitors.

Due to the idiosyncratic hepatotoxicity associated with tolcapone and adverse effects associated with peripheral inhibition of COMT, we sought to identify a non-nitrocatechol, brain penetrant MB-COMT inhibitor for the treatment of schizophrenia.<sup>29</sup> Additionally, to alleviate side-effects associated with peripheral inhibition, the human brain predominant isoform MB-COMT was utilized to conduct an HTS and complete lead optimization efforts to discover novel, non-nitrocatechol COMT inhibitors. Reported here is the identification and characterization of a series of 4-pyridinone compounds, some of which specifically or preferentially inhibit MB-COMT.

## RESULTS AND DISCUSSION

**COMT purification analysis.** Protein expression and purification methods were conducted to individually purify human MB-COMT, human S-COMT, rat MB-COMT, and rat S-COMT. Although S-COMT is soluble in the absence of detergent, all COMT preparations were prepared with detergent to avoid any false positive hits due to the presence of detergent. Additionally, preparation of S-COMT in the absence of detergent led to similar IC<sub>50</sub> values when a subset of compounds were screened (data not shown). The recombinant COMT samples were visually compared following gel electrophoresis using staining or Western blotting. The protein bands observed on the stained gel indicated that isoform specific COMT was purified (Figure 1A). S-COMT was not detected in the human or rat MB-COMT and similarly MB-COMT was not observed in the human or rat S-COMT purifications. To affirm the homogeneity of the purified COMT samples, the recombinant proteins were also analyzed by Western blotting using an antibody with a similar affinity for MB and S-COMT (Figure 1B). These results substantiated that each of the recombinant pools were composed of only one of the COMT forms. Additionally, light scattering as well as gel filtration revealed COMT as unaggregated (data not shown) and in vitro enzyme assays demonstrated that all of the COMT forms exhibited catalytic activity.



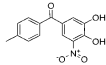
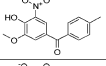
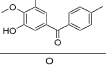
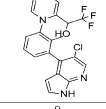
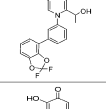
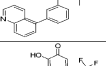
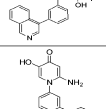
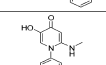
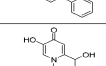
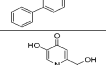
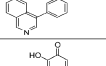
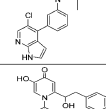
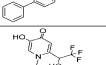
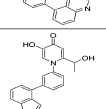
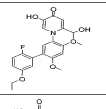
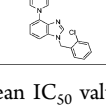
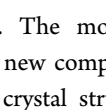
**Figure 1.** (A) 3.5  $\mu$ g recombinant proteins were resolved on a 4–12% Bis-Tris gel and stained with Instant Blue. From left to right: SeeBlue Plus2 Pre-Stained standard (molecular weight in kDa, Invitrogen), human MB-COMT (M51A, V158) with 3' EK and 6-HIS, recombinant human S-COMT (V158) with 3' EK and 6-HIS, recombinant rat MB-COMT (M44A) with 3' 10-HIS and recombinant rat S-COMT with 3' 10-HIS. (B) ~150 ng recombinant proteins were resolved on 4–12% Bis-Tris gels and subjected to Western blotting with a primary antibody to COMT from Millipore (AB5873).

**COMT Inhibitor Characterization.** As part of our drug discovery effort to treat cognitive deficit and negative symptoms associated with schizophrenia, an in vitro fluorescence polarization (FP) assay<sup>30</sup> was established to define the ability of novel compounds to inhibit the recombinant COMT forms. There are a number of advantages to the FP assay versus previously described HTS assays such as the fluorescence based esculetin assay,<sup>31</sup> including use of a long-wavelength rhodamine fluorophore, TAMRA, to avoid interference from fluorescent compounds as well as use of a physiologically relevant substrate. A series of 4-pyridinones possessing robust MB-COMT inhibitory activity were identified, and IC<sub>50</sub> values are presented in Table 1 (compounds 1–14). IC<sub>50</sub> values versus human MB-COMT ranged from 41 to 291 nM for this group of compounds. These 4-pyridinone compounds were also potent rat MB-COMT inhibitors. Compound IC<sub>50</sub> values against the rat enzyme varied from 13 nM to 388 nM (Table 1).

Relative to MB-COMT, the ability to inhibit S-COMT proved more variable among the novel compounds. IC<sub>50</sub> values for compounds 3, 4, 8, 11, and 12 as determined versus human S-COMT were within 3-fold of their human MB-COMT IC<sub>50</sub> values. Therefore, these compounds are deemed pan-COMT inhibitors. Compounds 2, 7, and 10 are considered MB-COMT specific since the human and rat S-COMT IC<sub>50</sub> values were all >5000 nM in the FP assay. Compounds 1, 9, 13, and 14 are MB-COMT preferring compounds since potencies in FP assays decreased at least 5-fold (compounds 9 and 13) or at least 17-fold (compounds 1 and 14) versus human S-COMT. Inhibition potency versus S-COMT was not defined for compounds 5 and 6 since these compounds selectively served as substrates for rat and human S-COMT (see COMT substrate evaluation below).

We find it intriguing that compounds identified here may either selectively or preferentially inhibit human and rat MB-COMT over human and rat S-COMT. We are unaware of the existence of COMT isoform specific non-nitrocatechol inhibitors. The ability of these novel molecules to differentiate between MB and S-COMT is somewhat surprising considering these two enzymes possess identical catalytic domains and the lone difference is a 50 amino acid hydrophobic region present

**Table 1. Tolcapone and Novel Compound in Vitro IC<sub>50</sub> Results (nM) versus Recombinant COMT Forms As Determined Using Fluorescence Polarization Based Assays<sup>a</sup>**

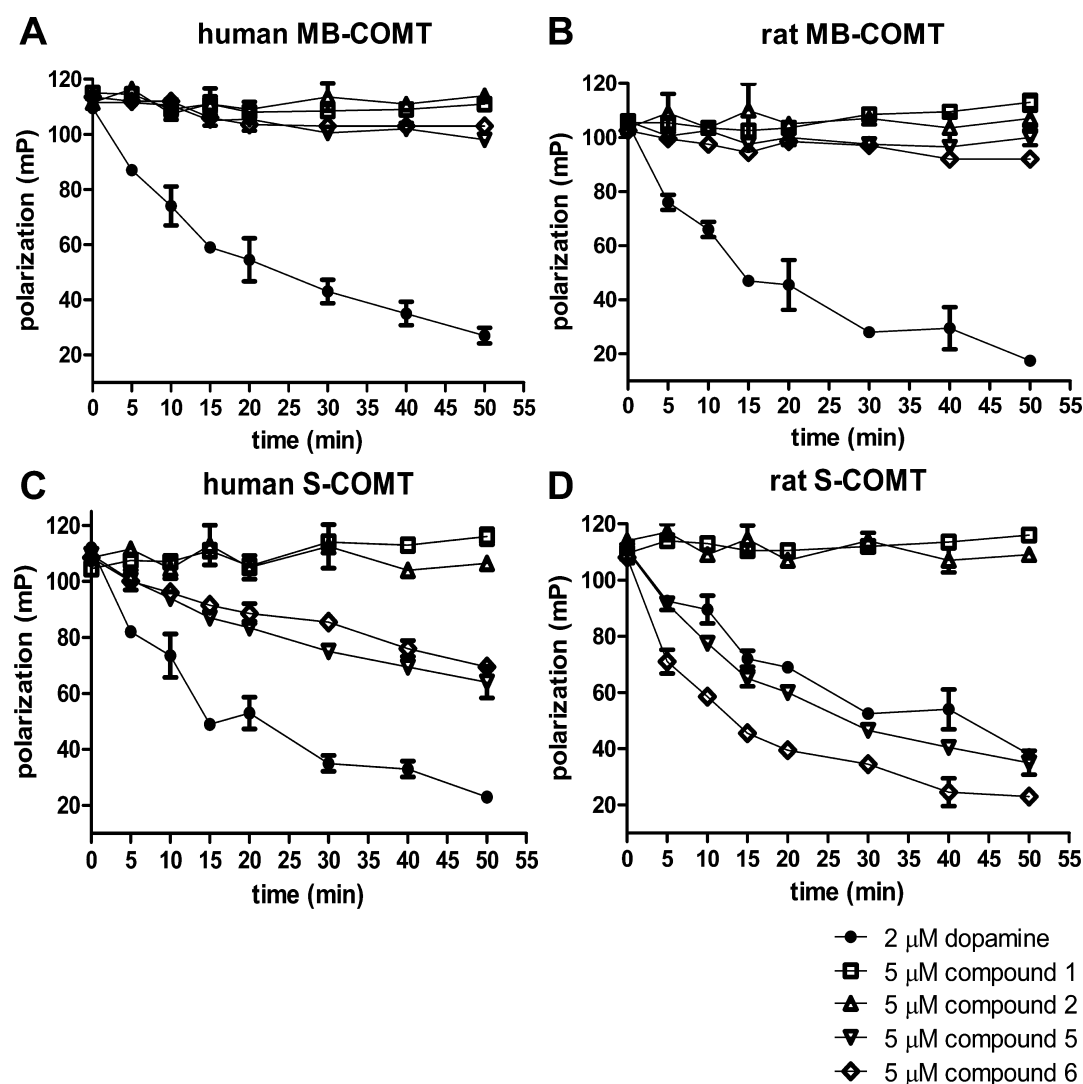
Compound	Structure	IUPAC name	Human MB-COMT IC <sub>50</sub> (nM) Mean ± S.E.M. (n)	Rat MB-COMT IC <sub>50</sub> (nM) Mean ± S.E.M. (n)	Human S-COMT IC <sub>50</sub> (nM) Mean ± S.E.M. (n)	Rat S-COMT IC <sub>50</sub> (nM) Mean ± S.E.M. (n)
Tolcapone		(3,4-dihydroxy-5-nitrophenyl)(4-methylphenyl)methanone	6.7 ± 0.3 (14)	substrate	substrate	substrate
3-methoxy-tolcapone		(4-hydroxy-3-methoxy-5-nitrophenyl)(4-methylphenyl)methanone	>5000 (2)	>5000 (2)	>5000 (2)	>5000 (2)
4-methoxy-tolcapone		(3-hydroxy-4-methoxy-5-nitrophenyl)(4-methylphenyl)methanone	29.0 ± 5.5 (5)	16.9 ± 2.4 (5)	185 ± 23.9 (5)	112 ± 22.6 (5)
1		1-[3-(5-chloro-1H-pyrrolo[2,3-b]pyridin-4-yl)-2-methylphenyl]-5-hydroxy-2-(2,2,2-trifluoro-1-hydroxyethyl)pyridin-4(1H)-one	63.6 (2)	20.5 (2)	1118 (2)	not determined
2		1-[3-(2,2-difluoro-1,3-benzodioxol-4-yl)phenyl]-5-hydroxy-2-(1-hydroxyethyl)pyridin-4(1H)-one	291 (2)	158 (2)	>5000 (2)	not determined
3		5-hydroxy-2-(1-hydroxyethyl)-1-(3-quinolin-5-ylphenyl)pyridin-4(1H)-one	109 ± 25.2 (4)	80.0 ± 16.4 (4)	242 ± 59.4 (5)	483 (1)
4		5-hydroxy-1-(3-isoquinolin-4-ylphenyl)-2-(2,2,2-trifluoro-1-hydroxyethyl)pyridin-4(1H)-one	63.0 (2)	13.0 (2)	175 ± 47.5 (3)	565 (1)
5		2-amino-1-biphenyl-3-yl-5-hydroxypyridin-4(1H)-one	122 ± 40.6 (6)	176 ± 64.4 (5)	substrate	substrate
6		1-biphenyl-3-yl-5-hydroxy-2-(methylamino)pyridin-4(1H)-one	100 ± 24.5 (4)	180 ± 77.4 (3)	substrate	substrate
7		1-biphenyl-3-yl-5-hydroxy-2-(1-hydroxyethyl)pyridin-4(1H)-one	228 ± 35.0 (8)	388 ± 37.5 (4)	>5000 (4)	>5000 (2)
8		5-hydroxy-2-(1-hydroxyethyl)-1-(3-isoquinolin-4-ylphenyl)pyridin-4(1H)-one	226 ± 29.9 (12)	88.8 ± 17.9 (8)	258 ± 47.3 (9)	356 (1)
9		1-[3-(5-chloro-1H-pyrrolo[2,3-b]pyridin-4-yl)phenyl]-5-hydroxy-2-(1-hydroxyethyl)pyridin-4(1H)-one	39.6 ± 6.7 (8)	27.4 ± 7.4 (6)	226 ± 48.8 (7)	237 (1)
10		1-biphenyl-3-yl-5-hydroxy-2-(1-hydroxy-2-phenylethyl)pyridin-4(1H)-one	93.7 ± 12.9 (7)	45.7 ± 15.4 (5)	>5000 (3)	>5000 (1)
11		5-hydroxy-1-(3-quinolin-5-ylphenyl)-2-(2,2,2-trifluoro-1-hydroxyethyl)pyridin-4(1H)-one	69.9 (2)	30.9 (2)	161 ± 8.0 (3)	292 (1)
12		5-hydroxy-2-(1-hydroxyethyl)-1-[3-(1H-indazol-4-yl)phenyl]pyridin-4(1H)-one	192 ± 59.9 (4)	80.0 (2)	338 (2)	1231 (1)
13		1-(5'-ethoxy-2'-fluoro-4,6-dimethoxybiphenyl-3-yl)-5-hydroxy-2-(1-hydroxyethyl)pyridin-4(1H)-one	73.4 ± 24.9 (4)	34.4 ± 5.2 (4)	482 ± 50.1 (4)	752 ± 57.3 (4)
14		1-[1-(2-Chloro-benzyl)-1H-benzimidazol-4-yl]-3-hydroxy-1H-pyridin-4-one	44.9 ± 15.0 (4)	26.5 ± 3.4 (4)	857 ± 81.7 (4)	881 ± 220 (4)

<sup>a</sup>Displayed are mean IC<sub>50</sub> values ± SEM (n); where applicable.

on MB-COMT. The molecular interactions between MB-COMT and the new compounds remain uncharacterized until an MB-COMT crystal structure becomes available. The fact that we identified MB-COMT specific compounds provides a possibility for reducing the adverse effects associated with pan-

COMT inhibition, but clinical data demonstrating efficacy and superior tolerability of MB-COMT specific inhibition versus pan-COMT inhibition is necessary.

Tolcapone and related methylated derivatives were additionally evaluated versus the recombinant COMT enzymes (Table

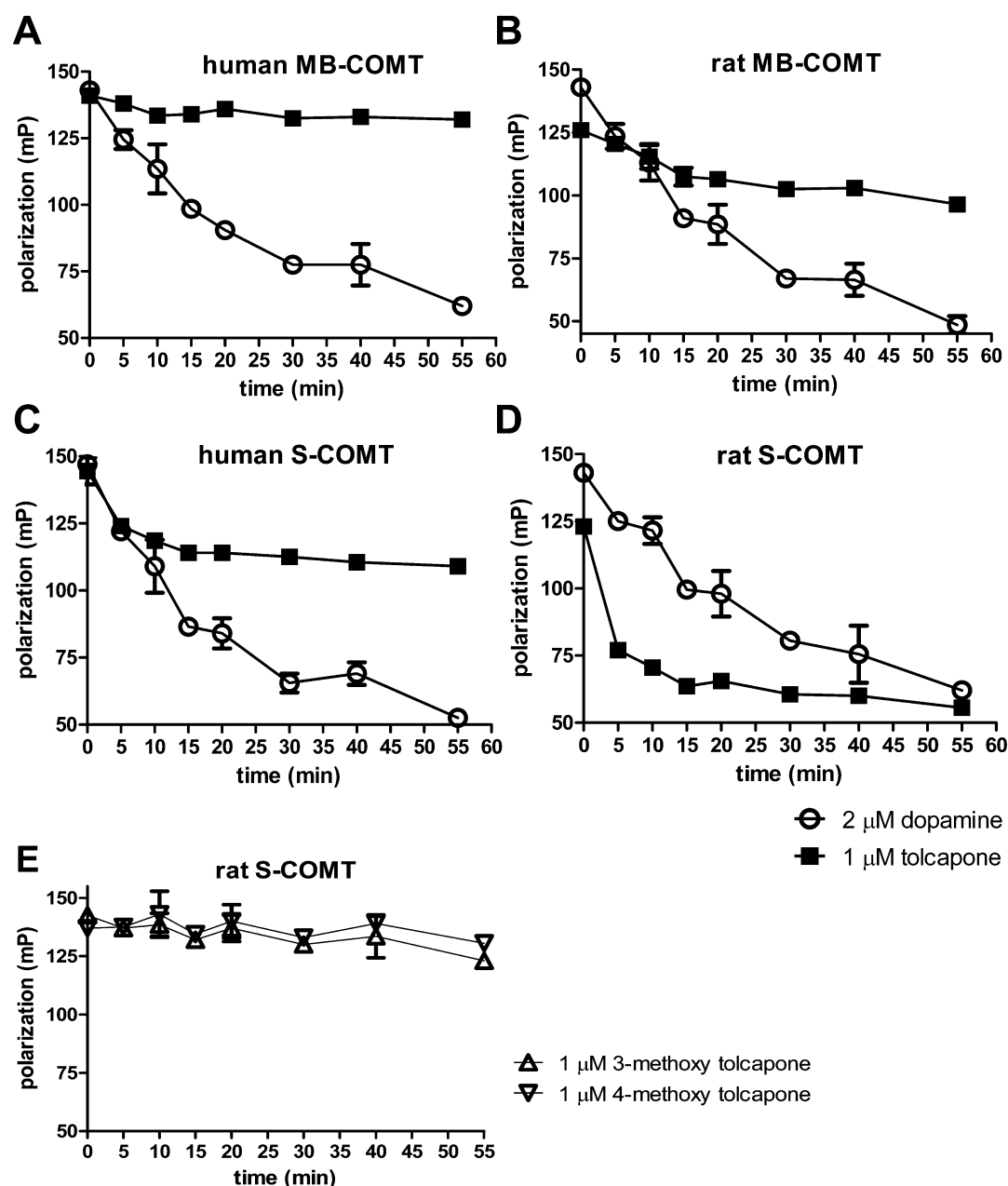


**Figure 2.** Modified fluorescence polarization based time course assays were used to identify novel compounds as COMT substrates. A 5  $\mu\text{L}$  mix comprising 40  $\mu\text{M}$  compound stocks, 8  $\mu\text{M}$  SAM, and 40 mM  $\text{MgCl}_2$  in assay buffer was placed into assay wells (black 96-well round-bottom polystyrene plates from Costar; catalog # 3792). Enzyme reactions were initiated at room temperature by adding 35  $\mu\text{L}$  of one of the COMT forms (1–6 ng of protein) also diluted into assay buffer. The final concentrations of the test compounds and SAM were 5 and 1  $\mu\text{M}$ , respectively. Additional assays replacing the compounds with a final 2  $\mu\text{M}$  concentration of dopamine were also included as a positive COMT substrate reference. The enzyme assays were quenched at various time points with 5  $\mu\text{L}$  of 250 mM EDTA and subsequently 20  $\mu\text{L}$  of a preformed complex containing SAC TAMRA tracer and anti-SAH antibody was added (described in the Methods section). The extent of SAH production was used to evaluate compounds as COMT substrates. Increasing levels of SAH inversely lowered polarization measurements of the SAC TAMRA/SAH antibody complex by displacing the fluorescence label from the antibody. Graphs A through D show time course assay results using 2  $\mu\text{M}$  dopamine or 5  $\mu\text{M}$  novel compounds as substrates, respectively, for human MB-COMT, rat MB-COMT, human S-COMT, and rat S-COMT.

1). Compared to the aforementioned non-nitrocatechol compounds, tolcapone ranked as the most potent inhibitor of human MB-COMT. Subsequent studies demonstrated that rather than behaving as a true inhibitor, tolcapone served as a substrate for the other COMT forms (described below). 4-methoxy-tolcapone was a pan-COMT inhibitor while 3-methoxy-tolcapone had no effect on human and rat MB or S-COMT activity.

**COMT Substrate Evaluation.** Dopamine was excluded in a modified version of the FP assay to provide a means of determining whether compounds served as COMT substrates. In Figure 2, each of the recombinant COMT forms was incubated with a 5  $\mu\text{M}$  concentration of novel compound sans dopamine and the conversion of *S*-adenosyl-*L*-methionine (SAM) to *S*-adenosyl-*L*-homocysteine (SAH) was monitored

over time. Routine FP assays which included COMT and 2  $\mu\text{M}$  dopamine, but excluded novel compounds, provided positive controls of product formation. In the absence of dopamine, a decrease in the polarization of the SAH antibody/*S*-adenosyl-*L*-cysteine (SAC) TAMRA tracer complex indicated SAH production and therefore compound methylation. No accumulation of SAH was detected when compounds 1 and 2 were tested in these assays. This confirmed that these two compounds were not utilized as substrates by any of the four COMT forms. The aminopyridinone compounds 5 and 6 were unusual because they were potent MB-COMT inhibitors, but also served as S-COMT substrates. SAH increase was not evident when the compounds were tested versus human and rat MB-COMT. However, compounds 5 and 6 (like dopamine) resemble traditional enzyme substrates when exposed to human



**Figure 3.** Modified fluorescence polarization (FP) based time course assays to evaluate tolcapone and methylated derivatives as COMT substrates. (A–D) 1  $\mu$ M tolcapone or 2  $\mu$ M dopamine provided as substrates for human MB-COMT, rat MB-COMT, human S-COMT, or rat S-COMT. (E) 1  $\mu$ M 4-methoxy-tolcapone or 1  $\mu$ M 3-methoxy-tolcapone provided as substrates for rat S-COMT.

or rat S-COMT as a gradual accumulation of product formation was observed over time until presumably the substrate and/or cofactor was exhausted. In particular, these two molecules were efficient substrates for rat S-COMT as 5  $\mu$ M concentrations of the compounds led to a more rapid depletion of SAM than was observed using 2  $\mu$ M dopamine.

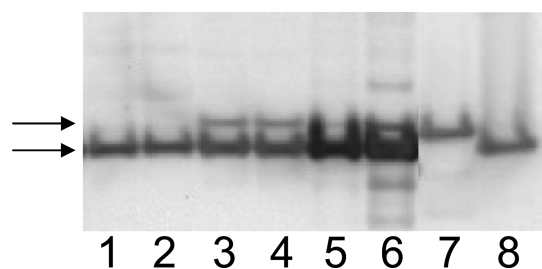
Tolcapone was also analyzed as a substrate for the recombinant COMT preparations. Graphs A through D in Figure 3 compare 1  $\mu$ M tolcapone and 2  $\mu$ M dopamine as substrates in FP time course assays. No substantial decrease in polarization was observed by incubating tolcapone with human MB-COMT indicating the drug is not an effective substrate for this COMT form. At the onset of combining tolcapone with human S-COMT or rat MB-COMT a modest increase in SAH levels was observed suggesting a limited capability to be utilized

as a substrate. However, the rates of SAH accumulation visualized following the combination of tolcapone with rat MB-COMT or human S-COMT become notably diminished prior to the depletion of substrate or SAM. Evidently, it is not simply the accumulation of methylated tolcapone which explains the time dependent reduction of rat MB-COMT or human S-COMT enzyme activity. A possible explanation comes from studies demonstrating that COMT inhibition potency of tolcapone increases the longer the enzyme and drug are allowed to interact and that tolcapone and COMT combine in a tight binding manner.<sup>32</sup> Taken together, this may initially allow for some product formation before enzyme catalytic activity ceases. Conversely, rat S-COMT incubated with tolcapone caused a substantial rapid decrease in assay polarization values. Based on the velocity of SAH accumulation,

it appears that 1  $\mu\text{M}$  tolcapone serves more readily as a substrate for rat S-COMT compared to dopamine tested at twice the concentration.

In hopes of utilizing a nonsubstrate tool compound, methylated versions of tolcapone were also evaluated. 4-methoxy- and 3-methoxy-tolcapone variants were allowed to react with rat S-COMT and then were examined for substrate potential (Figure 3E). Polarization readings remained essentially stable for the duration of the time course demonstrating that the modified tolcapone derivatives were no longer utilized as rat S-COMT substrates. Unfortunately, subsequent studies with 4-methoxy-tolcapone demonstrated that it had no effect in biomarker assays due to poor pharmacokinetic properties (data not shown). In fact, the principal tolcapone metabolite in vivo is 3-methoxy-tolcapone rather than 4-methoxy-tolcapone.<sup>33</sup> Thus, 4-methoxy-tolcapone does not contribute to the in vivo efficacy of tolcapone due in part to unfavorable methylation by COMT at this site as well as poor pharmacokinetic properties once formed.

**Biomarker and ex Vivo Assays.** Although free levels of dopamine are very low in CSF, the CSF dopamine metabolites homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) have been used historically to assess central dopaminergic function.<sup>34,35</sup> Following central COMT inhibition, HVA levels are expected to decrease and DOPAC increase, since COMT converts DOPAC to form HVA. Thus, DOPAC and HVA serve as preclinical biomarkers of COMT inhibition, while a COMT ex vivo assay in brain, blood, and liver serves as a direct measure of target engagement centrally and peripherally. However, based on the evolutionary progression moving from mouse to human of increased MB-COMT expression,<sup>16</sup> we expected, and observed, a small proportion of MB-COMT relative to S-COMT in fractions utilized for the COMT ex vivo rat brain assay. Total protein was utilized in ex vivo assays where analysis of representative fractions by Western blotting revealed that blood appears to solely express S-COMT, and rat brain and liver fractions contained a clear S-COMT majority (Figure 4).



**Figure 4.** Representative Western blot demonstrating the ratio of MB to S-COMT in blood (lanes 1–2), brain (lanes 3–4), or liver (lanes 5–6) fractions utilized in the COMT ex vivo assay from male Wistar rat. To demonstrate similar immunoreactivity for rat MB versus S-COMT, equal amounts of recombinant rat MB (lane 7) or S-COMT (lane 8) were also resolved. Immunoblotting with COMT antibody (Santa Cruz-135872, dilution 1:200) revealed immunoreactivity for MB (indicated by upper arrow) and S-COMT (lower arrow).














The effects of tolcapone or novel compounds 3–14 on total COMT activity and CSF dopamine metabolites were examined in male Wistar rats following 1 or 1.5 h treatment as detailed in Table 2. A separate set of vehicle treated rats were processed similarly for each time point and utilized for data normalization (data not shown). Plasma and CSF compound concentrations

were also determined following compound administration. Rats treated with 30 mg/kg tolcapone exhibited over a 3-fold increase in CSF DOPAC levels and an 80% reduction of HVA. Changes in CSF dopamine metabolites were significant as determined by two-way analysis of variance (ANOVA) followed by Dunnett posthoc comparison ( $p < 0.001$  versus vehicle), thus demonstrating central COMT inhibition following tolcapone treatment. To directly confirm COMT inhibition, ex vivo assays demonstrated the presence of minimal, if any, COMT activity in tissue and blood samples;  $p < 0.001$  versus vehicle using an unpaired  $t$  test. Tolcapone concentrations averaged 65  $\mu\text{M}$  in plasma and 0.9  $\mu\text{M}$  in CSF, which exceed the concentration of compound needed to completely inhibit MB-COMT in vitro. After confirming biomarker and ex vivo enzyme inhibition effects in our assays with tolcapone, we proceeded to analyze the novel non-nitrocatechol compounds in an attempt to establish a similar relationship.

Among the novel compounds, 100 mg/kg administration with S-COMT substrate compounds 5 and 6 or 100 mg/kg treatment using the preferential MB-COMT inhibitor compounds 13 and 14 induced significant biomarker changes that were closest to those observed with tolcapone (Table 2; at least  $p < 0.05$ ). In the ex vivo assay, each of these compounds resulted in >65% COMT inhibition in the periphery that was significant to at least  $p < 0.05$  by unpaired  $t$  test analysis. Despite significant changes in CSF biomarkers for these four compounds, ex vivo COMT activity in brain was only significantly affected following administration of compounds 6 and 14 (COMT activity  $50.2 \pm 11.7$  and  $48 \pm 12.2\%$ , respectively). Administration of compound 14 resulted in a CSF concentration of  $400 \pm 100$  nM, while 52% inhibition was observed in the rat brain COMT ex vivo assay. These results are consistent with a CSF concentration within 2-fold of the average in vitro S-COMT  $\text{IC}_{50}$  values. Thus, despite achieving significant effects on CSF biomarkers reminiscent of effects following 30 mg/kg administration of tolcapone, the effect of compounds 5, 6, 13, and 14 on COMT ex vivo activity were less robust than tolcapone.

Assays validated through the use of tolcapone as a tool compound were utilized to demonstrate preclinical efficacy of novel COMT inhibitors. Unlike tolcapone, significant changes in the levels of CSF dopamine metabolites did not necessarily result in the anticipated amount of brain COMT inhibition following administration of the non-nitrocatechol compounds. Changes in biomarker levels that rivaled those observed with tolcapone treatment were observed with compounds 5, 6, 13, and 14. However, rat brain COMT activity remained at 50% or higher relative to the near complete inhibition observed with tolcapone. For compounds 5 and 6, this was despite the fact that CSF levels were nearly 9-fold above in vitro  $\text{IC}_{50}$  values for rat MB-COMT. This may in part be explained by an evolutionary species change where the concentration of MB-COMT increases in brain moving from mouse to human.<sup>16</sup> Additionally, compounds 5 and 6 are readily metabolized by S-COMT and are less likely to be efficacious in an S-COMT predominant environment. Compounds 13 and 14, which preferably recognize MB-COMT, also produced a response in ex vivo assays that may be explained by the preponderance of S-COMT activity measured in the assay. For example,  $400 \pm 100$  nM of compound 14 in CSF led to  $48 \pm 12.2\%$  ( $p < 0.01$ ) brain activity in the ex vivo assay which corresponded more favorably

**Table 2. Effect of Single Dose Administration of Tolcapone or Novel Non-Nitrocatechol Compounds 3–14 on COMT Activity in Blood, Brain, or Liver as well as CSF DOPAC and HVA Levels in Male Wistar Rat<sup>a</sup>**

Compound	Structure	Dose and Vehicle	DOPAC (% of control) Mean ± S.E.M. (n)	HVA (% of control) Mean ± S.E.M. (n)	Compound plasma concentration (μM) Mean ± S.E.M. (n)	Compound CSF concentration (μM) Mean ± S.E.M. (n)	COMT activity blood (% of control) Mean ± S.E.M. (n)	COMT activity liver (% of control) Mean ± S.E.M. (n)	COMT activity brain (% of control) Mean ± S.E.M. (n)
Tolcapone		30 mg/kg, 2 mL/kg, p.o. 1h 10% Tween 80	326 ± 33.4*** (7)	19.6 ± 3.3*** (7)	65.0 ± 6.8 (8)	0.9 ± 0.09 (6)	0.6 ± 0.2*** (8)	0.6 ± 0.2*** (8)	3.3 ± 0.2*** (8)
3		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	143 ± 15.0* (5)	58.6 ± 7.8** (5)	18.2 ± 4.5 (5)	0.3 ± 0.09 (5)	11.5 ± 5.8*** (5)	5.0 ± 2.0*** (5)	51.4 ± 9.2** (5)
4		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	139 ± 7.0** (4)	75.0 ± 3.0** (4)	15.0 ± 1.0 (4)	0.1 ± 0.006 (3)	10.2 ± 1.5*** (4)	3.3 ± 0.2*** (4)	80.6 ± 12.4 (4)
5		100 mg/kg, 2 mL/kg, i.p. 1h 10% Tween 80	236 ± 20.3*** (5)	15.1 ± 0.9*** (5)	60.8 ± 11.0 (5)	3.8 ± 0.6 (4)	2.5 ± 0.5*** (5)	17.9 ± 4.7** (5)	67.4 ± 14.2 (5)
6		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	249 ± 23.7*** (5)	13.3 ± 5.2*** (5)	9.54 ± 2.4 (5)	1.6 (2)	18.0 ± 3.6** (5)	21.0 ± 3.1* (5)	50.2 ± 11.7** (5)
7		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	195 ± 26.5** (4)	41.7 ± 7.5** (4)	321 ± 121 (4)	3.4 ± 1.1 (4)	60.0 ± 7.6** (5)	not determined	63.2 ± 10.7* (5)
8		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	195 ± 31.6* (3)	47.6 ± 7.2* (3)	8.7 ± 0.5 (3)	0.3 ± 0.05 (3)	5.2 ± 1.2* (3)	3.7 ± 0.2*** (3)	not determined
9		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	136 ± 5.4 (5)	77.5 ± 2.4** (5)	10.2 ± 0.4 (5)	0.02 (1)	11.0 ± 0.8*** (5)	not determined	not determined
10		250 mg/kg, 5 mL/kg, p.o. 1.5h 10% Tween 80	107 ± 5.9 (5)	70.3 ± 4.1*** (5)	2.9 ± 0.5 (5)	0.06 (2)	79.0 ± 3.6 (5)	not determined	not determined
11		100 mg/kg, 2 mL/kg, i.p. 1h 10% Tween 80	140 ± 7.0 (5)	104 ± 4.7 (5)	14.7 ± 1.7 (5)	0.2 (1)	7.6 ± 4.6*** (5)	14.8 ± 9.6** (5)	121 ± 27.3 (4)
12		100 mg/kg, 2 mL/kg, i.p. 1h 90% PEG 400	160 ± 36.2 (5)	82.7 ± 7.7 (5)	27.8 ± 6.5 (5)	0.2 ± 0.02 (3)	47.0 ± 10.7** (5)	not determined	not determined
13		100 mg/kg, 2 mL/kg, i.p. 1h 10% Tween 80	294 ± 51.9* (3)	32.7 ± 8.4* (3)	24.0 ± 5.5 (4)	not determined	9.5 ± 3.2** (4)	5.3 ± 1.2*** (4)	63.5 ± 15.2 (4)
14		100 mg/kg, 2 mL/kg, i.p. 1h 10% Tween 80	264 ± 53.4** (3)	3.2 ± 1.4*** (3)	7.1 ± 2.7 (3)	0.4 ± 0.1 (3)	34.4 ± 12.2* (3)	not determined	48.0 ± 12.2** (3)

<sup>a</sup>Data were normalized to separate vehicle treated rats for each respective time point and data expressed as 100% of control. CSF biomarker values that significantly differed from vehicle were determined using regular two-way ANOVA followed by post-hoc tests using a Dunnett correction factor for individual group comparisons as indicated; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . A significant effect on COMT ex vivo activity versus vehicle was determined using an unpaired  $t$  test as indicated; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

with the rat S-COMT  $IC_{50}$  potency range of  $881 \pm 220$  nM than with the rat MB-COMT  $IC_{50}$  of  $27 \pm 3$  nM.

Treatment with pan-COMT inhibitors 3, 4, and 8 resulted in significant changes in CSF biomarkers, while administration of pan-COMT inhibitor compounds 11 and 12 did not. Plasma exposure for compounds 11 and 12 exceeded the  $IC_{50}$  values for both rat MB and S-COMT and a significant change in measurements of peripheral COMT activity were observed. However, CSF concentrations for both compounds ( $\sim 200$  nM) were below in vitro rat S-COMT  $IC_{50}$  values and no effect on brain COMT ex vivo activity was observed for compound 11. Based on lower in vitro S-COMT  $IC_{50}$  values, compound 12 effects on ex vivo COMT brain activity were not determined. In addition to significant effects on CSF dopamine metabolite levels, administration of compounds 3, 4, and 8 significantly reduced ex vivo COMT activity in blood and liver. In line with these data, plasma concentrations for compounds 3, 4, and 8 ( $18.2 \pm 4.5$ ,  $15 \pm 1$  and  $8.7 \pm 0.5$   $\mu$ M, respectively) exceeded rat S-COMT in vitro  $IC_{50}$  values (483, 565, and 356 nM, respectively). Ex vivo COMT brain activity was significantly decreased following administration of compound 3, but not compound 4. Similarly, CSF concentrations for compound 3 approached the in vitro  $IC_{50}$  value for rat S-COMT ( $300 \pm 90$  nM vs 483 nM) while compound 4 CSF concentrations were

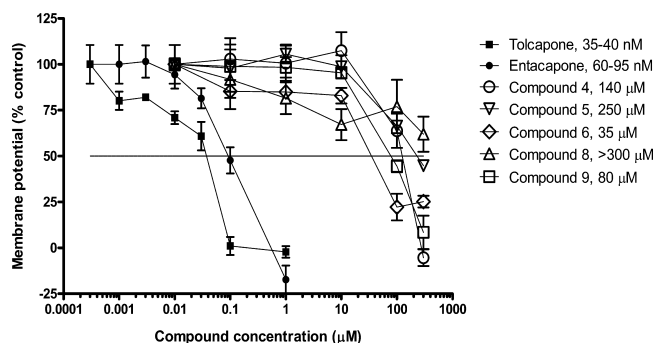
below the  $IC_{50}$  measurement for rat S-COMT ( $100 \pm 6$  nM vs 565 nM).

MB-COMT specific compound 7 significantly increased DOPAC ( $195 \pm 26.5\%$ ;  $p < 0.01$ ) and reduced HVA ( $41.7 \pm 7.5\%$ ;  $p < 0.01$ ), while compound 10 only significantly reduced HVA ( $70.3 \pm 4.1\%$ ;  $p < 0.001$ ). Additionally, compound 10 administration differed from the other non-nitrocatechol test compounds since the dose was 250 mg/kg p.o., whereas the remainder of novel compounds were administered 100 mg/kg i.p. Plasma concentrations ( $2.9$   $\mu$ M) following compound 10 treatment were the lowest observed and COMT activity was not significantly reduced in blood. Based on low compound levels in CSF, COMT activity in liver and brain were not determined. In contrast, plasma concentration among novel compounds was greatest for compound 7;  $321 \pm 121$   $\mu$ M while CSF concentration was  $3.4 \pm 1.1$   $\mu$ M. Compound 7 administration resulted in an  $\sim 40\%$  reduction of COMT activity in blood and brain that was significant ( $p < 0.05$ ).

In view of the fact S-COMT was the primary isoform in the ex vivo assays, a rational in vitro/in vivo correlation emerged for several of the novel compounds. The pan-COMT inhibitors 3, 4, 8, 11, and 12 achieved plasma concentrations that exceeded their in vitro rat S-COMT  $IC_{50}$  values by at least 20-fold and ex vivo COMT inhibition in blood and/or liver was 85% or better, except for compound 12 ( $\sim 50\%$ ). CSF

concentrations observed for compounds 3 and 4 were near or below their rat S-COMT  $IC_{50}$  potencies so brain COMT inhibition levels observed were reasonable. Compound 7, which was considered MB-COMT specific since in vitro  $IC_{50}$  values versus rat S-COMT were greater than  $5 \mu M$ , produced weak ex vivo COMT inhibition of  $\sim 40\%$  in brain and blood that was significant. In fact, these data are consistent with plasma and CSF concentrations of compound 7 ( $321 \pm 121 \mu M$  and  $3.4 \pm 1.1 \mu M$ , respectively). Compound 10 was also considered MB specific and with plasma levels of  $2.9 \pm 0.5 \mu M$  did not achieve significant inhibition in the ex vivo blood assay since only S-COMT activity is monitored. MB-COMT specific compounds 7 and 10 achieved significant effects on CSF DOPAC and/or HVA thus highlighting the possibility that despite an S-COMT majority ex vivo, the localization of COMT and affinity of dopamine for MB versus S-COMT may also be contributing factors.<sup>17,32</sup>

**Mitochondrial Membrane Potential Assessment.** To distinguish novel leads from the hepatotoxic liability associated with tolcapone, novel COMT inhibitors were tested alongside tolcapone and nonhepatotoxic entacapone in MMP assays.<sup>28</sup> Rat heart mitochondria were isolated and incubated with multiple concentrations of tolcapone, entacapone, or novel COMT inhibitors to determine the influence on the MMP. Evaluated novel compounds were approximately 1000- or 500-fold less potent than tolcapone and entacapone, respectively. Figure 5 demonstrates that 35–40 nM tolcapone leads to a



**Figure 5.** Effect of tolcapone, entacapone, or novel COMT inhibitors on rat heart mitochondrial membrane potential (MMP). Values listed next to compounds are concentrations that resulted in a 50% reduction of the mitochondrial membrane potential based on a vehicle treatment control representing maximum membrane potential and 3 mM carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) treatment representing minimum membrane potential.

50% reduction in membrane potential. In contrast, all of the novel COMT inhibitors tested were considerably less potent uncouplers of MMP compared to tolcapone. Moreover, all novel compounds tested were differentiated from entacapone, which is considered nonhepatotoxic. In fact, concentrations of the new compounds varying from  $35 \mu M$  to greater than  $300 \mu M$  were required to reduce the MMP by 50%.

Therefore, these in vitro data support the idea that non-nitrocatechol COMT inhibitors may be developed with reduced risk for hepatotoxicity. In pilot studies, we compared the tolcapone and entacapone MMP response in mitochondria isolated from rat liver with mitochondria isolated from rat heart (data not shown). In both liver and heart mitochondria, tolcapone was  $\sim 2$ – $5$  fold more potent MMP inhibitor when compared to entacapone, although MMP effects were observed

at  $\sim 100$ -fold lower concentrations in heart mitochondria as compared to liver mitochondria. These differences in the response could possibly result due to a greater basal level of coupled mitochondria isolated from heart as compared to liver. The tolcapone MMP response observed in the liver mitochondria was analogous to the response observed by Haasio and co-workers, although for entacapone a more potent response was observed as compared to what was previously reported.<sup>36</sup>

## CONCLUSIONS

In summary, our current research led to the discovery of novel, non-nitrocatechol COMT inhibitors with improved safety profiles. These compounds may provide a starting point for the development of a therapeutic that targets negative symptoms and cognitive deficit associated with schizophrenia. We recognize the limitation of using Wistar rats in our preclinical studies due to the predominance of brain S-COMT versus MB-COMT and that this ratio does not accurately reflect the evolutionary species change moving to human where MB-COMT is preferentially expressed in brain.<sup>16</sup> Future studies utilizing transgenic rats expressing human MB-COMT on a rat COMT knockout background, S-COMT deficient mice,<sup>37</sup> or mice overexpressing human MB-COMT (Val158)<sup>16</sup> are expected to improve correlations between biomarker and ex vivo data when evaluating MB-COMT specific and preferring compounds. Thus, along with the identification and evaluation of additional compounds, future exploratory work with the novel compounds utilizing a preclinical model with preferential MB-COMT brain expression will help determine whether MB-COMT specific or preferring compounds have similar biomarker and MB-COMT brain inhibition effects versus tolcapone.

## METHODS

**Compounds.** Tolcapone and entacapone were synthesized by Synfine (Richmond Hill, ON, Canada). 3-Methoxy-tolcapone and 4-methoxy-tolcapone along with novel compounds 1–14 were synthesized at Merck Research Laboratories (West Point, PA).

**Preparation of Recombinant COMT.** Bacmid expressing human MB-COMT (M51A, V158) with a C-terminal 6 histidine (HIS) tag was utilized to infect Sf-9 insect cells with a multiplicity of infection of 3 for 48 h (pFastBac vector, Invitrogen, Carlsbad, CA). For the preparation of rat MB-COMT (M44A) with a C-terminal 10 HIS tag/pET29b(+) (pET expression vectors, EMD Chemicals Inc., Gibbstown, NJ), human S-COMT (V158) with a C-terminal enterokinase cleavage site and 6-HIS/pET29b(+) and rat S-COMT with C-terminal enterokinase cleavage site followed by a 6-HIS tag/pET29b(+), approximately 50 ng DNA were transformed into BL21-Gold (DE3) or BL21-CodonPlus(DE3)-RIPL (for rat MB-COMT only) according to manufacturer protocols (Stratagene, La Jolla, CA). Colonies were selected for growth in YT media (Teknova, Hollister, CA) supplemented with 10 mM  $MgCl_2$ , 10 mM  $MgSO_4$ , 3 mM KCl, and 50  $\mu g/mL$  kanamycin, or Optigrow Superbroth (Fisher Scientific, Pittsburgh, PA) plus 50  $\mu g/mL$  kanamycin (for rat MB-COMT only) and incubated at  $37^\circ C$  until an  $OD_{600}$  of approximately 0.7. Temperature was then reduced to  $18^\circ C$  ( $20^\circ C$  for rat S-COMT only). When the  $OD_{600}$  reached approximately 0.7, cultures were induced with 0.4 mM IPTG and incubated at  $18$ – $20^\circ C$  for 16–18 h. Cells were then collected according to standard protocol and supernatant discarded.

Cell pellets were resuspended in Buffer A, pH 7.4 without detergent (50 mM Tris-HCl, pH 7.4, 150–200 mM NaCl, Roche complete, EDTA-free, protease inhibitor tablets (2 tablets/50 mL; Roche Applied Science, Indianapolis, IN), 1–2 mM tris[2-carboxyethyl] phosphine hydrochloride (TCEP, Pierce Biotechnology, Rockford,



IL), 30–40 mM imidazole (Fisher Scientific), 2 mM  $MgCl_2$  for MB-COMT constructs or with Buffer A plus detergent (1% Fos-choline 12 (FC-12, Anatrace, Maumee, OH, #F308)) for S-COMT constructs. Buffer A contained 25 units of benzonase nuclease (EMD Chemicals Inc.) per milliliter upon resuspension of cells. Pellets were homogenized by hand and then sonicated (power 5) with 10 s pulses for 3–4 min. In some cases, pellets were fluidized once at 14 000 psi. Suspension was collected at 23 800g with an SLA 1500 rotor at 4 °C for 1 h. Supernatant from S-COMT preparations was loaded onto a HIS Trap FF, 5 mL column (GE Healthcare, Piscataway, NJ) prewashed with Buffer A containing detergent using an Äktaxpress (GE Healthcare). Supernatant from MB-COMT preparations was discarded and pellet was resuspended in Buffer A without detergent, sonicated and then collected as above. Supernatant was again discarded and pellet was resuspended with detergent (1% FC-12) and sonicated as detailed above. Insoluble cell debris was removed by centrifugation at 32 000 or 48 000g for 1 h at 4 °C using a JA-20 rotor and then loaded onto a HIS Trap FF, 5 mL column as detailed above. After loading, column was washed with 3 column volumes (CVs) 100% Buffer A with detergent. After a system wash, elution of purified recombinant COMT occurred using a step elution with 50% Buffer B (Buffer A with detergent and 300–500 mM imidazole) for 10 CVs. Start and end level was 40 and 35 milli absorbance units, respectively. Aliquots of Äktaxpress fractions were separated using 4–12% Bis-Tris gels with 1× MES buffer (Invitrogen) as detailed below, stained with Instant Blue (Expediton, San Diego, CA) and fractions pooled for dialysis. Dialysis on pooled fractions containing recombinant COMT was completed at least 100 fold in Dialysis Buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.125 mM TCEP, 2 mM  $MgCl_2$ , 5% glycerol and 0.4% FC-12) to remove imidazole. For rat MB-COMT only, Buffer A and B, pH 7.5, contained 50 mM HEPES instead of 50 mM Tris-HCl. Also instead of dialysis, rat MB-COMT containing fractions were pooled and desalted with Buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM TCEP, 0.5% FC-12) using a 20 mL HiPrep 16/10 desalting column (GE Healthcare).

**Western Blotting.** Proteins were separated on 4–12% Bis-Tris gel with 1× MES buffer (Invitrogen) according to manufacturer protocol. Samples were heated 10 min at 70 °C prior to loading, except standards. Gels were transferred using an iBlot as directed by the manufacturer (Invitrogen). PVDF membranes were blocked for approximately 1 h at room temperature in blocking solution (5% milk (Bio-Rad, Hercules, CA), 0.1% Tween-20, in 1× TBS (Mediatech, Manassas, VA)) with agitation. Blots were incubated overnight at 4 °C with agitation in 10 mL primary antibody #sc-135872 recognizing COMT at a dilution of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) or COMT antibody #AB5873 at a dilution of 1:10 000 (Millipore, Billerica, MA) prepared in blocking solution. Blots were washed three times for 5 min in wash buffer (1× TBS plus 0.1% Tween-20) at room temperature with agitation. Blots were then incubated for 45 min to 1 h at room temperature with agitation in secondary antibody prepared in blocking solution at a 1:2000 dilution (anti-mouse IgG, HRP-linked antibody (#7076) or anti-rabbit IgG, HRP-linked antibody (#7074) from Cell Signaling Technology Danvers, MA. Prior to developing with ECL reagents (Pierce Biotechnology, #32106), blots were washed three times for 5 min in wash buffer with agitation. Blots were then developed using a VersaDoc imager (Bio-Rad) using the chemiluminescent setting.

**COMT FP Assay.** The activity of the compounds as COMT inhibitors may be readily determined using an FP methodology.<sup>30</sup> Compounds had activity in reference assays by exhibiting the ability to inhibit the production of SAH. Any compound exhibiting an  $IC_{50}$  below 1  $\mu M$  was considered a COMT inhibitor.

To determine  $IC_{50}$  values in the fluorescence polarization assay, 10 mM compound stock in DMSO was used to prepare 10 point 3-fold dilution series and 1  $\mu L$  of appropriate dilution was plated into assay wells (black 96-well, round-bottom, polystyrene plates from Corning Inc., Corning, NY, Costar # 3792). Recombinant enzyme was diluted in Assay Buffer (100 mM  $Na_2HPO_4$  pH 7.4, 1 mM DTT, 0.005% Tween-20), and 35  $\mu L$  of diluted enzyme was added to the assay wells containing 1  $\mu L$  of diluted compound. The final amount of enzyme in

the assay varied between 1 and 6 ng depending on the preparation of COMT utilized. Preincubation of COMT enzyme and compound progressed for 2 h at room temperature. Enzyme reactions were initiated upon the addition of 5  $\mu L$  of an 8× mix prepared in Assay Buffer containing 8  $\mu M$  SAM (USB Corporation, Cleveland, OH, # US10601), 16  $\mu M$  dopamine (Sigma-Aldrich, St. Louis, MO, # H8502), and 40 mM  $MgCl_2$ . After a 25 min incubation at room temperature, reactions were quenched with 5  $\mu L$  250 mM EDTA, pH 8.2. To quenched reactions, 20  $\mu L$  of a preformed complex containing a 1:80 000 dilution of SAC TAMRA tracer (2 mM from AnaSpec, Fremont, CA) and a 1:20 dilution of anti-S-adenosyl-L-homocysteine antibody (mouse monoclonal from IMX Homocysteine Reagent Pack, Abbott Laboratories, Abbott Park, IL, #7D29-20) was prepared in Assay Buffer B ( $Na_2HPO_4$  pH 7.2). Prior to combining with quenched enzyme assays, the SAH antibody/SAC TAMRA tracer complex was preformed at room temperature for 30 min while protected from light. Therefore, the final dilution of the SAH antibody/SAC TAMRA mix was 1:60 and 1:240 000, respectively. After a 2.5 h incubation at room temperature, protected from light, fluorescence polarization was measured using a Tecan Safire<sup>2</sup> plate reader using an excitation of 530 nm and an emission of 595 nm (Tecan US, Durham, NC). Titration curves and  $IC_{50}$  values were calculated using standard protocols.

**Animals.** All experimental protocols described in this study were approved by the Merck and Co., Inc. Institutional Animal Care and Use Committee and conducted in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Adult male Wistar rats were used for all in vivo experiments, while Sprague–Dawley rats were utilized as heart donors for mitochondrial membrane potential assays. Animals were maintained on a 12-h light/dark cycle (lights on 7:00 a.m.), and temperature and relative humidity were maintained at 22 to 24 °C and 50 to 55%, respectively.

**COMT Biomarker.** CSF concentrations of DOPAC and HVA were evaluated in male Wistar rats 7 to 10 weeks of age (220–250 g). Animals were administered test compounds or vehicle via intraperitoneal injection or, in the case of tolcapone and compound 10, by oral gavage and returned to home cages for a specified time determined from historical literature for tolcapone<sup>9</sup> or in-house in vitro and pharmacokinetic data (e.g., 1 h, 100 mg/kg for novel compounds). Animals were anesthetized with an inhalant anesthetic (isoflurane delivered at 5% with oxygen at 2 L/min) in an induction chamber. To ensure proper anesthesia, the animals were monitored and assessed by the lack of response to a toe pinch. CSF sampling was completed from the cisterna magna by needle puncture and aspiration using a 25–27 gauge butterfly needle with tubing attached to a syringe. While under anesthesia, rats were euthanized by decapitation using a small animal guillotine. The brain and liver were dissected and flash frozen on dry ice. Blood samples were obtained from the trunk and collected into tubes containing 0.5 M EDTA. CSF (15  $\mu L$ ) was diluted with 25  $\mu L$  of 0.1 M perchloric acid containing 0.1  $\mu M$  ascorbic acid and 4  $\mu L$  of internal standard (100 ng/mL 3,4-dihydroxybenzylamine hydrobromide (DHBA, Sigma-Aldrich) or 3-methoxytyramine hydrochloride (3-MT, Sigma-Aldrich)). Brain monoamine levels were quantitated using high performance liquid chromatography with electrochemical detection (HPLC-ED). Briefly, 20  $\mu L$  of the diluted sample was injected onto a C18 reverse phase column (MD150, 3 × 100 mm, 3  $\mu m$ , ESA; Chelmsford, MA) and the dopamine metabolites detected by an ESA Coulochem III controller (model 5200A, ESA) using a model 5020 guard cell (+350 mV) and a model 5014B microdialysis analytical cell (E1, –150 mV; E2, +220 mV). A commercially available mobile phase MD-TM (ESA) was used at a rate of 0.25 mL/min under isocratic conditions and DHBA was added to samples as an internal standard and sample run time was 35 min. The remaining CSF volume was diluted 1:1 with 100% acetonitrile and frozen at –80 °C for pharmacokinetic analysis. Whole blood was centrifuged (3000g for 10 min at 4 °C) and plasma removed and frozen at –80 °C for pharmacokinetic analysis. Red blood cells (following removal of plasma), brain and peripheral organs were processed in the COMT ex vivo assay.

**Pharmacokinetics.** For the determination of compound levels in CSF or plasma, samples were prepared and analyzed using ultrahigh performance liquid chromatography (UHPLC) combined with triple quadrupole mass spectrometry. Aliquots (50  $\mu\text{L}$ ) of control matrix (species specific EDTA plasma or CSF) were added into 0.75 mL tubes in a 96-well format. A 10  $\mu\text{L}$  aliquot of the appropriate intermediate solution of tolcapone (or other related compound) was added to each tube to generate a standard curve and replicate quality control samples. Each standard, quality control and unknown sample was precipitated with (150  $\mu\text{L}$  for plasma and 75  $\mu\text{L}$  for CSF samples) acetonitrile (HPLC grade; Sigma-Aldrich) + 0.1% formic acid (HPLC grade; Sigma-Aldrich) containing labetalol (US Pharmacopeia, Rockville, MD) as the analytical internal standard. The samples were sealed, vortexed for 2 min and collected at 2760g for 10 min. Following centrifugation, a 75  $\mu\text{L}$  aliquot of the extract was transferred to a plate containing 75  $\mu\text{L}$  of water +0.1% formic acid.

The samples were injected onto the UHPLC (Aria LX-2, Thermo Fisher Scientific, Waltham, MA) to determine linearity and reproducibility. Chromatographic separation was performed on a Supelco Ascentis Express (Sigma-Aldrich), C18–2.7  $\mu\text{m}$ , 5  $\times$  3 mm column. The mobile phase consisted of acetonitrile +0.1% formic acid and water +0.1% formic acid using a gradient at a flow rate of 1.0 mL/min. The gradient started at 90% aqueous and held for 60 s, ramped to 100% organic in 60 s, held at that rate for 75 s, followed by a ramp back to 90% aqueous and held for 75 s.

Following compound separation by UHPLC, samples were analyzed using an API 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) that was operated in the negative/positive ionization mode (MRM). The TurboV electrospray interface was set at an ionspray voltage of  $\pm 4000$  V. The temperature for the interface was set at 450  $^{\circ}\text{C}$ .

**COMT ex Vivo Assay.** COMT activity in blood, brain, and liver was evaluated by the ability to methylate norepinephrine (Sigma-Aldrich) to normetanephrine essentially as described<sup>38,39</sup> with modifications. Total COMT was obtained from liver or brain by homogenizing with FastPrep 24 Teen D Lysing Matrix tubes (MP Biomedicals, Solon, OH) 1:16 for liver and 1:4 for brain (w/v) in buffer 1 (100 mM sodium phosphate pH 7.4, 1 mM DTT and 5 mM  $\text{MgCl}_2$ ) followed by sonication on power 5, 10 s on and 10 s off for a total of five times for brain or eight times for liver. Homogenate was collected at 20 000g for 30 min at 4  $^{\circ}\text{C}$ , and the resulting supernatant used to evaluate COMT activity. Red blood cells were obtained following the removal of plasma and stored at  $-80$   $^{\circ}\text{C}$  without washing and hemolyzed as described previously.<sup>38</sup> Aliquots of 100  $\mu\text{L}$  of enzyme were then preincubated for 20 min at 37  $^{\circ}\text{C}$  with 92  $\mu\text{L}$  1 $\times$  assay buffer (100 mM sodium phosphate pH 7.4, 1 mM DTT, 5 mM  $\text{MgCl}_2$  and 100  $\mu\text{M}$  pargyline) for brain and liver or 2 $\times$  assay buffer for blood. The reaction was brought to a final volume of 200  $\mu\text{L}$  with 0.4 mM norepinephrine and 25  $\mu\text{M}$  SAM for brain and blood or with 0.05 mM norepinephrine and 100  $\mu\text{M}$  SAM for liver. Reactions were incubated at 37  $^{\circ}\text{C}$  for a further 15 min (liver), 15–35 min (brain), or 40 min (blood) and then quenched on ice with 50  $\mu\text{L}$  of perchloric acid. Prior to HPLC-ED, reactions were clarified by filtration (0.22  $\mu\text{m}$  pore, Corning Inc., Costar #8169). Norepinephrine metabolites were separated on a C18 reverse phase column and detected as detailed above except detector conditions were as follows: E1,  $-150$  mV; E2,  $+360$  mV.

**MMP Assay.** MMP was measured by modification of a previously described fluorometric method<sup>40</sup> and rationale.<sup>36</sup> Eight week old Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) served as heart donors. Hearts were removed from the animals rapidly following anesthesia (isoflurane delivered at 5% with oxygen at 2 L/min) and exsanguinations and minced and homogenized in Buffer C (120 mM KCl, 20 mM HEPES, 2 mM  $\text{MgCl}_2$ , 1 mM EGTA and 5 mg/mL BSA). Cellular debris were removed by centrifugation at 600g followed by mitochondria isolation through differential centrifugation at sequential speeds of 17 000g, 6000g, and 3500g. Isolated mitochondria were resuspended in Buffer D (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl and 0.1 mM EDTA), and protein concentration was determined by DC Protein Assay (Bio-Rad).

Isolated rat heart mitochondria (35  $\mu\text{L}$ ; protein concentration of 5 mg/mL) were incubated in Buffer D with 12.5 mM glutamate, 3.125 mM malate, 15  $\mu\text{M}$  safranin O, and selected COMT inhibitors. Additional assays included a vehicle treatment which served as a maximum membrane potential control, while treatment with 3 mM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) served as a minimum membrane potential control. The reaction temperature was maintained at 30  $^{\circ}\text{C}$  and fluorescence with excitation of 495 nm and emission of 584 nm was measured for 20 min. A single point of the plateau was chosen to calculate MMP as a percent of total membrane potential using the formula:  $[(\text{CCCP signal} - \text{COMT inhibitor signal})/(\text{CCCP signal} - \text{vehicle signal}) \times 100]$ .

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### Notes

The authors declare the following competing financial interest(s): Upon completion of the work, authors were employees of Merck Sharp & Dohme Corporation.

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## ■ ABBREVIATIONS

COMT, catechol-*O*-methyltransferase; MB-COMT, membrane bound COMT; S-COMT, soluble COMT; CSF, cerebrospinal fluid; HTS, high throughput screen;  $\text{IC}_{50}$ , half maximal inhibitory concentration; MMP, mitochondrial membrane potential; PFC, prefrontal cortex; SNP, single nucleotide polymorphism; DOPAC, dihydroxyphenylacetic acid; HVA,

homovanillic acid; FP, fluorescence polarization; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine

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