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Species differences in pharmacokinetic and pharmacodynamic properties of nebicapone

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ABSTRACT

The present study was designed to characterize pharmacodynamic and pharmacokinetic properties of nebicapone in rats and mice. Upon oral administration of nebicapone the extent of mouse liver catechol-O-methyltransferase (COMT) inhibition is half that in the rat. Nebicapone was rapidly absorbed reaching plasma C_{max} within 30 min and being completely eliminated by 8 h. Nebicapone was metabolized mainly by glucuronidation and methylation in both species, but rat had an extra major metabolite, resulting from sulphation. Administration of nebicapone by the intraperitoneal route significantly increased compound AUC in the rat while in the mouse a significant increase in AUC of metabolites was observed. These results show that nebicapone exhibited marked species differences in bioavailability and metabolic profile. Evaluation of COMT activity in rat and mice liver homogenates revealed that both had similar methylation efficiencies (K_{cat} values, respectively 7.3 and 6.4 min^{-1}), but rat had twice active enzyme units as the mouse (molar equivalency respectively 150 and 83). Furthermore, nebicapone inhibited rat liver COMT with a lower K_i than mouse liver COMT (respectively 0.2 nM vs. 1.2 nM). In conclusion, the results from the present study show that mice and rats respond differently to COMT inhibition by nebicapone. The more pronounced inhibitory effects of nebicapone in the rat may be related to an enhanced oral availability and less pronounced metabolism of nebicapone in this specie, but also concerned with the predominant expression of S-COMT over MB-COMT, the latter of which is less sensitive to inhibition by nebicapone than the former.

1. Introduction

Nebicapone (1-[3,4-dihydroxy-5-nitropheny]-2-phenyl-ethanone) is a catechol-O-methyltransferase (COMT) tight-binding inhibitor [1] that is in development as an adjunct of the L-3,4-dihydroxyphenylalanine (L-DOPA) plus peripheral aromatic L-amino acid decarboxylase (AADC) inhibitor treatment of Parkinsonian patients suffering from dyskinesias and motor fluctuations [2–4]. The combination L-DOPA plus a peripheral AADC inhibitor is efficacious in the replenishment of the dopaminergic deficiency, by supplying the dopamine precursor and averting its metabolism by peripheral decarboxylation. However, the proportion of L-DOPA reaching the brain is still about 5–10% of the orally administered dose. Under these circumstances, the 3-O-methylation of L-DOPA becomes a predominant elimination route, having a significant impact in the availability of L-DOPA to the brain, indicating that inhibition of this metabolic route should prove beneficial for Parkinsonian patients [5].

COMT is the enzyme responsible for the methylation of L-DOPA. It catalyses the transference of a methyl group from S-adenosyl-L-methionine (SAM) to catechol substrates, playing an important role in the elimination of biologically active or toxic catechol-based molecules such as catecholamines, their hydroxylated metabolites, catecholestrogens and xenobiotic catechols [6]. The enzyme is ubiquitously expressed in animal tissues, existing both as a soluble (S-COMT) and a membrane-bound (MB-COMT) form. Although the soluble to membrane-bound isoform ratios differ among tissues, S-COMT usually predominates [7]. Both isoforms share a similar primary structure with the membrane-bound form having an extra peptide in its amino-terminal that contains the residues responsible for membrane anchorage [8,9]. Furthermore, both S-COMT and MB-COMT have similar active sites sharing the same kinetic mechanism, with similar affinities for SAM, magnesium depen-

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dency, inhibition by calcium and optimal pH for activity; however, MB-COMT has a significantly higher affinity for catecholamines than S-COMT [10,11].

The administration of nebicapone to humans results in rapid and sustained inhibition of erythrocyte S-COMT, that recovers baseline levels of activity at 8–18 h post-dose [4,12,13]. The pharmacokinetics of the compound reveals a rapid absorption with maximum concentration in plasma reached within the first 2 h and apparent terminal elimination half-life between 2 and 4 h. Metabolic profiling in humans [14] showed that nebicapone is extensively metabolised, mainly by glucuronidation. It is also subject to methylation and although the 3-O-methyl-nebicapone (BIA 3-270) is present in human plasma in minor amounts it has a relatively long half-life. Metabolites resulting from sulphation and N-acetylation after nitro reduction were identified in residual amounts.

Preliminary experiments suggested that nebicapone was more potent in inhibiting rat COMT than mouse COMT upon oral administration to rats and mice. The purpose of the present study was to explore possible causes for such behaviour. The pharmacokinetic profile of nebicapone was evaluated in rats and mice after oral and intraperitoneal administrations. Kinetic properties of the MB- and S-COMT and their relative abundance were also evaluated in rat and mouse liver preparations.

2. Materials and methods

2.1. Reagents

Nebicapone (1-[3,4-dihydroxy-5-nitropheny]-2-phenyl-ethanone), its metabolites nebicapone glucuronide (BIA 3-476; 1–(3-O-β-D-glucopyranuronosido-4-hydroxy-5-nitrophenyl)-2-phenyl-ethanone), nebicapone sulphate (BIA 3-465; pyridinium-1-(4-hydroxy-5-nitro-3-O-sulphatophenyl)-2-phenyl-ethanone; prepared as a pyridinium salt), 3-O-methyl-nebicapone (BIA 3-270; 1-(4-hydroxy-3-methoxy-5-nitrophenyl)-2-phenyl-ethanone) and tolcapone (used as internal standard) were synthesized in the Laboratory of Chemistry, Department of Research and Development, BIAL (S. Mamede do Coronado, Portugal), with purities >99.5%. S-adenosyl-L-methionine, DL-metanephrine hydrochloride and L-adrenaline bitartrate were purchased from Sigma–Aldrich (St Louis, MO). Acetonitrile, dibutylamine and perchloric acid were from Merck KgaA (Darmstadt, Germany) and methanol was purchased Mallinckrodt Baker (Deventer, The Netherlands). All other reagents were from Sigma–Aldrich (St Louis, MO).

2.2. Animals

Adult male Wistar rats and NMRI mice, supplied by Harlan (Barcelona, Spain) were kept 5 per cage under controlled environmental conditions (12 h light/dark cycle, room temperature 22 ± 1 °C and humidity 50 ± 5%, food and tap water ad libitum). Rats weighed 200–250 g and mice weighed 24–40 g. All animal procedures were conducted in the strict adherence to the European Directive for Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609CEE), Portuguese legislation and the rules of the "Guide for the Care and Use of Laboratory Animals" 7th edition, 1996, Institute for Laboratory Animal Research, Washington, DC. The number of animals used was the minimum possible in compliance with current regulations and scientific integrity.

2.3. Animal treatments

Animals were fasted the night before administration. Nebicapone (12 mg/kg) was either given orally (p.o.) as a suspension in carboxymethylcellulose (0.5%, w/v; 4 ml/kg) or intraperitoneally (i.p.) as a solution in dimethyl sulfoxide (DMSO, 2 ml/kg). Anaesthesia was performed by i.p. administration of sodium pentobarbital (60 mg/kg). Blank plasma was obtained from animals not subject to any treatment.

2.3.1. Evaluation of nebicapone pharmacokinetics

In the experiments designed for pharmacokinetic evaluation of nebicapone, rats and mice were administered with nebicapone (12 mg/kg), either p.o. or i.p., and blood samples were collected from anaesthetized animals at various times after dosing (0.5, 1, 2, 4, 8, 16, and 24 h). Five animals were used for each time point: blood was collected from the vena cava with heparinised syringes and kept on ice until centrifuged at 1500 × *g* for 10 min at 4 °C. Plasma was stored at less than −20 °C until analysis.

2.3.2. Evaluation of COMT inhibition

In the experiments designed to evaluate the inhibition of COMT in vivo, mice were administered nebicapone p.o. (30 mg/kg for time dependency or 0.3, 1, 3, 10, 30, 60, and 100 mg/kg for ED_{50} determination) and then, either at defined intervals (0.5, 1, 3, 6 and 9 h) for time dependency or at 1 h for ED_{50} determination, animals were anaesthetized and perfused through left ventricle with 0.9% sodium chloride. Liver fragments were removed and frozen at −80 °C in sodium phosphate buffer (5 mM, pH 7.8). Homogenized tissues (Diax homogenizer, Heidolph GmbH & Co. KG, Schwabach, Germany) were used for the COMT activity determination.

2.4. Preparation of COMT fractions

The soluble fraction of COMT was obtained as previously described [15,16]. In brief, crude homogenates prepared as described above (total-COMT) 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 (S-COMT) and the pellets were washed and centrifuged again in the same conditions. The final pellets were resuspended in sodium phosphate buffer (5 mM, pH 7.8) and used as membrane-bound COMT (MB-COMT). The protein content in the samples was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard (50–250 μg/ml).

2.5. COMT assay

COMT activity was evaluated by measuring metanephrine, the product of adrenaline methylation, as previously described [16]. In brief, reaction mixture contained homogenate (4 mg/ml), 100 μM $MgCl_2$, 1 mM EGTA, 100 μM pargyline and either 500 μM (rat) or 250 μM (mouse) S-adenosyl-L-methionine in phosphate buffer (5 mM, pH 7.8). Reactions were initiated with adrenaline and proceeded for either 5 min (rat) or 10 min (mouse) at 37 °C. Reactions were terminated with the addition of 1/10 volume 2 M perchloric acid. Samples were centrifuged (16,000 × *g* for 3 min at 4 °C) and filtered through 0.22 μm pore size Costar Spin-X 200 filters (Corning Inc., Corning, NY). Metanephrine was then quantified by HPLC with electrochemical detection as previously detailed [16].

For K_m determinations, liver homogenates (4 mg/ml) were incubated with either 0.5–1500 μM (rat) or 0.5–100 μM (mouse) adrenaline. In the experiments designed to evaluate the methylation efficiency of the enzymes and inhibitor sensitivity, rat and mice liver homogenates (0.4–2 mg/ml protein) were incubated with either 1000 μM (rat) or 50 μM (mouse) adrenaline.

2.6. Extraction and quantification of nebicapone and metabolites from plasma

Nebicapone and metabolites were quantified in plasma samples as described before [14]. In brief, plasma samples were extracted along with an internal standard (tolcapone, 400 ng/ml) by solid phase extraction using Oasis HLB cartridges (30 mg, 1 ml, Waters Corporation, Milford, MA). The eluted samples were analysed by LC-(AP-ESI) MS (HP 1100 Series, Agilent Technologies, Santa Clara, CA) with negative ion detection. Separation was performed on a Zorbax SB-C_{18}, 3 μm, 30 mm × 4.6 mm, column (Agilent Technologies) using a mobile of water:1% formic acid (v:v) and acetonitrile:1% formic acid (v:v). Selected ion monitoring (SIM) with the detection of each compound of interest was used for quantification. The method was validated in accordance with FDA guidance for industry. The limits of quantification were 61.4 ng/ml for BIA 3-476 and 40.9 ng/ml for BIA 3-465, nebicapone and 3-O-methyl-nebicapone. The intra- and inter-batch coefficient of variation (CV) and accuracy were within 15.0% of the actual value for all the analytes and in all concentrations checked.

2.7. COMT immunoblotting

SDS-PAGE and Western blot analysis was performed according to a standard procedure of the laboratory [17]. In brief, rat and mouse liver tissues were homogenized (Diax homogenizer, Heidolph GmbH & Co. KG, Schwabach, Germany) in RIPA buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard. Samples containing 30 μg of protein were separated by SDS-PAGE with 10% polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were blocked for 1 h with 5% non-fat dry milk in PBS (10 mM phosphate-buffered saline) at room temperature with constant shaking. Blots were probed overnight at 4 °C, in 5% non-fat dry milk in PBS-T with a primary rabbit anti-COMT antibody (1:2000) obtained in-house. This antibody was raised against recombinant rat S-COMT [15] and it was further purified by affinity chromatography on a rat S-COMT HiTrap column. It displays cross-reactivity towards human [18] and mouse COMT. The immunoblots were subsequently washed and incubated with a fluorescently labeled secondary antibody (1:1.000; IRDye™ 800, Rockland Immunochemicals, Gilbertsvile, PA) for 60 min at room temperature and protected from light. The membrane was washed and imaged by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and band intensities were quantified with the Odyssey v2.0 software. The relative abundance of MB- and S-COMT isoforms was then calculated as the percentage of the sum of both isoforms intensities.

2.8. Data analysis

All data analysis was performed using GraphPad Prism software, version 4.0 (GraphPad Software Inc., San Diego, CA). The pharmacokinetic parameters of nebicapone and its metabolites were calculated from plasma concentration–time profiles and statistical significance was evaluated by two-tailed Student's *t*-test. ED_{50} value was determined by fitting experimental data to the sigmoidal-dose response curve (variable slope) with top and bottom fixed to 100 and zero, respectively. K_m and V_{max} values were calculated from non-linear regression analysis using the Michaelis–Menten equation. The catalytic number (K_{cat}), molar equivalency (ε), and inhibition constant (K_i) for nebicapone were obtained from non-linear regression analysis of the experimental values fitted to the equation [19,20]:

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

where E is the total enzyme concentration; ε represents the fraction of E that is active, thus the molar equivalency; I is the total inhibitor concentration; K_{cat} is the catalytic number, and K_i^* is a constant that is related to the K_i by the equation $K_i^* = K_i(1 + S/K_m)$ being S the substrate concentration.

3. Results

3.1. Rat and mouse liver COMT inhibition by nebicapone

The in vivo inhibitory profile of nebicapone evaluated in rat and mice liver homogenates is shown in Fig. 1. The maximal inhibitory effect obtained with 30 mg/kg nebicapone in both species was observed within 0.5 h after its administration (Fig. 1A). In the mouse an almost complete recovery of enzyme activity to baseline values was found to occur at 9 h post-dose, whereas in the rat recovery in enzyme activity at this time point achieved only 25% of baseline values. At 1 h post-dose, the ED_{50} calculated from the dose

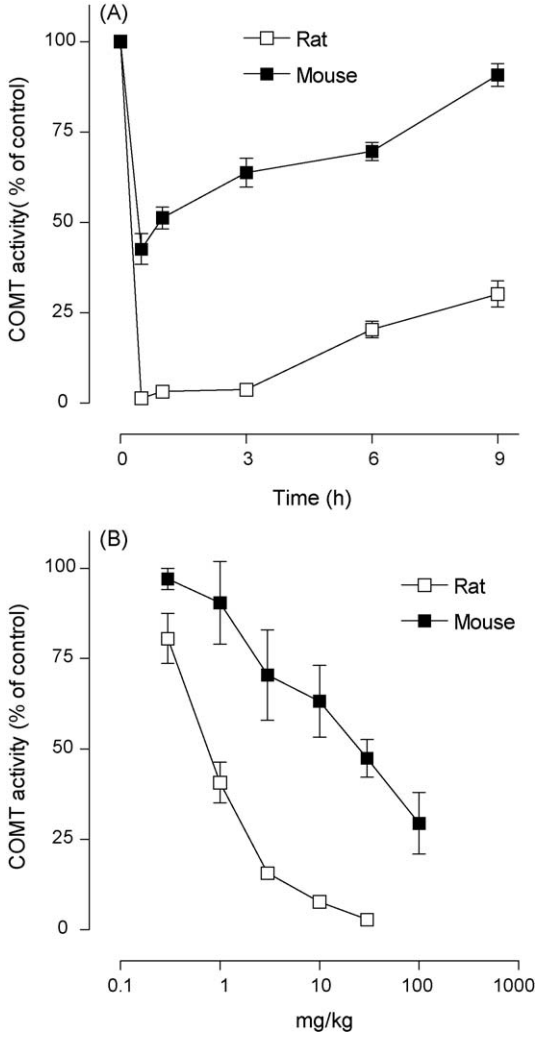

Fig. 1. Rat and mouse liver COMT inhibition by nebicapone. COMT activity was determined after (A) oral administration of nebicapone, 30 mg/kg at 0.5, 1, 3, 6 and 9 h and (B) 1 h after oral administration of nebicapone at 0.1, 0.3, 1, 3, 10, 30 and 100 mg/kg. Symbols represent means ± S.E.M. of $n = 5$ animals.

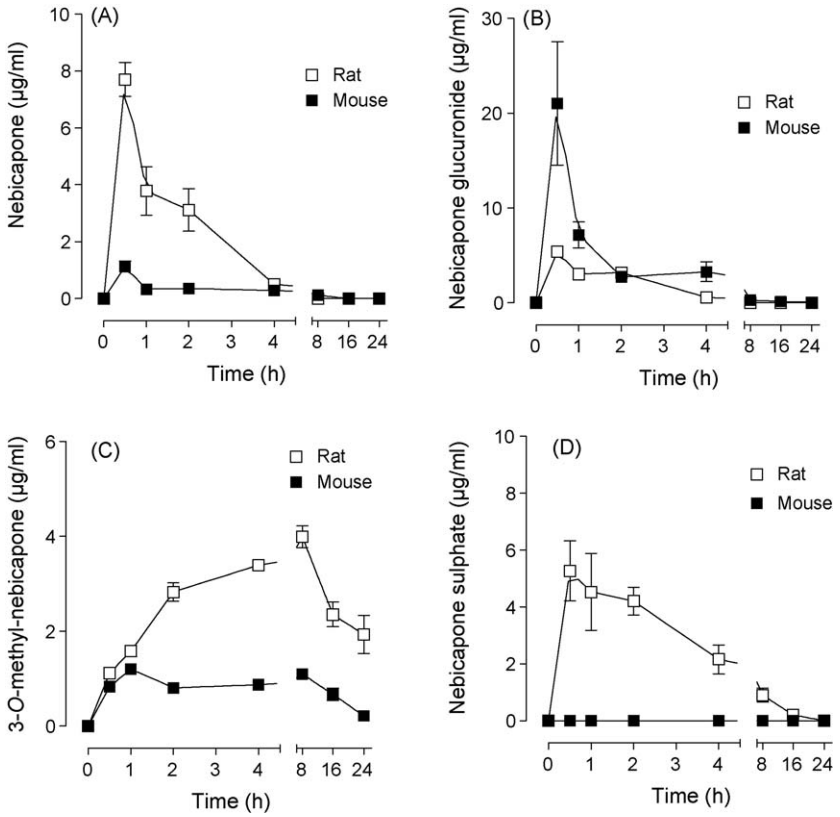

Fig. 2. Mean plasma concentration–time profile of (A) nebicapone, (B) nebicabone glucuronide, (C) 3-O-methyl-nebicapone and (D) nebicapone sulphate after oral administration of nebicapone (12 mg/kg) in the rat and mouse. Each point represents means ± S.E.M. of $n = 5$ animals.

response curves (Fig. 1B) was found to be 0.8 (0.5; 1.2) and 27.4 (23.1; 32.5) mg/kg in the rat and mouse, respectively.

3.2. Metabolism and elimination of nebicapone after oral administration

The metabolic profile and the elimination rate of nebicapone in rat and mouse plasma after oral administration of a dose of 12 mg/kg is shown in Fig. 2. The pharmacokinetic parameters derived from these curves are shown in Table 1. Maximal plasma concentration (C_{max}) values of nebicapone, following oral administration in both rat and mouse, were reached within 30 min and returned to baseline levels at 8 h. After oral administration, unchanged nebicapone represented about 11% and 6% of the total nebicapone and metabolites area under the curve (AUC_{0-24}) in rat and mouse, respectively. In the rat, the major metabolite of nebicapone was 3-O-methyl nebicapone (BIA 3-270), which accounted for 57% of the total nebicapone and metabolites AUC_{0-24}. In the mouse, by contrast, the major metabolite of nebicapone was the 3-O-β-glucuronic acid derivative (BIA 3-476)

Table 1
Pharmacokinetic parameters of nebicapone and respective metabolites (nebicapone glucuronide, 3-O-methyl-nebicapone and nebicapone sulphate) in mice and rat plasma after p.o. and i.p. administration of nebicapone (12 mg/kg).

	Rat		Mice	
	p.o.	i.p.	p.o.	i.p.
C_{max} (µg/ml)				
Nebicapone	7.7 ± 1.3	21.2 ± 4.5*	1.0 ± 0.4	3.9 ± 2.3*
Nebicapone glucuronide	5.4 ± 0.8	12.4 ± 2.9*	21.3 ± 14.2	120.6 ± 17.1*
3-O-methyl-Nebicapone	4.0 ± 0.5	4.7 ± 0.6	1.0 ± 0.2	2.4 ± 0.5*
Nebicapone sulphate	6.5 ± 2.8	11.5 ± 4.1	nd	nd
T_{max} (h)				
Nebicapone	0.5	0.5	0.5 (0.5–2)	0.5 (5–1)
Nebicapone glucuronide	0.5	0.5	0.5 (0.5–1)	0.5
3-O-methyl-Nebicapone	8.0 (4.0–8.0)	4.0	8.0 (2.0–8.0)	4.0 (4.0–8.0)
Nebicapone sulphate	1.0 (0.5–2.0)	0.5	nd	nd
AUC_{0-24} (h µg/ml)				
Nebicapone	13.1 ± 1.6	26.3 ± 9.1*	3.1 ± 0.7	3.4 ± 1.7
Nebicapone glucuronide	12.1 ± 2.2	17.1 ± 4.4	34.1 ± 11.7	141.0 ± 23.9*
3-O-methyl-Nebicapone	66.6 ± 7.3	65.1 ± 9.7	16.1 ± 3.3	27.8 ± 5.9*
Nebicapone sulphate	26.0 ± 4.3	24.4 ± 6.6	nd	nd

Values are means ± standard deviations for a n of 5. t_{max} values are median with range values in parentheses. nd: not detected.
* Significantly different from p.o. ($P < 0.05$).

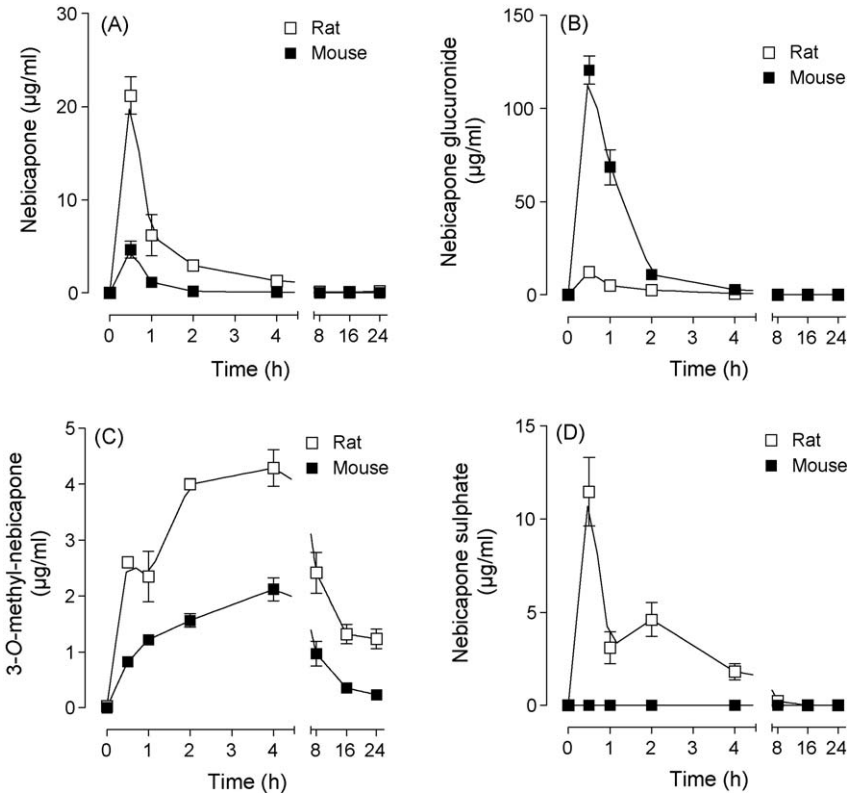

Fig. 3. Mean plasma concentration–time profile of (A) nebicapone, (B) nebicabone glucuronide, (C) 3-O-methyl-nebicapone and (D) nebicapone sulphate after intraperitoneal administration of nebicapone (12 mg/kg) in the rat and mouse. Each point represents means ± S.E.M. of $n = 5$ animals.

which accounted for 64% of the total nebicapone and metabolites AUC_{0-24}. Another difference in the metabolism of nebicapone between species concerned the formation of nebicapone sulphate (BIA 3-465), a metabolite that was not found in mice. Irrespective of differences in the pattern of nebicapone metabolism, a major finding is that total exposure to nebicapone and metabolites (using AUC_{0-24}) in the rat was twice that in the mouse (117.8 h μg/ml vs. 53.3 h μg/ml). In addition, nebicapone AUC_{0-24} values were 13.1 and 3.1 μg/ml after p.o. administration in the rat and mouse, respectively.

3.3. Metabolism and elimination of nebicapone after intraperitoneal administration

The data described above indicated marked differences in the metabolism of nebicapone between rat and mouse and suggests that oral bioavailability of nebicapone may be greater in the rat than in the mouse. In order to clarify this issue a series of experiments were performed with nebicapone being administered intraperitoneally (i.p.), to overcome differences in gastrointestinal absorption. The metabolic profile and the elimination rate of nebicapone in rat plasma after i.p. administration of 12 mg/kg nebicapone are shown in Fig. 3. The pharmacokinetic parameters derived from these curves are shown in Table 1. The plasma level vs. time profile of nebicapone and nebicapone metabolites after i.p. administration in both rats and mice was similar to that observed after oral administration of nebicapone. After i.p. administration, unchanged nebicapone represented about 20% and 2% of the total nebicapone and metabolites AUC_{0-24} in rat and mouse, respectively. Again, the major metabolite of nebicapone in the rat was 3-O-methyl nebicapone (BIA 3-270), which accounted for 49% of the total nebicapone and metabolites AUC_{0-24}. The major metabolite of nebicapone in the mouse was the 3-O-β-glucuronic acid derivative (BIA 3-476) which accounted for 82% of the total nebicapone and metabolites AUC_{0-24}. Similarly to that described in the previous section, nebicapone sulphate (BIA 3-465) after i.p. administration of nebicapone was not found detectable in mice. Contrariwise to that observed after oral administration of nebicapone, total exposure to nebicapone and metabolites (using AUC_{0-24}) after i.p. administration was found to be similar in the rat and in the mouse (132.9 h μg/ml vs. 172.2 h μg/ml). This suggests that differences in exposure observed after oral administration also relate to differences in the extent of gastrointestinal absorption of nebicapone in rat and mouse.

3.4. Kinetics of rat and mouse COMT

The kinetic behaviour of COMT in rat and mouse liver homogenates was evaluated towards a standard substrate, adrenaline. The saturation curves obtained for mouse and rat liver homogenates are shown in Fig. 4 and the kinetic parameters derived from these curves are given in Table 2. Mouse COMT has a higher affinity for the substrate adrenaline than rat COMT ($K_m = 5.0$ μM vs. 267.0 μM, respectively), but showed a lower O-methylation capacity ($V_{max} = 25.7$ nmol/mg protein/h vs. 93.8 nmol/mg protein/h, respectively).

3.5. Kinetics of COMT inhibition by nebicapone

COMT activity was measured in rat and mouse preparations homogenates (total COMT), soluble fraction (S-COMT) and membrane-bound fraction (MB-COMT) at several protein concentrations and inhibitor concentrations with a saturating concentration of substrate. The steady-state rates obtained were plotted against protein concentration (Ackermann–Potter plots) giving rise to the asymptotic concave curves represented in Fig. 5 for total COMT, which are characteristic of tight-binding inhibitors. The kinetic parameters ε, K_{cat} and K_i^* derived from non-linear regression

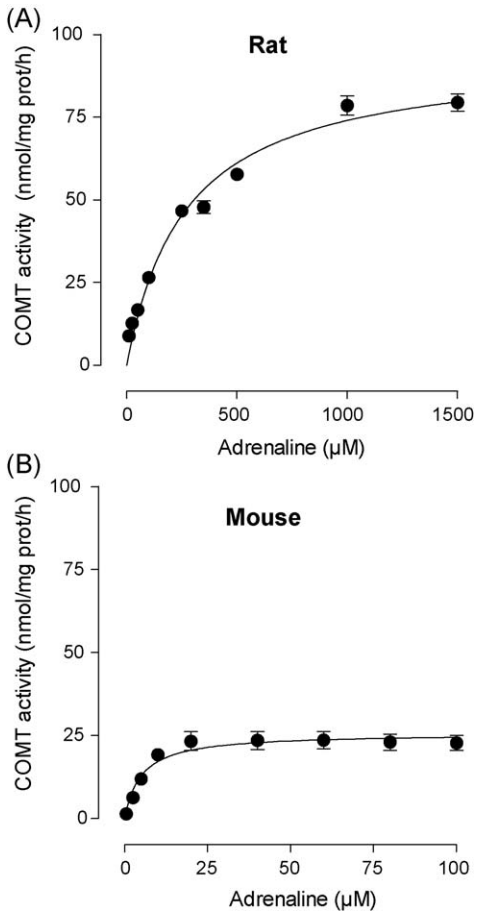

Fig. 4. Saturation curves of liver COMT. COMT activity is represented as the rate of metanephrine formation from increasing adrenaline concentrations by rat (A) and mouse (B) liver homogenates. Each point represents means ± S.E.M. of $n = 4$ animals.

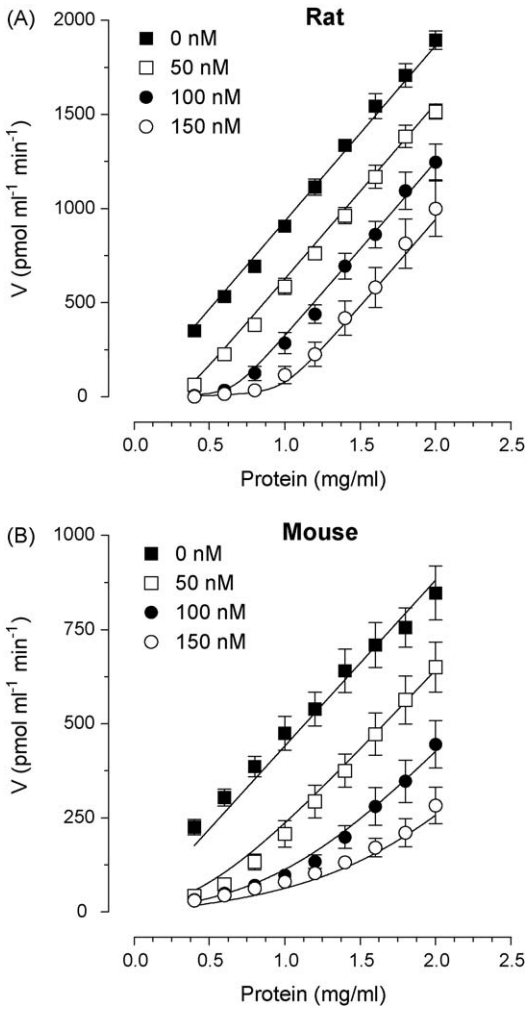

Fig. 5. Ackermann–Potter plots of rat (A) and mouse (B) liver COMT. Increasing amounts of liver homogenates were incubated with nebicapone (0–150 nM) in the presence of a saturating concentration of adrenaline (rat: 1500 μM; mouse: 50 μM). Each point represents means ± S.E.M. of $n = 4$ animals.

analysis of experimental data together with the true K_i value are shown in Table 3. Rat and mouse COMT share the same catalytic efficiency irrespectively of the preparation tested (K_{cat} between 5.6 and 8.6 min^{-1}), but the mouse total-COMT was found to be endowed with half of the active enzyme units as compared to the rat total COMT ($\varepsilon = 83$ vs. 150, respectively). In what concerns the potency of nebicapone, the inhibition constant obtained for rat S-COMT was identical to that obtained for mouse S-COMT ($K_i = 0.14$ nM for both preparations). However, the inhibition constant obtained for rat MB-COMT and total-COMT ($K_i = 0.04$ and 0.2 nM, respectively) was lower than that obtained for the mouse MB-COMT and total-COMT ($K_i = 2.55$ and 1.18 nM, respectively).

3.6. COMT expression

The relative abundance of MB- and S-COMT, evaluated by immunoblott analysis using a polyclonal rabbit anti-COMT antibody, was calculated as the percentage of total COMT in rat and mouse liver homogenates. In rat liver homogenates the abundance of S-COMT was 30-fold that of MB-COMT, while in mouse liver homogenates the abundance of S-COMT was slightly lower than MB-COMT (Fig. 6).

Table 2
Kinetic parameters for the interaction of COMT with adrenaline and with nebicapone.

	Mouse	Rat
Derived from Michaelis–Menten equation		
K_m (μM)	5.0 (3.8; 6.1)	267.0 (224.0; 311.0)
V_{max} (nmol/mg protein/h)	25.7 (24.4; 27.0)	93.8 (88.6; 98.9)

Values are means of 4 animals and were obtained by non-linear regression analysis of the equation mentioned above. 95% confidence intervals are given in parentheses.

Table 3
Kinetic parameters for the interaction of COMT with nebicapone.

	K_{cat} (min^{-1})	ε	K_i (nM)
Mouse			
Total	5.8 (4.7; 6.9)	83 (69; 98)	1.18 (0.55; 1.84)
S	8.1 (6.0; 10.3)	27 (20; 35)	0.14 (0.00; 0.31)
MB	5.6 (4.9; 6.4)	175 (153; 196)	2.55 (1.84; 3.25)
Rat			
Total	7.3 (6.7; 7.9)	150 (141; 160)	0.17 (0.00; 0.53)
S	8.6 (7.6; 9.7)	188 (171; 206)	0.14 (0.00; 0.46)
MB	6.6 (5.2; 7.9)	60 (50; 71)	0.04 (0.00; 0.09)

Values are means of 3–4 animals and were obtained by non-linear regression analysis of Eq. (1). 95% confidence intervals are given in parentheses.

4. Discussion

Evaluation of nebicapone pharmacodynamic properties, namely inhibition of peripheral COMT over time and over a range

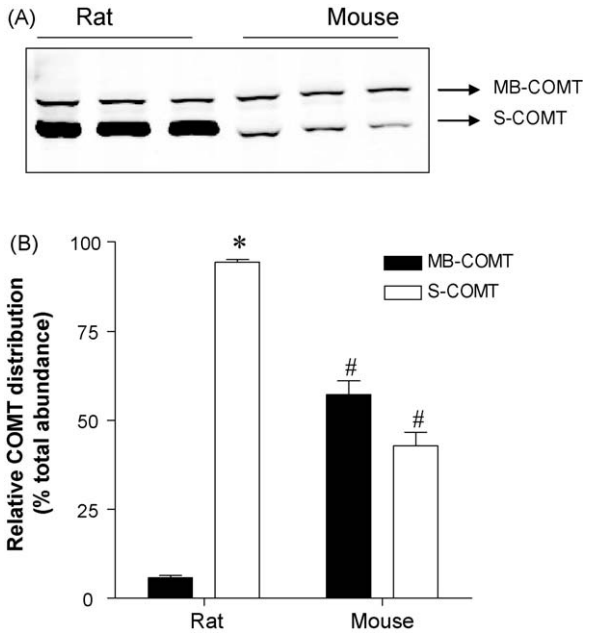

Fig. 6. Immunoblott analysis of MB- and S-COMT in rat and mouse liver homogenates. A 10% SDS-polyacrylamide gel was loaded with 30 μg total protein in each lane. Each column represents means ± S.E.M. of $n = 3$ animals. Significantly different from values for MB-COMT ($^{*}P < 0.01$). Significantly different from corresponding values in the rat ($^{\#}P < 0.01$).

of doses, revealed fundamental differences between the two species. Nebicapone markedly inhibited rat liver COMT activity at 30 min after administration and having still about 75% of the enzyme inhibited at 9 h. Under similar experimental conditions, mouse requires a 30-fold higher dose of nebicapone to achieve the same level of enzyme inhibition and the inhibitory effect was of shorter duration. While the mouse enzyme recovers to baseline values at 9 h, the rat enzyme recovered only at 24 h.

The analysis of the pharmacokinetic profile of nebicapone and elimination rate in both species contributes for the differences observed in the pharmacodynamic properties of nebicapone in rats and mice.

When comparing the metabolic pathways of nebicapone in the mouse and rat it can be concluded that the major metabolic pathway is phase II conjugation in the meta position to the nitro group of the catechol structure, which is similar to that observed in humans [14]. However, while in the mouse, similarly to what occurs in humans, the major metabolites were found to be the glucuronide and methylated derivatives, in the rat nebicapone sulphate is a major metabolite together with nebicapone glucuronide and 3-O-methyl-nebicapone. The metabolic pathways of nebicapone appear to be similar to those of other nitrocatechol inhibitors, such as entacapone and tolcapone, which primarily involve glucuronidation [21,22]. Catechols are metabolized by oxidative and conjugation reactions, with nitrocatechols being glucuronidated at lower rates than other catechols.

In addition to the different metabolic profile of nebicapone in the rat and mouse, the plasma bioavailability of nebicapone was also significantly different as evaluated after oral and i.p. administrations. A significant increase in the C_{max} of nebicapone and the corresponding glucuronide was observed in rat plasma after i.p. administration, but only the nebicapone AUC was significantly increased. In mice, on the other hand, after i.p. administration there was a significant increase in C_{max} values for nebicapone and metabolites, together with a significant increase in AUC values for the metabolites, but not for the parent compound.

In fact, glucuronidation of nebicapone in the mouse (both after p.o. and i.p. administration) accounts for 64 and 82%, respectively, of nebicapone metabolism, assuming AUC_{0-24} levels which contrasts with that in the rat, in which nebicapone glucuronide levels account for 10% and 13% of nebicapone metabolism (respectively after p.o. and i.p. administration). Mice do not sulphate nebicapone, whereas in the rat nebicapone sulphate accounts for 18–20% of nebicapone metabolism. Finally the O-methylation of nebicapone in the mouse, in opposition to nebicapone glucuronidation, accounts for approximately half of that observed in the rat.

Among phase II metabolism glucuronidation and sulphation represent the two most prevalent pathways, with UDP-glucuronosyltransferases and sulfotransferases often competing for the same substrates. The competition between the different conjugation reactions depends not only upon kinetics of the respective enzymes, subcellular localization and respective expression levels [23] but also on the available levels of co-factors [24]. Large differences between rat and mice on nebicapone glucuronidation are not unexpected, since species, strain and sex-differences of glucuronidation activities have been described for these species [25]. These differences probably result from variation in the UGT gene family such as the number of first exons and pseudogenes [26]. Differences between species concerning the metabolism of nitrocatechol derivatives have also been described for entacapone. Sulphated conjugates of entacapone and its metabolites were found in dog and rat urine, but only traces in human urine [27]. The complete lack of nebicapone sulphation in the mouse may reflect a low affinity of mouse sulfotransferase enzymes towards nebicapone.

Methylation, as a nebicapone elimination pathway, was more extensive in the rat than in mouse; the pharmacokinetics properties of the methylated derivatives, namely t_{max}, were quite different from the other phase II metabolites such as the glucuronides and sulphates. Methylated nebicapone is a long living metabolite with a late t_{max} (4–8 h in both species as compared to 0.5–1 h of glucuronides and sulphates). This most probably reflects the inhibitory nature of nebicapone towards COMT. Nebicapone, although it is a substrate for COMT it should be a poor substrate due to the electronegativity of the nitro group, similarly to what was found for other nitrocatechols [28].

The fact that in mice, but not in rat, there is an increase in the AUC of nebicapone metabolites, particularly of the glucuronide suggests that the extent of nebicapone metabolism in the mouse is greater than that in the rat. This, however, does not exclude potential differences in excretion rates in the two species.

The results reported here, may also suggest that the extent of absorption of nebicapone in the mouse may be lower than that in the rat. In fact, major differences between rat and mouse on the total exposure of nebicapone and metabolites (using AUC_{0-24}) were observed after oral administration, but not after i.p. administration of nebicapone. Oral vs. i.p. administration is not the best approach for evaluating the absorption process since it does not avoid first pass metabolism, though allows bypassing intestinal absorption. Ideally intravenous administration should be used, since it discriminates between metabolic pathways and first pass metabolism. However, this is not possible with nebicapone given its low solubility in water and DMSO toxicity limits the amount of nebicapone that can be administered.

In parallel to the pharmacokinetic studies, the kinetic properties of the interaction of mouse and rat COMT with nebicapone were studied using crude liver preparations. Nebicapone is a COMT tight-binding inhibitor [16], which means that it inhibits the enzyme at concentrations comparable to those of the enzyme; thus, the classical kinetic approaches cannot be used. Steady state rates determined at several enzyme and inhibitor concentrations can be used for the determination of the inhibition constant for this

type of inhibitors as well as for the determination of the active enzyme units in the preparation and the catalytic number of the enzyme [19,20]. The K_i value obtained for nebicapone in rat liver COMT was similar to that previously reported for rat S-COMT [19,20] and it was lower than obtained for mouse COMT (0.2 and 1.2 nM, respectively), indicating a species difference in the interaction of the inhibitor with the enzyme. Regarding the amounts of enzyme present in the rat and mouse preparations, the rat had almost twice the active enzyme units of the mouse, as shown by differences in molar equivalency values (150 and 83, respectively), which correlates with the differences in O-methylation of nebicapone in both species. The efficiency of the methylation reaction, however, was identical in both species, as revealed by identical K_{cat} values, which were similar to previously reported values for rat S-COMT [16].

The rate of O-methylation determined in this type of preparations results from a mixture of the S-COMT and MB-COMT activities, with a larger contribution by S-COMT. This isoform dominates over the MB-COMT in peripheral rat and human tissues, with the exception of adrenals and pheochromocytomas [7,29,30]. The analysis of adrenaline O-methylation kinetics in mouse and rat liver homogenates showed that the K_m value obtained for the rat COMT was in agreement with the values described in the literature for the methylation of catecholamines by S-COMT. However, the K_m value obtained for the mouse COMT was within the range of values described for MB-COMT [10,29,30]. Actually, the affinities of adrenaline O-methylation by mouse liver S-COMT and MB-COMT were found to be, respectively, 242 and 12 μM [7]. These results suggest that, in the mouse liver, MB-COMT dominates over S-COMT in opposition to what occurs in rats and humans. The western blot analysis of COMT expression in mouse and rat liver homogenates (Fig. 6) further supports this hypothesis, although differences in the affinity of the antibody used towards the rat and mouse COMT isoforms cannot be excluded. Rat S-COMT is about 30-fold more abundant than rat MB-COMT, while mouse S-COMT is about 0.8-fold mouse MB-COMT. The reason for this to occur is not apparent. In humans and rats there are two promoters that direct the synthesis of two COMT transcripts; a longer one constitutively expressed that translates MB-COMT and S-COMT by the leaky scanning mechanism of translational initiation, and a shorter one that is subject to tissue-specific transcription regulation and translates only S-COMT [31,32]. The mechanisms of transcriptional/translational regulation in the mouse have not yet been described but should be different from those described for rats and humans.

In conclusion, the results from the present study show that mice and rats respond differently to COMT inhibition by nebicapone. The more pronounced inhibitory effects of nebicapone in the rat may be related to an enhanced oral availability and less pronounced metabolism of nebicapone in this specie, but also concerned with the predominant expression of S-COMT over MB-COMT, the latter of which is less sensitive to inhibition by nebicapone than the former.

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