ELSEVIER

European Journal of Pharmacology 452 (2002) 1–10

www.elsevier.com/locate/ejphar

NMDA receptor antagonists to characterize rat renal organic cation transporter function

Jeanne Fourie [a,b], Miguel R. Escobar [a], Daniel S. Sitar [a,c,d,e,*]

[a] Department of Pharmacology and Therapeutics, University of Manitoba, A220-770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3
[b] Manitoba Institute of Cell Biology, Cancer Care Manitoba, University of Manitoba, Winnipeg, Manitoba, Canada
[c] Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada
[d] Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Manitoba, Canada
[e] Centre on Ageing, University of Manitoba, Winnipeg, Manitoba, Canada

Received 4 April 2002; received in revised form 10 July 2002; accepted 16 July 2002

Abstract

We hypothesized that uncompetitive NMDA glutamate receptor antagonists memantine (2,5-dimethyl-1-adamantanamine), and amino-alkyl-cyclohexane compounds: MRZ 2/579 (1-amino-1,3,3,5,5-pentamethylcyclohexane HCl), MRZ 2/600 (1-amino-1-ethyl-3,3,5,5-tetramethylcyclohexane HCl), and MRZ 2/615 (1-amino-1,3,5,5-tetramethyl-3-ethylcyclohexane HCl), all derivatives of amantadine (1-adamantanamine HCl), would inhibit the energy-dependent uptake of amantadine into rat renal tubules. All compounds displayed a concentration-dependent inhibition of amantadine uptake in the proximal and distal renal tubules. MRZ 2/579 showed a novel distal tubule selectivity of inhibition ($P < 0.001$). At a therapeutic amantadine concentration, bicarbonate-dependent transporter inhibition selectivity was observed with all compounds ($P < 0.05$) except MRZ 2/600, the only compound with a sterically bulky group next to the amino group of the cyclohexane ring structure. Steric hindrance around the ionized amino group of the cyclohexane ring appears to prevent bicarbonate-mediated organic cation transport. Furthermore, the distal tubule inhibition selectivity with MRZ 2/579 provides a novel tool to study the relative importance of organic cation transporters (OCTs) in proximal vs. distal renal tubules.

Keywords: Amantadine; Kidney; Memantine; MRZ 2/579; MRZ 2/600; MRZ 2/615; Organic cation transport

1. Introduction

The kidney plays an important role in drug elimination and contributes to the clearance of organic cationic drugs through glomerular filtration and tubular secretion (Rennick, 1981; Goralski et al., 1999). Organic cation renal tubule secretion is characterized by transporter-mediated movement from the peritubular capillaries across the renal tubule cell basolateral membrane and subsequent transport into the lumen of the kidney tubule. There are multiple organic cation transporters in the basolateral membrane of the renal tubule cell. Tetraethylammonium and amantadine are substrates for different organic cation transporters in the basolateral membrane, and have been utilized as probe substrates to characterize distinct renal organic cation transporters (Takano et al., 1984; Escobar and Sitar, 1995; Goralski and Sitar, 1999).

Cellular uptake of tetraethylammonium across the basolateral membrane of the proximal renal tubule is mediated by members of the organic cation transporter (OCT) family. These include rOCT1, rOCT1a, rOCT2, and rOCT3, all of which are expressed in rat kidney (Takano et al., 1984; Sokol and McKinney, 1990; Grundemann et al., 1994; Okuda et al., 1996; Zhang et al., 1997; Kekuda et al., 1998; Inui et al., 2000). Subsequent to basolateral transport, luminal transport of tetraethylammonium is facilitated by a saturable H^+/organic cation exchanger that uses a proton gradient derived from the luminal membrane Na^+/H^+ exchanger (Takano et al., 1984; Rafizadeh et al., 1987). Tetraethylammonium

* Corresponding author. Department of Pharmacology and Therapeutics, University of Manitoba, A220-770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3. Tel.: +1-204-789-3532; fax: +1-204-789-3932.
E-mail address: sitar@ms.umanitoba.ca (D.S. Sitar).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02121-0

transport studies in rat proximal and distal renal tubule segments in our laboratory identified two basolateral tetraethylammonium bicarbonate-independent organic cation transport sites, a high-affinity/low-capacity and a lower-affinity/higher-capacity tetraethylammonium transporter (Goralski and Sitar, 1999).

The organic cation drug amantadine undergoes electrogenic transport by hOCT2 using *Xenopus laevis* oocytes expressing hOCT2 (Busch et al., 1998). In addition, amantadine is used as a probe substrate in our laboratory to characterize renal tubule organic cation transport systems that are distinct from the OCT family of renal tubule transporters characterized by tetraethylammonium. These studies have shown that amantadine accumulates in proximal and distal renal tubules in rat and in human renal cortical tissue slices in an energy-dependent manner (Wong et al., 1990, 1992b). Furthermore, this process is subject to competitive inhibition by other organic cation substrates (Wong et al., 1990, 1991, 1992a,b). In humans, quinine and quinidine inhibit amantadine renal clearance exclusively in males (Gaudry et al., 1993). However, the interaction of these two inhibitors has been observed in rat renal cortical tissue from both sexes (Wong et al., 1992a). The basolateral energy-dependent amantadine transport in rat proximal and distal renal tubule segments is not yet fully characterized at a molecular level, but is known to be comprised primarily of a high affinity-capacity, bicarbonate-dependent and a lower affinity-capacity, bicarbonate-independent site(s) (Escobar et al., 1994; Escobar and Sitar, 1995; Goralski and Sitar, 1999). In contrast to electrogenic basolateral uptake of tetraethylammonium, amantadine transport appears to be mediated mainly by a non-electrogenic step at the basolateral membrane (Goralski and Sitar, 1999). Hence, amantadine basolateral transport may be subject to regulation by OCTs listed above, as well as other unidentified cation transporters. Similarly, the molecular basis of luminal transport of amantadine has not yet been characterized in full, and may occur through mechanisms similar and/or alternative to that for tetraethylammonium.

Uncompetitive *N*-methyl-D-aspartate (NMDA)-type glutamate receptor antagonists such as memantine (2,5-dimethyl-1-adamantanamine), and more recently developed aminoalkyl cyclohexane compounds: MRZ 2/579 (1-amino-1,3,3,5,5-pentamethylcyclohexane HCl), MRZ 2/600 (1-amino-1-ethyl-3,3,5,5-tetramethylcyclohexane HCl), and MRZ 2/615 (1-amino-1,3,5,5-tetramethyl-3-ethylcyclohexane HCl) are derivatives of the achiral primary aliphatic amine amantadine (1-adamantanamine HCl) (Fig. 1) (Kornhuber et al., 1991; Parsons et al., 1993, 1995, 1999; Danysz et al., 1997). These compounds are under investigation for their neuroprotective effects in the brain. Memantine is used in the treatment of Parkinson's disease, and is being evaluated for neuroprotective effects in a variety of other diseases (Parsons et al., 1995; Danysz et al., 1997), while MRZ 2/579, MRZ 2/600, and MRZ 2/615 are under investigation as putative antiepileptic agents (Parsons et al., 1999).

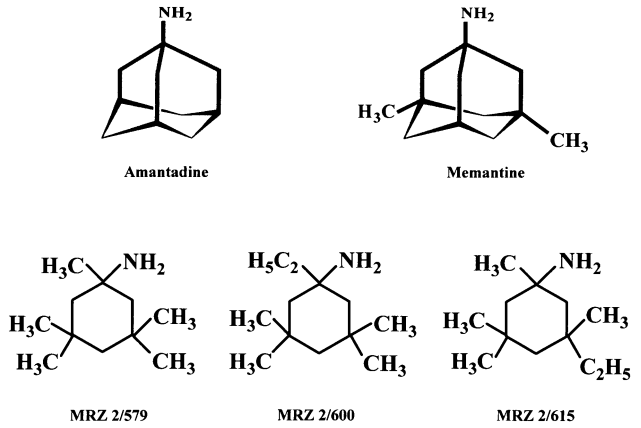

Fig. 1. Molecular structures for amantadine and its chemical derivatives, memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615.

We hypothesized that the structural similarity of these NMDA receptor antagonists to amantadine would allow them to inhibit organic cation transport through the transporters for which amantadine is a prototype substrate. The objectives of this study were twofold: (1) to evaluate the potential of memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 to be used as pharmacological tools to further characterize organic cation transporters for amantadine, and (2) to investigate the safety of these compounds from a drug–drug interaction perspective in the kidney. Hence, we determined the ability of memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 to inhibit energy-dependent amantadine uptake into rat renal tubules using an in vitro model.

2. Materials and methods

2.1. Renal tubule preparation

This study has been approved by the University of Manitoba Protocol Management and Review Committee. Proximal and distal renal tubule segments were purified based on the Percoll density-gradient centrifugation method (Vinay et al., 1981; Gesek et al., 1987), as modified and described previously by our laboratory (Wong et al.,1991, 1993). In brief, the modified protocol was as follows: Four male Sprague–Dawley rats (Charles River breeding stock; University of Manitoba, Canada) weighing 200–300 g were anaesthetized with a single dose of sodium pentobarbital (50 mg/kg, i.p.), and killed by severing the aorta. Kidneys were removed, decapsulated, and placed in ice-cold oxygenated (95% O_2; 5% CO_2) Krebs–Henseleit solution (KHS), (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 11 mM glucose. Dissection of the renal cortical tissue involved sectioning the medullary tissue approximately 1 mm from the corticomedullary boundary. Subsequently, the cortical tissue was finely minced using a tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK) and placed

in 10 ml ice-cold oxygenated KHS. The tissue–KHS mixture was then added to a KHS–collagenase solution (15 ml of KHS, 1 ml of 10% bovine serum albumin and 10 mg low-trypsin collagenase A (0.23 U/mg lysozyme) and oxygenated with 95% O_2/5% CO_2 for 2 min. The oxygenated tissue was incubated at 31 °C with shaking (100 oscillations/min) in a Dubnoff incubator (Precision Scientific, Chicago, IL, USA) for 35 min. During digestion (35 min in total), the tissue was gently pipetted in 5-min intervals with a large bore (5 ml) pipette tip at 15, 25, and 30 min into the digestion period, which aided in tissue breakup. Tissue was monitored by light microscopy (100 × magnification) of a small aliquot of the digestion mixture, starting at 30 min after the start of the incubation to insure that tissue was not over digested. Digestion was terminated by the addition of 30 ml ice-cold oxygenated KHS, and the tissue was filtered through a polyethylene mesh filter (pore size 292 μm) to remove undigested tissue. Subsequently, the tissue was washed three times by sequential resuspension in ice-cold oxygenated KHS, followed by low-speed centrifugation (4 °C, 60 × *g* for 1 min). The final pellet was resuspended in 40 ml of a 50% Percoll solution (20 ml each of Percoll and double-strength KHS at pH 7.4) and centrifuged for 30 min at 27,000 × *g* (4 °C). Bands IV and II, consisting of proximal and distal tubules, respectively, were then removed from the gradient, washed three times by sequential resuspension in ice-cold oxygenated KHS, followed by low-speed centrifugation (4 °C, 60 × *g* for 1 min).

Following the final wash, proximal and distal tubule fractions were routinely resuspended in the desired volume of oxygenated KHS. However, if the transport assays included measurements in the absence of bicarbonate, the last wash and the final resuspension of the tubule fragments would be done with Cross–Taggart (CT) buffer. CT buffer consisted of 135 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 15 mM sodium phosphate buffer (pH 7.4), 1.0 mM $CaCl_2$, and 11 mM glucose, and the pH was adjusted with NaOH. The tissue protein concentration was determined using the Biuret method (Gornall et al., 1949), and the resuspension volume was adjusted to give a final concentration of 6–8 mg/ml. The proximal and distal tubule suspensions were kept on ice and warmed to room temperature by a 20-min incubation in a 25 °C water bath just prior to the start of the transport assays. The purity of the tubule fractions was assessed by measuring the activity of enzyme markers (Guder and Ross, 1984), as well as by microscopic examination as previously described (Scholer and Edelman, 1979; Vinay et al., 1981; Wong et al., 1991).

2.2. Experimental protocol

Inhibition of [^{3}H]amantadine uptake (10 or 50 μM) by memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 (10–1000 μM) was determined in proximal and distal tubules in the presence of bicarbonate in oxygenated KHS buffer (pH 7.4), and in the absence of bicarbonate in phosphate based CT buffer (pH 7.4). Reactions were performed in triplicate in borosilicate tubes. Reaction mixtures consisted of a fixed amount of [^{3}H]amantadine (1 nM), unlabeled amantadine (final concentrations of 10 or 50 μM) and increasing concentrations of memantine, MRZ 2/579, MRZ 2/600, or MRZ 2/615 in a total volume of 150 μl of KHS or CT buffer. The proximal or distal tubule suspension (50 μl in the appropriate buffer) was added to each assay tube (final volume of 200 μl) to initiate the transport reaction. Subsequent to addition of the tubule suspension, the reaction mixture was incubated for 30 s in a 25 °C shaking water bath (100 oscillations/min). The transport assays were performed at 25 °C, as this ensures that transport occurs within the linear portion of the velocity vs. log[*S*] curve. The reactions were terminated by addition of 2 × 4 ml of ice-cold oxygenated KHS, followed by rapid filtration under negative pressure through glass fiber filters (no. 32; Schleicher and Schuell, Keene, NH, USA). The filters were immediately placed into scintillation vials containing 4 ml of Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA, USA) and counted using a Beckman model LS5801 scintillation counter.

Nonspecific uptake of [^{3}H]amantadine to the tissue and filters was determined by measuring uptake of [^{3}H]amantadine in the presence of unlabeled amantadine at a saturating amount (10 mM). The proximal or distal tubule suspension (50 μl in the appropriate buffer) was added to each assay tube (final volume of 200 μl) to initiate the transport reaction. Subsequent to addition of the tubule suspension, the reaction mixture was incubated for 30 s in a 25 °C shaking water bath (100 oscillations/min). Specific energy-dependent uptake of amantadine was then calculated by subtracting nonspecific uptake from the total radioactivity.

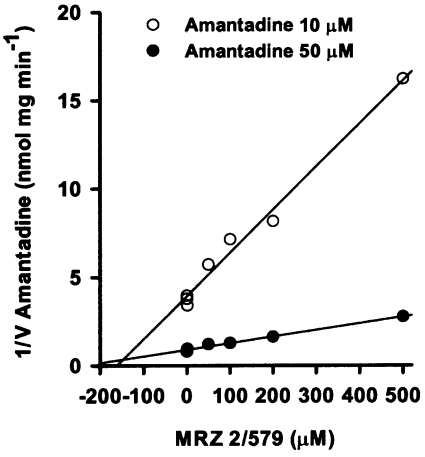

Fig. 2. Dixon plot of MRZ 2/579 inhibition of energy-dependent amantadine uptake by proximal rat renal tubule segments in the absence of bicarbonate, using amantadine concentrations of 10 and 50 μM. Data are mean ± S.E.M. of four to seven experiments.

Table 1
Calculated K_i values for memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 inhibition of energy-dependent amantadine accumulation in the proximal and distal renal tubule segments in Krebs–Henseleit solution (KHS) and Cross–Taggart (CT) buffer

Tubule	Buffer	Amantadine (μM)	Memantine K_i (μM)	MRZ 2/579 K_i (μM)	MRZ 2/600 K_i (μM)	MRZ 2/615 K_i (μM)
Proximal	KHS	10	$54 \pm 18^{bd,bf}$	$71 \pm 5^{af,cd}$	61 ± 7	35 ± 5^{cd}
Proximal	CT	10	168 ± 17^{bf}	163 ± 45^{af}	127 ± 14	110 ± 17
Distal	KHS	10	39 ± 6^{bf}	$9 \pm 3^{af,cd,ce}$	90 ± 69	26 ± 3^{cd}
Distal	CT	10	$45 \pm 16^{bf,ce}$	66 ± 14^{af}	74 ± 12	89 ± 22
Proximal	KHS	50	33 ± 5^{cd}	85 ± 17^{ad}	$56 \pm 6^{af,bd}$	37 ± 9^{cd}
Proximal	CT	50	177 ± 38	242 ± 89	154 ± 18^{af}	138 ± 13
Distal	KHS	50	38 ± 10^{cd}	50 ± 24^{ad}	72 ± 20^{af}	47 ± 1^{cd}
Distal	CT	50	99 ± 22	124 ± 44	98 ± 15^{af}	89 ± 20

K_i values were determined at therapeutic (10 μM) and toxic (50 μM) concentrations of amantadine. Values are means ± S.E.M. of four to seven experiments. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$, [d]KHS vs. CT buffer for memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 inhibition of amantadine uptake, [e]Proximal vs. distal tubule for memantine and MRZ 2/579 inhibition of amantadine uptake, [f]Tubule by buffer interaction for memantine, MRZ 2/579, and MRZ 2/600 inhibition of amantadine uptake.

2.3. Drugs

Unlabeled amantadine was obtained from Dupont Canada (Mississauga, ON, Canada). Memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 were supplied by Merz and Co. (Frankfurt am Main, Germany).

2.4. Chemicals

[^{3}H]Amantadine (350 mCi/mmol) was purchased from Amersham International (Buckinghamshire, UK). Collagenase was obtained from Boehringer Mannheim (Laval, Quebec, Canada). Percoll was obtained from Pharmacia Biotech-

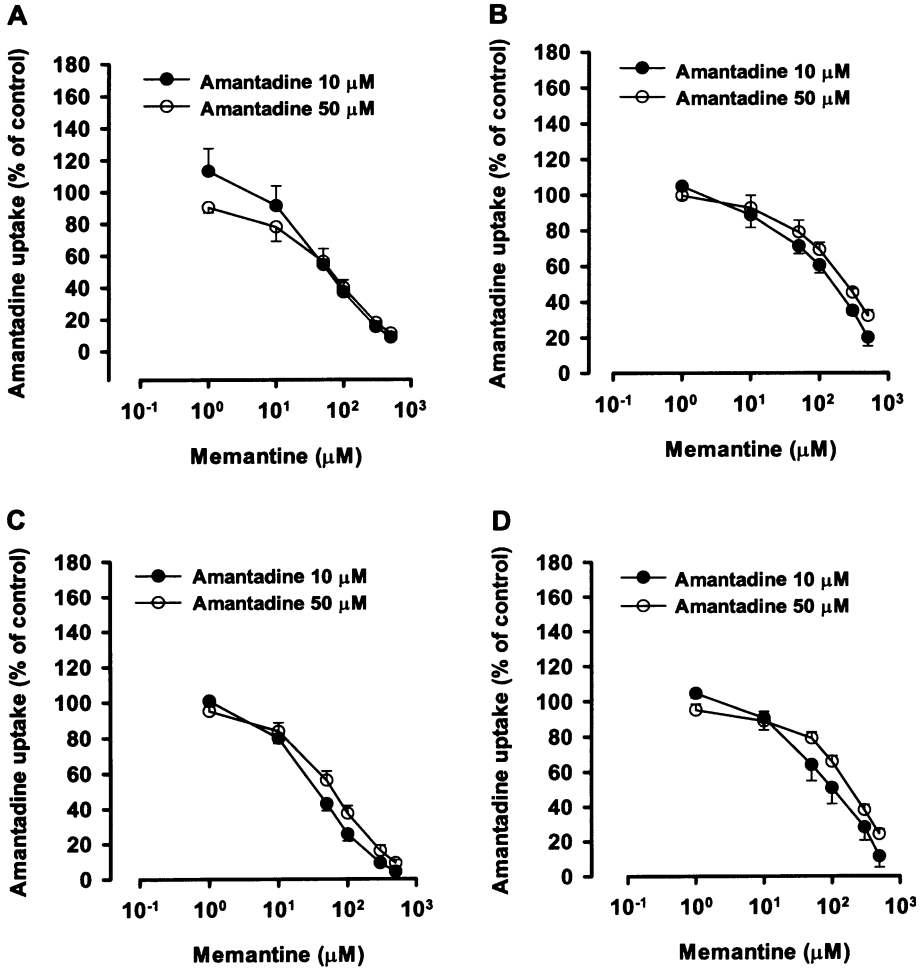

Fig. 3. Concentration-dependent inhibition of energy-dependent amantadine uptake by memantine in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to five experiments.

nology (Baie D'urfe, Quebec, Canada). All other chemicals were of the highest grade available from commercial suppliers.

2.5. Statistical analysis

The data analysis was conducted using the Statistical Package for Social Sciences (SPSS, Chicago, IL) version 10.0. A two-way analysis of variance (ANOVA) with two-way interaction was performed to test the contribution of the tubule and the buffer type to variability in inhibition constants in vitro (K_i). The two independent variables in the ANOVA were tubule (proximal vs. distal) and buffer (bicarbonate vs. phosphate), and the dependent variable was K_i value at 10 or 50 μM amantadine concentrations. When a deviation from normal distribution or lack of homogeneity of variances between each factor was found, the data were log transformed prior to ANOVA. The normal distribution of the dependent variable (K_i) and homogeneity of variance were tested using the Kolmogorov–Smirnov and the Levene tests, respectively. In cases where the Levene test suggested nonhomogeneity of variances, the results of the ANOVA were also ascertained with nonparametric tests (Kruskal–Wallis and Mann–Whitney *U*). A *P*-value of <0.05 was accepted as statistically significant in all analyses. Data points for each individual inhibition experiment are the mean of three individual determinations, using the same proximal or distal tubule preparations from four rats. Data are expressed as means ± S.E.M. of four to seven individual experiments. Transport rates are reported as specific uptake, which is determined by subtracting non-specific uptake from total uptake of amantadine by the tubules in nmol/min/mg of protein. IC_{50} values were determined from the amantadine inhibition profiles by increasing inhibitor concentrations using regressive probit analysis (Cheng and Prusoff, 1973). Dixon (1953) and Cornish-Bowden (1974) analyses were used to determine the nature of inhibition. The inhibition studies involved measuring amantadine accumulation in the KHS or CT buffers at different concentrations of amantadine (10 or 50 μM), under increasing concentrations of memantine, MRZ 2/579, MRZ 2/600, or MRZ 2/615. Representative K_i values were determined by using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

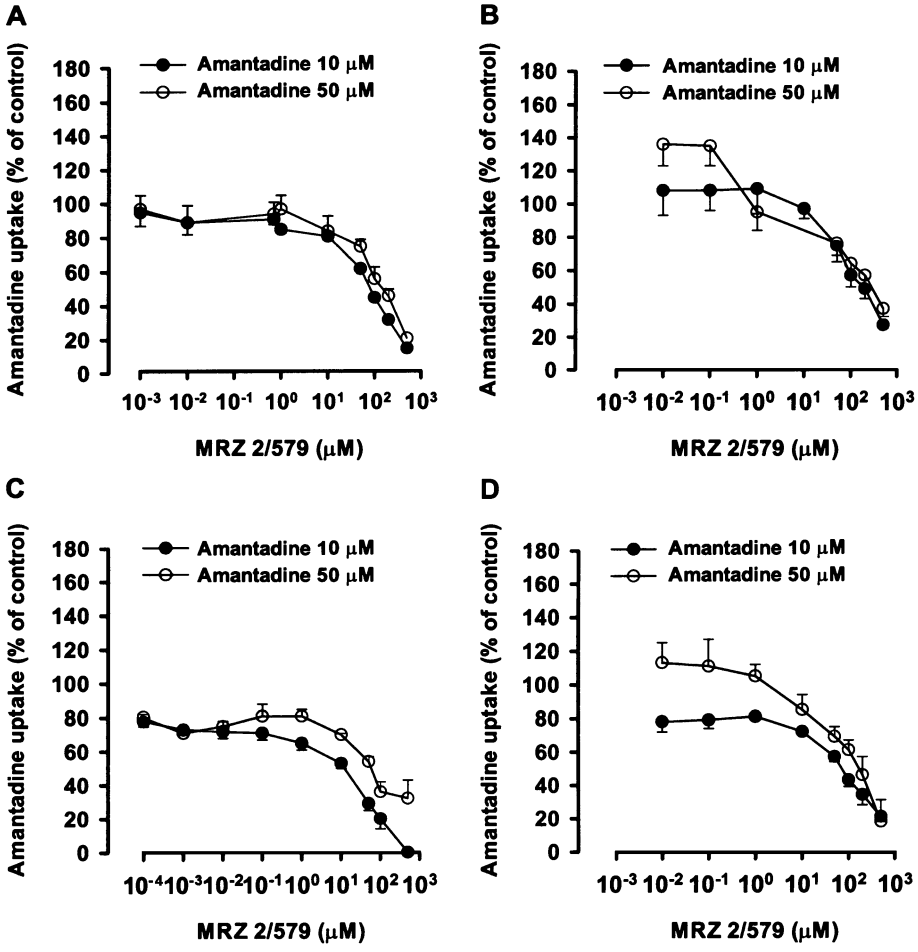

Fig. 4. Concentration-dependent inhibition of energy-dependent amantadine uptake by MRZ 2/579 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.

3. Results

3.1. Amantadine transport

In the absence of the NMDA receptor antagonists, and in the presence of bicarbonate (KHS), the rate of amantadine transport into proximal renal tubules was 0.55 ± 0.04 nmol/min/mg of protein (mean ± S.E.M.) at the therapeutic (10 μM) concentration of amantadine, and 1.89 ± 0.10 nmol/min/mg of protein at the toxic (50 μM) concentration of amantadine. In the absence of bicarbonate (CT buffer), in the proximal tubules, these values were 0.20 ± 0.01 and 0.77 ± 0.06 nmol/min/mg of protein at the therapeutic and the toxic concentrations of amantadine, respectively. The rate of amantadine transport into the distal renal tubules, in the absence of the NMDA receptor antagonists, and in the presence of bicarbonate (KHS), was 0.34 ± 0.02 nmol/min/mg of protein at the therapeutic (10 μM) concentration of amantadine, and 0.87 ± 0.09 nmol/min/mg of protein/min at the toxic (50 μM) concentration of amantadine. Similarly, in the absence of bicarbonate, (CT buffer), these values were 0.13 ± 0.01 and 0.40 ± 0.04 nmol/min/mg of protein at the therapeutic and toxic concentrations of amantadine, respectively.

3.2. Amantadine transport inhibition studies

The present in vitro study evaluated memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 with respect to their inhibitory potency on energy-dependent uptake of [^{3}H]amantadine into rat proximal and distal renal tubule segments in the presence of bicarbonate-based KHS, or phosphate-based CT buffer. Each compound displayed a concentration-dependent inhibition of amantadine uptake in both the proximal and distal renal tubule segments in the presence and absence of bicarbonate at pH 7.4 (Figs. 3–6).

Dixon and Cornish-Bowden analyses confirmed that all the NMDA receptor antagonists inhibited amantadine uptake in a competitive manner. For example, a representative Dixon plot ($1/V$ vs. i) for MRZ 2/579 in the proximal tubules in the

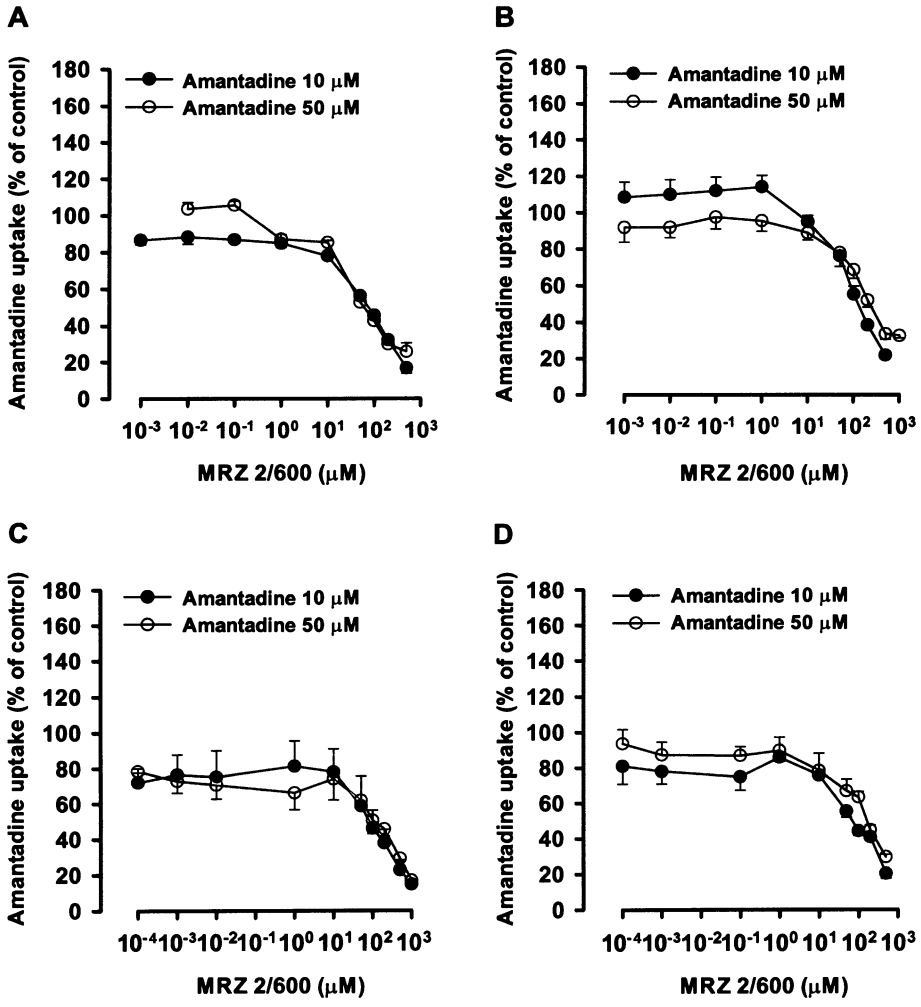

Fig. 5. Concentration-dependent inhibition of energy-dependent amantadine uptake by MRZ 2/600 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.

absence of bicarbonate is supportive of a competitive mode of inhibition of amantadine uptake (Fig. 2). Further regression analysis of the component slopes yielded an apparent K_i of 184 ± 29 μM for MRZ 2/579 in the proximal tubules in the absence of bicarbonate. These observations justified the determination of K_i values from IC_{50} values by utilizing the Cheng–Prusoff competition method (Cheng and Prusoff, 1973). The inhibitor dissociation constant (K_i) values for memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 inhibition of amantadine energy-dependent uptake at both therapeutically relevant (10 μM) and clinically toxic (50 μM) concentrations of amantadine are presented in Table 1.

For memantine, both a tubule (Table 1, $P<0.001$) and buffer (Table 1, $P<0.01$) effect were noted, as well as a tubule by buffer interaction (Table 1, $P<0.01$) at the therapeutic (10 μM) concentration of amantadine (Fig. 3). A comparison of the 95% confidence intervals (CI) showed that the inhibition by memantine in the proximal tubule was more potent in the presence of bicarbonate (KHS) (95% CI: 3–105 μM in KHS vs. 114–222 μM in CT). Furthermore, in the presence of phosphate (CT buffer), inhibition was more potent in the distal tubules (95% CI: 0–94 μM distal tubules in CT vs. 114–222 μM proximal tubules in CT). In the presence of a toxic amantadine concentration (50 μM), a clear buffer effect was demonstrated ($P<0.001$) with greater potency in the presence of bicarbonate.

For MRZ 2/579 inhibition of amantadine uptake, both a tubule and a buffer effect were observed at a therapeutic concentration of amantadine (Fig. 4, $P<0.001$), as well as a tubule by buffer interaction (Fig. 3, $P<0.05$). Further analysis using the 95% CI of the tubule effect at the therapeutic concentration of amantadine indicated that inhibition was most potent in the distal tubules, and in the presence of bicarbonate (KHS) (95% CI: 2–15 μM, distal tubules in KHS; 29–130 μM distal tubules in CT; 55–99 μM, proximal tubules in KHS; 62–344 μM, proximal tubules in CT). Notably, the K_i value for MRZ 2/579 inhibition of amantadine uptake was 9 ± 3 μM in the distal renal tubule segments in the presence of bicarbonate. (Fig. 4 and Table 1, $P<0.001$). In the presence of a clinically toxic concentration of amantadine, a buffer effect was indicated for MRZ 2/579 (Table 1, $P<0.05$), with more potent inhibition in the presence of bicarbonate.

The initial two-way ANOVA demonstrated that the pattern of energy-dependent amantadine uptake inhibition displayed by MRZ 2/600 was distinct from that of the other compounds studied. Specifically, there were no significant tubule or buffer effects observed at the therapeutic concen-

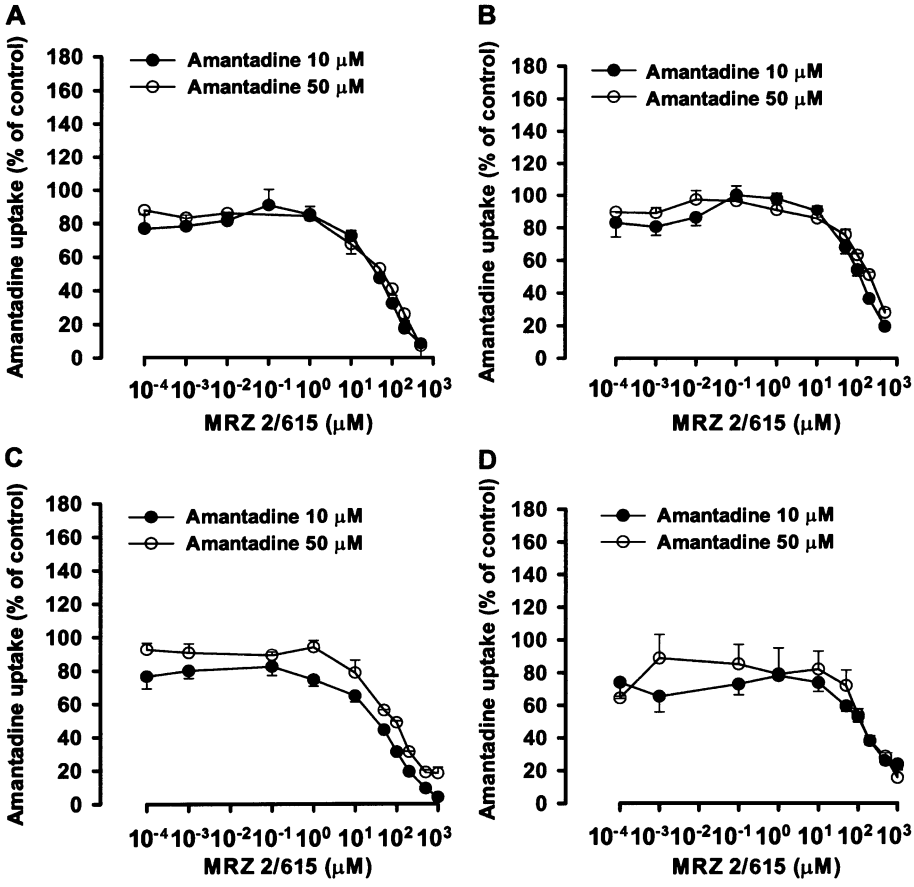

Fig. 6. Concentration-dependent inhibition of energy-dependent amantadine uptake by MRZ 2/615 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.

tration of amantadine (Fig. 5). However, at the clinically toxic concentration of amantadine, a buffer effect (Table 1, $P<0.01$) and a tubule by buffer interaction (Table 1, $P<0.05$) were found. Further analysis using the 95% CI indicated that the inhibition of amantadine uptake was more potent in the proximal tubules in the presence of bicarbonate (95% CI: 36–76 μM in KHS, vs. 97–211 μM in CT).

For MRZ 2/615 inhibition of amantadine uptake, a consistent buffer effect was demonstrated for both the therapeutic and the clinically toxic concentrations of amantadine (Fig. 6 and Table 1, $P<0.001$) with increased potency of inhibition in the presence of bicarbonate.

4. Discussion

There are several basolateral renal tubule organic cation transporter sites that mediate the clearance of organic cations. Amantadine has been used in our laboratory to identify two distinct basolateral organic cation transport sites. Specifically, amantadine energy-dependent uptake into rat proximal and distal renal tubule segments consists of a high-affinity capacity, bicarbonate-dependent transport site, and a lower affinity capacity, bicarbonate-independent transport site (Escobar et al., 1994; Escobar and Sitar, 1995; Goralski and Sitar, 1999). All four of the NMDA receptor antagonists produced concentration-dependent inhibition of amantadine uptake in rat proximal and distal renal tubules in the presence and absence of bicarbonate-based buffer. The present study was conducted at pH 7.4 and the NMDA receptor antagonists were mostly in the protonated form. The pK_a values for memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615 were 10.72, 10.72, 10.84, and 10.74, respectively, as calculated by use of the Pallas 3.0 program (CompuDrug Chemistry, Hungary). The pK_a values of these NMDA receptor antagonists are comparable amongst each other as well as to that of amantadine (10.1) (Aoki and Sitar, 1988). Thus, variability in pK_a values of the compounds is unlikely to confound the determination of the K_i values or the amount of protonated antagonist concentration.

However, a point to keep in mind is that the extent of inhibition of the renal tubule amantadine transporters in vivo depends on both the K_i values and the concentrations of the inhibitors in the kidney during treatment with typical clinical doses. Therefore, these results should be interpreted with caution in terms of the potential for drug–drug interactions in vivo, as the clinically relevant concentrations of these NMDA receptor antagonists in the protein bound and free forms at the transporter sites in humans are unknown at the present time.

Even though all four of the NMDA receptor antagonists inhibited the energy-dependent transport of amantadine, there were important differences in the inhibition profiles when specific buffer or tubule environments were considered. At a therapeutically relevant concentration of amantadine (10 μM), the potency of inhibition of amantadine uptake was most efficient on the bicarbonate-dependent organic cation transporter by memantine, MRZ 2/579, and MRZ 2/615. This may be of clinical relevance, as 80% of amantadine is transported through the bicarbonate-dependent, high-affinity capacity transporter site (Goralski and Sitar, 1999). Bicarbonate-dependent organic cation transporter selectivity of inhibition was also observed at the clinically toxic concentration of amantadine (50 μM) by memantine, MRZ 2/579, MRZ 2/600, and MRZ 2/615, but this observation may not be of clinical importance.

MRZ 2/579 displayed a novel distal tubule selectivity of inhibition at a therapeutic concentration of amantadine. In particular, the most potent inhibition of all compounds studied, was observed with MRZ 2/579 in the distal tubule segments in the presence of bicarbonate, with a K_i value of 9 ± 3 μM (mean ± S.E.M.). This unique selectivity of inhibition by MRZ 2/579 to the distal renal tubule segments has not been observed with any compound previously studied. The distal tubule selectivity of MRZ 2/579 may potentially be attributable to differences in molecular configuration of the organic cation transporters in different renal tubule segments and further molecular studies are therefore warranted. Alternatively, it is possible that MRZ 2/579 may inhibit unidentified amantadine transporters that are selectively expressed in the distal but not in the proximal renal tubule. We suggest that distal renal tubule organic cation transport may be of physiological importance as we have previously indicated that amantadine accumulation does occur in both proximal and distal renal tubule segments in rat kidney (Escobar and Sitar, 1995). Thus, MRZ 2/579 may provide us with a novel tool to assess the relative importance of proximal tubule vs. distal tubule organic cation transport systems.

MRZ 2/600 displayed a buffer effect only at a clinically toxic concentration of amantadine. Notably, most potent inhibition was observed on the bicarbonate-dependent organic cation transporter site in the proximal tubule segments. This observation may suggest the presence of multiple amantadine transporter sites, which are distinguished only by a clinically toxic concentration of amantadine.

MRZ 2/600 was unique in having similar potencies of inhibition at therapeutic concentrations of amantadine in both the proximal and distal renal tubule segments, irrespective of buffer environment. Unlike memantine, MRZ 2/579, and MRZ 2/615, this compound did not display selectivity of inhibition to the bicarbonate-dependent organic transporter sites at a therapeutic concentration of amantadine. This observation leads us to a novel hypothesis that bicarbonate-dependent organic cation transport selectivity requires specific structural characteristics from the substrate. The molecular structure of MRZ 2/600 is distinct from the other three NMDA receptor antagonists, in that this compound has a sterically bulky ethyl group close to the ionized amino group. We suggest that this ethyl group may produce steric hindrance around the ionized amino group, which may prevent bicarbonate-dependent organic cation transport selectivity. Due to the bulky ethyl group, MRZ 2/600 may not be a good

substrate for the bicarbonate-dependent organic cation transporter site. Therefore, this steric hindrance may explain the observation that MRZ 2/600 does not exhibit bicarbonate-dependent organic cation transporter selectivity of inhibition. Data from previously published studies from our laboratory are consistent with this observation. Specifically, inhibition studies in rat proximal and distal renal tubule segments indicated that tetraethylammonium did not inhibit amantadine transport through its bicarbonate-dependent transport site, suggesting that tetraethylammonium does not undergo transport through the bicarbonate-dependent organic cation transporter characterized by amantadine (Goralski and Sitar, 1999). Tetraethylammonium has four ethyl groups around its nitrogen, and these may produce a large amount of steric hindrance, preventing transport through the bicarbonate-dependent organic cation transport site. This interpretation is consistent with our hypothesis that bicarbonate-dependent organic cation transport selectivity is not affected by steric hindrance around the ionized amino group of the cyclohexane ring structures in memantine, MRZ 2/579, and MRZ 2/615 (Fig. 1).

In summary, these data demonstrate that all of the NMDA receptor antagonists studied displayed a concentration-dependent inhibition of amantadine uptake in both the proximal and distal renal tubule segments in the presence and absence of bicarbonate. At a therapeutically relevant concentration of amantadine, the potency of inhibition of amantadine uptake was most efficient on the bicarbonate-dependent organic cation transporter by memantine, MRZ 2/579, and MRZ 2/615, which may have clinical implications. MRZ 2/579 displayed a novel distal tubule selectivity of inhibition. This unique selectivity by MRZ 2/579 may provide us with a new tool to assess the relative importance of proximal vs. distal tubule organic cation transport mechanisms in future studies. At a therapeutic concentration of amantadine, MRZ 2/600 was unique in having similar potencies of inhibition in both proximal and distal renal tubule segments, irrespective of buffer environment. This observation leads us to a new hypothesis, which suggests that steric hindrance around the ionized amino group of the cyclohexane ring structure appears to prevent bicarbonate-dependent organic cation selectivity of inhibition. Further structure–activity studies are warranted to evaluate the initial observations in support of this hypothesis.

Acknowledgements

This study was funded by Merz and Co. We thank Mr. Harold Taylor of Merz and Co. for the calculation of pK_a values of the NMDA receptor antagonists used in our experiments. This work was presented at the Joint Meeting of the Australian, British, Canadian and Western Pharmacological Societies, Vancouver, 2001. J.F. is funded by a Canadian Institutes of Health Research Studentship.

References

Aoki, F.Y., Sitar, D.S., 1988. Clinical pharmacokinetics of amantadine hydrochloride. Clin. Pharmacokinet. 14, 35–51.

Busch, A.E., Karbach, U., Miska, D., Gorboulev, V., Akhoundova, A., Volk, C., Arndt, P., Ulzheimer, J.C., Sonders, M.S., Boumann, C., Waldegger, S., Lang, F., Koepsell, H., 1998. Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine and memantine. Mol. Pharmacol. 54, 342–352.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor, which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Cornish-Bowden, A., 1974. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem. J. 137, 143–144.

Danysz, W., Parsons, C.G., Kornhuber, J., Schmidt, W.J., Quack, G., 1997. Aminoadamantanes as NMDA receptor antagonists and antiparkinsonian agents—preclinical studies. Neurosci. Biobehav. Rev. 21, 455–468.

Dixon, M., 1953. The determination of enzyme inhibitor constants. Biochem. J. 55, 170–171.

Escobar, M.R., Sitar, D.S., 1995. Site-selective effect of bicarbonate on amantadine renal transport: quinine-sensitive in proximal vs. quinidine-sensitive sites in distal tubules. J. Pharmacol. Exp. Ther. 273, 72–79.

Escobar, M.R., Wong, L.T., Sitar, D.S., 1994. Bicarbonate-dependent amantadine transport by rat renal cortical proximal and distal tubules. J. Pharmacol. Exp. Ther. 270, 979–986.

Gaudry, S.E., Sitar, D.S., Smyth, D.D., McKenzie, J.K., Aoki, F.Y., 1993. Gender and age as factors in the inhibition of renal clearance of amantadine by quinine and quinidine. Clin. Pharmacol. Ther. 54, 23–27.

Gesek, F.A., Wolff, D.W., Strandhoy, J.W., 1987. Improved separation method for rat proximal and distal renal tubules. Am. J. Physiol. 253, F358–F365.

Goralski, K.B., Sitar, D.S., 1999. Tetraethylammonium and amantadine identify distinct organic cation transporters in rat renal cortical proximal and distal tubules. J. Pharmacol. Exp. Ther. 290, 295–302.

Goralski, K.B., Smyth, D.D., Sitar, D.S., 1999. In vivo analysis of amantadine renal clearance in the uninephrectomized rat: functional significance of in vitro bicarbonate-dependent amantadine renal tubule transport. J. Pharmacol. Exp. Ther. 290, 496–504.

Gornall, A.G., Bardawill, C.J., Donid, M.M., 1949. Determination of serum protein by means of the Biuret reaction. J. Biol. Chem. 177, 751–766.

Grundemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M., Koepsell, H., 1994. Drug excretion mediated by a new prototype of polyspecific transporter. Nature 372, 549–552.

Guder, W.G., Ross, B.D., 1984. Enzyme distribution along the nephron. Kidney Int. 26, 101–111.

Inui, K.I., Masuda, S., Saito, H., 2000. Cellular and molecular aspects of drug transport in the kidney. Kidney Int. 58, 944–958.

Kekuda, R., Prasad, P.D., Wu, X., Wang, H., Fei, Y.J., Leibach, F.H., Ganapathy, V., 1998. Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. J. Biol. Chem. 273, 15971–15979.

Kornhuber, J., Bormann, J., Hubers, M., Rusche, K., Riederer, P., 1991. Effects of the 1-amino-adamantanes at the MK-801-binding site of the NMDA-receptor-gated ion channel: a human postmortem brain study. Eur. J. Pharmacol. 206, 297–300.

Okuda, M., Saito, H., Urakami, Y., Takano, M., Inui, K., 1996. cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. Biochem. Biophys. Res. Commun. 224, 500–507.

Parsons, C.G., Gruner, R., Rozental, J., Millar, J., Lodge, D., 1993. Patch clamp studies on the kinetics and selectivity of *N*-methyl-D-aspartate receptor antagonism by memantine (1-amino-3,5-dimethyladamantan). Neuropharmacology 32, 1337–1350.

Parsons, C.G., Quack, G., Bresink, I., Baran, L., Przegalinski, E., Kostowski, W., Krzascik, P., Hartmann, S., Danysz, W., 1995. Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists in vitro with anticonvulsive and motor impairment activity in vivo. Neuropharmacology 34, 1239–1258.

Parsons, C.G., Danysz, W., Bartmann, A., Spielmanns, P., Frankiewicz, T., Hesselink, M., Eilbacher, B., Quack, G., 1999. Amino-alkyl-cyclohexanes are novel uncompetitive NMDA receptor antagonists with strong voltage-dependency and fast blocking kinetics: in vitro and in vivo characterization. Neuropharmacology 38, 85–108.

Rafizadeh, C., Roch-Ramel, F., Schali, C., 1987. Tetraethylammonium transport in renal brush border membrane vesicles of the rabbit. J. Pharmacol. Exp. Ther. 240, 308–313.

Rennick, B.R., 1981. Renal tubule transport of organic cations. Am. J. Physiol. 240, F83–F89.

Scholer, D.W., Edelman, I.S., 1979. Isolation of rat kidney cortical tubules enriched in proximal and distal segments. Am. J. Physiol. 237, F350–F359.

Sokol, P.P., McKinney, T.D., 1990. Mechanism of organic cation transport in rabbit renal basolateral membrane vesicles. Am. J. Physiol. 258, F1599–F1607.

Takano, M., Inui, K., Okano, T., Saito, H., Hori, R., 1984. Carrier-mediated transport systems of tetraethylammonium in rat renal brush-border and basolateral membrane vesicles. Biochim. Biophys. Acta 773, 113–124.

Vinay, P., Gougoux, A., Lemieux, G., 1981. Isolation of a pure suspension of rat proximal tubules. Am. J. Physiol. 241, F403–F411.

Wong, L.T.Y., Smyth, D.D., Sitar, D.S., 1990. Stereoselective inhibition of amantadine accumulation by quinine and quinidine in rat renal proximal tubules and cortical slices. J. Pharmacol. Exp. Ther. 255, 271–275.

Wong, L.T.Y., Smyth, D.D., Sitar, D.S., 1991. Differential effects of histamine H_2 receptor antagonists on amantadine uptake in the rat renal cortical slice, isolated proximal tubule and distal tubule. J. Pharmacol. Exp. Ther. 258, 320–324.

Wong, L.T.Y., Smyth, D.D., Sitar, D.S., 1992a. Stereoselective inhibition of renal organic cation transport in human kidney. Br. J. Clin. Pharmacol. 34, 438–440.

Wong, L.T.Y., Smyth, D.D., Sitar, D.S., 1992b. Interference with renal organic cation transport by (−)- and (+)-nicotine at concentrations documented in plasma of habitual tobacco smokers. J. Pharmacol. Exp. Ther. 261, 21–25.

Wong, L.T.Y., Escobar, M.R., Smyth, D.D., Sitar, D.S., 1993. Gender-associated differences in rat renal tubular amantadine transport and absence of stereoselective transport inhibition by quinine and quinidine in distal tubules. J. Pharmacol. Exp. Ther. 267, 1440–1444.

Zhang, L., Dresser, M.J., Chun, J.K., Babbitt, P.C., Giacomini, K.M., 1997. Cloning and functional characterization of a rat renal organic cation transporter isoform (rOCT1A). J. Biol. Chem. 272, 16548–16554.