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IN VITRO AND IN VIVO EVALUATION OF THE METABOLISM AND BIOAVAILABILITY OF ESTER PRODRUGS OF MSG0039, A POTENT MGLUR ANTAGONIST

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Running Title: Metabolism and Bioavailability of MGS0039 Prodrugs

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Abbreviations used are: mGluR(s) (metabotropic glutamate receptor(s)), cAMP (cyclic adenosine monophosphate), CHO (Chinese hamster ovary), DMSO (dimethyl sulfoxide), $NADP^+$ (nicotinamide adenine dinucleotide phosphate), Caco-2 (human colon carcinoma cell line), MDR1 (multi drug resistance), ESI (electro spray ionization), HPLC (high performance liquid chromatography), LC/MS/MS (liquid chromatography-mass spectroscopy), AUC_{inf} (The area under the curve of plasma concentration-time profile), F (oral bioavailability)

Abstract

MGS0039 has been identified as a potent and selective antagonist for metabotropic glutamate receptors (mGluRs). However, the oral bioavailability of MGS0039 is 10.9% in rats, due to low absorption. Several prodrugs, synthesized to improve absorption, exhibited 40 – 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 in order 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%). Cmax levels and F values following oral dosing increased to 4.1 – 6.3-fold and 2.4 – 6.3-fold, respectively, and a close relationship between transformation ratios and Cmax 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 (n-heptyl alkyl ester prodrug) appears to be a promising candidate among MGS0039 prodrugs.

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Introduction

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 (iGluRs) 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 Duvoision, 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). On the other hand, little is known of the therapeutic significance of group II mGluR antagonists, which may stem from the lack of potent and selective antagonists.

MGS0039 is a potent and selective antagonist for group II mGluRs as determined by attenuation of glutamate-induced inhibition of forskolin-evoked cAMP formation in CHO cells expressing mGluR2 (IC_{50} = 20 nM) or mGluR3 (IC_{50} = 24 nM) (Nakazato et al., 2004; Chaki 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). Following oral dosing in rats, MGS0039 exhibited higher plasma and brain concentrations than (2S)-amino-2-((1S,2S)-2-carboxycycloprop-1-yl)-3-(9-xanthyl)propionic acid (LY341495) (Ornstein et al., 1998a; Ornstein et al., 1998b), while oral bioavailability was 10.9% (Levine et al., 2002). This low bioavailability may be due to low

absorption, which may be attributable to a structural feature consisting of two carboxyl groups and an amino group. In the previous study (Submitted), we investigated the lipophilic prodrugs of MGS0039 in order to improve oral bioavailability. Based on in vitro metabolism and pharmacokinetic studies in rats, we found the following: a) Only the ester prodrugs, which were modified carboxyl group on C-6 position of bicyclo[3.1.0]hexane ring exhibited enzymatic transformation to an active substance in rat liver S9 fractions and plasma. b) Alkyl ester, cyclohexyl ester and cyclohexylmethyl ester prodrugs exhibited near-complete enzymatic transformation to active substances, without non-enzymatic degradation. However, significant non-enzymatic degradation was observed in alkoxycarbonylethyl ester prodrugs. c) Following oral dosing of these prodrugs to rats, improvement oral bioavailabilities (40 – 70%) were 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 in order to select the prodrug most likely to exhibit optimal pharmacokinetic profiles in humans.

Methods

Materials. MGS0039 (3-(3,4-Dichlorobenzyloxy)-2-amino-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid), its prodrugs (**Figure 1**) and (–)-11ay (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 (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 HPLC grade. Caco-2 cells and LLC-GA5-CoL300 cells were obtained from the American Type Culture Collection (Rockville, MD) and from the RIKEN Cell Bank (Tsukuba, Japan), respectively.

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

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, USA) and cynomolgus monkeys (XENOTECH LLC, Kansas City, KS, USA) and (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) were incubated with 3 μM MGS0039 prodrugs in the presence of an NADPH generating system (125 μg/mL $NADP^+$, 2.5 mM $MgCl_2$, 1.92 mM glucose-6-phosphate) in a 0.255 M phosphate buffer containing 0.575% (w/v) KCl (pH7.4) for 1 hour at 37ºC. All experiments were performed in triplicate. After incubation, a two-fold volume of DMSO was added to the incubation medium. The tube was then vortexed and centrifuged at 3000 rpm, 4ºC 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 μM), and then incubated for 2 hours at 37ºC. After incubation, 200 μL of internal standard working solution (250 ng/mL) with methanol or acetonitrile was added to 50 μL aliquot of plasma sample, and the tube was vortexed

and centrifuged at 11,200 g (4ºC) for 10 min. The resulting supernatant was analyzed by LC-MS/MS system.

Caco-2 study. Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, and a 1% antibiotic-antimycotic mixture (10,000 U/mL penicillin G, 10,000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B in 0.85% saline) at 37ºC in culture flasks in a humidified 5% CO_2 atmosphere. Caco-2 cells (passage number 50 to 60) were seeded on 24-well cell culture inserts (Transwell^{TM}, 0.3 µm pores, 6.5 mm id) at a density of 60,000 cells/cm^2. 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 post-seeding. The integrity of the cell monolayers was evaluated by measuring transepithelial electric resistance (TEER) and the ^{14}C-mannitol permeability of the monolayers before the transport experiment. Hank's balanced salt solution (HBSS) was used as the transport medium (TM) in all experiments after adjusting the pH to 6.5 for the apical side and the pH7.4 for the basolateral side. Stock solutions were diluted in TM (pH6.5) to obtain dosing solutions at final concentrations of 10 µM. After incubation for 20 minutes of both sides of the monolayers with drug-free TM, the test solution was added to the apical side of monolayer. At the time of initiation of transport (0 minute), dosing solutions were sampled to determine initial concentrations. Samples (50 µL) were taken from the basolateral side at 30, 60, 90, and 120 minutes after dose administration. Appropriate volumes of TM were added to the acceptor compartments. All experiments were performed in triplicate at 37ºC. 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 (Tanigawara et al., 1992) and Ueda et al. (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 µg/mL streptomycin sulfate in 0.85% saline) and 300 ng/mL colchicine at 37ºC in culture dishes in a humidified 5% CO_2 atmosphere. LLC-GA5-CoL300 cells were seeded on 12-well cell culture inserts (Transwell™, 0.3 µm pores, 12 mm id) at a density of 500,000 cells/cm^2. The cell monolayers were used 3 days post-seeding. 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 hours before the experiment. The integrity of the cell monolayers was evaluated by measuring the transepithelial electric resistance (TEER) and the ^{14}C-mannitol permeability of the monolayers before the transport experiment. Hank's balanced salt solution (HBSS, pH7.4) was used as the transport medium (TM) in all experiments. Stock solutions were diluted in TM to obtain dosing solutions at final concentrations of 10 µM. After incubation of both sides of the monolayers with drug-free TM for 20 minutes, 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 were then measured. At the time of initiation of transport (0 minute), dosing solutions were sampled to determine initial concentrations. Samples (50 µL) were taken from the acceptor side at 30, 60, 90, and 120 minutes after dose administration. Appropriate volumes of TM were added to the acceptor compartments. All experiments were performed in triplicate at 37ºC. Concentrations of compounds were determined by LC/MS/MS.

***In vivo* Study.** MGS0039 (1/15M phosphate buffer pH7.4 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-cycoldextrin 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 minutes, 1, 2, 3, 8, 24 hours after intravenous dosing, and 0.5, 1, 3, 8 and 24 hours after oral dosing. The plasma was separated by centrifugation (3000 rpm, 4ºC, 10 min) and kept at -80ºC until analysis.

Sample Analysis. Samples obtained from the *in vitro* study were analyzed by liquid chromatography with mass detection on an Agilent ZORBAX SB-C18 rapid resolution cartridge column (3.5 µm, 15 x 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 2.5 minutes at a flow rate of 500 µL/min. Tandem mass spectrometric detection was performed using TurboIonSpray (AB/MDS Sciex API3000) in positive ion mode. The lower limit of quantitation (LLOQ) was 1 µM.

Plasma samples (50 µL) were vortex mixed with 200 µL of the analytical internal standard (MGS0037) solution (250 ng/mL) with methanol, then centrifuged at 11,200 g (4ºC) for 10 minutes. The resulting supernatant was analyzed by liquid chromatography with mass detection on an Agilent ZORBAX SB-C18 column (5µm, 50 x 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 minutes at a flow rate of 250 µL/min. Tandem mass spectrometric detection was performed using an TurboIonSpray (AB/MDS Sciex API3000) in positive ion mode. The LLOQ were 1 ng/mL.

Metabolites Analysis. The mass spectrometer used was a PE SCIEX API3000 (Toronto, Canada) equipped with a Gilson 235P auto injector (Middleton, WI, USA), and a Shimadzu LC-10Advp pump (Kyoto, Japan). The interface between HPLC and mass spectrometer was accomplished through a PE SCIEX turbo ion spray (electro spray ionization (ESI) interface). The liver S9 fraction samples were analyzed on a Agilent ZORBAX SB-18 column (5 µm, 50 x 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 minutes, maintaining it thereafter for 8 minutes at a flow rate of 250 µL/min. The

metabolites were ionized in the ESI interface in positive or negative ion mode and detected using Q1 scan. A full-scan range was set up between 300 and 800. The ESI was performed at 5kV (positive) with a heated gas temperature of 425ºC, a nebulizer gas (Air) flow of 12 units, curtain gas (N_2) flow of 10 units, and collision gas (N_2) flow of 4 units. All data were processed by Analyst 1.1 software. 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 LC/MS chromatogram. Thus, the relative proportions 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 (t1/2) were calculated from the MGS0039 concentration in plasma by the non-compartmental analysis method (WinNonlin; Pharsight Co., Mountain View, CA, USA). Concentration and parameters were expressed as mol concentrations. Oral bioavailability (F) after oral dosing of each prodrug was estimated by dividing the dose normalized AUC_{inf}, MGS0039 following oral dosing of each prodrug by the dose normalized AUC_{inf}, MGS0039 value following 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 hours 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 (Submitted). For other prodrugs in group B (normal alkyls), C (branch alkyls), and D (cyclohexyls), transformation to active

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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%). While the cyclohexylmethyl ester prodrug (group D) was 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 non-enzymatic degradation was negligible (< 10%) for all prodrugs.

Metabolism Analysis in Liver S9 Fraction. The in vitro biotransformation of prodrugs, which exhibited good transformation, were studied using liver S9 fractions from monkeys and/or humans. After 60 minutes of incubation, at least four metabolite types could be proposed based on LC/ESI-MS/MS analysis: hydroxylated metabolite (M+16Da), carbonyl metabolite (M+14Da), carboxylic acid metabolite (M+30Da), and 2-fold hydroxylated metabolite (M+32Da), all of which were formed by oxidative metabolism of the alkyl group in each of the prodrugs. **Figure 2 and 3** shows the recovery of MGS0039 and unchanged prodrugs (quantitative value), and these 4 metabolites (estimation from peak height on LC/MS 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. And the amount recovered – that is, the sum of remaining prodrugs plus MGS0039 – ranged from 50 – 92% in monkeys and 76 – 99% in humans.

In monkeys, 1 – 4 types of metabolites for each prodrug, described above, were found in normal alkyl ester prodrugs (MGS0201, MGS0210, MGS0209, MGS0144). The sum of these metabolites and

unknown metabolites ranged from 27 – 40%. In the branched alkyl ester prodrugs (MGS0200, MGS0211, MGS0212, MGS0213), the amount of unknown metabolites in addition to these known metabolites appeared to increase (from 8% to 50%) with the length of the alkyl ester.

M+14Da and M+32Da types metabolites could not be found in humans for any prodrug. The amounts of these metabolites and unknown metabolites in MGS0201 and MGS0210 (normal alkey ester) and in MGS0200 and MGS0212 (branched alkyl ester) were very small (< 4%). On the other hand, relative 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 Papp for MGS0039 was 0.92 x 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 hours 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 cells assay. In the case of MGS0210 (Log D(7.4)= 3.32), the apparent permeability coefficient Papp for MGS0210 was low (0.78 x 10^6 cm/s). But active substances were detected on the basal side, and Papp, which was estimated by measuring MGS0210 plus the active substance, increased 5.8-fold (4.49 x 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 x 10^6 cm/s) or basal to apical (0.99 x 10^6 cm/s) in LLC-GAS-CoL300 cell lines (B→A/A→B ratio was 1.00), demonstrating that MGS0039 is low permeable across cell membranes and is not a substrate for P-gp.

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***In vivo* bioavailability.** Following intravenous administration of 1 mg/kg MGS0039, plasma concentrations in monkeys declined biphasically with a terminal half-life (t1/2) of 1.0 hour, sinking below the detection limit at 24 hours. A total plasma clearance of 352.8 mL/hr/kg, the volume of distribution was estimated to be 198.7 mL/kg, and the AUC_{inf} value to be 7.7 μM hr. After oral administration of 10 mg/kg MGS0039, plasma concentrations reached peak levels (Cmax) of 0.8 μM at 1.3 hours, and absolute bioavailability was 12.6%.

After oral administration of 10 mg/kg prodrugs (MGS0111, MGS0096, MGS0113, MGS0155, MGS0201, MGS0210, MGS0209, MGS0116, MGS0200, MGS0212, MGS0213) to monkeys, plasma concentrations of active substance reached Cmax of 0.8 – 5.0 μM at 1.3 – 4.7 hours. Oral bioavailability (F) determined using dose-normalized AUC_{inf} values following intravenous dosing of MGS0039 was 10.3 – 50.7% (**Table 2**), indicating increasing the absorption of MGS0039 in all prodrugs. Compared to MGS0039 oral dosing, increases in Cmax 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 ester).

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 (Submitted). 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 between monkeys and humans of ester prodrugs (Cook et al., 1995; Prueksaritanont et al., 1996), as well as the significant and comparable activity of drug-metabolizing enzymes (CYPs and carboxylesterases) in the humans and monkeys 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 monkeys and humans 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 rats plasma, a finding consistent with results obtained in monkeys and humans. However, other prodrugs (group 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 was 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 (Li et al., 1998; Fix et al., 1990). 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 (**A**) Cmax and (**B**) F values 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 cell, 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 suggests 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.

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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 humans was observed (**Figure 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 to rats.

The extent of non-productive ester hydrolysis as well as prodrug metabolism by a non-esterase 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* were 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 non-productive metabolites. The finding suggests that species differences for the optimal ester group as discussed above depends 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 ratio in humans (65.3 – 76.9%), indicating very low risk of generating non-productive 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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Figure legend

Figure 1. Chemical structure of MGS0039 and its prodrugs. R^1 and R^2 indicate the modified positions for prodrugs (see **Table1**).

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

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

Figure 4. The relationships between transformation ratio liver S9 fractions and (A) Cmax or (B) F values in monkey for prodrugs. Each point represents of mean of triplicate determinations (transformation ratio) and three animals (Cmax, F).

Figure 5. The relationships of transformation ratio in liver S9 fractions between monkeys and humans. Results are mean of triplicate determinations.

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Table 1. Transformation % from prodrugs to active substance (MGS0039) by plasma and liver S9 fractions from monkeys and humans

Group	Compound	R^1	R^2	Transformed %			
				Plasma		Liver S9 fractions	
				Monkey	Human	Monkey	Human
Active	MGS0039	H	H	N.T.	N.T.	N.T.	0.2[a)]
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	n-Propyl	H	0.2	1.5	39.2	39.2
	MGS0113	n-Butyl	H	0.4	1.5	64.2	52.1
	MGS0155	n-Pentyl	H	0.3	1.5	68.4	43.9
	MGS0201	n-hexyl	H	< 1.0	< 1.0	54.0	65.3
	MGS0210	n-heptyl	H	N.T.	N.T.	42.6	76.9
	MGS0209	n-octyl	H	N.T.	N.T.	43.2	68.8
	MGS0144	n-decyl	H	2.1	2.1	54.1	55.0
C	MGS0114	*i*-Propyl	H	0.6	0.9	5.9	5.4
	MGS0116	*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

Transformed % were obtained after 2 hours (plasma) and 1 hour (liver S9 fraction) incubation.

a) Metabolized % of MGS0039.

R^1, R^2: See **Figure 1**.

N.T.: Not tested

Data are expressed the mean of three experiments.

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Table 2. Pharmacokinetics parameters for active substance (MGS0039) following oral dosing (10 mg/kg) of MGS0039 or each prodrug to monkeys.

Group	Compound	Dose (μmol/kg)	Tmax (hr)	Cmax (μM)	F (%)
Active	MGS0039	26.4	1.3 ± 0.6	0.8 ± 0.5	12.6
B	MGS0111	23.3	3.3 ± 1.2	1.3 ± 0.6	16.9
	MGS0096	22.6	2.0 ± 0.0	1.4 ± 0.2	10.3
	MGS0113	21.2	2.7 ± 1.2	4.0 ± 1.0	29.7
	MGS0155	22.3	3.3 ± 1.2	3.3 ± 0.7	32.9
	MGS0201	21.6	2.3 ± 1.5	3.3 ± 1.4	30.9
	MGS0210	21.0	3.7 ± 3.8	4.0 ± 1.6	38.6
	MGS0209	20.4	1.3 ± 0.6	2.3 ± 0.6	25.2
C	MGS0116	21.2	4.7 ± 3.1	3.8 ± 1.0	50.7
	MGS0200	22.3	2.0 ± 0.0	5.0 ± 1.8	44.5
	MGS0212	21.0	4.3 ± 3.5	2.7 ± 0.3	44.0
	MGS0213	21.6	2.0 ± 0.0	2.8 ± 0.6	21.8

Data are expressed the mean ± SD of three animals.

Figure 1

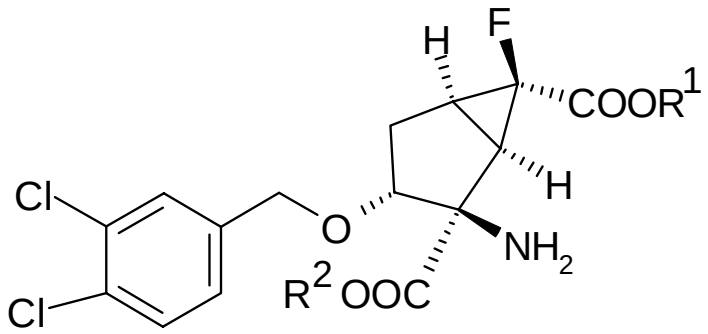

Figure 2

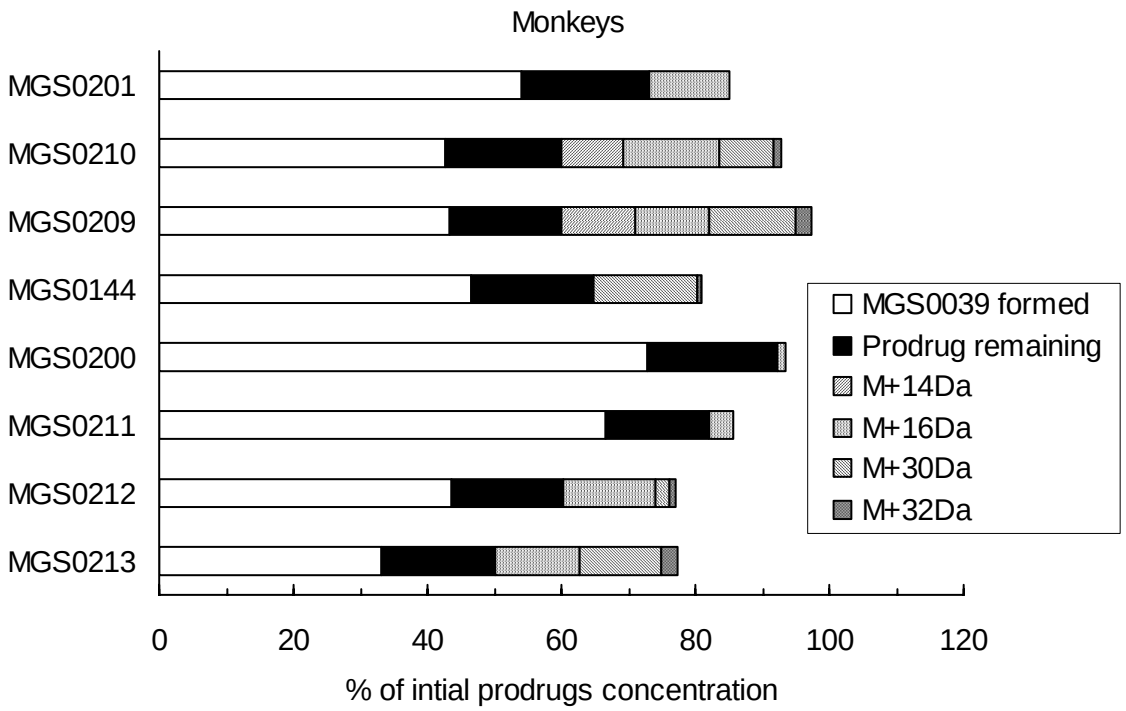

Figure 3

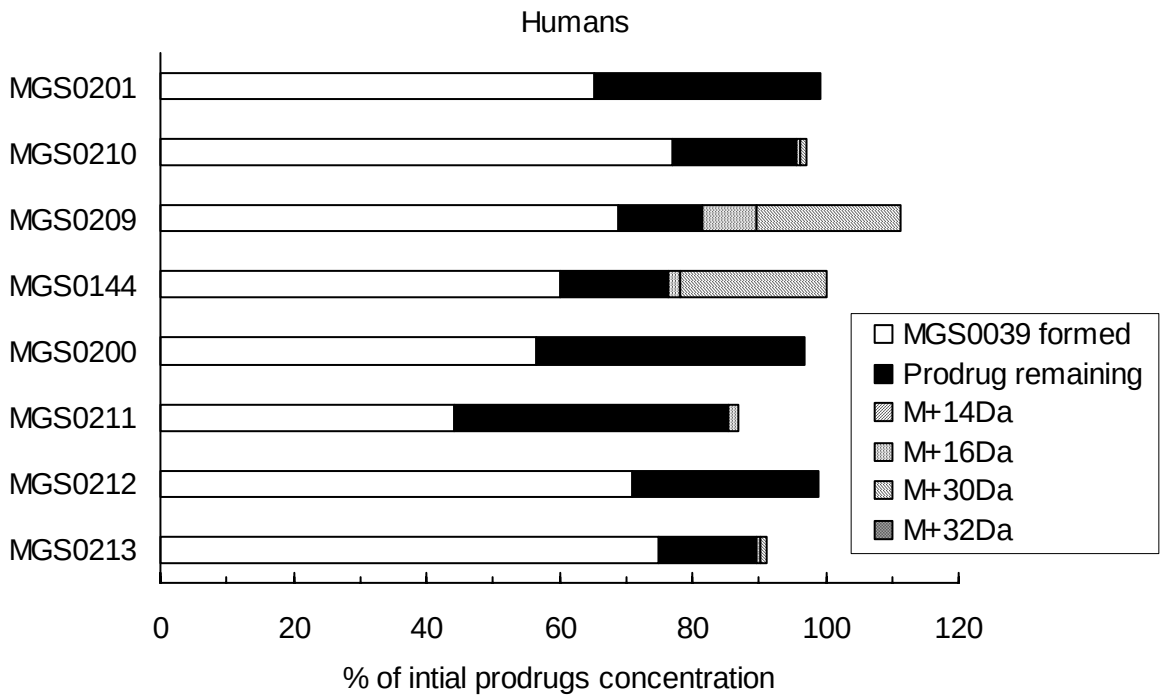

Figure 4

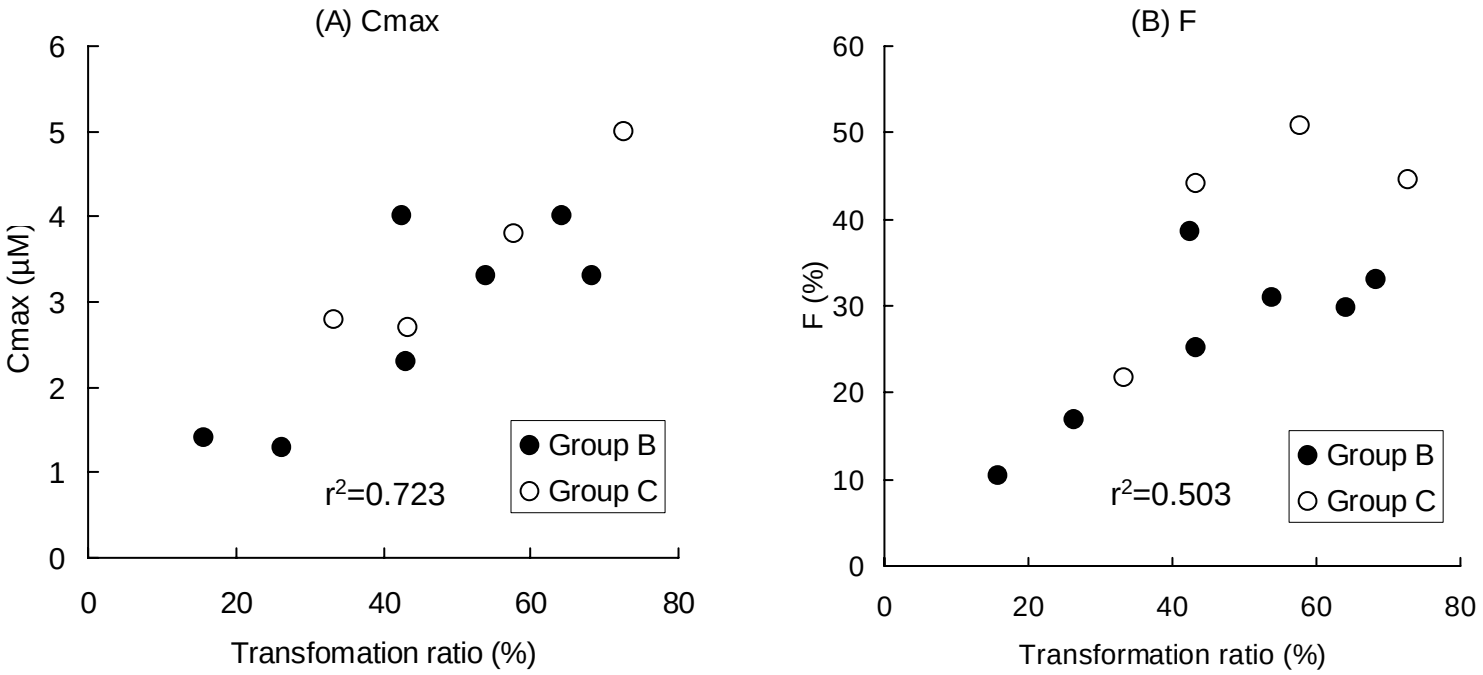

Figure 5