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ORIGINAL ARTICLE

Effect of structural modification on the gastrointestinal stability and hepatic metabolism of α-aminoxy peptides

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Abstract α-Aminoxy peptide AxyP1 has been reported to form synthetic chloride channel in living cells, thus it may have therapeutic potential for the treatment of diseases associated with chloride channel dysfunction. However, this study revealed significant gastrointestinal (GI) instability and extensive hepatic metabolism of AxyP1. To improve its GI and metabolic stability, structural modifications were conducted by replacing the isobutyl side chains of AxyP1 with methyl group (AxyP2), hydroxymethyl group (AxyP3), 4-aminobutyl group (AxyP4) and 3-carboxyl propyl group (AxyP5). Compared with AxyP1 (41 and 47 % degradation), GI stability of the modified peptides was significantly improved by 8-fold (AxyP2), 9-fold (AxyP3) and 12-fold (AxyP5) with no degradation for AxyP4 in simulated gastric fluid within 1 h, and by 12-fold (AxyP2) and 9-fold (AxyP3) with no degradation for AxyP4 and AxyP5 in simulated intestinal fluid within 3 h, respectively. The hepatic metabolic stability of the four modified peptides within 30 min in rat liver S9 preparation was also improved significantly with no metabolism of AxyP5 and threefold (AxyP2 and AxyP4) and eightfold (AxyP3) less metabolism compared with AxyP1 (39 % metabolism). Unlike hydrolysis as the major metabolism of peptides of natural α-amino acids, oxidation mediated by the cytochrome P450 enzymes, especially CYP3A subfamily, to form the corresponding mono-hydroxyl metabolites was the predominant hepatic metabolism of the five α-aminoxy peptides tested. The present findings demonstrate that structural modification can significantly improve the GI and metabolic stability of α-aminoxy peptides and thus increase their potential for therapeutic use in the treatment of chloride channel related diseases.

Keywords Structural modification · Hepatic metabolism · Gastrointestinal stability · α-Aminoxy peptide · Cytochrome P450 enzyme

Abbreviations

CYP	Cytochrome P450 enzyme
EDTA	Ethylene diamine tetraacetic acid
FMO	Flavin-containing monooxygenase
G-6-P	D-Glucose 6-phosphate
G-6-P-DH	Glucose-6-phosphate dehydrogenase
GI	Gastrointestinal
HLM	Human liver microsome
β-NADPH	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetra sodium salt hydrate
rCYP3A4	Recombinant CYP3A4
RLM	Rat liver microsome
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid

Introduction

Peptides containing natural α-amino acids have continuously demonstrated promising pharmacological activities in vitro, making them an important class of potential drug

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candidates. However, most of them fail to show convincing therapeutic efficacy in vivo. One major reason is their extensive metabolism, being catalyzed by a variety of enzymes, especially proteases in the liver and other organs (Gorris et al. 2009; Werle and Bernkop-Schnurch 2006). In addition, degradation in the gastrointestinal (GI) tract due to strong pH extremes and enzyme-mediated first-pass metabolism in the intestine are also responsible for their limited clinical application (Mahato et al. 2003). Many strategies have been used to improve the stability of natural peptides, and structural modification is one of the focuses in recent years. Among various classes of structurally modified peptides, peptidomimetics, which are small molecules mimicking the functions of natural peptides, have been developed and proved to have better structural and metabolic stability (Goodman et al. 2007; Hill et al. 2001; Li et al. 2008). In our previous studies, a series of peptidomimetics containing a mixed sequence of α-amino acids and α-aminoxy acids were synthesized. The α-aminoxy acids were analogs of β-amino acids in which the β-carbon was replaced by an oxygen atom. Our previous findings demonstrated that metabolic stability of these synthetic peptidomimetics was significantly improved compared to peptides composed of only natural α-amino acids, indicating that α-aminoxy acid moiety was resistant to proteases and other enzymes (Li et al. 2008; Li and Yang 2006; Chen et al. 2011). Subsequently, a series of α-aminoxy peptides containing only α-aminoxy acids were synthesized for further investigation of their metabolic stability and pharmacokinetic profile. It was revealed that these peptides self-assembled into synthetic ion channels in the lipid membranes and mediated ions flow across the membranes of living cells with remarkably high efficiency (Li et al. 2007, 2008, 2009). One representative α-aminoxy peptide AxyP1 (Fig. 1) formed anion channels in the lipid bilayers and MDCK cells. Quantitative assessments of the binding affinities of AxyP1 toward different anions revealed that AxyP1 was selective to chloride ions over other anions (Li et al. 2007). Therefore, they have potential to be developed as therapeutic agents for treatment of diseases associated with chloride ion channel dysfunction, such as cystic fibrosis.

In our systematic pharmacokinetic study of these α-aminoxy peptides, the first absorption study demonstrated that their intestinal absorption varied significantly with different structures due to different transport mechanisms. Although the representative AxyP1 had poor oral absorbability, its absorption was able to be dramatically improved by structural modifications (Ma et al. 2011). Consequently, the metabolism of the α-aminoxy peptides and their stability in the GI tract and the liver became a key issue for our follow-on study, and is the main focus in the present study. Our initial study on AxyP1 revealed that this

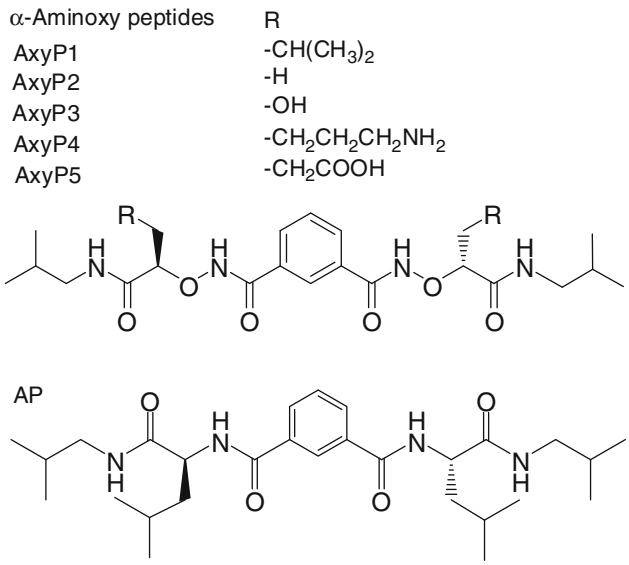


Fig. 1 Chemical structures of AP (α-amino acid peptide) and α-aminoxy peptides AxyP1, AxyP2, AxyP3, AxyP4 and AxyP5

representative α-aminoxy peptide underwent extensively hepatic metabolism and was unstable in the GI tract. Subsequently, structural modifications of AxyP1 were conducted and four analogs (AxyP2 to AxyP5, Fig. 1) were designed by replacing the isobutyl side chains of AxyP1 with methyl group (AxyP2), hydroxymethyl group (AxyP3), 4-aminobutyl group (AxyP4) and 3-carboxyl propyl group (AxyP5), respectively. The present study investigated the effects of structural modification on both hepatic metabolism of these peptides by rat hepatic subcellular fractions and human liver microsome (HLM), and the GI stability using intestinal S9 subcellular fractions, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). For the comparison, an α-amino acid peptide (AP in Fig. 1) was also studied in parallel.

Materials and methods

Materials

The synthesis of the α-aminoxy peptides (AxyP1 to AxyP5), and their structural identification and purity have been described previously (Li et al. 2007; Ma et al. 2011). Cimetidine, dexamethasone, diethyldithiocarbamate, ethylene diamine tetraacetic acid (EDTA), D-glucose 6-phosphate disodium salt hydrate (G-6-P), glucose-6-phosphate dehydrogenase from baker's yeast (*S. cerevisiae*), type VII ammonium sulfate suspension (G-6-P-DH), glycerol, methimazole, 8-methoxypsoralen, metyrapone, midazolam, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), quinine and SKF

525A were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Human liver microsomes, supersomes expressing recombinant cytochrome P450 3A4 (rCYP3A4) with supplementation of cytochrome b5 and P450 reductase, were obtained from BD Biosciences (Woburn, MA). All other compounds and reagents not listed were of analytical grade.

Instrumentation

Quantification of the peptides and their metabolites was performed by HPLC–UV using an Agilent 1100 series HPLC system coupled with a diode array detector. Separation was achieved using a Zorbax SB C18 column (150 mm × 4.6 mm i.d., 5 μm; Agilent, USA) with an Agilent C18 guard column (10 mm × 4.6 mm i.d., 5 μm) at 25 °C with a detecting wavelength at 230 nm. Identification of metabolites was performed by HPLC–MS using an Agilent 1200 HPLC system connecting with an API 2000 Q-Trap mass spectrometer (AB Sciex, USA). The chromatographic conditions are described in Supplementary Table 1. The eluate was directed into the mass spectrometer through a post-column flow splitter (40 % into the MS). The mass spectrometer was operated in positive ion mode using an ion spray interface with the following working parameters: ion spray voltage, 5,500 V; curtain gas, 20 psi; gas 1, 60 psi; gas 2, 20 psi; declustering potential, 80 V; entrance potential, 8 V; source temperature, 400 °C. Nitrogen gas was used. Analyst 1.4.2 (AB Sciex, USA) software system was used for HPLC–MS system operation, data acquisition and processing.

Preparation of rat intestinal and hepatic subcellular fractions

Male Sprague–Dawley (SD) rats (280–320 g) were supplied by the Laboratory Animal Service Center, The Chinese University of Hong Kong. The animal surgical procedures and experiments were approved and performed in accordance with the basic principles and guidelines of the Chinese University of Hong Kong for investigators conducting biomedical research involving animals. The rats were divided randomly into two groups (six animals in each group). One group (treated group) of rats were intraperitoneally administered with dexamethasone, a CYP3A inducer, at 100 mg/kg once daily for 3 consecutive days to induce CYP3A activity (Kishida et al. 2008), while the other group (normal group) of rats were treated similarly with saline. The rat hepatic subcellular fractions including S9, cytosol, microsome (RLM) and intestinal S9 fraction were prepared using standard methods described previously (Lin et al. 2007; Williams et al. 1989). The protein concentrations of all fractions were measured by assay kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. The content of cytochrome P450 in microsome was measured using the Omura methods (Omura and Sato 1964). The P450 contents in normal and induced RLM were determined to be 1.21 and 1.83 nmol/mg protein, respectively.

In vitro metabolic study

The reaction components (at their final concentrations) consisted of the subcellular fractions (rat liver S9 and microsome, 2 mg of protein/mL; cytosol, 0.5 mg of protein/mL; rat intestinal S9, 1 mg/mL; HLM, 1 mg of protein/mL; rCYP3A4, 50 pmol/mL), peptides (50 μM), NADPH-regenerating system (1 mM $NADP^+$, 10 mM G-6-P, 2 units/mL G-6-P-DH, 4 mM $MgCl_2$) and 50 mM Tris buffer containing 150 mM KCl, pH 7.4, in a total volume of 0.5 mL. Substrate and cofactor controls were also conducted in parallel. Reactions were initiated by the addition of NADPH-regenerating system or substrate in case of the control containing no NADPH-regenerating system. The samples were incubated for 30 min at 37 °C and terminated by the addition of 1 mL of cold methanol and chilled on ice. After centrifugation at 10,000*g* for 5 min, 50 μL aliquots of the supernatant were subjected to HPLC–UV for quantitative analysis. A full scan with *m*/*z* 200 ~ 900 was performed on HPLC–MS to detect putative metabolites, and a combination of precursor ion scan and product ion scan of the selected *m*/*z* value was used to further elucidate the structures of the metabolites.

Identification of enzymes mediating metabolism of peptides

To identify the enzymes mediating the metabolism of the α-aminoxy peptides in rat liver microsome, various enzyme inhibitors were utilized, including SKF 525A (a non-selective inhibitor of cytochrome P450 monooxygenases (CYPs), 100 μM), methimazole (a non-selective inhibitor of flavin-containing monooxygenases (FMOs), 100 μM) (Tsutsumi et al. 2004), 8-methoxypsoralen (an inhibitor of CYP2A2, 100 μM) (Aoki et al. 2000), metyrapone (an inhibitor of CYP2B, 50 μM) (Daniel et al. 2002), cimetidine (an inhibitor of CYP2C6/11, 100 μM) (Nakazawa et al. 2006), α-naphthoflavone (an inhibitor of CYP1A1/2, 50 μM), quinine (an inhibitor of CYP2D, 100 μM), diethyldithiocarbamate (an inhibitor of CYP2E1, 200 μM) and ketoconazole (an inhibitor of CYP3A, 10 μM) (Martignoni et al. 2006). All incubation conditions and sample preparation procedures are the same as described above except for the pre-incubation with individual inhibitors for 15 min.

Stability in the GI tract

The simulated gastric fluid (SGF) containing no pepsin and SIF containing no pancreatin were prepared according to the US Pharmacopeia (The United States Pharmacopeial Convention 2006). The pH values of SGF and SIF were adjusted to 3.9 and 6.0, which were the average pH values in the stomach and intestine of rats, respectively (McConnell et al. 2008). AP and the α-aminoxy peptides were spiked into SGF and SIF at a final concentration of 50 μM and incubated at 37 °C for 1 and 3 h, respectively. Aliquots (200 μL) of the incubated samples were collected at 0, 5, 15, 30, 45 and 60 min from SGF, and 0, 15, 30, 60, 90, 120 and 180 min from SIF. The collected samples were mixed with 200 μL of methanol, and 50 μL of the mixed solution was subjected to HPLC–UV for analysis. Each test was conducted in triplicate. Degradation of each peptide at each time point was calculated based on the comparison of its peak area with that at 0 min.

Data analysis

The percentage of metabolism of the peptides was calculated using the following equation:

$$\text{Metabolized } (\%) = [(C_0 - C_{30})/C_0] \times 100\%$$

where C_0 is the initial concentration of the peptide, and C_{30} is the concentration of peptide after incubation for 30 min.

The concentration of individual metabolites was determined from the calibration curve of the corresponding parent peptides. The metabolite formation rate was estimated using the following equation:

$$\text{Metabolite formation rate } (\text{nmol/min/mg protein}) = C_{\text{metabolite}}/(T \times C_{\text{protein}})$$

where $C_{\text{metabolite}}$ is the concentration of the metabolites after incubation for 30 min, T is the incubation time and C_{protein} is the protein concentration of the subcellular fraction.

All the data are presented as mean ± SD. Statistical analysis was conducted using one-way ANOVA followed by Dunnett post-test used for multi-group comparisons or two-tailed unpaired t test for the comparison between two groups. A p value less than 0.05 was considered to be statistically significant.

Results

GI stability

An α-amino peptide AP was studied in parallel for the comparison and, as expected, AP was unstable in the GI tract with 61 and 62 % degradation in SGF and SIF after incubation for 1 and 3 h, respectively (Table 1). Compared with AP, AxyP1 showed significantly better stability although it decomposed by 41 and 47 % in SGF and SIF, respectively. After structural modifications, the stability of the four peptides was dramatically improved with less than 6 % degradation in both fluids under the same incubation conditions (Table 1). The detailed degradation profiles of all tested peptides are shown in Supplementary Fig. 1. Furthermore, no obvious metabolism of all five α-aminoxy peptides was observed after incubation with intestinal S9 fractions, indicating that the modified four α-aminoxy peptides were stable in the GI tract, while the instability of AxyP1 in the GI tract was primarily caused by non-enzymatic degradation under different pH conditions rather than the metabolizing enzymes-mediated biotransformation. Therefore, the following metabolic studies focused on the hepatic metabolism.

Metabolic stability

AP was extensively metabolized by 39 % in rat liver S9 after incubation for 30 min, whereas the metabolic rate of AxyP1 was slower, with 31 % metabolized within 30 min (Table 2). The promising results of the four modified peptides demonstrated significant improvement of their stability with 11–5 % metabolism for AxyP2–AxyP4 and complete inertness of AxyP5 (Table 2).

As shown in Fig. 2, three metabolites of AxyP1 and one metabolite of AxyP2, AxyP3 and AxyP4 were observed in RLM, while no metabolite of AxyP5 was detected. Upon incubation with rat liver S9 preparation, the same result was also found except that only two metabolites (AxyP1-M1 and AxyP1-M2) of AxyP1 were generated. On the other hand, no metabolite was found when all five α-aminoxy peptides were incubated with rat hepatic cytosolic fraction. Furthermore, all metabolites of the α-aminoxy peptides were formed in the presence of NADPH-regenerating system, suggesting that their hepatic metabolism was mainly catalyzed by NADPH-dependent

Table 1 Stability of the α-amino peptide and α-aminoxy peptides in SGF and SIF

Peptide	Degradation (%)	
	SGF (pH 3.9)	SIF (pH 6.0)
AP	61.2 ± 6.74	61.7 ± 5.37
AxyP1	40.7 ± 6.91[b]	47.3 ± 5.41[a]
AxyP2	3.50 ± 1.41[b, c]	4.11 ± 1.14[b, c]
AxyP3	4.41 ± 2.21[b, c]	5.57 ± 0.654[b, c]
AxyP4	5.01 ± 1.75[b, c]	0
AxyP5	0	0

[a] $p < 0.01$ and [b] $p < 0.001$ compared with AP; [c] $p < 0.001$ compared with AxyP1 ($n = 3$)

Table 2 Percentage of the metabolism of the α-amino peptide and α-aminoxy peptides in rat liver S9 fraction

Peptide	Metabolized (%)		
	Rat liver S9	RLM	HLM
AP	39.3 ± 2.77	59.4 ± 2.23	45.1 ± 1.65
AxyP1	31.0 ± 2.33[a]	44.5 ± 5.18[a]	33.6 ± 1.27[a]
AxyP2	10.7 ± 1.17[a, b]	29.0 ± 1.45[a, b]	12.4 ± 2.11[a, b]
AxyP3	4.15 ± 0.16[a, b]	9.98 ± 0.73[a, b]	5.98 ± 0.76[a, b]
AxyP4	9.35 ± 0.64[a, b]	4.69 ± 0.28[a, b]	4.70 ± 0.85[a, b]
AxyP5	0	0	0

[a] $p < 0.001$ compared with the corresponding AP group; [b] $p < 0.001$ compared with the corresponding AxyP1 group ($n = 3$)

monooxygenases in the liver microsome. Subsequently, the metabolic rates of the α-aminoxy peptides in both RLM and rat liver S9 preparations were compared. The results (Table 2) showed that metabolic rates of all α-aminoxy peptides in RLM were significantly higher than that in rat liver S9, further confirming that the NADPH-dependent monooxygenases-mediated oxidation predominated in their hepatic microsomal metabolism. Moreover, compared with the microsomal metabolism of AxyP1, the metabolic stabilities of four modified peptides were improved significantly from 1.5-fold (AxyP2), 4.5-fold (AxyP3) and 9.5-fold (AxyP4) to complete inertness (AxyP5).

Metabolite identification

Three metabolites (AxyP1-M1, AxyP1-M2 and AxyP1-M3) of AxyP1 and one metabolite of AxyP2 (AxyP2-M1), AxyP3 (AxyP3-M1) and AxyP4 (AxyP4-M1) were generated in rat liver in vitro. The structures of all metabolites found were identified based on their MS and UV spectra. All MS spectra are available in Supplementary Fig. 2–11.

Metabolites of AxyP1

The structure elucidations of three metabolites were mainly based on the MS and MS/MS spectra analyzed by the

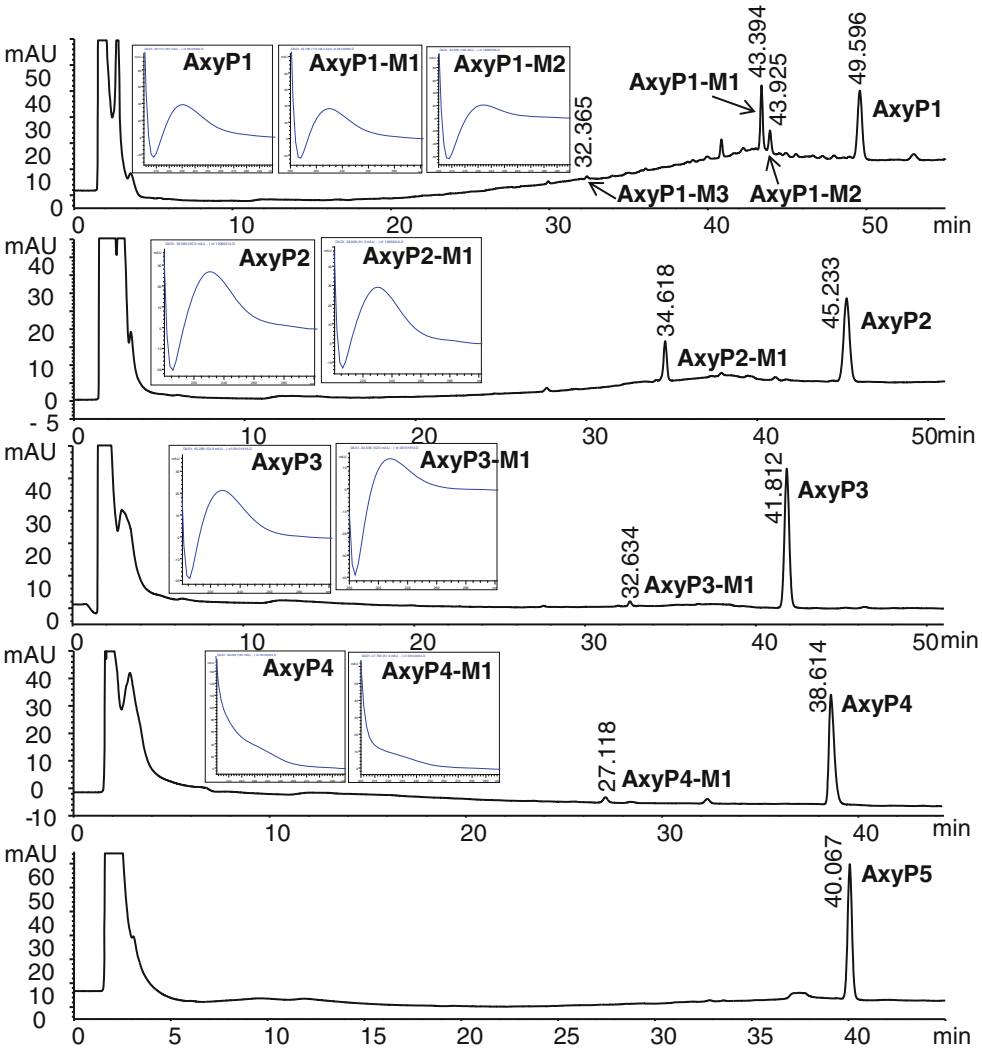


Fig. 2 HPLC–UV chromatograms and UV spectra of five α-aminoxy peptides after incubation with RLM for 30 min

HPLC–MS method. Firstly, MS and MS/MS spectra of AxyP1 were interpreted based on the protonated molecular ion ($[M + H]^+$) at *m/z* 535 plus a sodiated ion ($[M + Na]^+$) at *m/z* 557, and the major and characteristic fragment ions at *m/z* 462, 361, 333, 232 and 146 corresponding to the fragmentation pathways, respectively, as summarized in Table 3. Such major and characteristic ions were then used as diagnostic ions for the elucidation of structures of the metabolites.

The MS spectrum of AxyP1-M1 showed protonated ion $[M + H]^+$ at *m/z* 551 and sodiated ion $[M + Na]^+$ at *m/z* 573, indicating that molecular weight of AxyP1-M1 was 16 Da higher than that of AxyP1, suggesting an oxidized mono-hydroxylated metabolite of AxyP1. Compared with AxyP1, AxyP1-M1 had three similar fragment ions: two at *m/z* 462 and 361 indicating that the aryl ring and the isobutyl groups were intact; the other at *m/z* 232, further confirming an unchanged aryl ring (Table 3). The fragment ion at *m/z* 533, which was due to a loss of one molecule of water ($[M + H–H_2O]^+$), indicated the loss of water from a primary/secondary hydroxyl group, and fragment ion at *m/z* 184 further indicated that oxidation occurred at the *N*-isobutyl groups. Moreover, the UV spectrum of AxyP1-M1 was similar to that of AxyP1 (Fig. 2), further supporting that the chromophore of AxyP1 was intact and hydroxylation occurred at the side chain of the peptide. Therefore, AxyP1-M1 was identified as *N*-isobutyl-1-hydroxyl AxyP1 (Fig. 3a).

AxyP1-M2 exhibited the same protonated and sodiated ions of AxyP1-M1 at *m/z* 551 ($[M + H]^+$) and 573 ($[M + Na]^+$), and also had a fragment ion at *m/z* 533 corresponding to a loss of one molecule of water, indicating that it was another hydroxylated metabolite. The UV spectrum of AxyP1-M2 was similar to AxyP1, suggesting the presence of an unchanged chromophore of the parent compound. In its MS/MS spectrum, the characteristic fragment ion at *m/z* 478 was comparable to, but with 16 Da higher than, the ion at *m/z* 462 obtained from AxyP1 and AxyP1-M1, suggesting that hydroxylation occurred at one isobutyl side chain. The position of the hydroxyl group was further proved by fragment ion at *m/z* 460 due to a loss of a water molecule from fragment ion at *m/z* 478, and fragment ions at *m/z* 168 and 112 as indicated in Table 3. In addition, the fragment ion at *m/z* 333 further demonstrated the intact aryl ring. Based on all the data, AxyP1-M2 was identified as isobutyl-1′-hydroxyl AxyP1 (Fig. 3a).

The UV spectrum of AxyP1-M3 was unavailable due to its significantly low quantity in the incubated samples. Thus, its structure elucidation was solely based on MS data. The protonated ($[M + H]^+$) and sodiated ions ($[M + Na]^+$) at *m/z* 567 and 589 were 32 Da higher than AxyP1, suggesting a possible addition of two oxygen atoms in the parent compound. Two fragment ions at *m/z* 549 and 531 by losing one and two molecules of water, respectively, from primary hydroxyl groups formed a di-hydroxylated metabolite with hydroxylation at the same isobutyl, same *N*-isobutyl, or one of each group in either the same or different side chain. The same fragment ion at *m/z* 184 observed for AxyP1-M1 and AxyP1-M3 as well as the same fragment ions at *m/z* 460 observed for AxyP1-M2 and AxyP1-M3 confirmed that the two hydroxyl groups were present on one side chain isobutyl group and one *N*-isobutyl group on different sides of the isophthalamide unit and it was formed from further oxidation of AxyP1-M1 and/or AxyP1-M2 (Table 3). Therefore, AxyP1-M3 was proposed to be *N*-isobutyl-isobutyl-di-1,1′-hydroxyl P1 (Fig. 3a).

Metabolite of AxyP2

Similarly, both MS and UV spectra of AxyP2 were examined and compared for the structure elucidation of its metabolite. AxyP2 showed the protonated molecular ion ($[M + H]^+$) and a sodiated ion ($[M + Na]^+$) at *m/z* 451 and 473, respectively. The major and characteristic fragment ions at *m/z* 378, 291, 218, 190 and 146 were used as diagnostic ions for structural elucidation of its metabolite, as summarized in Table 3. For metabolite AxyP2-M1, its protonated ion $[M + H]^+$ at *m/z* 467 and sodiated ion $[M + Na]^+$ at *m/z* 489 were 16 Da higher than that of AxyP2, indicating that it was an oxidized metabolite of AxyP2. Its UV spectrum was similar to that of AxyP2, suggesting the unchanged chromophore (Fig. 2), which was further confirmed by the same fragment ion at *m/z* 378 observed for both AxyP2 and AxyP2-M1. The abundant fragment ion at *m/z* 449 ($[M + H–H_2O]^+$) indicated the loss of water from the primary/secondary hydroxyl group, and the fragment at *m/z* 142 demonstrated the hydroxylation at the *N*-isobutyl group (Table 3). Therefore, the structure of AxyP2-M1 was proposed as *N*-isobutyl-1-hydroxyl AxyP2 (Fig. 3b).

Metabolite of AxyP3

The protonated molecular ion ($[M + H]^+$) at *m/z* 483 and a sodiated ion ($[M + Na]^+$) at *m/z* 505 as well as the major fragment ions and their possible fragmentation pathway of AxyP3 are summarized in Table 3. In the case of AxyP3-M1, the protonated ion $[M + H]^+$ of AxyP3-M1 at *m/z* 499 was 16 Da higher than that of AxyP3, and the fragment ion $[M + H–H_2O]^+$ at *m/z* 481 corresponded to the loss of water from the primary/secondary hydroxyl group, indicating that it was a hydroxylated metabolite. Compared to MS/MS spectrum of AxyP3, AxyP3-M1 had the same fragment ions at *m/z* 410 and 307, suggesting that the aryl ring was intact, which was also supported by the same UV

Table 3 Summary of mass spectral data for α-aminoxy peptides and their in vitro hepatic metabolites

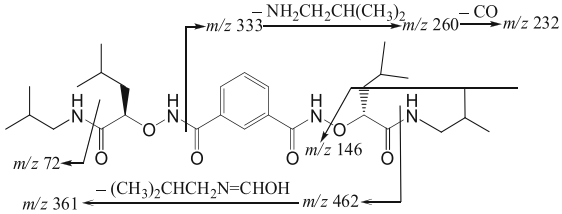
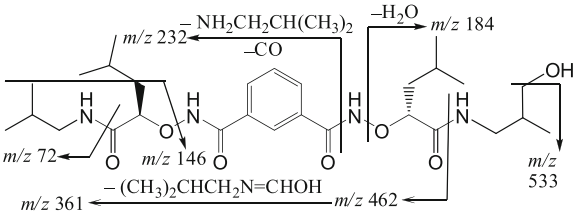
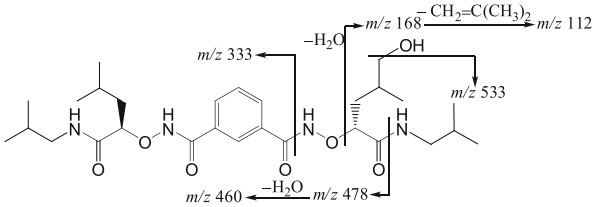
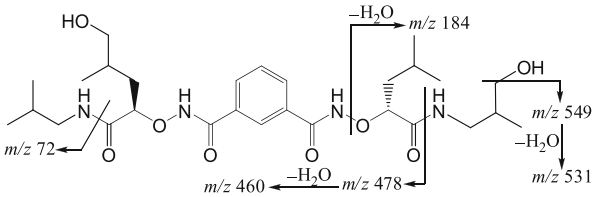
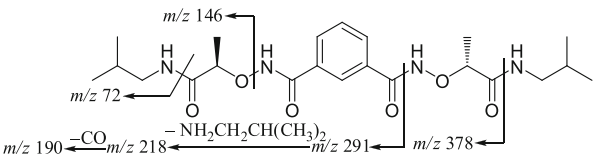
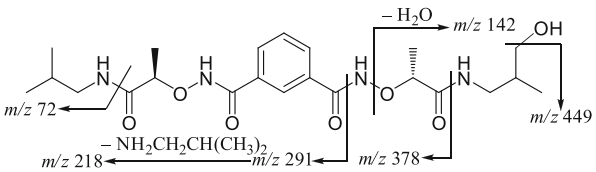
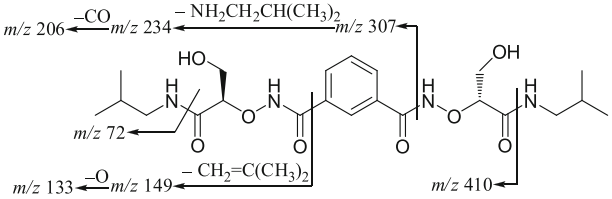
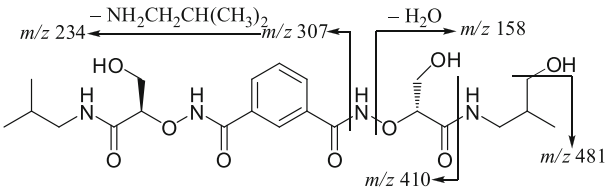
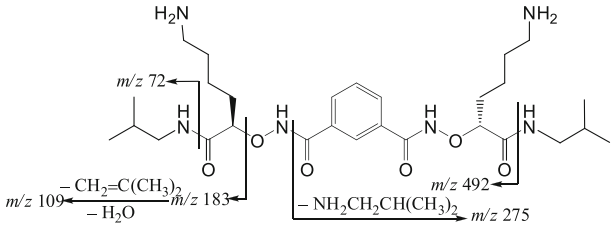
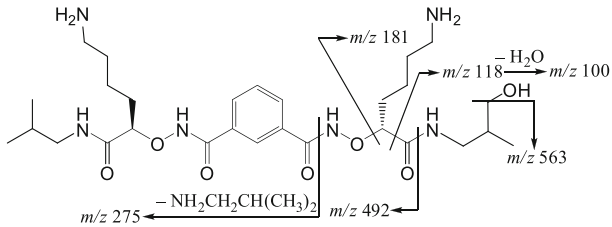
Peptide or metabolite	Structure and proposed fragmentation scheme	$[M + H]^+$/ $[M + Na]^+$	Major and characteristic fragment ion[a]
AxyP1		535/557	462, 361, 333, 260, 232, 146, 72
AxyP1-M1		551/573	533, 462, 361, 232, 184, 146, 72
AxyP1-M2		551/573	533, 478, 460, 333, 168, 112
AxyP1-M3		567/589	549, 531, 478, 460, 184, 72
AxyP2		451/473	378, 291, 218, 190, 146, 72
AxyP2-M1		467/489	449, 378, 291, 218, 142, 72
AxyP3		483/505	410, 307, 234, 206, 149, 133

Table 3 continued

Peptide or metabolite	Structure and proposed fragmentation scheme	$[M + H]^+$/ $[M + Na]^+$	Major and characteristic fragment ion[a]
AxyP3-M1		499/–	481, 410, 307, 234, 158
AxyP4		565/587	492, 275, 183, 109, 72
AxyP4-M1		581/–	563, 492, 275, 181, 118, 100

[a] Fragment ions with intensity >20 %

spectra obtained for both AxyP3 and AxyP3-M1. Furthermore, the fragment ion at *m/z* 158 demonstrated hydroxylation at the *N*-isobutyl group (Table 3). Therefore, AxyP3-M1 was identified as *N*-isobutyl-1-hydroxyl AxyP3 (Fig. 3b).

Metabolites of AxyP4

The MS spectrum of AxyP4 showed a protonated molecular ion ($[M + H]^+$) and a sodiated ion ($[M + Na]^+$) at *m/z* 565 and 587, respectively. The major fragment ions at *m/z* 492, 275, 183 and 109 were used as diagnostic ions for the elucidation of structures of its metabolite (Table 3). Comparing with its parent compound, the protonated molecule of AxyP4-M1 at *m/z* 581 was 16 Da higher. Its MS/MS spectrum yielded a fragment ion ($[M + H–H_2O]^+$) at *m/z* 563 corresponding to the loss of one molecule of water from the primary/secondary hydroxyl group, indicating a hydroxylated metabolite of AxyP4. The same characteristic fragment ion at *m/z* 492 observed in both AxyP4 and AxyP4-M1 indicated that both aryl ring and 4-aminobutyl groups were intact, and the oxidation might have occurred on the *N*-isobutyl groups. Moreover, the UV spectrum of AxyP4-M1 was similar to that of AxyP4, further suggesting the presence of a similar chromophore. The characteristic fragment ion at *m/z* 100 further indicated the hydroxylation at the *N*-isobutyl group (Table 3). Therefore, AxyP4-M1 was identified as *N*-isobutyl-1-hydroxyl AxyP4 (Fig. 3b).

Enzymes mediating the metabolism

As demonstrated above, all metabolites of the α-aminoxy peptides were formed via oxidation catalyzed by NADPH-dependent monooxygenases in the liver microsome, either CYPs or FMOs or both might be the enzymes mediating such metabolism. The results obtained from the inhibitory study in RLM demonstrated that SKF 525A (a non-specific CYPs inhibitor) significantly inhibited the formation of AxyP1-M1 (Fig. 4a) and AxyP1-M2 (Fig. 4b), while methimazole (a non-specific FMOs inhibitor) did not affect their formation, indicating that CYPs, but not FMOs, mediated the hepatic metabolism of AxyP1. Because only trace amount of AxyP1-M3 was formed, the enzymes mediating its formation were not investigated. Similarly, the metabolism of AxyP2, AxyP3 and AxyP4 were catalyzed by CYPs, but not FMOs (Fig. 4c–e).

Subsequently, the CYP isozymes involved in the hydroxylation were investigated using specific inhibitors. As demonstrated in Fig. 5, the formation of AxyP1-M1 and

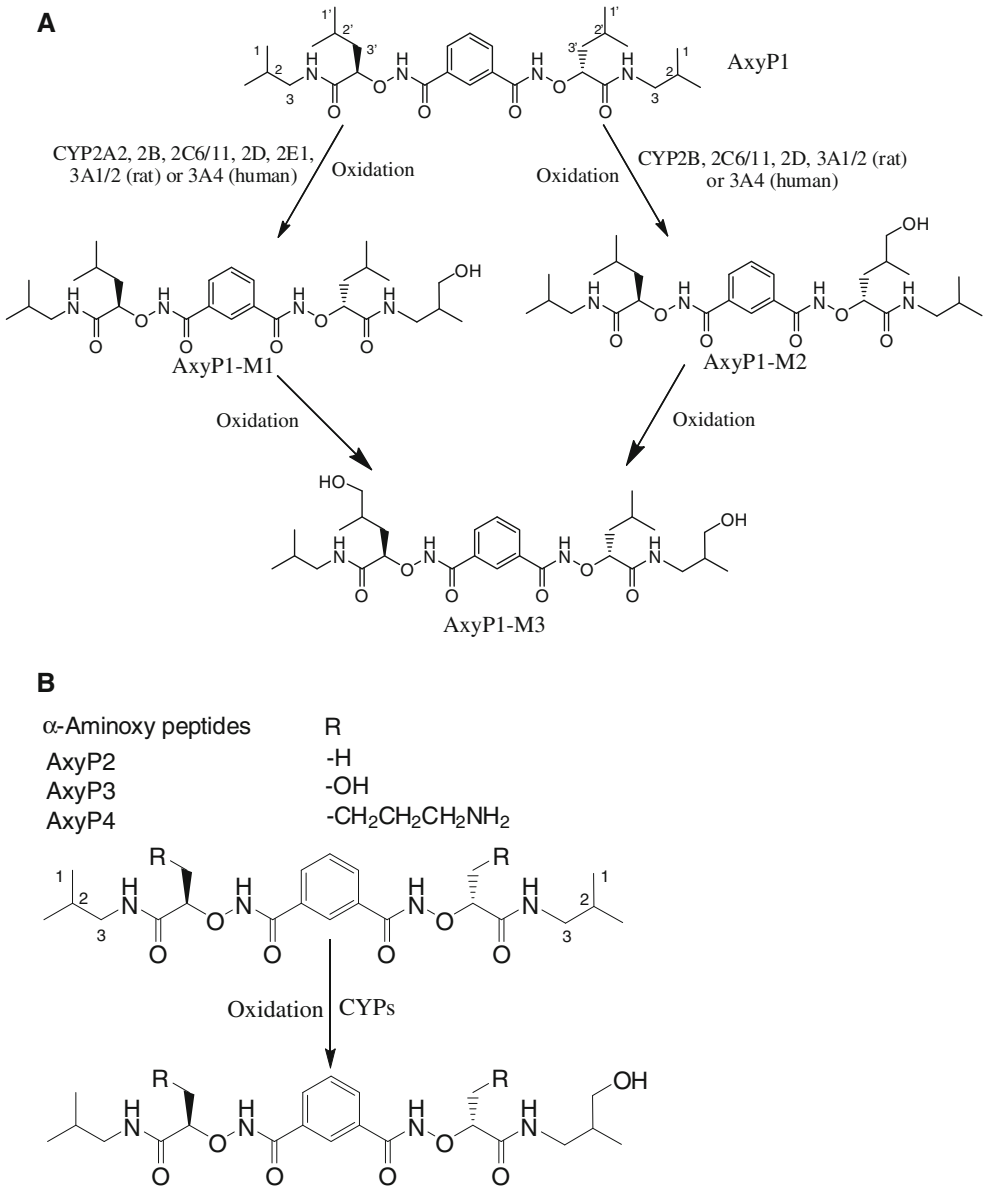


Fig. 3 The proposed in vitro hepatic metabolic pathways of AxyP1 (**a**), and AxyP2, AxyP3 and AxyP4 (**b**) in rat and human liver

AxyP1-M2 was catalyzed by CYP2A2, 2B, 2C6/11, 2D, 2E1, 3A (Fig. 5a) and CYP2B, 2C6/11, 2D, 3A (Fig. 5b), respectively. The formation of AxyP2-M1, AxyP3-M1 and AxyP4-M1 was mediated by CYP1A1/2, 2A2, 2B, 2C6/11, 2D, 2E1, 3A (Fig. 5c), CYP1A1/2, 2A2, 2C6/11, 2D, 2E1, 3A (Fig. 5d) and CYP1A1/2, 2A2, 2C6/11, 3A (Fig. 5e), respectively.

Among all CYP isozymes involved, CYP3A, in particular CYP3A1/2 in male rat microsome used in the present study, was found to catalyze the metabolism of all four α-aminoxy peptides. This isozyme corresponding to CYP3A4 in humans is the most important metabolizing enzyme responsible for the metabolism of variety of drugs (Zhou 2008). Its role in mediating the metabolism of all peptides was further investigated using the dexamethasone-induced RLM in which the CYP3A was significantly induced compared to the normal one (1.83 vs. 1.21 nmol/mg protein in RLM). The formation rates of all metabolites generated from four α-aminoxy peptides in the induced RLM were significantly higher than that in the normal RLM (Table 4). In the case of two metabolites of AxyP1, formation rates of both were similar in the induced RLM due to the significantly elevated rate of AxyP1-M2 (6.3-fold vs. 1.5-fold AxyP1-M1), while in the normal RLM formation rate of AxyP1-M1 was significantly higher than that of AxyP1-M2. These results demonstrated the important role of CYP3A1/2 in rat hepatic metabolism of these peptides.

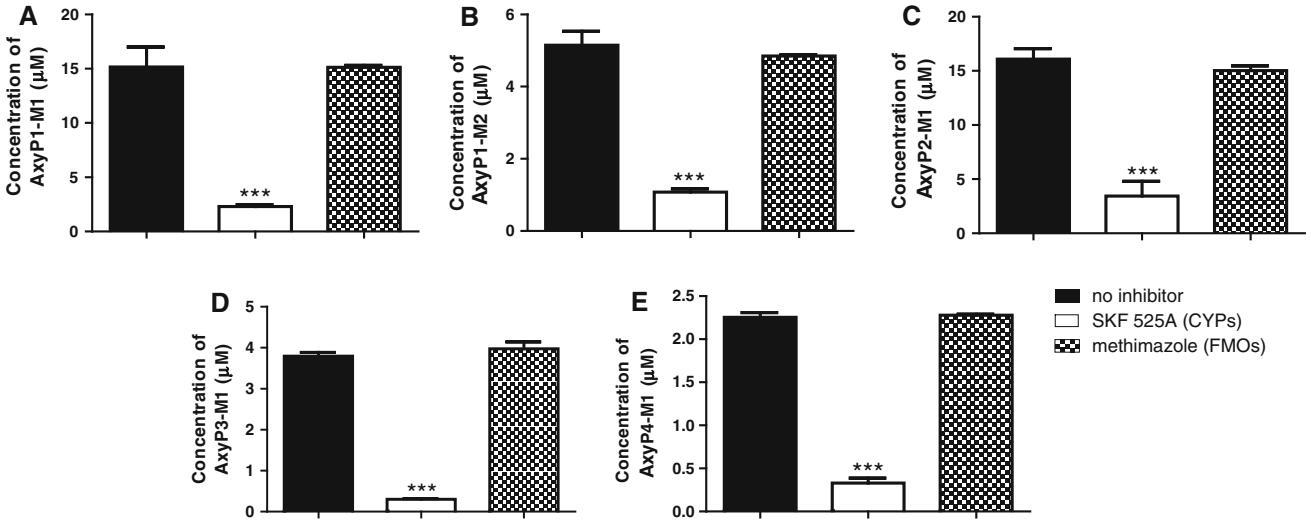


Fig. 4 The contents of the metabolites of AxyP1-M1 (**a**), AxyP1-M2 (**b**), AxyP2-M1 (**c**), AxyP3-M1 (**d**) and AxyP4-M1 (**e**) in RLM in the absence or presence of SKF 525A or methimazole. ***$p < 0.001$ compared with the control group without inhibitor ($n = 3$)

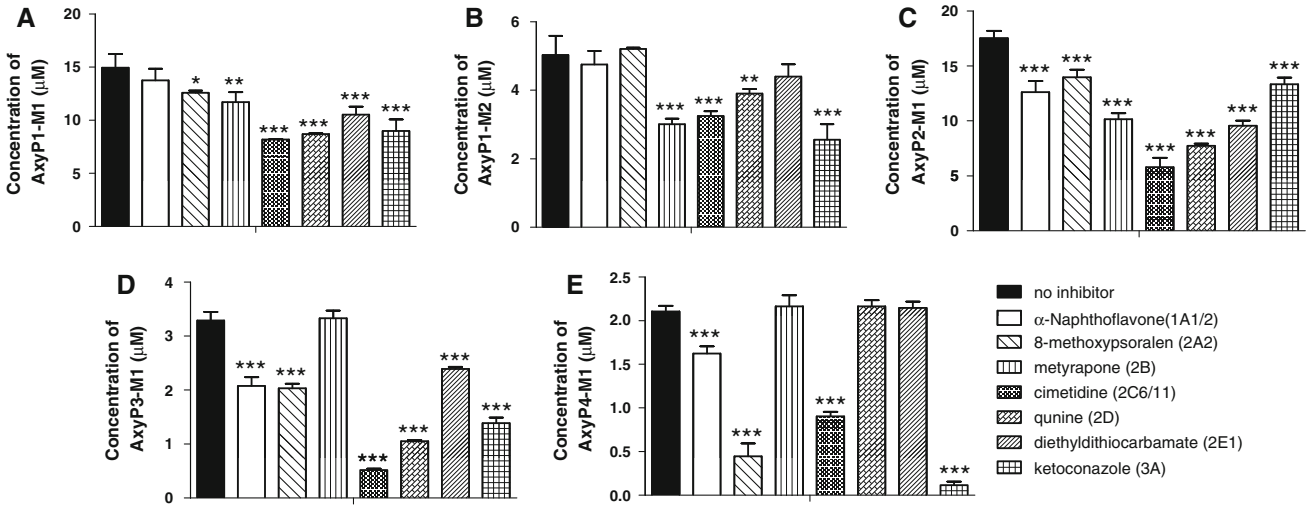


Fig. 5 The contents of the metabolites of AxyP1-M1 (**a**), AxyP1-M2 (**b**), AxyP2-M1 (**c**), AxyP3-M1 (**d**) and AxyP4-M1 (**e**) in RLM in the absence or presence of different CYP isozyme inhibitors. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the control group without inhibitor ($n = 3$)

Metabolism in HLM and rCYP3A4

The hepatic metabolism of all α-aminoxy peptides in human was also investigated using HLM. Similar to the metabolism in RLM, compared with AxyP1, the metabolic stabilities of four modified peptides in HLM were also significantly improved with the same trends as AxyP5 (not metabolized) > AxyP4 (7.1-fold) > AxyP3 (5.6-fold) > AxyP2 (2.7-fold). On the other hand, the metabolic rates of AxyP4 and AxyP5 were the same, but were remarkably less in HLM than that in RLM for AxyP1, AxyP2 and AxyP3 (Table 2). Furthermore, the metabolic pathway of each peptide in HLM was similar to that in RLM, but with different metabolic rates (Table 4). The formation rates of AxyP1-M2, AxyP2-M1 and AxyP4-M1 in HLM were significantly higher, while the rates of AxyP1-M1 and AxyP3-M1 in HLM were significantly lower than that in RLM. In addition, the involvement of CYP3A4 was also investigated using rCYP3A4 supersomes. The results revealed that the formations of the five metabolites were all mediated by CYP3A4 with very high activity, especially in AxyP1-M1 (5.1 ± 0.03 nmol/min/nmol CYP) and AxyP1-M2 (9.0 ± 0.13 nmol/min/nmol CYP) (Table 4), further demonstrating the important role of CYP3A4 in the

Table 4 Formation rate of the metabolites of the α-aminoxy peptides in normal and dexamethasone-induced RLM, HLM and rCYP3A4

Metabolite	Formation rate				
	(pmol/min/(mg protein))		(pmol/min/(nmol CYP))		
	Normal RLM	Induced RLM	RLM	HLM	rCYP3A4
AxyP1-M1	321 ± 56.7	492 ± 30.8[a]	998 ± 30.6	712 ± 51.8[d]	5108 ± 28.6
AxyP1-M2	70.9 ± 15.9	447 ± 21.0[b]	329 ± 35.5	1078 ± 72.4[e]	9016 ± 128
AxyP2-M1	113 ± 17.5	348 ± 26.4[b]	62.5 ± 5.24	100 ± 16.5[c]	643 ± 40.0
AxyP3-M1	250 ± 21.8	470 ± 2.51[b]	389 ± 16.7	212 ± 90.1[c]	680 ± 198
AxyP4-M1	30.9 ± 0.726	77.3 ± 3.07[b]	51.0 ± 2.03	219 ± 6.64[e]	595 ± 22.7

[a] $p < 0.05$ and [b] $p < 0.001$ compared with the normal RLM group; [c] $p < 0.05$, [d] $p < 0.01$ and [e] $p < 0.001$ compared with the RLM group ($n = 3$)

metabolism of these peptides. Interestingly, similar to the metabolism in RLM, CYP3A4 activity for the formation of AxyP1-M2 was significantly higher than that for AxyP1-M1.

Discussion

Most therapeutic peptides and proteins are currently used as parenteral formulations with relatively frequent administration (i.e., intravenous infusion) because of their low oral bioavailability and short biological half-lives due to their poor oral absorbability, instability in the GI tract and extensive metabolism (Mahato et al. 2003). To develop therapeutic peptide candidates with better oral bioavailability for potential oral administration, our research team prepared a series of α-aminoxy peptides by inserting an oxygen atom into the peptide backbone of α-amino acids. Our previous study proved that the backbone of α-aminoxy acids was more rigid than that of α-amino acids due to the repulsion between the lone pairs of electrons on the nitrogen and oxygen atoms (Wu et al. 1999). Recently, our studies have demonstrated significantly improved oral absorption of these α-aminoxy peptides (Ma et al. 2011). In our ongoing study to improve oral bioavailability of these peptides, we focused on their stability in the GI tract and metabolism in the liver.

The low oral bioavailability caused by instability of peptides of natural α-amino acids in the GI tract has been reported in many cases. For example, linaclotide, a 14-amino acid peptide developed for the treatment of constipation-predominant irritable bowel syndrome and chronic constipation, was reported to be degraded completely in jejunal fluid in 30 min in vitro (Bryant et al. 2010). Oral bioavailability of pepstatin and its glycine analog in mice and rats was only about 1 % due to their instability in the GI tract (Grant et al. 1982). Similarly, the representative peptide AP, consisting of natural α-amino acids, in the present study was also unstable in the GI tract with about 60 % degradation in both SGF and SIF within 1 and 3 h, respectively. In contrast, our α-aminoxy peptide AxyP1 showed significantly better GI stability (41 % degradation in SGF and 47 % degradation in SIF), although it was still unsatisfactory. To further improve the stability of AxyP1, structural modifications were conducted on its side chains to keep a minimum change in the core α-aminoxy acid moiety to retain its biological activity. Four analogs were synthesized by replacing the isobutyl side chain of AxyP1 with methyl group for AxyP2, hydroxymethyl group for AxyP3, 4-aminobutyl group for AxyP4 and 3-carboxypropyl group for AxyP5. Compared to AxyP1, the GI stability of four modified peptides was improved significantly by 8-fold (AxyP2), 9-fold (AxyP3) and 12-fold (AxyP5) with no degradation for AxyP4 in SGF, and 12-fold (AxyP2) and 9-fold (AxyP3) with no degradation for AxyP4 and AxyP5 in SIF, respectively. These results indicated that structural modifications could significantly improve the stability of the α-aminoxy peptides in the GI tract.

Another cautious factor affecting the bioavailability of oral and other administrative routes of α-amino peptides is the extensive hepatic metabolism, which results in the low oral bioavailability owing to extensive first-pass metabolism and also poor bioavailability of all administrative routes due to short half-lives. For example, it was reported that in general, peptides composed of natural α-amino acids were metabolically unstable and completely hydrolyzed within 15 min (Frackenpohl et al. 2001). This was also demonstrated in the present study of peptide AP with about 40 % metabolized in rat liver S9 within 30 min. Compared with AP, the prototype of α-aminoxy peptide AxyP1 showed significantly better hepatic metabolic stability, indicating that the α-aminoxy acid backbone was more resistant to metabolizing enzymes. However, about 30 % of AxyP1 was still metabolized in S9 within 30 min (Table 2), demanding further improvement. Since the α-aminoxy acid moiety was identified as the pharmacophore for construction of ion channel (Li et al. 2008;

Ma et al. 2011), structural modification of AxyP1 was conducted on the side chains to enhance metabolic stability based on the rationale of: the lower the lipophilicity, the better is the metabolic stability (Nassar et al. 2004). Lipophilicity of the compound is one of the key features determining substrate specificity, binding affinity and enzyme selectivity of the enzymatic metabolic biotransformation (Lewis 2000; Long and Walker 2003). After structural modifications, four AxyP1 derivatives were obtained with their log *P* values (AxyP2: 1.84; AxyP3: 0.13; AxyP4: 1.32; AxyP5: 0.84), which directly related to the lipophilicity, all lower than that of AxyP1 (4.31) (Ma et al. 2011). The results showed that the overall metabolic rates of the four modified peptides in the rat liver within 30 min were significantly reduced by threefold for AxyP2 and AxyP4 and eightfold for AxyP3, while no metabolism was observed for AxyP5. These findings demonstrated that the metabolic stability of the α-aminoxy peptides could be significantly improved by structural modifications of side chains with the reduction of lipophilicity.

It is interesting to note that different from hydrolysis, the primary metabolic pathways of peptides composed of natural α-amino acids (Bai and Amidon 1992), oxidation mediated by CYP isozymes was the predominant one for the α-aminoxy peptides to form the corresponding hydroxylated metabolites. For AxyP1 with the highest metabolic rate, two mono-hydroxylated and one di-hydroxylated metabolite were detected, and only one mono-hydroxylated metabolite was found for AxyP2, AxyP3 and AxyP4, while AxyP5 was not metabolized in the present study condition. The proposed metabolic pathways of the four peptides and their metabolites identified are illustrated in Fig. 3. Furthermore, the key role of CYP3A1/2 in mediating hepatic metabolism of the α-aminoxy peptides in RLM was proved by the significant increased formation rates of all metabolites of 1.5-fold (AxyP1-M1), 6.3-fold (AxyP1-M2), 3.1-fold (AxyP2-M1), 1.9-fold (AxyP3-M1) and 2.5-fold (AxyP4-M1) in the dexamethasone-induced RLM (Table 4).

The metabolic stability of the α-aminoxy peptides in humans was also investigated using HLM. A significant improvement of the metabolic stability of these peptides was also revealed in HLM with the same trend of AxyP5 $\gg$ AxyP4 > AxyP3 > AxyP2 in both species. The metabolic rates of individual peptides in both species were unable to be compared directly due to different contents of various metabolizing enzymes present in the two microsomal preparations. Furthermore, the same metabolic pathways for all five α-aminoxy peptides were determined in both species, while species difference in formation rates of different metabolites was observed between humans and rat. The formation rate ratio of AxyP1-M1 and AxyP1-M2 was 1:1.5 and 1:0.3 in HLM and RLM, respectively, and the formation rates were higher for AxyP2-M1 (1.6-fold) and AxyP4-M1(4.3-fold) but lower (0.54-fold) for AxyP3-M1 in HLM, which might be due to the relatively high activity of CYP3A4 in HLM compared with CYP3A1/2 in male HLM (Kim et al. 2005; Stiborova et al. 2003). Together with the observed higher formation rates of all metabolites generated by rCYP3A4, the results demonstrated the importance of CYP3A4 isozyme in the metabolism of these α-aminoxy peptides in humans.

In conclusion, the hepatic metabolic and GI stabilities of the α-aminoxy peptides investigated were significantly better than those of peptides consisting of natural α-amino acids, and such stabilities were further improved by structural modification. Moreover, the α-aminoxy peptides were mainly metabolized through oxidation mediated by CYP isozymes, especially CYP3A, to form hydroxylated metabolites in the liver. Our findings provided scientific evidences and rationale for the further design and development of better α-aminoxy peptide candidates with improved metabolic and gastrointestinal stability for potential clinical use.

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Conflict of interest The authors declare that they have no conflict of interest.

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