Biochemical Pharmacology, Vol. 38, No. 22, pp. 3953–3956, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INHIBITION OF RAT LIVER AND DUODENUM SOLUBLE CATECHOL-*O*-METHYLTRANSFERASE BY A TIGHT-BINDING INHIBITOR OR-462

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(*Received* 7 *December* 1988; *accepted* 1 *June* 1989)

Abstract—The inhibition kinetics of rat liver and duodenum soluble catechol-*O*-methyltransferase (COMT) with a disubstituted catechol OR-462 was studied. After preincubation of the enzyme and inhibitor in the presence of magnesium and *S*-adenyosylmethionine, an inhibition about thirty times greater than that without preincubation was observed. Reversible tight-binding inhibition was demonstrated with K_i values of 0.7 nM and 1.0 nM for liver and duodenum enzyme, respectively. K_m values of 53.4 μM and 56.9 μM for substrate 3,4-dihydroxybenzoic acid and 23.0 μM and 17.5 μM for *S*-adenosylmethionine were calculated for liver and duodenum enzyme, respectively. A catalytic number of 24/min for liver soluble COMT was calculated.

Inactivation of endogenous catechols like catecholamine neurotransmitters as well as exogenous catechols is catalysed by catechol-*O*-methyltransferase (COMT; EC 2.1.1.6), *S*-adenosylmethionine (SAM) serving as the methyl donor and magnesium as the cofactor. COMT activity varies greatly between different species and tissues. The specific activity in crude homogenates of animal tissues decreases in the order of liver, kidney, intestine, brain and red blood cells. Within the cell COMT is found mainly in soluble form (S-COMT) and a small fraction is in membrane bound form [1].

Although these enzyme forms have different affinities for catechol substrates, the reaction mechanism is the same [2]. The O-methylation reaction is believed to proceed via an ordered mechanism where SAM is bound to the magnesium–enzyme complex. The quaternary complex of both substrates, enzyme and magnesium, is formed before the release of products [2, 3]. This reaction is inhibited competitively by *S*-adenosyl-L-homocysteine which is formed from SAM [3]. Most substrates of COMT are O-methylated *in vitro* in both *meta*- and *para*-hydroxyl groups of the catechol ring [4], yielding two products, and the kinetic equations become complicated especially in the presence of an inhibitor.

The inhibition kinetics of COMT has been extensively studied using several catechol structured compounds [5–7]. Almost all inhibitors have been of the competitive type in respect to catechol substrate and uncompetitive to SAM. Dihydroisoquinoline derivative is an exception, since it has been reported to be an uncompetitive inhibitor of COMT when salsolinol-1-carboxylate, which was methylated only in one position, has been the substrate [6].

Several novel distributed catechol compounds have been designed and tested for COMT inhibitors *in vitro* as well as *in vivo* at Orion Pharmaceutica (Espoo, Finland) [8, 9]. One of these new compounds, OR-462 [3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione], showed an IC_{50} value of 18 nM for duodenum S-COMT [10]. This compound was also effective *in vivo*; after concomitant oral dosing of OR-462, L-dopa and carbidopa to rats, the 3-*O*-methyldopa level was reduced and the L-dopa and dopamine levels were increased in the striatum [11].

The main site of action was in the duodenum, while the COMT activity in the brain remained at the control level [10]. Here we present the results of OR-462 inhibition kinetics of soluble rat liver and duodenum COMT using 3,4-dihydroxybenzoic acid as substrate.

MATERIALS AND METHODS

Reagents. *S*-Adenosylmethionine (SAM) was purchased from Boehringer GmbH (Mannheim, F.R.G.) and was dissolved in 10 mM HCl. 3,4-Dihydroxybenzoic acid (DBA) and 3-methoxy-4-hydroxybenzoic acid (3MBA) were from Aldrich (Steinheim, F.R.G.). DBA was dissolved in dimethylsulfoxide and diluted with 10 mM sodium phosphate buffer, pH 7.4. OR-462, 3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione, was synthesized at the Research Center of Orion Pharmaceutica (Espoo, Finland). On the day of use the inhibitor was dissolved in dimethylsulfoxide as 10 mM stock solution and diluted with water as appropriate. Methanol (HPLC grade) was from Orion Pharmaceutica (Espoo, Finland). All other reagents were from commercial sources and of analytical grade.

Enzyme preparations. Wistar rats weighing about 200 g were killed by decapitation and the liver and the duodenum removed. The tissues were washed with cold 0.9% saline and stored at −80°. The tissues were homogenized in 10 mM sodium phosphate buffer, pH 7.4, 1:4 (w/v) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15,000 *g* for 20 min and then at 100,000 *g* for 60 min. The supernatant was concentrated with an ultra-

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filtration apparatus using a membrane with 10,000 cut-off (Amicon, Danvers, MA). These preparations, containing about 15 mg protein/ml, were stored at −20°. Before kinetic analyses they were gel filtrated through prepacked Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) and diluted with 10 mM phosphate buffer, pH 7.4. The protein concentration was determined according to the Lowry method using bovine serum albumin as standard.

Enzyme assay and chromatographic conditions. The assay was performed essentially as previously reported [12]. The reaction mixture containing 50 mM sodium phosphate buffer, pH 7.8, 5 mM $MgCl_2$, 200 μM SAM, 50 μl enzyme preparation and 400 μM DBA in a volume of 250 μl, was incubated at 37° for 10 min. The reaction was stopped with 25 μl of 4 M perchloric acid. After removing the protein precipitate by centrifugation the clear supernatant was analysed for O-methylated products using HPLC with electrochemical detection.

Kinetic measurements. Determination of K_m values for S-COMT of rat liver and duodenum was performed varying either SAM between 1 and 200 μM at saturating DBA concentration (400 μM) or varying DBA between 10 and 400 μM at saturating SAM concentration (200 μM). An aliquot containing 50 or 100 μg protein per sample of liver or duodenum preparation, respectively, was incubated for 10 min at 37°. A Lineweaver–Burk plot was used to calculate the K_m and V_{max} values.

IC_{50} values were determined at several enzyme concentrations with and without preincubation in the presence of inhibitor. Enzyme preparations containing 10–60 μg protein (activity 60–400 pmol product/min) were preincubated at varying inhibitor concentrations from 5 to 100 nM indicated as final concentrations of the reaction mixture including the substrate. Preincubations were performed in the presence of buffer, $MgCl_2$ and SAM at room temperature over several periods of time. The reaction was started by the addition of DBA except in the experiments without preincubation in which the enzyme was the last reagent added to the mixture. For calculation of the K_i value, the IC_{50} values were plotted against enzyme concentration [13]. All calculations were based on the measurement of 3-O-methylated product, although the 4-O-methylated derivative was separated and detected as well.

Dialysis experiments. OR-462 was incubated with the enzyme preparation at 37° for 30 min in the presence or absence of magnesium and SAM. The relative inhibition was determined and compared with the inhibition after dialysis of the incubation mixture against 1 mM sodium phosphate buffer, pH 7.4, at +4° for 5 or 24 hr.

RESULTS

Soluble COMT from rat liver and duodenum showed a Michaelian behaviour. K_m value of liver COMT was 53.4 ± 8.1 μM (V_{max} 6.0 ± 0.6 nmol/mg/min) for DBA and 23.0 ± 6.1 μM (V_{max} 6.4 ± 2.0 nmol/mg/min) for SAM. Duodenum COMT showed a K_m of 56.9 ± 6.1 μM (V_{max} 0.67 ± 0.1 nmol/mg/min) for DBA and 17.5 ± 4.0 μM (V_{max} 0.68 ± 0.01 nmol/mg/min) for SAM. The values are means of three determinations ± SD.

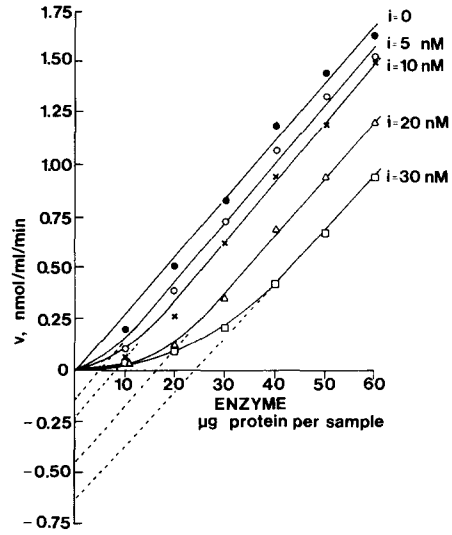

Fig. 1. Ackermann–Potter plot of rat liver S-COMT. Various amounts of enzyme were preincubated in the presence of 5 mM magnesium and 200 μM SAM and varying concentrations of OR-462. After 3 hr preincubation at room temperature the reaction was started by the addition of DBA (400 μM final concentration). Mixtures were incubated at 37° for 10 min. Proteins were removed by precipitation and 3 MBA measured by electrochemical detection.

After incubation of the enzyme with OR-462, the relative inhibition of both liver and duodenum enzyme decreased from the initial 90% to 60% when the samples were gel filtrated through Sephadex G-25 columns. When the same samples were dialysed, the inhibition decreased by 50% in 5 hr and it was over after 24 hr. This indicates that the inhibitor dissociates slowly from the enzyme and it is reversible.

Dialysis caused a gradual and marked loss of specific activity of control samples. The liver COMT enzyme lost about 50% of its activity during dialysis. Duodenum preparations were more sensitive since only 10% of the activity was left after 24 hr of dialysis.

The activity of rat liver COMT was measured in the presence of several concentrations of OR-462 using either DBA or SAM as the varied substrate. The data from the experiments gave nonlinear curves when $1/v$ was plotted against $1/S$ or inhibitor concentration (data not shown).

The reaction velocity of rat liver S-COMT after 3 hr of preincubation in the presence of varying inhibitor and enzyme concentrations (Ackermann–Potter plot) is shown in Fig. 1. The velocity increases progressively with increasing enzyme concentration. Also the velocity curve parallels to the control curve at sufficiently high enzyme concentrations, demonstrating clearly the tight-binding nature of the inhibition. A similar pattern of curves can be drawn from results without preincubation although the linear parts appear at higher enzyme concentrations.

The catalytic number k_{cat}, the moles of substrate that are converted to product per mole of active

Table 1. K_i values of OR-462 for rat liver and duodenum S-COMT with different preincubation times

Preincubation time (min)	K_i (nM)	
	Liver	Duodenum
0	23.1	19.9
10	4.6	3.6
30	1.2	2.6
60	0.70	1.0
180	0.65	—

Enzyme was preincubated with 5–100 nM of OR-462 in the presence of $MgCl_2$ and SAM (for other conditions see text).

center per min, can be calculated from the asymptotes of the curves from the Ackermann–Potter plot (Fig. 1). The linear parts of the curves intersect the velocity axis at a point $-k_{cat}I_tS/K_m + S$ where I_t is the total concentration of the inhibitor [13]. Using K_m of 53.4 μM in this equation, the numerical value of k_{cat} was 24/min for unpurified rat liver S-COMT.

K_i values were calculated by plotting IC_{50} values against enzyme concentration and by estimating the intercept at the IC_{50} axis [13] (Table 1). The inhibition constant for liver S-COMT was 23.1 nM without preincubation when the reaction was started by the addition of enzyme. The inhibition constant decreased with preincubation time. After 10 min of preincubation, a K_i value of 4.6 nM was calculated, after 1 and 3 hr it was 0.70 and 0.65 nm, respectively.

Duodenum COMT had a K_i value comparable to liver enzyme and the value of 1.0 nM was reached after 1 hr of preincubation (Table 1). Since COMT activity in the rat duodenum was unstable, longer preincubation times gave unreliable results. In the case of liver enzyme no stability problems were observed and the control samples kept their activity level during all preincubation tests.

IC_{50} values were determined at different substrate concentrations. The reactions were started by the addition of the enzyme (Fig. 2). An increase in the DBA concentration resulted in an increase in IC_{50} values like in the competitive type of inhibition at steady state [14]. When SAM was used as the varied substrate, a straight line was seen when the IC_{50} values were plotted against the reciprocals of the SAM concentrations as would be expected for uncompetitive inhibition (Fig. 2).

DISCUSSION

In the literature there is great variation in the kinetic data of COMT, especially concerning the K_m values for SAM [1]. Our value of liver S-COMT, 53.4 μM for DBA, is lower and 23.0 μM for SAM is higher than reported earlier [15]. It is worth noting that in this study the enzyme was unpurified, but the endogenous substrates were removed by ultrafiltration and gel filtration, and no stabilising agents were used. Whether there are essential variations in the kinetic parameters between species and tissues or between individuals is not clear. Until now, differences in kinetic parameters are mostly explained by assay methods.

The catalytic number calculated for liver COMT was 24/min. This is low and the procedure should be repeated with purified enzyme.

OR-462 is a potent inhibitor for liver and duodenum S-COMT having a K_i of 23 nM initially, and its efficacy still increases about 30 times during the preincubation of 1 hr (Table 1). Longer preincubation times caused only minor changes. So we conclude that the true K_i for S-COMT is 0.7 nM. Liver and duodenum S-COMT were inhibited to the same degree, and the instability of the enzyme is supposed to be the cause for the slightly higher K_i 1.0 nM for duodenum. The presence of magnesium and SAM in the preincubation medium is important and no increase in inhibition is seen if only the enzyme and the inhibitor are preincubated together. The inhibitor seems to bind to the magnesium–SAM–enzyme complex in the same manner as the substrate [2, 3].

Whether a tight-binding inhibitor is a competitive one, binding to the same binding site as the substrate, is more difficult to determine than for a less potent, readily reversible inhibitor. Here we have applied

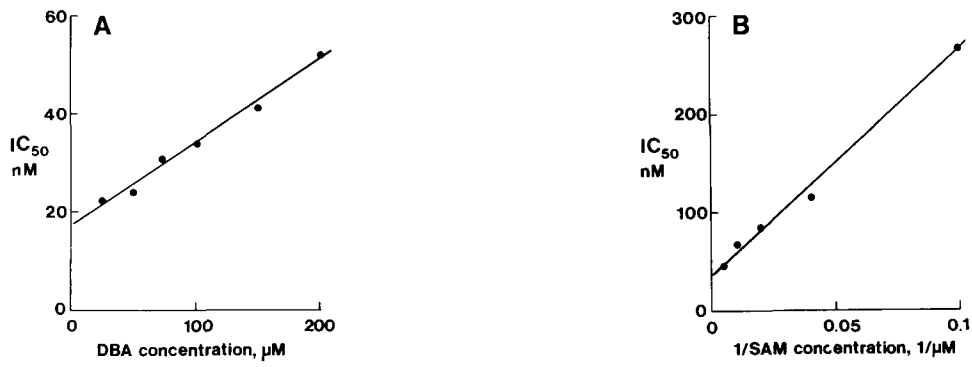

Fig. 2. The effect of varying substrate concentration on IC_{50} values of OR-462 at constant S-COMT concentrations. Reactions were started by the addition of rat liver preparation. For competitive inhibitor at steady state situation a straight line is obtained by plotting IC_{50} values against the various substrate concentrations (frame A for DBA) [10]. For uncompetitive type of inhibition a straight line is observed when IC_{50} values are plotted as a function of the reciprocal of the varied substrate (frame B for SAM).

the method which was derived for steady state and is useful also in a quasi steady state situation [13, 14]. Steady state between S-COMT and OR-462 was reached in about 1 hr at room temperature and a quasi steady state was certainly achieved in 10 min at 37°. The inhibition type was determined by varying either the DBA or SAM concentration. The IC_{50} values were calculated and plotted against either substrate concentration or the reciprocal of it. The binding of OR-462 to COMT was uncompetitive in respect to SAM and competitive to DBA (Fig. 2). This was a logical result since SAM binds to the enzyme–magnesium complex prior to the substrate [2]. OR-462 is a disubstituted catechol and is structurally enough related to DBA to compete for the same binding site, though OR-462 itself is a poor substrate for COMT [8].

The nature of the inhibitor did not change during the preincubation time (similar curves in Ackermann–Potter plots) and OR-462 behaved as a tight-binding inhibitor, irrespective of the preincubation time. The association reaction between the enzyme–SAM complex and the inhibitor must be relatively rapid since even without preincubation the enzyme activity was effectively blocked. If continuous measurement of formation of the reaction products were possible, at the very beginning of the association of enzyme and inhibitor, it would further elucidate this question.

All calculations were based on the production of 3MBA, and no attention was paid to the formation of the minor O-methylation product, 3-hydroxy-4-methoxybenzoic acid. Both products were detected, but the relative amounts of the O-methylation products remained the same in the presence and absence of the inhibitor.

We did not observe any differences in inhibition kinetics with OR-462 between soluble COMT from rat liver and duodenum. This does not rule out the possibility that the use of other substrates or tissues would give another result. As the most potent inhibitor of COMT so far, OR-462 offers a valuable tool to evaluate possible differences between soluble and membrane bound forms of COMT.

Acknowledgements—The authors wish to thank Annikki Alanko and Ulla Naukkarinen for excellent technical assistance.

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