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Characterization of *in vitro* biotransformation of the new oral anticoagulants, the factor VIIa inhibitors AS1927819-00 and AS1932804-00

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Received December 9, 2012, accepted January 4, 2013

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Pharmazie 68: 406–413 (2013) *doi: 10.1691/ph.2013.2906*

We recently developed a prodrug (AS1932804-00, CMP) of the novel FVIIa inhibitor AS1924269-00, which possesses a carbamate amidine backbone. In addition, we developed another type of prodrug (AS1927819-00, OXP) with an oxime amidine backbone. In this study, we investigated the efficiency of conversion of these novel FVIIa prodrugs to their active forms by evaluating the production of the active form in vitro by using microsomes, mitochondria, and cryopreserved hepatocytes, and compared it with the in vivo conversion mechanisms of the prodrugs (oxime amidine vs. carbamate amidine). We observed that OXP and CMP showed improved oral absorption, and the efficiency of conversion of CMP to the active form was higher than that of OXP. The in vivo rate of conversion of OXP to its active form was low in rats, and compared to liver microsomes and mitochondria, cryopreserved hepatocytes supplemented with serum and coenzymes were an appropriate metabolic test tool. On the other hand, the efficiency of conversion of CMP to its active from could be appropriately evaluated using small intestinal microsomes. The development of a prodrug can be optimized when information about the stability of carboxylic acid esters in the presence of serum esterases, membrane permeability of intermediate forms, and differential tissue specificity to metabolic activities for carbamate and oxime backbones of amidine can be obtained.

1. Introduction

Recently developed blood coagulation inhibitors such as sibrafiban (a glycoprotein IIb/IIIa receptor inhibitor) (Wittke et al. 1999), ximelagatran (an antithrombin agent) (Eriksson et al. 2003), and dabigatran etexilate (antithrombin agent) (Blech et al. 2008) possess an amidine backbone that retains their inhibitory activities but reduces their extent of oral absorption. Therefore, to improve the oral absorption of blood coagulation inhibitors, the hydroxyl or carbamate group was further introduced at the amidine position and the carboxylic acid was esterified. Despite these chemical modifications, the bioavailability (BA) of the active form was only 10–20% (Eriksson et al. 2003).

Microsomal and mitochondrial metabolism causes a reduction of oxime amidine *in vitro*, and ximelagatran is metabolized to the active form by reduction in the liver microsomes (Clement et al. 2005; Anderson et al. 2005, Fig. 1). We developed a blood coagulation inhibitor, AS1924269-00 (ACT), which is a low-molecular-weight amidine compound with an inhibitory effect on FVIIa but has low oral absorption. In this study, we attempted to develop a prodrug of this compound with improved oral absorption, as also applied for ximelagatran and dabigatran.

We recently developed a prodrug (AS1932804-00, CMP) of the novel FVIIa inhibitor (AS1924269-00, ACT), which possesses a carbamate and amidine backbone. The prodrug showed

Abbreviations: CES, carboxyl esterase; FXa, factor Xa; FVIIa, factor VIIa; GP IIb/IIIa, glycoprotein IIb/IIIa; HPLC, high- performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PAMPA, parallel artificial membrane permeability; AUC, area under the curve; BA, bioavailability; Cmax, maximum plasma concentration; Vss, volume of distribution at steady state; CLtot, total body plasma clearance; $T_{1/2}$, half life; Fa, fraction absorbed; PK, pharmacokinetic; AS1924269-00, (nominated as ACT) 2-{2-[({2-[(4-Carbamimidoylphenyl)carbamoyl]-4-methoxyphenyl}amino)methyl]-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy}acetic acid; AS1928272-00, (nominated as OXI) 2-(2-{[(2-{[4-(N'-hydroxycarbamimidoyl)phenyl]carbamoyl}-4-methoxyphenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy)acetic acid; AS1927819-00, (nominated as OXP) Ethyl2-(2-{[(2-{[4-(N'-hydroxycarbamimidoyl)phenyl]carbamoyl}-4-methoxyphenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy)acetate; AS1937090-FO, (nominated as CMI) 2-[2-({[2-({4-[(Amino[(ethoxycarbonyl)imino]methyl)phenyl]carbamoyl}-4-methoxyphenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy)acetic acid; AS1932804-00, (nominated as CMP) Ethyl2-[2-({[2-({4-[(amino[(ethoxycarbonyl)imino]methyl)phenyl] carbamoyl}-4- methoxyphenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy)acetate KDN-7429 (Internal standard) 2-[4-(Carbamoylmethyl)-2-{[(4-chloro-2-{[4-(N'-hydroxycarbamimidoyl) phenyl]carbamoyl}phenyl)-amino]methyl}-6-ethoxyphenoxy]acetic acid ;.

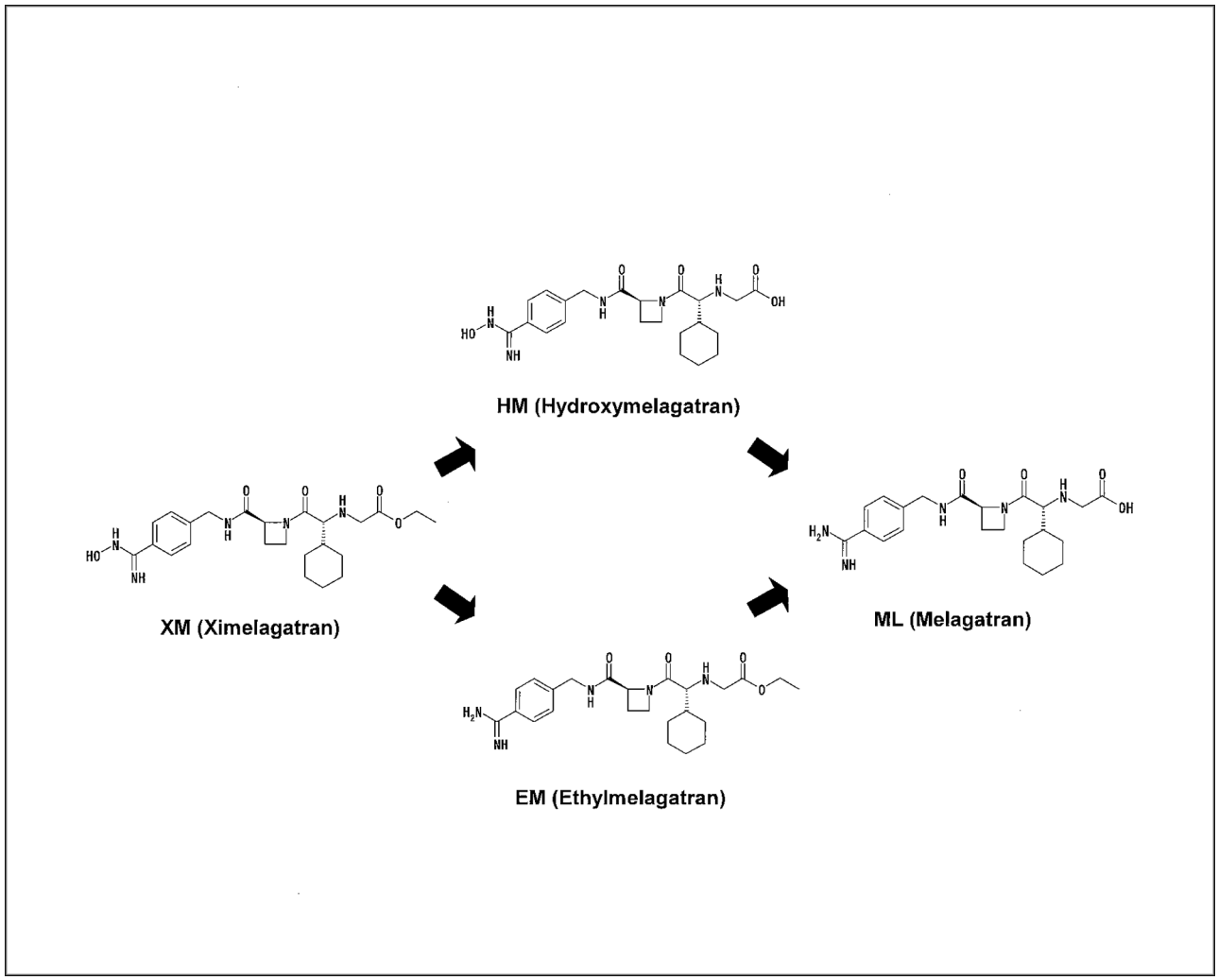

Fig. 1: Metabolic pathways of ximelagatran. Ximelagatran is converted to hydroxymelagatran and ethylmelagatran, and subsequently, these metabolites are converted to melagatran

increased Caco-2 membrane permeability and oral absorption in rats. The BA of the ACT was only 0.3%, but that after oral administration of a carbamate prodrug (CMP) was 36.1%, which suggested that the carbamate prodrug was rapidly hydrolyzed to its active form after oral absorption (Nakabayashi et al. 2013).
In this study, we investigated the efficiency of conversion of the novel FVIIa prodrugs to their active forms by evaluating the *in vitro* production of the active form using microsomes, mitochondria, and cryopreserved hepatocytes, and we compared the *in vivo* conversion mechanisms of the different prodrugs (oxime amidine vs. carbamate amidine) to the *in vitro* mechanisms.

2. Investigation and results

2.1. In vitro experiments

The active form of ximelagatran (i.e., melagatran) was produced in the liver microsomes and mitochondria of the rat at a rate of 50–120 pmol/min/mg (Table 1). AS1927819-00 (OXP) was metabolized in the liver microsomes and mitochondria to generate its active form; the rates of production of the active form were equivalent to or higher than those of ximelagatran in both rat liver microsomes and mitochondria. In contrast, no active form of the carbamate amidine prodrug, CMP, was detected in the liver microsomes or mitochondria.
We also investigated the production of the active form in small intestinal microsomes of the rat (Table 2). No active form was produced from an oxime amidine prodrug, ximelagatran, and small amounts were produced from OXP. In contrast, we observed marked conversion of CMP to its active form.
When the oxime amidine prodrugs, ximelagatran and OXP, were added to rat cryopreserved hepatocytes, the concentration of the active form measured after 1, 2, and 4 h showed a time-

Table1: *In vitro* bioactivation of OXP, CMP, and ximelagatran to theparent amidine drug by hepatic microsomes and mitochondria of the ratwith NADH or NADPH

	Source	Cofactor	Conversion rate (pmol/min/mg) [a]
OXP	Microsome	NADH	46.6 ± 7.2
		NADPH	35 ± 2.2
	Mitochondria	NADH	218 ± 5.4
		NADPH	135 ± 3.5
CMP	Microsome	NADH	ND
		NADPH	ND
	Mitochondria	NADH	ND
		NADPH	ND
Ximelagatran	Microsome	NADH	124 ± 2.9
		NADPH	100 ± 3.5
	Mitochondria	NADH	81 ± 7.5
		NADPH	53.6 ± 2.9

OXP, AS1927819-00; CMP, AS1932804-00
[a] Conversion rates are means ± standard deviation (SD) of 3 determinations
ND, not detectable

dependent increase, and the rate of production of the active form from OXP was faster than that from ximelagatran (Table 3).
In the presence of serum, the rate of active form production was higher in ximelagatran, whereas the active form was only weakly produced from OXP. In contrast, no active form was detected in cryopreserved hepatocytes of the rat treated with CMP.

2.2. In vivo experiments

The time-course of blood levels after the oral administration of ximelagatran, OXP, and CMP at 10 mg/kg to rats is shown in Fig. 2, and the pharmacokinetic parameters are shown in Table 4–1. After oral administration of ximelagatran, its active form, melagatran, was mainly detected, and the BAs of the active form and the intermediate (hydroxymelagatran) were 7.0% and 1.2%, respectively. The area under the curve (AUC) of the intermediate and active form as a percentage of the total AUC (conversion rate) was 85%. For OXP, the intermediate (OXI) was mainly detected. The BAs of OXI and the active form (ACT) were 32.2% and 4.0%, respectively, and the conversion rate was about 11%. The AUCs of OXI and the active form were 47.0 and 6.07 μmol·h/L, respectively.
The AUC of the active form after the oral administration of ximelagatran was 3.12 μmol·h/L, and that for OXP was 6.07 μmol·h/L, which indicated that OXP surpassed ximelagatran with regard to exposure to the active form.
After administration of CMP, the active form was mainly detected. The BAs of the intermediate (CMI) and the active form were 1.8% and 36.1%, respectively, and the conversion rate was about 95%. The AUC of the active form was 54.3 μmol·h/L, which was greater than that of orally administered ximelagatran. The AUC of the active form after the oral administration of CMP was about 9 times greater than that after the oral administration of OXP. Therefore, the rate of conversion of the carbamate amidine type prodrug to its active form was higher than that of the oxime amidine type prodrug.
The time-course of blood levels after intravenous administration of the active form, melagatran, and ACT at 1 mg/kg are shown in Fig. 3, and the pharmacokinetic parameters are shown in Table 4–2. The volume of distribution at steady state (Vss) of ACT was equivalent to that of melagatran, but its half-life was longer, systemic clearance was relatively low, and the AUC was about 4 times greater than that of ximelagatran.

3. Discussion

This study aimed to predict the production and metabolism of the active form of novel FVIIa inhibitors in humans. To address this issue, we validated the *in vitro* metabolic test systems. Detailed clinical and preclinical studies have described the metabolism of ximelagatran (an antithrombin agent); therefore, we evaluated the *in vitro* metabolism of ximelagatran using rat liver and small intestinal microsomes and mitochondria.

Table 2: *In vitro* bioactivation of OXP, CMP, and ximelagatran to the parent amidine drug by intestinal microsomes of the rat

	Conversion rate (pmol/min/mg) [a]
OXP	8.4 ± 0.3
CMP	39.3 ± 2.5
Ximelagatran	ND

OXP, AS1927819-00; CMP, AS1932804-00
[a] Conversion rates are means ± standard deviation (SD) of 3 determinations
ND, not detectable

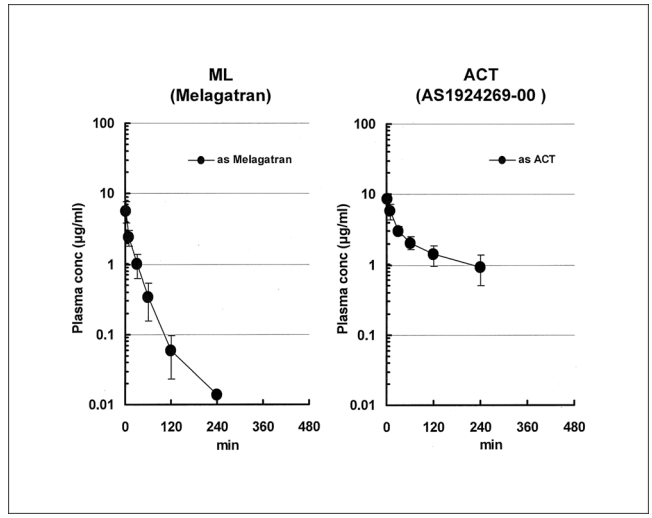

Fig.3: Pharmacokinetics profiles of ACT (AS1924269-00) and melagatran after intravenous administration to rats. Symbols and vertical bars represent the mean ± standard deviation (SD) from 3 rats. The vertical bars are not shown when SD falls within the symbol.

An oxime amidine prodrug, ximelagatran, was converted to its active form mainly by reduction in the rat and human hepatic samples (Clement et al. 2002). The rates of conversion of ximelagatran to its active form in rat microsomes and mitochondria (1 mg/mL) shown in Table 1 were similar to those reported previously (Clement et al. 2002). In addition, a major difference does not exist in the microsomal reduction of ximelagatran in the presence of NADH between aerobic and anaerobic conditions (Clement et al. 2005). Our study also showed no difference in the rate of production of the active form between aerobic and anaerobic conditions (data not shown), which indicated that aerobic conditions had a little effect on reductive metabolism. Thus, we used aerobic conditions for *in vitro* metabolic experiments.
We examined the microsomal reductions of oxime amidine prodrugs other than ximelagatran, including *N,N′*-dihydroxyamidine (Reeh et al. 2007), diacetyldiamidoxime (Clement et al. 2006), and benzamidoxime (Clement et al. 1997), and compared to NADH, NADPH had a higher requirement as a coenzyme. In addition, we compared the metabolism of a novel FVIIa inhibitor, OXP, in the microsomes and the mitochondria and observed that the reducing metabolic activity was higher in the group in which NADH was added than in the group in which NADPH was added (Table 1).
In addition, the rates of conversion of ximelagatran and OXP to the active form were investigated using microsomes and mitochondria, and the conversion of OXP was equivalent or greater than that of ximelagatran (Table 1).
However, examination of the blood levels of ximelagatran and OXP after oral administration in rats showed that about 85% of the administered ximelagatran was rapidly converted in to the active form, whereas only about 11% of OXP were converted in to the active form (Table 4–1).
The systemic clearance of the active form was lower than that of melagatran in rats. *In vitro* microsomal and mitochondrial production of the active form from OXP was favorable, but *in vivo* the conversion was insufficient. The low blood levels of the active form generated from the prodrugs *in vivo* in rats suggested that there is a discrepancy between the *in vivo* and *in vitro* conversion rates for OXP. Therefore, membrane permeability should be considered for the *in vivo* production of the active form. A permeability test using Caco-2 cells revealed that an intermediate with de-esterified carboxylic acid lost membrane permeability and was not readily incorporated into cells (Nakabayashi et al. 2013). The active form of a recently launched antithrom-

Table 3: ***In vitro*** **bioactivation of OXP, CMP, and ximelagatran to the parent amidine drug by cryopreserved hepatocytes of the rat with or without rat serum**

			Cryopreserved rat hepatocytes
			(nmol/h/10^6 cells) [a]
OXP	rat serum	without	2.42 ± 0.21
		with	0.89 ± 0.05
CMP	rat serum	without	0.38 ± 0.02
		with	0.13 ± 0.01
Ximelagatran	rat serum	without	2.92 ± 0.06
		with	3.49 ± 0.13

OXP, AS1927819-00; CMP, AS1932804-00

[a] CLint, *in vitro*, *in vitro* intrinsic clearances observed when the prodrugs were converted to parent drugs in the suspended hepatocyte with or without rat serum. Intrinsic clearances are expressed as means ± standard deviation (SD) of 3 determinations

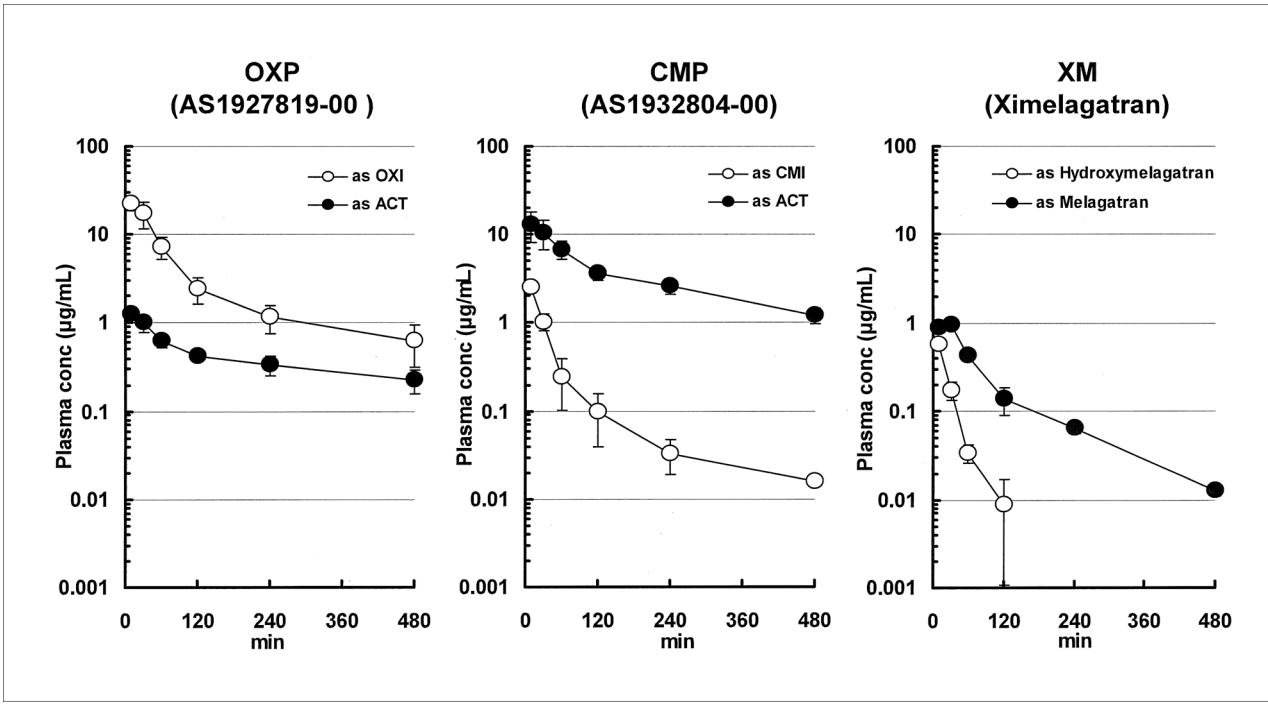

Fig.2: Pharmacokinetics profiles of CMP (AS1932804-00), OXP (AS1927819-00), and ximelagatran after oral administration to rats. Symbols and vertical bars represent the mean ± standard deviation (SD) from 3 rats. The vertical bars are not shown when SD falls within the symbol.

bin agent, dabigatran etexilate, is not a P-glycoprotein (P-gp) substrate, while the prodrug (dabigatran etexilate) is a P-gp substrate. Variable AUC and maximum plasma concentrations (C_{max}) have been reported clinically because of concomitant administration with a P-gp substrate inhibitor or inducer (Bendel et al. 2011). Therefore, the use of a comprehensive *in vitro* evaluation system using living cells will be more appropriate than the *in vitro* reaction systems involving suspended enzymes and enzyme fractions, such as microsomes and mitochondria.

Cryopreserved hepatocytes of various animal species have recently become available and widely used in pharmacokinetic studies. Suspension-type cryopreserved hepatocytes have also been used in some *in vitro* drug interaction tests (Lu et al. 2008, Shibata et al. 2008). The correlations of total body clearance (CL_{tot}) between metabolic tests using suspension-type cryopreserved hepatocytes and adhering cryopreserved hepatocytes have been reported (Jouin et al. 2006). Furthermore, a higher correlation with *in vivo* observation was obtained when serum was added to the reaction system (Blanchard et al. 2006).

In this study, we evaluated the validity of the *in vitro* metabolic test systems by comparing with *in vivo* conversion to the active form using suspension-type cryopreserved hepatocytes in the presence and absence of serum.

OXP, which is converted at a low rate *in vivo*, was rapidly converted in to its active form by metabolism in cryopreserved hepatocytes in the absence of serum (Table 2), similar to the rapid conversion by microsomes and mitochondria. Thus, it was suggested that an intermediate (OXI) was generated from OXP in the presence of serum, and OXI permeation through the membrane was low enough to generate the active form.

The rate of conversion of OXP in cryopreserved hepatocytes was markedly decreased in the presence of serum. In contrast, the rate of conversion of ximelagatran in cryopreserved hepatocytes was favorable regardless of the presence or absence of serum.

An intermediate, CMI or OXI, corresponding to the hydrolyzed form of the carboxylic acid ester was formed, but its stability in the presence of serum esterases is another problem. Stability of the carboxylic acid ester prodrug may also be important to improve the conversion rate, because the de-esterified intermediate form of the prodrug, CMI or OXI, may not easily permeate through the cells where the final active form will be produced. Ximelagatran was more stable than OXP and CMP (Fig. 4). In addition, studies using Caco-2 cells and parallel artificial membrane permeability assay (PAMPA) showed that the membrane permeability of the intermediate CMI was lower than that of the prodrug (Nakabayashi et al. 2013).

Table 4-1: Pharmacokinetic parameters after the oral administration of OXP, CMP, or ximelagatran to rats (10 mg/kg)

OXP	$AUC_{0\text{-}t}$ μmol·h/L	Cmax μmol/L	BA (%)	Ratio of parent drug (%)
as OXP	ND	ND	ND	
as OXI	47.0 ± 13.5	40.4 ± 6.44	32.2	
as ACT	6.07 ± 1.13	2.39 ± 0.47	4.0	11
CMP	**$AUC_{0\text{-}t}$ μmol·h/L**	**Cmax μmol/L**	**BA (%)**	**Ratio of parent drug (%)**
as CMP	ND	ND	ND	
as CMI	2.43 ± 0.60	4.12 ± 0.47	1.8	
as ACT	54.3 ± 12.1	24.2 ± 8.90	36.1	95
Ximelagatran	**$AUC_{0\text{-}t}$ μmo·h/L**	**Cmax μmol/L**	**BA (%)**	**Ratio of parent drug (%)**
as Ximelagatran	ND	ND	ND	
as Hydroxymelagatran	0.53 ± 0.01	1.28 ± 0.07	1.2	
as Melagatran	3.12 ± 0.28	2.27 ± 0.12	7.0	85

OXP, AS1927819-00; OXI, AS1928272-00; CMP, AS1932804-00; CMI, AS1937090-FO;
ACT, AS1924269-00
Cmax, maximum plasma concentration
$AUC_{0\text{-}t}$, area under the plasma concentration vs. time curve from zero to end point
BA(%), bioavailability
Ratio of parent drug, $AUC_{0\text{-}t}$ of parent drug/$AUC_{0\text{-}t}$ of total drugs
Each value is expressed as the mean ± standard deviation (SD) from 3 rats.

Table 4-2: Pharmacokinetic parameters after the intravenous administration of ACT and melagatran to rats

Melagatran	$AUC_{0\text{-}\infty}$ μmol·h/L	C0 μmol/L	T1/2 h	CLtot L/h/kg	Vss L/kg
as Melagatran	4.48 ± 1.08	16.7 ± 7.29	0.55 ± 0.20	0.54 ± 0.12	0.21 ± 0.01
ACT	$AUC_{0\text{-}\infty}$ μmol·h/L	C0 μmol/L	T1/2 h	CLtot L/h/kg	Vss L/kg
as ACT	15.1 ± 3.71	17.8 ± 1.11	2.82 ± 1.19	0.09 ± 0.04	0.28 ± 0.03

ACT, AS1924269-00
C0, maximum plasma concentration
$AUC_{0\text{-}\infty}$, area under the curve from zero to infinity
CL tot,p, total body plasma clearance
Vss, volume of distribution at steady state
T1/2, half-life
Each value is expressed as the mean ± standard deviation (SD) from 3 rats.

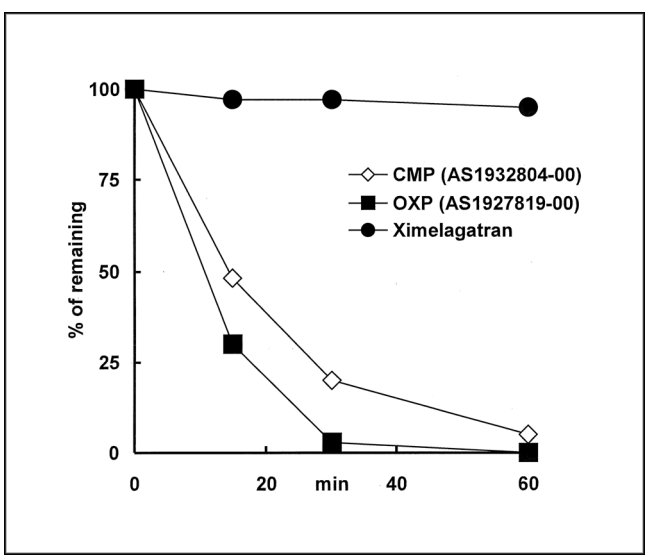

Fig.4: Stability of CMP (AS1932804-00), OXP (AS1927819-00), and ximelagatran in the rat plasma. The final concentration of the compounds used in the incubation was 10 μM. Each value is expressed as the mean (N = 2).

Because the activity of energy-dependent coenzyme-requiring metabolism in cryopreserved hepatocytes may be impaired during freezing and thawing, it may be desirable to supplement coenzymes (Baba 2005). Because our objective was to appropriately evaluate the production of the active form from FVIIa inhibitor prodrugs, we supplemented the cryopreserved hepatocyte suspension with coenzymes NADH or NADPH and identified that the prodrugs were converted favorably to their active forms in the living systems. Therefore, the evaluation system using cryopreserved hepatocytes in the presence of serum turned out to be useful for evaluating not only oxidation and conjugation but also reduction. Thus, compared to liver microsomes and mitochondria, this system of evaluation of drug metabolism is considered suitable for evaluating formation of metabolites *in vivo* from prodrugs.

In contrast, a carbamate amidine prodrug, CMP, did not react in the liver microsomes or mitochondria, and no conversion to the active form was observed in the test using cryopreserved hepatocytes (Table 3) although the active form production from CMP after oral administration occurred favorably *in vivo*. The small intestine and not the liver may be involved in the production of the active form from CMP; thus, we used small intestinal microsomes of the rat and found that the rate of conversion to the active form was higher for CMP than for OXP and ximelagatran. The hydrolysis of carbamate amidines was thus shown to be dominant in the small intestinal microsomes than in the liver.

Dabigatran etexilate is a carbamate amidine prodrug, which is converted to the active form, dabigatran by hydrolysis (Blech et al. 2008). BA of dabigatran itself was almost null, but the BA was improved to 7% after administration of the prodrug

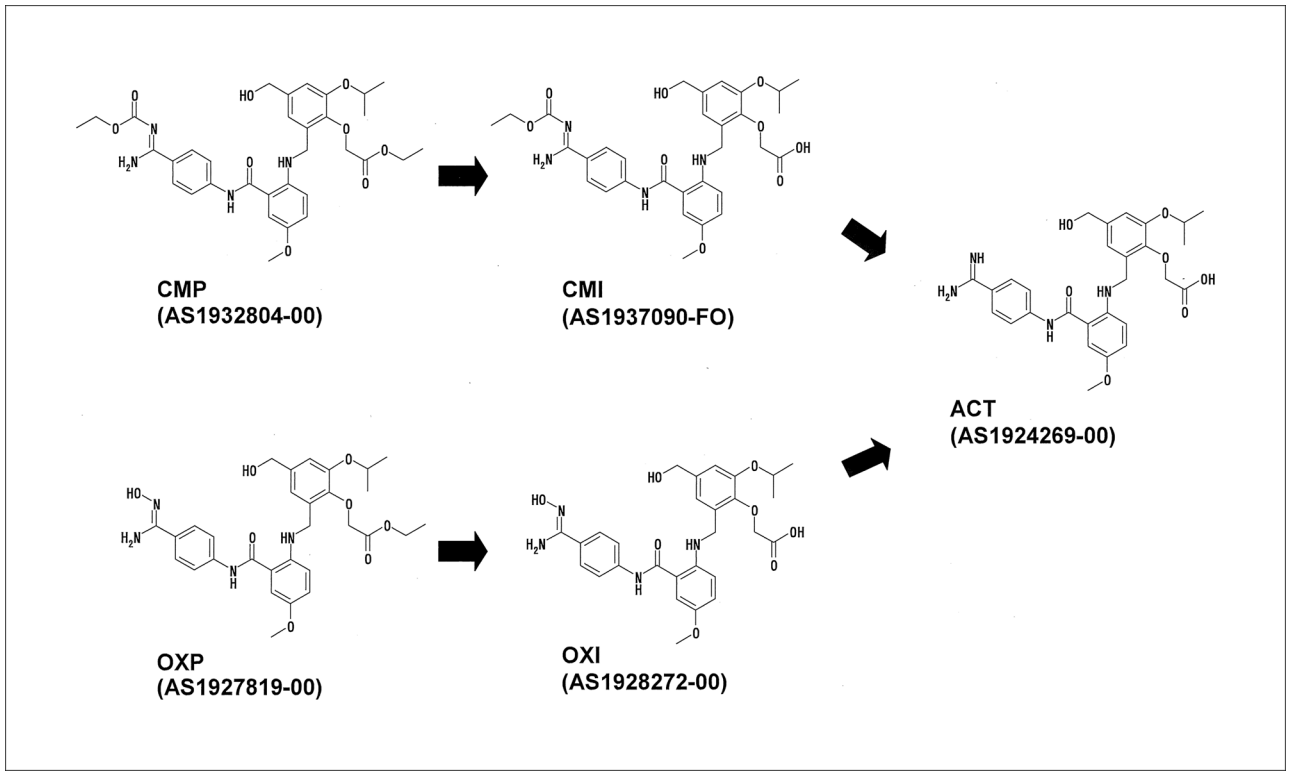

Fig.5: Metabolic pathways of CMP (AS1932804-00) and OXP (AS1927819-00). CMP (AS1932804-00) is converted to CMI (AS1937090-FO) and subsequently CMI is converted to ACT (AS1924269-00). OXP (AS1927819-00) is converted to OXI (AS1928272-00) and subsequently OXI is converted to ACT (AS1924269-00).

(Huttunen et al. 2011). Prodrugs with a carbamate amidine backbone, such as CMP, may be efficiently screened by evaluating the production of the active form using small intestinal microsomes. CPT-11 is a carbamate prodrug that is activated *in vivo* to SN-38 (active form). Carboxyl esterase 2 (CES2) is the most abundant CES that is responsible for CPT-11 hydrolysis (Sanghani et al. 2003). CES is a major hydrolyzing enzyme for detoxification of xenobiotics possessing carboxyl-ester, thioester, and amide bonds. Khanna et al (2000). have shown that the small intestine of humans has high levels of CES that could convert CPT-11 to SN-38. CES2 represents the major isoform of CES in the rat intestine, while the expression of CES1 is too low to influence the metabolism of ester prodrugs (Hosokawa M et al. 2008; Liu et al. 2011). Human and rat microsomes showed similar properties. The presence of only CES2 family enzymes in human and rat small intestine might be responsible for them having a similar hydrolytic profile (Taketani et al. 2007).

In this study, CMP was hydrolyzed by the small intestine of the rat, and it was considered that the enzyme responsible for CMP metabolism is CES2.

It is possible to increase the oral absorption of FVIIa inhibitors by developing oxime and carbamate amidine prodrugs, but the rate of conversion to the active form was low in rats (Riggs et al. 2006; Vijaykumar et al. 2006). Oral absorption may be improved by developing a prodrug, but only insufficient production concerning conversion to the active form has been obtained.

The BA of the ACT was only 0.3%, but that after the oral administration of a carbamate prodrug (CMP) was 36.1% (Nakabayashi et al. 2013). We observed that the oral absorbabilities of OXP and CMP, prodrugs of a novel FVIIa inhibitor, ACT, were improved in rats. The conversion efficiency of CMP to the active form was favorable, while the conversion rate of OXP was low both *in vitro* and *in vivo*. Prodrugs with an oxime amidine backbone, such as OXP, may well be developed by using living cell evaluating system as a tool to evaluate active form production i.e., cryopreserved hepatocytes supplemented with serum and coenzymes, not by using liver microsomes and mitochondria.

Although development of prodrugs is a useful method to increase the oral absorbability of drugs, the tissue specificity of hydrolysis as well as its chemical stability during pharmaceutical processing are some of the concerns.

However, the advantages of prodrugs could be fully realized in drug development if information about the stability of carboxylic acid esters in the presence of serum esterases, membrane permeability of intermediate forms, tissue specificity of metabolic enzymes (CES2 and reductases in intestine and liver, respectively), and differential metabolic activities for carbamate and oxime backbones of amidine is obtained. In this regard, our study provides valuable insights into prodrug development. The extent of production of the active form from oxime and carbamate amidines should be further investigated in humans.

4. Experimental

4.1. Materials

OXP, CMP, their intermediates (OXI, CMI), and ACT were supplied by Astellas Pharma Inc. and Kissei Pharmaceutical Co., Ltd. (purity > 98%). KDN-7429 was supplied by Kissei Pharmaceutical Co., Ltd. (purity > 98%) as the internal standard.

Ximelagatran, its intermediate metabolites (ethyl melagatran and hydroxyl melagatran), and melagatran were supplied by Kissei Pharmaceutical Co., Ltd. (purity > 98%).

The chemical structures of these compounds are shown in Fig. 1. All other chemicals were commercially available. Hepatic microsomes and cryopreserved hepatocytes of the rat were purchased from Biopredic Co., Ltd (Jean Pecker, Rennes, France). Rat intestinal microsomes were purchased from Xenotech Co., Ltd (Renekusa, KA, USA). Glucose parental 5% solution was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Heparin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise stated. All supplements and cell culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA)

4.2. In vitro experiments

4.2.1. Preparation of mitochondrial samples from rat liver

Rats were killed with ether, and their organs were excised, washed, weighed, and put in ice-cold 0.9% (w/v) NaCl solution. Tissue samples were homogenized in mitochondria buffer 10 mM Tris-HCl, pH 7.4; 0.25 M sucrose; and 0.5 mM EDTA. The liver and kidneys were homogenized either using a smooth Teflon pestle tissue grinder with approximately 4 strokes at 1000 rpm or minced. Mitochondrial fractions were prepared by a method described previously with some minor modifications (Vance 1990).

4.2.2. Assay for biotransformation using rat hepatic microsomes and mitochondria

The incubation mixture consisted of 0.5 to 1.0 mg/mL mitochondrial or microsomal protein from rat tissues and 1 mg/mL NADH in 100 mM potassium phosphate buffer pH 7. The final concentration of the compounds used in the incubation was 10 μM. The final DMSO concentration was 0.5% (v/v) in a total incubation volume of 0.2 mL. After 15, 30, and 60 min at 37 °C, the reaction was terminated by addition of 0.2 mL of ice-cold acetonitrile and 0.1 mL of internal standard solution (10 μM ketoconazole); the samples were briefly mixed and cooled on ice. Then, the samples were centrifuged on maximal speed (1600 × *g*) at 4 °C for 3 min to pellet the precipitate. Supernatants were subjected to HPLC for further analysis. The level of the active form was measured at 15, 30, and 60 min after metabolic reaction, and the rate of conversion to the active form was calculated.

4.2.3. Assay for biotransformation using rat intestinal microsomes

The incubation mixture consisted of 0.5 to 1.0 mg/mL of microsomal proteins from rat tissues, 1 mg/mL NADH, and 1 mg/mL NADPH in 100 mM potassium phosphate buffer (pH 7). The final concentration of these compounds used in the incubation was 10 μM. The final DMSO concentration was 0.5% (v/v) in a total incubation volume of 0.2 mL. After 30, 60, and 120 min at 37 °C, the reaction was terminated by addition of 0.2 mL of ice-cold acetonitrile and 0.1 mL of internal standard solution (10 μM ketoconazole); the samples were briefly mixed and cooled on ice. Samples were then centrifuged at 1600 × *g* at 4 °C for 3 min to pellet the precipitate. Supernatants were subjected to HPLC for further analysis. The level of the active form was measured at 30, 60, and 120 min after metabolic reaction, and the rate of conversion to the active form was calculated.

4.2.4. Assay for biotransformation using cryopreserved rat hepatocytes

We added 75 μL of 20 μM solution of the compounds to 75 μL of the hepatocyte suspension buffer containing 20% rat serum with 2 mg/mL NADH and NADPH. The final concentration of the compounds used in the incubation was 10 μM. The final DMSO concentration was 0.25% (v/v). The final concentration of the rat serum used in the incubation was 10% (v/v). The hepatocytes were incubated with the drug in a cell culture incubator maintained at 37 °C and 5% CO_2 in saturation humidity for 0, 1, 2, and 4 h. The reaction was stopped by the addition of ice-cold acetonitrile 200 μL and 0.1 mL of internal standard solution (10 μM ketoconazole) and then the samples were centrifuged at 1600 × *g* for 5 min. The compounds were determined by the following methods. The level of the active form was measured at 1, 2, and 4 h after metabolic reaction, and the rate of conversion to the active form was calculated (V0). The value calculated by dividing V0 by the initial substrate level (10 μM) was regarded as the intrinsic clearance of the active form.

4.2.5. Stability of the prodrugs in rat plasma

The stabilities of all prodrugs were determined *in vitro* in the rat plasma. The final concentration of the prodrugs used in the incubation was 10 μM. The final DMSO concentration was 0.5% (v/v) in a total incubation plasma volume of 0.2 mL. After 15, 30, and 60 min at 37 °C, the reaction was terminated by addition of 0.2 mL volume of ice-cold acetonitrile and 0.1 mL of internal standard solution (10 μM ketoconazole); the samples were briefly mixed and cooled on ice. Samples were then centrifuged at 1600 × *g* at 4 °C for 3 min to pellet the precipitate. Supernatants were subjected to HPLC for further analysis. The remaining percentages of the prodrugs in the rat plasma were calculated at 15, 30, and 60 min.

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