RESEARCH ARTICLE

Species differences in metabolism of ripasudil (K-115) are attributed to aldehyde oxidase

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Abstract
1. We examined the metabolism of ripasudil (K-115), a selective and potent Rho-associated coiled coil-containing protein kinase (ROCK) inhibitor, by in vitro and in vivo studies.
2. First, we identified metabolites and metabolic enzymes involved in ripasudil metabolism. Species differences were observed in metabolic clearance and profiles of metabolites in liver S9 fraction and hepatocytes. In addition, ripasudil was metabolised in humans and monkey S9 without nicotinamide adenine dinucleotide phosphate (NADPH). Studies using specific inhibitors and human recombinant enzyme systems showed that M1 (main metabolite in humans) formation is mediated by aldehyde oxidase (AO).
3. Therefore, we developed ripasudil as an ophthalmic agent. First, we compared the pharmacokinetic profiles of ripasudil in humans and rats. The results indicated rapid disappearance of ripasudil from the circulation after instillation in humans and its level remained relatively high only in M1. In contrast, we found six metabolites from M1 to M6 in plasma after oral administration to rats.
4. Analysis of enzyme kinetics using S9 showed that the formation of M1 is the major metabolic pathway of ripasudil in humans even though CYP3A4/3A5 and CYP2C8/3A4/3A5 were associated with the formation of M2 and M4, respectively. In conclusion, AO causes differences in ripasudil metabolism between species.

Keywords
Glaucoma, hepatocyte, instillation, non-cytochrome P-450, pharmacokinetics, ROCK, S9

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Introduction
The rho-associated coiled coil-containing protein kinase (ROCK) is a serine/threonine kinase that acts as a key downstream effector of Rho GTPase (Hall, 2005). ROCK controls multiple signalling pathways and many cellular responses involving the actin cytoskeleton (Riento & Ridley, 2003). ROCK inhibitor administration is effective for the treatment of a wide range of cardiovascular diseases, including cerebral and coronary vasospasm, angina, hypertension, pulmonary hypertension and heart failure (Shimokawa & Takeshita, 2005). In particular, ROCK inhibitors are expected to be useful in glaucoma therapy as they have different therapeutic targets than commercially available glaucoma drugs (Inoue & Tanihara, 2013). Evidence-based treatment for patients with glaucoma involves lowering the intraocular pressure (IOP) (Bahrami, 2006), and drugs acting on main outflow of the aqueous humour will provide effective treatment.

Ripasudil hydrochloride hydrate (K-115), a selective ROCK inhibitor, has been shown to reduce the IOP via increases in conventional outflow and was demonstrated to have good ocular penetration characteristics in an animal study (Isobe et al., 2014). We attempted to develop ripasudil not as a systemic agent but as an ophthalmic agent for the treatment of glaucoma and ocular hypertension due to its pharmacokinetic profiles. Phase-I clinical trials indicated that ripasudil is a topical IOP-lowering agent with a good adverse event profile in healthy volunteers (Tanihara et al., 2013a). Phase-II clinical trials demonstrated the IOP-lowering effect of ripasudil in 8-week treatment with twice-daily dosing in patients with primary open-angle glaucoma (POAG) (Tanihara et al., 2013b). Furthermore, the efficacy of ripasudil has been confirmed in phase-III clinical trials in both long-term monotherapy and combination therapy with 0.5% timolol or 0.005% latanoprost, both of which are representative first-line drugs (Tanihara et al., 2015a, 2015b). Ripasudil approved for use as a therapeutic agent in glaucoma and ocular hypertension in Japan.

Non-cytochrome P-450 (CYP) metabolism plays an important role in the drug development stage (Akabane et al., 2012). There are various types of non-CYP drug-metabolising enzyme, including the phase-I enzymes aldehyde oxidase (AO), esterase, and flavin-containing...
monooxygenase (FMO), and phase-II enzymes UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT). AO belongs to the molybdoflavoenzyme family that also includes xanthine oxidase (XO). Molybdoflavoenzymes require flavin adenine dinucleotide and a molybdopterin cofactor for their catalytic activity (Beedham, 1987; Garattini et al., 2008). AO is ubiquitously expressed in human tissues, and its expression level is especially high in the liver and adrenal gland, as determined by mRNA assay (Nishimura & Naito, 2006) and immunohistochemistry (Moriwaki et al., 2001). Whereas CYP is located in the microsomal fraction, AO is present in the cytosolic fraction and does not require NADPH as a cofactor (Beedham, 1985). AO catalyses the oxidation of many nitrogenous heterocyclic compounds as well as aldehyde, and remarkable species-related differences in its enzyme activity have been reported (Garattini & Terao, 2004; Pryde et al., 2010).

This study was performed to elucidate the species differences in metabolism, metabolite profile and enzymes involved in the metabolic pathway of ripasudil. We evaluated factors associated with metabolism of ripasudil in vitro using liver S9, hepatocytes and single-expression systems. Based on the in vitro data, we attempted to develop ripasudil as an ophthalmic agent and then compared the plasma concentration profiles after instillation in humans with those after oral administration to rats. Finally, we analysed enzyme kinetics and clarified the rate-limiting enzyme and the cause of the differences among species.

Materials and methods

Chemicals and reagents

Ripasudil, its metabolites and internal standards for measurement were synthesised in our laboratories. [14C]Ripasudil (2.07 GBq/mmol) was synthesised by Quotient Bioresearch (Fordham, Cambridgeshire, UK; formerly Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK). The chemical structure of ripasudil is shown in Figure 1. Allopurinol, menadione, raloxifene hydrochloride, phthalamazine and 1-phthalazinone were purchased from Sigma-Aldrich Corporation (St. Louis, MO). All other reagents and solvents were commercial products of analytical grade.

In vitro metabolic stability and metabolite profiling of ripasudil with liver S9 and hepatocytes

To determine the metabolism of ripasudil in humans and animals, we conducted in vitro metabolite profiling studies using radio high-performance liquid chromatography (HPLC) analysis. [14C]Ripasudil was incubated with or without an NADPH-regenerating system and pooled liver S9 (XenoTech LLC, Lenexa, KS) from male Sprague–Dawley rats, male New Zealand rabbits, male beagle dogs, male cynomolgus monkeys and male and female human subjects were prepared using a hepatocyte isolation kit according to the manufacturer’s protocol (XenoTech LLC). The cells were suspended in Krebs–Henseleit buffer to 1.5 × 10^6 cells/0.24 mL well. The hepatocyte suspension was preincubated for 30 min in a 5% CO_2 incubator at 37°C. Reactions were initiated by adding 0.01 mL of [14C]ripasudil (final concentration 5 μmol/L) and then incubated for 0, 1, 2 and 4 h. The reaction solution was collected into polypropylene tubes (rinsed with 0.25 mL of Krebs–Henseleit buffer) at predetermined time points, the reaction was terminated by adding 0.5 mL of ice-cold 0.1 mol/L NaOH, and samples were stored on ice.

The samples were loaded onto a solid-phase extraction column (Oasis HLB, 1 cc; Waters, Milford, MA) conditioned with 1 mL of methanol and 1 mL of purified water. The cartridge was washed with 1 mL of water/methanol/ammonia water (93/5/2, v/v/v) and 1 mL of purified water, and the analyte was eluted into a polypropylene tube with 1 mL of methanol. After evaporating the eluate, the residue was dissolved in 250 μL of mobile phase.

The samples were then subjected to radio-HPLC analysis using an Inertsil ODS-3 V (5 μm, 4.6 × 150 mm; GL Sciences, Tokyo, Japan) as the analysis column. The mobile phase consisted of 5 mmol/L formic acid containing 0.02% trifluoroacetic acid (TFA) adjusted to pH 3 with ammonia water (A) and acetonitrile (B). A gradient profile with a flow rate of 1 mL/min starting with 95:5 (A:B) and increasing linearly to 75:25 (A:B) over 28 minutes, 50:50 (A:B) with a 4-min hold before returning the starting mixture and re-equilibrating for 5 min. The injection volume was 100 μL. The eluate of HPLC consisted of 5 mmol/L formic acid containing 0.02% trifluoroacetic acid (TFA) adjusted to pH 3 with ammonia water (A) and acetonitrile (B). A gradient profile with a flow rate of 1 mL/min starting with 95:5 (A:B) and increasing linearly to 75:25 (A:B) over 28 minutes, 50:50 (A:B) with a 4-min hold before returning the starting mixture and re-equilibrating for 5 min. The injection volume was 100 μL. The eluate of HPLC was mixed with liquid scintillator Flo Scint II (PerkinElmer, Waltham, MA) at a ratio of 1:3 and radioactivity was detected with a FLO-ONE/525TR device (PerkinElmer). The ratio (%) of total of the area of each peak to the total area of all radioactivity peaks was calculated from the radiochromatograms. Each peak was identified by the retention time of the reference compound, and any peak that did not match any of the reference compounds was judged to be an unidentified metabolite.

The in vitro intrinsic clearance (CL_{int}, S9 or CL_{int}, Hep) calculated using the equations below was based on the first-order elimination rate constant of the unchanged drug.

\[
CL_{int} = \frac{k_e}{S9 \text{ protein concentration}}
\]

where \( k_e \) is the elimination rate constant.
Metabolic inhibition of ripasudil with liver S9 in vitro

To clarify the enzyme responsible for the elimination of ripasudil, we conducted an in vitro metabolic study using liver S9. 

\[ ^{14}C \text{ Ripasudil was incubated without an NADPH-regenerating system using pooled human liver S9 (XenoTech LLC) with or without chemical inhibitor. The chemical inhibitor allopurinol (Massey et al., 1970) was used to selectively inhibit xanthine oxidase (XO), whereas mephalone (Johns, 1967) and raloxifene (Obach, 2004a) were used to selectively inhibit aldehyde oxidase (AO). These chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added at final concentrations of 100 \mu M (final concentration of DMSO < 0.5% (v/v)). Reactions were initiated by adding S9 followed by incubation at 37°C for 60 minutes, terminated by adding 0.5 mL of ice-cold 0.1 M NaOH and stored on ice.}

The samples were subjected to radio-HPLC analysis after pretreatment similar to that described above.

Pharmacokinetics of ripasudil in humans

We conducted a randomised placebo-controlled double-masked group comparison phase-I clinical trial (K-115-01). The safety of single instillation of ripasudil ophthalmic solution (0.05%, 0.1%, 0.2%, 0.4% and 0.8%) was investigated in a stepwise manner in healthy adult Japanese men using placebo as a control. A total of 50 subjects aged between 20 and 35 years with BMI not less than 17.6 and not more than 26.4, consisting of 10 subjects (eight received active drugs, two received placebo) for each concentration, were included in this study.

Ripasudil ophthalmic solution was administered to both eyes as a single dose (one drop/eye/dose/day; one drop was approximately 36 \mu L). Blood samples were taken using heparin as anticoagulant at 0 (before dosing), 5, 15 and 30 min and 1, 2, 4, 6, 9, 12, 24 and 48 h after administration. All plasma samples were stored at −20°C until analysis.

Measurement of human plasma concentrations

Plasma samples were analysed for the presence of ripasudil and its two metabolites (M1 and M2) using validated liquid chromatography-tandem mass spectrometry (LC-MS/MS). Aliquots of 1 mL of human plasma after adding 1 mL of 0.1 M hydrochloric acid and 0.1 mL of internal standard (I.S.) solution prepared with purified water were applied to the solid-phase extraction cartridges (Oasis MCX, 60 mg, 3 cc; Waters Corp.) preconditioned with methanol (2 mL) followed by purified water (2 mL). The cartridges were washed with 0.1 M hydrochloric acid (2 mL), methanol (2 mL) and water/methanol/ammonia solution (85:10:5, v/v/v, 2 mL) and subsequently ripasudil, M1, M2 and their I.S. were eluted with methanol/ammonia solution (95:5, v/v, 2 mL). The eluates were evaporated to dryness at 40°C under a gentle stream of nitrogen, and the residues were dissolved with the mobile phase solvent (200 \mu L) followed by injection into the LC (Agilent 1100 systems; Agilent Technologies, Santa Clara, CA)-MS/MS (API4000, AB SCIEX). The conditions for LC-MS/MS were as described earlier, but MRM added m/z 340 > 99 [M + H]+ for M3, m/z 354 > 162 [M + H]+ for M4, m/z 347 > 112 [M + H]+ for M5 and m/z 343 > 112 [M + H]+ for M6. The standard curve ranged from 2.5 to 1000 ng/mL (specified concentration was half for M2 and double for M4) for each analyte.

Pharmacokinetic analysis

Pharmacokinetic parameters (T_{max}, C_{max}, T_{1/2} and AUC) were analysed using the plasma concentrations of individuals by the non-compartmental method using the analytical software Phoenix WinNonlin (Certara, Princeton, NJ).

In vitro enzyme kinetics analysis of ripasudil with human liver S9 and single-expression system

To clarify the metabolic pathway and the enzyme responsible for ripasudil elimination, we conducted in vitro enzyme kinetics analysis using liver S9 and a single-enzyme
expression system (cytosolic extract of Escherichia coli expressing recombinant human AO; Cypex Ltd., Dundee, UK).

When a metabolic reaction was confirmed in examination with each substrate, we evaluated substrate concentrations from 1.56 to 100 μmol/L. Ripasudil or its metabolites were incubated with or without an NADPH-regenerating system using pooled human liver S9 (Bioreclamation IVT, Baltimore, MD). The incubation mixture was prepared for each substrate in a total volume of 0.25 mL of 0.1 mol/L phosphate buffer (pH 7.4) containing 0.5 mg/mL S9 protein. The LC-MS/MS conditions were as described previously. For kinetic studies, the Michaelis–Menten constant (K_m) and maximum rate (V_max) of each substrate were calculated from a simple E_max model using the analytical software Phoenix WinNonlin (Certara).

Inhibitory effects of ripasudil on aldehyde oxidase using human liver cytosol

Phthalazine (final concentration 2 μmol/L), which is a marker substrate of AO (Obach et al., 2004b), was incubated with human liver cytosol (Corning Gentest; Corning, Tewksbury, MA) in the presence of ripasudil at 0.00064, 0.0032, 0.016, 0.08, 0.4, 2 and 10 μmol/L, and 1-phthalazinone, which is a generated marker metabolite, was quantified to examine the inhibitory effects of ripasudil on AO activity. The incubation mixture was prepared in a total volume of 0.2 mL of 25 mmol/L potassium phosphate buffer (pH 7.4) containing 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.025 mg/mL cytosol protein. Reactions were initiated by adding cytosol fraction and then incubated at 37°C for 2.5 min. The reactions were terminated by adding 50 μL of I.S. solution prepared with 1 mol/L formic acid. The positive control inhibitor used menadione at 0.03, 0.1, 0.3, 1 and 3 μmol/L, and raloxifene at 0.0003, 0.001, 0.003, 0.01 and 0.03 μmol/L.

Aliquots of 200 μL of 50% acetonitrile were added to the samples, followed by centrifugation at 11,000 × g at 4°C for 5 min. The supernatants were injected (5 μL) onto an ACQUITY UPLC HSS T3, 2.1 mm I.D. × 50 mm, 1.8 μm (Waters Corp.) column, with 0.1% formic acid and acetonitrile (B) as the mobile phase. The gradient was as follows: 5% B, maintained at 5% B to 0.8 min, increased to 60% B to 2.5 min and maintained at 60% B to 3.5 min, decreased to 5% B within 0.1 min and maintained at 5% B; total run time was 4 min. The flow rate was as follows: 0.5 mL/min initially, maintained at 0.5 mL/min to 2.5 min, increased to 0.8 mL/min to 2.6 min and maintained at 0.8 mL/min to 3.9 min, decreased to 0.5 mL/min within 0.1 min. The substances were ionised by electrospray ionisation (ESI) and detection was performed via MRM of the characteristic ion dissociation transition m/z 147 > 118 [M + H]+ for 1-phthalazinone, m/z 161 > 130 [M + H]+ for 4-methyl-1-phthalazinone as I.S. The standard curve ranged from 1 to 300 nmol/L. The 50% inhibitory concentrations (IC_50) of ripasudil and positive control inhibitor were calculated by logistic regression analysis using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

Kinase inhibition assay

Recombinant ROCK (ROCK 1 and ROCK 2), protein kinase A (PKA catalytic z/PPKACA) and calmodulin-dependent protein kinase (CaMKII) were purchased from Carna Biosciences (Kobe, Japan). Purified protein kinase C (PKC) was purchased from Promega (Madison, WI). ROCK 1 (0.75 ng/μL) and ROCK 2 (0.5 ng/μL) were incubated with various concentrations of ripasudil, M1, M2 or M6 at 25°C for 90 min in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 100 mmol/L KCl, 10 mmol/L MgCl_2, 0.1 mmol/L EGTA, 30 μmol/L long S6 kinase substrate peptide and 1 μmol/L ATP in a total volume of 40 μL. PKACz, PKC and CaMKII were also incubated with various concentrations of ripasudil, M1, M2 or M6. PKACz (0.0625 ng/μL) was incubated at 25°C for 30 min in 40 mmol/L Tris-HCl buffer (pH 7.5) containing 20 mmol/L MgCl_2, 1 mg/mL BSA, 5 μmol/L Kemptide peptide substrate and 1 μmol/L ATP in a total volume of 40 μL. PKC (0.025 ng/μL) was incubated at 25°C for 80 min in 20 mmol/L Tris-HCl buffer (pH 7.5) containing 20 mmol/L MgCl_2, 0.4 mmol/L CaCl_2, 0.1 mg/mL BSA, 0.25 mmol/L EGTA, 25 ng/mL phosphatidyserine, 2.5 ng/mL diacylglycerol, 0.0075% Triton-X-100, 25 μmol/L DTT, 10 μmol/L Neuroglinin (28–43) peptide substrate and 1 μmol/L ATP in a total volume of 40 μL. CaMKIIz (0.025 ng/μL) was incubated at 25°C for 90 min in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 10 mmol/L MgCl_2, 2 mmol/L CaCl_2, 0.04 mg/mL BSA, 16 μg/mL purified calmodulin from bovine testis, 500 μmol/L DTT, 50 μmol/L Autocamtide 2 and 1 μmol/L ATP in a total volume of 40 μL. After incubation, 40 μL of Kinase-Glo Luminescent Kinase Assay solution (Promega) was added, left to stand at 25°C for 10 min, and relative light units (RLU) were measured using a luminometer. The RLU without test compound was set as 100% (control value) and that without enzyme and compound was set as 0% (normal value). The reaction rate (% of control) was then calculated from the RLU with the addition of each concentration of test compound, and the IC_50 were determined by logistic regression analysis using SAS (version 8.2; SAS Institute Inc., Cary, NC).

Results

In vitro metabolic stability and metabolite profiling of ripasudil with liver S9 and hepatocytes

Ripasudil was metabolised in both the presence and absence of NADPH. Figure 2 shows that there were species differences in metabolite profile after incubation of [14C]ripasudil in rat and human liver S9. Six metabolites (M1–M6) derived from ripasudil were observed on the radiochromatograms. M1 was found at relatively high levels, while M3 and M5 were not found in humans, in contrast to rats. Moreover, M1, M2 and M6 were found independent of NADPH. The intrinsic clearance of [14C]ripasudil in liver S9 is shown in Table 1. In the presence of NADPH-regenerating system (NRS), CLint, S9 was highest in liver S9 of rabbits, followed by those of monkeys, humans, rats and dogs. On the other hand, in the absence of NRS, CLint, S9 was calculated in liver S9 of monkeys, humans and rabbits. Table 2 shows metabolite formation rate, which was calculated from % of total of each metabolite after 15-min reaction. The metabolic abundance of M1 was higher than the other metabolites in monkeys and humans and was approximately equivalent in the presence and absence of NRS. Metabolic abundances of
M2, M3 and M4 in rabbits were higher than those in the other animal species.

The intrinsic clearance of $[^{14}C]$ripasudil in cryopreserved hepatocytes is shown in Table 3. Among the animals, CL$_{int}$, Hep was highest in rabbits followed by monkeys, dogs and rats. For humans, three lots of hepatocytes from different individuals were used, and CL$_{int}$, Hep was found in the range of 15-fold. Table 4 shows the formation rate of metabolites calculated from % total of each metabolite after 1-hour reaction. Metabolic abundances of M1 were remarkably high in monkeys and humans for all metabolites. With regard to other metabolites, the metabolic abundances of M2 in rabbits, dogs and rats, M3 in rabbits, M4 in rabbits and M6 in monkeys were relatively high.

**Metabolite M1 is formed by aldehyde oxidase**

Figure 3 shows the effects of inhibitors on NADPH-independent metabolism using liver S9. Ripasudil was primarily metabolised to M1 and slightly metabolised to M2 in human liver S9 in an NADPH-independent manner. These metabolic reactions were inhibited by menadione and raloxifene as specific AO inhibitors but were not inhibited by allopurinol as an inhibitor of XO. Similar inhibitory effects were found in rabbit and monkey liver S9 (data not shown).

**Pharmacokinetics of ripasudil in humans**

M1 was found in human plasma as the major metabolite after single instillation of ripasudil, and the plasma concentration of M2 was below the limit of quantification at all-time points (Figure 4). Table 5 shows the pharmacokinetic parameters. The peak concentration of ripasudil was observed immediately after instillation (after 0.080 to 0.250 h), showing rapid distribution of ripasudil into systemic circulation after instillation in human subjects. In addition, we found rapid
disappearance of ripasudil from the circulation, and $T_{1/2}$ was 0.621–0.728 h. In contrast, high concentrations of M1 were observed in human plasma. The $C_{\text{max}}$ increased approximately threefold and its $AUC_{0-t}$ increased 22-fold compared to those of ripasudil (from 0.2% to 0.8% group).

**Pharmacokinetics of ripasudil in rats**

Six metabolites were present in rat plasma after single oral administration (Figure 5). Tables 6 and 7 show the pharmacokinetic parameters after single oral administration to male and female rats. In the 3 mg/kg male rat group, the plasma concentration of ripasudil arrived at $C_{\text{max}}$ immediately after oral administration ($T_{\text{max}} = 0.42$ h) and disappeared with a half-life of 0.546 h. Sex differences were found in exposure of metabolites; the concentration of M5 was high in males, while that of M2 was high in females.

**In vitro enzyme kinetics analysis of ripasudil with human liver S9 and human recombinant enzyme systems**

Enzyme kinetics analysis was performed on the metabolic reactions from ripasudil to M1 or M2, from M2 to M6 in human liver S9, and from ripasudil to M1 in the human AO expression system. The substrate concentration ($s$) was plotted against the metabolic formation rate ($v$), and the parameters obtained from fitting analysis are listed in Table 8. The reaction of ripasudil to M1 in human liver S9 and human AO showed similarly small $K_m$ values. The $V_{\text{max}}/K_m$ value of ripasudil to M1 in human liver S9, which represents hepatic intrinsic clearance, was higher than that of ripasudil to M2 and reached almost the same level as the clearance calculated from the decrement of ripasudil. These results indicated that ripasudil is mainly metabolised to M1 by AO, slightly metabolised to M2 by CYP3A4/3A5 and AO, and further metabolised to M6 by AO (Figure 6).

**Inhibitory effects of ripasudil on aldehyde oxidase**

The inhibitory effects of ripasudil on AO activity were investigated using the metabolic reaction from phthalazine to 1-phthalazinone as a marker. The concentration-dependent inhibition of AO activity in human liver cytosol in the presence of ripasudil was observed, and the IC$_{50}$ value of ripasudil was higher than those of the positive control inhibitors menadione and raloxifene (Table 9).

**Kinase inhibition by metabolites of ripasudil**

We evaluated the kinase inhibition properties of major metabolites of ripasudil in humans (Table 10). The IC$_{50}$ of M1 was one-sixth of ripasudil for ROCK 1, and one-ninth of ripasudil for ROCK 2. M2 and M6 showed very weak inhibitory activity in comparison with ripasudil.

**Discussion**

In this study, we evaluated the in vitro and in vivo pharmacokinetic profiles of ripasudil and investigated the...
metabolites and metabolic enzymes involved in the metabolic pathway of ripasudil. We developed ripasudil as an ophthalmic agent without developing an oral agent due to the metabolic properties of this compound.

First, we evaluated the metabolism of ripasudil using liver S9 fractions from several species, including humans. As indicated in Table 1, species differences were found in the intrinsic clearance of ripasudil, and ripasudil was metabolised independent of NADPH in human and monkey S9. The data clearly showed that non-CYP enzymes are associated with ripasudil metabolism. Therefore, we focussed on two major molybdenum hydroxylases that participate in drug metabolism, AO and XO. AO is a cytosolic enzyme and oxidises molybdenum hydroxylases that participate in drug metabol- 

Figure 3. Compositions of ripasudil and its metabolites after incubation of [14C]ripasudil in human liver S9 for 60 min at 37 °C without NADPH-regenerating system. The effects of the inhibitors (allopurinol was used to selectively inhibit xanthine oxidase, while menadione and raloxifene were used to selectively inhibit aldehyde oxidase) were compared with the control (without inhibitors).

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Figure 3. Compositions of ripasudil and its metabolites after incubation of [14C]ripasudil in human liver S9 for 60 min at 37 °C without NADPH-regenerating system. The effects of the inhibitors (allopurinol was used to selectively inhibit xanthine oxidase, while menadione and raloxifene were used to selectively inhibit aldehyde oxidase) were compared with the control (without inhibitors).
CYPs are suggested to contribute to the metabolism of ripasudil in rats, and the pharmacokinetics showed both nonlinearity and sex differences. These phenomena may be due to the metabolic saturation of CYPs and the differences in metabolic abundances of 3A and 2C, which are higher in males than in females (Imaoka et al., 1996). The in vivo data showed species differences in the metabolism of ripasudil similar to the results of in vitro studies. Species differences in AO activity have been well documented for many drugs. AO activity was reported to be high in humans and monkeys, but low in rats, and absent in dogs (Akabane et al., 2011; Diamond et al., 2010; Itoh et al., 2006; Kitamura et al., 1999). The differences in AO activity among species could be explained by the presence of different isozymes encoded by distinct genes (Kurosaki et al., 2013). Human liver expresses only AOX1, monkey liver expresses AOX1 and a small amount of AOX3, dogs theoretically do not have any AOX in the liver and rodents express both AOX1 and AOX3 in the liver. Furthermore, liver AOXs are strictly regulated in a gender-specific manner in mice (Kurosaki et al., 1999). With regard to the safety of metabolites, it can be assumed from the results of toxicological tests in rats that systemic toxicity involving metabolites occurs rarely as plasma exposure of metabolites after oral administration in rats (at the no observed adverse effect level (NOAEL) of ripasudil) was higher than that after instillation in humans.

We next evaluated the enzyme kinetics of metabolite formation using liver S9 and human AO recombinant systems (Table 8). The $K_m$ value of metabolism from ripasudil to M1 was the lowest, and its values in liver S9 and human AO expression system were approximately equal. In contrast, the $V_{max}$ values were different in liver S9 and human AO. These differences in $V_{max}$ are because the AO expression level is lower in the recombinant enzyme system than in liver S9. The $V_{max}/K_m$ value of M1 formation from ripasudil was close to the intrinsic clearance calculated by elimination of ripasudil in liver S9 (Table S1). We showed that AO is an enzyme contributing to the major metabolic pathway to M1. CYP3A4/3A5 and CYP2C8/3A4/3A5 were slightly associated with the formation of M2 and M4, respectively (Table S2). A compound with a similar structure, fasudil, is metabolised to M3 (the reported metabolite name) through oxidation (hydroxylation) at the C-1 position of the isoquinoline ring, similar to ripasudil. Fasudil is cleared quickly from the plasma with a half-life of less than 15 min, whereas its active metabolite, M3, remains in the plasma for 8 hours after infusion in humans (Nakashima et al., 1992). On the other
|                  | 1 mg/kg              |            |            | 3 mg/kg              |            |            | 10 mg/kg             |            |            |
|------------------|----------------------|------------|------------|----------------------|------------|------------|----------------------|------------|------------|
|                  | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) |
| Ripasudil        | 28.85 ± 8.56         | 16.71 ± 3.93 | –          | 265.01 ± 168.69       | 240.71 ± 118.33       | 0.546 ± 0.169 | 2109.15 ± 312.97      | 3066.59 ± 840.30      | 0.772 ± 0.147 |
| M1               | 11.04 ± 1.67         | 8.94 ± 3.51 | –          | 85.38 ± 38.78         | 101.58 ± 48.85        | 1.028 ± 0.308 | 528.74 ± 182.22       | 1144.00 ± 539.19      | 1.563 ± 0.334 |
| M2               | 14.27 ± 3.07         | 11.56 ± 2.33 | –          | 98.83 ± 67.34         | 203.97 ± 86.06        | 1.212 ± 0.346 | 402.45 ± 200.51       | 1288.29 ± 526.07      | 1.434 ± 0.199 |
| M3               | 43.97 ± 13.82        | 61.93 ± 22.10 | 0.847 ± 0.128 | 154.56 ± 79.62        | 126.54 ± 59.83        | –          | 591.34 ± 35.33        | 1085.30 ± 184.79      | 0.780 ± 0.076 |
| M4               | 21.93 ± 2.24         | 10.04 ± 4.89 | –          | 288.77 ± 110.31       | 505.76 ± 183.27       | 1.122 ± 0.314 | 930.44 ± 108.63       | 2744.21 ± 674.15      | 1.550 ± 0.116 |
| M5               | 73.29 ± 11.63        | 79.76 ± 15.82 | 0.885 ± 0.115 | 5.97 ± 4.01           | 6.18 ± 6.59          | –          | 22.09 ± 15.69         | 51.79 ± 43.89         | –          |
| M6               | –                    | –           | –          | 6.61 ± 3.38           | 8.66 ± 6.40          | –          | –                    | –          | –          |

Data represents the means ± SD ($n$ = 3).

–: Not calculated.

Table 6. Pharmacokinetic parameters of ripasudil and its metabolite after single oral administration to male rats.

Table 7. Pharmacokinetic parameters of ripasudil and its metabolite after single oral administration to female rats.

|                  | 1 mg/kg              |            |            | 3 mg/kg              |            |            | 10 mg/kg             |            |            |
|------------------|----------------------|------------|------------|----------------------|------------|------------|----------------------|------------|------------|
|                  | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) | $C_{\text{max}}$ (ng/mL) | $AUC_{\text{0-t}}$ (ng·h/mL) | $T_{1/2}$ (h) |
| Ripasudil        | 120.78 ± 18.01       | 84.14 ± 8.60 | 0.405 ± 0.004 | 632.07 ± 105.33       | 560.20 ± 126.06       | 0.521 ± 0.036 | 2347.06 ± 467.12      | 240.71 ± 118.33       | 0.546 ± 0.169 |
| M1               | 23.79 ± 4.58         | 28.07 ± 3.58 | –          | 34.02 ± 14.69         | 75.48 ± 27.04         | 1.433 ± 0.115 | 112.30 ± 30.76        | 300.79 ± 34.47        | 1.597 ± 0.172 |
| M2               | 95.66 ± 15.65        | 185.65 ± 25.53 | 1.307 ± 0.042 | 33.34 ± 16.05         | 857.45 ± 116.96       | 1.265 ± 0.040 | 1101.15 ± 145.46      | 4841.69 ± 1924.85     | 1.496 ± 0.488 |
| M3               | 37.94 ± 10.35        | 64.20 ± 10.50 | 1.053 ± 0.021 | 75.57 ± 34.60         | 175.17 ± 66.67        | 1.425 ± 0.420 | 194.42 ± 173.32       | 560.95 ± 358.25       | 1.434 ± 0.254 |
| M4               | 33.71 ± 4.27         | 25.66 ± 7.92 | –          | 46.19 ± 1.43          | 182.52 ± 4.06         | 1.514 ± 0.154 | 276.01 ± 22.73        | 663.88 ± 194.75       | 1.059 ($n$ = 2) |
| M5               | 14.61 ± 2.38         | 39.35 ± 5.21 | –          | 14.96 ± 6.79          | 39.45 ± 15.74         | –          | 168.23 ± 63.84        | 836.13 ± 446.52       | 1.572 ($n$ = 2) |
| M6               | 6.61 ± 3.38          | 8.66 ± 6.40 | –          | –                    | –                    | –          | 46.25 ± 10.46         | 213.25 ± 84.68        | 1.970 ± 0.729 |

Data represents the means ± SD ($n$ = 3).

–: Not calculated.
hand, M1 has inhibitory effects on ROCK1/2 and its strength ranges from about one-sixth to one-ninth that of ripasudil, and other metabolites of ripasudil have little activity (Table 10). Ripasudil was not suitable as a prodrug for M1 considering application as the unchanged form with high activity and individual differences in metabolism by AO. The plasma concentration of M1 was much lower than its IC50 value for ROCK inhibition because ripasudil was applied via eye drops. Furthermore, AO was shown to be associated with other metabolism; the conversion of M2 to M6 that occurred with oxidation of the same position as M1 and the conversion of ripasudil to M2 that occurred with oxidation at the C-5 position of the homopiperazine ring (Figure 6).

Lowering the IOP is an effective treatment for glaucoma, and prostaglandin analogues and β-blockers are regarded as representative first-line drugs because of their potent IOP-lowering effects. Ripasudil is expected to be a possible second-line choice similar to carbonic anhydrase inhibitors, α1-blockers and α2-agonists. The occurrence of drug–drug interactions (DDIs) even with topical instillation has been
reported (Edeki et al., 1995), and determination of the potency is necessary. To evaluate possible DDIs, we examined the inhibitory effects of ripasudil on metabolic enzymes; we found very weak effects on CYPs (data not shown) and the IC_{50} value of AO inhibition was 1.4 µmol/L (Table 9). The IC_{50} values of the positive control inhibitors in the AO inhibition assay were similar to those reported in the literature (Obach et al., 2004b), and ripasudil had a weaker inhibitory effect. The C_{max} after topical instillation of 0.4% ripasudil in humans was lower than the IC_{50} value, suggesting that ripasudil is less likely to cause DDIs. Although mainly AO-mediated clinical DDIs have not been reported and systemic exposure after instillation is low, the involvement of AO will have to be considered carefully in future studies.

Conclusions

Marked species differences were observed in the metabolism of ripasudil (K-115), a selective and potent ROCK inhibitor. This study clearly indicated that AO is involved in these species differences, and ripasudil was relatively highly metabolised to M1 in humans. The poor systemic exposure of ripasudil in humans is considered a risk for development as an oral agent. DDIs are thought to be less likely to occur based on the plasma concentration of ripasudil, and many drugs can be used in combination. We hope this study will contribute to the use of ripasudil in treatment of glaucoma as a topical ophthalmic agent.

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Declaration of interest

The authors of this work are employees of Kowa Co., Ltd. The authors are responsible for the content and writing of the paper.

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Table 10. Inhibitory effects of ripasudil and its metabolites on ROCKs.

| Compounds | ROCK 1 | ROCK 2 | PKAC/ε | PKC | CaMKII/ε |
|-----------|--------|--------|---------|-----|----------|
| Ripasudil | 0.051 (0.041–0.064) | 0.019 (0.017–0.021) | 2.1 (1.9–2.4) | 27 (23–33) | 0.37 (0.30–0.47) |
| M1        | 0.32 (0.28–0.36) | 0.17 (0.14–0.19) | 5.4 (4.7–6.2) | 43 (38–50) | 2.4 (1.8–3.2) |
| M2        | 1.4 (1.2–1.7) | 0.47 (0.34–0.66) | >50      | >50      | 5.6 (1.9–2.3) |
| M6        | 20 (17–23) | 7.1 (5.1–10) | >50      | >50      | >50       |

The 95% confidence intervals are shown in parentheses.
