Kinetic and inhibition studies on catechol-*O*-methyltransferase affinity labelling by *N*-(3,4-dihydroxyphenyl)maleimide

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Initial velocity and product inhibition studies have been performed on soluble catechol-*O*-methyltransferase which has been partially purified from pig liver. The results are consistent with an ordered reaction mechanism, in which *S*-adenosyl-L-methionine (AdoMet) is the leading substrate. The enzyme is irreversibly inhibited by maleimide derivatives in a biphasic manner, which suggests a differential reaction with two thiol groups. *N*-(3,4-Dihydroxyphenyl)maleimide, which has a reactive moiety (maleimide ring) and an affinity moiety (catechol ring), acts as an affinity labelling compound on the more reactive SH group; AdoMet and Mg²⁺ protect against this modification. Total protection of this SH group results in a pseudo-first-order inhibition of the enzyme, with the apparent rate constant being proportional to the inhibitor concentration. All the other maleimide derivatives studied inhibited the enzyme by reacting with one of the two SH groups in a non-specific manner. The reaction of the other, more reactive, SH group was either specific (active-site-directed) or non-specific, depending on the substituent present in the affinity moiety and also on the length of an intermediate chain of methylene groups present between this moiety and the reactive maleimide ring. In the presence of both AdoMet and Mg²⁺, 3,5-dinitrocatechol, a reversible inhibitor of the enzyme which is competitive with respect to the catechol substrate, protects the enzyme from inactivation by any of the maleimide derivatives. The adducts of these maleimide derivatives formed with dithiothreitol inhibit the enzyme reversibly, showing inhibition patterns that are consistent with the mechanism deduced from the initial velocity and product inhibition studies.

INTRODUCTION

Catechol-*O*-methyltransferase (EC 2.1.1.6; COMT) is a magnesium-requiring enzyme that catalyses the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to a phenolic hydroxyl group of a catechol substrate. Conflicting results have been published on the kinetic mechanism of COMT. A bi-bi random mechanism was first suggested by Flohe & Schwabe [1] using initial velocity studies; these studies were extended with product inhibition by Coward *et al.* [2], and supported the preliminary results. At the same time, Borchardt [3] proposed a ping-pong mechanism by using tropolone as a dead-end inhibitor. Rivett & Roth [4] studied initial velocity behaviour, product inhibition and inhibition by tropolone and concluded that a steady-state, compulsory-order, ternary complex mechanism was followed by membrane-bound COMT from human brain, in which AdoMet binds to the enzyme before dopamine. This suggested the existence of kinetic differences between the membrane-bound and soluble forms of the enzyme. However, the same mechanism was proposed later by Tunncliffe & Ngo [5] and by Jeffery & Roth [6] for the soluble COMT of rat and human brain respectively.

The loss of the activity of COMT in the presence of a reagent against thiol groups suggested that at least one thiol group was involved in the mechanism of transfer catalysed by the enzyme [7]. Several kinds of irreversible inhibitors of COMT have been described. *N*-Haloacetyl derivatives of 2-(3,5-dimethoxy-4-hydroxyphenyl)ethylamine and 2-(3,4-dimethoxy-5-hydroxyphenyl)ethylamine were first described as affinity labels of COMT

[8,9]. In our laboratory a series of *N*-haloacetyl-2-(3,4-substituted-phenyl)ethylamines [10] was developed and shown to inhibit COMT irreversibly. Other compounds used to label COMT included the oxidation products of 6-hydroxydopamine [11], 6-aminodopamine [12] and 5,6-dihydroxyindole [13]. The time-dependent inhibition by all these compounds gave pseudo-first-order kinetics of inactivation when incubated with COMT, suggesting that they modify one group, or more than one with similar reactivities, although Borchardt & Thakker [8] suggested that the *N*-haloacetyl derivatives of 2-(3,5-dimethoxy-4-hydroxyphenyl)- and 2-(3,4-dimethoxy-5-hydroxyphenyl)-ethylamines react with different groups of the enzyme. The inactivation of COMT by *N*-ethylmaleimide (NEM) follows biphasic kinetics with respect to time [14], which was interpreted as being caused by the modification of two different thiol groups by NEM, and suggested the existence of at least two thiol groups essential for the enzymic activity of COMT. Thus, the modification of only one group would not lead to a total loss of activity, but to a partially active enzyme.

The use of NEM coupled to an affinity group can direct the action of this reagent towards the active site of COMT. Thus, *N*-(3,4-dihydroxyphenyl)maleimide (NDIOHM) was proposed to be an active-site-directed inhibitor [15] which reacted with two different thiol groups in COMT. In this paper, we describe the influence of several modifications in the catechol moiety on the affinity for the active site of COMT. Moreover, we have studied the influence of spacers with different numbers of methylene groups on the mechanism of inactivation of COMT by the guaiacol derivative of maleimide. The reaction of the maleimide

Abbreviations used: COMT, catechol-*O*-methyltransferase; AdoMet, *S*-adenosyl-L-methionine; NEM, *N*-ethylmaleimide; NDIOHM, *N*-(3,4-dihydroxyphenyl)maleimide; DTT, dithiothreitol; AdoHcy, *S*-adenosyl-L-homocysteine; NDIOHS, *N*-(3,4-dihydroxyphenyl)succinimide; NDIOAcS, *N*-(3,4-diacetoxyphenyl)succinimide; NPhM, *N*-phenylmaleimide; NOMEAcM, *N*-(3-methoxy-4-acetoxyphenyl)maleimide; NOMEOHM, *N*-(3-methoxy-4-hydroxyphenyl)maleimide; NDIOAcM, *N*-(3,4-diacetoxyphenyl)maleimide; NOMEH2CM, *N*-(3-methoxy-4-hydroxyphenyl)-ethylmaleimide; NOMEH1CM, *N*-(3-methoxy-4-hydroxyphenyl)methylmaleimide; NOMEH3CM, *N*-(3-methoxy-4-hydroxyphenyl)propylmaleimide.

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compounds with dithiothreitol (DTT), leading to the formation of adducts, allowed us to use these compounds as reversible inhibitors of COMT, and to use them for kinetic studies, in addition to initial velocity and product inhibition studies, on COMT from pig liver, to clarify the existing controversy about the reaction mechanism followed by the enzyme.

MATERIALS AND METHODS

Materials

Catechol was obtained from Fluka. AdoMet hydrogen sulphate was purchased from Boehringer-Mannheim and [$Me-^3H$]AdoMet (specific radioactivity 15 Ci/mmol) was from Amersham. The maleimide derivatives were prepared in our laboratory [16]. All the other products and solvents used were of analytical grade.

COMT isolation

COMT was partially purified from pig liver by the method of Nikodejevic *et al.* [17] with minor modifications. The whole process was performed at 0–4 °C. Briefly, the tissue was homogenized in 2% (w/v) KCl and centrifuged at 12000 *g* for 30 min. After discarding the pellet, the supernatant was adjusted to pH 5.0 with 1 M-acetic acid and stirred for 15 min before undergoing centrifugation again as above. The supernatant obtained after this step was neutralized and fractionated with 30–50% (w/v) ammonium sulphate as follows: 0.16 g of ammonium sulphate/ml of supernatant was added, stirred for 15 min and the mixture was centrifuged at 17000 *g* for 20 min. The pellet was discarded and 0.12 g of ammonium sulphate per ml of supernatant was added. The precipitated protein was sedimented at 17000 *g* for 20 min, resuspended in 10 mM-phosphate buffer, pH 7.0, and desalted through a Sephadex-G25 column equilibrated with the same buffer. Finally, the eluate was concentrated to about 50 ml and applied to a Sephadex-G200 column (5 cm $\times$ 90 cm); the active fractions, eluted with 10 mM-phosphate buffer, pH 7.0, were pooled, distributed in 1 ml aliquots and frozen at –30 °C. This reproducible procedure gave a 35-fold degree of purification with respect to the crude homogenate. The COMT preparation obtained in this way was stable for several months. The protein concentration was measured using the Benedict reagent [18].

COMT assay

The method of Zürcher & Da Prada [19] was used and modified by us. In a final volume of 0.25 ml, the reaction mixture contained, unless otherwise stated: enzyme [1 unit (defined as the amount of enzyme which catalyses the transformation of 1 nmol of substrate/min)], 20 mM-catechol, 0.9 mM-[3H]AdoMet (1.6 Ci/mol), 1.5 mM-MgCl₂, 2.5 mM-DTT and 125 mM-phosphate buffer, pH 7.6. The reaction mixture was incubated at 37 °C for 10 min. The reaction was then stopped by the addition of 0.25 ml of 1 M ice-cold citric acid. Blanks were prepared without incubation or without enzyme. An aliquot (1.5 ml) of hexane/toluene (4:1, v/v) containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis(2-[5-phenyloxazolyl])benzene was added to each vial, and the mixture was vigorously vortexed for 30 s. The radioactivity present in the organic phase was then directly counted in an LKB Rackbeta scintillation counter.

All kinetic experiments were carried out with 10 μ g of enzyme preparation. The reaction was shown to be linear with protein concentration and with time of incubation for at least 10 min without the use of adenosine deaminase as described in the original method [19]. The concentrations of catechol and AdoMet given above represent saturating conditions.

Analysis of the kinetic data

The results from the kinetic assays were first analysed graphically by plotting the reciprocal velocities against reciprocals of the substrate concentrations. In all cases a linear relationship was obtained. The calculation of the kinetic constants was performed on a personal computer using the Fortran IV program described by Cleland [20].

For the analysis of the rate constants for the irreversible inactivation of COMT, we have used the equation described by Ray & Koshland [21] and the graphical approximation used by Borchardt & Thakker [14]. Having estimated the values for k_1 , k_2 and F , a non-linear regression computer program was used to calculate the real values for the constants of the equation.

RESULTS AND DISCUSSION

Kinetic mechanism

Double-reciprocal plots of initial velocity, obtained with varying concentrations of catechol and AdoMet (Fig. 1), gave families of lines which intersected to the left of the *y* axis and below the *x* axis, suggesting a sequential reaction mechanism [22]. Substrate inhibition at saturating concentrations of AdoMet or catechol was not observed under these conditions. The K_m values for AdoMet and catechol, and the k_s for AdoMet, were $(9.96 \pm 0.94) \times 10^{-5}$ M, $(2.22 \pm 0.17) \times 10^{-3}$ M and $(3.57 \pm 0.76) \times 10^{-5}$ M respectively.

S-Adenosyl-L-homocysteine (AdoHcy) was shown to be a

Fig. 1. Initial velocity patterns of the COMT reaction

(a) Plot of the reciprocal of initial velocity versus reciprocal of AdoMet concentration at fixed concentrations of catechol: $\diamond$, 0.625 mM; ∇ , 1 mM; $\triangle$, 1.6 mM; $\square$, 2.5 mM; $\circ$, 5 mM. The reaction was assayed as described in the Materials and methods section. (b) Double-reciprocal plot of initial velocity versus catechol concentrations at fixed AdoMet concentrations: $\diamond$, 0.0625 mM; ∇ , 0.08 mM; $\triangle$, 0.125 mM; $\square$, 0.4 mM; $\circ$, 0.625 mM.

Table 1. Product inhibition of COMT

Inhibition constants for the inhibition of COMT by the reaction products. COMT was assayed as described in the Materials and methods section, but at various catechol and AdoMet concentrations as indicated. Abbreviations: C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition. Values for inhibition constants are expressed as means $\pm$ S.E.M. S.E.M. refers to the standard error of the calculated value; kinetic constants are defined in the following scheme:

Inhibitor	AdoMet	Catechol	Inhibition	Inhibition constant (M)
AdoHcy	Variable	K_m	C	$K_{IS} = (3.33 \pm 0.28) \times 10^{-6}$
	Variable	Saturating	C	$K_{IS} = (1.24 \pm 0.47) \times 10^{-5}$
AdoHcy	K_m	Variable	NC	$K_{IS} = (6.86 \pm 0.45) \times 10^{-6}$
				$K_{II} = (5.34 \pm 0.70) \times 10^{-5}$
Guaiacol	Variable	K_m	NC	$K_{IS} = (4.0 \pm 0.59) \times 10^{-2}$
				$K_{II} = (6.96 \pm 0.68) \times 10^{-2}$
Guaiacol	Variable	Saturating	UC	$K_{II} = (4.08 \pm 0.35) \times 10^{-2}$
	K_m	Variable	NC	$K_{IS} = (4.15 \pm 0.68) \times 10^{-2}$
Guaiacol				$K_{II} = (7.44 \pm 0.66) \times 10^{-2}$
	Saturating	Variable	NC	$K_{IS} = (10.3 \pm 2.2) \times 10^{-2}$
				$K_{II} = (6.02 \pm 0.49) \times 10^{-2}$

competitive inhibitor with respect to AdoMet and a mixed inhibitor with respect to catechol. The inhibition by the product guaiacol was mixed with respect to AdoMet or catechol when the non-varied substrate was at its K_m concentration. However, when the catechol concentration was saturating, the inhibition with respect to AdoMet changed from mixed to uncompetitive. Table 1 shows the values of the kinetic constants for the product inhibition studies. These results suggest a sequential ordered mechanism in which AdoMet binds first to COMT:

This mechanism agrees with those proposed by Tunnicliff & Ngo [5] and Jeffery & Roth [6] for the soluble COMT from rat and human brain, and by Rivett & Roth [4] for the membrane-bound enzyme from human brain, rather than the random or ping-pong mechanisms proposed by others [1-3].

Reversible inhibition

The main purpose was the synthesis of several irreversible inhibitors bearing a reactive moiety (maleimide ring) and an affinity moiety (catechol-substituted moiety). Several maleimide derivatives were prepared [16] (Table 2), including other compounds without the reactive moiety, the dihydroxyphenyl- (NDIOHS) and diacetoxyphenyl- (NDIOAcS) succinimide derivatives. The maleimide-like compounds, present in the activity assay, in the presence of DTT form a DTT-maleimide-

derivative adduct the presence of which can be demonstrated by t.l.c. This adduct has the following structure:

The reaction appeared to occur instantly, even at 4 °C, when 1 mol of DTT was mixed with 1 mol of the maleimide derivative. Thus, under the COMT activity assay conditions, where an excess of DTT was present, all the maleimide compound would form the DTT-maleimide adduct. These adducts, as well as the succinimide analogues, are reversible inhibitors of COMT, except for the adducts of NEM and *N*-phenylmaleimide (NPhM), which had no effect on the enzyme activity at concentrations up to 2 mM. The inhibition patterns and the K_i values for the maleimide and the succinimide derivatives are given in Table 3. NDIOHM, and the corresponding NDIOHS, showed competitive inhibition with respect to catechol, and mixed inhibition with respect to AdoMet. The other compounds gave similar inhibition patterns to those seen with the product guaiacol, that is, mixed with respect to catechol and uncompetitive with respect to AdoMet

Table 2. Maleimide and succinimide derivatives assayed as inhibitors of COMT

Maleimide

Compound	<i>n</i>	R	R'	
NPhM	0	H	H	
NDIOHM	0	OH	OH	
NOMeOHM	0	OCH ₃	OH	
NOMeOAcM	0	OCH ₃	OCOCH ₃	
NDIOAcM	0	OCOCH ₃	OCOCH ₃	
NOMeOH1CM	1	OCH ₃	OH	
NOMeOH2CM	2	OCH ₃	OH	
NOMeOH3CM	3	OCH ₃	OH	

Succinimide

NDIOHS	0	OH	OH	
NDIOAcS	0	OCOCH ₃	OCOCH ₃	

(at saturating catechol concentrations). Inhibition by *N*-(3-methoxy-4-acetoxyphenyl)maleimide (NOMeOAcM) at varied catechol concentrations could not be determined, because very little inhibition was observed at concentrations as high as 1 mM, and higher concentrations produced a precipitate in the reaction mixture.

The results suggest that *N*-(3-methoxy-4-hydroxyphenyl)-

maleimide (NOMeOHM), NOMeOAcM, *N*-(3,4-diacetoxyphenyl)maleimide (NDIOAcM) and *N*-(3,4-diacetoxyphenyl)succinimide (NDIOAcS) act as analogues of the product guaiacol and support the sequential ordered mechanism suggested by the initial velocity and product inhibition studies.

Irreversible inhibition by maleimide derivatives

When COMT was incubated with NEM or any of the other maleimide derivatives, in the absence of DTT, irreversible inhibition occurred, and the activity was not recovered after dialysis or gel filtration on Sephadex G25 (results not shown). When the residual activity was measured as a function of the time of incubation, biphasic inhibition kinetic behaviour was seen (Fig. 2). According to Ray & Koshland [21], such biphasic behaviour suggests that at least two residues, presumably thiol groups [14], are being modified in the protein by the maleimide compounds, resulting in loss of enzyme activity, according to the mechanism shown in Scheme 1. In this scheme F_1 , F_2 and F_3 represent the partial enzyme activities after modification of groups 1, 2 and both residues respectively; k_1 and k_2 are the first-order rate constants for modification of residues 1 and 2 respectively.

Table 3. Reversible inhibition of COMT by the DTT-maleimide-derivative adducts and by the succinimide derivatives

Inhibition patterns and constant values for the reversible inhibition of COMT by the adducts of DTT and the maleimide derivatives and by the succinimide derivatives. Abbreviations: C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition; ND, not determined. Kinetic constants are as defined in Table 1.

Inhibitor	AdoMet	Catechol	Inhibition	Inhibition constant (M)
NDIOHM	Variable	K_m	NC	$K_{is} = (6.18 \pm 0.2) \times 10^{-5}$
	Saturating	Variable	C	$K_{ii} = (3.60 \pm 0.3) \times 10^{-5}$
NOMeOHM	Variable	Saturating	UC	$K_{is} = (2.02 \pm 0.18) \times 10^{-5}$
	Saturating	Variable	NC	$K_{ii} = (3.24 \pm 0.19) \times 10^{-4}$
NOMeOAcM	Variable	Saturating	UC	$K_{is} = (6.57 \pm 0.25) \times 10^{-4}$
	Saturating	Variable	ND	$K_{ii} = (3.39 \pm 0.78) \times 10^{-4}$
NDIOAcM	Variable	Saturating	UC	$K_{is} = (1.78 \pm 0.16) \times 10^{-3}$
	Saturating	Variable	NC	ND
NDIOHS	Variable	K_m	NC	$K_{is} = (2.08 \pm 0.12) \times 10^{-4}$
	Saturating	Variable	C	$K_{is} = (3.98 \pm 0.34) \times 10^{-5}$
NDIOAcS	Variable	Saturating	UC	$K_{ii} = (3.27 \pm 0.11) \times 10^{-4}$
	Saturating	Variable	NC	$K_{is} = (2.38 \pm 0.1) \times 10^{-4}$
	Variable	Saturating	UC	$K_{ii} = (9.57 \pm 0.15) \times 10^{-5}$
	Saturating	Variable	NC	$K_{is} = (6.16 \pm 0.1) \times 10^{-5}$
	Variable	Saturating	UC	$K_{ii} = (1.17 \pm 0.06) \times 10^{-3}$
	Saturating	Variable	NC	$K_{is} = (2.64 \pm 0.34) \times 10^{-4}$
	Variable	Saturating	UC	$K_{ii} = (2.29 \pm 0.12) \times 10^{-3}$
	Saturating	Variable	NC

Fig. 2. Inactivation of COMT by NDIOHM as a function of the time of incubation

COMT was incubated in phosphate buffer, pH 7.6, with different concentrations of inhibitor at 37 °C. At determined time intervals, an aliquot (25 μ l) (containing one unit of enzyme) was removed and assayed for COMT activity as described in the Materials and methods section. Inhibitor concentrations were: $\circ$, 12.5 μ M; $\square$, 25 μ M; $\triangle$, 37.5 μ M; ∇ , 50 μ M.

This mechanism of inactivation obeys eqn. (1):

$$A/A_0 = F_3 + (1 - F_1 - F_2 + F_3)e^{-(k_1+k_2)t} + (F_1 - F_3)e^{-k_1t} + (F_2 - F_3)e^{-k_2t} \quad (1)$$

This can be simplified if $F_2 = F_3 = 0$, when:

$$A/A_0 = (1 - F)e^{-(k_1+k_2)t} + Fe^{-k_2t} \quad (2)$$

where $F = F_1$ is the fraction of enzyme activity after modification of group 1.

Fig. 2 shows the logarithm of the residual activity as a function of time of incubation of COMT with NDIOHM, at several concentrations of inhibitor. Separate analysis of the apparent k_1 and k_2 values, as a function of inhibitor concentration (see Fig. 3), demonstrate that NDIOHM shows saturation kinetics with respect to the more reactive group; however, the modification rate of the slow group (k_2) is proportional to the concentration of the inhibitor. NEM, which is devoid of an affinity moiety,

shows non-saturating kinetics for both k_1 and k_2 with respect to the inhibitor concentration, as shown in Fig. 4.

Thus, NDIOHM modifies the more reactive group of COMT according to the following mechanism (specific):

which involves the formation of a reversible enzyme-inhibitor complex (COMT-I) that reacts to give to an irreversible one (COMT*I). k_a is the first-order rate constant and K_1 the steady-state constant of inactivation [23]. Under these conditions, the apparent first-order rate constant ($k_{app.}$) is related to the inhibitor concentration by the relationship $k_{app.} = k_a[I]/(K_1 + [I])$.

However, the slow group (k_2) is modified by NDIOHM according to the mechanism (non-specific):

without the formation of a reversible complex (COMT-I), where k_b is the first-order rate constant of inactivation. Under these conditions, the apparent first-order rate constant will be given by $k_{app.} = k_b[I]$. NEM modifies both groups (analysis of k_1 and k_2) in accordance with this mechanism. In this case, and others that follow this mechanism, the positive intercept on the x axis (see Fig. 4) was due to the use of a partially purified enzyme preparation, since the double bond of the maleimide is able to react with any available thiol groups present in other contaminating proteins.

The values of the inactivation constants obtained by analysis of k_1 and k_2 for the maleimide derivatives are given in Table 4. Whereas all the maleimide-like compounds modify the more slowly reacting group (group 2) in a non-specific manner, the

Fig. 4. Inactivation of COMT by NEM: analysis of $k_{app.}$ for groups 1 and 2

Modification of group 1 ($\circ$); $k_{app.}$ for group 2 ($\square$). The values of k'_b and k_b for both groups were obtained from the slope of the respective lines, as in Fig. 3(b).

Fig. 3. Analysis of the first-order rate constants ($k_{app.}$) for the modification of residues 1 and 2, as a function of NDIOHM concentration

(a) Double-reciprocal plot of $k_{app.}$ for group 1 versus NDIOHM concentration; the values of k_a and K_1 were first calculated from the intercept on the y axis and the slope respectively. (b) Plot of $k_{app.}$ for group 2 versus NDIOHM concentration; k_b was calculated from the slope.

Table 4. Irreversible inhibition of COMT by the maleimide derivatives

Rate constants are shown for the modification of group 1, indicating the inhibition mechanism, and group 2 of COMT by the maleimide derivatives. The inactivation assay was carried out as indicated in Fig. 2, at several concentrations of the inhibitor. Rate constants were calculated according to Kitz & Wilson [23]. The relationship k_a/K_i permits us to compare the reactivity of active-site directed inhibitors and non-specific inhibitors.

Inhibitor	Analysis of group 1		Analysis of group 2
	Inhibition mechanism	Inhibition constant $\pm$ S.E.M.	$k_b \pm$ S.E.M. ($M^{-1} s^{-1}$)
NEM	Non-specific	$k'_b = 58.5 \pm 1.2$ ($M^{-1} s^{-1}$)	9.74 ± 0.05
NPhM	Non-specific	$k'_b = 164.89 \pm 8$ ($M^{-1} s^{-1}$)	47.41 ± 0.11
NDIOHM	Specific	$K_i = (6.61 \pm 0.6) \times 10^{-5}$ (M) $k_a = 0.0379 \pm 0.002$ (s^{-1}) $k_a/K_i = 572.9$ ($M^{-1} s^{-1}$)	33.91 ± 0.06
NOMeOHM	Non-specific	$k'_b = 152.61 \pm 2.9$ ($M^{-1} s^{-1}$)	28.91 ± 0.11
NOMeOAcM	Specific	$K_i = (3.69 \pm 0.9) \times 10^{-4}$ (M) $k_a = 0.082 \pm 0.015$ (s^{-1}) $k_a/K_i = 222.05$ ($M^{-1} s^{-1}$)	41.13 ± 0.25
NDIOAcM	Specific	$K_i = (5.04 \pm 0.44) \times 10^{-4}$ (M) $k_a = 0.0983 \pm 0.007$ (s^{-1}) $k_a/K_i = 194.98$ ($M^{-1} s^{-1}$)	45.92 ± 0.09
NOMeOH1CM	Specific	$K_i = (8.34 \pm 0.58) \times 10^{-4}$ (M) $k_a = 0.136 \pm 0.007$ (s^{-1}) $k_a/K_i = 163.37$ ($M^{-1} s^{-1}$)	33.50 ± 0.07
NOMeOH2CM	Non-specific	$k'_b = 77.42 \pm 0.62$ ($M^{-1} s^{-1}$)	18.43 ± 0.08
NOMeOH3CM	Specific	$K_i = (8.44 \pm 0.9) \times 10^{-4}$ (M) $k_a = 0.072 \pm 0.006$ (s^{-1}) $k_a/K_i = 85.37$ ($M^{-1} s^{-1}$)	21.13 ± 0.13

modification of the more reactive group (group 1) varies with the group attached to the maleimide ring. For compounds which modify both groups in an unspecific manner, we will distinguish between k'_b and k_b for the modification of groups 1 and 2 respectively.

NPhM, like NEM, modifies group 1 according to the non-specific mechanism, as would be expected because of the lack of a group with affinity towards COMT. NOMeOHM, although bearing an affinity group (the guaiacol moiety), modifies the more reactive thiol group in a non-specific manner. When a spacer formed by one, two or three methylene groups is included between the guaiacol moiety and the maleimide ring, the mechanism of modification is dependent on the number of carbon atoms in the spacer, as shown in Table 4.

N-(3-Methoxy-4-hydroxyphenyl)ethylmaleimide (NOMeOH2CM), with a spacer formed by two methylene groups, reacts with group 1 in the same way as NOMeOHM, but with a considerably lower value of k'_b . However, *N*-(3-methoxy-4-hydroxyphenyl)methylmaleimide (NOMeOH1CM) and *N*-(3-methoxy-4-hydroxyphenyl)propylmaleimide (NOMeOH3CM), with one and three methylene groups respectively, modify group 1 in a specific manner, as might be expected because of the presence of an affinity moiety. The specificity constant for modification of group 1 by NOMeOH1CM (k_a/K_i) was similar to the k'_b value for the compound with no spacer between the substituted ring and the maleimide group (NOMeOHM), whereas the corresponding value for NOMeOH3CM was similar to that for NOMeOH2CM. These results may be explained when viewing the spatial conformation with Corey–Pauling–Koltun models of the guaiacol derivatives. As Fig. 5 shows, both the distance and the relative orientation of the reactive double bond, with respect to the guaiacol ring, change when a methylene group is inserted between the affinity moiety and the maleimide ring. Both NOMeOH1CM and NOMeOH3CM have the reactive double bond orientated in such a manner that it is able to react

with the thiol group when it forms a reversible complex with COMT through the guaiacol site. Both compounds have the same value for K_i . However, the length of the methylene chain influences the value of k_a , which is higher for NOMeOH1CM than for NOMeOH3CM. The effect of the distance between the

Fig. 5. Spatial conformation of the guaiacol derivatives and influence of the insertion of methylene groups between the reactive and the affinity moieties

reactive moiety and the affinity group is also seen in the modification of group 2, and NOMEHM and NOMEH1CM have higher k_b values than NOMEH2CM and NOMEH3CM. This may be owing to the effect of the neighbouring aromatic ring enhancing the reactivity of the maleimide ring towards thiol groups (compare the difference between NPhM and NEM; Table 4). Thus, an increased separation between the aromatic and the maleimide ring might be expected to decrease the reactivity of the double bond of the maleimide. A similar effect of the intermediate chain, reducing the rate constant for modification of thiol groups, has been observed with *N*-haloacetyl-2-(3,4-substituted-phenyl)ethylamines [10].

This explanation may be supported by the results obtained with NOMEoAcM and NDIOAcM. In these compounds the relative orientation of the double bond is the same as for NOMEHM, but the presence of a bulky group (the acetoxy group) in position 4 lengthens the distance between the extreme end of the affinity moiety and the maleimide ring. Moreover, this may alter the mechanism of binding of these inhibitors to the enzyme such that the double bond of the maleimide ring may be orientated in the correct manner for the covalent reaction to proceed. The values of K_i and k_a are slightly lower than for NOMEH1CM. All these results suggest that the catechol site is closer to group 1 than the guaiacol site. Substitution of the ethyl group in NEM by a phenyl group to give NPhM enhanced the reactivity of the double bond of the maleimide 3-fold towards group 1 and 5-fold towards group 2. Substitutions on the phenolic group did not greatly affect the reactivity of the inhibitor towards group 2. Only the lengthening of the spacer from zero or one to two or three methylene groups decreased the reactivity. Similar behaviour occurred with respect to group 1 (compare the results for the pairs of compounds NOMEH2CM–NOMEHM and NOMEH3CM–NOMEH1CM).

Substrate protection studies

Substrate protection studies were performed to elucidate the nature of the interaction of the maleimide derivatives and the enzyme. The inactivation studies were carried out in the presence of AdoMet, AdoHcy, 3,5-dinitrocatechol and/or Mg^{2+} . Table 5 shows the inactivation of COMT by NDIOHM and NEM in the presence of AdoMet or AdoHcy $\pm Mg^{2+}$. AdoMet (or AdoHcy) alone was able to protect the enzyme from inactivation by the

Fig. 6. Inactivation of COMT by NEM as a function of time of incubation in the presence of AdoMet and Mg^{2+} .

COMT was incubated in the presence of $50 \mu M$ -NEM without (□) or with 5 mM-AdoMet plus 5 mM- $MgCl_2$ (○). The extrapolation of the second slope (inactivation of the slow group) to the y ordinate (dashed line) gives a value of $F = 0.05$.

maleimide derivatives. The protection from the inactivation by NDIOHM was greatly increased in the presence of Mg^{2+} , suggesting that Mg^{2+} may facilitate the binding of AdoMet to the enzyme. When NEM was used instead of NDIOHM, the protection observed by AdoMet was not affected by the presence of Mg^{2+} . This result can be related to the fact that only the latter compound modifies group 1 by the mechanism involving initial non-covalent complex formation.

Incubation of COMT with NDIOHM in the presence of both AdoMet and Mg^{2+} , at a concentration of 100 times that of the inhibitor, gave pseudo-first-order kinetics, as shown in Fig. 6,

Table 5. Protection of COMT from inactivation by NDIOHM and NEM

COMT was incubated as described in Fig. 2, but in the presence of different concentrations of AdoMet, AdoHcy or $MgCl_2$. The activity remaining was measured after 1 min of incubation with 0.1 mM-NDIOHM or 8 min with 0.1 mM-NEM. Residual activity was determined with respect to a sample taken at 0 min of preincubation. The values of residual activity were calculated using the results from four experiments. Values are expressed as means $\pm$ S.E.M.

Inhibitor	AdoMet (mM)	AdoHcy (μM)	$MgCl_2$ (mM)	Residual activity (%)
NDIOHM	—	—	—	12.5 ± 0.1
	0.1	—	—	44.7 ± 0.8
	—	—	1	15.9 ± 0.6
	0.1	—	1	63.6 ± 2.3
	—	8	—	30.0 ± 0.1
	—	8	1	46.8 ± 4.7
NEM	—	—	—	8.3 ± 0.6
	0.1	—	—	41.4 ± 2.4
	—	—	1	9.4 ± 1.9
	0.1	—	1	41.3 ± 0.7

Table 6. Protection of COMT by compounds structurally related to AdoMet

Inactivation of COMT by 0.1 mM-NDIOHM was measured after 1 min of incubation as described in Fig. 3, but in the presence of several compounds structurally related to AdoMet $\pm Mg^{2+}$. Residual activity was determined with respect to a sample taken at 0 min of preincubation. The values were calculated using the results from four experiments, and are expressed as means $\pm$ S.E.M.

Addition (1 mM)	$MgCl_2$ (1 mM)	Residual activity (%)
—	—	11.3 ± 0.3
—	+	12.2 ± 0.1
Methionine	—	11.6 ± 1.2
Methionine	+	12.3 ± 0.1
Adenine	—	14.1 ± 0.2
Adenine	+	18.4 ± 0.6
Adenosine	—	32.5 ± 0.5
Adenosine	+	36.2 ± 1.3
Ribose	—	11.3 ± 0.6
Ribose	+	13.3 ± 0.1

Fig. 7. Inactivation of COMT by NDIOHM in the presence of substrates

COMT was incubated as in Fig. 2 with $40\ \mu\text{M}$ -NDIOHM alone ($\circ$) and in the presence of: $8\ \text{mM}$ - MgCl_2 ($\square$); $8\ \text{mM}$ -AdoMet ($\triangle$); $8\ \text{mM}$ - MgCl_2 plus $8\ \text{mM}$ -AdoMet (∇); the closed symbols represent the same conditions as the open symbols but with the addition of $1.5\ \mu\text{M}$ -3,5-dinitrocatechol in the incubation mixture.

with a slope similar to that obtained for the inactivation of group 2 in the absence of AdoMet/ Mg^{2+} . This suggests that AdoMet/ Mg^{2+} completely protects against the modification of group 1 by the maleimide derivative. This is then able to react only with group 2, suggesting that group 1 is located at, or near to, the binding site of AdoMet. The same result was obtained with the other maleimide derivatives.

When different compounds, structurally related to AdoMet, were present in the incubation mixture, the inactivation rate was not greatly affected (Table 6). Only when adenosine $\pm\ \text{Mg}^{2+}$ was present was some protection observed, suggesting that the adenosine moiety is one of the sites through which AdoMet is bound to COMT.

The presence of 3,5-dinitrocatechol, a competitive inhibitor of COMT with respect to catechol as the variable substrate ($K_i = 30.8\ \text{nM}$), with or without Mg^{2+} has no effect on the inactivation of the enzyme by NDIOHM, as can be seen in Fig. 7. However, when AdoMet, Mg^{2+} and 3,5-dinitrocatechol were included in the incubation mixture, a high degree of protection was achieved. A small degree of inactivation occurred during the first 4–6 min (10%) but after that there was no further inactivation. This result shows that the presence of 3,5-dinitrocatechol, together with AdoMet and Mg^{2+} , almost completely blocks the modification of group 2 by NDIOHM, suggesting that group 2, if located in the active centre of the enzyme, is close to the catechol binding site.

Fig. 7 also shows that 3,5-dinitrocatechol had no more effect on the inactivation than that afforded by AdoMet alone. It is necessary for both AdoMet and Mg^{2+} to be present for the protective effect of 3,5-dinitrocatechol to be seen. This may be related to the reaction mechanism of COMT discussed above; if Mg^{2+} as well as AdoMet is necessary for the binding of the catechol substrate. Although it has been proposed that Mg^{2+} bind COMT in a rapid equilibrium sequence before the addition of AdoMet [24], such a mechanism would predict no protection by AdoMet in the absence of Mg^{2+} . We have found a protective effect due to AdoMet alone, even in the presence of a chelating agent such as $1\ \text{mM}$ -EDTA (results not shown). This result is inconsistent with the ordered sequence of binding previously proposed [24].

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