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European Journal of Pharmacology 460 (2003) 163–170
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Kinetic inhibitory profile of BIA 3-202, a novel fast tight-binding, reversible and competitive catechol-*O*-methyltransferase inhibitor

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Received 13 August 2002; received in revised form 29 November 2002; accepted 6 December 2002

Abstract

The present study reports the kinetic inhibitory profile of 1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone (BIA 3-202), a novel inhibitor of soluble catechol-*O*-methyltransferase (COMT) in rat liver. After an oral single dose (30 mg kg^{-1}), there was a time-dependent recovery of enzyme activity from 98 ± 2% inhibition at 30 min to total recovery at 24 h after treatment. The inhibitory effect produced by BIA 3-202 on soluble COMT was reversible after gel filtration of the samples. BIA 3-202 acted as a fast inhibitor of rat liver soluble COMT, interacting immediately with the enzyme after mixing. No differences were observed in the metanephrine formation rates (in nmol mg $protein^{-1}$ min^{-1}) obtained without and with a 60-min preincubation with 30 nM of BIA 3-202 (1.30 ± 0.02 and 1.35 ± 0.03, respectively). The tight-binding nature of the inhibition produced by BIA 3-202 was evaluated by performing an Ackermann–Potter plot. The true K_i for BIA 3-202, derived from the nonlinear regression analysis, was 0.19 ± 0.02 nM. In substrate competition studies, an increase in the concentration of adrenaline resulted in a linear increase in IC_{50} values for BIA 3-202. In conclusion, BIA 3-202 behaves as a reversible, potent and fast tight-binding COMT inhibitor that acts competitively at the substrate binding site of rat liver soluble COMT.

Keywords: Catechol-*O*-methyltransferase inhibitor; Kinetic; Liver catechol-*O*-methyltransferase; BIA 3-202; Parkinson's disease

1. Introduction

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) was originally detected in rat liver extracts (Axelrod and Tomchick, 1958). Since then, COMT has been found in many species: animals, plants, and prokaryotes (Guldberg and Marsden, 1975). In mammals, the highest COMT activities have been found in the liver and kidney, but COMT is found in almost all mammalian tissues (Karhunen et al., 1994). The general physiological function of COMT is the inactivation of biologically active or toxic catechols. Physiological substrates of COMT are catecholamine neurotransmitters, dopamine, noradrenaline, and adrenaline, and some of their metabolites. COMT also inactivates catechol steroids such as 2-hydroxyestradiol, drugs with a catechol structure such as levodopa, and a large number of other catechol compounds. The enzyme catalyses the transfer of the methyl group from *S*-adenosyl-L-methionine (SAM) to one of the hydroxyl groups of the catechol substrate in the presence of Mg^{2+}, the reaction products being *O*-methylated catechol and *S*-adenosyl-L-homocysteine (Guldberg and Marsden, 1975). There is a single gene for COMT, which codes for both soluble COMT and membrane-bound COMT using different promoters (Lundström et al., 1991; Salminen et al., 1990).

In the past 25 years, levodopa has been routinely administered to patients afflicted with Parkinson's disease in combination with inhibitors of aromatic L-amino acid decarboxylase (AADC), to prevent its degradation to dopamine in the periphery. However, when administered with a peripheral decarboxylase inhibitor, a considerable amount of levodopa is converted by peripheral COMT to 3-*O*-methyldopa (3-OMD), so that only 10% of a given dose reaches the brain (Nutt and Fellman, 1984). Inhibition of peripheral COMT increases the amount of levodopa that is available to enter the brain by extending the half-life of levodopa, resulting in more stable levels in plasma (Kurth and Adler, 1998). As a result of this, inhibition of COMT was thought to provide a new therapeutic strategy for the treatment of Parkinson's disease. Tolcapone and entacapone have emerged from a large series of nitrocatechol derivatives as

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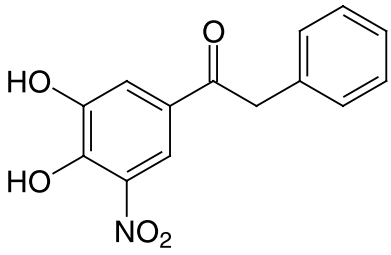
doi:10.1016/S0014-2999(02)02879-0



BIA 3-202

Fig. 1. Chemical structure of BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone).

highly selective and orally active COMT inhibitors that have been developed for clinical use (Bonifati and Meco, 1999; Jost, 2000; Kaakkola, 2000; Olanow et al., 2001). Subsequently, due to liver toxicity, the use of tolcapone has been severely restricted, and entacapone has emerged as the COMT inhibitor of choice (Olanow et al., 2001). However, the short half-life and the limited potency to inhibit peripheral COMT are considered the major reasons for the limited efficacy of entacapone as an adjunct to levodopa therapy in Parkinson's disease (Shepherd and Clegg, 1999).

1-[3,4-Dihydroxy-5-nitrophenyl]-2-phenyl-ethanone (BIA 3-202) (Fig. 1), a nitrocatechol, is a novel potent, orally active and long-acting COMT inhibitor (Learmonth et al., 2002). BIA 3-202 was also found to enhance, in a time- and dose-dependent manner, the availability of levodopa to the brain and reduce its *O*-methylation to 3-OMD (Parada et al., 2001), which may prove beneficial in parkinsonian patients treated with L-3,4-dihydroxyphenylalanine (L-DOPA) plus an aromatic amino acid decarboxylase inhibitor. The aim of the present study was to evaluate the kinetic inhibitory profile of BIA 3-202, and the type of inhibition exerted on the soluble form of COMT.

2. Methods

2.1. Animals

Male Wistar rats (Harlan-Interfauna Ibérica, Barcelona, Spain) aged 60 days and weighing 240–260 g were used in all experiments. Rats were kept under controlled environmental conditions (12-h light/dark cycle and room temperature 22 ± 1 °C) with food and tap water allowed ad libitum. All animals interventions were performed in accordance with the European Directive number 86/609, and the rules of the "Guide for the Care and Use of Laboratory Animals", 7th edition, 1996, Institute for Laboratory Animal Research (ILAR), Washington, DC.

2.2. In vivo studies

BIA 3-202 was given by gastric tube (0.3 to 30 mg kg^{-1} in 0.5% carboxymethylcellulose) to overnight fasted rats. Thereafter, at defined intervals (0.5, 1, 3, 6, 9,12 and 24 h), animals were anaesthetised with sodium pentobarbital (60 mg kg^{-1}) and perfused through the left ventricle with 0.9% (w/v) NaCl. Livers were immediately removed and homogenised in 5 mM sodium phosphate buffer, pH 7.8 1:4 (w/v) at 4 °C with a Teflon homogeniser (Heidolph). The crude homogenates were used for the determination of COMT activity.

2.3. Preparation of soluble COMT

The soluble fraction of COMT was obtained as previously described (Vieira-Coelho and Soares-da-Silva, 1999). In brief, crude homogenates prepared as described above were centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatants were then centrifuged at 100,000 × *g* for 60 min at 4 °C. The high-speed supernatants were used as soluble COMT. The protein content in the samples was determined by the method of Bradford (1976) with a standard curve of bovine serum albumin (50–250 μg ml^{-1}).

2.4. In vitro studies

Enzyme preparations (500 μl of crude homogenates or 100 μl of soluble COMT) were incubated with a concentration of adrenaline approaching saturation (1000 μM) and a saturating concentration of the methyl donor, *S*-adenosyl-L-methionine (500 μM). Reaction mixture contained also pargyline (100 μM), $MgCl_2$ (100 μM) and EGTA (1 mM) in 5 mM sodium phosphate buffer pH 7.8. Five-minute incubations were carried out at 37 °C under conditions of light protection with continuous shaking. In the experiments started by the addition of the substrate, the enzyme was preincubated for 20 min at 37 °C in the reaction mixture before the addition of adrenaline. In the reactions started by the addition of the enzyme, all the components were incubated at 37 °C for 5 min before the reaction was started with the enzyme. At the end of the incubation period, the tubes were transferred to ice and the reaction was stopped by the addition of 2 M perchloric acid.

2.5. Assay of COMT

COMT activity was determined by the ability of enzyme preparations to methylate adrenaline to metanephrine as previously described in crude homogenates and soluble COMT (Vieira-Coelho and Soares-da-Silva, 1999). In all experiments, after the reaction was stopped, the samples were kept at 4 °C for 2 h, centrifuged (200 × *g*, 4 min, 4 °C), filtered on 0.22-μm pore size Spin-X filter tubes (Costar) and used for the assay of metanephrine by means of high-pressure liquid chromatography (HPLC) with electrochemical detection, as previously described (Vieira-Coelho and Soares-da-Silva, 1996). The chromatography system consisted of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802 C) and a stainless

steel 5-μm ODS column (Biophase; Bioanalytical Systems, West Lafayette, IN) of 25-cm length. Samples were injected by means of an automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (10% v/v), adjusted to pH 3,5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml min^{-1}. Detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored using Gilson 712 HPLC software. The lower limits for detection of metanephrine ranged from 350 to 500 fmol.

2.6. Inhibition reversibility

The reversibility studies were performed as follows: samples containing soluble COMT (4 mg ml^{-1}) with or without 500 nM BIA 3-202 were preincubated for 20 min at 37 °C and then applied to PD-10 columns (Amersham Pharmacia Biotech). The eluate containing soluble COMT was collected and enzyme activity was determined in the samples collected before and after gel filtration.

2.7. Data analysis

K_m and V_{max} values for COMT activity were calculated by nonlinear regression analysis using the GraphPad Prism statistics software package (GraphPad Prism Software, San Diego). For the calculation of the IC_{50} values, the parameters of the equation for one-site inhibition were fitted to the experimental data (Motulsky et al., 1994). A nonlinear regression computer analysis was used to fit the steady-state rates obtained for various enzyme and inhibitor concentrations to the following equation (Cha, 1975; William and Hakala, 1979):

$$v = \frac{K_{cat}S}{2(K_m + S)} \times \left[(\varepsilon E - K_i^* - I) + \sqrt{(K_i^* + I + \varepsilon E)^2 - 4I\varepsilon E}\right] \quad (1)$$

In this equation, E is the total protein concentration; ε is the molar equivalency and represents the fraction of the total protein present as active enzyme; I is the total inhibitor concentration; K_{cat} is the catalytic number, which gives the number of molecules of substrate transformed into product per catalytic centre per unit of time; and K_i^* is the apparent enzyme-inhibitor dissociation constant value.

Another approach used to calculate the apparent $K_i(K_i^*)$ value for BIA 3-202 applied the following equation (Cha, 1975; Williams and Morrison, 1979):

$$IC_{50} = Et/2 + K_i^* \quad (2)$$

Et is the total enzyme concentration and K_i^* is the apparent enzyme-inhibitor dissociation constant value.

For competitive inhibition, the true K_i was obtained by dividing K_i^* by $(1 + S/K_m)$ (Cha, 1975; Williams and Morrison, 1979).

2.8. Statistical analysis

Geometric means are given with 95% confidence intervals and arithmetic means are given with S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons or Student's "*t*"-test. A *P*-value less than 0.05 was assumed to denote a significant difference.

2.9. Drugs

Drugs used were *S*-adenosyl-L-methionine (Sigma), L-adrenaline bitartrate (Sigma), DL-metanephrine hydrochloride (Sigma), pargyline hydrochloride (Sigma). BIA 3-202, tolcapone, entacapone and nitecapone were synthesised at Laboratory of Chemistry, Department of Research and Development, BIAL, with purities >99.0%. The synthesis and chemical characterization of BIA 3-202 (NMR, MS, UV and IR spectra) have been described elsewhere (Learmonth et al., 2002).

3. Results

3.1. BIA 3-202 inhibitory potency on rat liver soluble COMT

The in vivo inhibitory potency of BIA 3-202 was evaluated in experiments in which rats were given increasing doses of the compound (0.3 to 30 mg kg^{-1}). Animals were killed 1 h after test drug administration and COMT activity was determined. The results (Fig. 2A) show that BIA 3-202 produced a dose-dependent inhibition of liver COMT with an ED_{50} of 0.8 (0.5, 1.2) mg kg^{-1}. The inhibitory effect on rat liver COMT after the oral administration of 30 mg kg^{-1} BIA 3-202 showed a time-dependent recovery of enzyme activity from 98 ± 2% inhibition at 30 min to a total recovery at 24 h after treatment (Fig. 2B).

3.2. Kinetic parameters of rat liver soluble COMT

Incubation of the rat liver soluble COMT assay mixture in the presence of increasing concentrations of adrenaline resulted in the concentration-dependent formation of metanephrine (Fig. 3A), with K_m and V_{max} values of 286 (144, 427) μM and 124 ± 8 nmol mg $protein^{-1}$ h^{-1}, respectively. A saturation curve was also made for the methyl donor, SAM (Fig. 3B). The kinetic parameters, K_m and V_{max} obtained for SAM were 22 (10, 34) μM and 91 ± 5 nmol mg $protein^{-1}$ h^{-1}, respectively. From these results, the

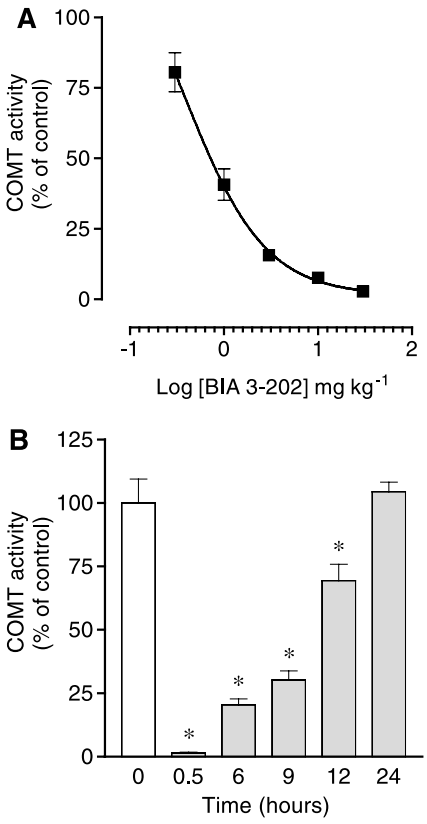


Fig. 2. (A) COMT activity in homogenates of rat liver, determined at 1 h after the oral administration of BIA 3-202 (0.3 to 30 mg kg^{-1}). (B) COMT activity in homogenates of rat liver, determined at 0.5, 3, 6, 9, 12 and 24 h after the oral administration of BIA 3-202 (30 mg kg^{-1}). Results are means ± S.E.M. of four experiments per group. Significantly different from corresponding control values (*$P<0.05$).

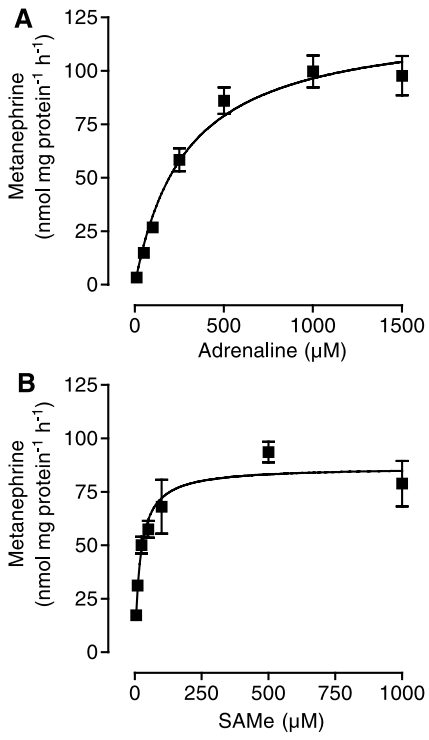


Fig. 3. Saturation curves of rat liver soluble COMT. COMT activity is shown as the rate of formation of metanephrine from increasing concentrations of adrenaline (A) and SAM (B). Symbols represent means of four experiments; vertical lines show S.E.M.

concentrations of adrenaline and SAM chosen for subsequent studies were 1000 and 500 μM, respectively.

3.3. Reversibility of BIA 3-202 effect

The reversibility of COMT inhibition by BIA 3-202 was studied under in vitro experimental conditions after gel filtration of the enzyme alone and in the presence of BIA 3-202 (500 nM) through PD10 columns. Rat liver soluble COMT activity was not affected by this procedure (Fig. 4). However, the effect of 500 nM BIA 3-202 (86 ± 4% inhibition) on soluble COMT (4 mg ml^{-1}) activity was significantly ($P<0.05$) reduced (22 ± 7% inhibition) by gel filtration of samples (Fig. 4).

3.4. In vitro kinetics of BIA 3-202

To determine whether BIA 3-202 acts as a fast or slow inhibitor, time curves were obtained either by starting the reaction with the protein (1 mg ml^{-1}) or, alternatively, by adding the substrate (adrenaline) to the enzyme (soluble COMT) preincubated for 15, 30 or 60 min with BIA 3-202. The inhibitory effect of BIA 3-202 (30 nM) was not changed by preincubation (data not shown). In addition, no differences were observed in the metanephrine formation rates obtained with no preincubation and with a 60-min preincubation with 3, 30 or 100 nM of BIA 3-202 (Fig. 5A). Though the linearity of time curves was independent of the BIA 3-202 concentration (Fig. 5A), the metanephrine formation rate in the control situation was significantly

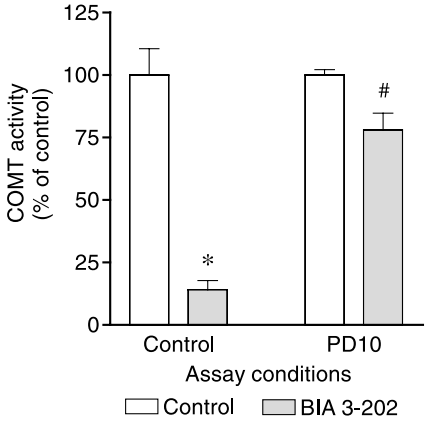


Fig. 4. Effect of BIA 3-202 (500 nM) on rat liver soluble COMT activity before and after passage of samples through PD10 columns. Bars represent mean of four experiments; vertical lines show S.E.M. Significantly different from corresponding control values (*$P<0.05$) and values for BIA 3-202 before passage through PD10 columns (#$P<0.05$).

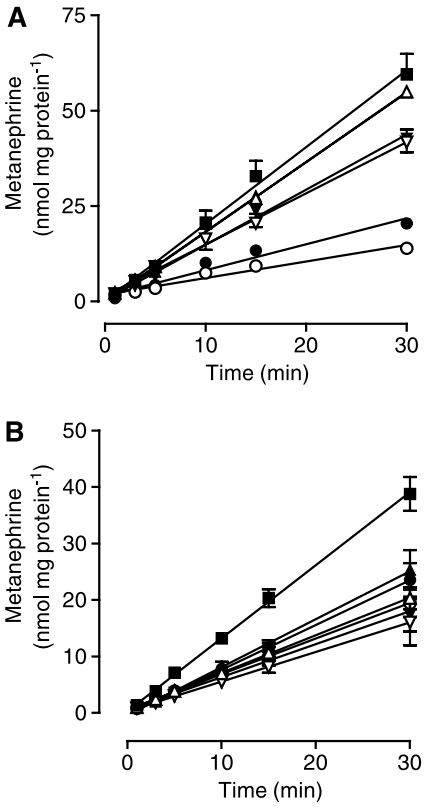


Fig. 5. (A) Time curves of rat liver soluble COMT in the absence (squares) and the presence of 3 nM (triangles), 30 nM (inverted triangles) and 100 nM (circles) of BIA 3-202, either by starting the reaction with the enzyme (no preincubation, open symbols) or with adrenaline added to soluble COMT preincubated for 60 min with BIA 3-202 (closed symbols). (B) Time curves of rat liver soluble COMT in the absence (squares) and the presence of (30 nM) tolcapone (triangles), (30 nM) entacapone (inverted triangles) and (30 nM) nitecapone (circles), either by starting the reaction with the enzyme (no preincubation, open symbols) or with adrenaline added to soluble COMT preincubated for 60 min with the nitrocatechol derivatives (closed symbols). Symbols represent means of four experiments; vertical lines show S.E.M.

lower in the presence of 3, 30 and 100 nM BIA 3-202 (Table 1). Fig. 5B also shows that metanephrine formation rates obtained with no preincubation and with a 60-min preincubation with tolcapone, nitecapone and entacapone (all at 30 nM) were identical. However, the metanephrine formation rate in the control situation was significantly greater than that in the presence of the COMT inhibitors (Fig. 5B).

Table 1
Metanephrine formation rate (nmol mg protein^{-1} min^{-1}) by rat liver soluble COMT in the absence (control) and in the presence of increasing concentrations of BIA 3–202 with no preincubation

BIA 3–202	0	3 nM	30 nM	100 nM
Metanephrine	1.92 ± 0.03	1.76 ± 0.01*	1.30 ± 0.02*	0.56 ± 0.03*

Values are means ± S.E.M. (n = 4).
* Significantly different from control values (P < 0.05).

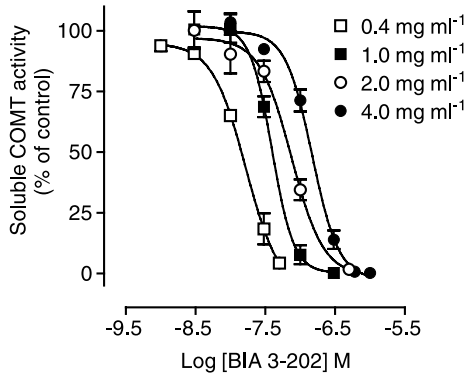


Fig. 6. Effect of increasing concentrations of BIA 3-202 on rat liver soluble COMT activity in the presence of 0.4, 1.0, 2.0 and 4.0 mg protein ml^{-1}. Symbols represent the mean of four experiments; vertical lines show S.E.M.

3.5. Tight-binding inhibition and Ackermann–Potter analysis

The inhibitory potency of BIA 3-202 on rat soluble COMT activity was dependent on the amount of the enzyme used in the assay. This is evidenced in Fig. 6, where increasing the concentration of total protein used in the assay (0.4, 1.0, 2.0 and 4.0 mg ml^{-1}) resulted in a parallel rightward shift of the concentration-dependent inhibition curves for BIA 3-202. IC_{50} values for BIA 3-202 obtained with the highest concentration of soluble COMT were 10-fold those obtained with the lowest concentration of soluble COMT (Table 2). Since this type of behaviour is indicative of tight-binding inhibition (Cha, 1975), BIA 3-202 was further analysed by testing different concentrations of the drug with different protein concentrations. The plot of enzyme velocity against protein concentration at different BIA 3-202 concentrations (Ackermann–Potter plot) was characterized by asymptotic concave curves (Fig. 7), which revealed the tight-binding or titrating nature of the inhibitor. This Ackermann–Potter representation shows that BIA 3-202 inhibition was dependent on the protein concentration, a result that confirms the tight-binding nature of BIA 3-202. With nonlinear regression analysis, it was possible to calculate the catalytic number (K_{cat} = 7.2 ± 0.6 min^{-1}), a measure of the efficiency of the enzyme, the molar equivalency (ε = 74.9 ± 2.6), which gives the active enzyme concentration in the

Table 2
Effect of protein concentration on the inhibitory potency of BIA 3–202 against soluble COMT activity

Protein (mg ml^{-1})	IC_{50} (nM)
0.4	12 (5, 27)
1.0	44 (11, 174)
2.0	73 (35, 152)
4.0	124 (47, 324)

Values are geometric means with 95% confidence intervals (n = 3).

sample, and the apparent inhibition constant ($K_i^* = 1.5 \pm 0.1$ nM) of BIA 3-202.

3.6. Interaction with the substrate and methyl donor binding sites

In experiments started with protein (1 mg ml^{-1}), IC_{50} values for BIA 3-202 were determined at different adrenaline and SAM concentrations. An increase in the adrenaline concentration resulted in a linear increase in IC_{50} values for BIA 3-202, giving a straight line when IC_{50} values were plotted against adrenaline concentrations (Fig. 8A). In contrast, when SAM was used as the varied substrate, a straight line was obtained when IC_{50} values were plotted against the reciprocal of the SAM concentration (Fig. 8B).

3.7. Determination of BIA 3-202 true K_i value

The apparent $K_i(K_i^*)$ values for BIA 3-202 were obtained using two different approaches. A nonlinear approach was used in which the steady-state velocities at different soluble COMT concentrations and for different inhibitor concentrations were fitted by nonlinear regression analysis to Eq. (1). As mentioned above, the K_i^* value obtained was 1.5 ± 0.1 nM. A linear approach was also used in which IC_{50} values (obtained with 20-min preincubation) were plotted against soluble COMT concentration and the intercept at the IC_{50} axis was estimated. The K_i^* value obtained for soluble COMT (15.1 ± 1.1 nM) was not significantly different from the K_i^* values obtained without preincubation or with a 10- and 60-min preincubation (18 ± 5.5, 12.2 ± 4.9, and 16.5 ± 4.6 nM, respectively), which is in agreement with the results obtained in the inhibition studies. The difference in values obtained when using different mathematical approaches has been described earlier (William and Hakala, 1979; Williams and Morrison, 1979). The true K_i value for BIA 3-202 (0.19 ± 0.02 nM) was calculated from the K_i^* obtained by the nonlinear approach, taking into consideration substrate concentration and K_m values.

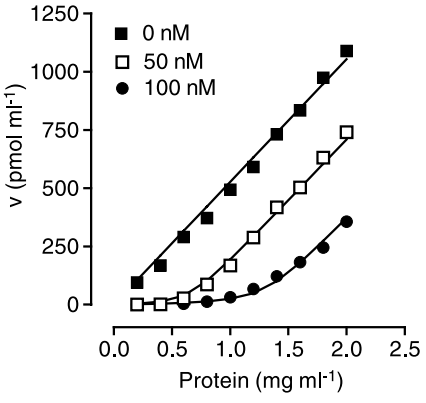


Fig. 7. Ackermann–Potter plot of rat liver soluble COMT. Increasing amounts of protein (0.2 to 2.0 mg ml^{-1}) were preincubated for 20 min in the absence and in the presence of 50 and 100 nM BIA 3-202. The reaction was started by addition of 1000 μM adrenaline. Mixtures were incubated at 37 °C for 5 min. Symbols represent the mean of four experiments.

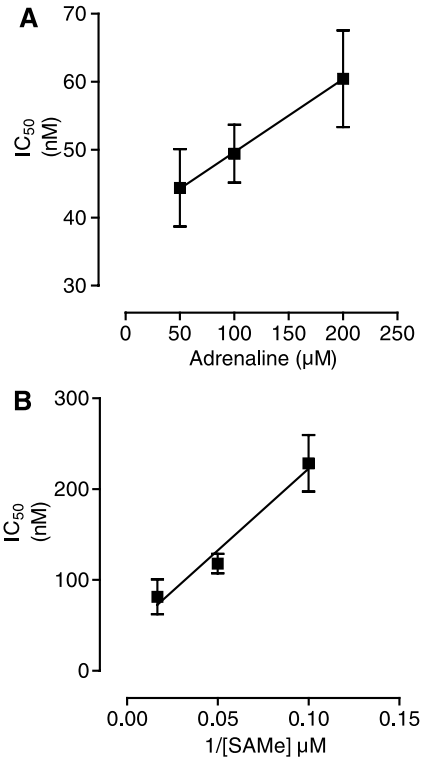


Fig. 8. The effect of varying substrate concentration, adrenaline (A) and SAM (B) on IC_{50} values of BIA 3-202 in rat liver soluble COMT (1 mg ml^{-1}). Symbols represent the mean of four experiments; vertical lines show S.E.M.

4. Discussion

The results obtained with the rat liver soluble COMT showed a Michaelis–Menten behaviour for both the catechol (adrenaline) and the methyl donor (SAM) substrates, with kinetic parameters similar to those described in the literature (Schultz and Nissinen, 1989). In fact, the affinity for catechol substrates found in the present study with nonpurified rat liver soluble COMT (K_m value of 286 ± 22 μM) was similar to that recently reported by Lautala et al. (2001) for the recombinant rat soluble COMT (K_m value of 250 ± 22 μM). The reason for choosing rat liver soluble COMT to evaluate the inhibitory properties of BIA 3-202 relates to the fact that the highest COMT activity in both rat and humans is found in the liver, where soluble COMT is the predominant form of COMT (Männistö and Kaakkola, 1999). Furthermore, the primary structure of human soluble COMT is 81% identical with the rat soluble COMT (Lundström et al., 1991), with all residues involved in catechol binding and catalysis being conserved in the two enzymes (Vidgren et al., 1994).

The results reported here for BIA 3-202 clearly show that this compound is a fast, potent, reversible and orally active COMT inhibitor. This is evidenced by the finding that an

oral dose of 30 mg kg^{-1} almost abolished rat liver COMT shortly after administration (at 30 min produced 98 ± 2% of inhibition) and that rat liver COMT activity was restored to pre-dose values 24 h after administration. The reversibility was verified under in vitro experimental conditions with the loss of the BIA 3-202 inhibitory effect after gel filtration through PD10 columns. Similar results were obtained with other nitrocatechol COMT inhibitors (Schultz and Nissinen, 1989). The inhibitory effect of BIA 3-202 (30 nM) was not changed by preincubation time. The formation of product (metanephrine) increased as a linear function of time and no differences were observed in rates of metanephrine formation with 0, 15, 30 or 60 min of preincubation. This indicates an almost immediate interaction with the enzyme after mixing, a characteristic of fast-binding kinetics (Cha, 1975). In addition, linearity of time curves was observed with increasing concentrations of BIA 3-202 (3, 30 and 100 nM) when the reaction was initiated with the enzyme. It should be noted that this shows that the steady-state velocities were reached immediately after mixing. Under our experimental conditions, all nitrocatechol COMT inhibitors (BIA 3-202, tolcapone, entacapone and nitecapone) were equally efficacious with and without preincubation with the enzyme. It appears, therefore, that this is a common feature of all nitrocatechol compounds. The present results, for nitecapone, differ from those reported by others (Schultz and Nissinen, 1989), where an increase in potency was observed with increasing preincubation time. Not only were the experimental approaches used different but the reaction conditions were also distinct. Furthermore, other authors (Lotta et al., 1995) have reported K_i values for tolcapone, nitecapone and entacapone with the recombinant human enzyme and using only a 5-min preincubation period.

The tight-binding kinetic behaviour of BIA 3-202 was evident from the finding that IC_{50} values for BIA 3-202 against rat soluble COMT were dependent on the amount of enzyme used in the assay. In fact, the IC_{50} value of 12 nM for BIA 3-202 with 0.4 mg ml^{-1} total protein in the assay increased by a factor of 10 (to 124 nM) when the amount of protein used in the assay was also increased 10-fold (to 4 mg ml^{-1}). Characteristically, the concentration of a tight-binding inhibitor required to give 50% inhibition (IC_{50}) depends on the concentration of enzyme. Therefore, IC_{50} values for tight-binding inhibitors become meaningless in the absence of information on the concentration of the enzyme used (Williams and Morrison, 1979). A reversible tight-binding inhibitor is one that exerts its effect on an enzyme-catalysed reaction at a concentration comparable to that of the enzyme. Therefore, the plot of velocity against enzyme concentration at different inhibitor concentrations (Ackermann–Potter plot) is a useful method for detecting tight-binding inhibition. The plot of the steady-state velocity against the total enzyme concentration at different BIA 3-202 concentrations was an asymptotic, concave curve and the velocity curve paralleled the control curve at sufficiently high enzyme concentrations, demonstrating the tight-binding nature of the inhibition (Cha, 1975). From these data, it is possible to determine several parameters: the catalytic number (K_{cat}), the molar equivalency (ε) of the enzyme and the apparent enzyme dissociation constant (K_i^*). The apparent K_i^* value obtained for soluble COMT was 1.5 ± 0.1 nM. The true K_i value obtained from K_i^* was 0.2 nM.

The K_i for soluble COMT is within the same range as reported before (Lotta et al., 1995) for nitecapone and entacapone with a recombinant form of COMT. Interestingly, in rat liver, K_i values of 145 and 0.7 nM have been reported for entacapone and nitecapone, respectively (Nissinen et al., 1992; Schultz and Nissinen, 1989). Because these authors used a different mathematical approach for the calculation of the K_i values, we thought it worthwhile to follow the same type of approach. Estimation of K_i^* values from the plot of IC_{50} values against enzyme concentration with a 20-min preincubation gave a value of 15.1 ± 1.1 nM for soluble COMT, which corresponded to a value for true K_i of 3.3 nM. Several authors working with tight-binding inhibitors have pointed out this type of discrepancy in K_i values. As a rule when comparing K_i values for tight-binding inhibitors, it is essential to examine the mathematical methods used for their estimation since different methods, even when applied to the same data, can yield K_i estimates differing by several orders of magnitude (William and Hakala, 1979).

Another difficulty in analysing the kinetic behaviour of a tight-binding inhibitor concerns the type of interaction with the enzyme, that is the inhibitory mechanism. The most appropriate procedure is to analyse the relationship between IC_{50} values and the concentration of the substrate. In this particular case, IC_{50} values for BIA 3-202 determined at different adrenaline and SAM concentrations showed that, in rat liver soluble COMT, the type of inhibition produced by BIA 3-202 was competitive with respect to the substrate, as revealed by the linear relationship between IC_{50} values and adrenaline concentrations. The type of inhibition produced by BIA 3-202 was uncompetitive with respect to the methyl donor SAM, as revealed by the linear relationship between IC_{50} values and the reciprocal of the SAM concentrations (Henderson, 1972). This is an expected finding, since SAM binds to the enzyme–magnesium complex prior to the catechol substrate and BIA 3-202 is structurally enough related to catechols to compete for the same binding site.

In conclusion, BIA 3-202 behaves as a reversible, potent and fast tight-binding COMT inhibitor that acts in a competitive manner at the substrate binding site and in an uncompetitive manner at the methyl donor binding site of rat liver soluble COMT.

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