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KINETICS OF THE REVERSIBLE TIGHT-BINDING INHIBITION OF PIG LIVER CATECHOL-O-METHYLTRANSFERASE BY [2-(3,4-DIHYDROXY-2-NITROPHENYL) VINYL]PHENYL KETONE.

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The inactivation of partially purified pig liver catechol-O-methyltransferase (COMT) by [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenylketone has been studied. The results demonstrated that COMT is inhibited in a reversible tight-binding fashion with an apparent K_i of 0.2 μM. IC_{50} values were determined at different concentrations of both substrates of the enzymatic reaction, pyrocatechol and S-adenosyl-L-methionine (AdoMet). The plot of IC_{50} versus pyrocatechol concentration gave a straight line, suggesting competitive inhibition. However the nitrocatechol derivative showed an uncompetitive inhibition pattern when measured as a function of AdoMet concentration.

KEY WORDS: COMT, tight-binding inhibitors, catechol-O-methyltransferase

INTRODUCTION

Catechol-O-methyltransferase (EC, 2.1.1.6, COMT) plays an important role in the metabolic inactivation of catecholamines and xenobiotic catechols[1,2]. The normal levels of circulating catecholamines have been shown to be altered in several clinical disorders, ie. Parkinson's disease which is correlated with a deficiency in dopaminergic neurons. The oral administration of L-dopa (a dopamine precursor) together with a peripheral inhibitor of dopa-decarboxylase (benserazide or carbidopa) is used as

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ABBREVIATIONS: AdoMet, S-Adenosyl-L-methionine; COMT, Catechol-O-methyltransferase; DTT, Dithiothreitol; E_t, Total enzyme concentration; I_t, Total inhibitor concentration; K_{app}, Apparent inhibition constant; k_{cat}, First-order rate constant; K_m, Michaelis constant; MAO, Monoamine oxidase; Nitecapone, 3-(3,4-Dihydroxy-5-nitrobenzylidene)-2,4-pentanedione; 3-OMD, 3-O-Methyl dopa.

current therapy in Parkinson's disease[3]. The monoamine oxidase B selective inhibitor l-deprenyl is also used together with L-dopa and carbidopa[4]. However, this therapy leads to an increase in circulating 3-methyldopa which is not beneficial for the disease. So, the administration of a COMT inhibitor would notably reduce the high doses of levodopa actually administered and improve the efficiency of this therapy[5].

The development of a COMT inhibitor clinically useful against Parkinson's disease has been one of the pursued goals for the last few years. Since the discovery of COMT, several compounds have been described as inhibitors of the enzyme[1,6-8]. However, most of them have been shown to be ineffective *in vivo*, toxic, or poorly selective towards COMT. In 1989, two research groups independently reported a new class of potent and selective inhibitors of COMT bearing a nitrocatechol structure[9,10]. These compounds were also orally active with a very low toxicity, and their discovery opened a new approach to the pharmacological study of COMT related disorders.

A nitro group at the 5-position of 1-substituted 3,4-dihydroxyphenyl derivatives has been shown to increase the activity of these products as inhibitors of COMT[7,10]. Also the hydrophobicity of the group substituted at position 1 is highly related to the activity of the compound as a COMT inhibitor. However, we have observed that the requirement necessary to increase this activity by introduction of a nitro group is that substitution occurs at an *ortho* position relative to one of both hydroxyl groups[11]. In order to obtain irreversible highly selective inhibitors of COMT, a series of novel 1-vinyl activated derivatives of nitrocatechol and nitroguaiacol were synthesized and tested as inhibitors of pig liver COMT[12]. Surprisingly, although bearing a potential sulfhydryl reagent, able to covalently react with essential thiol groups of COMT, most of the compounds tested inhibited the enzyme by a reversible process, except for the 3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentanedione. Here we report the reversible tight-binding inhibition of partially purified pig liver COMT by [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone, in an assay using pyrocatechol as the methyl acceptor substrate.

MATERIALS AND METHODS

Materials

Pyrocatechol was obtained from Fluka. S-Adenosyl-L-methionine hydrogen sulfate was purchased from Boehringer Mannheim and (^{3}H)-S-adenosyl-L-methionine (15 Ci/mmol) was obtained from Amersham. [2-(3,4-Dihydroxy-2-nitrophenyl)vinyl] phenyl ketone and nitecapone were synthesized in our laboratory as previously described.[12] The inhibitor was dissolved in dimethylsulfoxide and diluted in water as necessary; the final concentration of the organic solvent in the COMT activity assay was less than 4%; at these concentration this solvent did not affect the enzyme activity.

COMT isolation

COMT was partially purified from pig liver as previously described.[13] The isolation process was performed at 4°C. The tissue was homogenized in 2% KCl (w/v) and centrifuged at 12,000 × g for 30 min. The supernatant was adjusted to pH 5.0 with

1 M acetic acid, stirred for 15 min and centrifuged as above. The new supernatant was then neutralized with 1 M NaOH and fractionated with 30-50% of $(NH_4)_2SO_4$. The precipitated protein was sedimented at 17,000 × g for 20 min, re-suspended in 10 mM phosphate buffer (pH 7.0) and applied to a Sephadex G200 column (5 × 90 cm). The fractions with COMT activity were pooled and stored at −30°C. The protein concentration was determined using the Benedict reagent[14].

Determination of COMT activity

The enzyme activity was determined using the method of Zürcher and Da Prada[15] modified by us[13]. The reaction mixture (0.25 mL) contained: enzyme (1 U defined as the amount of protein that catalyzes the transformation of 1 nmol of substrate per min), 20 mM pyrocatechol, 0.9 mM [^{3}H]-S-adenosyl-L-methionine (1,6 Ci/mol), 1.5 mM $MgCl_2$, 2.5 mM DTT and 125 mM phosphate buffer pH 7.6. After incubation at 37°C for 10 min the reaction mixture was stopped by addition of 0.25 mL of 1 M ice-cold citric acid. Blanks were prepared without enzyme. Then, 1.5 mL of hexane-toluene (4:1) containing 0.4% (w/v) 2.5-diphenyloxazole and 0.01% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]benzene was added and the mixture was vortexed vigorously for 30 s. The radioactivity present in the organic phase was determined. The pyrocatechol and AdoMet concentrations given above represent saturating conditions.

Kinetic measurement

The inhibition was measured as a function of inhibitor concentration without pre-incubation and with pre-incubation of an enzyme-inhibitor mixture. The kinetic data were analysed according to a Lineweaver-Burk representation by plotting the reciprocal velocities against the reciprocal of the substrate concentration.

To determine the apparent K_i (K_{app}) values the IC_{50} values were measured for several enzyme concentrations, with or without pre-incubation of the enzyme and inhibitor alone or in the presence of $MgCl_2$, AdoMet and buffer. The estimated intercept observed in a plot of IC_{50} values against the enzyme concentration gave the value of the apparent constant.

RESULTS AND DISCUSSION

[2-(3,4-Dihydroxy-2-nitrophenyl)vinyl]phenyl ketone possesses a nitro group in a position *ortho* relative to one hydroxyl group, which we have shown increases the inhibitory activity of the parent compound[11]; at position-1 it contains a carbonyl group conjugated with the nitrocatechol ring and this is also known to increase the activity of related compounds as COMT inhibitors[9]. However, in despite of all these structural characteristics, the IC_{50} value obtained for this vinyl nitrocatechol derivative was 0.32 μM, higher than that reported for other compounds with similar structures[9]. This can be due to the source of the enzyme (pig liver) and/or to the nature of the catechol substrate (pyrocatechol) used in this study. We have observed[13] that the K_m for pyrocatechol, 2 mM, with pig liver COMT is 5 or 50 fold higher than that observed with COMT from rat liver[15], 0.4 mM, or human placenta[16], 0.04 mM respectively.

Then, in order to compare our results with those reported in the literature we synthesized 3-(3-4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione (nitecapone), which has been reported as a potent inhibitor of rat liver COMT[9], and assayed it as an inhibitor of pig liver COMT under our conditions. The IC_{50} value obtained under these conditions for nitecapone, 0.8 μM, was in the same order as that observed for our new compound, [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone, but 40-fold higher than that previously reported using 3,4-dihydroxybenzoic acid as substrate for rat liver COMT. This fact means that, under the same conditions, our new compound behaves as good an inhibitor of COMT as nitecapone.

When [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone or nitecapone were assayed without or with preincubation with COMT for 15 min at 37°C and the remaining activity determined, no time dependence was observed, suggesting a reversible inhibition mechanism. This reversibility was confirmed by a dilution assay; the remaining activity obtained after pre-incubation for 3 h at 37°C of a concentrated mixture of enzyme and inhibitor followed by appropriate dilution was similar to that measured for a unconcentrated none pre-incubated mixture (data not shown). However, the inhibition percentage was decreased by 80 to 50% from the initial value. This result indicates that although these compounds are reversible inhibitors of COMT, they dissociate relatively slowly from the enzyme.

The curves obtained had an asymptote described[18] by equation (1)

$$v = \frac{k_{cat} S}{K_m + S} E_t - \frac{k_{cat} I_t S}{K_m + S} \quad (1)$$

where k_{cat} is the first-order rate constant for the reaction ES $\longrightarrow$ E + P. The asymptote intersects the x-axis at $E_t = I_t$ and the y-axis at $v = - k_{cat} I_t S / (K_m + S)$, where, I_t is the total concentration of inhibitor. Accordingly, for a concentration of pyrocatechol equal to 20 mM and a K_m value of 2 mM[13], the value of k_{cat} obtained was 0.17/min for the vinylphenyl ketone derivative and 0.29/min for the nitecapone. Thus, the k_{cat} values were similar for both compounds, and the value for nitecapone 83-fold lower than that reported in the literature using unpurified rat liver COMT[20]. As above, this discrepancy could be due to the different source of COMT used in both studies and to the lower affinity of the pyrocatechol substrate towards COMT, comparing to other catechols.

The activity of both [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone and nitecapone as COMT inhibitors was then measured as a function of the catechol concentration. When the reciprocal velocities were plotted against the reciprocal of substrate concentration, non linear relationships were obtained with either compound (Figure 1). According to Morrison, this result suggests that both compounds behave as tight-binding inhibitors of COMT[17]. A plot of v against E_t at different concentrations of the inhibitor (Ackermann-Potter plot) was used to demonstrate the tight-binding nature of the process[18,19]. Different amounts of enzyme were pre-incubated with the inhibitor for 3 h at 37°C and an aliquot of the mixture was then assayed for enzyme activity at saturation of substrate catechol (20 mM). The data from these assays gave velocity curves parallel to the control without inhibitor only at sufficiently high concentration of enzyme, showing the tight-binding nature of the inhibition (Figure 2).

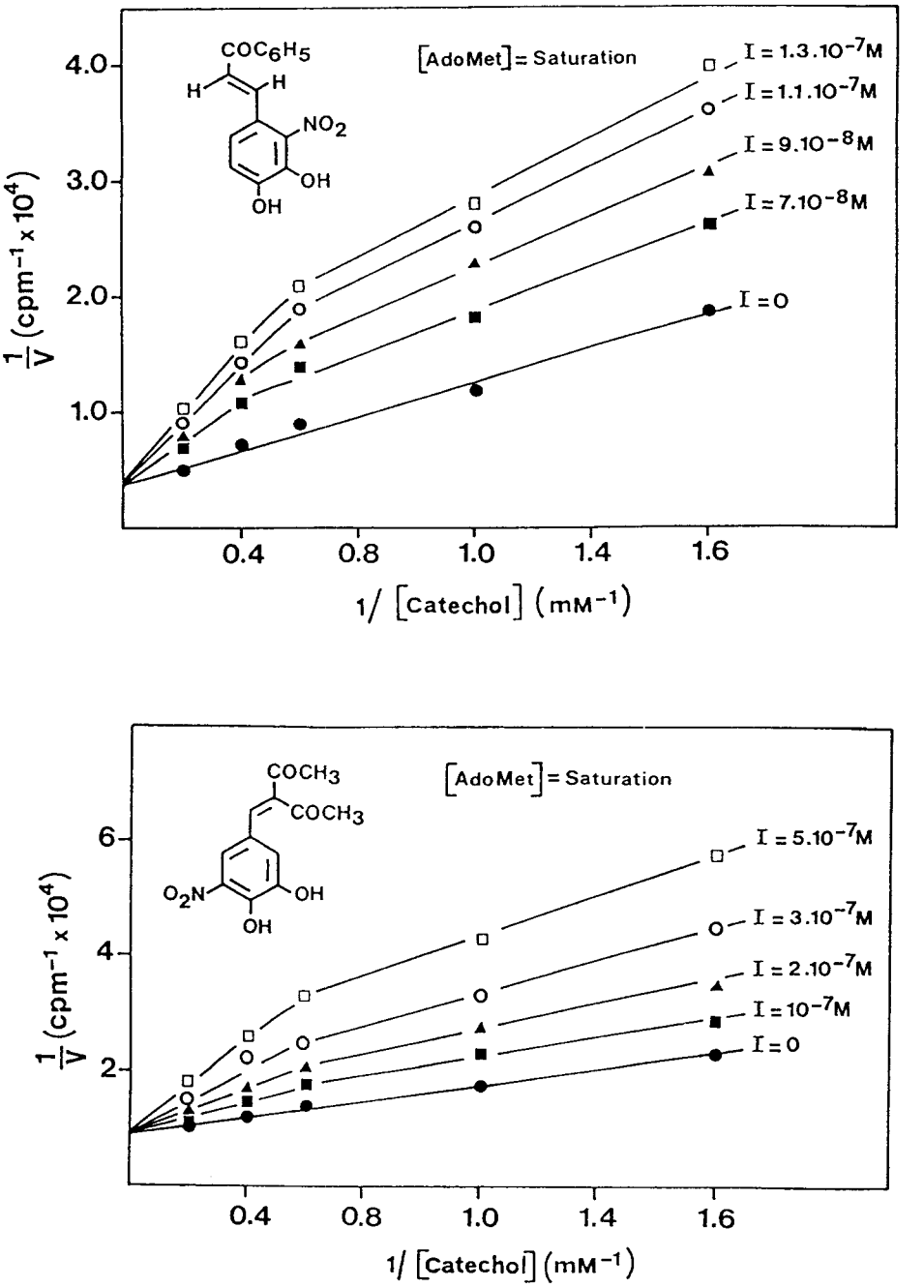

FIGURE 1 Reciprocal plot of initial velocity versus catechol concentration at AdoMet saturation and different concentrations of the inhibitors.

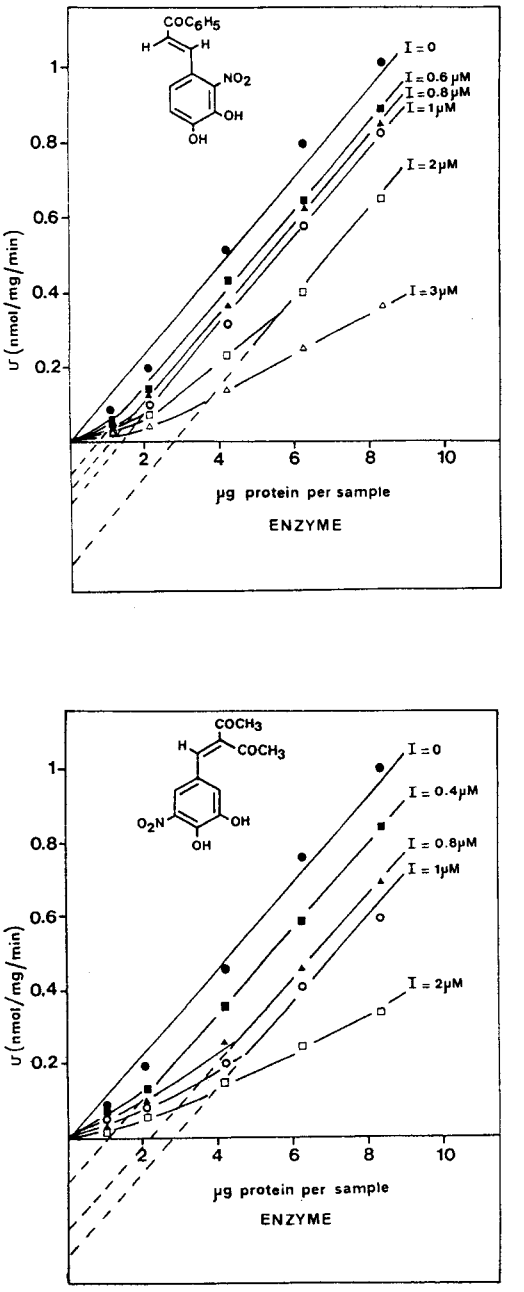

FIGURE 2 Ackermann-Plotter plot. Several concentrations of enzyme and inhibitor were pre-incubated at 37°C for 3 h. The reaction was started by the addition of pyrocatechol substrate.

When an enzyme exhibits a high affinity towards an inhibitor, the steady-state analysis of the mechanism involved is complicated because of the non-linearity of the usual plots. Several authors have described different equations for determining the inhibition pattern and the value of the kinetic constants for tight-binding inhibitors[18,19,21]. The apparent K_i values (K_{app}) can be obtained by plotting the IC_{50} values against different concentrations of enzyme[19], according to equation (2).

$$IC_{50} = E_t/2 + K_{app} \quad (2)$$

The K_{app} observed for the inhibition of pig liver COMT by the vinylphenylketone derivative was 0.2 μM without pre-incubation of the enzyme-inhibitor mixture. A similar value was obtained when determined with pre-incubation (1h, 37°C) of the enzyme with the inhibitor alone or in the presence of 1.5 mM $MgCl_2$, 0.9 mM AdoMet and 125 mM phosphate buffer pH 7.6.

The K_{app} for nitecapone obtained under our conditions with pig liver COMT was 0.7 μM; similarly, this value did not change when measured without or with pre-incubation of the enzyme and the inhibitor, in the absence or in the presence of substrates. This value was higher than that described in the literature for the inhibition of rat liver COMT (23 nM, without pre-incubation, or 0.7 nM after 1 h of pre-incubation in the presence of $MgCl_2$ and AdoMet)[20].

Finally, the IC_{50} for the inhibition of COMT by the vinylphenylketone derivative and nitecapone were measured as a function of the catechol and AdoMet concentrations. A similar inhibition pattern was obtained with both inhibitors. When pyrocatechol was the variable substrate, a competitive pattern was obtained, as a straight line was obtained for the IC_{50} values plotted against different concentrations of the catechol substrate (Figure 3A). However, the inhibition was uncompetitive when measured against the AdoMet concentration. In this case, in accord with Cha[18], a straight line was obtained in a plot of the IC_{50} values against the reciprocal of the AdoMet concentrations (Figure 3B).

In summary, we have presented here a new nitrocatechol derivative which shows a higher affinity towards COMT when compared to nitecapone[20], a potent and selective COMT inhibitor widely used in pharmacological studies and with high possibilities to be useful in a drug-combination therapy against Parkinson's disease[22-24]. Although both compounds showed a very similar catalytic number (k_{cat}), the apparent K_i (K_{app}), value is 3.5 times lower for the new [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone. We do not know if this higher affinity is due to the presence of the nitro group in position-2, instead of position-5, or to the presence of a bulky group attached to position-1, or both. Further studies with other substitutions at different positions are necessary in order to design new highly potent and selective COMT inhibitors with a potential therapeutic use.

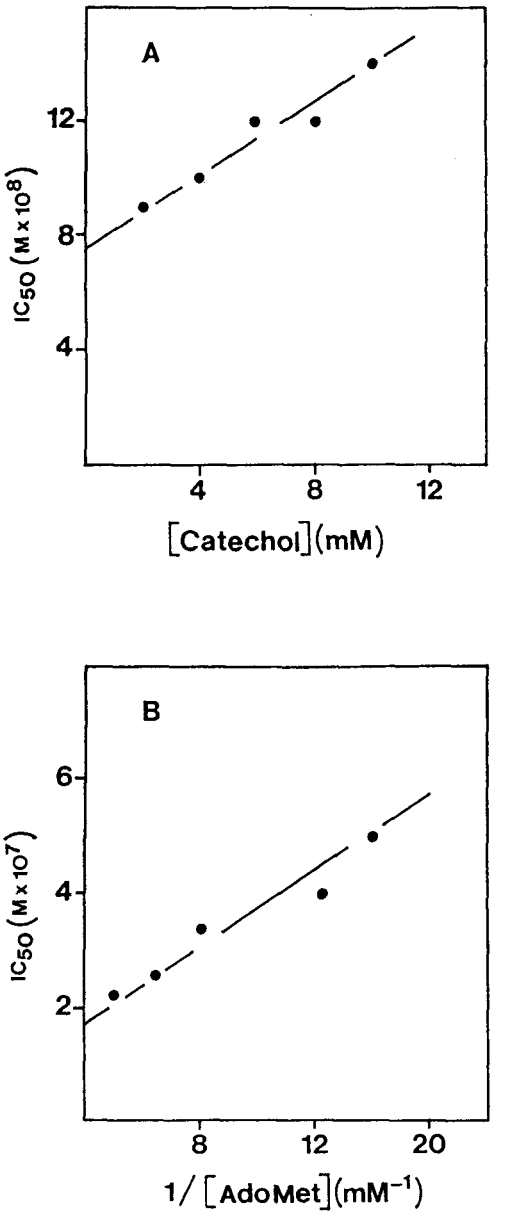

FIGURE 3 Inhibition of pig liver COMT by the vinylphenyl ketone derivative. **A.** Plot of the IC_{50} values against different pyrocatechol substrate concentration at constant COMT concentration. This representation shows a competitive inhibition of COMT with catechol as substrate. **B.** Plot of the IC_{50} values as a function of the reciprocal AdoMet concentration; an uncompetitive pattern was observed when AdoMet was used as variable substrate.

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