Metabolism of MRX-I, a Novel Antibacterial Oxazolidinone, in Humans: The Oxidative Ring Opening of 2,3-Dihydropyridin-4-One Catalyzed by Non-P450 Enzymes

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ABSTRACT

MRX-I is an analog of linezolid containing a 2,3-dihydropyridin-4-one (DHPO) ring rather than a morpholine ring. Our objectives were to characterize the major metabolic pathways of MRX-I in humans and clarify the mechanism underlying the oxidative ring opening of DHPO. After an oral dose of MRX-I (600 mg), nine metabolites were identified in humans. The principal metabolic pathway proposed involved the DHPO ring opening, generating the main metabolites in the plasma and urine: the hydroxyethyl amino propionic acid metabolite MRX445-1 and the carboxymethyl amino propionic acid metabolite MRX459. An in vitro phenotyping study demonstrated that multiple non–cytochrome P450 enzymes are involved in the formation of MRX445-1 and MRX459, including flavin-containing monoxygenase 5, short-chain dehydrogenase/reductase, aldehyde ketone reductase, and aldehyde dehydrogenase (ALDH). H218O experiments revealed that two 18O atoms are incorporated into MRX445-1, one in the carboxymethyl group and the other in the hydroxyl group, and three 18O atoms are incorporated into MRX459, two in the carboxymethyl group and one in the hydroxyl group. Based on these results, the mechanism proposed for the DHPO ring opening involves the metabolism of MRX-I via FMO5-mediated Baeyer-Villiger oxidation to an enol lactone, hydrolysis to an enol, and enol-aldehyde tautomerism to an aldehyde. The aldehyde is reduced by short-chain dehydrogenase/reductase, aldehyde ketone reductase, or oxidized by ALDH to MRX459. Our study suggests that few clinical adverse drug-drug interactions should be anticipated between MRX-I and cytochrome P450 inhibitors or inducers.

Introduction

Today, antibiotic resistance is still threatening the health of many people worldwide. Therefore, it is of great urgency for the pharmaceutical industry to develop new antibiotics with safer profiles for drug-resistant bacteria (Talbot et al., 2006). Oxazolidinone antibacterial agents bind to a distinct region of the 23S RNA adjacent to the peptidyl transferase center of the 50S ribosomal subunit and show activity against major Gram-positive pathogens (Shinabarger, 1999). Its distinct binding site reduces the cross-resistance between oxazolidinone and other antibiotic drugs. To date, linezolid is the only oxazolidinone antibacterial agent approved for the U.S. Food and Drug Administration for the treatment of serious Gram-positive infections, including those caused by antibiotic-resistant organisms. However, linezolid is subject to serious safety limitations. The primary safety concerns are myelosuppression and monoamine oxidase inhibition, which limit extended linezolid therapy (Vinich and Rubinstein, 2009).

MRX-I, (5S)-5-[(isoxazol-3-ylamino)methyl]-3-[2,3,5-trifluoro-4-(4-oxo-2,3-dihydropyridin-1-yl)phenyl]oxazolidin-2-one, is a novel oxazolidinone antibiotic currently in clinical trials in China. One of the important changes in the structure of the compound is the substitution of the morpholine heterocycle in linezolid with the more planar 2,3-dihydropyridin-4-one (DHPO) ring, which contributes to the increased antibacterial activity of MRX-I compared with that of linezolid (Gordeev and Yuan, 2014). In nonclinical studies, MRX-I demonstrated the same or better efficacy than linezolid in mouse infection models (Li et al., 2014). In preclinical toxicology studies and phase I clinical trials, MRX-I displayed less propensity to induce myelosuppression than did linezolid (Gordeev and Yuan, 2014).

A drug metabolism study is an integral and critical part of drug development. Information, such as the major circulating metabolites and enzymes responsible for their formation, is vital for evaluating drug safety and drug-drug interactions. A preliminary metabolism study in humans demonstrated that MRX-I is mainly metabolized by an unusual metabolic pathway involving the oxidative DHPO ring opening. Therefore, the objectives of the current study were to 1) characterize the metabolic pathways of MRX-I in humans following an oral dose of MRX-I (600 mg); 2) identify the enzymes involved in the DHPO ring opening of MRX-I; and 3) understand the DHPO ring-opening mechanism with H218O experiments.

Materials and Methods

MRX-I, HMS013199, HMS012569, HMS013876, HMI383, MRX-1314, and MRX-1315 were provided by MicuRx Pharmaceuticals Inc. (Shanghai, China). Methimazole, 1-aminobenzotriazole (ABT), 4-methylpyrazole (4-MP), ibuprofenic acid, raloxifene, allopurinol, and disulfiram were purchased from Sigma-Aldrich.

Abbreviations: 4-MP, 4-methylpyrazole; ABT, 1-aminobenzotriazole; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; ALDH, aldehyde dehydrogenase; AO, aldehyde oxidase; FMO, flavin-containing monoxygenase; IDA, information-dependent acquisition; P450, cytochrome P450; SDR, short-chain dehydrogenase/reductase; TOF MS, time-of-flight mass spectrometry; UPLC, ultraperformance liquid chromatography; XO, xanthine oxidase.
The mass range was set at 40–400 mass units. The flow rate was maintained at 0.5% formic acid in 20% aqueous methanol (500 μM final concentration, aldehyde dehydrogenase (ALDH) inhibitor), methimazole (100 μM final concentration, P450 (P450) inhibitor), methimazole (100 μM final concentration, ADH inhibitor), flufenamic acid [100 μM final concentration, ALDH inhibitor] (Stegos et al., 2010), after these inhibitors were incubated with human liver S9 fractions in the presence of NADPH for 15 minutes, the MRX-I in methanol stock solution was added to the incubation system. NADPH was also added to a final concentration of 2.0 mM. The mixture was preincubated at 37°C for 3 minutes. The reactions were initiated by the addition of ice-cold human liver microsomes at a final protein concentration of 0.5 mg/ml, human liver cytosol at a final protein concentration of 2.0 mg/ml, or human liver S9 fractions at a final protein concentration of 2.5 mg/ml. The mixtures were incubated at 37°C for 120 minutes. Each reaction was quenched by the addition of an equal volume of acetonitrile with subsequent centrifugation (11,000 g, 5 minutes, 4°C). The supernatant was dried under a stream of nitrogen at 40°C, reconstituted in 20% aqueous methanol (200 μl), and then analyzed by UPLC/triple TOF MS, as described above, for metabolite profiling. Enzyme Phenotyping Involved in DHPO Ring Opening. To determine the enzymes responsible for the formation of metabolites related to the MRX-I DHPO ring opening, various specific phenotypic inhibitors were added to the S9 fraction incubation system: ABT [1000 μM final concentration, cytochrome P450 (P450) inhibitor], methimazole (100 μM final concentration, P450 and ADH inhibitor, except FMOS), mesnadine [1–100 μM final concentration, aldehyde oxidase (AO) and short-chain dehydrogenase reductase (SDR) inhibitor] (Rosemond and Walsh, 2004; Morrison et al., 2012), raloxifene (0.001–100 μM final concentration, AO inhibitor), metyrapone [50 μM final concentration, xanthine oxidase (XO) inhibitor], allopurinol (100 μM final concentration, XO inhibitor) (Morrison et al., 2012), 4-MP [100 μM final concentration, alcohol dehydrogenase (ADH) inhibitor], flufenamic acid [100 μM final concentration, aldo-keto reductase (AKR) inhibitor] (Rosemond and Walsh, 2004), and disulfiram [50 μM final concentration, aldehyde dehydrogenase (ALDH) inhibitor] (Stegos et al., 2010). After these inhibitors were incubated with human liver S9 fractions in the presence of NADPH for 15 minutes, the MRX-I in methanol stock solution was added to the incubation system. NADP+ was also supplemented when 4-MP was used as an ADH inhibitor and NADPH for disulfiram as an ALDH inhibitor. To test the involvement of the thermally unstable FMOs in the oxidation reaction, human liver S9 fractions were preincubated at 50°C for 5 minutes in the absence of NADPH and then added to the incubation system. The samples were incubated at 37°C for 120 minutes and then processed and analyzed by UPLC/triple TOF MS, as described above. Incubation of MRX-I in Liver Cytosol–Fortified Recombinant FMOs. The MRX-I in methanol stock solution was added at a final concentration of 10 μM (final methanol 0.5%) to a final volume of 200 μl of potassium
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phosphate buffer (0.1 M, pH 7.4). A stock solution of NADPH and a stock solution of human liver cytosol were also added at final concentrations of 2.0 mM and 1 mg/ml, respectively. The mixtures were preincubated at 37°C for 3 minutes, and the reactions were initiated by the addition of ice-cold recombinant FMO1, FMO3, or FMO5 supersomes at a final protein concentration of 0.5 mg/ml. The mixtures were incubated at 37°C for 120 minutes, quenched by the addition of an equal volume of acetonitrile, and then centrifuged (11,000 g, 5 minutes, 4°C). The resulting supernatant was dried under a stream of nitrogen at 40°C, reconstituted in 20% aqueous methanol (200 ml), and then analyzed by UPLC/triple TOF MS, as described above.

H₂¹⁸O Incorporation into the DHPO Ring-Opened Metabolites. To probe the presence of the aldehyde intermediate, the incorporation of oxygen from the medium into the metabolites was assessed by incubation of MRX-I (10 µM) with liver cytosol–fortified FMO5 supplemented with NADPH (2.0 mM) containing H₂¹⁸O (90% v/v). The reactions were incubated at 37°C for 120 minutes and quenched by the addition of an equal volume of acetonitrile. For comparison, the DHPO ring-hydrogenated MRX-I, MRX-1314, was also subjected to the same in vitro experiment as MRX-I. The samples were processed and analyzed by UPLC/triple TOF MS, as described above, to assess the incorporation of H₂¹⁸O into the metabolites, including MRX445-1, MRX445-2, and MRX459.

Incubation of MRX-I and MRX-1314 with Human Hepatocytes. The metabolism of MRX-I and MRX-1314 was evaluated in human hepatocytes. The reactions were incubated in Williams’ E medium (Gibco-BRL, Invitrogen, Grand Island, NY) at a hepatocyte density of 1 × 10⁶ cells/ml. The final MRX-I or MRX-1314 concentration in the cell suspension was 10 µM in a volume of 200 µl. The reactions were incubated at 37°C for 120 minutes, quenched by the addition of an equal volume of acetonitrile, and then centrifuged (11,000 g, 5 minutes, 4°C). The resulting supernatant was dried under a stream of nitrogen at 40°C, reconstituted in 20% aqueous methanol (200 µl), and then analyzed by UPLC/triple TOF MS, as described above.

Results

UPLC/Triple TOF MS Analysis of MRX-I.

The chromatographic and MS fragmentation behaviors of the parent drug, MRX-I, was first investigated to identify MRX-I metabolites. MRX-I was eluted at 17.2 minutes under the chromatographic conditions employed. In the ESI (+) mode, MRX-I gave a protonated molecular ion at m/z 409.1111. The main characteristic product ions of m/z 409.1111 were observed at m/z 365.1212, 409.1095, (-2H₂O), 341.0855, 187.0465, 123.0544. The fragment ion at m/z 365.1212 was proposed to be the result of the loss of CO₂ from MRX-I, and the fragment ions at m/z 281.0899, 255.0749, 251.0790, and 243.0736 formed by the cleavage of the oxazolidinone ring, whereas the fragment ion at m/z 123.0549 was

<table>
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<th>Structure</th>
<th>tₚ</th>
<th>[M + H]⁺</th>
<th>Characteristic Fragment Ions</th>
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Fig. 1. Metabolite profile of human plasma 1.5 hours after the oral administration of 600 mg MRX-I, detected by UPLC/triple TOF MS (A) and UPLC-UV chromatograms of pooled plasma (B) and blank plasma (C). AU, arbitrary units. The inset is the expanded chromatogram in the region of 13–18 minutes.
from the part of the isoxazole amine of MRX-I based on high-resolution mass spectral information (Supplemental Fig. 1; Table 1).

**MRX-I Metabolite Profiling and Identification.**

MRX-I and its metabolites in humans were determined by UPLC-UV/triple TOF MS. In addition to MRX-I, seven metabolites were identified in the pooled human plasma (Fig. 1) and urine (Fig. 2), whereas nine metabolites were detected in human feces (Fig. 3). The metabolite profile of human plasma at 6 hours postdose was not shown, for it was similar to that at 1.5 hours. Although metabolic modifications of the parent drug usually result in a different ionization efficiency and a UV absorption coefficient, the MS response and UV absorbance roughly reflect the relative quantity of the metabolites. As shown in Fig. 1, the most abundant circulating drug-related component was the parent drug. The predominant metabolites in the plasma were MRX445-1 and MRX459, which were eluted at 16.2 and 16.1 minutes, respectively. The plasma concentrations of MRX-I, MRX445-1, and MRX459 were determined by a LC-MS/MS method, and the plasma exposures of MRX445-1 and MRX459 were 26.4% and 24.0% of the parent drug exposure (data not shown). Furthermore, MRX445-1 and MRX459 were also the main drug-related materials detected in the urine (Fig. 2). A large amount of unchanged parent drug was detected in feces, and the major metabolite detected was MRX413, eluted at 9.3 minutes (Fig. 3). Table 1 lists detailed information about these metabolites, including the proposed structures, protonated molecular ions, retention times, and characteristic fragment ions. The metabolites were named in accordance with their protonated molecular weights, and metabolites with the same molecular weight were named in the sequential order of their retention times. The metabolites were identified as follows:

**Parent Drug M0.** A chromatographic peak was detected at 17.2 minutes in human plasma, urine, and feces. It gave a protonated molecular ion at \( m/z \) 409.1111, indicating that its elemental composition was \( C_{18}H_{15}N_{4}O_{4}F_{3} \). The retention time and mass spectral fragmentation patterns were identical to the parent drug, MRX-I, indicating that this component was unmetabolized MRX-I, designated M0.

**MRX343.** Metabolite MRX343, found in plasma, urine, and feces, was eluted at 14.1 minutes, with a protonated molecular mass of 343.0895. The elemental composition of MRX343 was \( C_{15}H_{13}N_{2}O_{4}F_{3} \). The fragment ions at \( m/z \) 255.0737 and 251.0788 were the same as those of MRX-I. The fragment ion at \( m/z \) 123.0549 was not detected, indicating that the isoxazole amine was lost from the parent drug. The fragment ion at \( m/z \) 325.0795 was proposed to be the result of the neutral loss of \( H_2O \) from MRX343. The chromatographic retention time and mass spectral fragmentation patterns of MRX343 were identical to those of HMS013199, so MRX343 was accordingly confirmed as the deaminated metabolite of MRX-I.

**MRX413.** Metabolite MRX413, found in plasma, urine, and feces, was eluted at 9.3 minutes, with a protonated molecular mass of 413.0895. The elemental composition of MRX413 was \( C_{18}H_{15}N_{4}O_{4}F_{3} \). The fragment ions at \( m/z \) 255.0737 and 251.0788 were the same as those of MRX-I. The fragment ion at \( m/z \) 123.0549 was not detected, indicating that the isoxazole amine was lost from the parent drug. The fragment ion at \( m/z \) 325.0795 was proposed to be the result of the neutral loss of \( H_2O \) from MRX343. The chromatographic retention time and mass spectral fragmentation patterns of MRX343 were identical to those of HMS013199, so MRX343 was accordingly confirmed as the deaminated metabolite of MRX-I.
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MRX357. Metabolite MRX357, found in plasma, urine, and feces, was eluted at 15.1 minutes and had a protonated molecular mass of 357.0693, with an elemental composition of C_{18}H_{13}N_{4}O_{4}F_{3}. The fragment ion at m/z 251.0787 was the same as that of MRX-I. The fragment ion at m/z 123.0549 was not detected, indicating that the isoxazole amine was lost from the parent drug. The fragment ion at m/z 339.0587 was proposed to be the result of the neutral loss of H_2O from MRX357. The chromatographic retention time and mass spectral fragmentation pattern of MRX357 were identical to those of HM5012596, the authentic standard. MRX357 was therefore confirmed as the carboxylic acid metabolite of MRX-I, which was formed by oxidative deamination.

MRX407. Metabolite MRX407 was found in plasma, urine, and feces and eluted at 14.2 minutes. It had a protonated molecular mass of 407.0962, with an elemental composition of C_{19}H_{14}N_{4}O_{5}F_{3}, indicating that MRX407 was the dehydrogenated metabolite of MRX-I. The fragment ion at m/z 253.0553 was the same as that of MRX-I. The fragment ions at 363.1061, 279.0737, 253.0585, and 241.0579 were 2 Da smaller than those of the parent drug, indicating that dehydrogenation occurred on the DHPO ring. The chromatographic retention time and mass spectral fragmentation pattern of MRX407 were identical to those of HMS013876. MRX407 was accordingly confirmed as DHPO ring-dehydrogenated MRX-I.

MRX413. Metabolite MRX413 was only found in feces and was eluted at 9.3 minutes. It had a protonated molecular mass of 413.1428, with an elemental composition of C_{19}H_{15}N_{4}O_{5}F_{3}. The fragment ion at m/z 255.0733 was the same as that of MRX-I, indicating that the DHPO ring and trifluorobenzene moieties were not modified. The fragment ion at m/z 369.1528 was a result of the neutral loss of CO_2 from MRX413, and the fragment ion at m/z 339.1426 was proposed to result from the further loss of CH_2O from the fragment ion at m/z 369.1528. The fragment ion at m/z 123.0549 was not present, indicating that the isoxazole ring was modified. The structure of MRX413 was further characterized by NMR after the metabolite was isolated from human feces. The ^1H and ^13C NMR data are listed in Tables 2 and 3, respectively. Comparing the chemical shifts of MRX413 with those of MRX-I, it was shown that the DHPO ring and trifluorobenzene moieties were unchanged. However, the sp^3-hybridized methine signals of isoxazole disappeared and new signals from a methylene group attached to an oxygen atom (i.e., δ_C 58.2, δ_H 3.88, CH₂-18) and a methylene group (i.e., δ_C 35.9, δ_H 2.72, CH₂-17) appeared. The structure of MRX413 was further confirmed as the reduced isoxazole ring-opened metabolite with ^1H-^1H homonuclear correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear singular quantum correlation (HSQC) (Supplemental Fig. 5; Table 1).

MRX445-I. Metabolite MRX445-I was the most prominent metabolite in plasma and urine and was also found in feces. MRX445-I was eluted at 16.2 minutes and had a protonated molecular mass of 445.1322, with an elemental composition of C_{18}H_{19}N_{4}O_{6}F_{3}, indicating the dihydrogenated metabolite of MRX-I. The major fragment ions at m/z 365.1219, 281.0899, 255.0733, and 243.0736 of MRX-I were not found in MRX445-I, indicating that the DHPO ring might be modified. The structure of MRX445-I was further characterized by NMR after the metabolite was isolated from human urine. The ^1H and ^13C NMR data are listed in Tables 2 and 3, respectively. Comparison of the chemical shifts of MRX445-I with those of MRX-I showed that the trifluorobenzene, oxazolidinone, and isoxazole amine moieties were unchanged. However, the characteristic NMR signals of the MRX-I DHPO ring had disappeared in MRX445-I. Signals 1H NMR chemical shift assignments for MRX-I, MRX445-1, MRX445-2, and MRX413.
**TABLE 3**

$^{13}$C NMR chemical shift assignments for MRX-I, MRX445-1, MRX445-2, and MRX413

NMR spectra shown in Supplemental Figs. 2-5.

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$d$, CH or CF; $q$, CH$_3$; $s$, C$_2$ t, CH$_2$. 
of MRX445-1 are shown in Table 1. The fragment ions at m/z 341.0860 and 123.0555 were the same as those of MRX445-1, indicating that the trifluorobenzene, oxazolidinone, and isoxazole amine moieties were not modified. However, the fragment ion at m/z 385.1109 was not detected, indicating the loss of the hydroxyethyl group from MRX445-1. MRX401 was accordingly identified as N-dehydroxyethyl MRX445-1.

MRX459. Metabolite MRX459 was eluted at 16.1 minutes and was a major metabolite in plasma and urine and also found in feces. It had a protonated molecular mass of 459.1114, with an elemental composition of C16H13N4O5F3, indicating the introduction of an oxygen atom accompanied by dehydropenogenation relative to MRX445-1. The fragment ions at m/z 341.0860, 187.0473, and 123.0555 were the same as those of MRX445-1, suggesting that the trifluorobenzene, oxazolidinone, and isoxazole amine moieties were not modified. The fragment ion at m/z 385.1109 was not observed, but a fragment ion at m/z 399.0902 (+14 Da) appeared. A fragment ion derived from decarboxylation was also observed at m/z 413.1059. The chromatographic retention time and mass spectral fragmentation pattern of MRX459 were identical to those of HM1383. Therefore, MRX459 was identified as oxidized MRX445-1, where the hydroxyethyl group was converted to a carboxylic acid (Table 1).

MRX445-2. Metabolite MRX445-2, found in plasma, urine, and feces, was eluted at 16.7 minutes and had a protonated molecular mass of 445.1316, which was the same as that of MRX445-1. Although MRX445-2 had the same elemental composition as MRX445-1, it produced different fragment ions. The structure of MRX445-2 was characterized by NMR after the metabolite was isolated from human urine. The 1H and 13C NMR data are listed in Table 2 and 3. Comparison of the chemical shifts of MRX445-2 with those of MRX-I and MRX445-1 showed that the trifluorobenzene, oxazolidinone, and isoxazole amine moieties were unchanged. Like MRX445-1, the characteristic NMR signals of the DHPO ring of MRX-I had disappeared in MRX445-2. New signals from a carboxyl group (δC 173.5, C-2), methylene group (δC 23.4, and 2.27, C-1), and a methine group (δH 8.6, 6.5, CH-5) had appeared. The 1H NMR spectrum of MRX445-2 revealed the presence of two active NH protons (δN 6.59 and 5.61), indicating the opening of the DHPO ring at the C-N bond. The introduction of NH shifted the C-3 and C-11 signals upfield from δC 49.6 to 43.1 and 121.8 to 113.5, respectively.

## Table 4

<table>
<thead>
<tr>
<th>Inhibitor or Process</th>
<th>Inhibitor Concentration</th>
<th>Target Enzyme</th>
<th>Metabolite Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td></td>
<td>MRX445-1</td>
</tr>
<tr>
<td>No inhibitor (control)</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>ABT*</td>
<td>1000</td>
<td>P450</td>
<td>91.2</td>
</tr>
<tr>
<td>Methimazole*</td>
<td>100</td>
<td>P450, FMO1–FMO4</td>
<td>92.0</td>
</tr>
<tr>
<td>Heated</td>
<td>100</td>
<td>FMO1, FMO3, FMO4, and FMO5</td>
<td>7.22</td>
</tr>
<tr>
<td>Menadione*</td>
<td>10</td>
<td>AO and SDR</td>
<td>44.6</td>
</tr>
<tr>
<td>Raloxifene*</td>
<td>10</td>
<td>AO</td>
<td>83.1</td>
</tr>
<tr>
<td>Methotrexate*</td>
<td>50</td>
<td>XO</td>
<td>90.2</td>
</tr>
<tr>
<td>Allopurinol*</td>
<td>100</td>
<td>XO</td>
<td>110</td>
</tr>
<tr>
<td>Flu fenamic acid*</td>
<td>100</td>
<td>AKR</td>
<td>69.4</td>
</tr>
<tr>
<td>4-MIP*</td>
<td>100</td>
<td>ADH</td>
<td>103</td>
</tr>
<tr>
<td>Disulfiram*</td>
<td>50</td>
<td>ALDH</td>
<td>80.0</td>
</tr>
</tbody>
</table>

* NADPH (2.0 mM) was supplemented as a coenzyme.

* NADPH (2.0 mM) and NADP+ (2.0 mM) were supplemented as a coenzyme.
The reduction of the carbonyl group on C-5 shifted the C-4 signals downfield from $\delta_{C} 36.5$ to 42.3 (Supplemental Fig. 4). MRX445-2 was accordingly identified as the 5-amino-3-hydroxypentanoic acid metabolite of MRX-I formed by the opening of the DHPO ring (Table 1).

MRX427. Metabolite MRX427, found only in feces, was eluted at 13.6 minutes. It had a protonated molecular mass of 427.1227, with an elemental composition of C$_{18}$H$_{17}$N$_{4}$O$_{5}$F$_{3}$. The mass spectrum of $m/z$ 427.1 showed major fragment ions at $m/z$ 409.1119, 368.0851, 281.0889, 243.0727, and 123.0544. In the product ion spectrum of $m/z$ 427.1, the fragment ion at $m/z$ 409.1119 was a result of the neutral loss of H$_2$O ($\Delta m 18.0108$ Da) from MRX427, indicating that MRX427 might contain an alcoholic hydroxyl group. The fragment ions at $m/z$ 281.0889, 243.0727, and 123.0544 were the same as those of MRX-I. The exact structure of MRX427 needs to be further characterized.

Formation of MRX445-1 and MRX459 Cocatalyzed by Enzymes in Human Liver Microsomes and Cytosolic Fractions

After MRX-I was incubated with human liver microsomes in the presence of NADPH for 120 minutes, traces of oxidatively deaminated metabolites of MRX-I, such as MRX343, were detected by UPLC/triple TOF MS. The formation of DHPO ring-opened metabolites, including MRX445-1, MRX445-2, and MRX459, was not observed in the human liver microsomes (Fig. 4A). After MRX-I was incubated with human liver cytosolic fractions in the presence of NADPH for 120 minutes, only MRX445-2 was produced without the major metabolites in humans, MRX445-1 and MRX459 (Fig. 4B). However, all the DHPO ring-opened metabolites detected in humans, including MRX445-1, MRX445-2, and MRX459, were observed after MRX-I was incubated with human liver S9 fractions for 120 minutes (Fig. 4C). These results indicate that liver microsomal or cytosolic enzymes alone do not allow the biotransformation of MRX-I to MRX445-1 and MRX459, whereas S9 fractions provided the necessary enzyme systems to complete the metabolic pathways. The enzymes in liver microsome and cytosolic fractions cocatalyzed the opening of the DHPO ring of MRX-I.

Chemical Inhibition of the Formation of MRX445-1, MRX445-2, and MRX459 in S9 Fractions

MRX-I was incubated with the NADPH-supplemented human liver S9 fractions in the presence of various specific chemical inhibitors to investigate the enzymes involved in the formation of MRX445-1, MRX445-2, and MRX459. As shown in Table 4, ABT and methimazole did not significantly inhibit the formation of MRX445-1, MRX445-2, and MRX459, revealing that the oxidative opening of the DHPO ring was not catalyzed by P450 and FMO1–FMO4. However, the formation of MRX445-1 and MRX459 was reduced to 7.22% and 0%, respectively, after brief preincubation of liver S9 fractions at 50°C in the absence of the cofactor. This indicates that FMOs, other than FMO1–FMO4, are involved in the formation of MRX445-1 and MRX459. Menadione, flufenamic acid, and disulfiram reduced the formation of MRX445-1 to 44.6%, 69.4%, and 80.0%, respectively, in liver S9 fractions, whereas 4-MP had no effect on the formation of MRX445-1. This indicates that the reduction of the carbonyl group could be a step in the formation of MRX445-1 from MRX-I, which is catalyzed by multiple enzymes, including SDR, AKR, and ALDH. Furthermore, disulfiram also decreased the formation of MRX459 to 10.7%, suggesting that the oxidation of the aldehyde intermediate could be a step of the formation of MRX459, which is catalyzed by ALDH. The XO inhibitors methotrexate and allopurinol did not affect the formation of MRX445-1 from MRX-I, which is catalyzed by multiple enzymes, including SDR, AKR, and ALDH. Furthermore, disulfiram also decreased the formation of MRX459 to 10.7%, suggesting that the oxidation of the aldehyde intermediate could be a step of the formation of MRX459, which is catalyzed by ALDH. The XO inhibitors methotrexate and allopurinol did not affect the formation of MRX445-1 from MRX-I. However, the formation of MRX445-2 was concentration-dependently inhibited by the AO inhibitors menadione (IC$_{50}$ = 8.0 $\mu$M) and raloxifene (IC$_{50}$ = 0.66 $\mu$M), respectively, as shown in Supplemental Fig. 6. These indicated that AO is involved in the formation of MRX445-2.

Formation of MRX445-1, MRX445-2, and MRX459 by Incubation of MRX-I with Liver Cytosol–Fortified FMO5

After MRX-I was incubated with liver cytosol–fortified FMO1, FMO3, or FMO5 in the presence of NADPH for 120 minutes, MRX445-2 was produced from MRX-I (Fig. 5). This is consistent with the results of incubating MRX-I with human liver cytosolic fractions and the chemical...
inhibition of MRX445-2 formation, which demonstrated that the for-
motion of MRX445-2 was catalyzed by AO and XO in the liver cytosolic
fractions and not by oxidative enzymes in the liver microsomes. The
formation of MRX445-1 and MRX459 was not observed during the
incubation of MRX-I with liver cytosol
–
fortified FMO1 or FMO3, but
with liver cytosol–fortified FMO5. This confirms that FMO5 is involved
in the formation of MRX445-1 and MRX459, as shown in the inhibition
experiments for MRX445-1 and MRX459 formation in the human liver
S9 fractions.

Water Is the Source of Oxygen Atoms in DHPO Ring-Opened
Metabolites

To explore the mechanism of the oxidative DHPO ring opening, MRX-I and DHPO ring-hydrogenated MRX-I (MRX-1314) were in-
cubated with liver cytosol–fortified FMO5 in the presence of H218O.
UPLC/triple TOF MS analysis clearly showed that MRX445-1, derived
from MRX-I, had a protonated molecular weight of 449.1303 (Fig. 6B),
suggesting that two atoms of 18O from H218O were incorporated into
MRX445-1 by comparing the protonated molecular weight of MRX445-
1 (445.1322) in the absence of H218O (Fig. 6A). MS/MS analysis of the
protonated molecular ion at m/z 449.1 showed major fragment ions at
m/z 387.1065 and 341.0760. The fragment ion at m/z 387.1065 resulted
from the loss of CH3CO18OH, and the fragment ion at m/z 341.0760 arose
from the further loss of CHCH218OH. This reveals that one 18O atom
was incorporated into the hydroxylethyl group and the other 18O atom
was incorporated into the carboxylethyl group of MRX445-1 obtained from
MRX-I (Fig. 6B). The UPLC/triple TOF MS analysis also showed that
MRX445-1 obtained from MRX-1314 had a protonated molecular
weight of 449.1307 (Fig. 6C). Suggesting that two 18O atoms from H218O
were incorporated into MRX445-1. MS/MS analysis of the protonated
molecular ion at m/z 449.1 showed the major fragment ions at m/z 387.1065.
407.1017 and 341.0762. The fragment ion at m/z 387.1065 resulted from
the loss of CH3CO18OH, and the fragment ion at m/z 341.0760 arose
from the further loss of CHCH218OH. This reveals that one 18O atom
was incorporated into the hydroxylethyl group and the other 18O atom
was incorporated into the carboxylethyl group of MRX445-1 obtained from
MRX-I (Fig. 6B).
of the loss of CH$_3$C$^{18}$O$^{18}$OH. This indicates that two $^{18}$O atoms from H$_2$$^{18}$O were incorporated into the carboxylethyl group of MRX445-1 obtained from MRX-1314 (Fig. 6C). These results demonstrate that the ring opening of MRX-I and MRX-1314 occurred via different routes.

The UPLC/triple TOF MS analysis showed that MRX445-2 from MRX-I had a protonated molecular weight of 449.1296 (Fig. 6E). Comparison of the protonated molecular weight of MRX445 in the absence of H$_2$$^{18}$O (459.1114) (Fig. 6F) suggests that three $^{18}$O atoms from H$_2$$^{18}$O were incorporated into MRX459. MS/MS analysis of the protonated molecular ion at $m/z$ 465.1 showed major fragment ions at $m/z$ 415.0993, 403.0888, and 341.0768. The fragment ion at $m/z$ 415.0993 resulted from the loss of CH$_1^{18}$O$_2$H; the fragment ion at $m/z$ 403.0888 resulted from the loss of CH$_3$C$^{18}$OOH; and the fragment ion at $m/z$ 341.0758 was formed by the neutral loss of CH$_2$CO$^{18}$OHCH$_2$C$^{18}$O$^{18}$OH. This shows that one $^{18}$O atom was incorporated into the carboxylethyl group and that the other two atoms were incorporated into the carboxylmethyl group of MRX459 (Fig. 6G).

Incubating MRX-1314 with human liver cytosol–fortified FMO5 did not produce MRX459 or MRX445-2.

**Marked Metabolic Differences between MRX-I and MRX-1314 in Human Hepatocytes**

The formation of MRX445-1 in the H$_2$$^{18}$O experiments showed that the opening of the DHPO ring in MRX-I and MRX-1314 occurs via different routes. To clarify the possible mechanisms further, MRX-I and MRX-1314 were incubated with human hepatocytes, which contain all the liver enzymes in the liver and provide a more representative system than other in vitro liver models. After the incubation of MRX-I with human hepatocytes, all the DHPO ring-opened metabolites detected in humans were observed, including MRX445-1, MRX445-2, MRX459, and MRX401 (Fig. 7A). Furthermore, MRX445-1 was identified as the principle metabolite of MRX-I, which was observed in vivo. MRX445-1 was also present in the MRX-1314 incubation system at a similar level to that in the MRX-I incubation system (Fig. 7B). However, most of the MRX-1314 in the human hepatocyte incubation system was

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**Scheme 1.** Proposed metabolic pathway of MRX-I in humans.
reduced to MRX-1315, which was not observed in the MRX-I incubation system. MRX459 was also not detected in the MRX-1314 incubation system. This demonstrates further significant metabolic differences between MRX-I and MRX-1314.

**Discussion**

The metabolism of MRX-I, a novel antibiotic oxazolidinone for treatment of Gram-positive infections, was studied by UPLC-UV/triple TOF MS analysis after an oral dose of 600 mg of MRX-I to healthy male volunteers during phase I clinical trials. Nine metabolites were detected, and six were confirmed with synthesized or isolated reference standards. The proposed metabolic processes are shown in Scheme 1. The oxidative ring opening of the DHPO is the main metabolic pathway of MRX-I in humans via which the major metabolites MRX445-1, MRX445-2, and MRX459 were formed. To identify the enzymes involved in the DHPO ring opening, MRX-I was incubated with various in vitro human liver models, including liver microsomes, cytosol, S9 fractions, and primary hepatocytes. By co-incubating MRX-I with various specific chemical inhibitors in the human liver S9 fractions, the enzymes involved were identified as FMOs, AKR, SDR, ALDH, and AO. The involvement of FMO5 in the formation of MRX445-1 and MRX459 was further confirmed by incubating MRX-I with liver cytosol-fortified FMOs, including FMO1, FMO3, and FMO5.

As a group of important drug-metabolizing enzymes, FMOs catalyze oxidative reactions that are complementary to P450-mediated biotransformations (Strolin Benedetti et al., 2006). Among the isoforms identified, FMO3 and FMO5 are recognized as the major isoforms in adult human liver (Overby et al., 1997; Zhang and Cashman, 2006). Typical FMO-catalyzed reactions have long been recognized, including the mono-oxygenation of heteroatoms, such as nitrogen, sulfur, and phosphorus (Strolin Benedetti et al., 2006; Cashman 2008). However, recent studies have shown that FMO5 specifically catalyzes the Baeyer-Villiger oxidation of piperidine-4-one (Lai et al., 2011) and cyclic α,β-unsaturated

![Scheme 2. Proposed metabolic pathway for the formation of MRX445 from MRX-I by analogy with previous studies (Verhoeven et al., 1998; Lai et al., 2011).](image2)

![Scheme 3. Proposed mechanism for the formation of MRX445-1, MRX445-2, and MRX459 from MRX-I in the presence of H2^{18}O.](image3)
ketone moieties (Verhoeven et al., 1998). Verhoeven et al. also proposed that the ring-opening mechanism of cyclic \( \alpha,\beta \)-unsaturated ketones involved the reduction of the double bond of the unsaturated ketone, followed by Baeyer-Villiger oxidation catalyzed by FMOs. By analogy with previous reports, we proposed that the mechanism for the DHPO ring opening of MRX-I was as follows. MRX-I is first reduced to MRX-1314 by liver cytosolic reductases, followed by ring-expanding oxidation to a lactone via the Baeyer-Villiger reaction in liver microsomes. The lactone was then hydrolyzed to produce MRX445-1, which can be further oxidized to MRX459 (Scheme 2).

To test the proposed mechanism, the DHPO ring-hydrogenated metabolic intermediate, MRX-1314, was synthesized and incubated with human hepatocytes in vitro and compared with MRX-I. Although similar levels of MRX445-1 were produced independently when MRX-I and MRX-1314 were used as substrates, marked differences between MRX-I and MRX-1314 metabolism were observed. In the hepatocyte system, most of the MRX-1314 was converted to its carbonyl-reduced metabolite, MRX-1315, which indicates that the reduction of ketone is the principal metabolic pathway of MRX-1314. However, MRX-1315 was not detected in the MRX-I incubation systems. This suggests that MRX-1314 is not the metabolic intermediate in the formation of MRX445-1 from MRX-I. Furthermore, during its incubation with human hepatocytes, some MRX-I was transformed into MRX459, which could be formed by the further oxidation of the hydroxyethyl group on MRX445-1 because the oxidation of primary alcohols to carboxylic acids is one of the most common metabolic pathways of drugs in humans. However, no MRX459 was detected in the MRX-1314 incubation system, although the level of MRX445-1 produced in the MRX-1314 system was similar to that produced in the MRX-I system. This can be explained by the presence of an aldehyde intermediate in the MRX-I incubation system, from which MRX445-1 is formed by reduction and MRX459 is formed by oxidation. This is plausible because menadione (SDR inhibitor), flufenamic acid (AKR inhibitor), and disulfiram (ALDH) inhibited the formation of MRX445-1, and disulfiram (ALDH) inhibited the formation of MRX 459 from MRX-I (Table 4). Therefore, we propose a novel metabolic mechanism (Scheme 3) for the formation of MRX445-1 and MRX459. MRX-I is metabolized to an enol lactone via Baeyer-Villiger oxidation, hydrolyzed to an enol, and transformed to an aldehyde by enol-aldehyde tautomerism, after which the aldehyde intermediate is reduced to MRX445-1 or oxidized to MRX459.

In the Baeyer-Villiger oxidation of \( \alpha,\beta \)-unsaturated ketones, vinyl migration is generally favored over alkyl migration to produce ring-expanded enol lactones (Shono et al., 1974; Krafft and Katzenellenbogen, 1981), which supports the mechanism proposed in Scheme 3. Although the regiochemical selectivity of Baeyer-Villiger oxidation is recognized in chemistry, this is the first observation of the metabolism of xenobiotics to an aldehyde intermediate by direct Baeyer-Villiger oxidation of an \( \alpha,\beta \)-unsaturated ketone and hydrolysis of the enol lactone. To confirm the existence of the aldehyde intermediate, MRX-I or MRX-1314 was incubated in liver cytosol–fortified recombinant FM05 in the presence of H\(_2\)O\(_{18}\). The isotope experiment revealed that the MRX445-1 produced from MRX-I had incorporated two \( ^{18} \)O atoms derived from water: one in the carboxyethyl group and the other in the hydroxyethyl group. Because Baeyer-Villiger oxidation is catalyzed specifically by FM05, an oxygenase, the hydroxyethyl oxygen atom of MRX445-1 should be derived from \( \mathrm{O}_2 \) rather than from \( \mathrm{H}_2\mathrm{O} \). Therefore, the \( ^{18} \)O atom of the hydroxyethyl group of MRX445-1 indicates the existence of an aldehyde intermediate, which incorporated an \( ^{18} \)O atom from \( \mathrm{H}_2\mathrm{O} \) during nonenzymatic equilibrium with its geminal diol (Scheme 3). It is clear that the hydroxyl \( ^{18} \)O atom of the carboxylic acid was derived from \( \mathrm{H}_2\mathrm{O} \) during the hydrolysis of the enol lactone. The isotope experiments also demonstrated that the MRX445-1 produced from MRX-1314 incorporated two \( ^{18} \)O atoms derived from \( \mathrm{H}_2\mathrm{O} \). However, both atoms were incorporated in the carboxyethyl group, which differed from the results for the MRX445-1 produced from MRX-I. The carbonyl oxygen of the carboxyethyl group incorporated an \( ^{18} \)O atom because the ketone \( ^{16} \)O atom was exchanged with the \( \mathrm{H}_2\mathrm{O}^{18} \) \( ^{18} \)O atom during ketone-geminal diol equilibrium (Scheme 4). Furthermore, MRX-1315 with an \( ^{18} \)O-labeled hydroxyl group was the main metabolite of MRX-1314 in the presence of H\(_2\)O\(_{18}\), which also indicates that ketone-geminal diol equilibrium of MRX-1314 occurred (data not shown). MRX445-1 produced from MRX-1314 incorporated an \( ^{18} \)O atom in the hydroxyl group of the carboxyethyl group during lactone hydrolysis, which was identical to the results for MRX445-1 produced from MRX-I.

MRX459 incorporated three \( ^{18} \)O atoms from H\(_2\)O\(_{18}\) that was produced when MRX-I was incubated with liver cytosol–fortified FM05 in the presence of H\(_2\)O\(_{18}\). Two \( ^{18} \)O atoms were incorporated in the carboxyl hydroxyl groups of MRX459. The other \( ^{18} \)O atom was on the carboxymethyl carbonyl group, which could be incorporated during the oxidation of the aldehyde intermediate to the carboxylic acid (Scheme 3). This indicates that not oxygenases but oxidases, such as AO, OX, and ALDH (Uetrecht and Trager, 2007), are involved in the formation of MRX459 because the oxygen atom incorporated into the substrates by these enzymes is from \( \mathrm{H}_2\mathrm{O} \) rather than \( \mathrm{O}_2 \). The involvement of ALDH in the formation of MRX459 confirmed that disulfiram reduced the formation of MRX459 from MRX-I to 10.7%. The reaction

![Scheme 4. Proposed mechanism for the formation of MRX445-1 and MRX-1315 from MRX-1314 in the presence of H\(_2\)O\(_{18}\).](image-url)
phenotyping experiments showed that menadione and raloxifene reduced the formation of MRX445-2, with IC₅₀ values of 8.0 and 0.66 μM, respectively. This suggests that AO catalyzed the formation of MRX445-2. The involvement of AO in the formation of MRX445-2 was also supported by the H₂¹⁸O experiments. After MRX-I was incubated with human liver cytosol and H₂¹⁸O, two ¹⁸O atoms were incorporated into MRX445-2, including one in the carbonyl group of carboxyl acid, which indicates that the carbon was oxidized by AO (Scheme 3).

Finally, we have characterized the metabolism of MRX-I in humans. The major metabolic pathway of MRX-I involves the oxidative opening of the DHPO ring. The mechanism underlying the directed opening of the proposed DHPO ring is that MRX-I is first oxidized to an enol lactone via the Baeyer-Villiger ring-expanding reaction, which is specifically catalyzed by FMO5, followed by its hydrolysis to an enol and then transformation to an aldehyde intermediate by enol-aldehyde tautomerism. The aldehyde is reduced by SDR, AKR, and ALDH to MRX445-1 or oxidized to MRX459 by ALDH. Our study demonstrated that the main metabolic pathway of MRX-I via the opening of the DHPO ring is not catalyzed by P450s, the enzymes primarily responsible for metabolizing drugs and xenobiotics, but by multiple other enzymes, including FMO5, SDR, AKR, ALDH, and AO. Therefore, few clinical adverse drug-drug interactions should be anticipated between MRX-I and P450 inhibitors or inductors.

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References

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