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INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE BY *N*-(3,4-DIHYDROXYPHENYL) MALEIMIDE

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Catechol-*O*-methyltransferase (COMT) is inhibited rapidly and irreversibly by *N*-(3,4-dihydroxyphenyl) maleimide. S-adenosylmethionine (AdoMet) and magnesium ions protect the enzyme from inactivation by this compound, but no protection is observed by the catechol substrate. However, the corresponding succinimide analogue shows a reversible inhibition of COMT, which is competitive with pyrocatechol-phthalein and non-competitive with AdoMet. Amino-group reagents also inhibit COMT and this inhibition is protected by AdoMet, suggesting that sulphydryl and amino groups essential for activity are located in an AdoMet-binding site on COMT. The maleimide derivative may be considered to be an active-site directed inhibitor.

KEY WORDS: Catechol-*O*-methyltransferase, inhibitors, *N*-(3,4-dihydroxyphenyl) maleimide.

INTRODUCTION

Catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) plays an important role in the extraneural inactivation of endogenous catecholamines and the detoxification of many xenobiotic catechols. It is a *S*-adenosylmethionine (AdoMet)-dependent enzyme and requires Mg^{2+} to catalyze the transfer of a methyl group from AdoMet to a catechol substrate[1–3].

The lack of COMT inhibitors for clinical use, for example as possible antidepresants, has generated a considerable research interest in the study of COMT inactivation. Several classes of inhibitors of COMT have been described, however many of them show potent inactivation effects *in vitro* but are less effective *in vivo*[4–12].

The use of affinity labeling reagents might allow an elucidation of the relationship between the chemical structure of the inhibitor and the structure and function of the enzyme. Several compounds have been used to label COMT, including: *N*-haloacetyl-2-(3,5-dimethoxy-4-hydroxyphenyl)-ethylamines and *N*-haloacetyl-2-(3,4-dimethoxy-5-hydroxyphenyl)ethylamines[13–15], *N*-haloacetyl-2-(3,4-substituted-phenyl)-ethylamines[16], oxidation products of 6-aminodopamine[17], *N*-ethylmaleimide[18] and oxidation products of 5,6-dihydroxyindoles[19]. Studies with *N*-ethylmaleimide have suggested the existence of two sulfhydryl groups in the active site of COMT which are critical for the catalytic activity[18].

Since *N*-ethylmaleimide is known to be an irreversible inhibitor of COMT, we

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attempted to develop affinity labeling reagents for this enzyme by preparing maleimide derivatives bearing a catechol moiety so as to provide affinity for COMT[20]. These derivatives should have an affinity for the active site of COMT and, in addition, they should be capable of reacting to form a covalent bond with any nucleophiles present at, or near to, the binding site.

For comparative purposes, we also prepared some related compounds in which the reactive group (maleimide moiety) or, alternatively, the affinity group (catechol moiety) were lacking. The model compound *N*-ethylmaleimide was used as a reactive compound devoid of affinity, and we also used *N*-phenylmaleimide for this purpose. Compounds in which a succinimide group was substituted for the maleimide group were used as analogous structures devoid of reactivity. Open ring compounds, such as catechol derivatives of maleamic and succinamic acids, were also prepared for comparison.

In this paper we present the results of *in vitro* inhibition of COMT by *N*-(3,4-dihydroxyphenyl) maleimide and some other structurally related compounds. A preliminary report of part of this work has been published[20].

MATERIALS AND METHODS

Materials

AdoMet hydrogen sulfate was obtained from Boehringer Mannheim. 2,4-Dinitrofluorobenzene (2,4-DNFB) and *S*-methylisothiourea were purchased from Sigma. *S*-methyl-1-nitroisothiourea was synthesized according to the procedure of Fishbein and Gallaghan[21]. Pyrocatecholphthalein (PCPh) was prepared as described[22]. All the products used as inhibitors were prepared in our laboratory[20].

COMT Isolation

COMT was partially purified from pig liver according to methods previously described[7] through the Sephadex G-25 stage but omiting the 105000 × g centrifugation step and the refractionation of the protein precipitated with 50% ammonium sulfate. The enzyme, eluted with 10 mM phosphate buffer pH 7.0, was stored at −30 °C and was found to be stable for several months.

COMT Assay

Enzyme activity was determined using PCPh as substrate according to the method of Anderson and D'Iorio[23]. The incubation mixture (0.5 ml) contained: 1.5 mM AdoMet, 1.5 mM $MgCl_2$, 35 mM *Tris*-HCl buffer pH 7.9, 0.06 mM PCPh and enzyme solution (500–700 μg of protein). The mixture was incubated for 20 min at 37 °C and 0.25 ml of 0.5 M borate buffer, pH 10, was added to stop the reaction. Samples were then centrifuged at 4000 rmp for 10 min and absorbance was measured at 595 nm in a Hitachi Perkin-Elmer Model 139 spectrophotometer against a blank prepared without AdoMet.

Inhibition was measured as a function of inhibitor concentration without pre-incubation and with pre-incubation of an enzyme-inhibitor mixture. In the pre-incubation assay a concentrated mixture of COMT and inhibitor was maintained at 37 °C for specific periods before dilution for residual activity determination.

RESULTS AND DISCUSSION

The inhibition of COMT by *N*-(3,4-dihydroxyphenyl)-maleimide (Figure 1) and other maleimides (*N*-ethyl- and *N*-phenylmaleimide, data not shown) was time-dependent and not reversed by dilution. For an inhibitor concentration of 0.2 mM and a very short time of pre-incubation, the apparent pseudo-first-order rate constants observed were: 0.63, 1.95 and 2.27 min^{-1} for *N*-ethyl-, *N*-phenyl- and *N*-(3,4-dihydroxyphenyl)-maleimide, respectively.

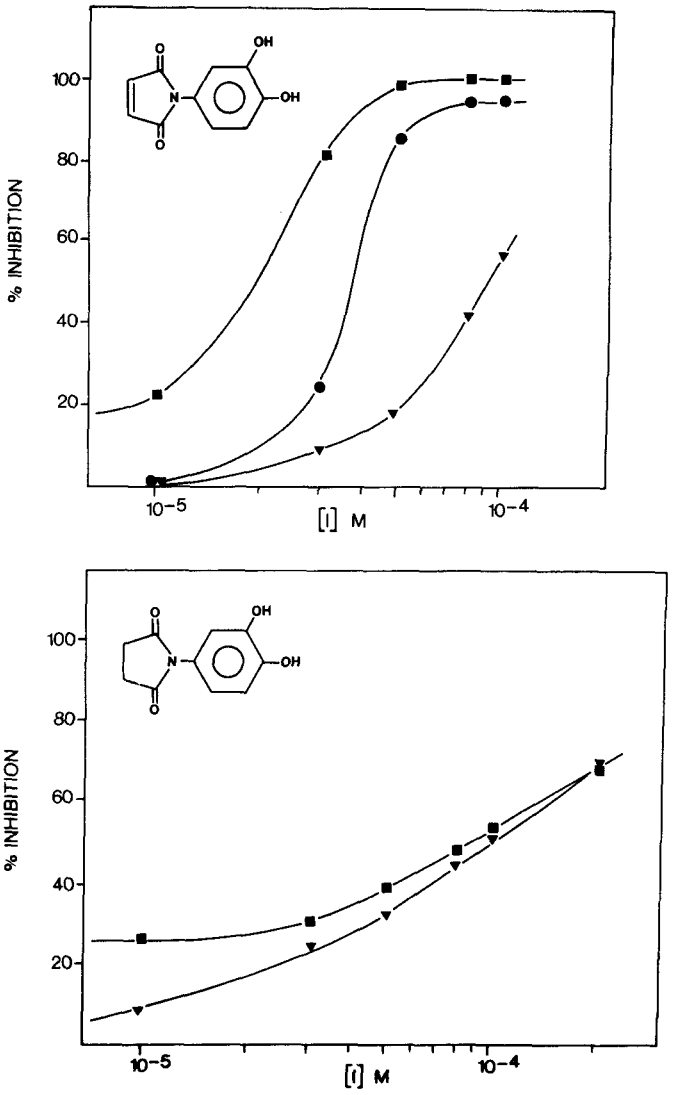

FIGURE 1 Inhibition of COMT as a function of the inhibitor concentration. ■—■ Pre-incubation for 30 min. at 37 °C of an enzyme-inhibitor mixture seven-fold concentrated with respect to the reaction mixture. ●—● Pre-incubation, as before, but including AdoMet in the pre-incubation mixture (5.10^{-5} M in reaction). ▼—▼ Assay without pre-incubation.

The irreversible inhibition, after pre-incubation with these maleimides, was confirmed by the failure to recover activity after dialysis for 20 h at 4 °C against 10 mM phosphate buffer, pH 7.0 (data not shown). The inhibition by the succinimide analogue (see Figure 1) showed some persistence after dilution of the samples pre-incubated at the lower inhibitor concentrations. However, as might be expected from the absence of a reactive double-bond in that compound, the inhibition obtained after enzyme-inhibitor pre-incubation was completely reversed by dialysis. This would

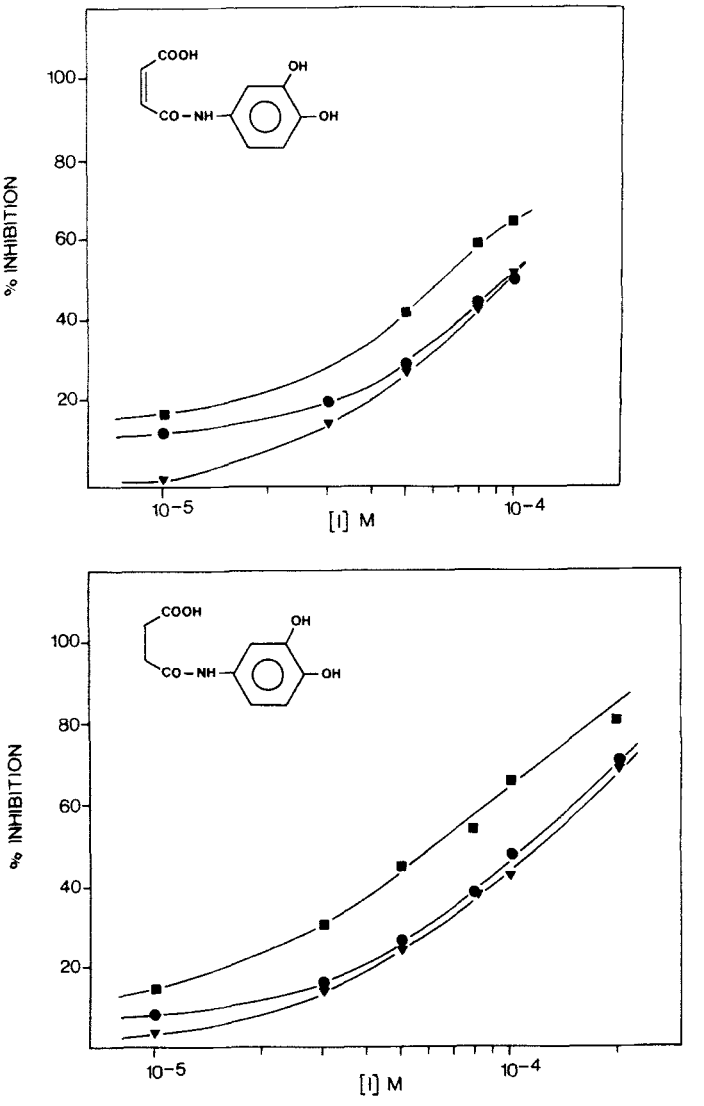

FIGURE 2 Inhibition of COMT as a function of the inhibitor concentration. The assay conditions were the same as in Figure 1, but the dilution was twelve-fold and the AdoMet concentration 8.10^{-5} M. Symbols as in Figure 1.

TABLE I
COMT inactivation by amino group reagents.

Compound	Concentration (mM)	Preincubation (h)	Residual activity %
S-methyl-isothiourea	5	6	85
	25	6	65
	100	6	41
S-methyl-1-nitroiso-thiourea	1	6	69
	5	6	27
	10	6	9
2,4-DNFB	1	2	4.5
	1 + 0.5 mM AdoMet	2	93

The standard pre-incubation mixture contains: 30 mM phosphate buffer pH 7.00, enzyme solution and water to a final volume of 140 μl. The pre-incubation was started by addition of the enzyme solution and was carried out at 37 °C for 2 or 6 h, when the samples were assayed for residual activity as described in Material and Methods. Residual activity was calculated relative to the activity of the control samples pre-incubated without inhibitors.

indicate that although the succinimide derivative is not an irreversible inhibitor of the enzyme, it dissociates relatively slowly from the enzyme-inhibitor complex.

The inhibitory behaviour of the N-(3,4-dihydroxyphenyl) maleamic and -succinamic acids, were very similar to each other (Figure 2) and showed some time-dependency to give an "irreversible component" which was eliminated by the presence of AdoMet in the pre-incubation mixture. This result was unexpected, since the two compounds differ at the reactive moiety but could be explained by a lower reactivity of the double bond of the maleamic moiety towards -SH groups because of electronic transitions due to the adjacent ionised carboxyl group. However, the reason for the presence of the "irreversible component" in the case of the succinamic acid derivative is unclear, although the carboxyl group could be involved through interaction with some essential amino group.

The existence of at least one amino group essential for COMT activity was indicated using free amino group reagents, such as 2,4-DNFB (Sanger's reagent), *S*-methyl-isothiourea and *S*-methyl-1-nitroisothiourea. Table I shows the results obtained when COMT was pre-incubated at 37 °C for a long period with different concentrations of these reagents. 2,4-DNFB was found to be the best reagent to inhibit COMT and a concentration as low as 1 mM inhibits enzyme activity almost completely in less than 2 h. However, the presence of 0.5 mM AdoMet in the pre-incubation mixture, protects the enzyme from inactivation by 2,4-DNFB, suggesting that the group(s) affected by this compound is located at the active site of COMT. The urea derivatives required higher concentrations and longer times of preincubation to produce a substantial inhibition of COMT. Because 2,4-DNFB is not entirely specific for amino groups, further experiments are needed to confirm the nature of these essential groups.

Our interest has been mainly centred on *N*-(3,4-dihydroxyphenyl) maleimide, due to the possibility of using this compound as an affinity labeling reagent of COMT. In order to explore the mechanism of the inhibition of COMT by *N*-(3,4-dihydroxyphenyl) maleimide, we have studied the time course of the enzyme inactivation by this compound. The activity remaining was calculated with respect to samples of enzyme

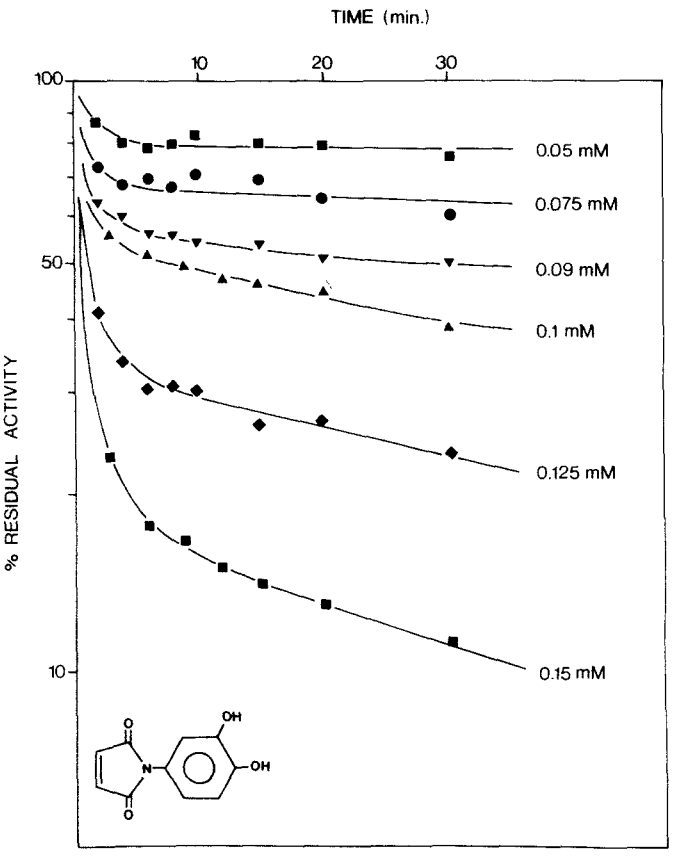

FIGURE 3 Effect of *N*-(3,4-dihydroxyphenyl) maleimide on COMT activity. The enzyme was pre-incubated at several inhibitor concentrations and the residual activity was determined as a function of time, in relation to samples pre-incubated without inhibitor, as described in Material and Methods.

pre-incubated without inhibitor. With the highest inhibitor concentration used, the inactivation of COMT without pre-incubation was less than 10%. When the logarithm of percentage activity remaining was plotted vs. pre-incubation time (Figure 3), we obtained biphasic kinetics similar to those observed with *N*-ethylmaleimide (according to Borchardt and Thakker[18]) and *N*-phenylmaleimide, with an initial sharp slope for the initial 5 min followed by a second slower step. This result differs from the pseudo-first order kinetics observed with some other affinity labeling reagents for COMT[13-19].

The non-linearity is not due to an inactivation of the inhibitor during the time of pre-incubation. This was demonstrated by incubating the inhibitor with phosphate buffer at 37 °C for variable times and evaluating its capacity to inhibit COMT. The inhibition obtained after 1 h of incubation of the inhibitor in phosphate buffer was the same as that obtained without incubation.

This biphasic kinetic behaviour suggests that modification of more than one residue in the active centre of COMT could lead to loss of the enzyme activity[24]. It is also

TABLE II
Protection of COMT from inactivation by *N*-(3,4-dihydroxyphenyl) maleimide.

Addition (mM)			Extent of inhibition (%)
AdoMet	Mg^{2+}	PCPh	
-	-	-	84
0.03	-	-	66
0.075	-	-	20
0.15	-	-	12
0.3	-	-	6
-	1	-	80
-	4	-	71
-	6	-	66
0.03	1	-	44
0.03	4	-	18
-	-	0.15	90
-	-	0.3	88
0.15	-	0.15	25

The inhibitor concentration was 0.15 mM and the pre-incubation mixture was as described in Table I except for the final volume (125 μl). The pre-incubation was started by addition of enzyme solution and was carried out at 37 °C for 30 min. Inhibition percentage was calculated with respect to control samples pre-incubated without inhibitor.

possible that two forms of COMT could exist in pig liver, as has been shown to be the case with rat liver[25], which have different affinities for the compound.

Analysis of both parts of the biphasic curve as independent first-order components shows that for the first part of the curve the apparent rate constants for *N*-ethyl- and *N*-phenylmaleimide are proportional to the inhibitor concentration, but the apparent rate constant for *N*-(3,4-dihydroxyphenyl) maleimide indicates saturation at high inhibitor concentration, as would be expected for an active site-directed inhibitor. In respect to the second part of the curve, the results obtained are not clearly conclusive, but it appears that they follow non-saturation kinetics by the inhibitor. Accordingly, these results indicate that *N*-ethyl- and *N*-phenylmaleimide interact in a non-specific manner with two or more reactive groups on COMT, whereas the catechol derivative of maleimide interacts specifically with a reactive group presumably located at, or near to, the active site of COMT, and non-specifically with any possible additional group.

In an attempt to further elucidate the nature of the interaction between *N*-(3,4-dihydroxyphenyl) maleimide and the enzyme, substrate protection studies were carried out. Inactivation studies were carried out in the presence of different concentrations of AdoMet, PCPh or Mg^{2+}. As shown in Table II (see also Figure 1), low concentrations of AdoMet show substantial protection of COMT from inhibition by the maleimide derivative, whereas Mg^{2+} only slightly protects the enzyme even at concentrations 40-fold greater than the inhibitor concentration. However, this weak protection by Mg^{2+} is potentiated by the presence of a low concentration of AdoMet (200 times less than Mg^{2+}) in the pre-incubation mixture. The protection observed with AdoMet and Mg^{2+} together is much greater than the protection produced by Mg^{2+} or AdoMet alone, and greater than that expected from a simple combination of both. This result would be expected if Mg^{2+} were involved in the binding of AdoMet to the enzyme. No protection by PCPh was observed. These results contrast with those of Borchardt and Thakker[15] with *N*-halo acetyl-2-(3,5-dimethoxy-4-

hydroxyphenyl) ethylamines, who observed protection by the catechol substrate but not by AdoMet.

N-(3,4-dihydroxyphenyl) succinimide was a reversible inhibitor which was competitive with respect to PCPh as the variable substrate (K_i = 0.068 mM). When AdoMet was the variable substrate, a noncompetitive pattern of inhibition was observed (K_i = 0.98 mM and K_i' = 0.48 mM).

All these results would be consistent with a site on COMT for AdoMet containing sulphydryl and amino groups, essential for activity, which react with compounds such as maleimide and amino-group reagents, respectively. An additional binding site for the catechol substrate would also bind compounds such as *N*-(3,4-dihydroxyphenyl) succinimide and *N*-(3,4-dihydroxyphenyl) maleimide. However the latter is also able to react with an essential residue in the AdoMet binding site, as shown by the protection studies. Thus, this compound may be considered to be an active site-directed inhibitor and further studies with it might prove to be of value in furthering our understanding of the molecular properties of COMT.

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