

IN VITRO AND IN VIVO EVALUATION OF THE METABOLISM AND BIOAVAILABILITY OF ESTER PRODRUGS OF MGS0039 (3-(3,4-DICHLOROBENZYLOXY)-2-AMINO-6-FLUOROBICYCLO[3.1.0]HEXANE-2,6-DICARBOXYLIC ACID), A POTENT METABOTROPIC GLUTAMATE RECEPTOR ANTAGONIST

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ABSTRACT:

MGS0039 (3-(3,4-dichlorobenzoyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid) has been identified as a potent and selective antagonist for metabotropic glutamate receptors. However, the oral bioavailability of MGS0039 is 10.9% in rats, due to low absorption. Several prodrugs, synthesized to improve absorption, exhibited 40 to 70% bioavailability in rats. This study investigated in vitro metabolism using liver S9 fractions from both cynomolgus monkeys and humans and oral bioavailability in cynomolgus monkeys to select the prodrug most likely to exhibit optimal pharmacokinetic profiles in humans. In monkeys, transformation to active substance was observed (5.9–72.8%) in liver S9 fractions, and *n*-butyl, *n*-pentyl, 3-methylbutyl, and 4-methylpentyl ester prodrugs exhibited high transformation ratios (>64%). C_{\max} levels and *F* values after oral dosing increased to 4.1- to 6.3-fold and 2.4- to 6.3-fold, respectively, and a close relationship between

transformation ratios and C_{\max} and *F* values was observed, indicating that the hydrolysis rate in liver S9 fractions is the key factor in determining oral bioavailability in monkeys. In humans, *n*-hexyl, *n*-heptyl, *n*-octyl, 5-methylbutyl, and 6-methylpentyl ester prodrugs exhibited high transformation ratios (>65%) in liver S9 fractions. With these prodrugs, *n*-hexyl, *n*-heptyl, and 5-methylpentyl ester, almost complete recovery (96–99%) was obtained. Given the transformation ratio, we anticipated that the *n*-heptyl alkyl ester prodrug would exhibit the highest oral bioavailability of active substances in humans, if the hydrolysis rate in liver S9 fractions is indeed the key factor in determining oral bioavailability in humans. On this basis, MGS0210 (3-(3,4-dichlorobenzoyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid *n*-heptyl ester) seems to be a promising candidate among MGS0039 prodrugs.

Glutamate, a major excitatory neurotransmitter in the brain, is involved in several physiological and pathological conditions (Parsons et al., 1998). Glutamate receptors are classified into one of two major types: ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs). However, mGluRs are classified into eight subtypes and organized into three, based on sequence homology, signal transduction mechanisms, and pharmacological properties (Pin and Duvoisin, 1992; Schoepp and Conn, 1993; Conn and Pin, 1997).

Studies involving animal models and clinical trials have shown that group II mGluR agonists may be effective in treating certain psychiatric disorders such as schizophrenia (Moghaddam and Adams, 1998; Cartmell et al., 2000; Nakazato et al., 2000), anxiety disorders (Monn et al., 1997; Helton et al., 1998; Tizzano et al., 2002; Grillon et al., 2003), and panic disorder (Levine et al., 2002). Conversely, little is

known of the therapeutic significance of group II mGluR antagonists, which may stem from the lack of potent and selective antagonists.

MGS0039 (3-(3,4-dichlorobenzoyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid) is a potent and selective antagonist for group II mGluRs as determined by attenuation of glutamate-induced inhibition of forskolin-evoked cAMP formation in Chinese hamster ovary cells expressing mGluR2 ($IC_{50} = 20$ nM) or mGluR3 ($IC_{50} = 24$ nM) (Chaki et al., 2004; Nakazato et al., 2004). We previously reported that group II mGluR antagonists exhibited antidepressant potential in experimental animal models such as the rat forced swimming and mouse tail suspension tests (Chaki et al., 2004). Moreover, we recently reported on the synthesis, in vitro pharmacological profiles, structure-activity relationships, and pharmacokinetics profiles of group II mGluR antagonists (Nakazato et al., 2004). After oral dosing in rats, MGS0039 exhibited higher plasma and brain concentrations than did (2*S*)-amino-2-((1*S*,2*S*)-2-carboxycycloprop-1-yl)-3-(9-xanthyl)propionic acid (LY341495) (Ornstein et al., 1998a,b), whereas oral bioavailability was 10.9% (Levine et al., 2002). This low bioavailability may be due to low absorption, which

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; MGS0039, 3-(3,4-dichlorobenzoyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid; MGS0210, 3-(3,4-dichlorobenzoyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid *n*-heptyl ester; ESI, electrospray ionization; LY341495, (2*S*)-amino-2-((1*S*,2*S*)-2-carboxycycloprop-1-yl)-3-(9-xanthyl)propionic acid; LC-MS/MS liquid chromatography-tandem mass spectrometry; TM, transport medium; P-gp, P-glycoprotein; AUC_{inf}, area under the curve of plasma concentration-time profile; *F*, oral bioavailability.

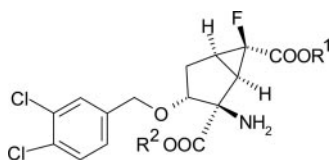


FIG. 1. Chemical structure of MGS0039 and its prodrugs. R¹ and R² indicate the modified positions for prodrugs (see Table 1).

may be attributable to a structural feature consisting of two carboxyl groups and an amino group. In the previous study (A. Yasuhara, M. Nakamura, K. Sakagami, T. Shimazaki, R. Yoshikawa, S. Chaki, H. Ohta, and A. Nakazato, manuscript submitted for publication), we investigated the lipophilic prodrugs of MGS0039 to improve oral bioavailability. Based on *in vitro* metabolism and pharmacokinetic studies in rats, we found the following. 1) Only the ester prodrugs, which were a modified carboxyl group on the C-6 position of the bicyclo[3.1.0]hexane ring exhibited enzymatic transformation to an active substance in rat liver S9 fractions and plasma. 2) Alkyl ester, cyclohexyl ester, and cyclohexylmethyl ester prodrugs exhibited near-complete enzymatic transformation to active substances, without non-enzymatic degradation. However, significant nonenzymatic degradation was observed in alkoxy-carbonyl-ethyl ester prodrugs. 3) After oral dosing of these prodrugs to rats, improvement in oral bioavailabilities (40–70%) was confirmed.

Ester prodrugs are most commonly used to improve oral bioavailability (Beaumont et al., 2003), and hydrolyzed esterase is present in the intestinal mucosa (Inoue et al., 1979), liver (Morikawa et al., 1976; Luttrell and Castle, 1988) and plasma/blood (Quon et al., 1988). Esterase activities are known to show species differences (Satoh et al., 2002; Buchwald and Bodor, 2002). The usefulness of monkeys in the development of prodrugs has been demonstrated in recent years (Cook et al., 1995; Prueksaritanont et al., 1996a; Prueksaritanont et al., 1996b). In the present study, we investigated *in vitro* metabolism using liver S9 fractions from both cynomolgus monkeys and humans and oral bioavailability in cynomolgus monkeys to select the prodrug most likely to exhibit optimal pharmacokinetic profiles in humans.

Materials and Methods

Materials. MGS0039, its prodrugs (Fig. 1), and MG0037 (3-(3,4-difluorobenzyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid) (internal standard) were synthesized in Taisho Research Laboratories as described previously (Nakazato et al., 2004). NADP⁺ and glucose 6-phosphate were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals were commercially available and of analytical grade, except acetonitrile and methanol, which were of high performance liquid chromatography grade. Caco-2 cells and LLC-GA5-CoL300 cells were obtained from The American Type Culture Collection (Manassas, VA) and from the RIKEN Cell Bank (Tsukuba, Japan), respectively.

Animals. Male cynomolgus monkeys weighing 3 to 7 kg were fasted for 17 h before dosing and 8 h thereafter. The animals were given access *ad libitum* to water and a standard laboratory diet (AS; Oriental Yeast Co., Ltd) during acclimation. Constant environmental conditions were maintained during breeding (relative humidity 55%, temperature 23°C).

All animal studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee to ensure compliance with the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments (1987).

In Vitro Study. Liver S9 fractions (1 mg protein/ml) from humans (BD Gentest, Woburn, MA) and cynomolgus monkeys (XenoTech LLC, Kansas City, KS and Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) were incubated with 3 μ M MGS0039 prodrugs in the presence of an NADPH-generating system (125 μ g/ml NADP⁺, 2.5 mM MgCl₂, 1.92 mM glucose 6-phosphate) in a 0.255 M phosphate buffer containing 0.575% (w/v) KCl (pH 7.4) for 1 h at 37°C. All experiments were performed in triplicate. After incubation, a

2-fold volume of dimethyl sulfoxide was added to the incubation medium. The tube was then vortexed and centrifuged at 3000 rpm, 4°C for 10 min. The resulting supernatant was analyzed by an LC-MS/MS system. Human and monkey plasma and human blood were spiked with MGS0039 prodrugs at concentrations of 1000 ng/ml (approximately 2.3 μ M) and then incubated for 2 h at 37°C. After incubation, 200 μ l of internal standard working solution (250 ng/ml) with methanol or acetonitrile was added to a 50- μ l aliquot of plasma sample, and the tube was vortexed and centrifuged at 11,200g (4°C) for 10 min. The resulting supernatant was analyzed by the LC-MS/MS system.

Caco-2 Study. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, and a 1% antibiotic-antimycotic mixture (10,000 U/ml penicillin G, 10,000 μ g/ml streptomycin sulfate, and 25 μ g/ml amphotericin B in 0.85% saline) at 37°C in culture flasks in a humidified 5% CO₂ atmosphere. Caco-2 cells (passage number 50–60) were seeded on 24-well cell culture inserts (Transwell; 0.3- μ m pores, 6.5-mm i.d.) at a density of 60,000 cells/cm². The culture medium (0.1 ml in the insert and 0.6 ml in the well) was replaced the day after seeding and every other day thereafter. The cell monolayers were used 21 days postseeding. The integrity of the cell monolayers was evaluated by measuring transepithelial electric resistance and the ¹⁴C-mannitol permeability of the monolayers before the transport experiment. Hanks' balanced salt solution was used as the transport medium (TM) in all experiments after adjusting the pH to 6.5 for the apical side and 7.4 for the basolateral side. Stock solutions were diluted in TM (pH 6.5) to obtain dosing solutions at final concentrations of 10 μ M. After incubation for 20 min of both sides of the monolayers with drug-free TM, the test solution was added to the apical side of the monolayer. At the time of initiation of transport (0 min), dosing solutions were sampled to determine initial concentrations. Samples (50 μ l) were taken from the basolateral side at 30, 60, 90, and 120 min after dose administration. Appropriate volumes of TM were added to the acceptor compartments. All experiments were performed in triplicate at 37°C. Concentrations of compounds were determined by LC-MS/MS.

P-gp Study. Interactions with P-glycoprotein (P-gp) were examined by the method of Tanigawara et al. (1992) and Ueda et al. (1992). LLC-GA5-CoL300 cells, a human MDR1-transfected porcine kidney epithelial cell line (LLC-PK1), were grown in Medium 199 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution (10,000 U/ml penicillin G and 10,000 μ g/ml streptomycin sulfate in 0.85% saline), and 300 ng/ml colchicine at 37°C in culture dishes in a humidified 5% CO₂ atmosphere. LLC-GA5-CoL300 cells were seeded on 12-well cell culture inserts (Transwell; 0.3- μ m pores, 12-mm i.d.) at a density of 500,000 cells/cm². The cell monolayers were used 3 days postseeding. The culture medium (0.5 ml in the insert and 1.5 ml in the well) was replaced by fresh culture medium without colchicine 6 h before the experiment. The integrity of the cell monolayers was evaluated by measuring the transepithelial electric resistance and the ¹⁴C-mannitol permeability of the monolayers before the transport experiment. Hanks' balanced salt solution (pH 7.4) was used as the TM in all experiments. Stock solutions were diluted in TM to obtain dosing solutions at final concentrations of 10 μ M. After incubation of both sides of the monolayers with drug-free TM for 20 min, the test solution was added to the donor side of the monolayer. The apical to basal and basal to apical transport of the test compounds was then measured. At the time of initiation of transport (0 min), dosing solutions were sampled to determine initial concentrations. Samples (50 μ l) were taken from the acceptor side at 30, 60, 90, and 120 min after dose administration. Appropriate volumes of TM were added to the acceptor compartments. All experiments were performed in triplicate at 37°C. Concentrations of compounds were determined by LC-MS/MS.

In Vivo Study. MGS0039 (1/15 M phosphate buffer, pH 7.4 isotonic with NaCl) was administered intravenously at a dose of 1 mg/kg (0.2 ml/kg) and orally at a dose of 10 mg/kg (2 ml/kg). The prodrugs of MGS0039 were administered at a dose of 10 mg/kg (2 ml/kg) in 10% to 20% hydroxypropyl- β -cyclodextrin solution slightly acidified with 0.03 N hydrochloric acid.

Blood samples of 1.5 ml were collected from the cephalic vein at 7 and 30 min, or 1, 2, 3, 8, and 24 h after intravenous dosing, and at 0.5, 1, 3, 8, and 24 h after oral dosing. The plasma was separated by centrifugation (3000 rpm, 4°C, 10 min) and kept at –80°C until analysis.

Sample Analysis. Samples obtained from the *in vitro* study were analyzed by liquid chromatography with mass detection on an Agilent ZORBAX SB-

TABLE 1

Transformation percentages from prodrugs to active substance (MGS0039) by plasma and liver S9 fractions from monkeys and humans

Transformed percentages were obtained after 2 h (plasma) and 1 h (liver S9 fraction) of incubation. Data are expressed as the mean of three experiments.

Group	Compound	R ^{1a}	R ^{2a}	Transformed Percentage			
				Plasma		Liver S9 Fractions	
				Monkey	Human	Monkey	Human
Active	MGS0039	H	H	N.T.	N.T.	N.T.	0.2 ^b
A	MGS0103	H	Alanine	<0.1	<0.1	<1.0	<1.0
	MGS0105	Leucine	H	0.2	0.2	<1.0	<1.0
	MGS0097	H	Ethyl	0.2	0.2	<1.0	<1.0
B	MGS0111	Methyl	H	4.1	7.1	26.4	44.2
	MGS0096	Ethyl	H	0.4	1.5	15.8	28.2
	MGS0112	<i>n</i> -Propyl	H	0.2	1.5	39.2	39.2
	MGS0113	<i>n</i> -Butyl	H	0.4	1.5	64.2	52.1
	MGS0155	<i>n</i> -Pentyl	H	0.3	1.5	68.4	43.9
	MGS0201	<i>n</i> -hexyl	H	<1.0	<1.0	54.0	65.3
	MGS0210	<i>n</i> -heptyl	H	N.T.	N.T.	42.6	76.9
	MGS0209	<i>n</i> -octyl	H	N.T.	N.T.	43.2	68.8
	MGS0144	<i>n</i> -decyl	H	2.1	2.1	54.1	55.0
	C	MGS0114	<i>i</i> -Propyl	H	0.6	0.9	5.9
MGS0116		<i>i</i> -Butyl	H	0.2	0.9	57.8	31.1
MGS0200		3-Methylbutyl	H	<1.0	<1.0	72.8	56.4
MGS0211		4-Methylpentyl	H	N.T.	N.T.	66.4	44.2
MGS0212		5-Methylpentyl	H	N.T.	N.T.	43.4	71.1
MGS0213		6-Methylheptyl	H	N.T.	N.T.	33.3	74.9
D	MGS0115	Cyclohexyl	H	1.3	1.6	19.4	12.3
	MGS0117	Cyclohexylmethyl	H	0.5	1.1	69.0	10.0

N.T., not tested.

^a R¹, R², see Fig. 1.^b Metabolized percentage of MGS0039.

C18 rapid resolution cartridge column (3.5 μ m, 15 \times 2.1 mm) (Agilent Technologies, Palo Alto, CA). The analytes were eluted with a linear gradient mobile phase system by increasing the acetonitrile concentration in 0.1% acetic acid from 5% to 95% over 2.5 min at a flow rate of 500 μ l/min. Tandem mass spectrometric detection was performed using TurboIonSpray (AB/MDS Sciex API3000; Applied Biosystems/MDS Sciex, Concord, ON, Canada) in positive ion mode. The lower limit of quantitation was 1 μ M.

Plasma samples (50 μ l) were vortex-mixed with 200 μ l of the analytical internal standard (MGS0037) solution (250 ng/ml) with methanol, then centrifuged at 11,200g (4°C) for 10 min. The resulting supernatant was analyzed by liquid chromatography with mass detection on an Agilent ZORBAX SB-C18 column (5 μ m, 50 \times 2.1 mm). The analytes were eluted with a linear gradient mobile phase system by increasing the acetonitrile concentration in 0.1% acetic acid from 5% to 95% over 4 min at a flow rate of 250 μ l/min. Tandem mass spectrometric detection was performed using a TurboIonSpray (AB/MDS Sciex API3000) in positive ion mode. The lower limit of quantitation was 1 ng/ml.

Metabolite Analysis. The mass spectrometer used was a PE SCIEX API3000 (PE SCIEX, Toronto, Canada) equipped with a Gilson 235P auto injector (Gilson Company, Inc., Middleton, WI), and a Shimadzu LC-10Advp pump (Shimadzu, Kyoto, Japan). The interface between the high performance liquid chromatograph and the mass spectrometer was accomplished through a PE SCIEX TurboIonSpray [electrospray ionization (ESI) interface]. The liver S9 fraction samples were analyzed on an Agilent ZORBAX SB-18 column (5 μ m, 50 \times 2.1 mm). The analytes were eluted with a linear gradient mobile phase system by increasing the acetonitrile concentration in 0.1% acetic acid from 5% to 95% over 7.5 min, maintaining it thereafter for 8 min at a flow rate of 250 μ l/min. The metabolites were ionized in the ESI interface in positive or negative ion mode and detected using a Q1 scan. A full-scan range was set up between 300 and 800. The ESI was performed at 5 kV (positive) with a heated gas temperature of 425°C, a nebulizer gas (air) flow of 12 units, curtain gas (N₂) flow of 10 units, and collision gas (N₂) flow of 4 units. All data were processed by Analyst 1.1 software (Applied Biosystems/MDS Sciex). The amounts of MGS0039 and each prodrug were determined by each calibration curve. The amounts of metabolites were estimated from the ratios of the peak heights of each metabolite to that of each unchanged prodrug on the liquid chromatography/mass spectrometry chromatogram. Thus, the relative por-

tions of the metabolites may change if analyzed against a calibration curve for each synthetic standard.

Calculation of Pharmacokinetic Parameters. AUC_{inf} (time to infinity) and half-life (*t*_{1/2}) were calculated from the MGS0039 concentration in plasma by the noncompartmental analysis method (WinNonlin; Pharsight, Mountain View, CA). Concentration and parameters were expressed as molar concentrations. Oral bioavailability (*F*) after oral dosing of each prodrug was estimated by dividing the dose-normalized AUC_{inf} MGS0039 after oral dosing of each prodrug by the dose-normalized AUC_{inf} and MGS0039 value after intravenous MGS0039 dosing.

Results

Metabolism in Plasma. No transformation to active substance from any of the prodrugs in plasma from monkeys and humans or whole blood from humans was observed during 2 h of incubation (Table 1).

Metabolism in Liver S9 Fractions. MGS0039 was stable in liver S9 fractions from both monkeys and humans, indicating negligible first-pass metabolism of the active substance. No transformation to active substance from the prodrugs of group A (amino acids or ethyl) was observed in liver S9 fractions from either monkeys or humans, corresponding to results obtained in rats (A. Yasuhara, M. Nakamura, K. Sakagami, T. Shimazaki, R. Yoshikawa, S. Chaki, H. Ohta, and A. Nakazato, manuscript submitted for publication). For other prodrugs in group B (normal alkyls), C (branch alkyls), and D (cyclohexyls), transformation to active substance was observed in liver S9 fractions from both monkeys (5.9–72.8%) and humans (5.4–76.9%), although complete transformation to active substances was not observed in either species.

In monkeys, *n*-butyl and *n*-pentyl ester prodrugs (group B) and 3-methylbutyl and 4-methylpentyl ester prodrugs (group C) exhibited high transformation ratios (>64%). In humans, *n*-hexyl, *n*-heptyl, and *n*-octyl ester prodrugs (group B) and 5-methylbutyl and 6-methylpentyl ester prodrugs (group C) exhibited high transformation ratios (>65%). Whereas the cyclohexylmethyl ester prodrug (group D) was

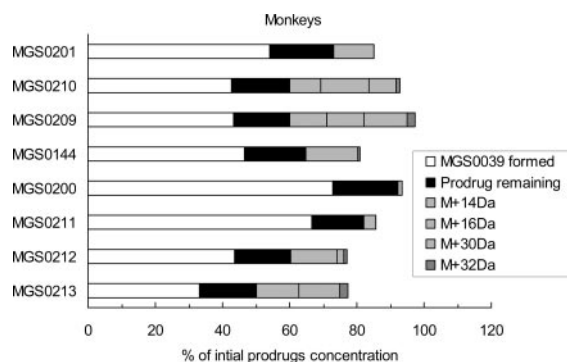


FIG. 2. In vitro metabolism of prodrugs in liver S9 fractions obtained from monkeys. Results are the mean of triplicate determinations.

transformed efficiently (69%) in monkeys, favorable transformation ratios were not observed in humans (Table 1). The tested prodrugs were also incubated with boiled liver S9 fractions, but nonenzymatic degradation was negligible (<10%) for all prodrugs.

Metabolism Analysis in Liver S9 Fraction. The in vitro biotransformation of prodrugs, which exhibited good transformation, was studied using liver S9 fractions from monkeys and/or humans. After 60 min of incubation, at least four metabolite types could be proposed based on LC/ESI-MS/MS analysis: hydroxylated metabolite (M + 16 Da), carbonyl metabolite (M + 14 Da), carboxylic acid metabolite (M + 30 Da), and 2-fold hydroxylated metabolite (M + 32 Da), all of which were formed by oxidative metabolism of the alkyl group in each of the prodrugs. Figures 2 and 3 show the recovery of MGS0039 and unchanged prodrugs (quantitative value), and these four metabolites (estimation from peak height on liquid chromatography/mass spectrometry chromatogram) in monkeys and humans, respectively.

These prodrugs were metabolized to MGS0039 with a transformation ratio ranging from 33% to 73% in monkeys and 44% to 77% in humans. The remaining prodrug ratios were 16% to 19% in monkeys and 13% to 41% in humans. The amount recovered, that is, the sum of remaining prodrugs plus MGS0039, ranged from 50 to 92% in monkeys and 76 to 99% in humans.

In monkeys, one to four types of metabolites for each prodrug, described above, were found in normal alkyl ester prodrugs (MGS0201, MGS0210, MGS0209, and MGS0144). The sum of these metabolites and unknown metabolites ranged from 27 to 40%. In the branched alkyl ester prodrugs (MGS0200, MGS0211, MGS0212, and MGS0213), the amount of unknown metabolites in addition to these known metabolites seemed to increase (from 8% to 50%) with the length of the alkyl ester.

M + 14 Da- and M + 32 Da-type metabolites could not be found in humans for any prodrug. The amounts of these metabolites and unknown metabolites in MGS0201 and MGS0210 (normal alkyl ester), and in MGS0200 and MGS0212 (branched alkyl ester) were very small (<4%). In contrast, relatively large amounts of metabolites (>18%) were found in MGS0209 and MGS0144, which have long-chain normal alkyl ester groups (*n*-octyl and *n*-decyl), and MGS0211 and MGS0213 (branched alkyl ester) were metabolized to these and other unknown metabolites (>10%).

Caco-2 Study. The apparent permeability coefficient, P_{app} , for MGS0039 was 0.92×10^6 cm/s, indicating low permeability due to low lipophilicity ($\log D(7.4) = -1.29$) (Beaumont et al., 2003). Complete recovery was obtained after 2 h of incubation. For the prodrugs, it should be noted that the compound did show low recovery (<38%; MGS0210) due to plastic sticking or retention in Caco-2 cell assays. In the case of MGS0210 ($\log D(7.4) = 3.32$), the apparent permeability coefficient, P_{app} , for MGS0210 was low (0.78×10^6

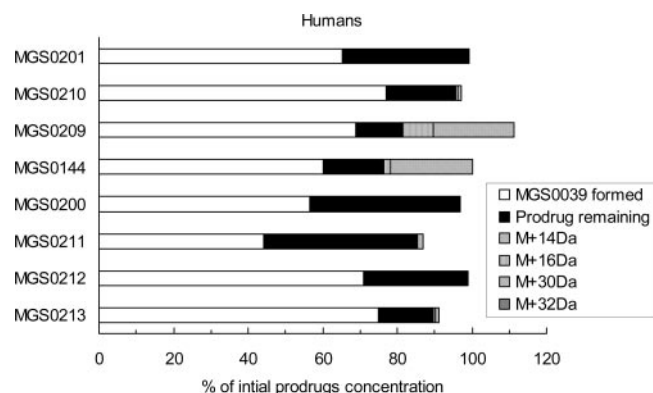


FIG. 3. In vitro metabolism of prodrugs in liver S9 fractions obtained from humans. Results are the mean of triplicate determinations.

cm/s). But active substances were detected on the basal side, and P_{app} , which was estimated by measuring MGS0210 plus the active substance, increased 5.8-fold (4.49×10^6 cm/s), indicating high permeability. These data indicated that MGS0210 was well absorbed and transformed to an active substance by the Caco-2 cells.

P-gp Study. The potential of MGS0039 as a substrate for P-gp-mediated efflux transport was evaluated. No significant transport of MGS0039 was observed from either apical to basal (1.00×10^6 cm/s) or basal to apical (0.99×10^6 cm/s) in LLC-GAS-CoL300 cell lines (B → A/A → B ratio was 1.00), demonstrating that MGS0039 has low permeability across cell membranes and is not a substrate for P-gp.

In Vivo Bioavailability. After intravenous administration of 1 mg/kg MGS0039, plasma concentrations in monkeys declined biphasically with a terminal half-life ($t_{1/2}$) of 1.0 h, sinking below the detection limit at 24 h. A total plasma clearance of 352.8 ml/h/kg, the volume of distribution, was estimated to be 198.7 ml/kg, and the AUC_{inf} value was estimated to be 7.7 μ M h. After oral administration of 10 mg/kg MGS0039, plasma concentrations reached peak levels (C_{max}) of 0.8 μ M at 1.3 h, and absolute bioavailability was 12.6%.

After oral administration of 10 mg/kg prodrugs (MGS0111, MGS0096, MGS0113, MGS0155, MGS0201, MGS0210, MGS0209, MGS0116, MGS0200, MGS0212, and MGS0213) to monkeys, plasma concentrations of active substance reached a C_{max} of 0.8 to 5.0 μ M at 1.3 to 4.7 h. Oral bioavailability (F) determined using dose-normalized AUC_{inf} values after intravenous dosing of MGS0039 was 10.3 to 50.7% (Table 2), indicating increasing absorption of MGS0039 in all prodrugs. Compared with MGS0039 oral dosing, increases in C_{max} levels (4.1- to 6.3-fold) and F values (2.4- to 6.3-fold) were observed for MGS0113, MGS0155, MGS0201, and MGS0210 (normal alkyls), and MGS0116 and MGS0200 (branched alkyl esters).

Discussion

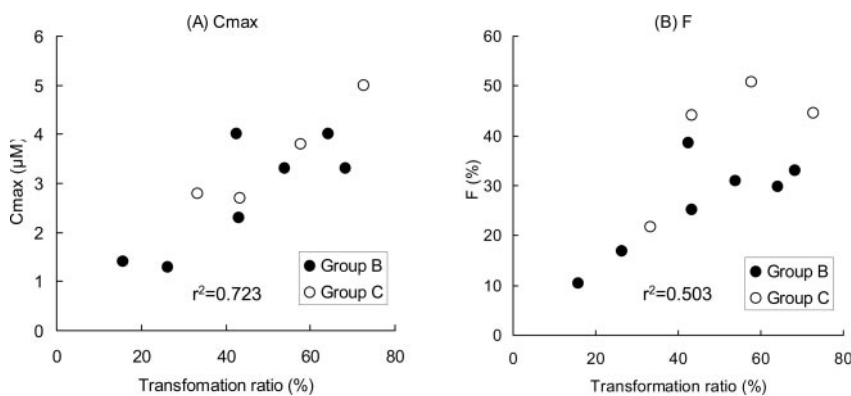
Our previous study demonstrated that prodrugs in groups B, C, and D transform almost completely to active substances in plasma and liver S9 fractions in rats, exhibiting good bioavailability after oral dosing in rats (A. Yasuhara, M. Nakamura, K. Sakagami, T. Shimazaki, R. Yoshikawa, S. Chaki, H. Ohta, and A. Nakazato, manuscript submitted for publication). However, it remains unclear which enzyme plays the key role in improving bioavailability in these tissues and plasma. Nor is it known whether the results obtained can be extrapolated to humans. Recent investigations have demonstrated similarities in in vitro metabolism of ester prodrugs between monkeys and humans (Cook et al., 1995; Prueksaritanont et al., 1996), as well

TABLE 2

Pharmacokinetics parameters for active substance (MGS0039) after oral dosing (10 mg/kg) of MGS0039 or each prodrug to monkeys

Data are expressed as the mean \pm S.D. of three animals.

Group	Compound	Dose	T_{\max}	C_{\max}	F	
		$\mu\text{mol/kg}$	h	μM	%	
Active B	MGS0039	26.4	1.3 \pm 0.6	0.8 \pm 0.5	12.6	
	MGS0111	23.3	3.3 \pm 1.2	1.3 \pm 0.6	16.9	
	MGS0096	22.6	2.0 \pm 0.0	1.4 \pm 0.2	10.3	
	MGS0113	21.2	2.7 \pm 1.2	4.0 \pm 1.0	29.7	
	MGS0155	22.3	3.3 \pm 1.2	3.3 \pm 0.7	32.9	
	MGS0201	21.6	2.3 \pm 1.5	3.3 \pm 1.4	30.9	
	MGS0210	21.0	3.7 \pm 3.8	4.0 \pm 1.6	38.6	
	MGS0209	20.4	1.3 \pm 0.6	2.3 \pm 0.6	25.2	
	C	MGS0116	21.2	4.7 \pm 3.1	3.8 \pm 1.0	50.7
		MGS0200	22.3	2.0 \pm 0.0	5.0 \pm 1.8	44.5
MGS0212		21.0	4.3 \pm 3.5	2.7 \pm 0.3	44.0	
MGS0213		21.6	2.0 \pm 0.0	2.8 \pm 0.6	21.8	

FIG. 4. The relationship between transformation ratios of liver S9 fractions and C_{\max} (A) or F values (B) in monkey for prodrugs. Each point represents the mean of triplicate determinations (transformation ratio) and three animals (C_{\max} , F).

as the significant and comparable activity of drug-metabolizing enzymes (cytochromes P450 and carboxylesterases) in human and monkey intestines (Prueksaritanont et al., 1996b). The present study examined the relevance of esterase activity for in vitro and in vivo studies using monkeys, as well as species differences between monkeys and humans in vitro, with the goal of selecting the optimal prodrug.

Not all tested prodrugs were converted into active substance in monkey and human plasma/blood. In our previous study, prodrugs (group A) with alanine and leucine incorporated at the C-2 position and C-6 position of bicyclo[3.1.0]hexane ring resisted metabolism in rat plasma, a finding consistent with results obtained in monkeys and humans. However, other prodrugs (groups B, C, and D) of alkyl esters at the C-6 position of this ring gave high ratios (>95%) of the active substance in rat plasma. Species differences in transformation were observed in plasma/blood from rats, monkeys, and humans. Some of these differences may be attributable to the high levels of carboxylesterases in the plasma of rodents (Fix et al., 1990; Li et al., 1998). The results suggest that esterase in plasma/blood is unlikely to play an important role in the transformation to active substances in monkeys or humans.

Prodrugs of MGS0039 were transformed in liver S9 fractions, but not in the plasma of either monkeys or humans. Figure 4 shows the close relationships between transformation ratios and C_{\max} (Fig. 4A) and F values (Fig. 4B) in monkeys for prodrugs (normal alkyl esters and branched alkyl esters), indicating that the hydrolysis rate in liver S9 fractions is the most important factor in determining oral bioavailability in monkeys.

MGS0039 is metabolically stable. The low intestinal permeability

of MGS0039 was demonstrated using in vitro Caco-2 cells, and no indication of efflux-mediated transport was observed. The permeability of prodrugs could not be evaluated, although transformation to active substance was observed qualitatively. These results suggest that some absorbed prodrugs may be transformed into an active substance in the small intestine, with this active substance entering systemic circulation without efflux or first-pass metabolism in the small intestine and liver. Thus, transformation in the small intestine must improve rather than impede bioavailability.

To compare transformation ratios of species differences, optimal ester groups were observed from *n*-butyl to *n*-pentyl or from 3-methylbutyl to 4-methylpentyl in monkeys, and from *n*-hexyl to *n*-octyl or from 5-methylbutyl to 6-methylpentyl in humans. Low correlation ($r^2 = 0.138$) between the transformation ratios in monkeys and

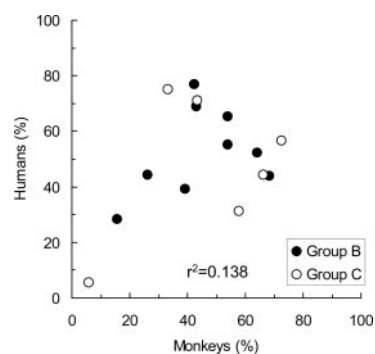


FIG. 5. The relationship of transformation ratios in liver S9 fractions between monkeys and humans. Results are the mean of triplicate determinations.

humans was observed (Fig. 5). Several prodrugs, with transformation ratios ranging up to 54% in monkeys, exhibited a positive correlation with humans ($r^2 = 0.704$). However, this correlation was lost, and the transformation ratio in humans did not increase for the prodrugs, which were transformed above 57% in monkeys. These results suggest that modifying length from *n*-hexyl to *n*-octyl or from 5-methylbutyl to 6-methylpentyl will likely increase effectiveness in humans, assuming formation of active substance from these prodrugs in humans is due primarily to liver metabolism. In the previous study, transformation ratios have been determined for rats in the same condition, and high transformation ratios (82–105%) of these prodrugs were observed. A wide range of transformation ratios depending on the modifying length in monkeys was observed as compared with rats.

The extent of nonproductive ester hydrolysis as well as prodrug metabolism by a nonesterase enzyme will determine the potential success of a prodrug approach (Beaumont et al., 2003). For this reason, the recovered sum of remaining prodrugs plus active substances and metabolites *in vitro* was examined with respect to *n*-hexyl to *n*-decyl groups and 3-methylbutyl to 6-methylbutyl groups. Comparisons of monkeys and humans for each alkyl group showed higher recovery rates for humans (76–99%) than for monkeys (50–92%). Metabolite analysis confirmed low recovery in monkeys, indicating formation of nonproductive metabolites. The finding suggests that species differences for the optimal ester group as discussed above depend on the rate of oxidative metabolism as well as transformation to active substance. Near-complete recovery (96–99%) was also achieved in these studies for three prodrugs, MGS0201, MGS0210, and MGS0212, which exhibited high transformation ratios in humans (65.3–76.9%), indicating very low risk of generating nonproductive and unknown human-specific metabolites. Given the transformation ratio, MGS0210 would exhibit the highest oral bioavailability of active substances in humans, assuming that the hydrolysis rate in liver S9 fractions is the most important factor in determining oral bioavailability in humans. In conclusion, MGS0210 (*n*-heptyl alkyl ester prodrug) represents a promising candidate prodrug for the mGluR receptor antagonist, exhibiting favorable oral bioavailability characteristics in humans.

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