CHARACTERIZATION OF IN VITRO BIOTRANSFORMATION OF NEW, ORALLY ACTIVE, DIRECT THROMBIN INHIBITOR XIMELAGATRAN, AN AMIDOXIME AND ESTER PRODRUG

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ABSTRACT:

N-Hydroxylated amidines (amidoximes) can be used as prodrugs of amidines. The prodrug principle was developed in our laboratory for pentamidine and had been applied to several other drug candidates. One of these compounds is melagatran, a novel, synthetic, low molecular weight, direct thrombin inhibitor. To increase the poor oral bioavailability due to its strong basic amidine functionality selected to fit the arginine side pocket of thrombin, the less basic N-hydroxylated amidine was used in addition to an ethyl ester-protecting residue. The objective of this investigation was to study the reduction and the hydrolytic metabolism of ximelagatran via two mono-prodrugs (N-hydroxy-melagatran and ethyl-melagatran) to melagatran by in vitro experiments. New high-performance liquid chromatography methods were developed to analyze all four compounds. The biotransformation of ximelagatran to melagatran involving the reduction of the amidoxime function and the ester cleavage could be demonstrated in vitro by microsomes and mitochondria from liver and kidney of pig and human, and the kinetic parameters were determined. So far, one enzyme system capable of reducing N-hydroxylated structures has been identified in pig liver microsomes, consisting of cytochrome b$_5$, NADH-cytochrome b$_5$ reductase, and a P450 isoenzyme of the subfamily 2D. This enzyme system also reduces ximelagatran and N-hydroxy-melagatran. The participation of recombinant human CYP1A2, 2A6, 2C8, 2C9, 2C19, 2C6, and 3A4 with cytochrome P450 5 reductase, and a P450 isoenzyme of the subfamily 2D. This enzyme system also reduces ximelagatran and N-hydroxy-melagatran are easily reduced by several enzyme systems located in microsomes and mitochondria of different organs.

Melagatran is the active form of the novel, oral thrombin inhibitor ximelagatran (Gustafsson et al., 2001). Melagatran has suboptimal bioavailability because of the presence of a carboxylic acid, a secondary amine, and an amidine residue resulting in a charged molecule at physiological pH values. The prodrug ximelagatran was developed with a view to improving absorption. Ximelagatran comprises an ethyl ester group in place of the carboxylic acid and an N-hydroxymidamine group in place of the amidine. After protonation of amidines at the double-bonded nitrogen, cations are formed that are highly stabilized by mesomerism. Amidines are very strong bases (Albert et al., 1948) and protonated under physiological conditions. By introduction of an oxygen atom in the amidine functional group, the basicity is lowered by 5 pK$_a$ value. Amidoximes have been used as prodrugs for certain amidine-containing drugs, including pentamidine derivatives (Clement, 1993) and sibrafiban (Weller et al., 1996). It has been shown that certain amidoximes are rapidly reduced in vitro and in vivo to the amidines (Clement et al., 1988, 1992; Hauptmann et al., 1988). More recently, it has been demonstrated that the model compound benzamiadoxime is reduced by microsomes and mitochondria from liver and other organs like kidney, lung, and even brain (Clement and Mau, 1999; Clement and Deters, 2000).

So far one enzyme system capable of reducing N-hydroxylated derivatives of strongly basic functional groups has been identified in pig liver microsomes consisting of cytochrome b$_5$, NADH-cytochrome b$_5$ reductase, and a P450 isoenzyme of the subfamily 2D (Clement et al., 1997).

Current orally active anticoagulants on the market are vitamin K antagonists, ADP antagonists, and the thromboxane A$_2$ antagonist acetyl salicylic acid. These agents show some disadvantages and limitations in antithrombotic therapy (Hauptmann, 2002).

In particular, coumarins such warfarin or phenprocoumon, which achieve their anticoagulant effect by modulating the synthesis of vitamin K-dependent proteins resulting in the synthesis of defective coagulation factors without any coagulation activities (Hull et al., 1979), exhibit large variations in pharmacokinetics and a slow onset and offset of action (Hirsh et al., 1998). Additionally, drugs and food influence metabolism in particular in the case of changes in the vitamin K content of the diet. Consequently, extensive and expensive anticoagulation monitoring is necessary to maximize efficacy and safety of coumarins. The shortcomings of current available oral anticoagulants have stimulated great efforts to develop new oral drugs. One of these new anticoagulation drug candidates is melagatran, a new direct, low molecular weight thrombin inhibitor (Eriksson et al., 1999).

Melagatran has a strong basic amidine structure, a free carboxylic acid, and in addition a less basic amine function, implying that melagatran will be positively charged under physiological conditions. The oral bioavailability is only 3 to 7%. By N-hydroxylating melagatran at the amidine function in addition to the inclusion of an ethyl group to protect the carboxylic acid functionality (Fig. 1), the bio-
availability of melagatran is increased to 18 to 24% (Gustafsson et al., 2001). The resulting amidoxime has a lower pK_a value than the amidine function, and the carboxylic ester group, which is uncharged, reduces the pK_a of the secondary amine, too (Gustafsson et al., 2001).

From in vivo studies, it is clear that ximelagatran is reduced and hydrolyzed very efficiently to the active principle melagatran. There is low intersubject variability of pharmacokinetic parameters and no signs of toxicity (Gustafsson et al., 1999). However, not much is known about the enzymes metabolizing prodrugs such as ximelagatran. This study is directed toward the elucidation of the enzymatic bases of ximelagatran bioactivation, which means the reduction of an amidoxime function and the cleavage of an ethyl ester. Because two protecting groups are present ximelagatran has properties of a double prodrug.

Materials and Methods

Ximelagatran and metabolites were obtained from Astra (Hässle, Mölndal, Sweden). NADH, NADPH, DLPC, and unspecific carboxylic esterases from pig liver were obtained from Sigma Chemie (Deisenhofen, Germany). All other chemicals were commercially available and of analytical grade, except acetonitrile and methanol, which were of HPLC grade.

Human liver samples were obtained from medicinal departments of several universities. They came from patients that were subjected to a partial hemihepatectomy because of secondary liver tumors. Prior consent of the local medical ethics committee and from the donors was obtained for these studies. Human and pig mitochondria were prepared by differential centrifugation as described previously (Clement et al., 1996). The kidney microsomes were prepared analogously.

Human and pig mitochondria were prepared by differential centrifugation as described previously (Beattie, 1968; Kline et al., 1986) with slight modifications (Clement and Deters, 2000). To account for the biological variability liver samples from pigs or human organs were pooled (from at least three individuals per pool).

Mitochondria were checked for microsomal impurities by assessing rotenone-insensitive NADH cytochrome c reductase and succinate-cytochrome c reductase (Sottocasa et al., 1967). Microsomes were checked for mitochondrial impurities by assessing NADPH-cytochrome c reductase (Yasukochi and Masters, 1976).

Protein was assayed according to the method of Smith et al. (1985) using a bicinchoninic acid procedure, according to the manufacturer’s directions (BCA reagent kit; Pierce Chemical, Rockford, IL).

Cytochrome P450 concentrations were determined by measuring the carbon monoxide difference spectra after reduction with dithionite (Omura and Sato, 1964). NADH-cytochrome b_5 reductase was purified from pig liver microsomes to homogeneity by affinity chromatography on 5’AMP-Sepharose 4B (Pharmacia, Freiburg, Germany) similar to the procedure described for the purification of NADPH-P450 reductase (Yasukochi and Masters, 1976) with modifications (Clement et al., 1997). Cytochrome b_5 was purified from pig liver microsomes according to a published method (Taniguchi et al., 1984). Pig benzamidoxime reductase was purified from liver microsomes described by Clement et al. (1997).

Recombinant CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, recombinant and purified CYP3A4, and NADPH-cytochrome P450 reductase were obtained from BD Gentest (Woburn, MA).

Calculation of Apparent Kinetic Parameters. To determine N-hydroxy-melagatran reduction kinetics, activities were measured at a minimum of 0.05 mM substrate concentrations with two to four replicates at each concentration level. Apparent kinetic parameters K_m and V_max were estimated using nonlinear regression analysis (SigmaPlot 5.0; SPSS Science, Chicago, IL).

Assay for Activation of Ximelagatran and N-Hydroxy-melagatran. The incubation mixture consisted of 0.05 to 0.3 mg/ml microsomal or mitochondrial protein of liver or kidney (human or pig), 0.5 or 1 mM ximelagatran (H376/95), or 2 mM N-hydroxy-melagatran (H415/04) as substrate in 100 mM phosphate buffer pH 6.3, 7.0, and 7.4. A time course was run for all incubations. The reactions were linear for more than 30 min (up to 60 min). To obtain sufficient amounts of metabolites an incubation time of 30 min was chosen. After preincubation for 5 min at 37°C under aerobic conditions the reaction was started by the addition of NADH (final concentration 1 mM) to a total volume of 250 μl and maintained for 30 min. The reaction was terminated by the addition of 250 μl of cold methanol and vortexing. After centrifugation at 10,000 U/min (48 g) (Mikroliterzentrifuge Hettich, Tutlingen, Germany), 15 μl of the supernatant was analyzed by HPLC.

The standard incubation mixture with unspecific carboxylic esterases consisted of 0.5 U of esterases from pig liver and 2 mM ximelagatran in 100 mM phosphate buffer pH 6.3, 7.0, and 7.4. A time course was run for all incubations. The reactions were linear for more than 30 min (up to 60 min). To obtain sufficient amounts of metabolites an incubation time of 30 min was chosen. After preincubation for 5 min at 37°C under aerobic conditions the reaction was started by the addition of NADH (final concentration 1 mM) to a total volume of 250 μl and maintained for 30 min. The reaction was terminated by the addition of 250 μl of cold methanol and vortexing. After centrifugation at 10,000 U/min (48 g) (Mikroliterzentrifuge Hettich, Tutlingen, Germany), 15 μl of the supernatant was analyzed by HPLC.
The incubation mixtures have been optimized for protein content, NADH concentration, and substrate concentration. An incubation mixture consisted of 0.3 mg/ml microsomal or mitochondrial protein of liver or 0.15 mg/ml of kidney (human or pig), 0.5 mM ximelagatran as substrate, and 1 mM NADH as cosubstrate in 100 mM potassium phosphate buffer pH 7.0. For incubation conditions and HPLC analysis, see Materials and Methods.

### Materials and Methods

#### HPLC Method for the Reduction of N-Hydroxy-Melagatran to Melagatran

An isocratic separation was carried out by the first mobile phase described above, with the same retention times for melagatran and N-hydroxy-melagatran. Standard curves with 0 to 250 μM for melagatran were constructed and found to be linear over this range with a correlation coefficient >0.99. The recovery of melagatran from incubation mixture was 98.5 ± 3.8%. The determination limit of melagatran was about 0.125 μM.

#### HPLC Method for the Reduction of Ximelagatran to Ethyl-Melagatran

The separation was carried out isocratically by phosphate buffer (20 mM, 10 mM tetramethylammoniumchloride, pH 4.5) and acetoni triole in the proportion of 80:20 (v/v) by a LiChrospher RP-select B column and a corresponding precolumn. The retention times were 8.3 ± 0.6 min (ethyl-melagatran) and 15.4 ± 0.5 min (ximelagatran).

Standard curves with 10 to 600 μM melagatran were constructed and found to be linear over this range with a correlation coefficient >0.98. The recovery of melagatran from incubation mixture was 102.5 ± 6.4%. The determination limit of melagatran was about 1.25 μM.

### Results

#### Activation of Ximelagatran

The in vitro activation of ximelagatran to melagatran via two intermediate mono-prodrugs, ethyl-melagatran (H338/57) and N-hydroxy-melagatran (H415/04), by liver and kidney microsomes and mitochondria from pig and human is demonstrated for the first time (Table 1). A new HPLC analytical method was developed to separate and quantify the metabolites of ximelagatran. A representative chromatogram, recorded after the incubation of ximelagatran with pig liver mitochondria, is shown in Fig. 2. The retention times of the metabolites are in accordance with those of the reference compounds: 7.4 ± 0.3 min (melagatran), 11.7 ± 0.6 min (N-hydroxy-melagatran), and 19.7 ± 0.7 min (ethyl-melagatran). The determination limits are 0.15 μM for melagatran, 1.25 μM for N-hydroxy-melagatran, and 10 μM for ethyl-melagatran.

#### Standard curves and found to be linear with correlation coefficients >0.99. Precision of the assays and accuracy were assessed by adding different concentrations (0–100 μM melagatran, 0–1000 μM N-hydroxy-melagatran, 0–600 μM ethyl-melagatran) of metabolites to standard incubation mixtures (without cosubstrate NADH). After the usual working up procedure, standard curves were constructed by linear regression analysis. The recoveries of melagatran, N-hydroxy-melagatran, and ethyl-melagatran from incubation mixture were 100 ± 5% of that obtained using samples that contained the same amount of each metabolite dissolved in phosphate buffer. The determination limits were about 0.15 μM for melagatran, 1.25 μM for N-hydroxy-melagatran, and 10 μM for ethyl-melagatran.
basic pH and the N-reduction has its pH optimum at 6.0 (Fig. 3, a and b).

The preferred cosubstrate for the reduction was NADH. If NADPH was used as cosubstrate, the rates were 50% lower. The ester cleavage was independent of any cosubstrate (NADH or NADPH). There was no significant effect of additional 3.3 mM MgCl₂ on both reactions (data not shown).

Ester Cleavage of Ximelagatran to N-Hydroxy-melagatran. The in vitro ester hydrolysis of ximelagatran to N-hydroxy-melagatran could be shown by unspecific carboxylic esterases from pig liver (Fig. 4).

The activation of ximelagatran by esterases was linear over more than 100 min and followed Michaelis-Menten kinetics. Esterases/incubation mixture (0.5 U) was sufficient to obtain linear formations of N-hydroxy-melagatran. The optimized substrate concentration was 2 mM, and the pH dependence shows higher product formation at higher pH values. A physiological pH of 7.4 was selected for further studies. The ester cleavage of ximelagatran to N-hydroxy-melagatran by unspecific carboxylic esterases does not require NADH or NADPH as cosubstrate, the addition of 3.3 mM MgCl₂ had no significant effect. The kinetic parameters were determined: V₉₅ amounts to 398 nmol of N-hydroxy-melagatran/min/mg esterases and Kₘ is 2.16 mM. The catalytic efficiency was 1.84 × 10⁻⁴ l/min/mg protein.

To exclude the participation of CYP3A4 in the hydrolysis of the ester functionality of ximelagatran, it was tested on esterase activity. Recombinant, purified CYP3A4 in combination with NADPH-cytochrome P450 reductase was not capable of cleaving the carboxylic ester of ximelagatran to N-hydroxy-melagatran.

N-Reduction of N-Hydroxy-Melagatran to Melagatran. The in vitro N-reduction of N-hydroxy-melagatran by microsomes and mitochondria of liver and kidney from pig and human was also demonstrated (Table 2) and showed very high rates. In particular, the activities for pig liver and kidney mitochondria were 2 to 4 times higher than for microsomes. An isocratic HPLC analytical method was used to separate and quantify the reduced metabolite melagatran of H415/04. The retention time of melagatran 7.4 ± 0.3 min also agreed with the retention time of the reference compound. The determination limit of melagatran was 0.125 μM. The reduction showed linearity over 60 min, followed Michels-Menten-kinetics. The Kₘ values of all enzyme sources were in the same range (Table 2). The optimized pH value for the N-reduction was pH 6.3 and the addition of MgCl₂ did not significantly increase the reductions (data not shown). The reaction required either NADH or NADPH as cosubstrate, whereas considerably higher conversion rates were detected in the presence of NADH (data not shown).

Reconstitution Studies with Ximelagatran and N-Hydroxy-melagatran. To decrease the determination limit in the reconstitution experiments, a new HPLC method was developed for the reduction of the double prodrug ximelagatran to ethyl-melagatran. Ethyl-melagatran was eluted isocratically at 8.3 ± 0.6 min and ximelagatran at 15.4 ± 0.5 min. The reduction of N-hydroxy-melagatran to melagatran was measured with the same HPLC method as used for microsomes and mitochondria.

The reduction of the double prodrug ximelagatran and the monoprodrug N-hydroxy-melagatran to their corresponding amidines ethyl-melagatran and melagatran could be demonstrated by pig purified cytochrome P450 isoenzyme of the subfamily 2D in the presence of...
cytochrome b₅ and NADH-cytochrome b₅ reductase (Table 3). Omission of cytochrome P450 considerably reduced the conversion rates. Cytochrome b₅ and b₅ reductase alone were not capable of reducing the amidoximes. Furthermore, a chemical formation of amidine metabolites could be excluded by omitting of any protein.

To exclude the participation of CYP3A4, the xenobiotic-metabolizing cytochrome P450 enzyme with the highest concentration in human liver, and other P450s in the reduction of N-hydroxylated amidines, reductase activity by a group of recombinant human P450s was tested. None of the recombinant P450s (CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, and 3A4) tested together with cytochrome b₅ and NADH-cytochrome b₅ reductase were capable of reducing the melagatran prodrugs (data not shown).

**Discussion**

The introduction of modern technologies, such as combinatorial chemistry and high-throughput pharmacological screening in drug discovery, has resulted in a vast increase in the number of lead compounds identified. However, the compounds generated in high-throughput drug discovery programs very often possess properties that are not compatible with oral administration, which is desired because of the convenience of this administration route. So oral absorption, which means the transport of a drug molecule across the mucosal membrane, is one goal of drug development (van der Waterbeemd et al., 2001). In fact, the clinical development of new drugs is often terminated due to unfavorable pharmacokinetic characteristics, such as poor bioavailability of the drug after oral administration (Clement, 2002). Bioavailability can be improved by using amidoximes instead of amidines (Clement, 1993) as prodrugs. This principle has been applied to drug candidates such as sibrafiban (Weller et al., 1996) and melagatran (Eriksson et al., 1999), and further compounds under development (Kitamura et al., 2001; Schipper et al., 2001).

The enzymatic basis of the prodrug principle, reduction of the amidoxime to the amide, was mainly studied with benzamidoxime as a model compound. This is the first study that clearly demonstrates that more complicated molecules such as the double-prodrug ximelagatran as well as the intermediate N-hydroxy-melagatran are metabolized in liver and kidney by microsomal and mitochondrial systems. Reduction of an amidoxime could be shown previously for model compounds such as benzamidoxime by pig liver and kidney microsomes (Clement and Mau, 1999) or mitochondria (Clement and Deters, 2000).

The formation of amidines by microsomal impurities in mitochondria and vice versa could be excluded by using established corresponding marker reactions (Sottocasa et al., 1967; Yasukochi and Masters, 1976) (data not shown). Both ximelagatran and N-hydroxy-melagatran were substrates for the reducing systems so that the activation follows the two pathways as shown in Fig. 1. It is clear that the simple melagatran and N-hydroxy-melagatran only represent intermediates that even in vitro studies are sometimes not detectable (Table 1). The activation of the double prodrug ximelagatran to the active principle melagatran was catalyzed by all enzyme sources, except for human kidney microsomes. However, both mono-prodrugs were formed again. There were no indications for metabolites other than ethyl-melagatran, N-hydroxy-melagatran, and melagatran.

A pH of 7.0 and the use of NADH as cosubstrate constitute optimum incubation conditions for the complete activation of ximelagatran via two intermediate mono-prodrugs to melagatran. The preference for NADH has also been observed for the model compound benzamidoxime (Clement et al., 1997). This is in agreement with an electron transfer by NADH-cytochrome b₅ reductase. The redox potential for transfer of electrons from b₅ to a P450 is unfavorable (Guengerich, 2001). The first reported reduction catalyzed by this system was thus unexpected and the mechanism needs further clarification. It is possible that by complexation, reduction potentials are changed, which has been reported for cytochrome b₅ and P450 iron in the ferric state. Electrons are donated via the formation of a complex between the P450 enzyme in the resting state is regenerated. Oxygen cannot interfere because it is bound by ferrous P450 (Clement, 2002). It can also not be ruled out that the reduction is actually performed by b₅ reductase and b₅ and that the role of the P450 isozyme is to increase the interaction between b₅ and its reductase (Clement, 2002).

The reduction in the presence of oxygen is in contrast to the known reductions performed by P450 enzymes alone or/and NADPH-cytochrome P450 reductase used, for example, for the bioactivation of N-oxide prodrugs in tumor cells with low oxygen pressure (Patterson, 2002).

In particular microsomes were able to form higher concentrations of the intermediate N-hydroxy-melagatran than mitochondria, whereas mitochondria formed higher concentrations of the mono-prodrug ethyl-melagatran. This can be explained by higher concentrations of esterases in microsomal preparations than in mitochondria. On the other hand, the reducing enzymes are present in mitochondria more than in microsomes as evidenced by the higher activity of mitochondria in reducing N-hydroxy-melagatran (Tables 1 and 2).

The ester cleavage of ximelagatran to N-hydroxy-melagatran cata-

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**Fig. 4.** Characterization of the ester hydrolysis of ximelagatran to N-hydroxy-melagatran by unspecific carboxyl esterases from pig liver. A standard incubation mixture consisted of 2 mM ximelagatran and 0.5 U of unspecific carboxyl esterases in 100 mM phosphate buffer pH 7.4. In the other experiments, 3.3 mM MgCl₂ or 1 mM NADH or 1 mM NADPH was added. After a 3-min preincubation period at 37°C in a shaking water bath, the reaction was initiated by the addition of thermotolerated esterases. After 20 min, the reaction was stopped by the addition of cold methanol, and the supernatant was analyzed by HPLC. Each column represents the means ± S.D. of four determinations (⁎, p < 0.05).
In vitro N-reduction of N-hydroxy-melagatran to melagatan by liver and kidney microsomes and mitochondria of different species and kinetic data

Incubation mixtures have been optimized for protein content, NADH concentration, and substrate concentration. An incubation mixture consisted of 0.15 mg/ml microsomal or mitochondrial protein of pig or human liver or 0.05 mg/ml of pig kidney or 0.2 mg/ml of human kidney preparations, 2 mM N-hydroxy-melagatran as substrate, and 1 mM NADH as cosubstrate in 100 mM potassium phosphate buffer pH 6.3. For incubation conditions and HPLC analysis, see Materials and Methods.

### Table 2

<table>
<thead>
<tr>
<th>Activity</th>
<th>N-Hydroxy-melagatran</th>
<th>Ethyl-melagatran</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/mg protein</td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>Complete system, pig</td>
<td>34.40 ± 1.54 *</td>
<td>17.13 ± 0.08</td>
</tr>
<tr>
<td>Without CYP2D</td>
<td>&lt;DL</td>
<td>[7.35 ± 0.02]</td>
</tr>
<tr>
<td>Without protein</td>
<td>&lt;DL</td>
<td>0.07 ± 0.04</td>
</tr>
</tbody>
</table>

DL, determination limit.

* Conversion rates are means ± S.D. of four determinations.

#### Table 3

<table>
<thead>
<tr>
<th>Species and System</th>
<th>V_{max} nmol/min/mg protein</th>
<th>V_{max}/K_{m} \times 10^{-6}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig liver microsomes</td>
<td>0.69 ± 0.04</td>
<td>5.60 ± 0.08</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>1.59 ± 0.04</td>
<td>2.26 ± 0.00</td>
</tr>
<tr>
<td>Pig liver mitochondria</td>
<td>0.30 ± 0.02</td>
<td>15.06 ± 0.67</td>
</tr>
<tr>
<td>Human liver mitochondria</td>
<td>0.98 ± 0.05</td>
<td>8.68 ± 0.15</td>
</tr>
<tr>
<td>Pig kidney microsomes</td>
<td>0.78 ± 0.06</td>
<td>13.83 ± 0.17</td>
</tr>
<tr>
<td>Human kidney microsomes</td>
<td>1.08 ± 0.14</td>
<td>3.93 ± 0.18</td>
</tr>
<tr>
<td>Pig kidney mitochondria</td>
<td>0.47 ± 0.03</td>
<td>20.67 ± 0.41</td>
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<tr>
<td>Human kidney mitochondria</td>
<td>1.40 ± 0.11</td>
<td>3.47 ± 0.07</td>
</tr>
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Conversion rates are means ± S.D. of four determinations.

In conclusion, it can be summarized that orally available double produgs such as ximelagatran are metabolized by enzymes present in several organs and cell organelles. One responsible enzyme system seems to consist of cytochrome bs, b5 reductase, and a P450 enzyme and is not influenced by oxygen.

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**References**


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