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Synthesis and analysis of novel catecholic ligands as inhibitors of catechol-*O*-methyltransferase

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ABSTRACT

L-DOPA, a dopamine precursor, is commonly used as a treatment for patients with conditions such as Parkinson's disease. This therapeutic L-DOPA, as well as the dopamine derived from L-DOPA, can be deactivated via metabolism by catechol-*O*-methyltransferase (COMT). Targeted inhibition of COMT prolongs the effectiveness of L-DOPA and dopamine, resulting in a net increase in pharmacological efficiency of the treatment strategy. Following the completion of a previous *ab initio* computational analysis of 6-substituted dopamine derivatives, several novel catecholic ligands with a previously unexplored neutral tail functionality were synthesized in good yields and their structures were confirmed. The ability of the catecholic nitriles and 6-substituted dopamine analogues to inhibit COMT was tested. The nitrile derivatives inhibited COMT most effectively, in agreement with our previous computational work. pKa values were used to further examine the factors involved with the inhibition and molecular docking studies were performed to support the *ab initio* and experimental work. The nitrile derivatives with a nitro substituent show the most promise as inhibitors, confirming that both the neutral tail and the electron withdrawing group are essential on this class of inhibitors.

KEYWORDS: catecholamine, catechol-*O*-methyltransferase, Parkinson's disease, pKa determination, dopamine

Abbreviations: AE, 6,7-dihydroxycoumarin; BBB, blood brain barrier; CHES, 2-(cyclohexylamino)ethanesulfonic acid; COMT, catechol-*O*-methyltransferase; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; DNC, 3,5-dinitrocatechol; equiv, equivalent; EtOAc, ethyl acetate; EWG, electron withdrawing group; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectrometry; MB-COMT, membrane bound catechol-*O*-methyltransferase; MeOH, methanol; NBS, N-bromosuccinimide; PD, Parkinson's

disease; PDB ID, protein data bank identifier; SAM, S-adenosyl-L-methionine; SC, scopoletin;

S-COMT, soluble catechol-*O*-methyltransferase; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

Most of the central nervous system's chemical communication occurs with molecules originating from naturally-occurring amino acids. Derived from the amino acid tyrosine, catecholamines, such as dopamine, epinephrine and norepinephrine, are synthesized in the adrenal medulla and certain nuclei of the brain [1]. These compounds act as hormonal signals or neurotransmitters, inducing activity in the body and brain, respectively. Catechol-*O*-methyltransferase (COMT) is an enzyme involved in the metabolism of catecholamines and other catechols, producing inactive, *O*-methylated metabolites of its substrates and thereby affecting the ability of the molecule to act as a signaling agent in the body [2].

The structure of COMT, first elucidated by Rodrigues et al. [3] and later further studied by Palma and coworkers [4], shows the active site located between two dimerized subunits with both contributing residues to the binding pocket (Fig. 1). It has organometallic character due to the presence of a magnesium ion, which in one monomer is coordinated with Asp141, Asp169, and Asn170 [4-6]. The magnesium is near Glu199 and Lys144, which complete a hydrophilic pocket of charged amino acids [4-6]. The opposite side of the active site consists of non-polar and aromatic residues, creating a hydrophobic region [4]. Catecholic ligands bound in the active site coordinate with the magnesium ion via one of the two phenolic hydroxyl groups [7]. The other hydroxyl group is oriented toward Lys144, which extracts a proton [4]. The deprotonated ligand then interacts with an electron-deficient region of co-factor S-adenosyl-L-methionine (SAM), resulting in the transfer of a methyl group from SAM onto the ligand and producing a methylated metabolite [4].
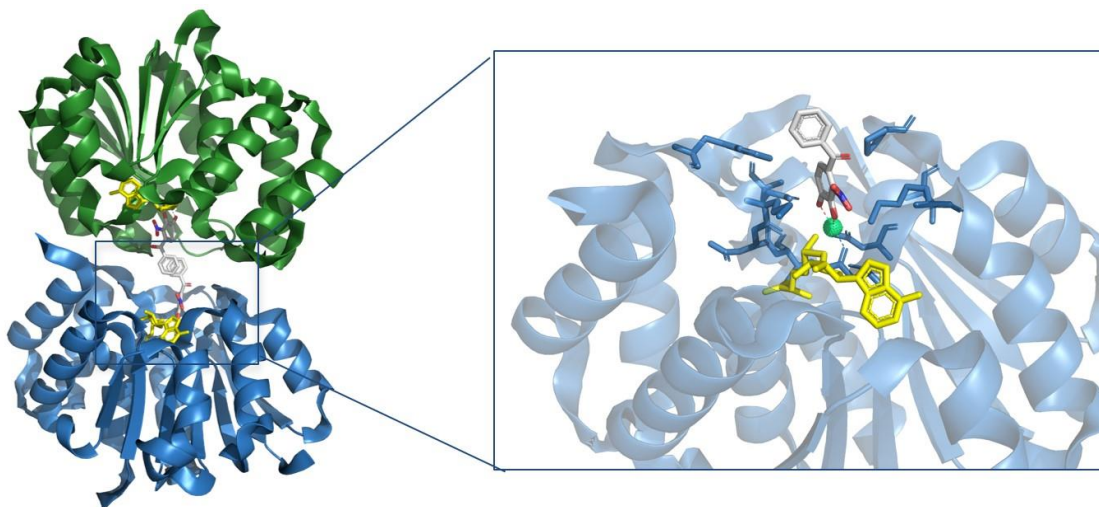
Figure 1. The structure of the COMT dimer (PDB ID: 2CL5 [4]) with the active site residues highlighted in blue and substrate shown in white, blue and red, SAM shown in yellow and the Mg^{2+} ion shown in green (inset).

Competitive inhibition of COMT can prevent the metabolism of catechols into methylated metabolites, and the strategic inhibition of COMT can be used in treatment strategies for conditions resulting from catecholic dysfunctions, like Parkinson's disease (PD). PD results from the degeneration of the substantia nigra pars compacta, a region of the midbrain [8]. Traditionally, PD is treated with the oral administration of 3,4-dihydroxy-L-phenylalanine (L-DOPA), a dopamine precursor [8, 9]. Dopamine cannot cross the blood brain barrier (BBB), but L-DOPA is able to cross via the LAT1 transporter, which transports large, neutral amino acids across the BBB [5, 10]. L-DOPA, naturally present in the brain or administered as a therapeutic, is converted to dopamine by DOPA decarboxylase [11]. However, orally administered L-DOPA is not a targeted treatment, and very little of the administered dose actually reaches the brain [5]. To circumvent this early metabolism of L-DOPA, treatments typically involve co-administration with a DOPA decarboxylase inhibitor, such as carbidopa or benserazide [12].

The effectiveness of L-DOPA treatments can be further prolonged by the administration of a COMT inhibitor in conjunction with L-DOPA and a decarboxylase inhibitor. COMT is

involved, with monoamine oxidase and aldehyde dehydrogenase, in a two-step metabolism of dopamine into homovanillic acid, and the enzyme can also metabolize L-DOPA into 3-*O*-methyldopa [6, 9, 13]. COMT is ubiquitously expressed in both the brain and the body. Thus, inhibition of COMT increases the neurological levels of L-DOPA available to be converted to dopamine, by preventing its premature metabolization in the periphery, and slowing its undesired metabolization within the brain.

The progression of COMT inhibitor design has led to the identification of inhibitors with drastic variability in the structure. Entacapone is a widely prescribed peripheral COMT therapy, acting as a fully reversible, competitive inhibitor that exhibits nearly complete inhibition at 30 mg/kg in rat duodenal COMT [14]. However, its low bioavailability and its short-lasting ability as an inhibitor have made it necessary to have elevated and repeated doses throughout the day. Additionally, the precursor to entacapone, tolcapone, revealed severe hepatitis during phase III trials, raising concern for hepatotoxicity in these drugs [5, 7]. These inefficiencies prompt the continued exploration of other molecule classes that could be used as effective, longer-lasting enzyme inhibitors. Therefore, this investigation proposed and analyzed two generations of potential COMT inhibitors, with the first generation characterized as dopamine derivatives (Fig. 2A) and the second generation with a neutral nitrile tail (Fig. 2B). Compounds **1-9** and **11** were first examined via an *ab initio* study of ligand binding energies in the enzymatic active site [15]. In that work, we developed models of increasing accuracy and found that the second generation of compounds (with nitrile tails) were predicted to be better inhibitors than the catecholic compounds. This prediction is in line with the structure of entacapone, which has a nitrile tail and the nitro group at the 5th position.
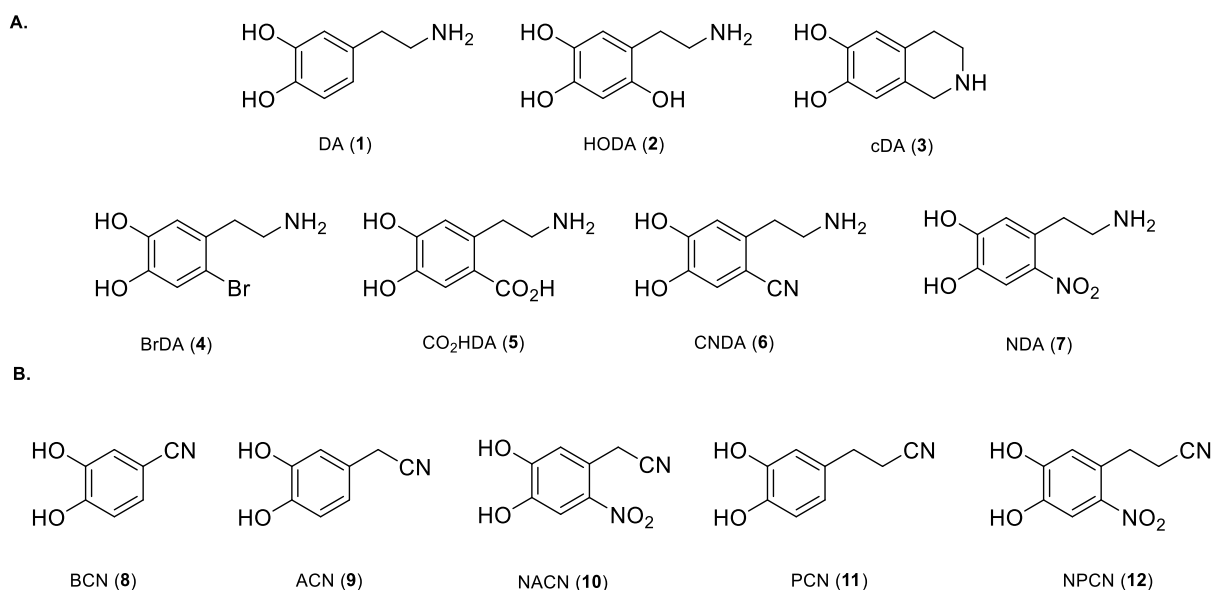
Figure 2. The structures of A) dopamine (**1**), dopamine analogues (**2-7**) and B) nitrile derivatives (**8-12**) investigated in this study.

Herein, we report the synthesis of five catechol nitrile derivatives **8-12**. After the structure and purity of the synthesized molecules were confirmed, all dopamine and nitrile derivatives were analyzed for stability before examining the molecules as inhibitors of COMT through an *in vitro* inhibition assay. These results, taken with our measured pKa values, the previous *ab initio* work, and new supporting docking studies provide a clear picture of the activity of these compounds as COMT inhibitors.

In our previous work, compounds **1-9** and **11** were analyzed computationally in COMT [15]. Interestingly, in an implicit solvation and a non-rigid amino acid model, HODA (**2**) was the most favorable ligand of the dopamine derivatives and the catechol nitriles had similar average interaction energies to the dopamine derivatives [15]. However, when desolvation energies were calculated and included with the interaction energies to determine the total electronic binding energies, the neutral catechol nitriles were significantly more favorable than the dopamine derivatives (average -234 kcal/mol vs. -196 kcal/mol, respectively) [15].

The dopamine derivatives **2-7** (Fig. 2A) have various electron-donating and withdrawing substituents at the 6-position. The electronic effects were confirmed first by examining the chemical shift of the proton *meta* to the substituent correlated with the Hammett meta constant for the substituent [16] and subsequently by determination of the pKa [17]. Therefore, we expected these derivatives to be potential inhibitors, especially compounds with electron withdrawing substituents (cyano and nitro) due to the effect on the pKa. Although the catecholamines showed favorable binding energies in COMT in the computational studies, analysis of the COMT active site led to the design of the neutral catechol nitriles **8**, **9** and **11** based on the hydrophobic, aromatic residues at the "back" of the active site, including Trp38, Met40 and Trp143. A nitro group was added to form catechols **10** and **12** due to the success of nitrated catechols in the literature [7]; *ab initio* electronic binding energies for these compounds were calculated and are presented here for comparison.

The dopamine derivatives **3-7** were prepared as described [16]. The nitrile derivatives, 3,4-dihydroxybenzonitrile (BCN, **8**) and 2-(3,4-dihydroxyphenyl)acetonitrile (ACN, **9**) were successfully synthesized according to Scheme 1. BCN (**8**) was synthesized from commercially available starting material, 3,4-dimethoxybenzonitrile, via deprotection with boron tribromide [18]. Treatment of 2-(3,4-dimethoxyphenyl)acetonitrile with boron tribromide yielded ACN (**9**) as an off-white solid in a 57% yield.

The literature describes several successful inhibitors with nitro groups on the ring, which was confirmed by our previous computational work. We attempted to synthesize the nitrated analogue of **8**, but it was not isolated as it seemed to oxidize almost immediately. However, **14** was nitrated with acetic and nitric acid (Scheme 1) to yield **15** followed by subsequent deprotection with boron tribromide, providing 2-(4,5-dihydroxy-2-nitrophenyl)acetonitrile (NACN, **10**) in a 38% yield overall.

Scheme 1
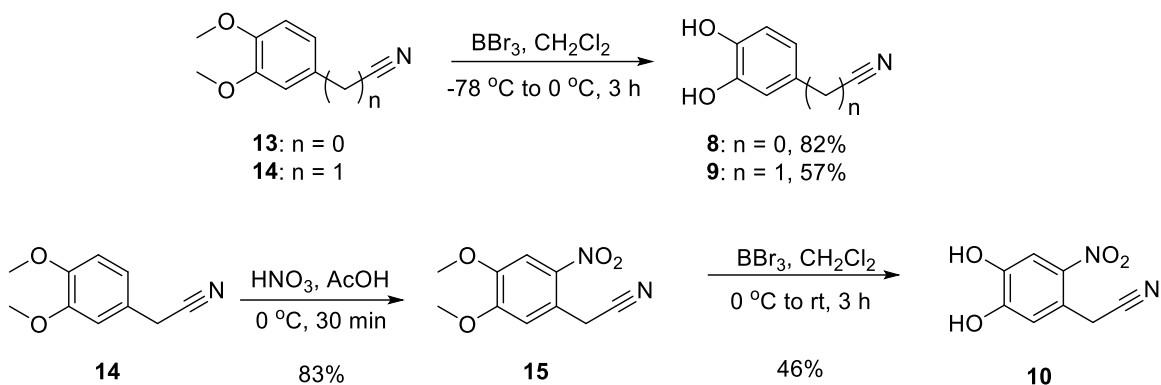
3-(3,4-Dihydroxyphenyl)propanenitrile (PCN, **11**) was synthesized in three steps from the commercially available alcohol according to Scheme 2. 2-(3,4-Dimethoxyphenyl)ethan-1-ol (**16**) was first brominated using triphenylphosphine and N-bromosuccinimide in tetrahydrofuran to produce intermediate **17** in a 64% yield. The reaction produces triphenylphosphine oxide as a byproduct, which was partially removed through precipitation following the addition of diethyl ether. Flash chromatography was used to remove any residual triphenylphosphine oxide and yield **17** as a pure product. 4-(2-Bromoethyl)-1,2-dimethoxybenzene (**17**) was cyanated by the addition of potassium cyanide in DMF to yield **18**, which was purified through flash chromatography with a 1:1 hexanes:ethyl acetate solvent system. Finally, **18** was treated with boron tribromide to remove the methyl ethers giving the final compound **11** in 61% yield.

In a similar manner, 3-(4,5-dihydroxy-2-nitrophenyl)propanenitrile (NPCN, **12**) was synthesized in four steps from the commercially available starting material 2-(3,4-dimethoxyphenyl)ethan-1-ol (**16**) according to Scheme 2. A nitro group was added to **18** using acetic and nitric acid, yielding a light yellow solid (**19**). Treatment of **19** with boron tribromide resulted in NPCN (**12**) as a yellow solid in a 93% yield. The structures of all final compounds were determined via ^{1}H NMR and ^{13}C NMR (Fig. S33-S48) and high-resolution mass spectrometry (HRMS). Purity was confirmed via high performance liquid chromatography (HPLC; Fig. S49-S53).

Scheme 2
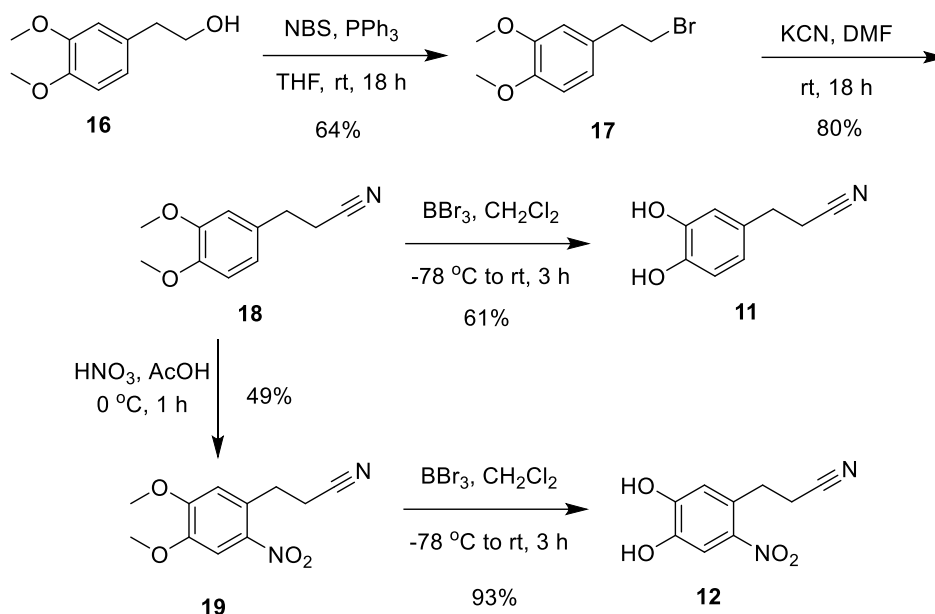
All the compounds were studied at the assay conditions (37 °C and pH 7.4) to ensure their stability before analysis of *in vitro* COMT inhibition. To this end, the compounds were incubated in a phosphate buffer at pH 7.4 for 1 hour and then analyzed by HPLC. Of the 11 analogues analyzed, all showed little to no degradation over time except for four: HODA (**2**), cDA (**3**), NACN (**10**) and PCN (**11**). Out of these four, NACN (**10**) displayed only a small amount of degradation (<5%). HODA (**2**), cDA (**3**), and PCN (**11**) all displayed a more sizable area difference, whereas HODA (**2**) had over 40% degradation during the 1-hour analysis. Although degradation was observed for compounds **2**, **3**, **10** and **11**, we continued with the enzymatic testing of them along with the others.

The ability of the compounds to inhibit COMT activity was determined through an *in vitro* microplate screening assay. Using an assay first described by Kurkela et al., COMT activity was determined via analysis of fluorescence [19]. When 6,7-dihydroxycoumarin (AE) is methylated by COMT, it becomes scopoletin (SC), which gives a large fluorescent signal. By tracking the change of AE to SC through fluorescent readings over 60 minutes, we are able to determine the

in vitro COMT activity by compounds **1-12** by comparing the fluorescence observed in the presence of the catechols versus no inhibitor. There are two different types of COMT found in mammals: membrane bound COMT (MB-COMT) and soluble COMT (S-COMT). In humans, S-COMT is found in peripheral tissues, while MB-COMT is dominant in the brain [20, 21]. Additionally, MB-COMT has 50 extra hydrophobic amino acids [22]. This experiment used S-COMT for the *in vitro* testing.

DA (**1**) and the first generation of dopamine-derived inhibitors **2-7** were tested at two concentrations, 10 μM and 100 μM (Fig. S29 and 3). A known, tight-binding inhibitor 3,5-dinitrocatechol (DNC) was used as a positive control (IC_{50} of 12 nM in rat brain [23]). At 10 μM, the dopamine compounds (**2-7**) showed no inhibition of COMT activity (Fig. S29), but at 100 μM, the catechols followed the trend observed computationally [15] with CNDA (**6**) and NDA (**7**) inhibiting about 30% to 50% of COMT activity (Fig. 3). Despite the instability, HODA (**2**) showed some inhibition at 100 μM (Fig. 3). The nitriles **8-12** were also tested as inhibitors of COMT (Fig. 3). At 10 μM, only NACN (**10**) showed a significant decrease in COMT activity (Fig. S29) among all compounds tested. However, at 100 μM, all the nitrile derivatives inhibited COMT activity to some degree with NACN (**10**) and NPCN (**12**) demonstrating complete inhibition of COMT (Fig. 3).
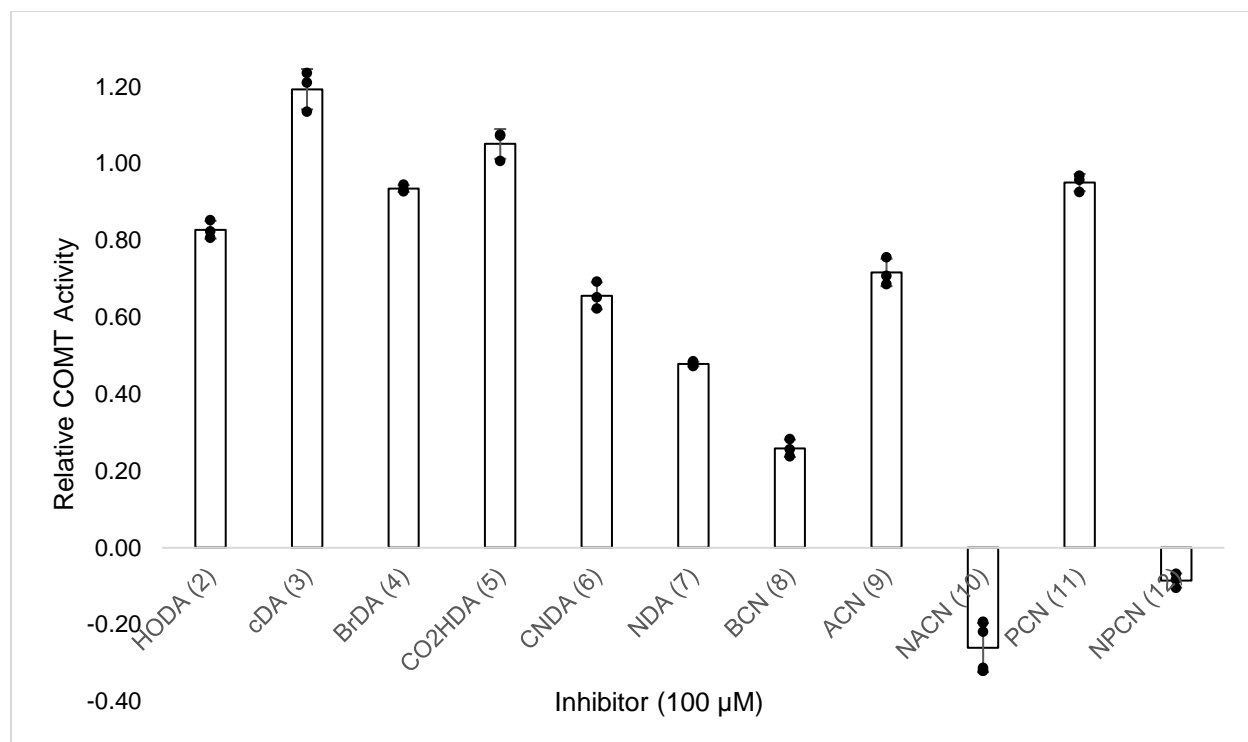
Figure 3. I*n vitro* COMT activity after incubation with 100 µM catecholamine or nitrile derivative relative to 6 µM aesculetin. The dopamine derivatives **2-5** showed no to modest inhibition of COMT. However, CNDA (**6**) and NDA (**7**), both derivatives with electron withdrawing substituents, showed the best inhibition of COMT among the dopamine derivatives. The nitrile derivatives **8-12** were generally more effective at inhibiting COMT, but the compounds with an electron withdrawing group directly attached to the ring showed the best inhibition of COMT activity. Each compound was assayed in at least triplicate and was normalized to the fluorescence of aesculetin with no inhibitor.

The results from the COMT activity assay demonstrate that in these assay conditions, the nitrile compounds with a neutral tail are stronger inhibitors, which is supported by our computational data [15] and likely results in more favorable interactions with the Trp38, Met40 and Trp143 at the "back" of the active site (Fig. 4). Furthermore, compounds with a strong electron withdrawing substituent, such as -CN or -NO$_2$, were most effective at inhibiting COMT (NDA (**7**), BCN (**8**), NACN (**10**) and NPCN (**12**), Fig. 3). The results of this assay agree with the results of the previous computational studies of these compounds in COMT [15]. Interestingly, ACN (**9**) and

PCN (**11**), with the cyano group separated from the ring, did not show as good of inhibition compared to their nitrated counterparts, indicating that the neutral tail alone is not enough for strong inhibition. It is not surprising that the nitriles NACN (**10**) and NPCN (**12**) were the best inhibitors of COMT activity.

The determination of the pKa values for compounds **1, 7-10** was achieved using a five-buffer system as described previously [24]. It was hypothesized that boron in the Borax buffers was interacting with the catechol hydroxyl groups to form complexes that interfere with the correct determination of pKa for catechols [25] and resulted in inconclusive data. Therefore, Borax buffers in the pH range of 8.2 to 10.8 were substituted with CHES buffers for more accurate data. Additionally, the KCl buffers were utilized to ensure that a wider pH range was covered. The compounds were dissolved in buffers ranging from 3.01 to 12.36 to a final concentration of 0.2 mM and absorbance was determined. Absorbance data was normalized and analyzed using Origin 6.0 to determine the pKa of each compound (Table 1). The second pKa value was also determined for several of the derivatives. The pKa values of the dopamine derivatives have recently been reported using a similar method [17]. However, the pKa values reported here differ slightly from those recently reported, as previous work used the buffer system outlined by Martinez and Dardonville [24]. In addition, previously published experimental data on pKa values of related catechols [26] differ in both concentration and buffer system, therefore resulting in different pKa values. The pKa values follow the trend expected based on the electron withdrawing ability of the substituent or tail (Table 1). The presence of the nitro group significantly lowered the pKa (DA (**1**) vs. NDA (**7**) and ACN (**9**) vs. NACN (**10**)).

Table 1: Summary of experimentally determined pKa values for compounds **1, 7-10** for both catechol hydroxyl groups.

Compound	Experimentally Determined First pKa	Experimentally Determined Second pKa
DA (**1**)	8.66 ± 0.05	> 12.51
NDA (**7**)	6.33 ± 0.02	11.19 ± 0.08
BCN (**8**)	7.45 ± 0.03	11.61 ± 0.10
ACN (**9**)	$9.60 \pm 1.4 \times 10^{-9}$	10.51 ± 0.13
NACN (**10**)	6.23 ± 0.04	$10.83 \pm 1.7 \times 10^{-9}$

An analysis of the literature reveals that the best COMT inhibitors all share a similar moiety – a nitrocatechol (1,2-dihydroxy 3-nitrobenzene). The placement of the nitro group relative to the two hydroxyl groups has been previously explored, but the most successful inhibitors have the nitro group at the 5-position relative to a substituent or *meta* to the 3-OH that is methylated by COMT [7, 14]. The presence of the electron withdrawing group helps to stabilize the phenolate anion, making it a poorer substrate for *O*-methylation [7]. The trends observed for the pKa values (Table 1) were as expected and correlated well with the σ_{para} Hammett constants for the appropriate substituent [27]. Stabilization of the conjugate base by resonance can be observed by comparing the pKas (Table 1) of DA (**1**) and NDA (**7**), which were determined to be 8.66 ± 0.05 and 6.33 ± 0.02, respectively. This trend is consistent with ACN (**9**) and NACN (**10**) (Table 1). The decrease in pKa value leads to a greater percentage of the anion at physiological conditions and significantly affects the binding and subsequent inhibition of COMT. This effect is observed when comparing the inhibition of COMT (Fig. 3) by ACN (**9**) and NACN (**10**), which suggests that NACN binds tightly and is a poor substrate. These data illustrate, however, that a lower pKa is not the only factor involved in inhibiting COMT as NDA (**7**) is not as strong of an inhibitor as NACN (**10**) despite a similar pKa value.

In silico docking calculations were performed in AutoDock Vina [28] to support the previous computational and current experimental work. During the dockings, attention was given to the

orientation of the catechol hydroxyl groups and the novel ligands toward the magnesium and the methyl group of SAM (S-adenosyl-L-methionine), components of COMT's (PDB ID: 2CL5 [4]) active site. Although there are more recent crystal structures available, this crystal structure was used to compare with our previous computational results [15]. It should be noted that this crystal structure has a structural anomaly wherein Asn170 binds the Mg^{2+} through the side-chain N rather than the O. While this was corrected for the higher accuracy *ab initio* calculations presented here and in the previous work, it was not corrected for the docking calculations. As this residue only has indirect or longer-range interactions with the ligand, the docking score should not be affected qualitatively.

Much of the structure of the compounds line up with BIE (the inhibitor bound in the crystal structure) and each other, such as the hydroxyl groups of the catechol and the similar orientation of the nitro group despite being substituted at a different position (Fig. S31). Both dopamine (**1**, Fig. 4A) and ACN (**9**, Fig. 4C) in addition to their nitrated counterparts NDA (**7**, Fig. 4B) and NACN (**10,** Fig. 4D) were examined. Addition of the nitro group increased binding across both pairs (docking score DA = -6.3; NDA = -6.7, ACN = -6.9; NACN = -7.4), supporting the experimental results, and the neutral nitrile compounds produced stronger docking scores than the dopamine analogues. Both of the nitrated molecules had strong interactions with Trp38 and Trp143, which is consistent with the literature [7]. These docking scores also support the fact that these nitrated inhibitors can act as competitive inhibitors due to their increased binding interactions relative to dopamine. Further, the reduced pKas of the nitrated analogues confirmed the additional stabilization of the phenolate anion, making those analogues even poorer substrates for the enzyme.
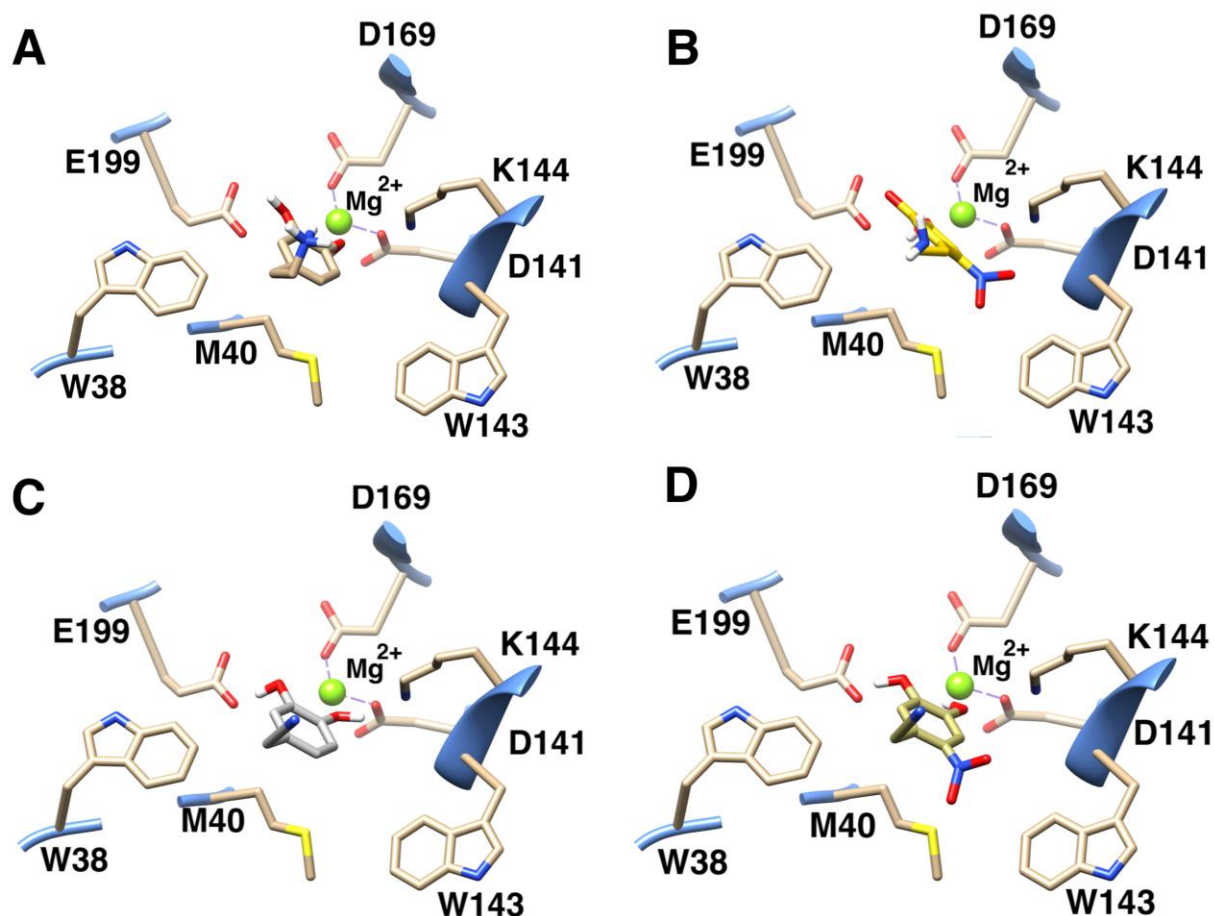
Figure 4. Molecular interactions of A) DA (**1**) B) NDA (**7**), C) ACN (**9**) and D) NACN (**10**) with the binding site residues (PDB ID: 2CL5 [4]). The catechol OH groups interact strongly with the Mg^{2+} ion. The hydrophobic residues, Trp38, Met40 and Trp143, at the "back" of the active site interact more favorably with the catechol nitriles.

The electronic binding energies for compounds **1-9** and **11** can be found in our previous work [15]. The new calculations for NACN (**10**) and NPCN (**12**) (performed with the same methods as the previous work)[15] show some interesting results. While the total electronic binding energies for NACN (**10**) and NPCN (**12**) are comparable to the other compounds (-215.4 and -211.9 kcal/mol, respectively), the interaction energies with Mg^{2+} (-323 and -317 kcal/mol, respectively) are stronger than that of any of the DA-based compounds other than CO_2HDA (**5**) (which has a stronger interaction due to the negatively-charged carboxylate). Further, the desolvation penalties for these compounds (19 and 15 kcal/mol, respectively) are one-quarter to

one-third the magnitude of the desolvation penalties for the dopamine-based compounds. We thus suggest that these two factors—interaction energy with Mg^{2+} and desolvation penalty—may be the main predictors of overall binding energy.

To confirm the importance of the interactions between the ligand and the residues in the active site, additional docking calculations wherein the protein was modified via *in silico* site-directed mutagenesis of both Trp38 and Trp143 to Ala were performed. Although the docking score for NACN (**10**) became less favorable when Trp143 was mutated to Ala (-7.4 WT vs. -6.2 W143A), a more significant loss of binding was observed when Trp28 was mutated (-7.4 vs. -5.0; Fig. S32). As these residues are relatively far from the ligand and the *ab initio* work shows that they contribute about 1-5 kcal/mol to the total electronic binding energy [15], these docking results suggest that they may have a steric or entropic influence on binding.

The novel catechols **8-12** were synthesized in good yields, ranging from 46-93% overall. All the compounds were tested for chemical stability, and subsequently examined in a COMT activity assay. While **2** showed over significant degradation, which is not surprising due to the ease with which it oxidizes, it may explain the poorer activity observed in the *in vitro* assay. The enzymatic assay demonstrated the ability of certain compounds to inhibit the activity of COMT, especially those with neutral tails and nitro substituents: NACN (**10**) and NPCN (**12**). These results agree with our previous computational data, but underscore the importance of considering multiple factors in determining the inhibitory ability of these catechols. The pKa values and docking studies support the behavior of these compounds as inhibitors in the active site of COMT – compound **10** binds more strongly than compounds **8** and **9** because of its electron withdrawing nitro group, which also helps to lower the pKa by stabilizing the anion, but the neutral nitrile tail interacts more favorably with the residues at the back of the active site. This work confirms the trends predicted by computational modeling and highlights its potential in developing inhibitors of COMT. While more work needs to be done to determine if these

compounds are worth pursuing *in vivo*, the efforts reported herein provide insight into the structure-activity relationships of potential COMT inhibitor scaffolds.

SUPPLEMENTARY MATERIALS: Supplementary data (full synthetic details, absorbance vs. concentration spectra for determination of pKa values, fluorescence vs. time spectra for the compounds, ^{1}H and ^{13}C NMR spectra of **8-19;** HPLC chromatograms of **8-12**) associated with this article can be found, in the online version, at

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18. In initial attempts to remove the methyl ethers, the product was not isolated through an aqueous workup. Rather, the reaction solution was quenched with a small volume of water or methanol and precipitation through the addition of diethyl ether was used to isolate the product. However, unlike the dopamine derivatives already prepared, the product did not precipitate out of solution. Therefore, the workup was modified to remove byproducts through aqueous washes, allowing for the isolation of a pure product (**8**).

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