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Development of new Catechol-*O*-methyltransferase inhibitors

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“Celebrate every tiny victory.
Anything is possible if you have got enough nerve.”

- J.K. Rowling

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Que tenhamos sempre saúde para as histórias nunca acabarem.

Resumo alargado

A Catecol-*O*-metiltransferase (COMT, EC 2.2.1.6)) é a enzima responsável pela *O*-metilação de substratos catecólicos, como as catecolaminas e os estrogénios com estrutura catecólica. Considerando as suas funções fisiológicas e a existência de polimorfismos, vários estudos associam a COMT com a patogénese de várias desordens neurológicas, especialmente com a Doença de Parkinson (DP) e também com doenças cardiovasculares e cancros hormono-dependentes, como cancros da mama. Devido à importância que a COMT tem no metabolismo das catecolaminas e dos estrogénios catecólicos, a COMT tornou-se nas últimas décadas num importante alvo terapêutico. Atualmente, a terapia mais eficaz e clinicamente aprovadas pela *Federal Drug Administration* e *European Medicines Agency* para a doença de Parkinson consiste no uso de Levodopa, combinada com inibidores da COMT. Uma vez que os inibidores desta enzima comercialmente disponíveis ainda apresentam diversas desvantagens, como hepatotoxicidade, dificuldade em alcançar o cérebro, entre outras, o objetivo principal deste trabalho foi desenvolver novos inibidores da COMT com potencial clínico para a terapia da DP. Para isto, foram preparados triazolopirimidínicos através da reação de Biginelli, os quais podem ser considerados bioisósteros de catecóis, podendo por isso ter potencial para interagir com a COMT. Esta hipótese foi confirmada através de *docking* molecular, prevendo-se interações moleculares semelhantes às dos substratos catecóis com o centro ativo da COMT. As suas propriedades inibitórias foram avaliadas em lisados recombinantes da enzima, após incubação dos compostos nas concentrações de 10 e 100 μM. Contrariamente ao expectável, os compostos aumentaram a atividade específica da enzima, podendo ser considerados estabilizadores da COMT. Foi ainda avaliada a citoxicidade dos mesmos em células dopaminérgicas neuronais de rato (N27) nas mesmas concentrações. A grande maioria dos compostos a 10 μM não mostrou citotoxicidade, observando-se valores semelhantes aos dos inibidores comerciais da COMT, Entacapone e Tolcapone, na linha celular N27. Como esperado com o aumento da concentração (100 μM) ocorreu um decréscimo na proliferação celular, atingindo valores já considerados citotóxicos. No geral, os compostos sintetizados, na concentração de 10 μM estabilizaram a COMT e não induziram citoxicidade nas células N27. Em suma, as moléculas sintetizadas podem ser úteis para estudos de estabilidade térmica, de cristalografia, de relação estrutura-atividade e apresentam potencialidade para ser estudados em linhas celulares especificas do cancro da mama.

Palavras-chave

Catecol-*O*-metiltransferase; Desordens neurológicas; Inibidores da COMT; Triazolopirimidinas.

Abstract

The Catechol-*O*-methyltransferase (COMT, EC 2.2.1.6) is an enzyme responsible for the *O*-methylation of catechol substrates, such as catecholamines and catechol estrogens. Considering its physiological functions and the existence of polymorphisms, several studies associate COMT with the pathogenesis of several neurological disorders, especially with Parkinson's Disease (PD) as well as with cardiovascular and hormone-dependent cancers, like breast cancers. Given the important role that COMT has in the catecholamines and catechol estrogens metabolism, COMT has become a relevant therapeutic target. Currently, the most effective and clinically approved by the Federal Drug Administration and the European Medicines Agency for the PD therapy consists of the use of Levodopa, combined with COMT inhibitors. Since the commercially available inhibitors for this enzyme still display a lot of disadvantages, like hepatoxicity, difficulty to reach the brain, among others, the main goal of this work was to develop new COMT inhibitors with potential clinical interest for the PD therapy. For this, triazolopyrimidinics were prepared through the Biginelli reaction, that can be considered catechol bioisosteres, therefore have a higher potential to interact with COMT. This hypothesis was confirmed through molecular docking, being predicted similar interactions as the ones that the catecholic substrates forms with the COMT active site. Their inhibitory properties were evaluated in human recombinant COMT lysates, after the compounds' incubation at 10 and 100 μM. Contrary to what was expected, the compounds increased the enzyme specific activity, being considered COMT stabilizers. The compounds cytotoxicity was also evaluated in neural dopaminergic rat cells (N27), in the same concentrations. The vast majority of compounds at 10 μM did not exhibited cytotoxicity, being observed similar values to those of the commercial COMT inhibitors, Entacapone and Tolcapone, in the studied cell line. As expected, with the increase in compounds' concentration (100 μM) a decrease in the relative cell proliferation was observed, reaching values considered to be cytotoxic. Altogether, the synthesized compounds at the concentration of 10 μM stabilized COMT and did not induce cytotoxicity in the N27 cells. In sum, these compounds may be useful for thermal stability assays, crystallography, structure-activity relationship studies and display potential to be studied in specific breast cancers cell lines.

Keywords

Catechol-*O*-methyltransferase; Neurological disorders; COMT inhibitors; Triazolopyrimidines.

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List of Abbreviations

2D	Two-dimensional
3,5-DNC	3,5-dinitrocatechol
3-*O*MD	3-*O*-methyldopa
3D	Three-dimensional
43G	1-(biphenyl-3-yl)-3-hydroxypyridin-4(1H)-one
5-FU	5-Fluorouracil
637	*N*-[(*E*)-3-[(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(6-methylpurin-9-yl)oxolan-2-yl]prop-2-enyl]-5-(4-fluorophenyl)-2,3-dihydroxy-benzamide
Aa	Amino acids
AADC	Aromatic acid decarboxylase
Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid
ATLC	American Type Culture collection
BBB	Blood-brain barrier
BIA 9-1067	Opicapone
BIE	(3,4-dihydroxy-2-nitro-phenyl)-phenyl-methanone
BMG	Buffered Minimal Glycerol Medium
BMM	Buffered Minimal Methanol Medium
BSA	Bovine Serum Albumin
BSM	Basal Salt Medium
CADD	Computer-Aided Drug Design
CL4	*N*-[(*E*)-3-[(2*R*,3*S*,4*R*,5*R*)-5-(6-aminopurin-9-yl)-3,4-dihydroxy-oxolan-2-yl]prop-2-enyl]-2,3-dihydroxy-5-nitro-benzamide
CNS	Central Nervous System
COMT	Catechol-*O*-methyltransferase
$COMT^{Val/Val}$	Valine at codon 108/Valine at codon 158
$COMT^{Val/Met}$	Valine at codon 108/Methionine at codon 158
$COMT^{Met/Met}$	Methionine at codon 108/Methionine at codon 158
d	Duplet
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ED	Electrochemical detector
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid
FBS	Fetal Bovine Serum
gDNA	Genomic DNA
Gln	Glutamine

Glu	Glutamic acid
HBHK	Hamster baby kidney fibroblasts
HeLa	Human embryonic kidney cells
HPLC	High-performance liquid chromatography
Hz	Hertz
Ile	Isoleucine
IMAC	Immobilized metal affinity chromatography
J	Coupling constant
K. pastoris	*Komagataella pastoris*
K_i	Inhibition constant
L-DOPA	Levodopa
Leu	Leucine
LU1	5-(4-fluorophenyl)-N-[(*E*)-3-[(2*S*,4*R*,5*R*)-5-(6-methylpurin-9-yl)-4-oxidanyl-oxolan-2-yl]prop-2-enyl]-2,3-bis(oxidanyl)benzamide
Lys	Lysine
m	Multiplet
MBCOMT	Membrane-bound isoform of COMT
MCR	Multicomponent reaction
MPD	(4*S*)-2-methylpentane-2,4-diol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nH	number of protons
NMR	Nuclear magnetic resonance
OD	Optical density
OD_{600}	Optical density at 600 nm
P_{500}	Lysis resulting pellet after centrifugation at 500G
PBS	Phosphate-buffered saline solution
PCR	Polymerase-chain reaction
PD	Parkinson's Disease
PDB	Protein data bank
Pro	Proline
PSA	Ammonium persulfate
q	Quartet
RMSD	Root mean square deviation
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
s	Singlet
S_{500}	Lysis resulting supernatant after centrifugation at 500G
SAH	S-adenosyl-$_L$-homocysteine
SAM	S-adenosyl-$_L$-methionine
SAR	Structure-activity relationship

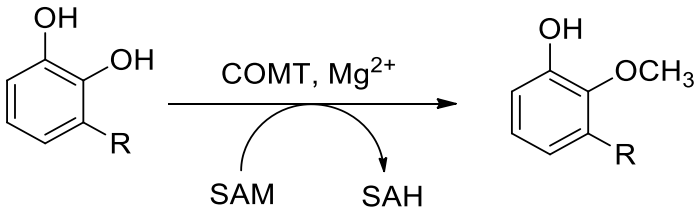
SBDD	Structure-based drug design
SCOMT	Soluble isoform of COMT
SDS	Sodium-dodecyl-sulfate
Ser	Serine
Sf9	*Spodoptera frugiperda* cells
SMT	Trace metal solution
SNP	Single nucleotide polymorphism
t	Triplet
TEMED	Tetramethylethylenediamine
TLC	Thin-layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
UV	Ultraviolet
Val	Valine
$Val^{108}Met$	Valine for a Methionine at codon 108
$Val^{158}Met$	Valine for a Methionine at codon 158
V_{max}	Enzymatic capacity
WR	Working reagent

Chapter 1 - Introduction

1.1. Catechol-*O*-methyltransferase

1.1.1 Biochemical Characterization

In 1958, Axelrod and co-workers identified and described the function of the enzyme responsible for the *O*-methylation of catecholamines [1]. The catechol-*O*-methyltransferase (COMT, E.C. 2.1.1.6) is a monomeric enzyme that in the presence of a magnesium cation (Mg^{2+}) catalyzes the transference of a methyl group from the cofactor S-adenosyl-$_L$-methionine (SAM) to a catechol substrate, resulting in *O*-methylated products and S-adenosyl-$_L$-homocysteine (SAH) [1,2] **(Scheme 1).**



***Scheme 1* - The *O*-methylation of a standard catechol catalysed by COMT.**

This ubiquitous enzyme has already been found and characterized regarding its structure and function in a large number of organisms across several phylogenetic levels, specifically in bacteria, yeasts, plants, insects, fish, amphibians, birds, and mammals [3–5]. The main function of COMT is to *O*-methylate biologically active endogenous and/or exogenous catechol molecules, acting as an enzymatic detoxifying barrier [6]. In humans, COMT is present in two distinct molecular isoforms: a soluble form (SCOMT) and a membrane-bound form (MBCOMT) [7]. The COMT gene, located in the band q11.21 of chromosome 22, is composed of six exons and encodes both isoforms [8] (**Figure 1**). The translation initiation codons for both isoforms are located in the third exon, while the first two are non-coding [9]. Their expressions are regulated by two distinct promoters, a short transcript of 1.3 kb translates SCOMT and a longer transcript of 1.5 kb translates MBCOMT [8].

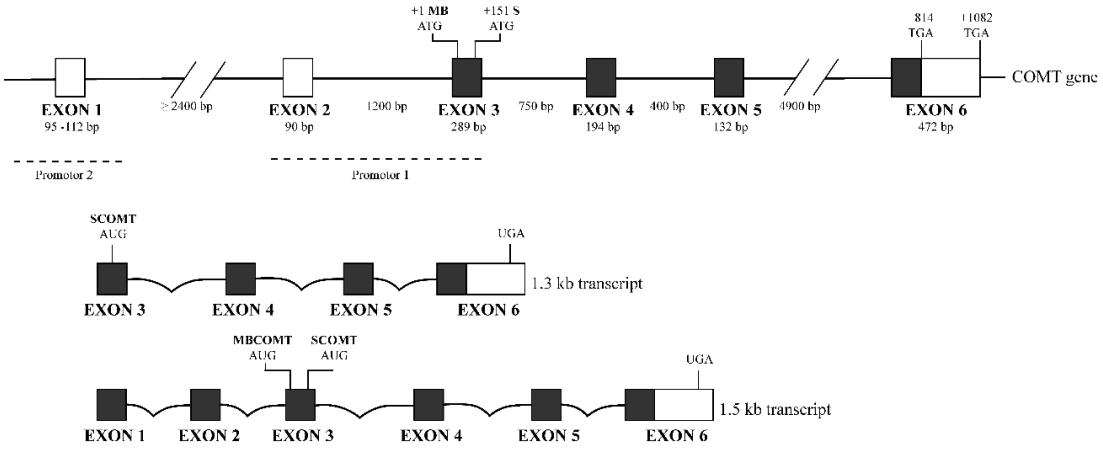


***Figure 1* - Physical maps of the human COMT gene and its transcripts: Depiction of the exon-intron organization (not in scale) of the gene and isoforms transcripts, adapted from [9].**

This enzyme is expressed in almost all human tissues, primarily in the liver, kidneys, and gastrointestinal tract, in the peripheric tissues the levels of SCOMT are considerably higher than the levels of MBCOMT [4]. However, in the central nervous system (CNS), the expression levels of MBCOMT surpass SCOMT, representing approximately 70% of the total COMT [8]. The cellular localization of these isoforms are also different: while SCOMT can be found in the cytoplasm, with a reported molecular weight of 24.7 kDa and a total of 221 amino acids (aa), MBCOMT with a molecular weight of 30 kDa and an extra 50-residue long amino-terminal signal sequence, forms a membrane anchor associated with the membrane of the rough endoplasmic reticulum, generally oriented towards the cytoplasmic side of the membrane [4]. Despite the kinetic similarities shared by both isoforms, the Mg^{2+} and SAM dependency, the substrate affinity is completely distinct. Typically, the physiological substrates for this enzyme include catecholamines, namely dopamine, epinephrine, norepinephrine and their hydroxylated metabolites, catechol estrogens, such as 2- and 4-hydroxyestradiol, 2- and 4-hydroxyestrone, ascorbic acid, and other dietary substances [10]. Lotta and coworkers studied the affinity of both COMT isoforms for several catecholamines, using COMT obtained from rat liver [11]. Actually, for dopamine, SCOMT displayed a K_m of 207 μM while MBCOMT had a K_m of 15 μM; for norepinephrine, SCOMT presented a K_m of 369 μM while MBCOMT a K_m of 24 μM [11]. In terms of enzymatic velocity (V_{max}), SCOMT was reported to have a greater velocity than MBCOMT [11], once SCOMT presents a V_{max} of 37.2 min^{-1} for dopamine and MBCOMT 16.9 min^{-1}; while for norepinephrine the values were 35 min^{-1} against 18 min^{-1} [11], respectively. These findings clearly support the claims that MBCOMT is the isoform responsible for the *O*-methylation at physiologically low concentrations of catecholamines, particularly in the CNS. On the other hand, SCOMT is mostly responsible for the *O*-methylation of catechol compounds throughout the peripheric tissues at higher substrate concentrations [12].

1.1.2 Three-dimensional structure of COMT

The three-dimensional (3D) structures of human and rat COMT are characterized by a very similar fold, sharing approximately 81% of the genomic sequence [13]. Structurally, human COMT is composed by a single domain of a mixed α/β-protein structure, consisting of a seven stranded β-sheet core, of which β1-β6 stands are parallel and stand β7 is antiparallel, sandwiched between two sheets of α-helices [14]. The catalytic site is shaped to fit the catechol substrate and the Mg^{2+} binding site in a shallow groove on the outer surface of the enzyme, while the SAM binding site is located within the enzyme structure, in a more buried cleft. In this S_N2 transfer reaction, the binding sequence needs to follow a crucial order to successfully *O*-methylate catechol substrates. Thus, SAM must be the first to bind, followed by Mg^{2+}, and lastly the substrate. The major molecular interactions of the cofactors with the protein active site are displayed in **Figure 2**.

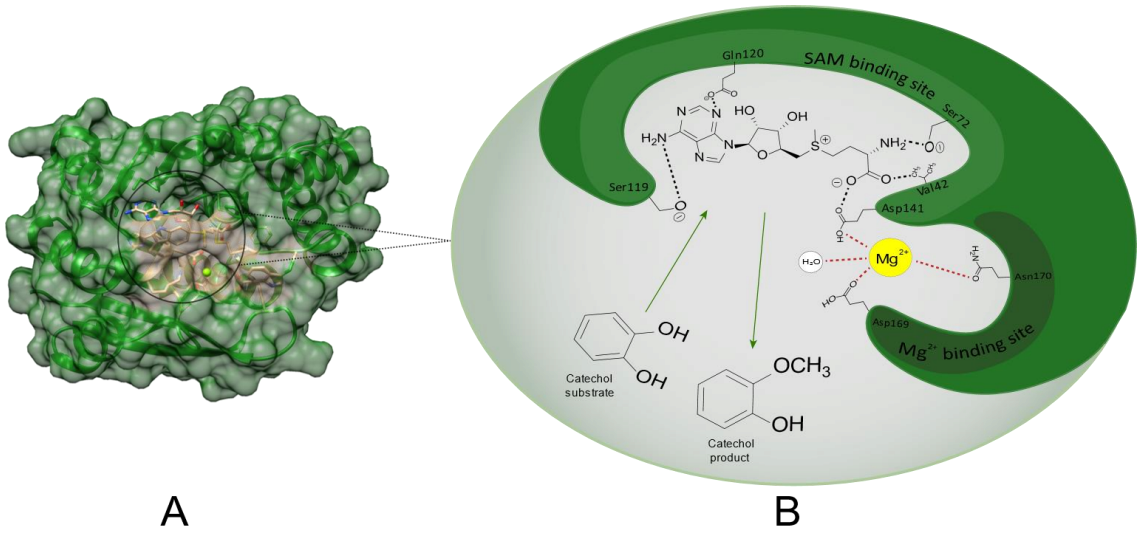


***Figure 2* - A- 3D structure of COMT (PDB#6I3C), adapted from [15]. B- Major molecular interactions that take place in the COMT active site (PDB#6I3C), black dashes represent hydrogen bonds, red dashes represent metallic bonds with Mg^{2+}.**

The adenine ring of SAM is hydrogen-bonded to Ser119 and Gln120, forming Van der Waals interactions with residues Ile91, Ala118, and Trp143 [2]. Also, the methionine fragment of SAM and SAH is oriented toward the substrate-binding site, promoting hydrogen bonds with Val42, Ser72, and Asp141 [16]. Despite the Mg^{2+} binding site being in the center of the catalytic site, it does not directly interact with SAM. It is octahedrally linked to the oxygen atoms of the side chains of residues Asp141, Asp169, Asn170, and to one crystallographic water molecule. The other two free coordination sites allow the chelation to each of the catechol hydroxyl groups of the substrate. In this context, the interaction with Mg^{2+} lowers the substrate pK_a, facilitating the deprotonation of the catechol hydroxyls [17]. The hydroxyl groups of the catechol ring form hydrogen bonds with the side chains of the residues Glu199 and Lys144, and interact with the "gatekeeper" residues Trp38, Trp143, Pro174, and Leu198 by hydrophobic interactions, that help maintain the correct ring position for the *O*-methylation [18]. These residues are also responsible for the selectivity and *O*-regioselectivity of COMT to different substrates and inhibitors [11,19]. The interaction with the residue Trp38 was found to be essential for a higher substrate binding affinity [20].

1.1.3 Genetic polymorphisms of COMT and their relationship with human diseases

In mammals, the COMT genotype distribution is known to differ interspecies and even intraspecies. Over 900 genetic variants have been reported for the COMT gene, but the majority did not show any physiological importance [21–24]. One of the most studied and characterized COMT polymorphism is the rs4680, a single nucleotide (SNP) transition in the gene code involving a Guanine to an Adenine in the genetic code. This alteration leads to an aa substitution, a Valine (Val) for a Methionine (Met) in the polypeptide chain at codon 108, in SCOMT ($Val^{108}Met$) and at codon 158 in MBCOMT ($Val^{158}Met$) [11,14]. This functional SNP is genetically

polymorphic with a trimodal distribution with high ($COMT^{Val/Val}$), intermediate ($COMT^{Val/Met}$), and low ($COMT^{Met/Met}$) enzymatic activity, respectively. The polymorphic variant $COMT^{Met/Met}$ when compared with the native form of COMT ($COMT^{Val/Val}$), exhibits a decrease of approximately 40% in enzyme activity, is more prone to active-site distortion and protein aggregation, as well as having a decreased thermal stability [11]. The $Val^{158}Met$ SNP can cause dysregulations in the catecholamine levels and, therefore, many studies established a link of this SNP to several diseases. Some studies evidenced a connection between this SNP and multiple neuropsychiatric disorders, for example: depression [24], autism spectrum disorder [24], schizophrenia [25–29], obsessive-compulsive disease [28], panic [30–32], bipolar disease [33–35], attention deficit hyperactivity disorder [24,36,37], anxiety [38], nervous anorexia [39–41] and Parkinson's Disease (PD) [42]. Considering its physiological function and polymorphic activity, COMT is considered a relevant therapeutical target, especially for PD therapy [43,44]. This neurodegenerative diseases widespread worldwide, being a major age-related health problem, especially in people over 80 years old [45]. It is characterized by a progressive degradation of the dopaminergic neurons in the CNS, specifically in the *substantia nigra pars compacta* of the brain. In the early stages of the disease, due to the decline of dopamine levels, the patient's motor system is unable to control movement and coordination, leading to characteristic motor parkinsonian signals. In the later stages of PD, cognitive and behavioral problems may also arise, along with postural instability, dysphagia, dementia, and other severe complications [46,47]. Despite the advances and breakthroughs achieved by science, the etiology of PD is still unknown, but it is thought to be a multifactorial cause, linked to oxidative damage, environmental toxins, accelerated aging, gene mutations, and genetic factors [42,48]. Currently, there is no cure for PD, however, some drugs can help to control the symptoms. The dopamine replacement using the dopamine precursor Levodopa (*L*-DOPA), used in clinical practice since the 1960s, still remains as the first-line of pharmacologic treatment for PD [43]. Orally administered *L*-DOPA can compensate for the deficit of dopamine, as it can cross the blood-brain barrier (BBB) and later be converted into dopamine in the CNS. However, *L*-DOPA is rapidly metabolized after oral administration by peripheral enzymes, particularly by aromatic amino acid decarboxylase (AADC) and COMT [49]. To maximize its bioavailability and to prevent early metabolization, *L*-DOPA is usually administered together with AADC and COMT inhibitors. In the absence of both inhibitors, approximately only 1% of the administered dose would reach the brain, and without the COMT inhibitor, 90% would be converted to 3-*O*-methyldopa (3-*O*MD) by COMT, and to dopamine by AADC before reaching the brain (**Figure 3**) [50].

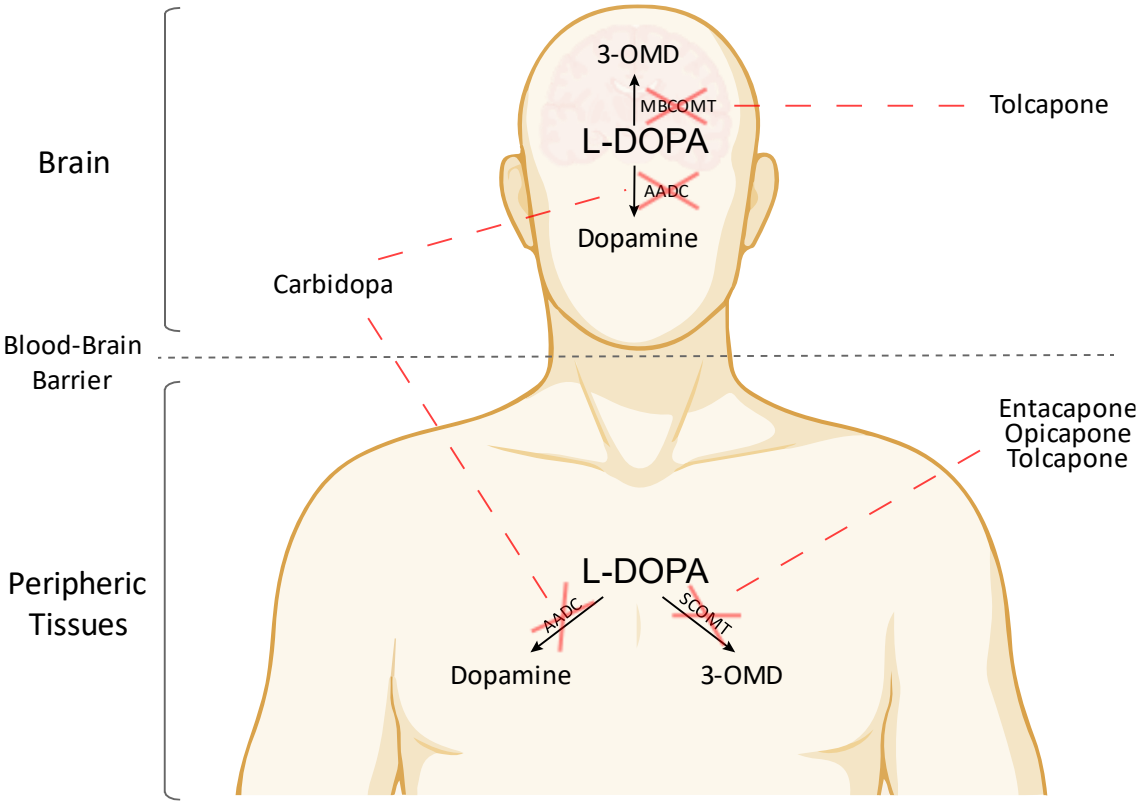


***Figure 3 - L*-DOPA metabolism after oral administration in brain and peripheric tissues, and the effects of commercial AADC and COMT inhibitors, adapted from** [51].

Therefore, the use of COMT inhibitors as adjuvants in this therapy significantly improves the clinical benefits in PD [2]. In the last years, the use of central COMT inhibitors in the treatment of other neuropsychiatric diseases has significantly increased, especially in schizophrenia. Recently, COMT has also been associated with cardiovascular [52,53] and hormone-dependent diseases, namely breast [54] and colorectal cancers [55]. In fact, Wu and collaborators and coworkers studied the role of COMT in colorectal cancer and demonstrated to have tumor-suppressive functions [56]. In this context, *in vitro* and *in vivo* experiments showed that COMT inhibited cancer cell proliferation and decreased tumor growth in human colorectal carcinoma (HCT116) COMT overexpressing cell line [56]. The major catechol estrogen metabolites, 2- and 4-hydroxyestrone, which are highly carcinogenic for the peripheric tissues, combined with lower COMT activity, can increasing risk of developing hormone-dependent diseases [3]. However, 2-methoxyestradiol, a 2-hydroxyestradiol metabolite originated by COMT-mediated methylation, exhibited very interesting antitumoral properties, and its action was studied in several clinical trials [57–59]. Therefore, considering the data previously presented, COMT became a relevant target for new therapeutic approaches for different diseases.

1.1.4 Recombinant human Catechol-*O*-methyltransferase biosynthesis and recovery

Since the discovery of COMT and its pharmacological significance, the scientifical community focus switched to the development of bioprocesses capable of obtaining high amounts of the target protein with a high degree of purity for structural and functional studies. Axelrod and collaborators were the firsts to successfully isolate this enzyme, employing a combination of ammonium sulphate fractioning with gel filtration in a partially purified rat liver COMT [1]. Ever since, many productions and recovery methodologies were described [60,61]. In mammal tissues, most of the COMT activity is generally found in the soluble fraction of the lysates, especially from rat liver [61]. However, the recovery and isolation of COMT its complicated requiring multiple steps, which can affect the enzyme specific activity [61]. The advances in the recombinant DNA technology allowed the development of bioprocess with higher production yields and easier recovery of human recombinant proteins, including COMT [5,62]. The choice of the expression system is a factor that must be taken into consideration. In fact, the host's capacity to process and translate the transcript RNA, along with the ability to modify the translated protein with the correct folding and bioactivity is also extremely important. Particularly for membrane proteins, including MBCOMT, due to its hydrophobic nature, the production process can lead to protein misfolding and aggregation. To avoid that, biological chaperons or fusion constructs, such as solubility tags and signal sequences can be employed in the production step [63]. In **table 1** are displayed the expressions systems, both prokaryotic and eukaryotic, used for the biosynthesis of S- and MBCOMT, as well as the corresponding bibliographic references.

***Table 1* - Overview of the main expression system applied for COMT biosynthesis.**

Human recombinant COMT			
Expression System	**Organism**	**Isoform**	**Reference**
Prokaryotic	*Escherichia coli*	S-	[60,64–69]
		MB-	[60]
	Brevibacillus choshinensis	S- and MB-	[70]
	Komagataella pastoris	S-	[71]
		MB-	[72]
	Spodoptera frugiperda cells	S- and MB-	[73,74]
Eukaryotic	Hamster baby kidney fibroblasts	S- and MB-	[7]
	Human embryonic kidney	S- and MB-	[7]

Over the years, multiple methods were reported regarding COMT isolation using both recombinant and the animal source of the enzyme. The number of steps employed in this step can affect the enzyme specific activity, therefore a compromise has to be done to obtain high amounts

of purified enzyme in high biologically active state. One of the first SCOMT purification strategies reported was developed by Lundtröm and collaborators using ionic exchange chromatography, with Q-Sepharose columns [61,65]. Afterwards, several other methodologies were reported using other types of chromatography to purify both isoforms [61]. For the SCOMT, multiple strategies were studied: a combination of affinity chromatography plus anion-exchange chromatography [61], immobilized-metal affinity chromatography (IMAC) plus gel filtration [14], and IMAC for both polymorphic variants, $COMT^{Val/Val}$ and $COMT^{Met/Met}$ [67]. Our research group also studied the effect of hydrophobic interaction chromatography, using different ligands [75,76], the combination of hydrophobic interaction with gel filtration [64], aa affinity chromatography [77] and IMAC [78], anion-exchange chromatography [79]. In addition several purification flowsheets for MBCOMT were also developed. For instance, Robinson and collaborators used an IMAC strategy involving a His Trap FF column [74]. Passarinha and coworkers studied the effect of hydrophobic interaction chromatography [80], anion-exchange chromatography [79], arginine-affinity chromatography [81] and finally using IMAC [72] to isolate MBCOMT. The vast majority of the described methodologies obtained high amounts of COMT, with a high degree of purity, to be used for further kinetic, bio-interaction and structural studies.

1.1.5 Analytical methods for the assessment of catechol-*O*-methyltransferase activity

In general, *in vitro* enzymatic tests to the measurement of the enzyme activity are used to evaluate the inhibitory effect of new compounds, including in structure-activity relationship (SAR) studies [82]. Specifically for COMT specific activity determination the incubation conditions, the pH of the enzyme solution, temperature, the substrate/inhibitor and co-factors concentration and any other supplements to stabilize or increment the target activity, are very important parameters that need to be fully optimized to obtain reliable and reproducible results [82,83]. Typically, the analysis of *O*-methylated products is mostly performed by liquid chromatography, particularly by high-performance liquid chromatography (HPLC) [84]. This technique employs the use of different stationary and mobile phases, based on the sample properties, and various detection systems, to guarantee that all the components are successfully detected and quantified [85]. It is a non-destructive process coupled with excellent specificity and high precision sample analysis. This technique allows the use of different detectors coupled with the HPLC system. The most used detectors are: the ultraviolet (UV), diode array (DAD); refractive index; and electrochemical detector (ED), suitable to substances that are either oxidable or reducible [82]. During the last decades, several analytical methods have been proposed for COMT activity analysis [4]. The first methods involved the direct quantification of the substrate or the product [4]. Abdel-Latif reported a colorimetric method for the quantification of *O*-methylated catecholamine-hydroxylamine derivatives in the presence of COMT [86]. Despite the simplicity and precision of the proposed method, it was not capable of fully quantifying the products formed [86]. So, more precise and sensitive methods were developed. Firstly, using liquid-liquid extraction with organic solvents to separate the *O*-methylated products from the catechol substrate, followed by an

analysis using fluorometric approaches [1] or radiochemical detection [87–92]. This last one can improve the sensitivity of the assay, but it is often restricted to special cases and specific types of samples. This approach was usually combined with paper or thin-layer chromatography (TLC), to separate and identify the products faster [87]. More recently, other strategies have been developed, namely gas chromatography, specifically in the separation and quantification of 3,4-dihydroxybenzoic acid and its metabolites, using flame ionization detection [93] and mass spectrometry to analyse *O*-methylated catechol estrogens [94] and catecholamines [95]. Despite the developments, HPLC is still viewed as the standard analytic technique for the analysis of the *O*-methylated catecholamines. There are reports of HPLC coupled with several types of detectors for COMT assays, such as UV, but displayed poor selectivity [96,97]; fluorometric, but the analytes must have native fluorescence or include fluorogenic reagents, which could affect the enzyme's kinetic parameters [98–100]; radiochemical, an highly sensitive method, but it is restricted due to the use of radioactive material [19]; and ED, that allows the possibility to work in amperometric or coulometric mode, depending on the analyte, exhibiting high sensitivity and a lower detection limits (0.5 pmol/injection) [84,101]. Generally, the ED is the most used for this analysis, since the phenolic hydroxyls of the catechol substrates are easily oxidized [11,82,102–104]. Passarinha and co-workers separated catecholamines products using reversed-phase matrices onto HPLC systems with ion-pairing reagents. With this strategy the research group was able to detect the O-methylated products by their reversible oxidation using amperometric [84] and coulometric cells [105]. One of the major differences between coulometric and amperometric detection is that in the first one, virtually, all of the target analyte in the column effluent is oxidized or reduced in the working electrode at a constant potential, while in the amperometric only part of the analyte is converted.

1.2 Medicinal Chemistry of COMT inhibitors

1.2.1 "First Generation"

Following the report of the first studies using partially purified rat COMT and its characterization by Axelrod and Tomchick in the late 1950s, several families of COMT inhibitors started to be developed. In the earlies 1960s, the "first generation" of COMT inhibitors was synthesized, primarily formed by catechol and pyrogallol systems [83]. These compounds contained primarily a catechol ring in their structure, or some related bioisosteric moiety, including flavonoids, benzoic acids, catechol ketones, and pyrogallol derivatives, among others [106,107]. This class of competitive substrates of COMT also included 2-hydroxyoestrone, quercetin, rutin, gallic acid, caffeic acid, U-0521, and non-catecholic compounds, such as ascorbic acid, tropolones, derivatives of 8-hydroxyquinolines, 3-hydroxylated pyrones, and pyridines [83,108] (**Figure 4**). The *in-vitro* studies demonstrated that these compounds have low selectivity and inhibitory potency towards COMT [4]. Despite the disappointing results, some compounds advanced to *in vivo* studies and even to clinical trials, but they showed very limited pharmacological usefulness, namely due to poor bioavailability and a very-short lasting biological effect [10,109–111]. Due to

their low efficacy *in vivo,* larger doses of these compounds were required to inhibit COMT, increasing their toxicity [83].

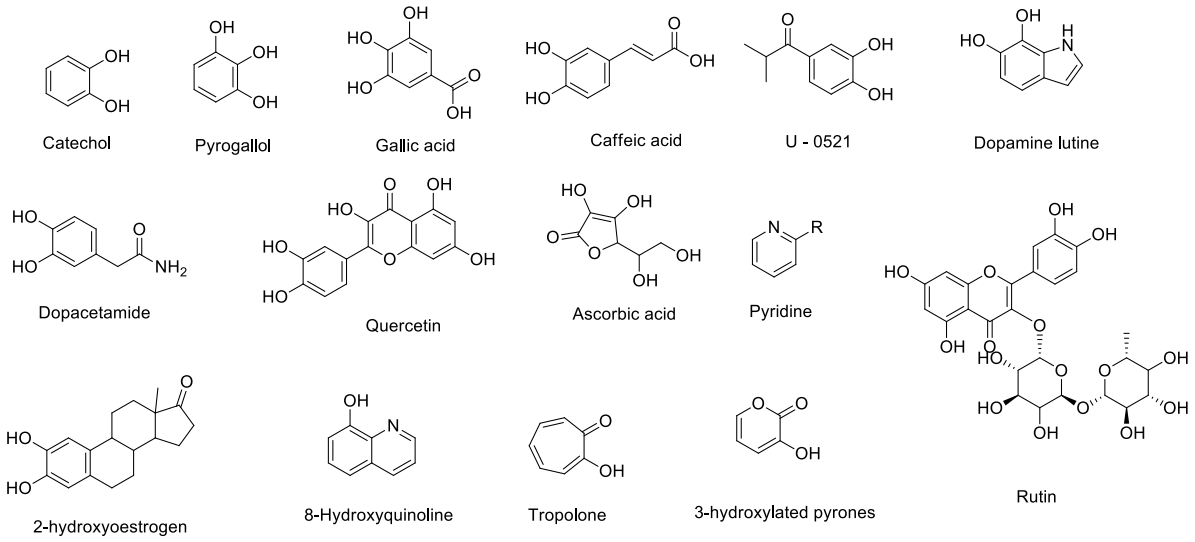


***Figure 4* – Structures of the "first generation" COMT inhibitors.**

1.2.2 "Second Generation"

To overcome the handicaps that the first generation exhibited, a "second generation" of di-substituted catechol COMT inhibitors was developed. Taking into account the information retrieved from numerous SAR studies, the best results were observed when a nitro group is present at position 5 of the catechol ring, displaying a high potency, selectivity, and better pharmacokinetics than the other substituents tested [4,6]. Consequently, several highly potent, selective, and orally active inhibitors were intensely studied to assess their safety throughout *in-vitro*, *in-vivo* studies and even clinical trials [4,10]. One of the most promising nitro-catechol COMT inhibitor prepared was 3,5-dinitrocatechol (3,5-DNC) (**Figure 5**), bearing two nitro groups in the *ortho* and *para* positions of the hydroxyl groups of the catechol moiety [2]. Despite showing high potency *in vitro*, during *in vivo* studies this molecule displayed high toxicity and unfavourable pharmacokinetics, restricting its clinical use [4]. Further studies demonstrated that the compound's potency, bioavailability, and toxicity, could be adjusted by using a second substituent in these molecules, specifically at the *para* position to the same hydroxyl group, for example a carbonyl group or a carbon-carbon double bond, resulting in the development of novel nitrocatechol molecules [112,113]. The K_i of the most promising nitrocatechol in COMT lysates was in the low nanomolar range (nM), while U-0521, a "first generation" compound, displayed values in the micromolar range (mM), a difference of three orders of magnitude [112]. Nitrocatechol molecules are generally kinetically characterized as reversible tight-binding inhibitors of COMT [11]. Despite being poor substrates for the enzyme, they compete with the substrate for the binding site and are uncompetitive with the SAM binding site [114]. In addition, these compounds displayed more selectivity towards COMT over other enzymes involved in catecholamines metabolism [112]. In accordance with the data obtained in the *in-vitro* and *in vivo*

studies, only Tolcapone, Nitecapone, and Entacapone (**Figure 5**) were fully characterized in further studies [2]. They were the most promising compounds of this class, inhibiting SCOMT with an K_i of 1.02, 0.30, 0.27 nM for, and the MBCOMT K_i values of 2.00, 1.37, and 0.29 nM for Nitecapone, Entacapone, and Tolcapone, respectively [11]. These values may vary according to the different experimental conditions, enzymatic preparations, and assay conditions. In humans, despite the compounds structural similarities they inhibited COMT in different manners. Nitecapone, mostly affected COMT duodenum activity, while Entacapone showed higher inhibition in the liver and erythrocytes [115], being both considered strictly peripheral inhibitors. In contrast, Tolcapone exhibited higher potency and a longer period of action than the previously mentioned, it is considered a central COMT inhibitor, due to its ability to cross the BBB and influence brain COMT activity [116,117]. However, Tolcapone is associated with increases in the plasmatic levels of liver transaminases, causing severe hepatoxicity [118,119]. Nowadays, only Entacapone and Tolcapone are approved for use in clinical setting [110]. However, the medical prescription of Tolcapone requires continuous hepatic monitoring, restricting its use in patients whose therapy with other COMT inhibitors had previously failed [119]. The use of these COMT inhibitors together with Carbidopa, a commercial AADC inhibitor, as adjuvants to the *L*-DOPA therapy, significantly improved the bioavailability of *L*-DOPA, with a significant reduction of 3-*O*MD levels and a significant increase in the levels of *L*-DOPA reaching the brain [115,120,121].

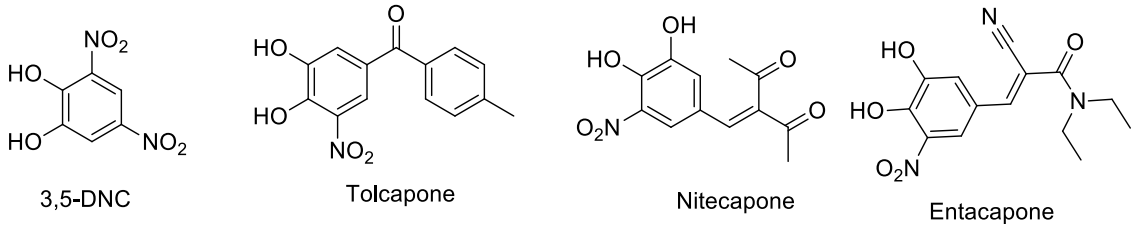


***Figure 5* – Structure of the main nitrocatechol "second generation" COMT inhibitors.**

More recently, novel inhibitors were designed using computational tools based on protein-inhibitor atomic interactions by computer-aided drug discovery (CADD) approaches [122–125]. Based on the results of several SAR studies, novel nitrocatechol inhibitors were prepared, namely nebicapone and BIA 3-335 (**Figure 6**) [126,127]. Both are considered potent and reversible competitive inhibitors with the characteristic tight-binding properties of other nitrocatechols. These studies demonstrated that the nitrocatechol moiety was responsible for the binding of the compound to the enzyme catalytic site, but the variations on the side chain of the substituents heavily influenced the selectivity and inhibition. *In vivo,* both compounds showed similar potency and duration of effect similar to the observed in Tolcapone. Moreover, despite having limited access to the CNS, they displayed a longer duration of peripheral COMT inhibition when compared with Entacapone [128]. It was also demonstrated that the position of attachment of the substituents to the nitrocatechol pharmacophore also had an important effect on the potency, selectivity, and duration of action of these molecules, more specifically, substituents containing a heteroatom, such as oxygen, were considered to be more effective [129]. Both compounds were

successfully co-crystallized with rat recombinant SCOMT. The crystal structure of rat SCOMT with BIA 3-335 (PDB#1h1d) was the first complexed co-crystallized structure with a potential clinically relevant COMT inhibitor [16]. The determination of the crystal structure was very important to better understand the interactions of other clinically relevant COMT inhibitors, such as Tolcapone, Entacapone, or Nebicapone. In 2016, Palma and co-workers successfully co-crystallized recombinant rat SCOMT with a novel nitrocatechol COMT inhibitor, BIA 8-176 (PDB#2cl5) (**Figure 6**) [130]. This molecule has a nitro group in the *ortho* position relative to one of the catechol hydroxyls and side-chain substituents. This substitution lead to a profound alteration on the *O*-regioselectivity of the methylation reaction [130].

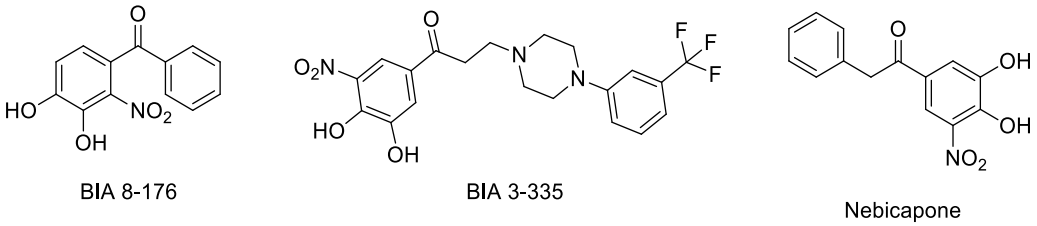


***Figure 6* – Structures of CADD generated nitrocatechol COMT inhibitors.**

1.2.3 "Third-generation"

More recently, the Portuguese Pharmaceutical Company Bial – Portela & C^{a}, S.A. developed a new COMT inhibitor, BIA 9-1067 (Opicapone) (**Figure 7**) [118,131]. Opicapone was approved to be used in a clinical setting in 2016 as an adjuvant for *L*-DOPA in PD therapy [132]. This "third-generation" nitrocatechol COMT inhibitor demonstrated a longer duration of action, a higher binding affinity and bioavailability *in vivo* [133,134]. The pharmacokinetics of this molecule allowed a once-daily administration, being observed over 24h of stable and sustained *L*-DOPA plasma levels in the patients [118]. In addition, this compound displayed high potency and a low propensity to cause toxicity. Opicapone mostly inhibited erythrocytes COMT activity, but it cannot penetrate the BBB, therefore is considered a strictly peripheral inhibitor [118,134–136]. Even with the improvements that these inhibitors brought to the PD and other neurological diseases therapies, there is still the need for improvements and, therefore, new classes of COMT inhibitors continue to be explored [43].

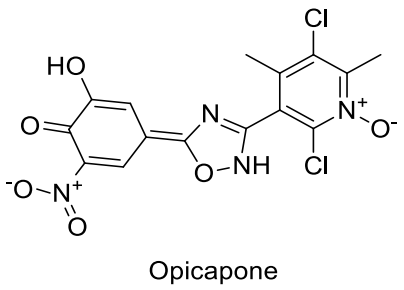


***Figure 7* – Structure of Opicapone, a "third generation" COMT inhibitor.**

1.2.4 Other COMT inhibitors

Alongside with the development of the nitrocatechol-based COMT inhibitors, some other classes of compounds were studied. A pyridine derivative, CGP-28014 (**Figure 8**) , increased the levels of catecholamines in the brain after oral administration in rats [137,138]. Despite, lacking the catechol moiety in its structure and not showing significant *in vitro* COMT inhibition, in *in vivo* mimicked the physiological effects of Tolcapone, reducing the formation of 3-*O*MD after *L*-DOPA administration [10]. Another class of compounds heavily studied were the bisubstrate inhibitors and many molecules were prepared targeting simultaneously the catechol and the SAM binding sites [139]. Nevertheless, only more recently promising compounds were obtained, using computational tools combined with the information gathered from the 3D structures of COMT. Paulini and co-workers, studied the interactions of these compounds with an adenine moiety, due to the great abundance of this structure in biological systems, resulting in the design and synthesis of new bisubstrate inhibitors with adenine replacements [140,141]. The compounds CL4 was most potent displaying an IC_{50} of 9 nM in the *in-vitro* studies, using 6-amino-3,4-dihydropyrimidin-4-one as the adenine replacement and a catechol moiety covalently linked to the C(5')-OH group of the adenosine fragment of SAM, using propene as a spacer (**Figure 8**). The SAR studies demonstrated that the size and rigidity of the spacer group were essential for a higher binding affinity, further kinetic studies showed a competitive mechanism for both SAM and substrate binding sites. These findings were demonstrated with the determination of the rat COMT 3D structure complexed with Cl4, where the compound occupied the SAM and the catechol binding sites. The replacement of the nitro moiety with hydrophobic groups (for example, R = 4-methyl-phenyl) was also hypothesized to increase the compound binding affinity [90], but the *in vitro* studies proved the contrary and this substitution resulted in lower binding affinity and a higher IC_{50} (23 nM) (**Figure 8**). However, the results refined the SAR studies, which resulted in the synthesis of novel compounds incorporating other substituents, with less toxicity and higher inhibitory potency than Tolcapone *in vitro* [90]. Another class of compounds bearing two catechol pharmacophores in the same molecule was synthesized to increase the inhibitory potency, Bis-nitrocatechol inhibitors. Depending on the nature of the substituents, these bifunctional compounds displayed a similar *in vitro* potency ($R_1 = NO_2$; $R_2 = OH$; n = 2) and K_i values (3.48 μM), when compared with the monofunctional analogues (R = Et) with a corresponding K_i of 3.41 μM [142,143] (**Figure 8**). Despite the similarities in inhibitory potency, the insertion of the second catechol pharmacophore induced changes in the mechanisms of the enzyme activity. Mazumder and collaborators reported that garcinol (**Figure 8**), a benzophenone derivative with antioxidant and anti-inflammatory properties, could potentially inhibit COMT activity, based on *in-silico* studies [144–146].

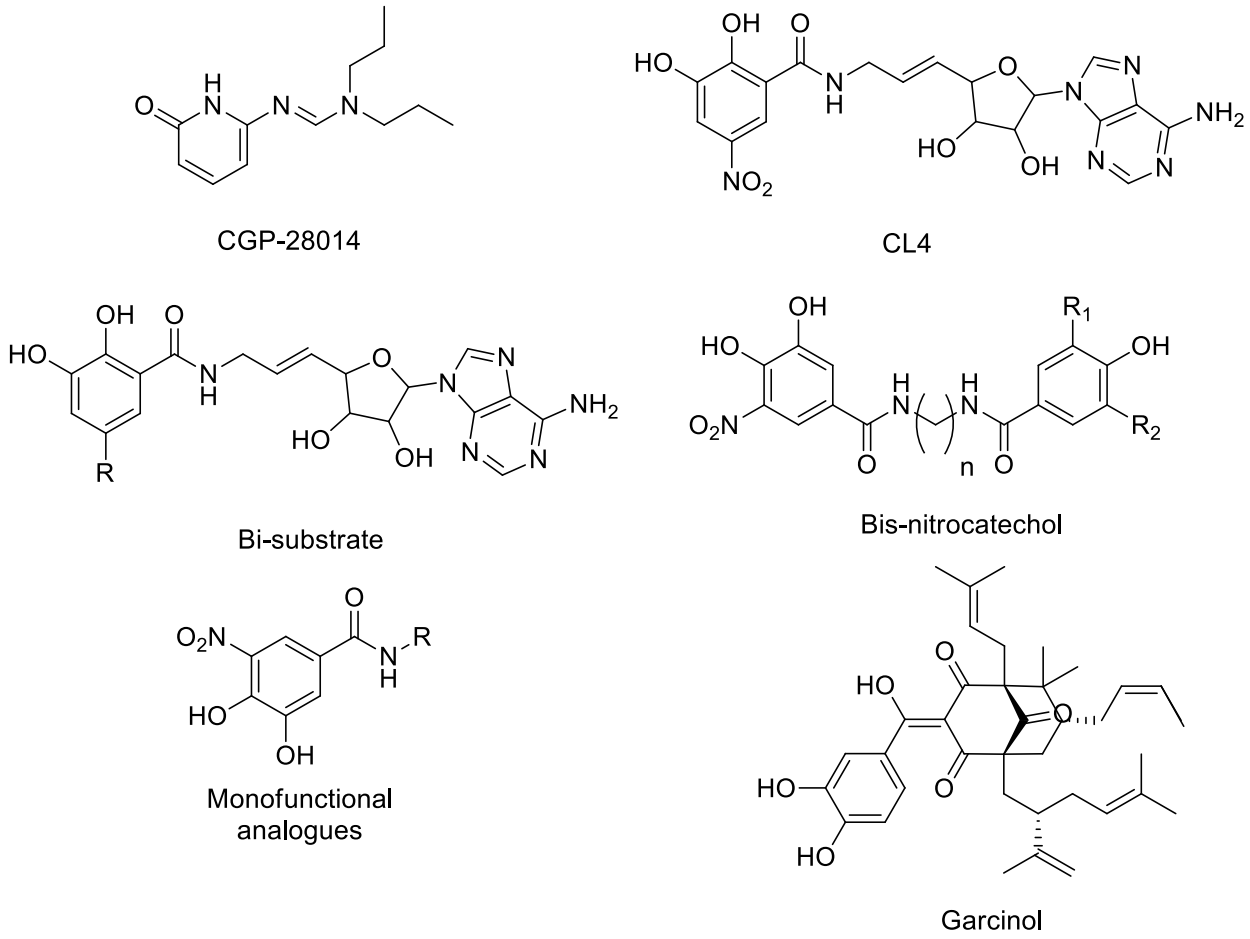


***Figure 8* - Structure of other classes of COMT inhibitors.**

1.3 Structure-Based drug design

The process of bringing a new drug to the market requires a huge investment in time and money [147]. Thanks to the technological advances it is now possible to combine computational tools to support drug discovery, helping in the selection of potential targets and potentially bioactive compounds, using fewer resources and less time [148,149]. Structure-based drug design (SBDD) is a well-known part of modern medicinal chemistry that combines the data found in the protein 3D structure with computational algorithms to predict the affinity of a certain ligand to key binding pocket sites in the proteins structure [18,150,151]. These tools can be used to sort out big libraries of molecules into smaller groups with higher potential to bind in specific biologically bindings sites and to accelerate the design of new compounds [152]. In recent years, an increasing number of COMT 3D structures, both from rat and human, have been deposited in the Protein Data Bank (PDB) (**Table 2**).

***Table 2* - Crystal COMT structures deposited in the PDB (checked 29/10/2020).**

Human COMT						
COMT	**Source**	**Form**	**PDB ID**	**Atomic Resolution**	**Ligands**	**Reference**
S	Recombinant	Holo	3BWM	1.98 Å	DNC + SAM + Mg^{2+}	[14]
			4XUC	1,8 Å	43G + Mg^{2+} + SAM	[153]
			6I3C	1.336 Å	DNC + Mg^{2+} + SAM	[15]
S (Met^{108})			3BWY	1.3 Å	DNC + SAM + MPD + Mg^{2+}	[14]
S	Humanized rat	Apo	4PYI	1.35 Å	Na^{+}	[154]
Rat COMT						
COMT	**Source**	**Form**	**PDB ID**	**Atomic Resolution**	**Ligands**	**Reference**
S	Native	Holo	1JR4	2.63 Å	CL4 + Mg^{2+}	[140]
			4PYL	2.2 Å	Tolcapone + Sinefungi + Mg^{2+}	[154]
			3S68	1.85 Å	Tolcapone + Mg^{2+} + SAM	[91]
			3NW9	1.65 Å	637 + Cl^{-} + Mg^{2+}	[155]
			5FHQ	1.63 Å	DNC + Mg^{2+} + SAM	[156]
			2CL5	1.6 Å	BIE + Mg^{2+} + SAM	[130]
			5K01	1.383 Å	6P4 + K^{+}	[157]
			3R6T	1.2 Å	LUI + Cl^{-} + Mg^{2+}	[91]
	Humanized rat	Apo	4PYM	1.19 Å	SO_4^{-} + K^{+}	[154]

With the information retrieved from the resolved structures, it is possible to better understand how molecules interact with the protein active site. The information collected from these structures indicated that the hydrophobic interactions with the "gatekeeper" residues Trp38, Trp143, Pro174, and the hydrogen bonds formed with the residues Lys144 and Glu199, can be used to filter and recognize molecules with a higher potential to inhibit COMT [4,18,111]. Vidgren and co-workers used quantitative structure-activity relationship (QSAR) studies with substituted catechols to demonstrate the importance of the acidity of the hydroxyl groups and the relevance of the lipophilicity of the compounds side chain for binding affinity [158]. The first crystal structure successfully resolved was reported by the same research group , the rat COMT structure, complexed with 3,5-DNC (PDB#1VID), provided new insights into the mechanism of the *O*-methylation reaction catalyzed by COMT [158]. The described atomic arrangement was later confirmed with the determination of several other COMT structures complexed with other molecules, demonstrating that the shallow pocket in the active site of COMT allows the long side chains of potential inhibitors to reach the surface of the enzyme [16,130]. Overall, this structural data demonstrated the crucial role of Mg^{2+} in the binding of substrates and inhibitors in the

methyl group transfer. In this context, molecular docking can be used to study the *O*-regioselectivity of potential COMT inhibitors. Palma and collaborators compared two nitrocatechol-type inhibitors, BIA 3-228 and BIA 8-176 (**Figure 9**), and studied the differences in the interactions formed between both and the protein active site [130].

BIA 3-228 BIA 8-176

***Figure 9* – Structures of nitrocatechol COMT inhibitors studied using SBDD.**

The only structural difference between the two compounds is that in BIA 3-228 the side chain (R = Benzaldehyde) was placed in a *meta*-position, whereas in BIA 8-176 was at the *ortho* position of the hydroxyl groups of the catechol ring. The *in-silico* studies demonstrated that the side-chain position influences the interactions that the compound forms with the enzyme active site, with the *ortho*-substituted derivative presenting a higher binding *O*-regioselectivity than the derivative with the *meta* substituent. However, the nitrocatechol moiety was far more important for the compound binding. Garcinol (**Figure 8**), a compound with the potential to be used in PD therapy due to its neuroprotective properties, was studied using SBDD approaches to assess the potential to interact with the COMT active site [159]. In these studies, the interactions that Tolcapone formed with COMT were compared with the ones that garcinol formed [159]. The authors demonstrated, using molecular docking, that garcinol formed four hydrogen bonds with the active site of COMT, while Tolcapone only performed three. The binding free energies were very similar and interacted with the same aa residues [159]. Combining other computational tools like comparative homology modeling or pharmacophore maps with molecular docking models to design and identify new potential inhibitors can be very useful to study the molecules interactions with the protein active site [160]. The study of the ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties using computational algorithms is another tool to complement the *in-silico* studies [161]. Nowadays, more than 250 potential COMT inhibitors have been prepared with the support of these methodologies to help in the design of new molecules, using the information collected from more than 100 resolved COMT structures deposited in PDB (**Table 2**) [14,20,154,156,162,163].

1.4 Biginelli reaction

As referred, over the years, several different classes of compounds with the potential to inhibit COMT in a selective manner were developed [2]. Of these, hydroxypyridin-4-ones (**Figure 10**) caught our attention due to the clear structure similarity with different 3,4-dihydropyrimidin-2(1*H*)-ones or -thiones (DHPMs), compounds with recognized anticancer or anti-convulsive properties already synthesized by our research group [164,165].

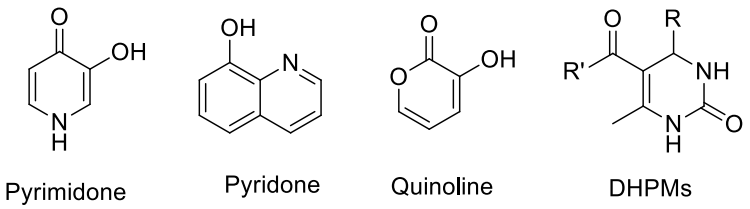
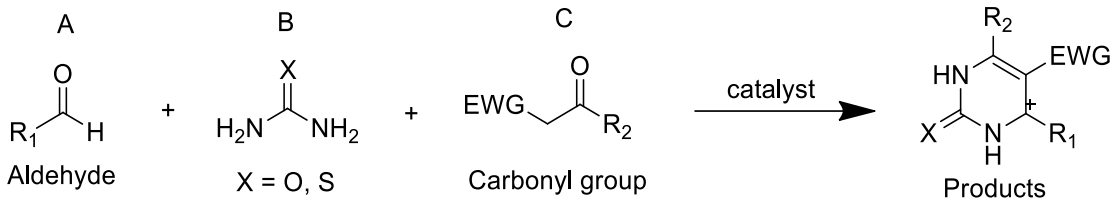


***Figure 10* – Structures of pyrimidone, pyridone, quinoline based inhibitors and DHPMs.**

These products can be prepared through the Biginelli reaction. This reaction was firstly reported by Pietro Biginelli in 1893, via a three-component condensation reaction between an aldehyde, a β-ketoester, and urea or thiourea in the presence of an acid catalyst [166] (**Scheme 2**).

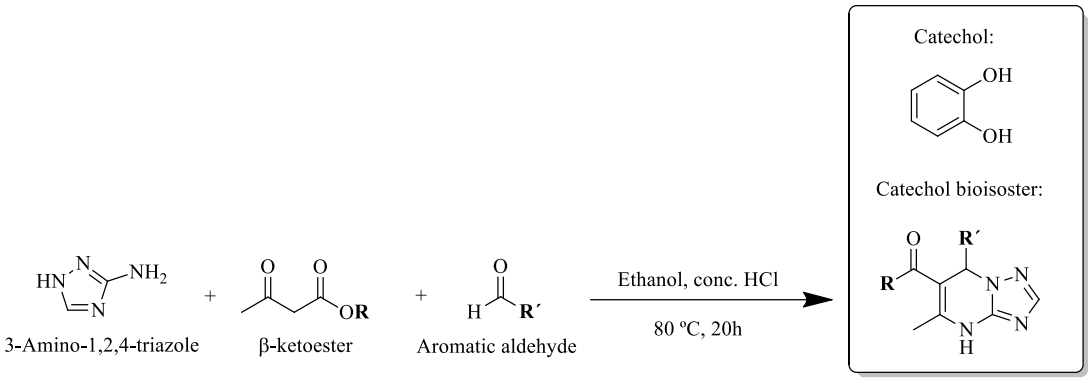


***Scheme 2* - Biginelli reaction, A: Aldehyde, B: Urea or Thiourea, C: Carbonyl group, EWG – electron withdrawing group.**

Several methodologies, namely using microwaves, sonication [167], ionic liquids [168], and different catalysts [169] are reported to be employed to perform this reaction. These DHPMs, prepared through this reaction, possess a broad spectrum of biological and pharmacological properties, such as anticancer [170], antimalarial [171], anti-inflammatory [172], antioxidant [173], among others [174,175]. This multi-component reaction (MCR) allows the preparation of a broad variety of highly structural diverse molecules, generally purer than the final products from multi-step reactions, combining three reactants in a single step to form a final product incorporating structural characteristics of each reagent [176–178] (**Scheme 2**). The structural diversity allows the obtainment of very interesting SAR data in the development of new bioactive compounds.

1.4.1 Triazolopyrimidine derivatives

When 3-amino-1,2,4-triazole is used instead of the classical urea or thiourea, it is possible to prepare triazolopyrimidine derivatives (**Scheme 3**) [179].



***Scheme 3* - Preparation of triazolopyrimidines derivatives.**

Interestingly, this triazolopyrimidine bicyclic system can be considered a catechol bioisoster, and therefore, we decided to evaluate their potential to interact COMT and inhibit this enzyme. In addition, our research group showed that several DHPMs can penetrate the BBB and also may have good intestinal permeability [161]. This data is extremely pertinent, indicating that the proposed molecules could also have good oral absorption and reach significant concentrations within the brain. Within this context, SBDD methodologies to support the design of novel molecules with the potential to inhibit COMT were employed.

Chapter 2 – Objectives

The objectives of this dissertation were:

- To synthesize triazolopyrimidine derivatives with high structural diversity and with potential clinical interest.

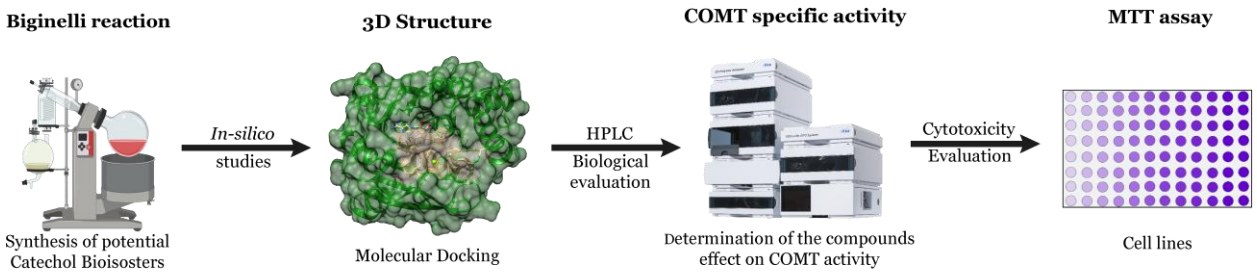
- To understand through molecular docking how the synthesized molecules interact with the COMT active site.

- To biologically evaluate the compounds' effect on COMT specific activity and their cytotoxicity in cell lines.

- To compare the results obtained in the *in-silico* with the *in vitro* results.

- To understand the substituent effect by SAR studies.

Graphical Abstract:



Chapter 3 - Materials and Methods

3.1 Materials

Ultrapure reagent-grade water was obtained with a Mili-Q system (Millipore/Waters). Yeast nitrogen base, glucose, agar, yeast extract, peptone, biotin, di-potassium hydrogen phosphate, potassium dihydrogen phosphate monobasic, phosphoric acid, calcium chloride, magnesium sulfate heptahydrate, potassium hydroxide, dimethyl sulfoxide (DMSO), methanol, ammonium hydroxide, sulphuric acid, sodium hydroxide, sodium dodecyl sulfate (SDS), ammonium persulfate (PSA), tetramethylethylenediamine (TEMED), magnesium di-chloride ($MgCl_2$), ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), perchloric acid, 3-amino-1,2,4-triazole, benzaldehyde, 4-methoxybenzaldehyde, 4-nitrobenzene, furfural, 1,3-dichlorobenzene, thiophene, pyridine, ethyl- and methyl-acetoacetate, hydrochloric acid, 1-*n*-hexane, urea, thiourea, bovine serum albumin (BSA), dithiothreitol (DTT), SAM, epinephrine (bitartrate salt), deoxyribonuclease (DNase), protease inhibitor cocktail, *L*-cysteine, sucrose, glycerol, dl-metanephrine hydrochloride, citric acid monohydrate and glass beads (500 μm) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide 40%/Bis solution was obtained from Bio-Rad (Hercules, CA). Tris(hydroxymethyl)aminomethane (Tris) and CAPS was obtained from Fisher scientific (Epson, United Kingdom). The NZYcolour Protein Marker II used for estimation of subunit molecular weight was purchased from NZYTech (Lisbon, Portugal). Anti-rabbit IgG alkaline phosphatase secondary antibody was purchased from GE Healthcare Biosciences (Uppsala, Sweden), while the monoclonal rabbit COMT antibody was acquired from Abcam (Cambridge, England). All chemicals used were of analytical grade, commercially available, and applied without further purification.

3.2 *In-silico* studies

3.2.1 Molecular Docking

The molecular docking was performed between the original ligand used for method validation (3,5-DNC), the triazolopyrimidine derivatives synthesized, and the COMT active site. The 2D structures were drawn, and the 3D energy minimization was performed using the Cambridge Soft's ChemOffice v14.0 software. The crystal structure of COMT was obtained through the PDB code 6I3C (1.34 Å resolution) [15]. The protein and the ligand (3,5-DNC) were isolated using Chimera 1.10.2 software. AutoDock Tools 1.5.6 software was used for the preparation of the molecules for docking, to set the docking parameters, and for the result analysis. All docking calculations were obtained using the AutoDock 4.0 software. To visualize the interactions between the ligands and the protein active site the software Discovery Studio Visualizer v16.1 was used. The protein was prepared by removing the water molecules, adding protons, Gasteiger charges, and by removing non-polar hydrogens. The ligand parameters were maintained unchanged. The method validation was performed with the re-docking of COMT with 3,5-DNC. The grid box used had the dimensions of 35 x 35 x 35 Å with a spacing of 0.300 Å around the centre of the co-crystalized ligand, ensuring that the docking is realized in the active site of the enzyme. The

Lamarckian Genetic algorithm was used as the docking research method and the 10 best conformations were generated.

3.2.2 ADMET properties prediction

The pharmacokinetic and toxicity profile of a compound defines its absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. Ensuring that the drug can reach the target site in acceptable concentrations safely to produce the physiological effect is crucial. pkCSM (http://biosig.unimelb.edu.au/pkcsm/prediction) uses graph-based structural signatures to study and predict a range of ADMET properties, that can be used for the analysis and further optimization of pharmacokinetic and toxicity properties. SwissADME (http://www.swissadme.ch/index.php), can also be used to complement the *in-silico* analysis, predicting physicochemical properties, pharmacokinetics, drug-likeness and medicinal chemistry friendliness.

3.3 Organic Synthesis

3.3.1 General Considerations

The reactions were performed under heating and constant magnetic stirring using Heidolph plates. TLC analysis was performed using 0.20 mm Al-backed silica-gel plates (Macherey-Nagel 60 F254). The silica-gel plates were first visualized under UV light (254 nm) and then revealed with the mixture ethanol/sulfuric acid (95:5) followed by heating at 120°C. The eluent used are indicated as a v/v proportion in the experimental procedure. The UV revelatory chamber used was CN-15.LC. The evaporation of solvents was achieved by using a rotary vacuum drier from Büchi (R-215). Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance 400 MHz spectrometer (^{1}H NMR at 400 MHz) and were processed with the software TOPSPIN 3.1 (Bruker, Fitchburg, WI, USA). The hexa-deuterated dimethyl sulfoxide (DMSO-d_6) was used as solvent and as internal standard (DMSO-d_6, δ = 2.50 ppm in ^{1}H-NMR). In the description of the compounds, the data obtained are indicated in the following order: solvent; chemical shift (δ, in ppm); signal multiplicity (s, d, t, q, and m); coupling constant (J, in Hz); relative intensity (nH, number of protons); and attribution of the proton in the molecule, if possible. For each synthesized compound is presented the two-dimensional (2D) structure and its SMILES notation, which were obtained with ChemBridgeSoft® Chembiodraw 14.0 drawing software.

3.3.2 Synthesis of triazolopyrimidine derivatives

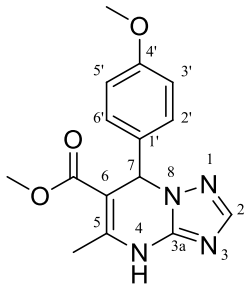
The pyrimidines **1a-l** were obtained using the following conditions:

Condition A: To a stirred solution of 3-amino-1,2,4-triazole (4 mmol, 0.35 g) in ethanol (20mL) was added a solution of methyl/ethyl acetoacetate (4 mmol) and an aldehyde (4 mmol). The mixture was heated with continuous stirring at 80°C and 4 drops of hydrochloric acid (HCl) were added. The reaction lasted 20h and was followed by TLC (Ethyl acetate: *n*-Hexane: methanol; 6:3:1). Once completed, the reaction was cooled down to room temperature, forming a precipitate. The precipitate was filtered under suction and washed with ice-cold methanol (99.9%), dried and then recrystalized with methanol to afford the pure product.

Condition B: To a stirred solution of urea/thiourea (13 mmol, 0.9995 g) in ethanol (20mL) was added a solution of ethyl acetoacetate (10 mmol) and an aldehyde (10 mmol). The mixture was heated with continuous stirring at 70°C and then the catalyst $ZrCl_4$ (1 mmol, 0.12 g) was added. The reaction was followed by TLC (Ethyl-acetate: *n*-Hexane: methanol; 6:3:1) and was completed after near 1h. Then, the reaction was cooled down to room temperature, forming a precipitate. The precipitate was washed with ice-cold water and filtered under suction and washed with ice-cold ethanol (99%), dried and then recrystalized with ethanol to afford the pure product.

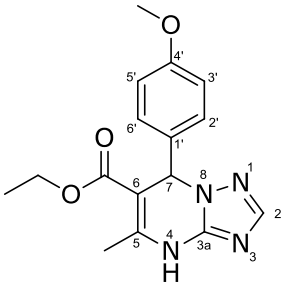
Methyl-7-(4-methoxyphenyl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1a**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 4-methoxybenzaldehyde (4mmol, 0.8625 mL) and methyl acetoacetate (4 mmol, 0.44 mL); Condition A; Completed in 20 hours; Yield 16%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H, 4-NH), 7.64 (s, 1H, 2-CH), 7.13 (d, J = 8.7 Hz, 2H, Ar-CH), 6.86 (d, J = 8.7 Hz, 2H, Ar-CH), 6.23 (s, 1H, 7-CH), 3.71 (s, 3H, 4'-COCH_3), 3.52 (s, 3H, ester-OCH_3), 2.41 (s, 3H, 5-CCH_3). SMILES CC(N1)=C(C(OC)=O)C(C2=CC=C(OC)C=C2)N3C1=NC=N3.



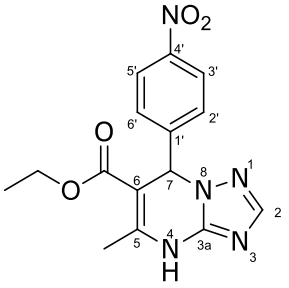
Ethyl-7-(4-methoxyphenyl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1b**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 4-methoxybenzaldehyde (4mmol, 0.8625 mL) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 11%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.76 (s, 1H, 4-NH), 7.64 (s, 1H, 2-CH), 7.13 (d, J = 8.7 Hz, 2H, Ar-CH), 6.85 (d, J = 8.9 Hz, 2H, Ar-CH), 6.22 (s, 1H, 7-CH), 4.00 – 3.92 (m, 2H, ester-OC$\underline{H}_2$CH$_3$), 3.71 (s, 3H, 4'-COC$\underline{H}_3$), 2.41 (s, 1H, 5-CC$\underline{H}_3$), 1.06 (t, J = 7.1 Hz, 3H, ester-OCH$_2$C$\underline{H}_3$). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C(OC)C=C2)N3C1=NC=N3.



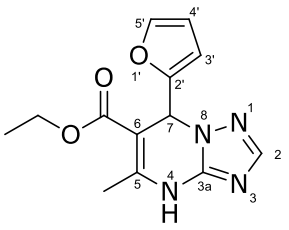
Ethyl-5-methyl-7-(4-nitrophenyl)-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1c**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 4-nitrobenzaldehyde (4mmol, 0.556 g) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 17%; yellow crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ δ 10.99 (s, 1H, 4-NH), 8.19 (d, J = 8.6 Hz, 2H, Ar-CH), 7.70 (s, 1H, 2-CH), 7.53 (d, J = 8.8 Hz, 2H, Ar-CH), 6.44 (s, 1H, 7-CH), 4.00 – 3.92 (m, 2H, ester-OC$\underline{H}_2$CH$_3$), 2.44 (s, 3H, 5-CC$\underline{H}_3$), 1.05 (t, J = 7.1 Hz, 3H, ester-OCH$_2$C$\underline{H}_3$). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C([N+]([O-)=O)C=C2)N3C1=NC=N3.



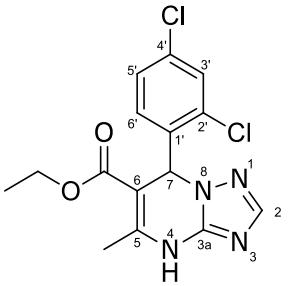
Ethyl-7-(furan-2-yl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1d**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), furfuraldehyde (4mmol, 0.688 g) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 26%; brown crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H, 4-NH), 7.70 (s, 1H, 2-CH), 7.52 (s, 1H, 7-CH), 6.38-6.36 (m, 2H, Ar-CH), 6.31 (d, J = 3.2, 1H, Ar-CH), 4.08 – 3.97 (m, 2H, ester-OCH_2CH$_3$), 2.40 (s, 3H, 5-CCH_3), 1.11 (t, J = 7.1 Hz, 3H, ester-OCH$_2$CH_3). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=CO2)N3C1=NC=N3.



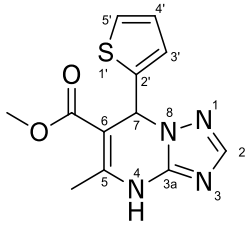
Ethyl-7-(2,4-dichlorophenyl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1e**)

From 3-amino-1,2,4-triazole (4mmol, 0.35g), 2 4-dichlorobenzaldehyde (4mmol, 0.71 g) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 22%; greenish white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H, 4-NH), 7.65 (s, 1H, 2-CH), 7.58 (d, J = 1.5 Hz, 1H, Ar-CH), 7.42 – 7.32 (m, 2H, Ar-CH), 6.70 (s, 1H, 7-CH), 3.99 – 3.86 (m, 2H, ester-OCH_2CH$_3$), 2.43 (s, 2H, 5-CCH_3), 1.00 (t, J = 7.1 Hz, 3H, ester-OCH$_2$CH_3). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C(Cl)C=C2Cl)N3C1=NC=N3.



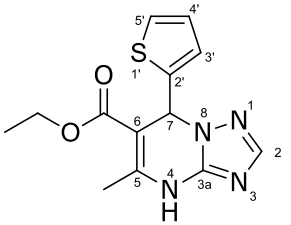
Methyl-5-methyl-7-(thiophen-2-yl)-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1f**)

From 3-amino-1,2,4-triazole (4mmol, 0.35g), 2-thiophenecarboxaldehyde (4mmol, 0.38 mL) and methyl acetoacetate (4 mmol, 0.44 mL); Condition A; Completed in 20 hours; Yield 18%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H, 4-NH), 7.73 (s, 1H, 2-CH), 7.41 (t, J = 3.2 Hz, 1H, Ar-CH), 6.94-6.92 (m, 2H, Ar-CH), 6.59 (s, 1H, 7-CH), 3.59 (s, 3H, ester-OCH3), 2.41 (s, 3H, 5-CCH3). SMILES CC(N1)=C(C(OC)=O)C(C2=CC=CS2)N3C1=NC=N3.



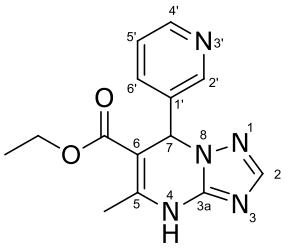
Ethyl-5-methyl-7-(thiophen-2-yl)-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1g**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 2-thiophenecarboxaldehyde (4mmol, 0.38 mL) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 41%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.92 (s, 1H, 4-N<u>H</u>), 7.73 (s, 1H, 2-C<u>H</u>), 7.41 (t, *J* = 2.9 Hz, 1H, Ar-C<u>H</u>), 6.93 (s, 2H, Ar-CH), 6.59 (s, 1H, 7-C<u>H</u>), 4.11 – 3.96 (m, 2H, ester-OC<u>H</u>$_2$CH$_3$), 2.41 (s, 3H, 5-CC<u>H</u>$_3$), 1.11 (t, *J* = 7.1 Hz, 3H, ester-OCH$_2$C<u>H</u>$_3$). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=CS2)N3C1=NC=N3.



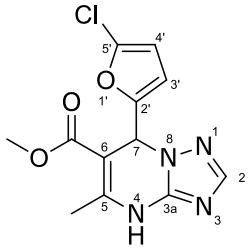
Ethyl-5-methyl-7-(pyridin-3-yl)-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1h**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 3-pyridinecarboxaldehyde (4mmol, 0.588 g) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 6%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.91 (s, 1H, 4-NH), 8.51 (d, J = 1.9 Hz, 1H, Ar-CH), 8.47 (dd, J = 4.8, 1.6 Hz, 1H, Ar-CH), 7.69 (s, 1H, 2-CH), 7.60 (dt, J = 7.9, 1.9 Hz, 1H, Ar-CH), 7.35 (dd, J = 7.8, 4.8 Hz, 1H, Ar-CH), 6.35 (s, 1H, 7-CH), 4.02 – 3.89 (m, 2H, ester-OCH_2CH$_3$), 2.44 (s, 3H, 5-CCH_3), 1.02 (t, J = 7.0 Hz, 3H, ester-OCH$_2$CH_3). SMILES CC(NC1=NC=NN12)=C(C(OCC)=O)C2C3=CC=CN=C3.



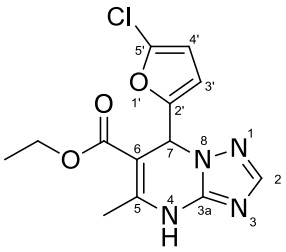
Methyl-7-(5-chlorofuran-2-yl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1i**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 5-chloro-2-furaldehyde (4mmol, 0.53 g) and methyl acetoacetate (4 mmol, 0.44 mL); Condition A; Completed in 20 hours; Yield 16%; yellow crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.96 (s, 1H, 4-NH), 7.74 (s, 1H, 2-CH), 6.45 (d, J = 3.3 Hz, 1H, Ar-CH), 6.39 (d, J = 3.4 Hz, 1H, Ar-CH), 6.34 (s, 1H, 7-CH), 3.59 (s, 3H, ester-OCH3), 2.41 (s, 3H, 5-CCH3). SMILES CC(N1)=C(C(OC)=O)C(C2=CC=C(Cl)O2)N3C1=NC=N3.



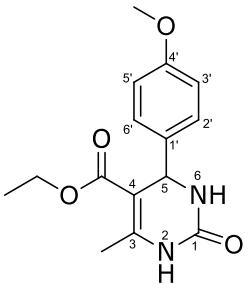
Ethyl-7-(5-chlorofuran-2-yl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (compound **1j**)

From 3-amino-1,2,4-triazole (4mmol, 0.35 g), 5-chloro-2-furaldehyde (4mmol, 0.53g) and ethyl acetoacetate (4 mmol, 0.512 mL); Condition A; Completed in 20 hours; Yield 30%; yellow crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.93 (s, 1H, 4-NH), 7.74 (s, 1H, 2-CH), 6.44 (d, J = 3.4 Hz, 1H, Ar-CH), 6.40 (d, J = 3.4 Hz, 1H, Ar-CH), 6.35 (s, 1H, 7-CH), 4.13 – 3.94 (m, 2H, ester-OCH2CH3), 2.41 (s, 3H, 5-CCH3), 1.13 (t, J = 7.1 Hz, 3H, ester-OCH2CH3). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C(Cl)O2)N3C1=NC=N3.



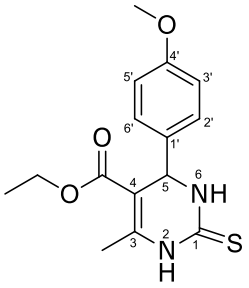
Ethyl 4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (compound **1k**)

From urea (13mmol, 0.9995 g), 4-methoxybenzaldehyde (10mmol, 1.276 mL) and ethyl acetoacetate (10 mmol) and $ZrCl_4$ (1 mmol, 0.12 g); Condition B; Completed in 58 mins; Yield 54%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.14 (s, 1H, 2-N<u>H</u>), 7.66 (s, 1H, 6-N<u>H</u>), 7.15 (d, *J* = 8.6 Hz, 2H, Ar-C<u>H</u>), 6.88 (d, *J* = 8.6 Hz, 2H, Ar-C<u>H</u>), 5.09 (d, 1H, *J* = 3.2 Hz, 5-C<u>H</u>), 3.98 (q, 2H, ester-OC<u>H</u>$_2$CH$_3$), 3.72 (s, 3H, 4'-COC<u>H</u>$_3$), 2.24 (s, 1H, 3-CC<u>H</u>$_3$), 1.11 (t, *J* = 7.1 Hz, 3H, ester-OCH$_2$C<u>H</u>$_3$). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C(OC)C=C2)NC1=O.



Ethyl-4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (compound **1l**)

From thiourea (13mmol, 0.9995 g), 4-methoxybenzaldehyde (10mmol, 1.276 mL) and ethyl acetoacetate (10 mmol) and $ZrCl_4$ (1 mmol, 0.12 g); Condition B; Completed in 15 mins; Yield 75%; white crystals; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H, 2-NH), 9.59 (s, 1H, 6-NH), 7.13 (d, J = 8.7 Hz, 2H, Ar-CH), 6.91 (d, J = 8.6 Hz, 2H, Ar-CH), 5.11 (d, 1H, J = 3.6 Hz, 5-CH), 4.01 (q, 2H, ester-OCH_2CH$_3$), 3.73 (s, 3H, 4'-COCH_3), 2.29 (s, 1H, 3-CCH_3), 1.11 (t, J = 7.1 Hz, 3H, ester-OCH$_2$CH_3). SMILES CC(N1)=C(C(OCC)=O)C(C2=CC=C(OC)C=C2)NC1=S.



3.4 Biological evaluation

3.4.1 Recombinant MBCOMT production and recuperation

The construction of the expression vector pPICZα-hMBCOMT, the *K. pastoris* transformation and selection for positive clones was previously done by our research group [8][180]. Unless otherwise stated, recombinant MBCOMT was carried out according to the following protocol. Cells containing the expression construct were grown at 30°C in YPD plates. A single colony was inoculated in 62.5 mL of BMG in 250 mL shake flasks. Cells were grown at 30°C and 250 rpm overnight when the optical density at 600 nm (OD_{600}) typically reached 6.0. Subsequently, since the inoculation volume was fixed to achieve an initial OD_{600} of 1.0; an aliquot was collected and centrifuged at room temperature for 5 min at 500G. After centrifuging the cells and ensure that all glycerol was removed, the cells were resuspended in the induction medium BMM and added to 500.0 mL shake flasks to a total volume of 100.0 mL. The fermentations were carried out during 24 h at 30°C and 250 rpm. For a larger scale of Batch and fed-batch processes, MBCOMT biosynthesis was carried out in mini-bioreactors (Infors HT, Switzerland) with a working volume of 0.25 L in modified basal salts medium (BSM) containing 4.35 mL/L of trace metal solution (SMT) [71]. The pH was adjusted to 4.7 to avoid salts precipitation and, consequently, undesired operational problems such as starvation of nutrients and optical densities measurement interferences. *K. pastoris* cultivations in bioreactor were initiated with a glycerol batch phase (30 g/L glycerol) that ends when glycerol was depleted, indicated by a sharp increase in the dissolved oxygen, to at least 40%. After this stage, a fed-batch growth on glycerol [20% (v/v) at 18.54 mL/L/h] for 2 hours, followed by the methanol induction phase where *K. pastoris* was cultivated on a methanol fed-batch mode. To promote the derepression of the AOX promoter before induction, 1 h before starting the induction phase, methanol was added to the bioreactor at a flow-

rate of 18.16 mL/L/h. Finally, the cells were harvested by centrifugation (1500×g, 10 min, 4°C) and stored at −20.0°C until further use. *K. pastoris* lysis was accomplished through the application of a sequential procedure involving glass beads consisting of 7 cycles of vortexing for 1 min with 1 min of interval on ice. Therefore, cell suspensions were lysed in lysis buffer (150mmol L^{-1} NaCl, 10mmol L^{-1} DTT, 50mmol L^{-1} Tris, 1mmol L^{-1}, $MgCl_2$, pH8.0) containing protease inhibitor cocktail at a ratio of 1:2:2 (1 g cells, 2 mL lysis buffer and 2 g glass beads) and, after the lysis process was completed, the mixture was centrifuged (500 g, 5min, 4°C). Finally, the supernatant was removed, and the pellet obtained was resuspended in the same lysis buffer but without DTT, DNase I was added (250 μgmL^{-1}), and the samples were stored at 4°C until used for further assays.

3.4.2 Western-Blot

Total protein was resolved by 12% SDS-APGE gel at 95V for 1h30 at room temperature in Running Buffer (Glycine 190 mM, Trizma-base 25 mM, SDS 1 mL, Mili-Q H_2O), and then, electro-transferred to a Polyvinylidene difluoride membrane (GE Healthcare, Sweden) at 0.75A for 1h45 in Transference Buffer (CAPS 10x, Methanol, Mili-Q H_2O). Membranes were blocked for 1h in a 5% (w/v) milk solution and incubated overnight with a rabbit polyclonal antibody against MBCOMT (GE Healthcare Biosciences), diluted at 1:1000, at 4°C with constant stirring. The membranes were then washed in TBS-T buffer [Tris (0.2M), NaCl (1.4M) and H_2O, pH 7.6] and incubated with a polyclonal antibody anti-rabbit (GE Healthcare Biosciences), diluted 1:40000, for 1 h at room temperature with constant stirring. Finally, the membranes were once more washed with TBS-T buffer, exposed to ECL substrate for 5 minutes protected from light and visualized on the Molecular Imager FX (Bio-Rad, Hercules, USA) [79].

3.4.3 Total Protein quantification

The protein quantification assay was carried out according to the instructions of the Pierce BCA Protein Assay kit (Thermo Scientific, USA) on a 96 well plate. A specific volume of working reagent (WR) was prepared based on the number of wells used in the assay from solutions A and B from the kit as represented in **Equation 1**.

Equation 1: $$V_{WR}(\mu L) = [(number\ of\ samples + number\ of\ blanks) \times 3] \times 200$$

The proportion of each solution used for the mixture was calculated based on **Equations 2** and **3**:

Equation 2: $$V_B\ (\mu L) = \frac{V_{WR}}{50}$$

Equation 3: $$V_A(\mu L) = V_{WR} - V_B$$

To quantify the sample proteins, calibration curves were needed. Therefore, several solutions with increasing concentrations of BSA (25, 125, 200, 500, 750, 1000, 1500, and 2000 μg/mL; n=3) were prepared in lysis buffer (150mmol L^{-1} NaCl, 10mmol L^{-1} DTT, 50mmol L^{-1} Tris, 1mmol L^{-1},

$MgCl_2$, pH8.0) diluted 1:100. For the blank, a solution of lysis buffer diluted 1:100 was used. Then, 20 µL of the prepared solutions were added to each well, followed by 80 µL of WR and slowly homogenized. The plate was covered in aluminium foil to protect it from light and incubated at 37ºC for 30 mins. After the incubation period the absorbances were measured at 562 nm. For the data analysis, from the value of the average of the sum of three replicates of each concentration the blank value was withdrawn, and the graphs were plotted (**Figure 11**).

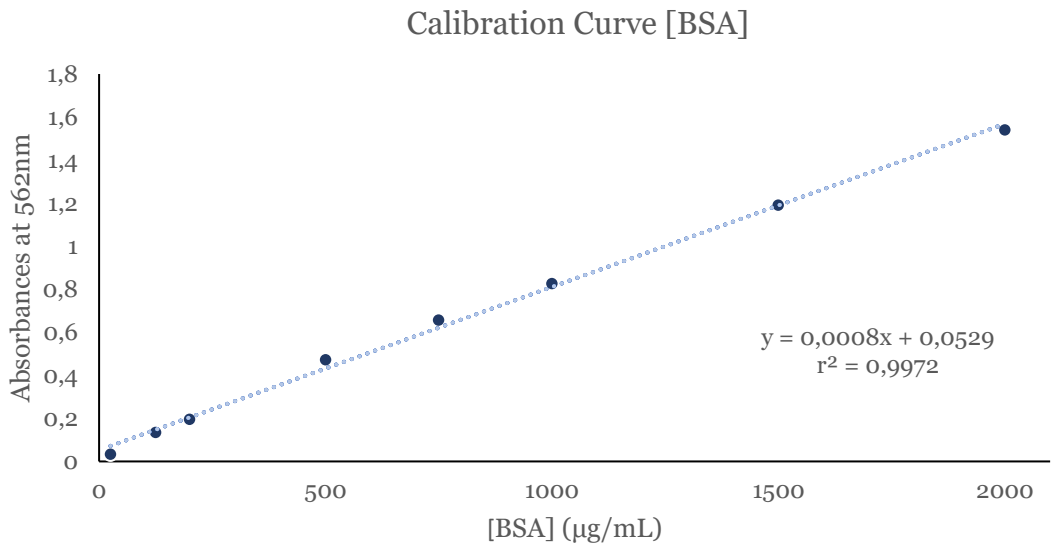


***Figure 11* - Calibration Curve for BSA, with the respective trendline equation and r².**

For the samples quantification the same protocol was employed with minor modifications. The sample was diluted in the same diluted lysis buffer. However, in the well, the sample was diluted 1:20 in lysis buffer, therefore firstly 19 µL of Mili-Q water was added to the well, followed by 1 µL of the sample and finally 80 µL of WR. The plate was also covered in aluminium foil and incubated for 30 mins at 37ºC before the absorbances were measured at 562 nm. The sample protein concentration was later calculated using the trendline equation in **Figure 11**.

3.4.4 MBCOMT enzymatic assay

To evaluate the methylation efficiency of human recombinant MBCOMT lysates, the amount of metanephrine formed from the COMT substrate epinephrine was quantified as previously described [105]. For this assay all the samples used were diluted to a final concentration of 1 mg/mL of protein, using the calibration curve trendline mentioned above (**Equation 4**).

$$\text{Equation 4: } [Sample]_{initial} \times V_{supernant} = 1_{mg/mL} \times V_{final}$$

The experiments were carried out in previously cooled and covered in aluminium foil test tubes. The assay consisted in the addition of 250 µL of the diluted lysate containing recombinant MBCOMT and 200 µL of incubation solution [0.272 mg/mL SAM, 1 µL/mL $MgCl_2$ (0.2M), 40

µL/mL EGTA (50 mM) and 959 µL/mL phosphate buffer pH 8.0] for 5 minutes in a water bath at 37ºC in constant stirring at 83 rpm. After 5 minutes of pre-incubation, 50 µL of epinephrine (3.331 µg/mL dissolved in incubation solution) were added. The enzymatic reaction occurred for 15 minutes. After this time, the reaction was stopped with the addition of 100 µL of perchloric acid (2M) and the tubes were transferred into ice. The tubes were then stored at 4ºC for 1h, after which the resulting samples were transferred to eppendorfs and centrifuged for 10 minutes at 4ºC at 6000 rpm. The resulting supernatant was filtered through a 0.22 µm pore size filter to remove precipitants and contaminants and injected to an HPLC system coupled with coulometric detection. Briefly, the chromatographic analysis was performed using an HPLC Agilent 1260 system (Agilent, Santa Clara, USA) equipped with an autosampler and quaternary pump coupled to an ESA Coulochem III detector (Milford, MA, USA). Chromatographic separation was achieved on an Hypersil™ ODS C18 Columns analytical column (250 × 4.6mm i.d. 5 µm) (Thermo Fisher Scientific, USA). The chromatographic method was developed using as mobile phase [0.1mol L^{-1} NaH_2PO_4, 0.024 mol L^{-1} citric acid monohydrate, 0.5 mmol L^{-1} sodium octyl sulfate, and 9% (v/v) acetonitrile] and the column effluent was monitored with an ED in the coulometric mode. This detector was equipped with a 5011 high sensitivity dual-electrode analytical cell (electrodes I and II) using a procedure of oxidation/reduction (analytical cell #1: +410mV; analytical cell #2: −350mV). The method sensitivity was set at 1 µA, the flow-rate applied was 1mL min^{-1} and the column temperature was optimized to 30ºC [105]. The chromatograms were obtained by monitoring the reduction signal of the working electrode where metanephrine retention time was around 12 mins in. Finally, the metanephrine content in samples was measured using metanephrine standards (1–15 nmol/L) as a calibration control. To quantify the exact concentration of metanephrine formed, a calibration curve was performed with various concentrations of metanephrine (0.25, 0.5, 1, 2, 4, 8, 12, and 15 nmol/mL) (**Figure 12**).

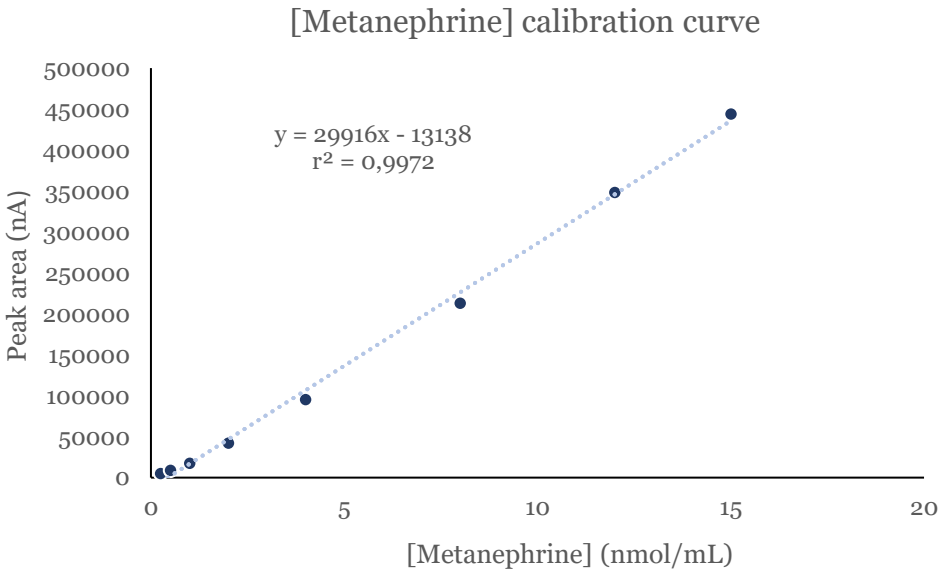


***Figure 12* – Calibration curve for metanephrine, with the respective trendline equation and r^2.**

The corresponding peak of metanephrine (elution time – 12.3 mins) was manually integrated and the area value was used to calculate the specific MBCOMT activity, using the trendline mentioned above.

3.4.5 MBCOMT inhibition

This assay was performed with minor modifications from the previous mentioned protocol [181]. To study the inhibitory profiles of commercial COMT inhibitors in human recombinant MBCOMT lysates, all the samples were diluted to a final concentration of 1 mg/mL. The assay consisted in the addition of 250 µL of the diluted lysate containing recombinant MBCOMT and 200 µL of incubation solution [0.272 mg/mL SAM, 1 µL/mL $MgCl_2$ (0.2M), 40 µL/mL EGTA (50 mM) and 959 µL/mL phosphate buffer pH 8.0] and 200 µL of the compounds **1a-l** and entacapone. After 20 minutes of incubation, 50 µL of epinephrine (3.331 µg/mL dissolved in incubation solution) were added. The enzymatic reaction occurred for 15 minutes. The reaction was stopped with the addition of 100 µL of perchloric acid (2M) and the tubes transferred into ice. The samples were later stored at 4ºC for 1 hour and the same protocol was employed as described in section 3.4.4.

3.4.5.1 Preparation of samples solutions

All the solutions were always freshly prepared for the assay and never reused. All the tested compounds were dissolved in DMSO in a final concentration of 10 mM and stored at 4ºC, protected from light. From this solution, several dilutions were prepared in incubation solution before each experiment. For the inhibition assays, final concentrations of 10 and 100 µM were prepared for each compound in a total volume of 1 mL. The maximum DMSO concentration used in these studies was 5% (v/v), a concentration with no significant effect on MBCOMT activity (data not shown).

3.4.6 Cell viability assays

A rat dopaminergic neuronal (N27) cell line was used, and the culture medium was changed every day. These cells were obtained from American type culture collection (ATCC), while culture media, reagents, and other required supplements were purchased from Sigma-Aldrich. To evaluate the effects on cell proliferation, the compounds in study were compared with 5-fluorouracil (5-FU) as the positive control.

3.4.6.1 Cell culture

The cells were maintained in an incubator at 37°C under humidified atmosphere containing 5% of CO_2. The N27 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), *L*-glutamine (2mM), and 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B).

3.4.6.2 Preparation of sample solutions

All the tested compounds were dissolved in DMSO in a final concentration of 10 mM and stored at 4°C protected from light. From this solution, several dilutions were prepared with the respective complete culture medium before each experiment. For the cytotoxicity assays concentrations of 10 and 100 µM were guaranteed in each well. The maximum DMSO concentration used in these studies was 1% (v/v), a concentration with no significant effect on cell proliferation (data not shown).

3.4.6.3 MTT assay

The cell proliferation was evaluated by quantifying the extent of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described in [182]. Cells were seeded in 96-well plates ($2x10^4$ cells/ml, 100µL in each well) in the respective culture medium and incubated at 37°C. After 48 hours of cell adherence they were incubated at 37°C with the compounds, in 10 and 100 µM, for 72 hours. Untreated cells were used as negative control, whereas 5-FU was used as positive control. At the end of the 72 hours of incubation, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS) solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, and KH_2PO_4 1.8 mM at pH 7.4), and further incubated for 4 hours with MTT solution (5 mg mL^{-1} prepared with 20% PBS and 80% incomplete culture medium). Lastly, the medium containing MTT was removed and the formazan crystals were dissolved in DMSO followed by absorbance readings at 570 nm. The cytotoxicity was expressed as relative cell proliferation in percentage in comparison with the negative control cells.

Chapter 4 - Results and Discussion

4.1 *In-silico* studies

Based on the structure of the compounds that we aimed to prepare, can be considered catechol bioisosteres and, therefore, could have a specific affinity to interact with COMT. An *in-silico* analysis was performed to evaluate this hypothesis and to determine which interactions are formed with the protein active site. For this a molecular docking study was conducted in AutoDock Tools 4 to study the compound's binding affinity and interactions with COMT. The crystal COMT structure used was retrieved from the PDB (10/05/2019). In addition, the ADMET properties as well as the druglikeness were also predicted for these compounds.

4.1.1 Molecular Docking

The crystal structure of human SCOMT complexed with 3,5-DNC, SAM, and Mg^{2+} (PDB#6I3C) with a resolution of 1.336Å is the crystal structure of a human COMT with the best resolution submitted to date in PDB (29/10/2020) [15]. The docking methodology used was validated by re-docking the ligand 3,5-DNC, obtaining an RMSD value of 1.60 Å (lower than 2 Å) (**Table 3**). The simulations were performed using the same protocol described previously in the materials and methods section. The main outputs are displayed in **Table 3**.

***Table 3* - Molecular docking results for the tested compounds. In bold are highlighted the main hydrogen and hydrophobic bond interactions.**

Docking results			
Ligand	**Binding energy (kcal/mol)**	**Molecular interactions**	
		Hydrogen bonds	**Hydrophobic bonds**
3,5-DNC	-4.85	Asn170, **Glu199**	Met40, **Pro174**
Entacapone	-6.1	His142, **Lys144**, Asn170, **Glu199**	**Trp38**, Met40, Cys173, **Pro174**, Leu198
1a	-6.65	Trp143, Asn170, **Lys144**	Met40, **Pro174**, Glu199
1b	-6.6	**Lys144**, Asn170	Met40, **Trp143**, **Pro174**, Leu198
1c	-7.94	**Lys144**, Asn170	**Trp38**, Met40, **Pro174**, Glu199
1d	-6.44	**Lys144**, **Glu199**	Met40, **Pro174**, Leu198
1e	-6.94	**Lys144**, **Glu199**	Met40, **Trp143**, **Pro174**, Leu198
1f	-6.42	**Lys144**, **Glu199**	**Trp38**, Met40, **Trp143**, **Pro174**, Leu198
1g	-6.65	**Lys144**, **Glu199**	Met40, **Trp38**, **Trp143**, **Pro174**, Leu198
1h	-6.13	**Lys144**	Met40, **Trp143**, **Pro174**
1i	-6.7	**Lys144**, **Glu199**	**Trp38**, Met40, **Trp143**, **Pro174**, Leu198
1j	-6.86	**Lys144**, **Glu199**	**Trp38**, Met40, **Pro174**, Leu198
1k	-5.6	**Lys144**, Asn170	Met40, **Trp143**, **Pro174**, Glu199
1l	-6.1	Asn170, **Glu199**	**Trp38**, Lys144

According to the literature, the most important interactions between the protein active site and the commercial COMT inhibitors are hydrogen bonds with residues Lys144 and Glu199, and hydrophobic interactions with residues Trp38, Trp143, and Pro174 [18]. The substrate forms with the protein active site the majority of these interactions as well. So, if a compounds binds to the same aa as the substrate, they might compete with it the binding site. Based on the information displayed in **Table 3,** most of the compounds interact with these aa and with a high binding energy. Comparing with 3,5-DNC and Entacapone, only **1l** of the tested compounds exhibits a higher binding energy than the screened COMT inhibitors. This behaviour might be due to the lack of interactions formed with the Sulfur atom in its structure. The specific interactions of the various compounds with the protein active site are displayed in **Figures 13-26**.

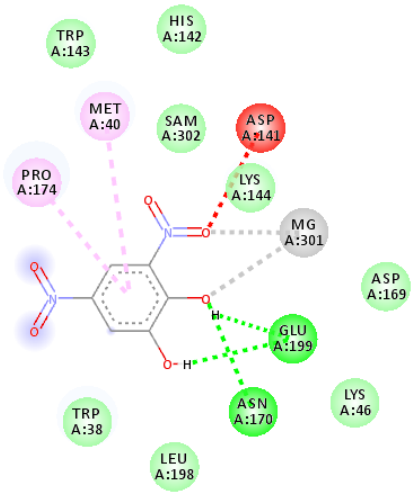


Figure 13 **- Interactions of 3,5-DNC with SCOMT. Green dashes - Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Gray dashes – Metal interaction; Red dashes – Unfavorable bump.**

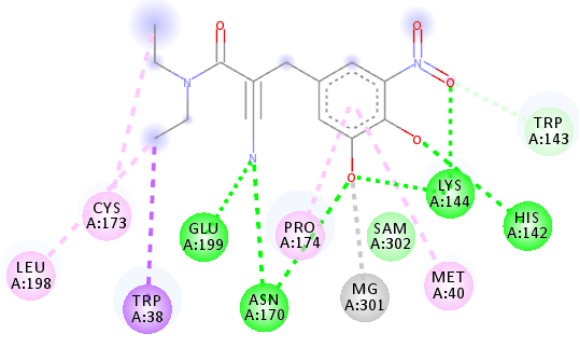


Figure 14 **- Interactions of Entacapone with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Gray dashes – Metal interaction.**

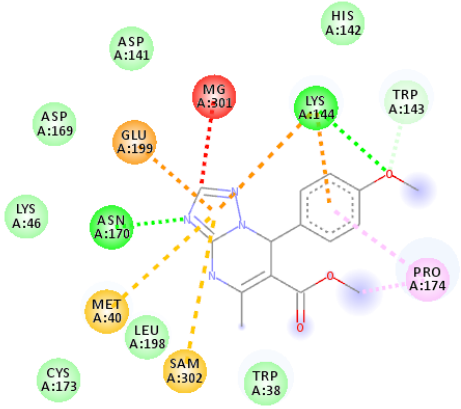


Figure 15 **- Interactions of 1a with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

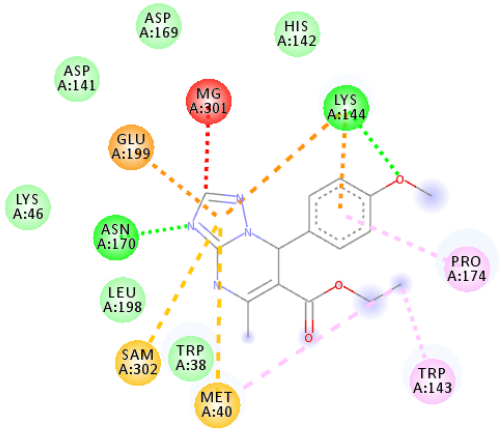


Figure 16 **- Interactions of 1b with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

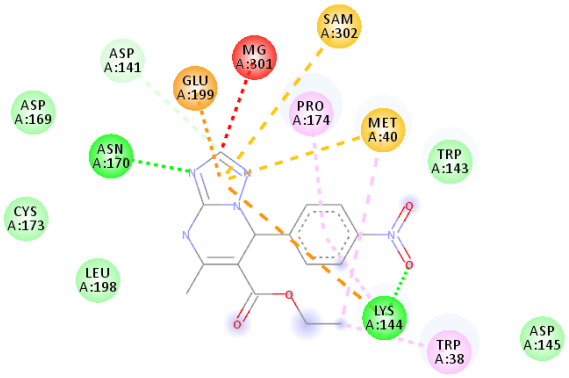


Figure 17 **- Interactions of 1c with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

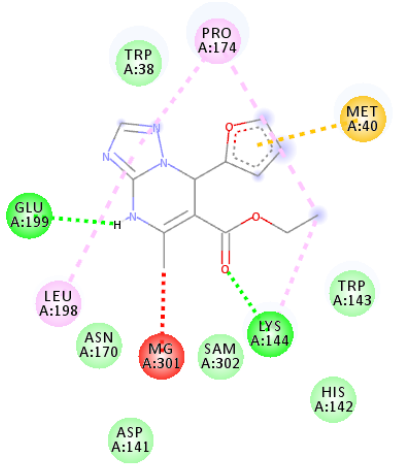


Figure 18**- Interactions of 1e with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump**

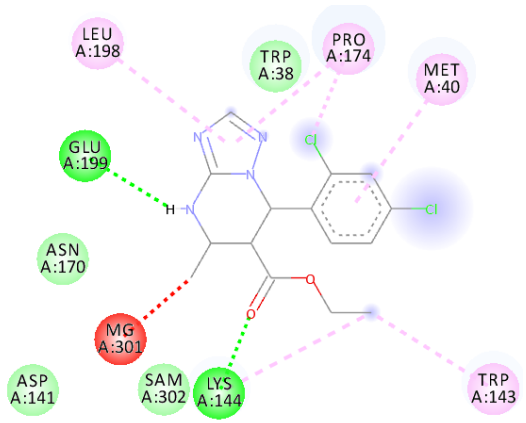


Figure 19 **- Interactions of 1d with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump**

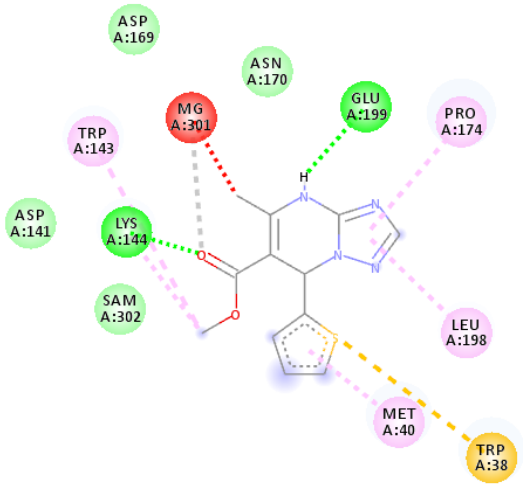


Figure 20 **- Interactions of 1f with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

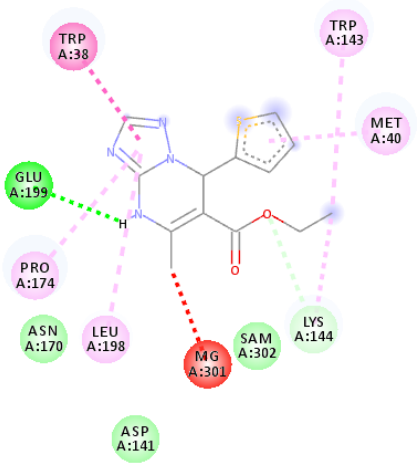


Figure 21 **- Interactions of 1g with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

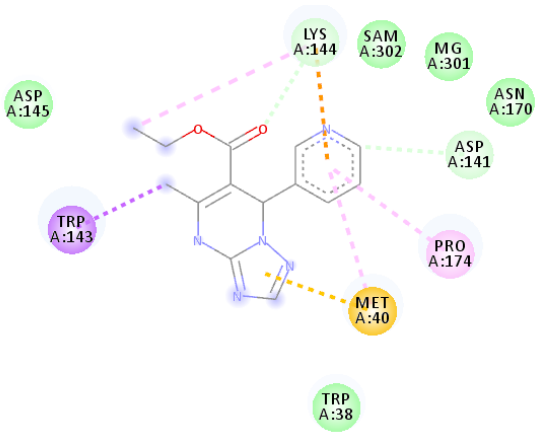


Figure 22 **- Interactions of 1h with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

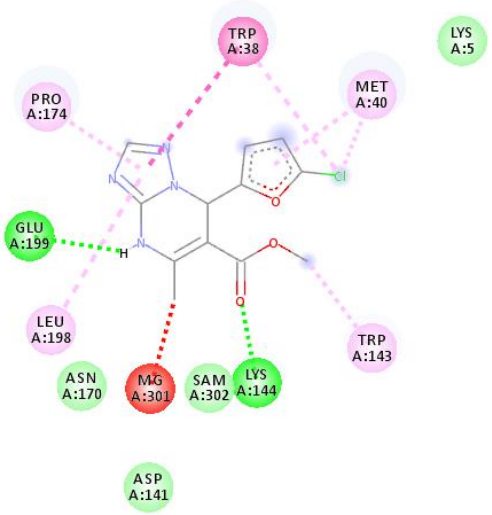


Figure 23 **- Interactions of 1i with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

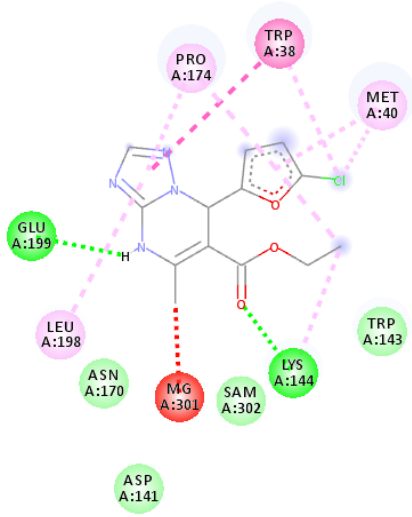


Figure 24 **- Interactions of 1j with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Red dashes - Unfavorable bump.**

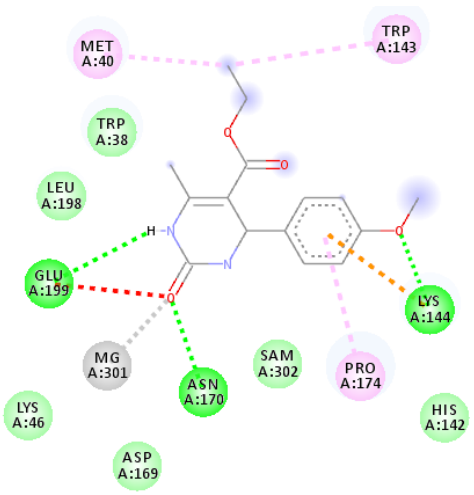


Figure 25 **- Interactions of 1k with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Gray dashes – Metal interactions.**

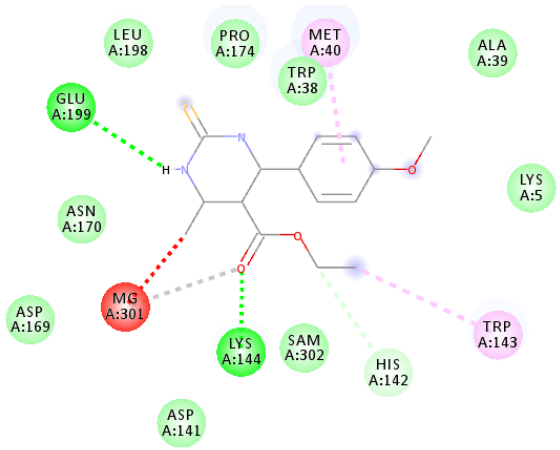
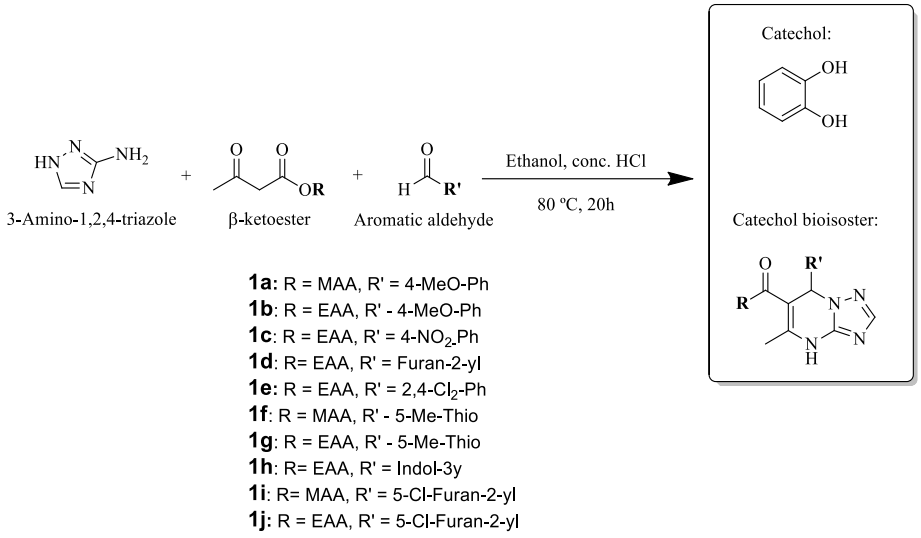


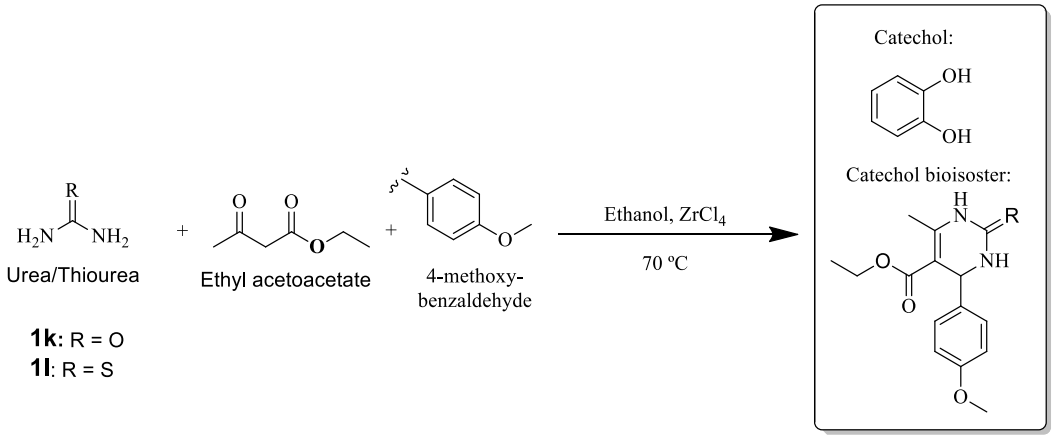
Figure 26 **- Interactions of 1l with SCOMT. Green dashes – Hydrogen bonds; Light green dashes – van der Waals interactions; Yellow dashes – Pi-Sulfur interactions; Orange – Pi-Cation/Anion interactions; Pink – Pi-Alkyl interactions; Gray dashes – Metal interactions.**

4.2 Chemical Synthesis

In this topic the experimental results obtained in the preparation of the compounds are presented (**Table 4**) and discussed. In a first instance, the synthesis of triazolopyrimidines **1a-j** (**Scheme 4**) will be presented, followed by the synthesis of compounds **1k** and **1l** (**Scheme 4** and **5**), prepared for comparison purposes.



Scheme 4 **- Synthesis of triazolopyrimidines 1a-j.**



Scheme 5 **- Synthesis of 3,4-dihydropyrimidin-2-(1H)-one/thione 1k and 1l.**

Table 4 - Conditions used for the synthesis of the compounds 1a-l.

Synthesis conditions				
Compound	**Condition**	**Temperature** (ºC)	**Time** (h)	**Yield** (%)
1a	A	80	20	16%
1b	A	80	20	11%
1c	A	80	20	17%
1d	A	80	20	26%
1e	A	80	20	22%
1f	A	80	20	18%
1g	A	80	20	41%
1h	A	80	20	6%
1i	A	80	20	16%
1j	A	80	20	30%
1k	B	70	58 mins	54%
1l	B	70	15 mins	75%

In Table 4 are summarized the chemical conditions employed for the preparation of the compounds 1a-1l. Although the conditions were very similar in most reactions, the yields are very different. One of the reasons for lower yields can be due to the selection of the acid catalyst used, in this case we used hydrochloric acid, but there were other alternatives that could result in higher yields [166,168,169,174], unfortunately they were unavailable at the moment for our research group. The compounds 1k and 1l exhibit much higher yields when compared with the majority of the other compounds, this is because this reaction was previously optimized by our research group, using other substituents and catalysts [183,184]. In addition to that, all the final products were successfully characterized and identified by NMR and obtained in sufficient amounts for further assays. Also, the prepared compounds possess a chiral centre, which can influence the molecules binding affinity with the protein [185].

4.3 Biosynthesis and Isolation of MBCOMT

As previously stated, similarly to the majority of membrane proteins, MBCOMT is present in cells at lower concentrations than the soluble form in most tissues [70]. However, for structural and functional studies, high amounts of highly stable and biologically active protein are required. Therefore, it is essential to employ a biotechnological bioprocess capable of producing high levels of MBCOMT with a high purity degree. Recently, our research group developed a bioprocess using K.pastoris with high production yields. This single-cell microorganism is easy to manipulate, cultivate, and has the capacity to perform posttranslational modifications as higher eukaryotic cells [186]. Considering previous studies developed by Passarinha's research group, the MBCOMT

biosynthesis was successfully performed in a *K. pastoris* expression system, using as a construct the plasmid pPICZα-hMBCOMT [180]. So, the first part of this work consisted in the production and further validation of MBCOMT protein. For the production, we used the conditions and methodologies previously described [180]. A pre-fermentation process was carried out in BMG medium inoculated with a single colony for approximately 18/20h until the OD_{600} typically reached 5/6. The fermentation process was carried out in a BMM medium with the initial OD_{600} at 1.0 and for 24 h. After this, the medium was centrifuged at 1000G for 5 minutes and the corresponding pellet was stored at -20ºC for further studies. The resulting pellets were lysed using the previously mentioned protocol in the materials and methods section 3.4.4. To confirm that the lysis was efficient, a western blot analysis was performed in both the obtained supernatant (S_{500}) and the corresponding pellet (P_{500}) of the lysis of MBCOMT. Using a specific antibody against MBCOMT, two immunoreactive bands were found approximately at 30 and 35 kDa. Considering that the molecular weight of MBCOMT is approximately 30 kDa, our results suggest that the first band detected corresponds to MBCOMT protein. The band at 35 kDa, an increase of 5 kDa in the molecular weight of the target protein, may correspond to MBCOMT with the alpha mating factor still attached, as previously demonstrated by our research group [51]. This may be due to the inefficient cleavage of the alpha mating factor by the Kex2 protease, displaying an increase in the molecular weight, which is consistent to the obtained in this work (**Figure 26**). With this study we also demonstrated that the lysis method employed was efficient and the P500 presented the higher amount of MBCOMT, demonstrated by a higher intensity band in **Figure 26**.

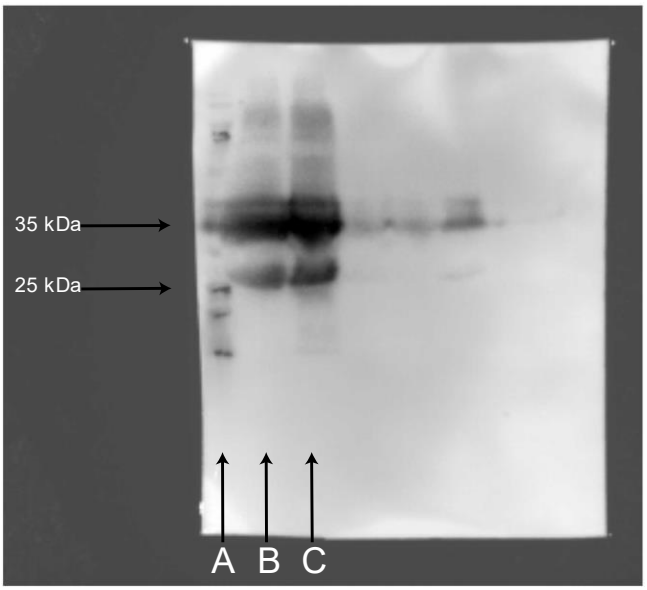


Figure 27 **- Western blot analysis of MBCOMT. A – Protein Molecular weight marker; B – S_{500}; C – P_{500}.**

4.4 Evaluation of Biological Activity

In this section the experimental results obtained in the *in vitro* screening of the biological evaluation of the synthesized compounds **1a-l**, are presented and discussed. In the subtopic 4.3.1. the results of the MBCOMT specific activity after incubation with the compounds **1a-l** in human recombinant MBCOMT lysates will be discussed, followed by the subtopic 4.3.2 in which the results of the cell viability assays in N27 cells will be discussed. Lastly, the general SAR of the synthesized compounds **1a-l** for the different biological assays tested in this work will be presented. The results are presented as average with their standard error of the means (SEM) and the difference between groups was considered statistically significant at $p < 0.05$ (Student's *t*-test).

4.4.1 MBCOMT specific activity assay

The effect of compounds **1a-l** on the specific activity of MBCOMT lysates was evaluated by quantifying the amount of metanephrine formed by injecting the samples in a HPLC coupled with an ED, using recombinant MBCOMT lysates as the negative control and entacapone as positive control. The corresponding peak of metanephrine (elution time – 12.3 mins) was manually integrated and the area value was used to calculate the specific MBCOMT activity, using the trendline mentioned above. For all the samples the resulting lysis pellet (P_{500}) was used, and the same methodology was employed. In **Figure 28-33** are represented the respective chromatograms, in **Figure 34** and **Table 5** are represented the specific activities after incubating with the compounds **1a-l** and with the commercial COMT inhibitor entacapone.

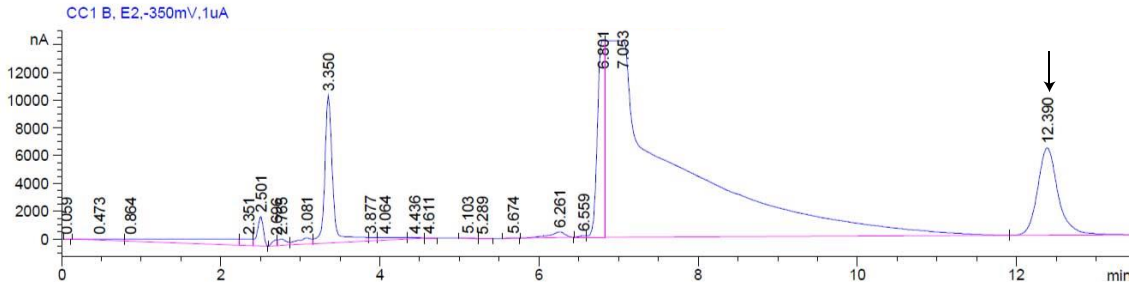


Figure 28 **- Chromatogram of MBCOMT lysate (Control). Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.390 min.**

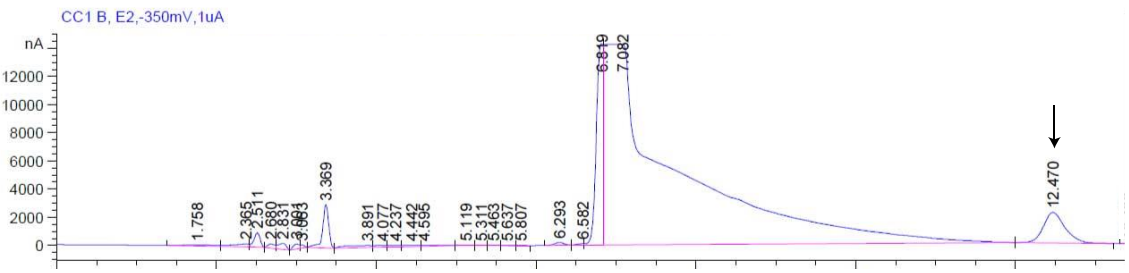


Figure 29 **- Chromatogram of MBCOMT lysate + Entacapone 10 µM. Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.470 min.**

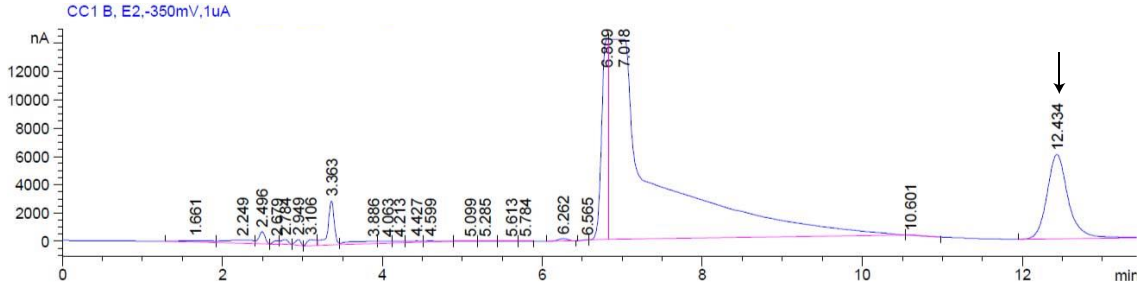


Figure 30 **- Chromatogram of MBCOMT lysate + 1a 10 µM. Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.415 min.**

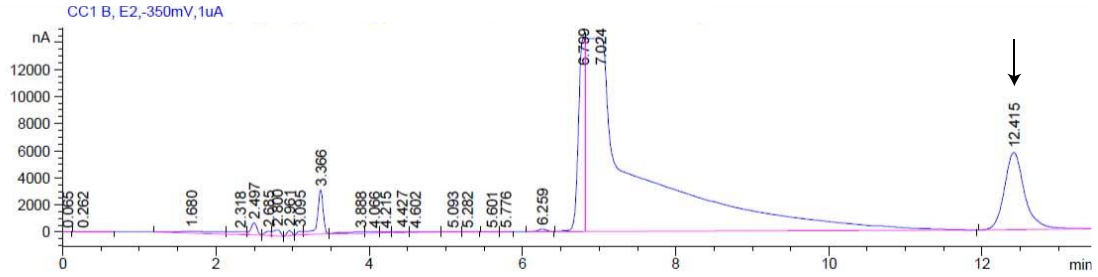


Figure 31 **- Chromatogram of MBCOMT lysate + 1a 100 µM. Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.434 min.**

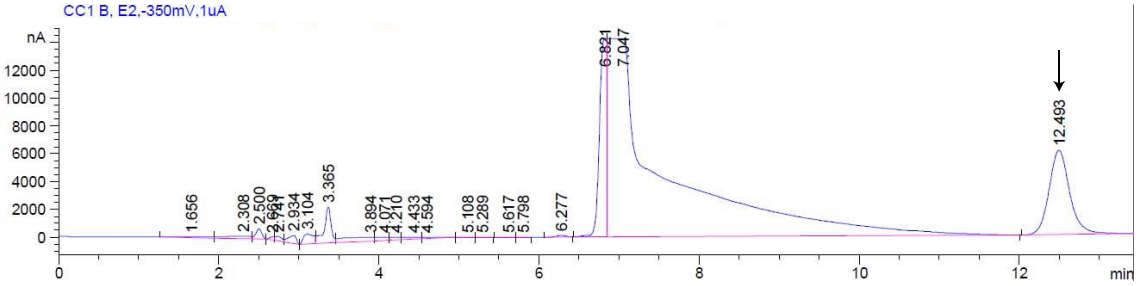


Figure 32 **- Chromatogram of MBCOMT lysate + 1b 10 µM. Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.493 min.**

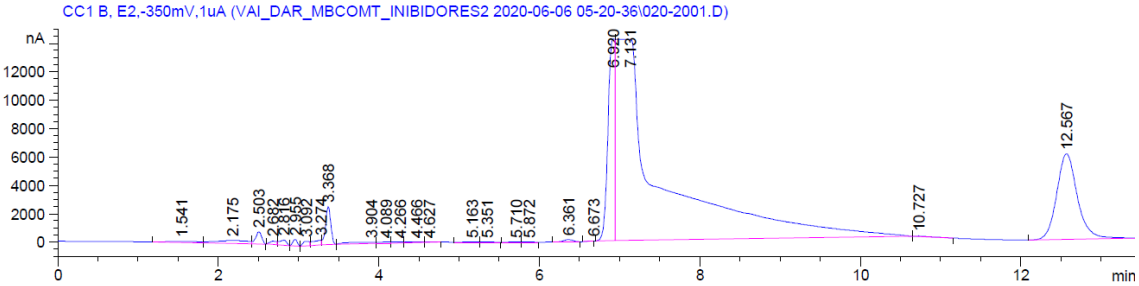


Figure 33 **- Chromatogram of MBCOMT lysate + 1b 100 µM. Electrode 2 (reduction channel: -350 mV). Retention time (metanephrine) of 12.567 min.**

***Table 5* – Compounds effect on MBCOMT specific activity.**

COMT activity	
Condition tested	**Specific activity** (nmol/h/mg of protein)
MBCOMT lysate (**Control**)	34.62 ± 1.96
MBCOMT lysate + **Entacapone** 10 µM	14.32 ± 4.97
MBCOMT lysate + **1a** 10 µM	45.12 ± 1.93
MBCOMT lysate + **1a** 100 µM	43.04 ± 1.26
MBCOMT lysate + **1b** 10 µM	47.42 ± 3.32
MBCOMT lysate + **1b** 100 µM	43.24 ± 2.80

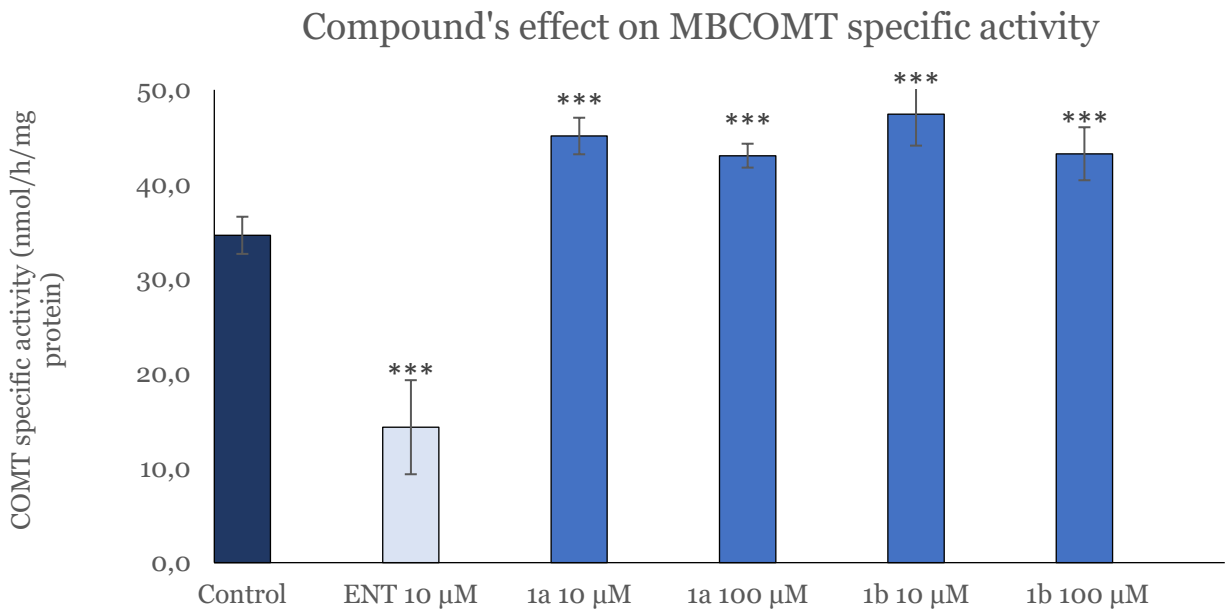


***Figure 34* – MBCOMT specific activity values in nmol/h/mg of protein. Control -Dark blue; Entacapone (ENT) – Light blue; Compounds – Blue.**

The compounds were incubated in MBCOMT human recombinant lysates at 10 and 100 µM. At both concentrations, the compounds were expected to decrease the enzyme specific activity, with a more significant inhibition at 100 µM. Due to the cost and time spent for the assay, the compounds were divided and tested separately. A compound would be considered COMT inhibitor if at any of the tested concentrations a decrease in the MBCOMT specific activity was shown after incubation with the compound. To validate the results, the negative control had to display significant activity, if only exhibited residual activity the assay would not be considered valid. The results for these assays are summarized in **Table 5** and **Figure 34**. Of all the tested compounds only the results for compounds **1a** and **1b** were validated. For all the other compounds the control displayed residual activity (for example 5.12 ± 0.63 nmol/h/mg of protein) and the activity values with and without compounds incubation were very similar, and no conclusions

could be draw from this assay (data not shown). However, compounds **1a** and **1b** at both concentrations increased the enzyme activity. Surprisingly, with both compounds at the concentration of 10 μM a higher increase was observed in MBCOMT activity when compared with 100 μM. However, as expected with an increase in the compound concentration there is a slight decrease in the enzyme activity. Due to the compounds structure similarity, **1a** and **1b** induced very similar effects on MBCOMT activity. Based on these results **1a** and **1b** can be considered, at this experimental phase, as MBCOMT stabilizers

4.4.2 Cell viability assays

To evaluate the effect of compounds **1a-l** on cell proliferation, a MTT assay was performed, after 72 hours of incubation of these compounds in the cells. The effects on the cell proliferation were compared to the observed with 5-fluorouracil (5-FU) as a positive control in N27 cell lines. To determine the cytotoxic potential of compounds **1a-l** in N27 cells, a screening at concentrations 10 and 100 μM was also performed.

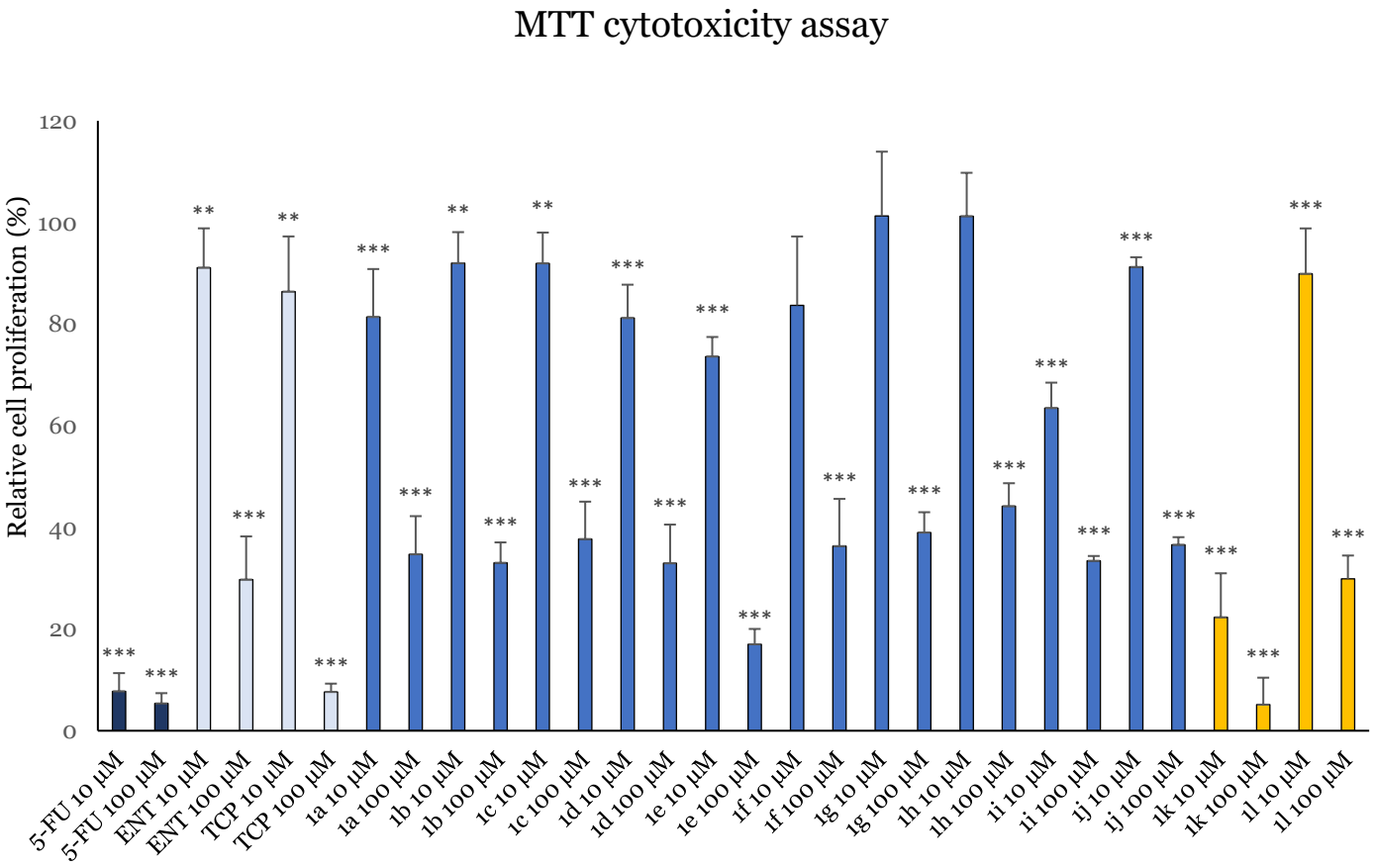


Figure 35 **- Percentage of relative cell proliferation of N27 cells after 72 hours exposure to 5-fluorouracil (5-FU), Entacapone (ENT), Tolcapone (TCP), and compounds 1a-l at concentrations of 10 and 100 μM. The data are presented as average values with their SEM. Compounds prepared using condition A are represented in blue. Compounds prepared using condition B are represented in yellow.**

Based on the results displayed in **Figure 35** it can be concluded that: all compounds at the concentration of 10 μM showed no cytotoxicity, except for **1k** that was toxic, with a relative cell proliferation of 22%, approximately; as expected, at 100 μM, due to the increase in compounds concentration, a sharp decrease in the relative cell proliferation was observed. The majority of compounds exhibited a similar or even higher relative cell proliferation than the commercial COMT inhibitors, Entacapone and Tolcapone. As expected Entacapone and Tolcapone did not exhibit cytotoxicity at 10 μM similarly to the results obtained by other researchers [104,187]. In addition, other groups performed cytotoxicity studies of with other similar triazolopyrimidines derivatives in cancerous cell lines, namely colorectal carcinoma (HCT-116), hepatocarcinoma (HepG2), breast cancer (MCF-7), some of these compounds inhibited the growth of the tested cell lines with IC_{50} values in the sub micromolar range and high selectivity towards tumorous cells [188]. Additionally, our research group also studied the cytotoxicity of compounds **1k** and **1l** and other similar structures in N27, hepatic (HepaRG), normal dermal fibroblasts (NHDF), intestinal (Caco-2), and breast cancer (MCF-7) cell lines [184]. The tested concentration was 30 μM. The results in N27 cells were very similar to the ones obtained in this work and in HepaRG cells, the compound **1k** did not exhibit marked cytotoxic activity. On the other hand the structure of **1l** exhibited strong inhibition of cell proliferation (IC_{50} – 41.48 μM). Both compounds showed no marked cytotoxicity for the Caco-2 cell line. Finally, these compounds were studied in NHDF cells and did not show marked cytotoxicity in these dermal cells.

4.5 Structure-Activity Relationship

A brief systematization of SAR information for compounds **1a-l** is displayed in **Figures 36** and **37** for a straightforward interpretation of the obtained results. Overall, from the analysis of the *in vitro* results, compounds incorporating an ethyl acetoacetate (EAA) moiety stabilized more MBCOMT than the ones with methyl acetoacetate (MAA), but the contrary happens in cell cytotoxicity for the triazolopyrimidines derivatives. Specifically, in terms of cell cytotoxicity, compounds **1e** and **1i**, both incorporating a chlorine atom, were the more toxic for the cells. For the compounds **1k** and **1l**, we can conclude that the thiourea derivate (1l) was less toxic than the urea derivative (1k).

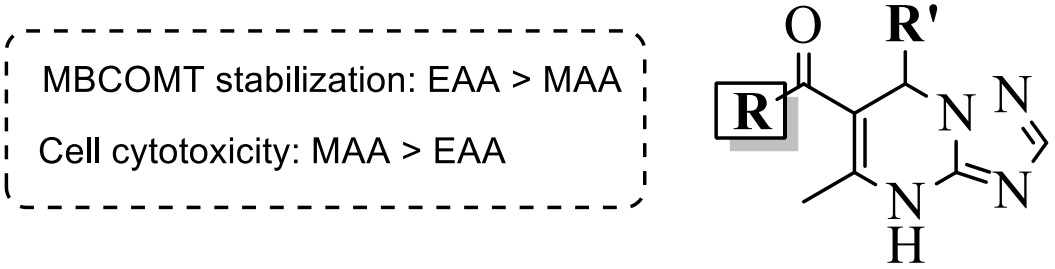


Figure 36 **- SAR of triazolopyrimidines 1a-j for MBCOMT stabilization and N27 cell cytotoxicity.**

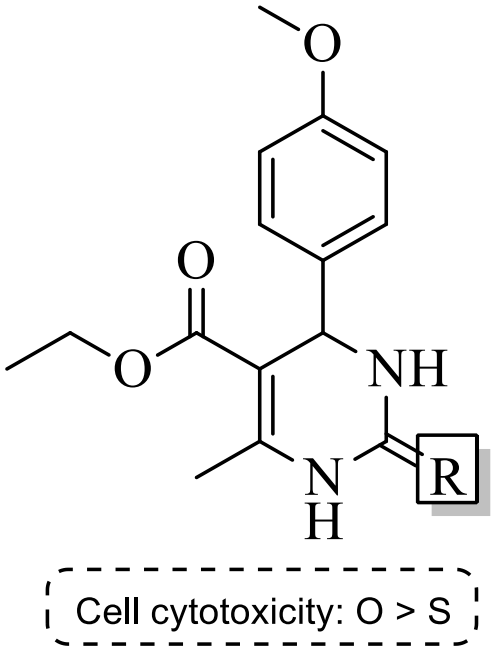


***Figure 37* - SAR of compounds 1k and 1l for N27 cell cytotoxicity.**

4.6 Pharmacokinetics, toxicity and druglikeness prediction

To acquire additional information about the most relevant pharmacokinetic properties for the synthesized compounds, an online analysis was performed in the software's pkCSM and SwissADME. The results are presented in **Tables 6-11**. None of the screened molecules violated any of Lipinski's rules (Molecular mass under 500 Daltons, lipophilicity Log P o/w < 5, less than 5 hydrogen bonds donors and less than 10 hydrogen bond acceptors) nor Veber rules (10 or fewer routable bonds and polar surface area no greater than 140 A^2) as represented in **Table 6**. This data indicates that these molecules may have good oral bioavailability. Concerning the absorption properties (**Table 7**), none of the screened molecules displayed good water solubility, as expected from the groups in their chemical structures. However, all compounds exhibited an intestinal absorption higher than 30% and, therefore can be absorbed. The derivative **1c**, Entacapone, and 3,5-DNC were predicted as potential P-glycoprotein substrates, and only **1c** was considered to be a P-glycoprotein I inhibitor. This result can be due to the presence of nitro and phenyl groups, which can increase the formation of hydrophobic interactions with the protein. Regarding the distribution properties (**Table 8**), the majority of the compounds demonstrated to have low potential to penetrate both the BBB and the CNS, with exception of compounds **1k** and **1l** which might be potential BBB^+. Concerning the metabolism properties (**Table 9**), none of the compounds was considered to be CYP2D6 substrate or inhibitor, neither CYP3A4 inhibitor. However, the majority of the molecules were predicted as CYP3A4 substrate and CYP1A2 inhibitors, with **1e** being also a CYP2C19 inhibitor. Only compounds **1k** and **1l** did not show any negative result in this category. This data can indicate that the cytochrome P450 isoenzymes can dramatically interfere in the molecules' pharmacokinetics. In terms of excretion properties (**Table 10**), none of these compounds was considered a renal OCT2 substrate. Concerning the

toxicity properties (**Table 11**), Entacapone, 3,5-DNC, and **1c** might be mutagenic, as AMES toxicity can occur, possibly due to the nitro groups. In addition, all compounds display a low maximum tolerated dose, which might confer a potential risk in clinical use. However, none of the compounds was considered to be hERG I and II inhibitors, but all of them could cause potential hepatoxicity. This data can be very relevant for the developing of future studies, despite the potential differences with the real properties.

***Table 6* - Predicted physicochemical and druglikeness properties, COMT inhibitor (grey cells), positive result (green cells), and negative result (red cells)**

Compounds	Physiochemical properties					Druglikeness	
	Molecular Weight	Routable Bonds	H-Bond Acceptors	H-Bond Donors	Lipophilicity	Lipinski's Rule	Veber Rule
	Violates > 500 Daltons	Violates > 10	Violates > 10	Violates > 5	Violates: Log $P_{o/w}$ > 5	Nº of Violations	Nº of Violations
1a	300,32	3	7	1	1,61	0	0
1b	314,35	4	7	1	1,93	0	0
1c	329,32	4	8	1	1,2	0	0
1d	274,28	3	7	1	1,26	0	0
1e	353,21	3	6	1	2,97	0	0
1f	276,32	2	7	1	0,95	0	0
1g	290,35	3	7	1	1,91	0	0
1h	285,31	3	7	1	1,11	0	0
1i	294,70	2	7	1	1,59	0	0
1j	308,73	3	7	1	1,9	0	0
1k	290,32	4	4	2	1,52	0	0
1l	306,39	4	4	2	2,05	0	0
3,5-DNC	200,11	2	6	2	-0,38	0	0
Entacapone	305,29	5	6	2	0,93	0	0

***Table 7* - Predicted Absorption Properties, COMT inhibitor (grey cells), a positive result (green cells), and negative result (red cells)**

Compounds	Absorption properties				
	Water Solubility	Intestinal absorption	P-glycoprotein substrate	P-glycoprotein I inhibitor	P-glycoprotein II inhibitor
		Absorbs: % > 30%	Yes/No	Yes/No	Yes/No
1a	-3,25	97,68	No	No	No
1b	-3,55	97,15	No	No	No
1c	-4,09	86,34	Yes	Yes	No
1d	-3,29	96,24	No	No	No
1e	-4,80	93,28	No	No	No
1f	-3,01	94,81	No	No	No
1g	-3,34	94,25	No	No	No
1h	-2,80	97,01	No	No	No
1i	-3,78	95,01	No	No	No
1j	-4,09	94,48	No	No	No
1k	-3,34	94,40	No	No	No
1l	-3,66	94,41	No	No	No
3,5-DNC	-2,39	78,37	Yes	No	No
Entacapone	-3,15	70,52	Yes	No	No

Table 8 **- Predicted Distribution properties, COMT inhibitor (grey cells), BBB^+/CNS^+ (green cells), and BBB^-/CNS^- (light yellow cells)**

Compounds	Distribution properties	
	BBB permeability	**CNS permeability**
	BBB^-: logBB < -1	CNS^-: logPS < -3
	BBB^+: logBB > 0.3	CNS^+: logPS > -2
1a	-0,70	-3,06
1b	-0,72	-3,06
1c	-0,90	-2,71
1d	-0,76	-3,11
1e	-0,79	-2,96
1f	-0,63	-3,00
1g	-0,65	-3,00
1h	-0,81	-3,13
1i	-0,93	-3,12
1j	-0,95	-3,11
1k	0,03	-2,99
1l	0,02	-2,97
3,5-DNC	-0,76	-2,66
Entacapone	-0,89	-2,79

***Table 9* - Predicted Metabolism properties, COMT inhibitor (grey cells), a positive result (green cells), and negative result (red cells).**

Compounds	Metabolism properties					
	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No
1a	No	Yes	No	No	No	No
1b	No	Yes	No	No	No	No
1c	No	Yes	No	No	No	No
1d	No	No	Yes	No	No	No
1e	No	Yes	Yes	Yes	No	No
1f	No	No	Yes	No	No	No
1g	No	Yes	Yes	No	No	No
1h	No	Yes	No	No	No	No
1i	No	No	Yes	No	No	No
1j	No	No	Yes	No	No	No
1k	No	No	No	No	No	No
1l	No	No	No	No	No	No
3,5-DNC	No	No	No	No	No	No
Entacapone	No	Yes	Yes	No	No	No

Table 10 **- Predicted Excretion properties, COMT inhibitor (grey cells), a positive result (green cells).**

Compounds	Excretion properties
	Renal OCT2 substrate
	Yes/No
1a	No
1b	No
1c	No
1d	No
1e	No
1f	No
1g	No
1h	No
1i	No
1j	No
1k	No
1l	No
3,5-DNC	No
Entacapone	No

Table 11 - Predicted Toxicity properties, COMT inhibitor (grey cells), a positive result (green cells), and negative result (red cells)

Compounds	Toxicity properties				
	AMES toxicity	Max. tolerated dose	hERG I inhibitor	hERG II inhibitor	Hepatotoxicity
	Yes = Mutagenic	Low < 0.477 < High	Yes/No	Yes/No	Yes/No
1a	No	0,12	No	No	Yes
1b	No	0,06	No	No	Yes
1c	Yes	-0,46	No	No	Yes
1d	No	0,28	No	No	Yes
1e	No	0,09	No	No	Yes
1f	No	0,05	No	No	Yes
1g	No	-0,03	No	No	Yes
1h	No	0,10	No	No	Yes
1i	No	0,32	No	No	Yes
1j	No	0,24	No	No	Yes
1k	No	-0,12	No	No	Yes
1l	No	-0,20	No	No	Yes
3,5-DNC	Yes	-0,41	No	No	No
Entacapone	Yes	0,30	No	No	No

Chapter 5 – Conclusions

Given the physiological relevance of COMT in the *O*-methylation of catecholamines and catechol estrogens, as well as the number of diseases associated with this enzyme, in the last decades, COMT is seen as a therapeutic target of high clinical impact. The current commercial COMT inhibitors have disadvantages, and particularly harmful side effects. So, novel molecules need to be designed and evaluated in terms of pharmaceutical performance. In this work, a total of twelve potential catechol bioisosters were successfully synthesized and their COMT inhibitory and cytotoxic effects evaluated. Through molecular docking their potential to interact with the COMT active site was confirmed. The interactions formed were similar to the catecholic substrates, indicating that compounds might compete with it for the binding site. These findings suggested *a priori* that the new molecules could be potential COMT inhibitors. However, regarding the compounds' effect on MBCOMT specific activity, after incubation at 10 and 100 μM, the products **1a** and **1b** increased MBCOMT specific activity. Regarding the other compounds (**1c**-**1l**), due to the lack of COMT activity in the negative control, their inhibitory or stabilizing effect was not conclusive. The stabilization effect was not anticipated due to the *in-silico* studies results, suggesting that the synthetized molecules may not bind in the enzyme active site, but interact with the protein facilitating the substrate binding and the corresponding *O*-methylation. Further studies are needed to study this hypothesis, including more advanced *in-silico* methodologies involving more potent and advanced molecular docking and molecular dynamics software's. Additionally, more *in vitro* studies can be performed to fully validate the stabilizing role of these compounds in COMT, including thermal stability assays and crystallographic studies. Concerning the MTT assay results, most of the compounds displayed no marked cytotoxicity for the N27 cell line at 10 μM. However and as expected, at 100 μM a sharp decrease in the relative cell proliferation was observed. The majority of the tested compounds displayed similar or lower cytotoxicity than the commercial COMT inhibitors understudy, Tolcapone and Entacapone at 10 μM. This result suggests that if these compounds can penetrate the BBB they will not cause toxicity for the neural dopaminergic cells. The predicted ADMET properties indicate that most of the compounds might have good oral bioavailability, high intestinal absorption, low probability to be both CNS^+ and BBB^+, interact with several isoforms of the cytochrome P450, which would alter the molecules' pharmacokinetics, and finally all of them could induce hepatoxicity. These results are very promising and can be further improved with the use of new substituents to enhance their ADMET properties. Although this study was very preliminary, the main results suggest that these new molecules are MBCOMT stabilizers and might have potential clinical interest. These findings could also be useful for hormone-dependent cancers therapy, namely in breast cancers, because the exposure to 2- and 4-hydroxyestrone is associated with an increased risk of developing some cancers, and some or their metabolites can be *O*-methylated by COMT into, for instance, 2-metoxyestradiol, which exhibits anti-tumoral properties. As future work we intend to expand the cell cytotoxicity studies to other cell lines from both tumoral and non-tumoral tissues, especially in COMT overexpressed hormone-dependent cancer cell lines, such as MCF-7, to study the antiproliferative and growth inhibition properties of the synthesized compounds.

Chapter 6 – References

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Chapter 7 – Publications

Oral communication fulfilled within this dissertation

Cruz-Vicente, P. , Gonçalves, A.M. , Passarinha, L.A. , Silvestre, S. , Synthesis of new catechol-O-methyltransferase stabilizers., Abstract book of the International School of Chemistry: Chemistry of everyday life web edition (Attachment 1).

Cruz-Vicente, P. , Gonçalves, A.M. , Passarinha, L.A. , Silvestre, S. , Synthesis of new catechol-O-methyltransferase stabilizers., Abstract book of the XV Annual CICS-UBI Symposium (Attachment 2).

Scientific articles in international journals peer-reviewed

Barroca-Ferreira J., Cruz-Vicente P., Santos MFA., Rocha SM., Santos-Silva T., Maia CJ., Passarinha LA., An Innovative Integration Between Hydrophobic Interaction Chromatography and Co-Immunoprecipitation Towards STEAP1 Isolation (Attachment 3)

Cruz-Vicente P, Gonçalves AM, Barroca-Ferreira J, Silvestre SM, Passarinha LA, Unveiling the biopathway for the design of novel COMT inhibitors (Attachment 4)

Chapter 8 – Attachments

Attachment 1 – International School of Chemistry: Chemistry for everyday life web edition

Synthesis of new catechol-O-methyltransferase stabilizers

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KEYWORDS: *Catechol-O-methyltransferase, Cancer, Structure-based drug design, chemical synthesis, Stabilizers*.

ABSTRACT: Catechol-O-methyltransferase (COMT) is an enzyme responsible for the O-methylation of catechol substrates, such as catecholamines and catechol estrogens (CE). Considering its physiological functions and polymorphic activity, several studies associated COMT with the pathogenesis of several neurological disorders, cardiovascular diseases, and hormone-dependent cancers [1]. In fact, the major CE metabolites, 2- and 4-hydroxyestrone, are carcinogenic in peripheric tissues and it has been demonstrated that lower COMT activity increases the risk of developing hormone-dependent diseases. However, 2-methoxyestradiol, a 2-hydroxyestradiol metabolite originated by COMT-mediated methylation, exhibited antitumor properties. Hence, the main goal of this work is to develop new COMT stabilizers, to maintain or increase COMT activity with potential clinical interest. For this, we prepared triazolopyrimidine derivatives, through the Biginelli reaction using 3-amino-1,2,4-triazole instead of urea [2], which can be considered catechol bioisosteres with the potential to bind/stabilize the enzyme. To select the compounds with a higher potential to bind COMT, we performed an *in-silico* screening using molecular docking (AutoDock Tools) and studied the pharmacokinetics using predictive models (pkCSM and SwissADME). The most relevant molecules were synthesized, and their stabilizing properties were evaluated using recombinant human COMT lysates, followed by an MTT cytotoxicity assay in N27 cell lines [3]. Overall, the selected compounds displayed stabilizing effects on COMT activity, and no marked cytotoxicity was observed. To sum up, these findings can be useful for crystallographic studies, thermal stability assays and structure-activity relationship studies.

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Attachment 2 – XV Annual CICS-UBI Symposium

SYNTHESIS OF NEW CATECHOL-*O*-METHYLTRANSFERASE STABILIZERS

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ABSTRACT

Catechol-*O*-methyltransferase (COMT) is an enzyme responsible for the *O*-methylation of catechol substrates, such as catecholamines and catechol estrogens (CE). Considering its physiological functions and polymorphisms, several studies associated COMT with the pathogenesis of several neurological disorders, cardiovascular and hormone-dependent diseases. In fact, the major CE metabolites, 2- and 4-hydroxyestrone, are carcinogenic in peripheric tissues and it has been demonstrated that lower COMT activity increases the risk of developing hormone-dependent diseases, specifically breast cancer. However, 2-methoxyestradiol, a 2-hydroxyestradiol metabolite originated by COMT-mediated methylation, exhibited antitumor properties. Hence, the main goal of this work is to develop new COMT stabilizers, to maintain or increase COMT activity with potential clinical interest. For this, we prepared triazolopyrimidine derivatives, through the Biginelli reaction using 3-amino-1,2,4-triazole instead of urea, which can be considered as catechol bioisosteres with the potential to interact and stabilize the enzyme. Their stabilizing properties were evaluated using recombinant human COMT lysates, followed by an MTT cytotoxicity assay in N27 cell lines. To better understand how the molecules interact with COMT, an *in-silico* screening was performed using molecular docking (AutoDock Tools) and pharmacokinetics and toxicity properties were predicted using web software (pkCSM and SwissADME). Overall, the selected compounds displayed stabilizing effects on COMT activity, and no marked cytotoxicity was observed. To sum up, these findings can be useful for crystallographic studies, thermal stability assays, and structure-activity relationship studies should be performed to disclose new molecules with potential for biomedical applications.

Keywords: *Catechol-O-methyltransferase, Structure-based drug design, chemical synthesis, Stabilizers.*

Attachment 3 – An Innovative Integration Between Hydrophobic Interaction Chromatography and Co-Immunoprecipitation Towards STEAP1 Isolation (Manuscript)

Biotechnology Journal

An Innovative Integration Between Hydrophobic Interaction Chromatography and Co-Immunoprecipitation Towards STEAP1 Isolation.

Journal:	*Biotechnology Journal*
Manuscript ID	Draft
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Primary Keywords:	Downstream processing, Medical biotechnology
Secondary Keywords:	Bioseparation, Protein purification, Protein stability
Additional Keywords:	Co-Immunoprecipitation, Hydrophobic Interaction Chromatography, STEAP1

Wiley-VCH

An Innovative Integration Between Hydrophobic Interaction Chromatography and Co-Immunoprecipitation Towards STEAP1 Isolation

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Abbreviations

Co-IP, Co-Immunoprecipitation; Cryo-EM, Cryogenic Electron Microscopy; EDTA, Ethylenediamine Tetra-Acetic Acid; HIC, Hydrophobic Interaction Chromatography; MPs, Membrane Proteins; PCa, Prostate Cancer; NP-40, Nonidet P-40; PMSF, Phenylmethylsulfonyl Fluoride; PVDF, Polyvinylidene Difluoride; SDS - Sodium Dodecyl Sulfate; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; STEAP1, Six Transmembrane Epithelial Antigen of the Prostate 1; T_m, Melting Temperature; TSA – Thermal Shift Assay; WB, Western Blot.

Attachment 4 – Unveiling the biopathway for the design of novel COMT inhibitors (Manuscript)

Unveiling the biopathway for the design of novel COMT inhibitors

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Abstract

Catechol-*O*-methyltransferase (COMT) is the enzyme responsible for the *O*-methylation of biologically active endogenous and xenobiotic catechol-based molecules. Considering its physiological functions and polymorphic activity, COMT is associated with neurological disorders, especially with Parkinson's Disease (PD). Given the relevant role that COMT holds in the catecholamines metabolism, this enzyme is considered an important therapeutic target for central nervous system disorders. The aim of this paper is: i) to summarize the bio-characteristics and therapeutical perspective of COMT; ii) to review the medicinal chemistry behind the development of COMT inhibitors and the importance of structure-based drug design for the design of novel molecules; iii) to discuss the current methodologies for biosynthesis, isolation and purification of the target enzyme; and iv) to revise the existing bioanalytical approaches for the determination of COMT activity.

Keywords: Catechol-*O*-Methyltransferase; Isolation; Structure-Based Drug Design; Bioanalytical Methods.

Outline

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1. Introduction

In 1958, Axelrod and collaborators identified and described the function of the enzyme responsible for the *O*-methylation of catecholamines [1]. This enzyme was later partially purified and characterized from rat liver and named catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) [2]. This enzyme is essential for the *O*-methylation of biologically active catechol molecules, including catecholamines (e.g. dopamine, norepinephrine and epinephrine), catechol estrogens, and catechol drugs [3]. The discovery of the involvement of COMT in the metabolism of the antiparkinsonian drug Levodopa (*L*-DOPA) brought a growing interest concerning this enzyme [2]. The administration of *L*-DOPA, used in clinical practice since the 1960s, still remains nowadays as the first-line of pharmacologic treatment for Parkinson's Disease (PD) [4–6]. The *L*-DOPA can be used for the manipulation of the central levels of dopamine, because it can cross the blood-brain barrier (BBB) [7]. However, this biological dopamine precursor is rapidly metabolized by peripheric enzymes before entering the CNS, particularly by the aromatic amino acid decarboxylase (AADC) and COMT [8]. Therefore, the use of COMT inhibitors, as adjuvants, can significantly improve the amount of *L*-DOPA that reaches the brain, preventing the conversion to 3-*O*-methyldopa (3-*O*MD) and consequently increasing its bioavailability [9]. Thus, the inhibition of COMT became an attractive strategy for the manipulation of the endogenous levels of dopamine [3]. The "first generation" of inhibitors was developed in the earlies 1960s, primarily formed by catechol and pyrogallol systems, containing a catechol ring or a bioisosteric-related moiety [3]. The *in vivo* studies demonstrated that their pharmacological usefulness was very limited, demanding higher concentrations for an effective COMT inhibition [10]. Nevertheless, it was reported that these enhanced concentrations caused cytotoxicity [10]. In the late 1980s, a new series of di-substituted catechol inhibitors was developed to overcome the drawbacks the "first generation" displayed. The addition of a nitro group at position 5 of the catechol ring resulted in higher inhibitory potency, increased selectivity and improved pharmacokinetics [2,11]. This "second generation" of novel nitrocatechol COMT inhibitors showed very promising results, with some compounds advancing for clinical trials [2,10,11]. However, only Tolcapone and Entacapone were approved for clinical use for PD therapy [12]. In 2016, a "third generation" COMT inhibitor, BIA 9-1067 (Opicapone) was also accredited for use as adjuvant in PD therapy [12–14]. The use of this drug resulted in stable and sustained *L*-DOPA plasma levels for over 24 h after administration which allowed an once-daily administration [12]. Despite the improvements that these drugs brought for the management of PD patients, new methodologies must be employed for the development of novel COMT inhibitors with potential clinical interest. In this perspective, we intend to analyze throughout this review the biochemical and structural characteristics of COMT, computational tools that can be employed in the design of new potential inhibitor molecules and the current biotechnology platforms for the biosynthesis and purification of this enzyme.

2. Pathological and Therapeutic Perspective of Catechol-*O*-Methyltransferase

2.1. Biomolecular Characteristics

The Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) is a monomeric enzyme that catalyzes the transference of a methyl group from S-adenosyl-$_L$-methionine (SAM) to a catechol substrate, resulting in *O*-methylated products and S-adenosyl-$_L$-homocysteine (SAH), in the presence of the magnesium (Mg^{2+}) co-factor [1,3]. This enzyme has already been found in a large number of species, in particular bacteria, yeasts, plants, insects, fish, birds, and mammals, and characterized regarding its structure and function [2,15]. The main role of COMT is to *O*-methylate biologically active endogenous and/or exogenous catechol molecules, acting as an enzymatic detoxifying barrier [11]. In humans, COMT is present in two distinct molecular isoforms: a soluble (SCOMT) and a membrane-bound form (MBCOMT) [16]. The COMT gene, located in the band q11.21 of chromosome 22, is composed of six exons and encodes both isoforms [17]. The translation initiation codons for both isoforms are located in the third exon, while the first two are non-coding [18]. Their expression are regulated by two distinct promoters: a short transcript of 1.3 kb translates SCOMT while a longer transcript of 1.5 kb translates MBCOMT [17]. This enzyme is expressed in several human tissues, particularly in liver, kidneys, and gastrointestinal tract, where the levels of SCOMT are considerably higher in comparison with MBCOMT [2]. Curiously, only in the central nervous system (CNS), the expression levels of MBCOMT surpasses SCOMT, representing approximately 70% of the total COMT [17]. The cellular localization of these isoforms are also different: while human SCOMT can be found in the cytoplasm, with a reported molecular weight of 24.7 kDa and a total of 221 amino acids (aa) [19], MBCOMT has a molecular weight of 30 kDa and an extra 50-residue long amino-terminal signal sequence that forms a membrane anchor associated with the membrane of the rough endoplasmic reticulum, oriented towards the cytoplasmic side of the membrane [2]. Despite the kinetic similarities and Mg^{2+} and SAM dependency shared by both isoforms, the substrate affinity is substantially different [20]. The physiological substrates for this enzyme include catecholamines, namely dopamine, epinephrine, norepinephrine, catechol estrogens, such as 2- and 4-hydroxyestradiol, 2- and 4-hydroxyestrogen, ascorbic acid, and other dietary products [10]. Lotta and co-workers studied the affinity of both COMT isoforms for several catecholamines, using S- and MBCOMT obtained from rat liver [20]. Indeed, for dopamine, SCOMT has a K_m of 207 μM while MBCOMT has a K_m of 15 μM; for norepinephrine, the SCOMT has a K_m of 369 μM while MBCOMT has a K_m of 24 μM [20]. In terms of enzymatic velocity (V_{max}), SCOMT was reported to have a greater velocity than MBCOMT [20], once SCOMT presents a V_{max} of 37.2 min^{-1} for dopamine and MBCOMT 16.9 min^{-1}, while for norepinephrine the values were 35 min^{-1} against 18 min^{-1} [20], respectively. These findings clearly support that MBCOMT is the isoform responsible for the *O*- methylation at physiologically low concentrations of catecholamines, particularly in the CNS [21]. On the other hand, SCOMT is mostly responsible for the *O*-methylation of catechol compounds throughout the peripheral tissues at higher substrate concentrations [21]. Given the importance of COMT in many essential biological functions several studies tried to establish a connection

between this enzyme and CNS-related diseases, as detailed described in the following subtopic of this manuscript.

2.2. Genetic Polymorphisms of COMT and their Relationship with CNS Disorders

Although the enzyme function has been established for a long time, an ever expanding COMT role in biological pathways and diseases has become a subject of intense study in the past years. In mammals, over 900 genetic variants for the COMT gene have been reported [22]. However, the majority did not show any physiological importance [22–25]. Nevertheless, one of the most studied and characterized COMT polymorphism is the rs4680, which consist in a single nucleotide (SNP) transition of a guanine to an adenine. Consequently, this genetic modification leads to an aa substitution, where a Valine (Val) origins a Methionine (Met) in the polypeptide chain at codon 108 in SCOMT ($Val^{108}Met$) and at codon 158 in MBCOMT ($Val^{158}Met$) [20,26]. This functional SNP is genetically polymorphic with a trimodal distribution with high ($COMT^{Val/Val}$), intermediate ($COMT^{Val/Met}$) and low ($COMT^{Met/Met}$) enzymatic activity [2]. The polymorphic variant $COMT^{Met/Met}$, when compared with the native form of COMT ($COMT^{Val/Val}$), exhibits a decrease of approximately 40% in its enzymatic activity [20]. This aa substitution makes the protein active site more prone to distortion, aggregation and a significant decrease in the enzyme thermal stability [20]. Currently, it is widely reported that the $Val^{158}Met$ can induce dysregulations in the catecholamine levels and, therefore, many studies tried to establish a connection between this SNP and multiple neuropsychiatric disorders, including in depression [22], autism spectrum disorder [22], schizophrenia [27–30], obsessive-compulsive disease [29], panic [31–33], bipolar disease [34–36], attention deficit hyperactivity disorder [22,37], anxiety [38], nervous anorexia [39–41], and PD [42]. Nevertheless, this association may differ according to the ethnicity, gender and age of the population [43]. These findings and the role of COMT in the development of novel potential pharmacological strategies can be better understood with the determination of the protein crystal three-dimensional (3D) structure.

2.3. Catalytic Mechanism and 3D Crystal Structure of COMT

The COMT is composed by a single domain of a mixed α/β-protein structure which consists of a seven stranded β-sheet core (β1-β6 stands are parallel and the β7 stand is antiparallel) integrated between two sheets of α-helices [3]. The catalytic site is shaped to fit the catechol substrate and the Mg^{2+} binding site in a shallow groove on the outer surface of the enzyme, while the SAM binding site is located within the enzyme structure, in a more buried cleft [26]. In this S_N2 transfer reaction, the binding sequence needs to follow a crucial order for the reaction to occur [3]. Therefore, SAM must be the first to bind, followed by Mg^{2+}, and lastly the substrate [3]. The major bio-interactions of the co-factors with the protein active site are displayed in **Figure 1**. Specifically, the adenine ring of SAM is hydrogen-bonded to Ser119 and Gln120,

forming Van der Waals interactions with residues Ile91, Ala118, and Trp143 [3]. The methionine fragment of SAM and SAH is oriented towards the substrate binding site, promoting hydrogen bonds with residues Val42, Ser72, and Asp141 [44], as shown in **Figure 1**. Although the Mg^{2+} binding site is in the center of the catalytic site, it does not directly interact with SAM [44]. The cation Mg^{2+} is octahedrally linked to the oxygen atoms of the side chains of residues Asp141, Asp169, Asn170 and to one crystallographic water molecule [3]. The other two free coordination sites allow the chelation to each of the hydroxyl groups of the catechol [3]. As a consequence, the interaction with the Mg^{2+} lowers the substrate acid dissociation constant (pK_a), facilitating the deprotonation of the catechol hydroxyls [45]. The substrate forms important hydrogen bonds with the side chains of the residues Glu199 and Lys144, and interacts with the "gatekeeper" residues Trp38, Trp143, Pro174, and Leu198 by hydrophobic bonds [46]. The interaction with the residue Trp38 was found to be essential for a higher binding affinity [47]. These residues are also responsible for the selectivity and the *O*-regioselectivity of COMT [20,48]. Considering that rat SCOMT shares an 81% aa sequence similarity with human SCOMT, including all the important residues for the substrate binding in the catalytic site, it has been widely used to study the interactions in the protein active site [49,50]. From these findings the first crystallized structure of recombinant rat SCOMT was obtained [49]. Since its determination, 16 structures of human and 94 rat COMT structures have been deposited in the Protein Data Bank (PDB) (checked: 29/10/2020) with atomic resolutions, resulting in a better understand of the interactions happening in the protein active site, in both the Holo and Apo forms of the enzyme (**Table 1**) [26]. With the constant improvements in technology, new tools for the design of new molecules, based on the information found in the protein 3D structure have been developed.

2.4. COMT inhibitors and the relevance of SBDD in their development

The combination of the data of a protein 3D structure with computational algorithms to support drug discovery allows a quicker selection of potential therapeutic drugs [51,52]. In this context, Structure-Based Drug Design (SBDD) involves the prediction of the binding affinity of a ligand to the corresponding protein target, as well as the key binding sites and the ligand interactions with the protein. Interestingly, extensive libraries of compounds can be analyzed, being possible to accelerate the drug discovery, saving time and money [53]. Specifically for COMT, a molecule that forms hydrophobic interactions with the residues Trp38, Trp143, and Pro174 and/or hydrogen bonds with the residues Lys144 and Glu199 has a good possibility to become a potential inhibitor of this enzyme [54–56]. In recent years, Entacapone and Tolcapone (**Figure 2**), considered "second generation" inhibitors have been introduced into clinical practice as adjuvants to the *L*-DOPA therapy in patients with motor fluctuations [57]. Despite being effective and selective through oral administration, these compounds have several limitations in pharmacokinetics and pharmacodynamics properties, in clinical efficacy and even in safety for patients [58,59]. In contrast with Entacapone, which only inhibits peripheral COMT, Tolcapone can inhibit both peripheral and central COMT, due to its ability to penetrate the BBB [60]. However, Tolcapone is associated with increases in plasmatic levels of liver transaminases and can cause severe

hepatoxicity [61]. This side effect limits the extensive application of this inhibitor in therapeutic routine. Recently, the Portuguese Pharmaceutical Company Bial – Portela & C^{a}, S.A. developed Opicapone (**Figure 2**) [62]. Opicapone is a nitrocatechol COMT inhibitor considered more effective and safer, with higher binding affinity and, most importantly, a longer period of action and enhanced bioavailability, than the previously mentioned drugs [63]. The pharmacokinetics of this novel inhibitor allowed a once-daily administration, allowing over 24 hours of stable and sustained *L*-DOPA plasma levels, requiring fewer administrations than the previous drugs [64–67]. This "third generation" inhibitor is considered a strictly peripheral COMT inhibitor, once it mainly acts by modulating the erythrocytes COMT activity and does not have the ability to penetrate the BBB [12,14,68].

Despite the improvements in PD therapy using these inhibitors, novel molecules that can inhibit central COMT activity and with residual cytotoxicity are required. Thus, the use of SBDD can allow the design and structural optimization of novel compounds with higher potential and selectivity to inhibit COMT [55]. This tool has been used over the years and contributed for the design and development of more than 250 novel compounds from several chemical classes, as summarized in **Figure 3**. The main compounds synthetized are summarized in **Figure 3** which include: *N*-heterocyclic pyridones [69], nitrocatechol-substituted heterocycles [70],nitrocatechol hydroxycinnamic acid derivatives [71], bifunctional nitrocatechols [72] nitrocatechol bisubstrates [73] and [74], non-nitrocatechol bisubstrates [75], pyrazole-thiazole derivatives [76], 7,8-dihydroxyflavone [77], *Z*-vallesiachotamine [78], 3-hydroxypyridin-4-ones [79], catechins [80,81], benzotropolones [82], baicalein [83], morin molecule [84], rosmarinic acid [85], oleacein [86], 8-hydroxyquinolines [87] and bicyclic hydroxypyridones [88], and others [89,90].

3. Preparative and Analytical methodologies for COMT isolation and activity assessment

3.1. Strategies for Expression and Isolation of COMT

In the last decades, several research groups have cloned and expressed the human recombinant S- and MBCOMT [91,92] and studied their biochemical characteristics for the *O*-methylation of representative classes of endogenous and exogenous catechol substrates. The recombinant COMT has been produced in *Escherichia coli* (*E. coli*) [93], in insect cells using a baculovirus expression system [94] and in mammalian cell cultures using expression vectors based on Epstein-Barr virus, a herpesvirus [94], and Simian virus 40, a polyomavirus [16]. In spite of all of the aforementioned systems have produced functional forms of the enzyme, the method suits essentially for shaking flasks on rich mediums by induction at low cell density (0.2-0.5 g dry weight/L) [93] and enables to produce up to 1 g of target protein. However, for the past twenty years with the development of recombinant technology and the structural refinement of the expression vectors, new hosts have been described as potential sources of the enzyme COMT, as

summarized in **table 2**. Typically, when COMT is obtained from animal tissue homogenates, a multiplicity of steps are usually performed to recover the target enzyme, generally with consecutive centrifugations, acid and salt precipitation, hydroxyapatite adsorption and numerous other chromatographic techniques to ensure a highly pure protein fraction [95]. In spite of this strategy, the COMT yields and bioactivity levels associated to these multiple stages, were not suitable to perform structure determination [95,96]. To circumvent this drawback, the recombinant DNA technology was considered an interesting alternative, due to its capacity to insert genes into a variety of prokaryotes and eukaryotes organisms, which are able to produce enhanced amounts of the functional target [97]. In fact, these production systems had already demonstrated promising results, once the first reported resolved structure of rat SCOMT and human $SCOMT^{Val/Met}$ was expressed in E. *coli* [98,99]. In turn, due to the hydrophobic nature of the membrane, the MBCOMT is more difficult to handle, when compared to the soluble counterpart, mainly because it induce toxicity into the cells of bacterial host systems [100]. Surprisingly, there is one report of a prokaryotic organism, *Brevibacillus choshinensis* [101], frequently used to produced high levels of human soluble proteins [102,103], that was also capable to produce an active MBCOMT enzyme. Nevertheless, the production of this isoform are deeply associated with eukaryotic expression system, such as *Spodoptera frugiperda* (Sf9) insect cells [94,104], transfected human embryonic kidney fibroblast cell lines [105] and *Komagataella Pastoris (K. pastoris)* [19,100,106]. The optimization of the induction phase parameters (induction temperature, methanol flow-rate and dimethyl sulfoxide concentrations) in *K. pastoris* mini-bioreactors cultures allowed an improvement of 6.4 fold in MBCOMT specific activity over the best results previously reported in shake-flasks [107]. Directly coupled to the upstream stage, an alternative strategy to further increase functional and active MBCOMT biosynthesis may involve its recombinant production through cell free (CF) expression systems. Indeed, we believe it will be worthwhile to try to overexpress MBCOMT using a eukaryotic CF expression system based on wheat germ or insect cells and insert it in a co-translational form into pre-formed lipid bilayers such as liposomes or nano discs. Moreover, it should be explored not only the stabilizer potential of different sugars and polyols [108] during the CF expression of MBCOMT but also the effect of SAM and the Mg^{2+} [2]. After the establishment of a proper procedure for the expression of COMT, a suitable downstream processing must be designed in order to obtain higher recovery yields of the protein. [109,110]After cell disruption by mechanical or non-mechanical methods (**table 2)**, SCOMT is usually found on a complex protein supernatant, while MBCOMT is in the membrane cell debris fraction. This last compartmentalization requires the use of surfactants to mimic the native lipidic membrane, to solubilize the protein and then maintain the membrane-bound enzyme in its active and stable conformation to the main downstream steps [110]. Several purification data suggested that COMT is fairly labile and loses its activity rapidly during the isolation process and storage [96], probably due to the oxidation of the free cysteine-SH groups [111]. In practice it turned out that human SCOMT activity was completely lost at the very beginning of the purification if cysteine was omitted from the buffers [112]. Moreover, experimental observations reveal that EDTA and MgCl2 in equimolar concentrations conjugated with the reducing agents dithiothreitol (DTT) or mercaptoethanol [19,111,113] into buffers, has a stabilizing effect on all enzyme preparations allowing its purification and partial characterization; nevertheless highly purified fractions show an accentuated decrease in activity [95]. In order to improve the performance of the isolation stage, several chromatographic techniques from hydrophobic interaction

chromatography (HIC), anion exchange chromatography (AEC), cation exchange chromatography (CEC), and also affinity chromatography (AC) with immobilized amino acids or immobilized metals (IMAC) were employed to recover COMT isoforms. The **Table 2** summarizes this data. Concerning the HIC methodology, the proteins are separated owing to the differential hydrophobic interactions between immobilized ligands onto the chromatographic resin and the non-polar regions exposed on the surface of proteins [114]. The most promising results, regarding the specific activity of the recovered fraction of COMT, were obtained using Butyl- and Octyl-Sepharose matrices [113]. The base principal of HIC are intrinsically related with the manipulation of the ionic strength in both binding and elution buffers [114]. As expected, considering the biochemical features and structural rearrangement of S- and MBCOMT, different salt concentrations were needed to promote the adsorption of the target protein to the resins. Therefore, it was demonstrated that SCOMT required higher hydrophobic strength to be adsorbed onto the column, when compared to the MBCOMT isoform. Furthermore, the elution process was also different, between these two COMT counterparts. Indeed, a successful desorption of SCOMT was achieved by a decrease in the ionic strength, while the MBCOMT demand the supplementation of the elution buffer with a detergent, namely Triton X-100, FC-12 or DDM [113,115]. Regarding AEC, the proteins are retained at low salt concentrations and then eluted by an increasing in the NaCl concentration, as we can see in table 2 for both isoforms of COMT. Similarly to HIC, the recovery of an active fraction of MBCOMT is deeply dependent on the addition of a surfactant, in order to ensure a complete protein solubilization. Lastly, we can also find several reports that used AC to purify the COMT isoforms. Usually, this technique is used when the recombinant protein is fused at the N-or C- terminal with a specific tag that have affinity to an immobilized ligand, aa or metal. Despite the particular features associated with the different chromatographic approaches exposed in this section of the article, the purification of the protein will on the conformation of the isolated target, which is directly affect by the environmental and operational conditions used throughout these procedures, such as the buffer pH and its ionic strength variation [116–118]. Although the different chromatographic techniques could be separately employed, a combination between two or more strategies can promote a selective elution of the protein of interest and also enhance its purity degree. However, the specific activity of the enzyme could be compromised and then this may impair further trials. To sum up, there are four major reports concerning the chromatographic purification of MBCOMT by hydrophobic adsorbents [115], using Q-sepharose as an anion-exchanger [108] or using affinity chromatography, either by Immobilized-Metal Affinity Chromatography (IMAC) [104] or arginine-affinity chromatography [117]. Specifically, while the highest MBCOMT purity degree was obtained through IMAC, the use of tag sequences often interferes with the kinetic parameters of the target enzyme. Therefore, there is still room to design a suitable chromatographic strategy for MBCOMT purification in a native, untagged form. This is usually accomplished in immunoaffinity purification where a target antibody is immobilized into a matrix to form a solid absorbent. Again, it will be worthwhile to integrate the upstream strategy previously reported [100], with the development of a chromatographic matrix with an immobilized antibody anti-COMT. The wide research conducted with recombinant MBCOMT allowed a deep characterization of its biochemical properties, catalytical mechanisms and the determination of its kinetic constants not only for different substrates but also for a wide range of commercial inhibitor molecules (3,5-dinitrocatechol, Tolcapone, Entacapone or Opicapone). However, the

application of this enzyme as a therapeutic protein or the development of MBCOMT-specific inhibitors still relies in the improvement of the downstream processing.

3.2. Parameters which Influence COMT Activity

The activity of COMT enzyme is usually expressed as a specific activity i.e., activity units (nmol/min) in mg of protein. As referred in the previous section, this parameter is corelated to the protein source and its purity degree. Indeed, the use of different approaches in the sample handling, isolation and purification processes could result in substantial variations of SCOMT and MBCOMT specific activity values (table 2). The activity of a target protein is affected since its production in animal tissues or recombinant expression hosts. Here, the operational and environment conditions have a considerable influence in the biosynthesis yield, and to avoid protein aggregation or misfolding, particularly the cell host, growth media, chaperones, feeding strategies, temperature and pH [119]. One of the most important parameters that may influence the activity of COMT is the lysis strategy used to recover the target from the cells of the expression host. For instance, if the target is internally produced by the host system, an enzymatic lysis with lysozyme incubation or mechanical cell disruption by sonication, freeze/thaw or glass beads can ensure the recovery of a bioactive fraction of COMT [113,117]. Moreover, it is frequent to combine several lysis procedures in order to maximize the amounts of total protein recovered [93,116,120]. However, and as mention in the previous section, the multiplicity of steps may negatively affect the protein activity. Pedro and co-workers reported that for the MBCOMT production in *K. pastoris,* a combination of freeze/thaw with glass beads or glass beads followed by sonication increased the total values of recovered protein, when compared to a procedure based on just a single technique [117]. Nevertheless, it was verified that sonication compromises the specific activity of COMT, probably due to temperature increments and the shearing effect promote by the ultrasounds [109,121]. Particularly for MPs, the incorporation of surfactant to the lysis buffer and ensuring the complete solubilization of the protein is mandatory [108,118]. However, according to their nature, these surfactants may promote different results. For instance, the ionic Sodium Docecyl ulfate (SDS) is considerably harsh to the proteins, then promoting denaturation and almost null bioactivity [118]. In turn, non-ionic detergents such as Tween-20, Triton X-100 and Digitonin were able to fully solubilize MBCOMT with high values of biological activity even at low concentration [110]. Likewise, it is frequent to supplement both binding and elution buffers of chromatographic trails for MBCOMT purification with low concentrations of detergents (Triton X-100,FC-12) in order to maintain the desirable biological activity [21,104,108,115] . Furthermore, the salt type and ionic strength of the chromatographic buffers and their pH can also inactivate the enzyme [116]. The most used salts are sodium phosphate and tris in a concentration ranging 20 to 50 mM, frequently supplemented with protein stabilizers as cysteine, dithiothreitol (DTT) and 2-mercaptoethanol to ensure the maintenance of the active form of COMT [122]. Also, the divalent ion used in the buffers lead to variations in the enzymatic activity of the target, mainly through the substitution the Mg^{2+} for other divalent ions, including Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+} and Sn^{2+}. The most relevant are the replacement for Fe^{2+}, which

showed decreased binding affinity and poorer catalysis [REFs]; Co^{2+} and Mn^{2+}, which revealed increased effectiveness in the catalysis process; and finally Ca^{2+}, which unveil a repackage of the enzyme in its binding site. Additionally, COMT may be deactivated due to the loss of collinearity of the substrate, the methyl group-to-transfer and the methyl-donor S atom of the cofactor SAM. As a result of these impairment in the transfer of methyl group, the activation barrier and the energy of reaction are increased. A similar behaviour is found with Fe^{3+}, which is capable to induce loss of activity to COMT based only on electronic effect of the metal, specifically due to the impact of the partial charge of the metal on the basicity of the catechol oxygen atoms [123,124].

3.3. Bioanalytical Methods for the Determination of COMT activity

In this section of the review, we intend to discuss the bioanalytic methods for the determination of COMT activity. Currently, the analysis of *O*-methylated products found in biologic samples is mostly performed by chromatographic approaches, particularly by liquid chromatography as high-performance liquid chromatography (HPLC) [125]. This technique allows the use of different detection systems, based on the substrate properties to guarantee that all the components are analysed, and it is a non-destructive methodology that provides high specificity and precision [126]. The most used detectors are the UV detector, which are capable of simultaneously monitoring a wide range of wavelengths, the diode array detector (DAD), the refractive index detector, mainly used when the analytes have restricted or none UV absorption, and the electrochemical detector (ED), which responds to substances that are either oxidable or reducible [127,128]. Particularly for COMT 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 mass spectrometry (UV/MS), gas chromatography (GC) and ELISA kits, as described in **Table 4**. In general, radiochemical detection improves the sensitivity of COMT assays, but it is often restricted to special cases [129]. Also, the poor selectivity and moderate sensitivity of HPLC coupled to a UV detector make this method less used [129]. In contrast, fluorescence detection provides higher sensitivity, being especially useful when tissues with less COMT activity are analysed [129]. In addition, ED detection has a significantly low limit of detection (LLOD), along with high sensitivity and selectivity for catecholamines [130]. In most procedures developed for these assays, HPLC coupled with an ED detector is the technique of choice for many researchers. Indeed, Lotta and co-workers were the first research group to use bioanalytical methods to quantify the formation of *O*-methylated products [131]. The methodology here reported used HPLC coupled to an ED detector 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 [20]. Furthermore, the methods developed to quantify COMT activity uses HPLC with a reversed-phase C_{18} column [120,127,130]. The majority of these assays

are reported in **Table 4** and used physiological substrates, namely catecholamines, such as dopamine, norepinephrine, epinephrine, catechol oestrogens, like 2- and 4-hydroxyestrone (2- and 4-OHE1), *L*-DOPA, and a few natural and synthetized compounds. The data obtained from these assays allowed the determination of the enzyme kinetic parameters, namely K_m and V_{max}, as well as the K_i, K_{cat} and IC_{50} of the different tested inhibitors, as outlined in **Table 5**. Despite the differences in substrate concentrations and enzyme source, the kinetic discrepancies between S- and MBCOMT are evident. As previously stated, SCOMT exhibits a higher V_{max}, in some cases 10 fold higher than MBCOMT, and considerable less substrate affinity, particularly for catecholamines, than SCOMT [132]. This information can be confirmed with the data on **Table 5**, in which is possible to notice that the recombinant source of COMT has a higher K_m and V_{max} then the native form, maybe due to structural differences related with the production bioprocess [REFs]. Regarding COMT extracted from rat brain, it has a greater substrate affinity than rat liver, despite its lower value for V_{max}. Concerning the inhibitory effect, the results showed a tendency for a higher K_i and K_{cat} in SCOMT than in MBCOMT, which can be explained by structural differences in the catalytic site of both isoforms [REFs]. Lotta and collaborators studied the effect of multiple inhibitors in both recombinant isoforms. As expected, Tolcapone displayed higher inhibitory effect for both isoforms [20], as similarly obtained by Silva [71] and Forsberg [133] using rat tissues homogenates. Despite the promising results here explored, it was evident that the development of novel COMT inhibitors with increased efficiency and selectivity as well as residual levels of induced toxicity are of main importance for the management of patients with PD and other dementia-related disorders.

4. Conclusions and future perspectives

In a near future, we intend to refine the bioprocesses regarding the biosynthesis and isolation of COMT to improve the yields associated with the production and purification steps, to obtain active and stable fractions of the target with higher degree of purity. The highest goal consists in developing further structural and functional trials aiming to gather a deeper understanding about the interaction mechanisms of this enzyme with novel molecules with potential clinical interest for a highly effective response to neurological disturbances.

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Table 1 – A comparative analysis of atomic resolution and ligands of human and rat COMT crystal structures currently deposited onto PDB date (05/10/2020)

Human COMT						
COMT	Source	Form	PDB ID	Atomic Resolution	Ligands	Reference
S	Recombinant	Holo	3BWM	1.98 Å	DNC + SAM + Mg^{2+}	[26]
			4XUC	1,8 Å	43G + Mg^{2+} + SAM	[134]
			6I3C	1.336 Å	DNC + Mg^{2+} + SAM	[135]
S (Met^{108})			3BWY	1.3 Å	DNC + SAM + MPD + Mg^{2+}	[26]
S	Humanized rat	Apo	4PYI	1.35 Å	Na^{+}	[136]
Rat COMT						
COMT	Source	Form	PDB ID	Atomic Resolution	Ligands	Reference
S	Native	Holo	1JR4	2.63 Å	CL4 + Mg^{2+}	[137]
			2CL5	1.6 Å	BIE + Mg^{2+} + SAM	[138]
			3NW9	1.65 Å	637 + Cl^{-} + Mg^{2+}	[139]
			3R6T	1.2 Å	LUI + Cl^{-} + Mg^{2+}	[140]
			3S68	1.85 Å	Tolcapone + Mg^{2+} + SAM	[140]
			5FHQ	1.63 Å	DNC + Mg^{2+} + SAM	[141]
			5K01	1.383 Å	6P4 + K^{+}	[142]
			4PYL	2.2 Å	Tolcapone + Sinefungi + Mg^{2+}	[136]
S	Humanized rat	Apo	4PYM	1.19 Å	SO_4^{-} + K^{+}	[136]

Table 2: Isolation methodologies used for COMT isoforms recovery

SCOMT								
Source	**Protein Recovery**	**Chromatographic methodology**		**Elution strategy**	**Specific activity (nmol/h/mg of protein)**	**Purification factor (fold)**	**Bioactivity recovery (%)**	**Ref.**
		Nº of Steps	**Chromatography**					
Rat liver	1. Cell homogenized 2. Centrifuged (10000 g, 25 min;), (30000**g**, 25 min), (100000 g, 1 h); 3.Supernatant collect and pH adjusted to 5.1 with acetic acid (30min at 0°C) 5.Centrifuged (15000x g, 20 min). 6. Supernatant collected and pH adjusted to 7.2 with 1 M sodium hydroxide 7. Solution mixed with hydroxyapatite and stirred (20 min); 8. Centrifuged (15000g, 20 min); 9. Ammonium sulfate precipitation (65% saturation at 0°C); 10. Centrifuged (30000 g, 20 min); 11.Precipitate dissolved in 20 mM sodium acetate, pH 4.8 and applied on Bio Gel P-10 column; 12.Fractions with COMT activity were pooled and concentrated by ultrafiltration	**3**	**CEC** (Mono-S**)** **AEC** (Mono Q) **RP-chromatography.**	**NR**	NR	1330	11	[96]
	1. Cell homogenized 2.Centrifuged (15000g, 20 min), (100000g, 60min, 4°C); 2. Sequential ammonium sulfate precipitation (40% and 65% saturation); 3. Precipitate dissolved in 20 mM triethanolamine acetate, pH 7.2 and dialyzed against the same buffer;	**3**	**AEC** (Hiload Q-Sepharose fast flow XK 26/10) **SEC** (Superdex 75) **CEC** (Mono-S)	**Equilibrium:** 20 mM triethanolamine acetate, pH 7.2 **Elution:** step gradient (0.05, 0.2, 1M) of NaCl **Equilibrium:** 20 mM triethanolamine acetate, pH 7.2 **Equilibrium:** 20 mM sodium acetate, pH 4.8 **Elution**: linear gradient (0–0.5M) of NaCl	NR	NR	NR	[93]
E. coli	1.Cell disrupted by sonication (40 cycles of 15 s at 100W followed by 30s on ice);	**5**	**SEC** (Bio-Gel P-100)	**Equilibrium:** 20 mM Bistris/CL, pH 5.8	14.3 U/mg of protein (recombinant rat SCOMT)	5.7 (recombinan	NR	

	2.Centrifuged (10000g); 3.Ammonium sulphate precipitation (65% at 0°C); 4.Centrifuged; 5. Precipitate dissolved				11.2 U/mg of protein (recombinant human placental SCOMT)	t rat SCOMT) 6.6 (recombinant human placental SCOMT)		[143]
			AEC (Q-Sepharose HL 16/16)	**Equilibrium:** 20 mM Bistris/CL, pH 5.8 **Elution:** linear gradient of NaCl, 0–0.5 M in 20 mM Bistris/CL, pH 5.8	109 U/mg of protein (recombinant rat SCOMT) 87 U/mg of protein (recombinant human placental SCOMT)	NR		
			SEC (Bio-Gel P-6DG)	**Equilibrium:** 20 mM bisTris-HCl pH 5.8, lo mM dithiothreitol	NR			
			AEC (Mono-Q HR5/5)	**Equilibrium:** 20 mM bisTris-HCL, pH 5.8 lo mM dithiothreitol **Elution:** linear gradient of NaCl, 0–0.35 M 20 mM bisTris-HCL, pH 5.8 lo mM dithiothreitol				
			RP-chromatography	**NR**	330U/mg of protein, 500U/mg of protein, 240U/mg of protein (recombinant rat SCOMT)			
	1. Cell pellets resuspended; 2. Cell disrupted by lysozyme treatment (0.5 mg/mL) followed by sonication on ice (10min, 90% amplitude with 0.5s interval); 3. Centrifuged at 16000 g for 20 min at 4 °C; 4. Supernatant recovered; 5.Ammonium sulfate precipitation (55% on ice); 6. Centrifuged (13000g, 20 min); 7. Pellet dissolved	**1**	**HIC** (Butyl-sepharose 4FF)	**Adsorption**: 0.6 M ammonium sulphate in 10 mM Tris at pH 7. 8 **Elution: setp gradient of ammonium sulfate** (0.2, 0.075 to 0M) in 10mM Tris at pH 7.8	1461±30	3.9	23	[120]
			HIC (Epoxy-sepharoseCL-6B)	**Adsorption**: 0.6 M ammonium sulphate in 10 mM Tris at pH 7. 8 **Elution:** 10mM Tris at pH 7.8	NR	NR	NR	
			HIC (Phenyl-sepharose 6FF)					
			HIC					

	1. Cell pellets resuspended; 2. Cell disrupted by **lysozyme** treatment (0.5 mg/mL) followed by cycles of **freeze/thaw** (in liquid nitrogen at −120 ◦C and a water bath at 42 ◦C); 3. Centrifugation at 16,000 g for 20 min at 4 ◦C; 4. Supernatant recovery.	**1**	(Octyl-sepharose 6FF)	**Adsorption**: 0.025 M ammonium sulphate and 0.025 M sodium Citrate in 10 mM Tris at pH 7.8 **Elution:** 10mM Tris at pH 7.8	677±10	1.5	22	[142]
				Adsorption: 0.025 M ammonium sulphate and 0.025 M sodium Citrate in 10 mM Tris at pH 7.8 **Elution:** 10mM Tris at pH 7.8 (5°C)	1688 ± 11	5,9	13	
	1. Cell pellets resuspended; 2. Cell disrupted by lysozyme treatment (0.5 mg/mL) followed by sonication on ice (10min, 90% amplitude with 0.5s interval); 3. Centrifuged at 16000 g for 20 min at 4 ◦C; 4. Supernatant recovered; 5.Ammonium sulfate precipitation (55% on ice); 6. Centrifuged (13000g, 20 min); 7. Pellet dissolved	**2**	**HIC** (Butyl-sepharose 4FF)	**Adsorption**: 0.6 M ammonium sulphate in 10 mM Tris at pH 7. 8 **Elution:** 0.2 M ammonium sulphate followed by 10mM Tris at pH 7.8	1688	1,8	14	[113]
			SEC (Superose™ 12)	**Equilibrium: 10mM Tris at pH 7.8**	5500	5,9	1	
	1. Cell pellets resuspended; 2. Cell disrupted by **lysozyme** treatment (0.5 mg/mL) followed by cycles of **freeze/thaw** (in liquid nitrogen at −120 ◦C and a water bath at 42 ◦C); 3. Centrifugation at 16,000 g for 20 min at 4 ◦C; 4. Supernatant recovery.	**2**	**AEC** (Q-sepharose)	**Adsorption**: 10 mM Tris at pH 7,8 **Elution:** step gradient of 350 mM and 1 M NaCl in 10 mM Tris at pH 7.8	250	3,6	107	[143]
	1.Cell pellets resuspended; 2.Cell disrupted by **lysozyme** treatment (0.5 mg/mL) followed by cycles of **freeze/thaw** (in liquid nitrogen at −120 ◦C and a water bath at 42 ◦C); and homogenized; 3.Centrifugation at 14,000 g for 15 min at 4 ◦C; 4. Supernatant recovery.	**2**	**AC** (calmodulin-affinity XK-16/20)	**Equilibrium:**300 mM NaCl, 10 mM DTT, 1 mM $MgCl_2$, 1mM imidazole, 2 mM $CaCl_2$, 50 mM Tris pH 8.0 and 10% glicerol **Elution:** 300 mM NaCl, 2 mM EGTA in 50 mM Tris buffer pH 8.0, containing 10 mM DTT and 10% glycerol	9759		30	[93]
			SEC (Superdex 75XK 16/60)	**Equilibrium**: 150 mM NaCl, 1 mM MgCl2 in 20 mM Tris at pH 8.5, containing, 10mM DTT, and 10% glycerol.	12113		8	

	NR	**2**	**IMAC** (Talon)	**Adsorption**: 100 mM Tris, 200 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol at pH 7.5 **Elution:** gradient step of 7 and 100 mM imidazole in 100 mM Tris, 200 mM NaCl, 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol at pH 7.5	NR	NR	NR	[26]
			SEC (Superdex 75)	**Equilibrium**: 50 mM Tris, 200 mM NaCl, and 10 mM DTT at pH 8				
	1.Cell pellets resuspended; 2.Cell disrupted by **lysozyme** treatment (0.5 mg/mL) followed by cycles of **freeze/thaw** (in liquid nitrogen at −120 ◦C and a water bath at 42 ◦C); and homogenized; 3.Centrifugation at 14,000 g for 15 min at 4 ◦C; 4. Supernatant recovery.	**1**	**AC** (L-histidine) **AC** (L-aspartate) **AC** (L-methionine) **AC** (L-leucine) **AC** (L-arginine) **AC** (L-glutamine)	**Elution:** stepwise gradient from 0 to 100% buffer A (buffer A: 10 mM Tris–HCl, pH 7.8 (l-arginine), 4 (l-aspartate), 6.5 (l-glutamine), 5 (l-methionine and l-histidine), 5.7 (l-leucine); buffer B: SC 1 M in 10 mM Tris–HCl, pH 7.8)	NR	NR	NR	[116]
***K. Pastoris* X-33**	1. Cells resuspended; 2. Cells disrupted by **glass beads** mechanical lysis 3. Centrifugation; 4. Pellet resuspended	**1**	**IMAC** (His Trap FF crude)	**Adsorption**: 500 mM NaCl, 50 mM Tris, 1mM $MgCl_2$ and 5mM imidazole at pH 7.8 **Elution:** step gradient of imidazole of 50mM,,70mM 300mM and 500mMin 500mM NaCl, 50 mM Tris and 1mM$MgCl_2$ at pH 7.8	121,1 ± 5,1	81	57,4	[19]
Sf9 insect cells	1.Cells Disrupted 2.Centrifuged 1000g 3.Supernatant collected	**5**	**SEC** (Bio-Gel P-100)	**Equilibrium:** 20 mM Bistris/CL, pH 5.8	**NR**	**NR**	**NR**	[94]
			AEC (Q-Sepharose HL 16/16)	**Equilibrium:** 20 mM Bistris/CL, pH 5.8 **Elution:** linear gradient of NaCl, 0–0.5 M in 20 mM Bistris/CL, pH 5.8				
			SEC (Bio-Gel P-100)	**Equilibrium:** 20 mM bisTris-HCl pH 5.8, lo mM dithiothreitol				
			AEC (Q-Sepharose HL 16/16)	**Equilibrium:** 20 mM bisTris-HCL, pH 5.8 lo mM dithiothreitol				

				Elution: linear gradient of NaCl, 0–0.35 M 20 mM bisTris-HCL, pH 5.8 lo mM dithiothreitol				
			RP-Chromatography	**NR**				
MBCOMT								
Source	**Protein Recovery**	**Chromatographic methodology**		**Elution strategy**	**Specific activity (nmol/h/mg of protein)**	**Purification factor (fold)**	**Bioactivity recovery (%)**	**Ref.**
		Nº of Steps	**Chromatography**					
Rat and liver	1.Cells homogenized 2. Centrifuged (1500g, 20min), (100000g, 60min, 4ºC) 3. Pellets whashed and centrifuged (1000000g 60min, 4ºC) 4.sSolubilez with 1.0% (v/v) Triton X-100 in 5 mM sodium phosphate buffer, pH 7.8 (30 min at 0°C) 5. Centrifuged (100,000*g*, 30min, 4°C) 6. Supernatant collected	**1**	**AEC** (Resource Q column)	**Equilibrium:** 20 mM Tris, pH 7.8, 0.5% Triton X-100. **Elution:** linear gradient of NaCl, 0–0.5 M in 20 mM Tris, pH 7.8, 0.5% Triton X-100	NR	NR	NR	[21]
Brevibacillus choshinesis	1.Cell resuspended 2.Cell disrupted by **lysozyme** treatment (10.0 mg/ml) (15 min at room temperature) followed by six **freeze** (−196 ◦C in liquid nitrogen)/**thaw** (42 ◦C) cycles. 3. DNase (1.0 mg/ml) was added to the lysate 4. Soluble material removed by centrifugation (16000g, 20 min, 4 ◦C).	**1**	**HIC** (butyl-sepharose 4FF)	**Adsorption**: 250 mM NaH_2PO_4 in mM of Tris pH 7.8 **Elution**10 mM of Tris pH 7.8 followed by a linear detergent 0 to 1% of Triton X-100 in 10mM of Tris-HCL pH7.8	NR	NR	NR	[115]
			HIC (octyl Sepharose 6FF)	**Adsorption**: 375 mM NaH_2PO_4 in mM of Tris pH 7.8 **Elution:** 10 mM of Tris pH 7.8 followed by a linear detergent gradient from 0 to 1% of Triton X-100 in 10mM of Tris-HCL pH7.8	NR	NR	NR	
		1	**AEC** (Q-sepharose)	**Adsorption**: NaCl linear gradient (0 to 100mM) in 10mM Tris at pH 7.8 **Elution:** NaCl step gradient (300mM,and 350)ollow by NaCl linear gradient (350 to 1M) and a step of 1M NaCl in in 10mMTris pH 7.8	496	7	67	[143]
				Adsorption: 0.5 % of Triton X-100 in 10mMTris at pH 7. 8	331	4.3	91	

				Elution: NaCl step gradient of 300 mM, followed by a NaCl linear gradient(350 to 1 M) follow by a step of 1 M NaCl, and 0.5% of Triton X-100 in 1 mM Tris pH 7.8				
Sf9 insect cells	1.Cell resuspended; 2.Cell disrupted by sonication (10s pulses for 3-4min); 3. Centrifuged (23800g at 4°C, 1h); 4. Pellet resuspended without detergent andsonicated and collected as previously; 5. Supernatant discarted; 6. Pellet resuspended with detergent (1% FC-12) and sonicated; 7. Centrifuged (32000g, at 4°C ,1h); 8. Supernatant recover	**1**	**IMAC** (His Trap FF crude)	**Adsorption**: Buffer A (50 mM Tris, 150-200mM NaCl, 1-2 mM tris, 30-40 mM imidazole, 2mM $MgCl_2$ at pH 7.4 **Elution:** 50% of Buffer A with detergent linear gradient of 300 mM to 500 mM imidazole	NR	NR	NR	[104]
***K. Pastoris* X-33**	1. Cells resuspended; 2. Cells disrupted by glass beads mechanical lysis 3. Centrifugation; 4. Pellet resuspended with detergent and incubated 3h at 4°C; 5. Centrifugation	**2**	**IMAC** (His trap FF crude with Ni^{2+})	**Adsorption:** 50 mM Hepes, 500mM NaCl, 0.03% DDM and 1mM$MgCl_2$ at pH 7.8 **Elution:** imidazole step gradient of 10, 50, 175 and 500 mM in 50 mM Hepes, 500 mM NaCl, 0.03% DDM and 1mM $MgCl_2$ at pH 7.8	67 ± 67	1,53	47,3	[118]
			AEC (Q-sepharose)	**Adsorption:** 50 mM, 50 mM NaCl at pH 7.3 **Elution:** NaCl step gradient of 300, 500 and, 1M in 50 mM at pH 7.3				

Table 4: Overall operational conditions of several currently used analytical methods for the determination of COMT activity

Analyte	COMT	Source	Substrate	Technique	Detection	Column	Mobile phase	LLOQ	LLOD	Reference
3-*O*-methyldopamine	S and MB	Recombinant	Dopamine	HPLC	ED	ODS-2 Spherisorb	Citric acid (27.5 mM) + Sodium acetate (50 mM) + EDTA (1 mM) + 1-octanesulfonic acid (1 mM) + Methanol (13%) - pH 2.5	NR	10 nA	[20]
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-*O*MD			*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 Kidney	Human	Epinephrine	HPLC	ED	NR	Citric acid (0.1 mM) + Sodium octylsulphate (0.5 mM) + Methanol (8%) - pH 3.5	NR	350 to 500 fmol	[144]
3-*O*-methyldopamine	Liver and Brain	Rat	Dopamine	HPLC	ED	Brownlee C18 Velosep (3.2 mm x 10 cm, 3 µm)	NR	NR	NR	[145]
Vanillic acid	Liver, Duodenum and Renal Cortex	Human	DBA	Radiochemical/ TLC	NR	NR	NR	NR	NR	[146]
Metanephrine	Liver and Brain	Rat	Epinephrine	HPLC	ED	ODS (5mm x 25 cm)	Citric acid (0.1 mM) + Sodium octylsulphate (0.5 mM) + Methanol (8%) - pH 3.5	NR	350 to 500 fmol	[132,147]
Vanillic acid	S	Recombinant	DBA	Radiochemical/ HPLC	λ – 260 nm	Hypersil BDS C18 (125 x 4 mm, 5 µm)	Phosphate/Citrate buffer (50 mM Na_2HPO_4) + Citric acid (20 mM) + Na_2EDTA (0,15 mM) + 1-octanesulphonic acid (1.25 mM) + Methanol (3%) - pH 3.2	0,45 pmol	9 mol	[148]
Sinapyl aldehyde	NR	Recombinant	5-hydroxyconiferyl aldehyde	HPLC UV/MS	Diode array - 344 nm	Supelcosil ABZ-Plus (25 cm x 2.1 mm, 5 µm)	NR	NR	NR	[149]
Vanillic acid	S and MB	Recombinant	DBA	HPLC	ED	LiChrospher 100 RP C18 (125 x 4mm, 5 µm)	Na_2HPO4 (0.1M) + EDTA (0.15 mM) + Methanol (15%) - pH 3.2	NR	NR	[150,151]
2-MeOE1	NR	Recombinant	2-OHE1	GC	NR	NR	5% phenyl methyl silicone stationary phase fused silica capillary column (30 m x 0,2 mm x 0,5 µm)	NR	400 to 800 fmol	[152]

2-MeOE1	S	Recombinant	2-OHE1	HPLC	ED	YMC-Pack FL-oDS3 AM	Citric acid (0.75 mM) + Ammonium acetate (25 mM) + Acetonitrile (35%)	NR	NR	[153]
3-*O*-methyldopamine	Intestine	Rat	Dopamine	HPLC	ED	NR	pH 8.6	NR	350 to 1000 fmol	[154]
Vanillic acid	Liver	Rat	DBA	HPLC with diode array	ED	Purospher RP-18e (4 x 125 mm)	Disodium hydrogen phosphate (0.1M) + Na_2-EDTA (0.15mM) + Methanol (15%) - pH 3.2	NR	NR	[133]
Metanephrine	Adrenal gland	Rat	Epinephrine	HPLC with fluorescence detection	ED	TKS-gel ODS-80TsQa (150 x 4.6 mm)	Potassium acetate buffer (75mM) + Potassium buffer (100 mM) + Sodium l-hexane sulfonate (4mM) + Acetonitrile (5%) - pH 3.2	NR	0,2 pmol	[155]
Methylated catechol	Brain	Human	Catechol	Radiochemical	NR	NR	NR	NR	NR	[156,157]
3-*O*-methyldopamine	S and MB	Recombinant	Dopamine	HPLC/Radiochemical	UV/radioactive	Spherisorb ODS column (250 x 4.6 mm, 5 µm)	Ammonium formate (100 mM) + Water + Acetonitrile (10%) - pH 3.0	NR	NR	[158]
2-MeOE1	Liver	Human	2-OHE1	Radiochemical	NR	NR	NR	NR	NR	[140,159–161]
Vanillic acid	S	Human	DBA	Radiochemical	NR	NR	NR	NR	NR	[162]
Metanephrine	S	Human	Epinephrine	HPLC	ED	ODS2 (25 cm x .,6 mm)	Citric acid (0.1 mM) + Sodium octylsulphate (0.5 mM) + Sodium acetate (0.1M) + Na_2EDTA (0.17 mM) + Dibultylamine (1mM) + Methanol (10%) - pH 3.5	NR	350 to 1000 fmol	[163]
3-*O*-methyldopamine	Liver	Human	Dopamine	LC-MS	NR	ODS BP (2.1 x 150 mm, 5 µm)	CH_3CN + Formic acid (0.2%)	NR	NR	[164]
3-*O*-methyldopamine	Brain	Rat	Dopamine	HPLC	ED	ODS Reverse phase (4.6 x 150 mm)	NR	NR	NR	[165]
SAH	Liver	Porcine	SAM	fluorescein isothiocyanate	NR	NR	NR	NR	0.15 pmol	[166]
Metanephrine	Brain	Rat	Epinephrine	HPLC	ED	ODS2 (25 cm x 4.6 mm, 5 µm)	Citric acid (0.1 mM) + Sodium octylsulphate (0.5 mM) + Sodium acetate (0.1M) + Na_2EDTA (0.17	NR	350 to 1000 fmol	[14]

							mM) + Dibultylamine (1mM) + Methanol (10%) - pH 3.5			
Metanephrine	Brain	Rat	Epinephrine	HPLC	ED	ODS (25 cm x 4.6 mm, 5 µm)	Citric acid (0.1 mM) + Sodium octylsulphate (0.5 mM) + Sodium acetate (0.1M) + EDTA (0.17 mM) + Dibultylamine (1mM) + Methanol (6%) - pH 3.5	NR	350 to 1000 fmol	[71]
Normetanephrine	Liver	Porcine	Norepinephrine	HPLC	Fluorescence (283/315 nm)	Phenomenex USP L1 Luna C18 (250 x 4.6 mm, 5 µm)	Sodium phosphate buffer (10 mM) + Boric acid (80 mM) + Sodium 1-hex-anelsulfonate (4 mM) + Acetonitrile (10%)	NR	NR	[79]
Scopoletin	NR	Porcine	Aesculetin	HPLC	Fluorescence (335/460 nm)	NR	NR	NR	NR	[78]
2-MeOE1	Liver	Human	2-OHE1	HPLC	ED	Supelcosil C18 (15 cm x 4.6 mm, 5 µm)	Acetonitrile (33%) + THF (6%) + Phosphate buffer (15 mM) - pH 3.4	NR	NR	[167]
Vanillic acid	Placenta	Human	DBA	HPLC-LC	λ – 280 nm	PerfectSIL C18 ace-EPS (250mm x 4.6 mm, 5 µm)	Sodium phosphate monobasic buffer (17.1 mM) + Acetonitrile (33%) + Tetrahydrofuran (6%) - pH 3.4	NR	NR	[168]
2-MeOE1	Liver	Human	2-OHE1	HPLC	ED	Supelcosil C18 (250 mm x 4.6 mm, 5 µm)	Sodium phosphate monobasic buffer (17.1 mM) + Acetonitrile (33%) + Tetrahydrofuran (6%) - pH 3.4	NR	NR	[167]
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 x 4.6 mm, 5 µm)	Water + Methanol + Trifluoroacetic acid (30:70:0,2)	NR	NR	[72]
-	NR	Recombinant	4-nitrocatechol-Alexa Fluor 488	Fluorescence	475 nm/535 nm	NR	NR	NR	NR	[140]
SAH	MB	Recombinant	SAM	HTRF	Fluorescence	NR	NR	NR	NR	[88,169]
3-*O*-methyldopamine	Brain	Rat	Dopamine	HPLC	ED	LiChrospher 100 RP C18 (125 x 4mm, 5 µm)	Na2HPO4 (0.1M) + EDTA (0.15 mM) + Methanol (15%) - pH 3.2	0.5 pmol	0.1 pmol	[170]

Metanephrine	S	Recombinant	Epinephrine	HPLC	ED	Xterra MS C18 ODS (250 x 4.6 mm, 5 µm)	EDTA (0.145 mM) + Sodium acetate (0.1 M) + Citric acid (0.1 mM) + Sodium octyl sulphate (0.5 mM) + Dibutylamine (1 mM) + Methanol (5%) - pH 3.5	NR	100 nA	[130]
Metanephrine	MB	Recombinant	Epinephrine	HPLC	ED	Zorbax 300SB C18 RP	Sodium dihydrogen phosphate (0.1 M) + citric acid monohydrate (0.024 M) + Sodium octyl sulphate (.5 mM) + Acetonitrile (9%) - pH 2.9	0.25 nmol/ mL	1 uA	[127]
Normetanephrine	Erythrocytes	Human	Norepinephrine	Normetanephrine ELISA kit	NR	NR	NR	NR	NR	[171]

Table 5: The kinetic parameters (K_m, V_{max}) of the COMT enzyme and its affinity to catechol substrates in the presence or absence of inhibitors (K_i, K_{cat} and IC_{50})

Substrate	COMT	Source	V_{max}	K_m (μM)	Inhibitor	K_i (nM)	K_{cat} (min^{-1})	IC_{50}	Reference
Dopamine (300 μM)	S	Recombinant	37.2 ± 1.3 (min^{-1})	207 ± 4 (μM)	Entacapone (10 - 30 nM)	0.30	31.1	NR	[20]
	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		
Epinephrine (500 μM)	Liver	Rat	5.3 ± 0.5 (nmol/mg protein/h)	3.3 (μM)	Tolcapone (0,5 - 10000 nM)	NR	NR	41 (nM)	[144]
	Kidney		2.6 ± 0.1 (nmol/mg protein/h)	2.7 (μM)				8 (nM)	
Dopamine (50 mM)	Liver	Rat	49.0 ± 9.8 (nmol 3-*O*-methyldopamine/gm wet tissue x min)	NR	Entacapone (2,5 - 30 mg/kg)	NR	NR	NR	[145]
	Brain		0.4 ± 0.04 (nmol 3-*O*-methyldopamine/gm wet tissue x min)		3,5-dinitrocatechol (2,5 - 30 mg/kg)				
DBA (242 μM)	Liver	Human	NR	NR	Entacapone (62.5 - 1000 nmol/L)	NR	NR	151 (nM)	[146]
					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)	
Epinephrine (500 μM)	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)	[132,147]
	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		58 ± 4 (μM)	3,5-DNC	NR	NR	28 (nM)	

			345 ± 78 (nmol/mg protein/h)					3 (nM)	
	MB		3.0 ± 0.6 (nmol/mg protein/h)	24 ± 1 (μM)		NR	NR	356 (nM)	
								11 (nM)	
DBA (240 μM)	Liver	Rat	5210 (μM)	156 (μM)	Entacapone	10.7	NR	20.1 (nM)	[133]
					Tolcapone	10.0		19.7 (nM)	
Epinephrine (1000 μM)	Liver	Rat	NR	NR	Entacapone	NR	NR	30.59 ± 4.85 (nM)	[71]
					Tolcapone			34.45 ± 9.01 (nM)	
	Brain		NR	NR	Entacapone			0.91 ± 0.17 (nM)	
					Tolcapone			3.47 ± 0.48 (nM)	
Norepinephrine (250 μM)	Liver	Porcine	NR	379 (μM)	Entacapone (0.0003 – 1 μM)	NR	NR	0.00047 (μM)	[79]
					Tolcapone (0.0003 – 1 μM)			0.0068 (μM)	

List of Figures

Figure 1. A- 3D structure of COMT (PDB#6I3C), adapted from [130]. B- Major molecular interactions that take place in the COMT active site (PDB#6I3C), black dashes represent hydrogen bonds, red dashes represent metallic bonds.

Figure 2. Structure of the commercial COMT inhibitors.

Figure 3. Structure of other COMT inhibitors.

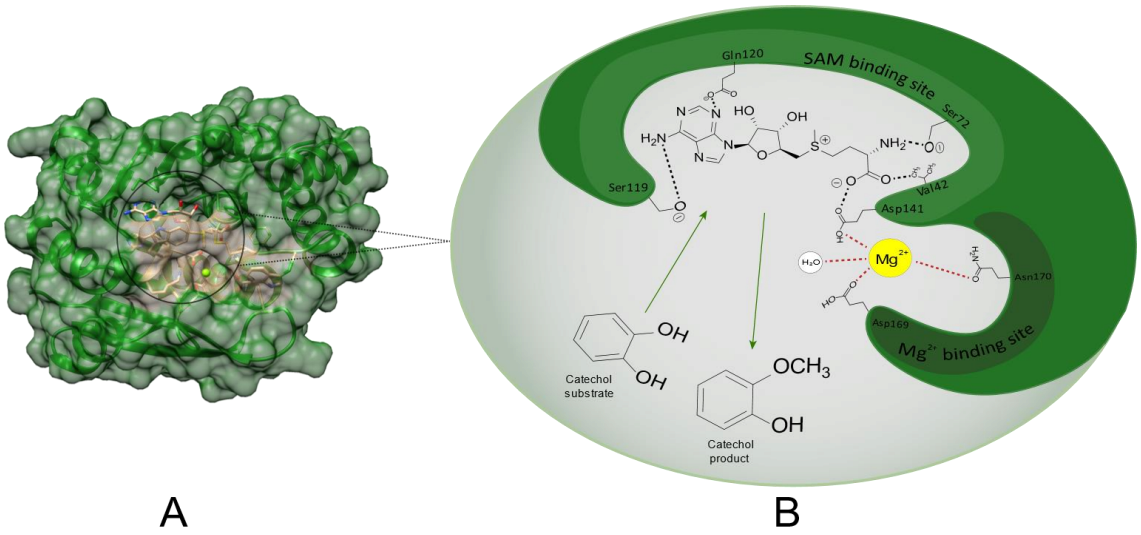
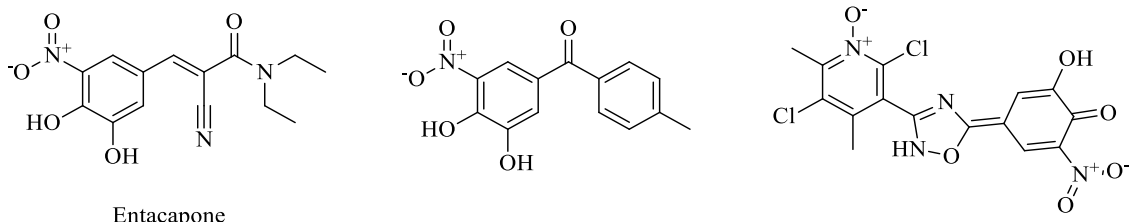


Figure 1 **– A- 3D structure of COMT (PDB#6I3C), adapted from [130]. B- Major molecular interactions that take place in the COMT active site (PDB#6I3C), black dashes represent hydrogen bonds, red dashes represent metallic bonds with Mg^{2+}.**



Entacapone Tolcapone Opicapone

Figure 2 **– Structure of the commercial COMT inhibitors.**

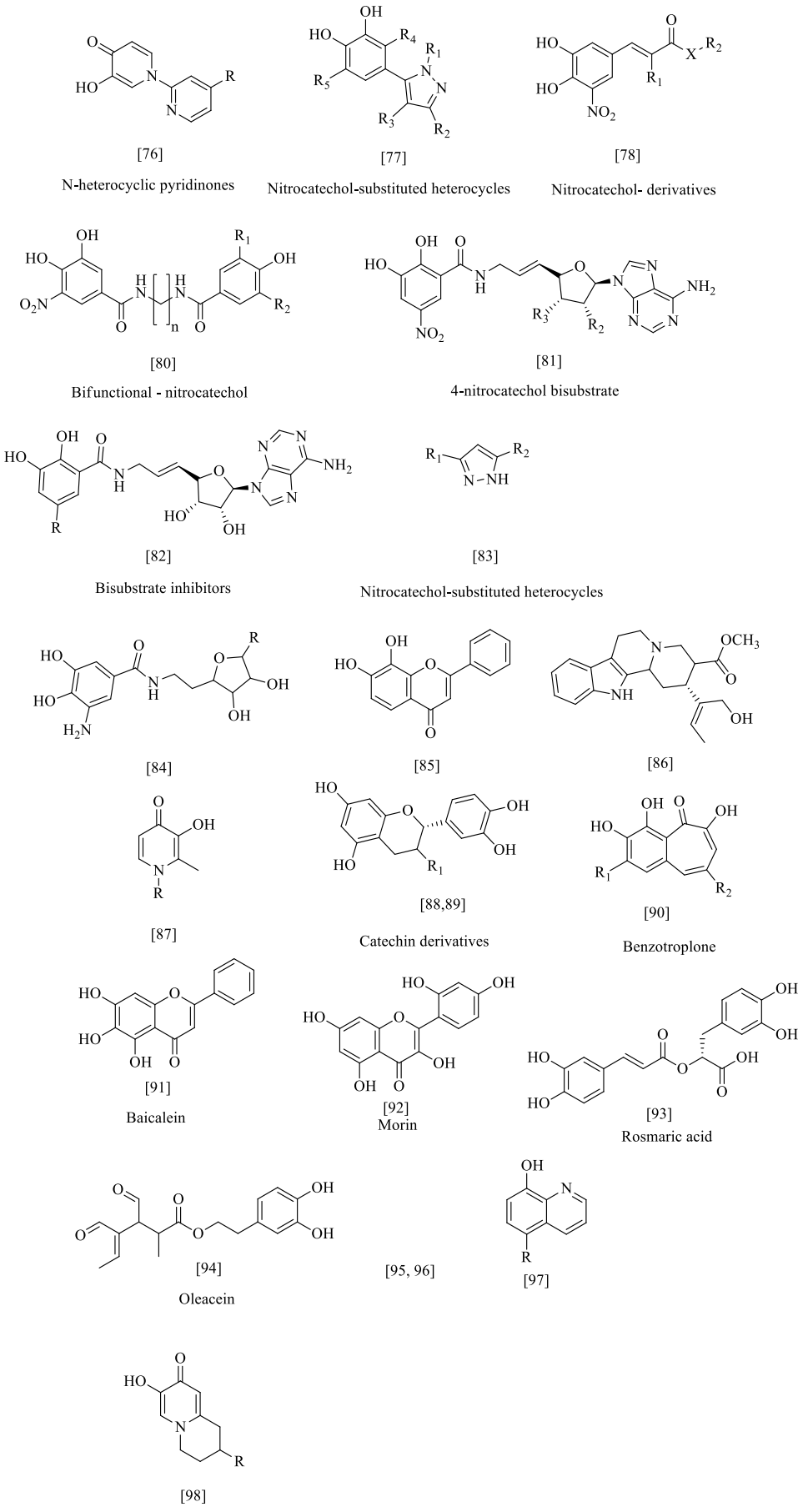


Figure 3 – Structure of other COMT inhibitors.