Unveiling the biopathway for the design of novel COMT inhibitors

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Catechol-*O*-methyltransferase (COMT) is an enzyme responsible for the *O*-methylation of biologically active catechol-based molecules. It has been associated with several neurological disorders, especially Parkinson's disease (PD), because of its involvement in catecholamine metabolism, and has been considered an important therapeutic target for central nervous system disorders. In this review, we summarize the biophysical, structural, and therapeutical relevance of COMT; the medicinal chemistry behind the development of COMT inhibitors and the application of computer-aided design to support the design of novel molecules; current methodologies for the biosynthesis, isolation, and purification of COMT; and revise existing bioanalytical approaches for the assessment of enzymatic activity in several biological matrices.

Keywords: Catechol-O-methyltransferase; COMT inhibitors; Computer-aided drug design; Up-/down-stream processing; Bioanalytical methods

Introduction

COMT is the enzyme responsible for the *O*-methylation of catechol substrates, including catecholamines, catechol estrogens, and catechol drugs.[1] The discovery of COMT involvement in the metabolism of the antiparkinsonian drug levodopa (*L*-DOPA) increased interest in this enzyme.[2] Administration of *L*-DOPA remains one of the first-line approaches for the treatment of PD.[3–5] However, *L*-DOPA is rapidly and extensively metabolized into 3-*O*-methyldopa (3-*O*MD) by COMT in the peripheral tissues before reaching the brain, limiting its efficacy.[6] To increase *L*-DOPA bioavailability, several COMT inhibitors are also administered as adjuvants, aiming to overcome limitations associated with the slight amount of *L*-DOPA that reaches the brain.[7] Thus, inhibition of COMT has become an attractive strategy to increase catecholamine levels.[1] Many classes of compound have been studied as potential COMT inhibitors and are divided into first-, second-, and third-generation compounds.[1,8–11] Despite the improvement that these drugs brought for PD therapy, disadvantages and adverse effects that affect the life quality of patients remain, which turn mandate the development of novel COMT inhibitors.

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Pathological and therapeutic perspective of catechol-*O*-methyltransferase

Biomolecular characteristics

COMT is a monomeric enzyme that catalyzes the transference of a methyl group from *S*-adenosyl-$_L$-methionine (SAM) to a catechol substrate in the presence of the cofactor Mg^{2+}, resulting in *O*-methylated products and *S*-adenosyl-L-homocysteine (SAH).[12] The enzyme has been found in numerous species, including bacteria, yeasts, plants, insects, fish, birds, and mammals.[13] The main function of COMT is to *O*-methylate biologically active and/or exogenous catechol molecules, acting as an enzymatic detoxifying barrier.[1,2] In humans, COMT has two distinct molecular isoforms: a soluble (SCOMT) and a membrane-bound (MBCOMT) form.[14] Both isoforms are expressed in almost all human tissues, although levels of SCOMT are considerably higher than MBCOMT in peripheral tissues, mostly in the liver and kidneys, whereas the expression levels of MBCOMT surpasses those of SCOMT in the central nervous system (CNS).[15,16] The cellular localization of these isoforms is also distinct: SCOMT (~24.7 kDa, 221 amino acids) is found in the cytoplasm.[17] by contrast MBCOMT (~30 kDa) has an extra 50 residue-long N-terminal signal sequence that forms a membrane anchor associated with the membrane of the rough endoplasmic reticulum.[2] Although both isoforms share identical Mg^{2+} and SAM dependency and a similar catalytic mechanism, these isoenzymes display distinct substrate affinities.[18] For dopamine, SCOMT displayed a K_m of 207 μM, whereas, for MBCOMT, a K_m of 15 μM was reported; for norepinephrine, SCOMT showed a K_m of 369 μM and MBCOMT a K_m of 24 μM.[18] In terms of enzymatic velocity (V_{max}), SCOMT was reported to have a higher V_{max} compared with MBCOMT. SCOMT exhibits a V_{max} of 37.2 min^{-1} for dopamine and MBCOMT 16.9 min^{-1}, whereas, for norepinephrine, the values were 35 min^{-1} and 18 min^{-1},[18] respectively. These results indicate that MBOCMT is the isoform responsible for *O*-methylation at lower physiological concentrations of catecholamines, particularly in the CNS, whereas SCOMT acts in higher substrate concentrations, generally in the peripheral tissues.[19] Given these relevant COMT physiological functions, many researchers have focused on establishing a connection between this enzyme and CNS-related disease.

Genetic polymorphisms of COMT and their relationship with CNS disorders

In humans, over 900 genetic variants of *COMT* have been reported, although most did not show any physiological significance.[20–23] One of the most studied and characterized *COMT* polymorphisms is rs4680, which involves a guanine-to-adenine nucleotide transition.[2] This functional polymorphism leads to a substitution of Met by Val in the polypeptide chain at codon 108 in SCOMT and at codon 158 in MBCOMT.[18–24] When compared with the native form of COMT ($COMT^{Val/Val}$), the polymorphic variant ($COMT^{Met/Met}$) exhibits a decrease of ~40% in enzymatic activity, and the active site appears more prone to distortion, which could lead to a significant decrease in the thermal stability of the enzyme.[18–24] In addition, it is widely reported that this polymorphism can induce dysregulation of catecholamine levels. Consequently, many studies have established a connection between $COMT^{Met/Met}$ and neuropsychiatric disorders, including depression,[23] autism spectrum disorder,[23] schizophrenia,[25–28] obsessive–compulsive disease,[27] panic,[29–31] bipolar disease,[32–34] attention deficit hyperactivity disorder,[23,35] anxiety,[36] nervous anorexia,[37–39] and PD.[40] These interconnections highlight the relevance of understanding the interactions between COMT with substrate/inhibitors at the active site to further design novel enzyme inhibitor molecules.

Catalytic mechanism and crystal structure of COMT

COMT comprises a single domain with a mixed α/β topology consisting of a seven-stranded β-sheet flanked by two sets of α-helices. The protein catalytic site is shaped to fit the catechol substrate, the Mg^{2+} and the SAM interaction sites,[24] wherein SAM is the first to bind, followed by Mg^{2+} and then substrate binding.[1] In particular, the adenine ring of SAM is hydrogen bonded to the residues Ser119 and Gln120, and also forms van der Waals interactions with residues Ile91, Ala118, and Trp143,[1] whereas the methionine fragment of SAM/SAH forms hydrogen bonds with residues Val42, Ser72, and Asp141.[41] Mg^{2+} is octahedrally linked to the oxygen atoms of the side chains of residues Asp141, Asp169, and Asn170 and to one crystallographic water molecule.[1] The two remaining free coordination sites allow chelation of each of the hydroxyl groups of the catecholic substrate and this interaction deceases the substrate acid dissociation constant (pK_a), facilitating catechol hydroxyl deprotonation.[42] The substrate mainly interacts by hydrogen bonds with the residues Lys144 and Glu199, as well as through hydrophobic interactions with the 'gatekeeper residues' Trp38, Trp143, Pro174, and Leu198, which are vital for the *O*-regioselectivity of COMT.[43,44] Since the determination of the first crystal structure of rat SCOMT,[45,46] 18 structures of human and 95 of rat COMT have been deposited in the Protein Data Bank (PDB) (https://www.rcsb.org/).[47–53] The careful analysis of these structures obtained by X-ray crystallography, alone or combined with computational approaches, will support the design of new molecules, optimizing the drug discovery pipeline.

COMT inhibitors and the relevance of structured-based drug design in their development

The first COMT inhibitors were reported to be used as *L*-DOPA adjuvants for PD therapy and are now divided into three main generations.[1] The 'first generation' of COMT inhibitors were primarily catechol and pyrogallol derivatives or some related bioisosteric moieties and simple substituents.[1] *In vivo* studies showed that most of these compounds displayed poor bioavailability, high toxicity, and a very short-lasting effect, impairing their clinical approval.[1].

Subsequently, a new series of di-substituted catechol inhibitors was developed, forming the 'second generation'.[1] In this case, it was demonstrated that the presence of a nitro group at position 5 of the catechol ring led to molecules that were more potent and more selective and with improved pharmacokinetics (PK). The most effective nitrocatechol inhibitors of this generation were entacapone and tolcapone (Fig. 1), inhibiting recombinant SCOMT with an inhibition constant (K_i) of 0.30 and 0.27 nM, respectively and 1.37 and 0.29 nM for MBCOMT,

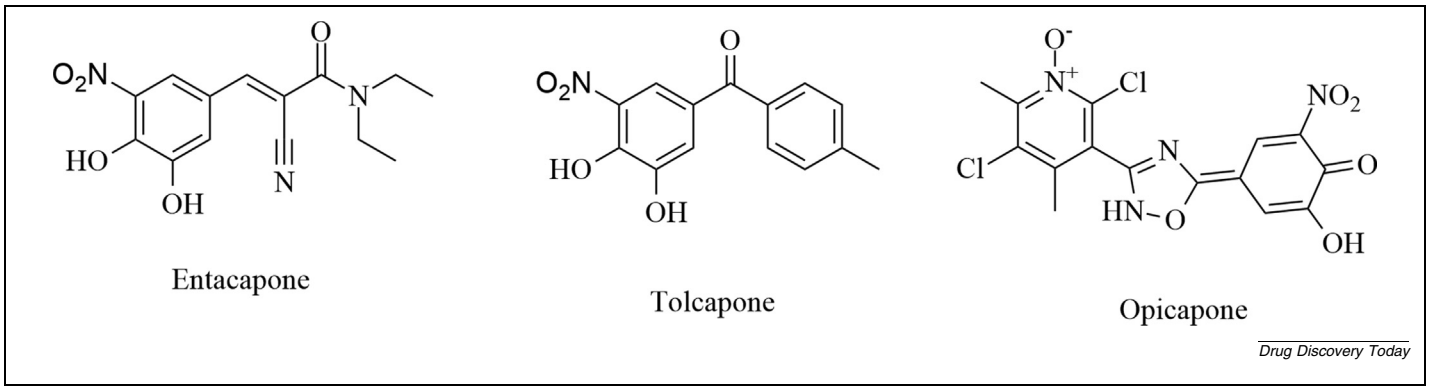

FIGURE 1
Structures of commercial catechol-O-methyltransferase (COMT) inhibitors.

respectively.[18] Entacapone mostly affects COMT activity in liver and erythrocytes, being considered a strictly peripheral COMT inhibitor because of its inability to penetrate the blood–brain barrier (BBB). By contrast, tolcapone can cross the BBB and, therefore, is capable of inhibiting COMT in the brain.[2] Despite interest in this drug, tolcapone is also associated with severe hepatoxicity, limiting its clinical use.[54,55].

Recently, a 'third generation' COMT inhibitor, opicapone (Fig. 1), was approved for clinical use.[56] This compound was described as being more effective and less toxic, and displaying a longer period of *in vivo* effects and enhanced bioavailability, allowing once-daily administration and more than 24 h of stable *L*-DOPA plasma levels in patients with PD.[10] It mainly acts in the modulation of COMT activity in erythrocytes, and other tissues, such as liver and kidney, despite not being able to cross the BBB. As a consequence, this compound is considered a strictly peripheral COMT inhibitor.[7].

Given this evidence, there is a need to develop more effective COMT inhibitors with synergic effects and, as a result, the pharmaceutical industry is using new methodologies for the design of novel bioactive molecules. In this context, advances in biomolecular and structural techniques, such as NMR and X-ray crystallography, as well as the determination of more than 100 3D COMT crystal structures, have provided essential information about the interactions between the protein active site and several substrates/inhibitors.[57] The synergy of such information with computational algorithms to support drug discovery is viewed as a fundamental part of modern medicinal chemistry.[58] When 3D structures of targets are available, structure-based drug design (SBDD) approaches, which include molecular docking, structure-based virtual screening, and molecular dynamics, become highly relevance, particularly in drug development.[59] For instance, the prediction of key binding sites and the determination of molecule binding affinities toward certain macromolecular targets make this approach attractive for the design of novel compounds.[60] Ligand-based drug design (LBDD) is frequently applied when 3D structures of targets are not available and can rank ligands from extensive libraries of molecules based on their similarity.[61] In this context, the identification of potential novel COMT inhibitor by using quantitative structure–activity relationships studies (QSAR) modeling, ligand-based virtual screening, similarity searching, and pharmacophore generation has also been performed.[59].

Vidgren and coworkers use computational tools to study the molecular interactions of potential inhibitors with the COMT catalytic site.[62] The acidity of both catechol hydroxyl groups and the lipophilicity of the inhibitors side chains were demonstrated to have an important role in the binding affinity of the molecules, later confirmed with the determination of the first COMT crystal structure.[45] By comparing unsubstituted catechol/pyrogallol and their substituted derivatives, Lautala and team demonstrated that the turnover rate modification of the tested compounds was inversely proportional to their binding affinity to the protein active site.[43] The inhibitory potential of flavonoids through molecular docking,[63] dynamic simulations,[64] and QSAR studies[65] was also studied.

A similar strategy was used for second-generation inhibitors. Palma and coworkers applied molecular docking to understand the molecular interactions of nebicapone,[66] a nitrocatechol inhibitor of COMT, and the *O*-regioselectivity of the enzyme for BIA 3–228 and BIA 8–176, two other nitrocatecholic-type COMT inhibitors.[47] Another class of inhibitors that was exhaustively studied using these tools was the bisubstrate COMT inhibitors, molecules that target simultaneously both the SAM and the substrate binding sites.[54] Both Lerner and coworkers[48] and Paulini and coworkers[67] used molecular docking to evaluate the effect of several substituents on the binding affinity with COMT. Through homology modeling using the 3D structure of rat COMT, Lee and coworkers studied the molecular docking of several bisubstrate inhibitors to analyze putative binding affinities.[68] Furthermore, Ellerman and collaborators used SBDD methodologies to evaluate the binding affinity of multiple compounds with the COMT 3D structure.[49,69] Recently, garcinol, a natural product with *in vitro* effects similar to those observed with tolcapone, was also studied *in silico* and shown to form identical atomic interactions with the protein compared with tolcapone.[70] A slightly different approach was used by Jatana and collaborators, who used pharmacophore modeling and ligand screening analysis to obtain molecules with a high potential to interact with COMT.[71] There is an urgent demand for the design of new drug candidates that surpass the limitations of current dopamine replacement therapies.

Given the involvement of several biological pathways and proteins, and the complexity of the affected organs, multitarget drugs (MTD) have been attracting increasing attention as potential PD drug candidates.[72] In particular, dual COMT and MAO-B inhibitors could significantly increase the bioavailability of *L*-DOPA and prevent adverse effects associated with single-target drug administration.[72] Recently, *in vitro* studies described the potential dual-inhibitory properties of several xanthones, such as mangiferin,[73] and anthraquinones, such as alizarin,[74] toward COMT and MAO-B, with promising results compared with clinically used drugs. In the coming years, an increase in the evaluation of novel hydroxylated xanthones, anthraquinones, and fluorenones as dual COMT and MAO-B inhibitors MTD for PD treatment is expected. Several structural modifications could be explored to prepare novel analogs with improved pharmacological profiles, for instance, aromatic electrophilic substitutions, such as nitration, given their high structural similarity with the COMT inhibitor tolcapone, sulfonation, and Mannich reactions.

Preparative and analytical methodologies for COMT isolation and activity assessment

Reported strategies for the expression and isolation of COMT isoforms

During the previous century, pioneering studies used COMT extracted from mammalian tissues, mainly from the brain and rat liver.[18] This procedure involved several preliminary steps to recover a highly pure protein fraction,[75] namely consecutive centrifugations, acid and salt precipitation steps, and hydroxyapatite adsorption combined with chromatographic techniques (Table 1).[75] However, the enzyme yields and bioactivity levels obtained were not suitable for bio-interaction and structural analysis studies.[75–77] Attempting to attain higher biosynthesis levels, *Escherichia coli* was the first system described for SCOMT production,[78] resulting in well-ordered crystals of rat SCOMT, which enabled determination of its 3D structure and a detailed kinetic characterization.[45] Over the following decades, multiple bioprocesses targeting recombinant S- and MBCOMT were developed (Table 1). In terms of SCOMT, *E. coli* was the most frequently used expression system for the biosynthesis of both the native[79–82] and the polymorphic variant.[24] Given that *E. coli* presents a complete lack of homologous COMT, has low-cost production, and rapid growth, allowing moderate to high protein yield, it has been considered an ideal system for the production of large quantities of SCOMT.[83] In addition, *Komagataella pastoris*[84] and transfected human embryonic kidney (HEK) fibroblasts[14] were also used for this purpose. Given that these systems perform all human post-translational modifications, they are frequently used when more complex proteins need to be produced, including MBCOMT. For its expression, hosts such as *Spodoptera frugiperda* (Sf9) insect cells,[85,86] transfected HEK fibroblasts,[14,87] and also *K. pastoris*[17,88] are often used. Both *Brevibacillus choshinensis*[89] and *E. coli*[80] prokaryotic systems were also explored to achieve higher yields of MBCOMT, even though they can induce toxicity.[88]

Such strategies for the biosynthesis of both the S- and MBCOMT isoforms allowed researchers to produce the enzyme in an active, functional form, although they require distinct recovery and isolation procedures.[90,91] Typically, after cell disruption by mechanical (glass beads or sonication) and/or non-mechanical methods (freeze/thaw or enzymatic digestions), SCOMT is usually found in the homogenate extract, whereas MBCOMT is mostly found in the cellular compartment, which requires the use of solubilizing agents for the protein to maintain an active and stable conformation.[90,91] However, when using micellar strategies, the nature of the detergent and its concentration must be taken into consideration, because micelles are highly dynamic assemblies the intrinsic instability of which could result in protein unfolding, misfolding, and aggregation.[92] Alternatively, several techniques have been developed to stabilize membrane proteins in native-like environments, namely with amphipols, lipid micelles, lipid nanodiscs, and liposomes.[93] To our knowledge, MBCOMT has not yet been recovered through the implementation of these non-micellar procedures.

A suitable purification procedure must be used to isolate the target enzyme, which is a crucial step to characterize its biochemical properties, catalytical mechanisms, and kinetic parameters. In terms of SCOMT purification, it is reported that COMT is fairly labile and loses its activity,[76] probably because of the oxidation of the free cysteine-SH groups in its structure.[94] Interestingly, in one of the first reports of COMT purification, in which the binding and elution buffers did not contain cysteine, SCOMT partially lost its activity.[95] Furthermore, it was observed that ethylenediamine tetraacetic acid (EDTA) and magnesium chloride ($MgCl_2$), when combined with dithiothreitol (DTT) or 2-mercaptoethanol, promoted reduction of the loss of COMT activity.[17,75,81] Following advances in buffer formulations for COMT stabilization, upon the optimization of environmental conditions (pH, salt concentration, detergent type and concentration, presence of additives, column resin, and temperature), its purification was widely described using typical chromatographic strategies, including hydrophobic interaction chromatography (HIC), anion-exchange chromatography (AEC), cation-exchange chromatography (CEC), and affinity chromatography (AC) with immobilized amino acids or immobilized metals (IMAC) (Table 1). HIC is the most reported approach for the purification of both COMT isoforms.[81] Given the distinct properties of the enzyme, a higher ionic strength is required for the adsorption of SCOMT compared with MBCOMT.[90] Therefore, additional amino acids of the anchor region of MBCOMT might reinforce the hydrophobicity of the protein. In terms of AEC, SCOMT is adsorbed onto the column at lower ionic strength, whereas MBCOMT requires the application of a linear salt gradient. In turn, the elution of both S and MB isoforms can be performed with an increase in the ionic strength coupled to supplementation with Triton X-100, FC-12, or DDM.[81,90] Here, the extra anchor region amino acids of MBCOMT contribute to enhanced electrostatic interactions between the protein and the anionic ligands.[80] In addition, in IMAC, the presence of the metallic tag and its further removal result in a significant decrease in yield.[96] Regarding the final purification yield, HIC and AEC had similar results for SCOMT (5.9× for HIC and 3.6× for AEC), although higher bioactivity recovery was obtained when AEC was used (107% compared with 13% for HIC).[79,80] The most promising results were obtained using IMAC, with 81× purification yield and a bioactivity recovery of

TABLE 1

Isolation methodologies used for COMT isoform recovery.[a]

Isoform	Source	Protein recovery	Chromatography	Elution	Specific activity	Purification factor (fold)	Yield/ bioactivity recovery	Refs
Rat COMT								
S	Liver	1. Cells homogenized 2. Centrifugations (10 000 ***g*** 25 mins,	–	–	1.0 U/mg of protein	–	–	[67]
		30 000 ***g*** mins, 100 000 ***g*** 1 h)	SEC (Bio Gel P-10)	Isocratic (20 mM sodium acetate, pH 4.8)	NR	1330	11	
		3. Pellet discarded and supernatant pH adjusted to 5.1	CEC (Mono S) AEC (Mono Q)	Linear gradient (0–0.65 M sodium chloride, pH 7.2)				
		4. Centrifugation (15 000g 20 mins)	RP (TSK TMS 250)	Linear gradient (0–100% acetonitrile)				
		5. Supernatant pH adjusted to 7.2 6. Solution mixed and stirred with hydroxyapatite (20 mins) 7. Centrifugation (15 000 ***g*** 20 mins) 8. Ammonium sulfate precipitation (65% saturation) 9. Centrifugation (30 000 ***g*** 20 mins) 10. Precipitate dissolved in sodium acetate (20 mM pH 4.8)						
MB	Liver and brain	1. Tissues homogenized in sodium phosphate (5 mM, pH 7.8)2. Solubilized in sodium phosphate buffer (5 mM, pH 7.8) with 1% (v/v) Triton X-1003. Centrifugation (100 000 ***g*** 30 mins)4. Pellet dissolved in Tris buffer (20 mM, pH 7.8) with 0.5% (v/v) Triton X-100	AEC (Resource Q)	Linear gradient (0–0.5 M NaCl + 20 mM Tris + 0.5% Triton X-100, pH 7.8)	NR	NR	NR	[14]
Recombinant COMT								
S	*Escherichia coli* BL21 (DE3)	1. Cell pellets thawed at 42 °C and resuspended in calcium binding buffer 2. Cell disruption by lysozyme treatment	–	–	6592 nmol/h/mg	–	–	[68]
			AC (XK-16/20)	Isocratic (50 mM Tris + 0.3 M NaCl + 2 mM EGTA + 10 mM DTT + 10% glycerol, pH 8.0)	9759 nmol/h/mg	NR	30	
		3. Four cycles of freeze/thaw and homogenization4. Centrifugation (14 000 ***g*** 15 min)	SEC (Superdex 75)	Isocratic (20 mM Tris + 150 mM NaCl + 1 mM MgCl2 + 10 mM DTT + 10% glycerol, pH 8.5)	12113 nmol/h/ mg	NR	8	
		1. Cells disrupted by lysozyme treatment 2. Sonication on ice for 10 min3. Centrifugation (16 000 ***g*** 20 min) 4. Ammonium sulfate precipitation (55% saturation) 5. Centrifugation (13 000 ***g*** 20 min) 6. Pellet homogenized in sonication buffer	–	–	491 ± 26 nmol/ h/mg	1	–	[80]
			HIC (butyl-Sepharose 4FF)	Stepwise gradient [0.6–0 M $(NH_4)_2SO_4$]	1461 ± 30 nmol/ h/mg	3.9	23	
		1. Cell disrupted by lysozyme treatment 2. Sonication on ice for 10 min3. Centrifugation (16 000 ***g*** 20 min)	–	–	518 nmol/h/mg	–	–	[69]
			HIC (butyl Sepharose 4FF)	Stepwise gradient [0.6–0.2–0 M $(NH_4)_2SO_4$]	1688 ± 8	1.8	14	
			HIC (octyl	Stepwise gradient [0.025–0 M	1668 ± 11	5.9	13	

(continued on next page)

POST-SCREEN (GREY)

TABLE 1 (*CONTINUED*)

Isoform	Source	Protein recovery	Chromatography	Elution	Specific activity	Purification factor (fold)	Yield/ bioactivity recovery	Refs
		4. Ammonium sulfate precipitation (55% saturation) 5. Centrifugation (13 000 ***g*** 20 min) 6. Pellet homogenized in sonication buffer	Sepharose 6FF)	$(NH_4)_2SO_4$ + 0.025–0 M sodium citrate]				
		1. Cells homogenized 2. Cells disrupted by lysozyme treatment 3. Cycles of sonication–ice or freeze/thaw4. Centrifugation (16 000 ***g*** 20 min) 5. Ammonium sulfate precipitation (55% saturation)6. Centrifugation (13 000 ***g*** 20 min) 7. Pellet homogenized	HIC (butyl-Sepharose 4FF) SEC (Superose^{TM}12)	Isocratic [0.6 M $(NH_4)_2SO_4$ + 10 mM Tris-HCl, pH 7.8] Isocratic (0.15 M NaCl + 10 mM Tris-HCl + 20 mM DTT, pH 7.8)	1688 nmol/h/mg 5500 nmol/h/mg	1.8 5.9	14 1	[71]
		1. Cells homogenized 2. Cells disrupted by lysozyme treatment 3. Cycles of freeze/thaw4. Centrifugation (16 000 ***g*** 20 mins) 5. Pellet homogenized	AEC (Q-Sepharose)	Stepwise gradient (0–0.35–1 M NaCl + 10 mM Tris-HCl, pH 7.8)	250 nmol/h/mg	3.6	107	[70]
	E. coli	1. Cells disrupted by sonication2. Centrifugation (12 000 **g** 20 min)3. Supernatant filtered (0.22-μm pores)	IMAC (Talon)	NR	NR	NR	NR	[19]
	P. pastoris X33 cells	1. Cells homogenized2. 7 cycles of vortex with glass beads/ice (1 min/1 min)3. Centrifugation (500 **g** 5 mins) 4. Pellet homogenized	IMAC (His Trap FF)	Stepwise gradient (0–0.5 M imidazole + 0.15 M NaCl + 50 mM Tris + 1 mM $MgCl_2$, pH 7.8)	121.1 ± 5.1	81	57.4	[12]
MB	*Brevibacillus choshinensis*	1. Cells homogenized 2. Cells disrupted by lysozyme treatment3. Centrifugation (16 000 **g** 20 min) 4. Pellet solubilization at 4 °C	HIC (butyl-Sepharose) HIC (epoxy-Sepharose) HIC (octyl-Sepharose)	Stepwise gradient (0.25, 0.7, and 1% of Triton X-100 in 10 mM Tris-HCL buffer, pH 7.8) Linear gradient (0–1% Triton X-100 in 10 mM of Tris-HCL buffer pH 7.8)	NR	NR	NR	[76]
		1. Cells homogenized 2. Cell disrupted by lysozyme treatment3. Centrifugation (16 000 ***g*** 20 min) 4. Pellet solubilization at 4 °C		Stepwise gradient (0–1 M NaCl + 10 mM Tris-HCl + 0.5% Triton X-100, pH 7.8)	331 nmol/h/mg	4.3	91	
	Human kidney cell lines	1. Cells homogenized 2. Centrifugation 600 ***g*** × 20 min 3. Cycles of freeze/thaw 4. Pellet washed and resuspended	NR	NR	206 U/mg[b]	NR	NR	[75]
	P. pastoris X33 cells	1. Cells homogenized2. 7 cycles of vortex with glass beads/ice (1 min/1 min)3. Centrifugation (500 **g** 5 min) 4. Pellet homogenized.	l-Arginine-Sepharose (2 mL gel) 20 °C, pH 7 l-Arginine-Sepharose (2 mL	Stepwise gradient (0–500 mM NaCl, subsequently, to 3 M in 10 mM tris buffer (pH 7), 10 mM DTT and 0.5% (v/v) Triton X-100)	187.3 ± 6.5 47.5 ± 5.7	1.69 ± 0.11 1.19 ± 0.21	61.4 ± 3.8 58 ± 4.5	[79]

TABLE 1 (*CONTINUED*)

Isoform	Source	Protein recovery	Chromatography	Elution	Specific activity	Purification factor (fold)	Yield/ bioactivity recovery	Refs
			gel) 20 °C, pH 6					
			l-Arginine-Sepharose (2 mL gel) 20 °C, pH 8		65.5 ± 6.7	0.98 ± 0.08	24.8 ± 1.9	
			l-Arginine-Sepharose (6 ml gel) 4 °C		341.1 ± 9.7	1.01 ± 0.07	58 ± 4.5	
			l-Arginine-Sepharose (6 ml gel) 20 °C		533.7 ± 44	2.28 ± 0.12	23 ± 3.2	
					164.9 ± 7.6	4.95 ± 0.23	24 ± 2.2	
	P. pastoris X33 cells	1. Cells homogenized2. 7 cycles of vortex with glass beads/ice (1 min/1 min)3. Centrifugation (500 ***g*** 5 min) 4. Pellet homogenized	IMAC (His Trap FF)	Linear gradient (0–0.5 M imidazole + 0.15 M NaCl + 50 mM Tris + 0.03% DDM + 1 mM $MgCl_2$, pH 7.8)	67 ± 6.6 nmol/h/mg	1.53 ± 0.17	47.3 ± 11.5	[78]
S-Val[108] S-Met[108]	*E. coli* BL21 (DE3)	1. Cells disrupted by sonication2. Centrifugation (12 000 ***g*** 20 mins)3. Supernatant filtered (0.22-μm pores)	IMAC (Talon)	Stepwise gradient (0–-0.1 M imidazole + 0.1 M Tris-HCl + 0.2 M NaCl + 5 mM $MgCl_2$ + 5 mM β-mercaptoethanol, pH 7.5	16.6 ± 0.8	NR	NR	[77]
			SEC (Superdex 75)	Isocratic (50 mM Tris-HCl + 0.2 M NaCl + 10 mM DTT, pH 7.5)				

[a] Abbreviation: NR, not reported.
[b] Activity value increased over untransfected cells.

TABLE 2

Overall operational conditions of currently used analytical methods for the determination of COMT activity.[a]

Analyte	COMT	Source	Substrate	Technique	Detection	Column	Mobile phase	Refs
3-*O*-methyldopamine	S and MB	Recombinant	Dopamine	HPLC	ED	ODS-2 Spherisorb C18 (50 × 4.6 mm, 3 μm)	Citric acid (27.5 mM) + sodium acetate (50 mM) + EDTA (1 mM) + 1-octanesulfonic acid (1 mM) + methanol (13%), pH 2.5	13
Normetanephrine			Norepinephrine				Citric acid (27.5 mM) + sodium acetate (50 mM) + EDTA (1 mM) + 1-octanesulfonic acid (1 mM) + methanol (3%), pH 5.0	
3-OMD			*L*-DOPA				Citric acid (27.5 mM) + sodium acetate (50 mM) + EDTA (1 mM) + 1-octanesulfonic acid (1 mM) + methanol (13%), pH 5.0	
Vanillic acid			DBA				Sodium phosphate (86 mM) + EDTA (0.13 mM) + methanol (14%), pH 3.2	
Metanephrine	Liver and brain	Rat	Epinephrine	HPLC	ED	ODS (5 mm × 25 cm)	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + methanol (8%), pH 3.5	43,95
2-MeOE1	NR	Recombinant	2-OHE1	GC	MS	5% Phenyl methyl silicone stationary phase fused silica capillary column (30 m × 0.2 mm × 0.5 μm)	–	103
	Liver	Human	2-OHE1	Radiochemical	NR	NR	NR	58,94,106,107
				HPLC	ED	Supelcosil C18 (15 cm × 4.6 mm, 5 μm)	Acetonitrile (33%) + THF (6%) + phosphate buffer (15 mM), pH 3.4	52
						Supelcosil C18 (250 mm × 4.6 mm, 5 μm)	Sodium phosphate monobasic buffer (17.1 mM) + acetonitrile (33%) + tetrahydrofuran (6%), pH 3.4	52
3-*O*-methyldopamine	Liver and brain	Rat	Dopamine	HPLC	ED	Brownlee C18 Velosep (3.2 mm × 10 cm, 3 μm)	NR	99
	Intestine	Rat	Dopamine	HPLC	ED	NR	NR	104
	Liver	Human	Dopamine	LC	MS	ODS BP (2.1 × 150 mm, 5 μm)	CH_3CN + formic acid (0.2%)	Reenilä, I. PhD thesis, University of Helsinki, 1999
	Brain	Rat	Dopamine	HPLC	ED	ODS reverse phase (4.6 × 150 mm)	NR	108
						LiChrospher 100 RP C18 (125 × 4 mm, 5 μm)	Na_2HPO_4 (0.1 M) + EDTA (0.15 mM) + methanol (15%), pH 3.2	109
4-[(4-hydroxy-3-methoxyphenyl) azo]benzene-sulfonate	Liver	Pig	3-((E)-(4-((E)-(3,4-dihydroxyphenyl)diazenyl)-3-methoxyphenyl)diazenyl) benzenesulfonic acid	HPLC	Visible light detection (370 nm)	Microsorb-MV C18 (250 mm × 4.6 mm, 5 μm)	Water + methanol + trifluoroacetic acid (30:70:0.2)	110

TABLE 2 (*CONTINUED*)

Analyte	COMT	Source	Substrate	Technique	Detection	Column	Mobile phase	Refs
Metanephrine	Adrenal gland	Rat	Epinephrine	HPLC	ED fluorescence detection (505 nm and 430 nm)	TKS-gel ODS-80TsQa (150 × 4.6 mm)	Potassium acetate buffer (75 mM) + potassium buffer (100 mM) + sodium l-hexane sulfonate (4 mM) + acetonitrile (5%), pH 3.2	105
	Brain	Rat	Epinephrine	HPLC	ED	ODS2 (25 cm × 4.6 mm, 5 μm)	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + sodium acetate (0.1 M) + Na_2EDTA (0.17 mM) + dibutylamine (1 mM) + methanol (10%), pH 3.5	111
						ODS (25 cm × 4.6 mm, 5 μm)	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + sodium acetate (0.1 M) + EDTA (0.17 mM) + dibutylamine (1 mM) + methanol (6%), pH 3.5	96
	Liver and brain	Rat	Epinephrine	HPLC	ED	ODS (5 mm × 25 cm)	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + methanol (8%), pH 3.5	43,95
	Liver and kidney	Human	Epinephrine	HPLC	ED	NR	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + methanol (8%), pH 3.5	98
	MB	Recombinant	Epinephrine	HPLC	ED	Zorbax 300SB C18 RP (250 × 4.6 mm i.d. 5 mm)	Sodium dihydrogen phosphate (0.1 M) + citric acid monohydrate (0.024 M) + sodium octyl sulfate (0.5 mM) + acetonitrile (9%), pH 2.9	87
	S	Human	Epinephrine	HPLC	ED	ODS2 (25 cm × 0.6 mm)	Citric acid (0.1 mM) + sodium octylsulfate (0.5 mM) + sodium acetate (0.1 M) + Na_2EDTA (0.17 mM) + dibutylamine (1 mM) + methanol (10%), pH 3.5	112
		Recombinant	Epinephrine	HPLC	ED	Xterra MS C18 ODS (250 × 4.6 mm, 5 μm)	EDTA (0.145 mM) + sodium acetate (0.1 M) + citric acid (0.1 mM) + sodium octyl sulfate (0.5 mM) + dibutylamine (1 mM) + methanol (5%), pH 3.5	92
Methylated catechol	Brain	Human	Catechol	Radiochemical	NR	NR	NR	106,107
Normetanephrine	Liver	Porcine	Norepinephrine	HPLC	Fluorescence (283/315 nm)	Phenomenex USP L1 Luna C18 (250 × 4.6 mm, 5 μm)	Sodium phosphate buffer (10 mM) + boric acid (80 mM) + sodium 1-hexanelsulfonate (4 mM) + acetonitrile (10%)	113
	Erythrocytes	Human	Norepinephrine	ELISA kit	NR	NR	NR	114
SAH	Liver	Porcine	SAM	Fluorescein isothiocyanate	NR	NR	NR	115
	MB	Recombinant	SAM	HTRF	Fluorescence	NR	NR	116,117
Scopoletin	NR	Porcine	Esculetin	HPLC	Fluorescence (335/460 nm)	NR	NR	114

(continued on next page)

POST-SCREEN (GREY)

TABLE 2 (*CONTINUED*)

Analyte	COMT	Source	Substrate	Technique	Detection	Column	Mobile phase	Refs
Sinapyl aldehyde	NR	Recombinant	5-hydroxyconiferyl aldehyde	HPLC	Diode array detector, mass spectral detector	Supelcosil ABZ-Plus (25 cm × 2.1 mm, 5 μm)	Acetonitrile (20%) + formic acid (10 mM), pH 2.5	100
Vanillic acid	Liver	Rat	DBA	HPLC	ED	Purospher RP-18e (4 × 125 mm, 3 μm)	Disodium hydrogen phosphate (0.1 M) + Na_2-EDTA (0.15 M) + methanol (15%), pH 3.2	97
	S	Recombinant	DBA	Radiochemical/ HPLC	UV (λ– 260 nm)	Hypersil BDS C18 (125 × 4 mm, 5 μm)	Phosphate/citrate buffer (50 mM Na_2HPO_4) + citric acid (20 mM) + Na_2EDTA (0.15 mM) + 1-octanesulfonic acid (1.25 mM) + methanol (3%), pH 3.2	38
		Human	DBA	Radiochemical	NR	NR	NR	118
	S and MB	Recombinant	DBA	HPLC	ED	LiChrospher 100 RP C18 (125 × 4 mm, 5 μm)	Na_2HPO4 (0.1 M) + EDTA (0.15 mM) + methanol (15%), pH 3.2	101,102

[a] Abbreviations: HTRF, homogenous time-resolved fluorescence; LC, liquid chromatography; MS, mass spectrometry; NR, not reported.

57.4%.[17] Similar outputs were achieved for MBCOMT upon AEC (4.3× purification and 91% bioactivity recovery).[80] However, when IMAC was used for the purification of this protein isoform, an increase of only 1.53× was observed, with 4.95 × for arginine AC.[96].

Major factors influencing COMT activity

Sample handling, isolation, and storage interfere with the specific activity of the protein, although the enzyme source and its purity degree are considered the main influencing factors. Operational conditions used for protein production, such as the cell host, growth media, feeding strategies, temperature, and pH, have a significant influence on protein stability and specific activity.[97] Subsequently, the method used for cellular lysis and further protein recovery is also crucial: if the target protein is internally produced by the host system, a more aggressive method, such as mechanical methods (e.g., sonication or glass beads, or enzymatic lysis) might be needed to ensure the recovery of a bioactive fraction of COMT.[81,98] Moreover, it is often necessary to combine several lysis strategies to maximize the amount of recovered total protein, despite the risk of decreasing the specific activity rate.[82,99] Indeed, the combination of freeze/thaw with glass beads, or glass beads followed by sonication, increases the recovery levels, although a decrease in the enzyme specific activity was observed when compared with the results obtained with a single lysis step.[98] This decrease could be explained by potential increments in the solution temperature and the shearing effect promoted by ultrasound.[81,88] Particularly for MBCOMT extraction, it was demonstrated that low concentrations of non-ionic detergents, such as Tween-20, Triton X-100, and digitonin, were able to increase MBCOMT solubilization, while maintaining high biological activity.[89] By contrast, the use of ionic detergents, such as sodium dodecyl sulfate (SDS), resulted in almost no MBCOMT bioactivity.[89] The use of a strong salt solution might also influence protein activity and folding.[82] Generally, the most used are sodium phosphate and Tris in concentrations ranging from 20 to 50 mM, frequently supplemented with protein stabilizers, such as cysteine, DTT, and 2-mercaptoethanol, to maintain the functional conformation of COMT.[80] The concentrations of the cofactors SAM and Mg^{2+} and of the substrate/inhibitors also has an effect on COMT intrinsic kinetic parameters. In terms of the dependence of COMT on the concentrations of SAM and Mg^{2+}, each research group optimizes the concentrations to be used depending on the source of COMT under study. From our own experience, studies to optimize the concentrations of COMT cofactors in enzyme activity assays showed a dose–response increment regardless of the isoform of the protein.

Bioanalytical methods for the determination of COMT activity

Currently, the analysis of *O*-methylated products found in biological samples is mostly performed by high-performance liquid chromatography (HPLC).[100] This technique allows the use of different detection systems [e.g., ultraviolet (UV), diode array detector (DAD), and electrochemical detector (ED)], based on the substrate properties to guarantee that all the components are analyzed, given that it is a no-destructive methodology that provides high specificity and precision.[101] Particularly for COMT

TABLE 3

Kinetic parameters (K_m, V_{max}) of COMT and its affinity to catechol substrates in presence or absence of inhibitors (K_i, K_{cat}, and IC_{50}).[a]

Substrate	COMT	Source	V_{max}	K_m (μM)	Inhibitor	K_i (nM)	K_{cat} (min^{-1})	IC_{50}	Refs
Dopamine (300 μM)	S	Recombinant	37.2 ± 1.3 (min^{-1})	207 ± 4 (μM)	Entacapone (10–30 nM)	0.30	31.1	NR	[13]
	MB		16.9 ± 0.5 (min^{-1})	15.1 ± 0.9 (μM)		2.00	14.1		
	S		37.2 ± 1.3 (min^{-1})	207 ± 4 (μM)	Nitecapone (10–30 nM)	1.02	26.2		
	MB		16.9 ± 0.5 (min^{-1})	15.1 ± 0.9 (μM)		1.37	17.8		
	S		37.2 ± 1.3 (min^{-1})	207 ± 4 (μM)	Tolcapone (10–30 nM)	0.27	30.9		
	MB		16.9 ± 0.5 (min^{-1})	151 ± 0.9 (μM)		0.29	17.4		
Dopamine (50 mM)	Liver	Rat	49.0 ± 9.8 (nmol 3-*O*-methyldopamine/gm wet tissue × min)	NR	Entacapone (2.5–30 mg/kg)	NR	NR	NR	[99]
	Brain		0.4 ± 0.04 (nmol 3-*O*-methyldopamine/gm wet tissue × min)	NR	3,5-dinitrocatechol (2.5–30 mg/kg)	NR	NR	NR	
Epinephrine (1000 μM)	Liver	Rat	NR	NR	Entacapone	NR	NR	30.59 ± 4.85 (nM)	[96]
					Tolcapone			34.45 ± 9.01 (nM)	
	Brain		NR	NR	Entacapone	NR	NR	0.91 ± 0.17 (nM)	
					Tolcapone			3.47 ± 0.48 (nM)	
Epinephrine (500 μM)	Liver	Rat	5.3 ± 0.5 (nmol/mg protein/h)	3.3 (μM)	Tolcapone (0.5–10 000 nM)	NR	NR	41 (nM)	[98]
	Kidney		2.6 ± 0.1 (nmol/mg protein/h)	2.7 (μM)				8 (nM)	
	S	Rat brain	168 ± 37 (nmol/mg protein/h)	1.4 ± 0.1 (μM)	Tolcapone (0.3–30 mg/kg)	NR	3.3 ± 0.2	3 (nM)	[95]
	MB		0.9 ± 0.1 (nmol/mg protein/h)	4.8 ± 0.1 (μM)			7.1 ± 0.3	2 (nM)	
	S	Rat liver	345 ± 78 (nmol/mg protein/h)	58 ± 4 (μM)		NR	4.5 ± 0.2	795 (nM)	
	MB		3.0 ± 0.6 (nmol/mg protein/h)	24 ± 1 (μM)			3.6 ± 0.3	123 (nM)	
	S	Rat liver	345 ± 78 (nmol/mg protein/h)	58 ± 4 (μM)	3,5-DNC	NR	NR	28 (nM)	
								3 (nM)	
	MB		3.0 ± 0.6 (nmol/mg protein/h)	24 ± 1 (μM)		NR	NR	356 (nM)	
								11 (nM)	
DBA (242 μM)	Liver	Human	NR	NR	Entacapone (62.5–1000 nmol/l)	NR	NR	151 (nM)	[119]
					Tolcapone (125–4000 nmol/l)			773 (nM)	
	Kidney		NR	NR	Entacapone (62.5–1000 nmol/l)	NR	NR	134 (nM)	
					Tolcapone (125–4000 nmol/l)			981 (nM)	
DBA (240 μM)	Liver	Rat	5210 (μM)	156 (μM)	Entacapone	10.7	NR	20.1 (nM)	[97]
					Tolcapone	10.0		19.7 (nM)	
Norepinephrine (250 μM)	Liver	Porcine	NR	379 (μM)	Entacapone (0.0003–1 mM)	NR	NR	0.00047 (μM)	[113]
					Tolcapone (0.0003–1 mM)			0.0068 (μM)	

[a] Abbreviation: NR, not reported.

POST-SCREEN (GREY)

assays, various analytical methods have been proposed to assess catecholamines and their *O*-methylated products, namely radiochemical assays, HPLC with ED or DAD detectors, fluorescence detection, thin-layer chromatography (TLC), UV and mass spectrometry (UV/MS), gas chromatography (GC), and ELISA (Table 2). Despite improving the assay sensitivity, radiochemical detection is often restricted to specific cases.[102] By contrast, HPLC coupled to UV detector is used less often because of its poor selectivity and only moderate sensitivity regarding the analysis of these metabolites.[102] However, because of its higher sensitivity, fluorescence detection is generally used when the analyte is a tissue with low COMT activity.[102] ED detection provides a significantly lower limit of detection (LOD), along with high sensitivity and selectivity for catecholamines.[100] In most cases, HPLC coupled with ED is the technique of choice for the analysis of *O*-methylated analytes in COMT assays. Lotta and coworkers described a bioanalytical quantification method using HPLC coupled to ED to study the kinetic parameters of recombinant SCOMTVal[108]- and Met[108]-, and native S- and MBCOMT, using dopamine as substrate and in the presence or absence of COMT inhibitors.[18] Most methods were developed using a reversed-phase C_{18} column,[91,99,100] and various physiological substrates, such as dopamine, norepinephrine and epinephrine, catechol oestrogens, like 2- and 4-hydroxyestrone (2- and 4-OHE1), *L*-DOPA have been used. The data obtained allowed determination of K_m and V_{max}, as well as the K_i, K_{cat} and IC_{50} of the different inhibitors tested (Table 3). Despite the differences in the substrate concentrations and the source of the enzyme, the kinetic discrepancies between S- and MBCOMT are evident: SCOMT exhibits a higher V_{max}, in some cases tenfold higher than MBCOMT, and a considerably lower substrate affinity, particularly for catecholamines, compared with SCOMT.[103] Likewise, the recombinant source of COMT has a higher K_m and V_{max} than the native form, which might be because of structural differences related to the production bioprocess (Table 3). COMT extracted from rat brain presents a higher K_m compared with that from rat liver, despite its lower V_{max} values. In terms of the inhibitory effects, the results showed a tendency for a higher K_i and K_{cat} in SCOMT than in MBCOMT, which can be explained by kinetic differences between the isoforms.[2] Lotta and collaborators studied the effect of several inhibitors in both recombinant isoforms[18] and, as expected, tolcapone displayed a higher inhibitory effect in both isoforms,[18] as also reported by Silva *et al.*[104] and Forsberg *et al.*[105] using rat tissue homogenates.

Concluding remarks and future perspectives

The crucial role of COMT in the inactivation of molecules bearing catechol moieties, along with its potential role in the pathogenesis of several neurological disorders, makes this protein an appealing research target. This enzyme has distinct isoforms (SCOMT and MBCOMT) with similar catalytic mechanism but distinct subtract affinities, endogenous biodistribution, and several polymorphisms associated with catechol dysregulation. Thus, the development of therapeutic molecules against COMT has become an urgent need, which has encouraged researchers to implement several biotechnological platforms to produce, recover, and purify both COMT isoforms. One of the biggest challenges in the future will be to develop a biotechnology integrative system to obtain high yields of protein without compromising COMT stability, structure, and enzymatic activity. To achieve this goal, new host expression systems (e.g., HEK or Sf9), along with an efficient recovery processes through the application of non-micellar structures, followed by an isolation step with affinity chromatography exploiting new combination of tags (e.g., MBP, GRP, or SUMO) must be considered. Furthermore, because tolcapone is the only commercially available MBCOMT inhibitor with the ability to cross the BBB and inhibit the *O*-methylation process performed by MBCOMT in the CNS, where this isoform is predominantly expressed, a deeper understanding of the interaction mechanisms of this enzyme with new inhibitors and substrate/analogs is crucial. This will be the starting point for the creation of novel, highly effective, and safer drugs able to block the effects of the protein in the CNS and PD, thus improving both the quality of life, and outcome, of patients with PD.

Declaration of interests

The authors declare no conflict of interest.

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