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Crystal Structure of Catechol *O*-Methyltransferase Complexed with Nitecapone

Hiroshi Iijima,^{*a} Katsuki Takebe,^b Mamoru Suzuki,^c Hiroko Kobayashi,^a Tomoko Takamiya,^a Hiroaki Saito,^a Norio Niwa,^a and Takao Kuwada-Kusunose^d

^aSchool of Pharmacy, Nihon University; 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan; ^bOral and Maxillofacial Surgery II, Osaka University Graduate School of Dentistry; Suita, Osaka 565-0871, Japan; ^cInstitute for Protein Research, Osaka University; 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan; and ^dSchool of Dentistry at Matsudo, Nihon University; 2-870-1 Sakaemacho Nishi, Matsudo, Chiba 271-8587, Japan.

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Catechol *O*-methyltransferase (COMT) is known as an important drug-target protein in the field of Parkinson's disease. All clinically approved COMT inhibitors bring a 5-substituted-3-nitrocatechol ring as a pharmacophore, and they bind to COMT with *S*-adenosylmethionine (SAM) and an Mg²⁺ ion to form a quaternary complex (COMT/SAM/Mg²⁺/inhibitor). However, structural information about such quaternary complexes is only available for a few inhibitors. Here, a new crystal structure of COMT complexed with nitecapone (5), SAM and Mg²⁺ is revealed. Comparison of the structures of these complexes indicates that conformation of the catechol binding pocket is almost constant regardless of structure of the inhibitors. The only restriction of the side chain of inhibitors (*i.e.*, the substituent at the 5-position of 3-nitrocatechol) seems to be that it does not make steric repulsion with COMT. However, recent crystallographic and biochemical studies suggest that COMT is a flexible protein, and its conformational flexibility seems crucial for its catalytic process. Based on this information, implications of these quaternary inhibitor complexes were investigated. Met 40 in the $\alpha 2\alpha 3$ -loop makes atomic contacts with SAM or *S*-adenosylhomocysteine and the 3-position of the catechol inhibitor. This interaction seems to play a critical role in the affinity of the inhibitor and to stabilize the COMT/SAM/Mg²⁺/nitrocatechol inhibitor complex by fixing the flexible $\alpha 2\alpha 3$ -loop.

Key words catechol *O*-methyltransferase; nitecapone; crystal structure

Introduction

Catechol *O*-methyltransferase (COMT) is an important enzyme that catalyzes *O*-methylation of a wide variety of endogenous and exogenous catechol substrates. COMT catalyzes the transfer of a methyl group from *S*-adenosylmethionine (SAM) to catechol substrates such as noradrenaline, adrenaline and dopamine as well as 2-hydroxyestadiol.¹⁾

COMT inhibitors are used to enhance the uptake of levodopa in patients with Parkinson's disease.²⁾ Levodopa, a precursor of dopamine in the central nervous system, is the most efficacious and tolerated medicine for Parkinson's disease. Levodopa is taken up through the neutral amino acid transporters to pass the blood–brain barrier, but it is highly susceptible to decarboxylation catalyzed by dopa decarboxylase in the peripheral circulation. To prevent the decrease of levodopa in the circulation, dopa decarboxylase inhibitors (DDIs) are used with levodopa. This treatment enhances the bioavailability of levodopa by extending its half-life, which enhances its clinical effect. However, inhibition of the decarboxylation pathway by DDIs leads to enhanced formation of 3-*O*-methyldopa by COMT as an alternative pathway. Use of COMT inhibitor in combination with levodopa/DDI is rationalized with the following four major reasons: (1) inhibitors protect levodopa in circulation and improve its bioavailability; (2) because the COMT metabolite, 3-*O*-methyldopa, competes with levodopa in the amino acid transporters in the gut and blood–brain barrier, suppression of the generation of 3-*O*-methyldopa is advantageous; (3) COMT inhibition is proven effective to avoid the wearing-off phenomenon; and (4) COMT produces

S-adenosylhomocysteine (SAH), which is related with many cardiovascular events.³⁾ The inhibition of COMT reduces the undesirable generation of SAH. It should be noted that the usual dose of levodopa is 600–750 mg/d when used with a DDI. Given that 600 mg (3 mmol) of levodopa is exclusively metabolized by COMT, 1.2 g of SAH would be produced.

Today, COMT inhibitors attract certain interest in the field of schizophrenia as the membrane-bound form of COMT plays a critical role in the clearance of dopamine in the prefrontal cortex in contrast to that in the mid-brain, in which major clearance of dopamine is made by its transporters.⁴⁾ COMT inhibition could be important for the treatment of psychotic disorder in the future.

Selective and orally active COMT inhibitors have been developed since the mid-1980s, and most of them are 3-nitrocatechol derivatives (Fig. 1). Among them, entacapone (1) and tolcapone (2) have been used clinically since the mid-1990s.⁵⁾ Entacapone is a peripherally active COMT inhibitor whereas tolcapone penetrates the blood–brain barrier, though with low efficiency, and works in brain, too. Tolcapone occasionally causes hepatotoxicity and is not approved in Japan. Entacapone is regarded as being safer, but its half-life is shorter such that frequent dosing is necessary. Opicapone (3), also carrying a 3-nitrocatechol pharmacophore, was approved in the European Union in 2016.⁶⁾ The effect of opicapone is reported to last longer than that of entacapone and tolcapone. Administration of opicapone once a day is regarded adequate to reduce the wearing-off phenomenon. As 3-nitrocatechol structurally resembles 2,4-dinitrophenol, which is known as a uncoupler

* To whom correspondence should be addressed. e-mail: iijima.hiroshi@nihon-u.ac.jp

Fig. 1. Chemical Structures of COMT Inhibitors

The Protein Data Bank IDs are indicated if the structure of COMT complexed with the compounds are available in the Protein Data Bank.

of mitochondrial proton transfer,⁷⁾ scaffolds other than 3-nitrocatechol have been under development to reduce hepatic toxicity. To design a new type of inhibitor, understanding the structural features of the interaction between nitrocatechol inhibitors and COMT is important.

Here, we report a crystal structure of soluble form of rat COMT (S-COMT) complexed with SAM, Mg²⁺ and nitecapone (**5**) to investigate the interaction between the enzyme and the inhibitor.

Experimental

Expression and Purification Rat S-COMT was expressed as a fusion protein with glutathione *S*-transferase. Details of the construction of the expression plasmid, protein expression and purification are supplied as supplemental material.

Recombinant rat S-COMT protein carries a wild-type rat S-COMT sequence with two additional amino acid residues (GS) derived from the expression plasmid at the *N*-terminus. The amino acid sequence of the recombinant rat S-COMT

used in this study is as follows: GSMGDTKEQRILRYVQQN AKPGDPQSVLEAIDTYCTQKEWAMNVGDAKGQIMDAV IREYSPSLVLELGAYCGYSAVRMARLLQPGARLLTMEM NPDYAAITQQMLNFAGLQDKVTILNGASQDLIPQLKKKY DVDTLDMVFLDHWKDRYLPDTLLLEKCGLLRKGTVLL ADNVIPTGTPDFLAYVRGSSSFECTHYSSYLEYMKVVD GLEKAIYQGPPSPDKS, where the underlined M represents Met 1 of the native sequence. In this paper, we adopted amino acid numbering based on the native S-COMT sequence.

Crystallization, Diffraction Measurement and Structure Refinement Set-up of the crystallization experiments was completed within 2 d after the purification.

To a solution of rat S-COMT (1.4 mg/mL, 55 μ M, in phosphate buffered saline (PBS) containing 1 mM dithiothreitol (DTT)) was added 10 mM aqueous solution of SAM, 50 mM dimethyl sulfoxide (DMSO) solution of nitecapone and 2 M aqueous solution of MgCl₂ to give final concentrations of 150 μ M, 750 μ M and 2 mM, respectively. The mixture was incubated for 2 h at 20°C and then concentrated to 9.1

Table 1. Crystallographic Data Collection

Diffraction source	SPring-8 BL41XU
Wavelength (Å)	1.000
Temperature (K)	100
Detector	Eiger-4M
Rotation range per image (°)	0.1
Total rotation range (°)	180
Exposure time per image (s)	0.1
Space group	$P2_12_12_1$
a, b, c (Å)	49.665 53.298 80.786
α, β, γ (°)	90.00 90.00 90.00
Resolution range (Å)	47.57–1.60 (1.63–1.60)
Total No. of reflections	177424 (5248)
No. of unique reflections	28732 (1262)
Completeness (%)	99.2 (92.2)
Redundancy	6.2 (4.2)
$\langle I/\sigma(I) \rangle$	13.7 (3.6)
R_{merge}^a (%)	7.2 (31.0)
$CC_{1/2}^b$	0.998 (0.907)

Values in parentheses are for the highest resolution shell. a) $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measured for the i th reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflection with indices hkl . b) $CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data.

Table 2. Refinement Statistics

Resolution range (Å)	9.92–1.60 (1.66–1.60)
Completeness (%)	98.39 (92.94)
No. of reflections, working set	28518 (2646)
No. of reflections, test set	1431 (140)
Final R_{work}	0.1476 (0.1989)
Final R_{free}^a	0.1611 (0.2393)
No. of non-H atoms	
Protein	1734
Ligand	42
Water	230
Total	2006
R.m.s. deviations	
Bonds (Å)	0.016
Angles (°)	1.51
Average B factors (Å ²)	
Protein	17.80
Ligand	29.93
Water	34.49
All atoms	19.96
Ramachandran plot ^b	
Most favoured (%)	96.7
Allowed (%)	3.3
Disallowed (%)	0

Values in parentheses are for the highest resolution shell. a) To calculate R_{free} , a subset of reflections (5.0%) was randomly chosen as a test set. b) Ramachandran plot statistics is based on Rampage software available in the CCP4 software package.

mgprotein/mL by using Spin-XTM UF 6 (MW cut-off 10kDa) at 4°C. Crystallization was conducted at 20°C by the sitting-drop vapor diffusion method. The concentrated protein was mixed in a 1 : 1 volume ratio with reservoir solution consisting of 0.1 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) sodium pH 7.5, 10% (v/v) 2-propanol and 20% (w/v) polyethylene glycol 4000. After 2 d, orange rod-shaped crystals were grown. The crystals were cryoprotected with a solution consisting of 0.1 M HEPES sodium pH 7.5, 10% (v/v) 2-propanol and 40% (w/v) polyethylene glycol 4000 and flash-cooled with liquid nitrogen.

Data Collection and Processing The X-ray diffraction experiments were performed in the BL41XU beamline of SPring-8 (Harima, Hyogo, Japan) with a wavelength of 1.000 Å and diffraction data were collected from 0° to 180° with an oscillation range of 0.1°.

The datasets were integrated and processing using the *XDS* Package⁸⁾ and scaled using *Aimless* from the *CCP4* suite.⁹⁾ The crystal structure was solved by molecular replacement methods with *MOLREP* using the structure of rat S-COMT (Protein Data Bank ID: 1VID¹⁰⁾) as a search model. The structure model was refined using *PHENIX*¹¹⁾ and *REFMAC5*.¹²⁾ Manual model building was performed using *Coot*.¹³⁾

The atomic coordinates and the structure factors of rat S-COMT/SAM/Mg²⁺/nitecapone complex are deposited in the Protein Data Bank under accession code 6LFE.

Results

Crystallography The crystals of COMT/SAM/Mg²⁺/nitecapone complex belonged to space group $P2_12_12_1$ with unit-cell parameters: $a = 46.67$ Å, $b = 53.23$ Å, $c = 80.79$ Å and $\alpha = \beta = \gamma = 90.00^\circ$. We determined the structure at 1.6 Å resolution and refined it to R_{work} and R_{free} values of 14.47 and 16.95%, respectively. The crystallographic summary is given in Table 1 (data collection) and Table 2 (refinement).

Overall Structure The structure of COMT/SAM/Mg²⁺/nitecapone complex was solved at 1.6 Å resolution. Figure 2

shows a schematic diagram of the complex. Nitecapone chelates with a six-coordination Mg²⁺ ion, which is coordinated by a water molecule, the side chain carbonyl oxygen atom ($O_{\delta 1}$) of Asn 170 and carboxylates ($O_{\delta 1}$) of Asp 141 and Asp 169.

The overall structure of the COMT/SAM/Mg²⁺/nitecapone complex overlaps well with the known COMT structures carrying SAM, Mg²⁺ and 3,5-dinitrocatechol complexes (Protein Data Bank ID: 1VID, 2A7E, 3BWM, 3BMV; Hereafter, a 4-character identification code represents the Protein Data Bank ID). The root-mean-square deviation of Ca fit value between the COMT/SAM/Mg²⁺/nitecapone complex and 1VID was 0.30 Å (the smallest) and that with 3BMV was 0.35 Å (the largest).

Discussion

Several crystal structures of COMT complexed with a nitrocatechol inhibitor are known: 3,5-dinitrocatechol (**4**, 1VID, 3A7E, 3BWM, and 5LSA), tolcapone (**2**, 3S68), BIA 3–335 (**6**, 1HID) and BIA 8–176 (**7**, 2CL5). Including the nitecapone complex, these structures well overlap each other. Figure 3 shows a comparison of the structure of COMT/nitecapone complex with the structure of COMT complexed with known nitrocatechol inhibitors. We aligned the structures by fitting the Ca positions of the β -1 (L61:L65) and β -4 (M137:L140) strands as the topology of these two β -strands are most conserved among COMT crystal structures. Figure 3(a) shows the superimposed Ca traces. The overall structure is highly conserved. Slight conformational differences were observed at the $\alpha 7\beta 4$ - and $\beta 6\beta 7$ -loops. Figure 3(b) shows a close-up view of the surrounding environment of inhibitors. Positions of the nitrocatechol scaffold, the Mg²⁺ ion and the surrounding amino acid residues are highly conserved. The side chain (*i.e.*, the substituted group at the 5-position) of nitrocatechol

Fig. 2. Schematic Diagram of COMT Folding

COMT structure consists of seven β -strands and nine α -helices. This diagram represents the COMT/SAM/Mg²⁺/nitecapone complex. Two loops, the $\alpha 2\alpha 3$ - and $\beta 6\beta 7$ -loops, are very flexible and change their conformation depending on the combination of ligands. Often, the atomic coordinates of the $\alpha 2\alpha 3$ -loop cannot be determined due to its flexibility. Domain swap dimers are known to arise from large conformational changes of these loops.

rings extends to outside of the binding pocket. Conformation of the flexible loops, the $\alpha 2\alpha 3$ - and $\beta 6\beta 7$ -loops, are conserved. However, the interactions between the loops and the side chain moiety of inhibitors are not obvious, and this is common among the COMT/SAM/Mg²⁺/nitrocatechol inhibitor complexes. This fact suggests that the nitrocatechol scaffold of inhibitors contributes more to the binding energy than the side chain moiety.

Recently, new COMT inhibitors that have catechol scaffolds but no nitro substitution at the 3-position were reported. Examples are 4-aryl-7,8-dihydroxycoumarin (**8**, 2ZVJ), 6-aryl-4-hydroxyquinazolin (**9** and **10**, 5P9Z and 5P9O) and 5-aryl-3-hydroxy-1-methyl-2-pyridone (**11**, 5PA0). These inhibitors substitute the 3-nitro group of the dinitrocatechol ring with small fragments. For example, inhibitor **8** replaces the nitro group with a lactone functional group. It should be noted that these non-nitrocatechol-type inhibitors are all complexed with a Mg²⁺ ion and a SAM molecule. The volume of the methyl group of SAM was occupied by the fragment atoms that supersede the nitro group of nitrocatechol-type inhibitors. The conformation of COMT bound with SAM, Mg²⁺ and these non-nitrocatechol-type inhibitors is similar to the known nitrocatechol inhibitor complexes. Met 40 in the $\alpha 2\alpha 3$ -loop is the only amino acid residue that is near the 3-substitution moiety. The side chain of Met 40 is located over the SAM/SAH and the 3-substitution moiety (Fig. 3(b)).

A crystal structure of COMT with a catechol-type inhibitor that lacks the 3-substitution (**12**, 4XUC) was also revealed. Although compound **12** was synthesized as an inhibitor for the membrane-bound form of COMT, this compound was crystallized with human S-COMT.¹⁴⁾ Due to the lack of the substitution at the 3-position, compound **12** co-crystallized with SAM, and the side chain atoms of Met 40 interact with them. However, the inhibitory potency of **12** against S-COMT is much lower ($IC_{50} = 0.5\text{--}2\text{ }\mu\text{M}$ ¹⁴⁾) than that of the other ni-

Fig. 3. The Structure of COMT/SAM/Mg²⁺/Nitecapone Complex

The complex is compared with other COMT structures holding various type of nitrocatechol inhibitors (wall-eye stereo presentation). 1VID: 3,5-dinitrocatechol (**4**), 3S68: tolcapone (**2**), 1HID: BIA3-335 (**5**) and 2CL5: BIA8-176 (**6**), where 1VID, 3S68, 1HID and 2CL5 are the IDs of the PDB. Panel (a): α traces, the position of the Mg²⁺ ion and inhibitor are compared. α atoms of the $\beta 1$ strand (Leu61:Leu65) and the $\beta 4$ strand (Met137:Leu14) were rms-fitted. COMT/SAM/Mg²⁺/Nitecapone complex: white, 1VID: red, 3S68: blue, 1HID: green, and 2CL5: orange. Panel (b): Close-up view of inhibitor binding site. The side chain atoms of Met 40 make contacts with SAM and the 3-nitro group. Color scheme is same as in panel (a). Panel (c): Interaction of Met 40 with SAM and nitecapone. Molecular volumes of nitecapone and SAM are represented in green and orange. Side chain of Met 40 is represented in yellow. The volumes were calculated using MOLCAD software available in SYBYL-X 2.1.1 software package (Cortara, LP, Princeton, NJ, U.S.A.). Hydrogen atoms of hydroxy and carboxylic groups are not included.

trocatechol inhibitors whose IC_{50} is in a low nM range. This fact suggests that the tight packing between SAM/SAH, inhibitor and Met 40 is very important. This Met 40 interaction is observed only in the presence of both the catechol inhibitor and SAM/SAH. As Met 40 is located in the center of the $\alpha 2\alpha 3$ -loop, Met 40 interaction should be related with the elasticity of the $\alpha 2\alpha 3$ -loop and play an important role in binding Mg²⁺-chelating catechol inhibitors. The interaction made among Met 40, SAM (or SAH) and the substituent at the 3-position of catechol inhibitor would stabilize the $\alpha 2\alpha 3$ -loop conformation.

Compound (**13**, 5P98) carries a large fragment at the 3-position of nitrocatechol ring. This compound is demonstrated to form COMT/Mg²⁺/**13** complex in a crystalline state, suggesting that the hydroxyethyl substitution is too large to ac-

commodate with SAH or SAM. Unfortunately, the inhibitory potency of **13** has not been revealed yet.

The speculation that the interaction between Met 40, SAM and the catechol inhibitor stabilizes the $\alpha 2\alpha 3$ -loop and brings tight binding of the inhibitor to COMT might be rationalized by the success of the bi-substrate type inhibitors such as **14–17**.¹⁵⁾ The structures of COMT complexed with bi-substrate-type inhibitors also show the interaction of Met 40 in a similar manner as was observed in the 3-substituted catechol inhibitors. In contrast, in the complex of COMT/SAM/Mg²⁺ (Z2TH), the $\alpha 2\alpha 3$ -loop is disordered and the atomic coordinates of Met 40 could not be determined. In the complex of COMT/SAH (3U81), the $\alpha 2\alpha 3$ -loop is located away from SAH. Met 40 plays an important role in the interaction between the 3-substituted moiety of catechol inhibitor and SAM/SAH.

Domain swap dimers of COMT/SAM/Mg²⁺/dinitrocatechol are known (5FHR, 5FHQ). Although the $\beta 7$ strands of the two COMT molecules are swapped, the $\alpha 2\alpha 3$ -loop keeps almost the same conformation of the monomer complex, and the Met 40-dinitrocatechol-SAM contact is conserved. In the same way, Met 40-SAH-8-hydroxyquinazolin-4-one interaction was conserved in a domain swap dimer structure (5P9U).

Besides Met 40, Pro174 which is located on the $\beta 5\alpha 9$ -loop makes interactions with the catechol ring of the inhibitors. In the crystal structures of COMT with any catechol-type inhibitor, including nitecapone, Pro174 creates a hydrophobic interaction with the catechol ring. Any catechol-type inhibitor would gain binding affinity by chelating Mg²⁺.

In this study we have determined a new crystal structure of COMT complexed with nitecapone. Through investigation of a series of COMT inhibitors, we suggest the importance of the $\alpha 2\alpha 3$ -loop, especially the contribution of the side chain atoms of Met 40 as well as the gatekeeper residue Pro174 on the $\beta 5\alpha 9$ -loop and chelate formation with Mg²⁺. The side chain of the nitrocatechol did not seem to contribute much to the binding affinity and thus modification of the side chain can be a rational strategy to improve properties such as solubility, metabolism or target specificity of the 3-substituted catechol-type inhibitors.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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