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ABSTRACT:

In previous studies, it was shown that liver microsomes from rabbit, rat, pig, and human are involved in the reduction of N-hydroxylated amidines, guanidines, and amidinohydrzones of various drugs and model compounds (Drug Metab Rev 34: 565–579). One responsible enzyme system, the microsomal benzamidoxime reductase, consisting of cytochrome b5, its reductase, and a cytochrome P450 isoenzyme, was isolated from pig liver microsomes (U Biol Chem 272:19615–19620). Further investigations followed to establish whether such enzyme systems are also present in microsomes of other organs such as brain, lung, and intestine. In addition, the mitochondrial reduction in human and porcine liver and kidney preparations was studied. The reductase activities were measured by following the reduction of benzamidoxime to benzamide, guanoxabenz to guanoxabenz, and Ro 48-3656 (([1-[2S]-2-[4-[(aminoiminomethyl)benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy]-acetic acid) to Ro 44-3888 (([1-[2S]-2-[4-[aminoiminomethyl]benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy]-acetic acid). Interestingly, preparations of all tested organs were capable of reducing the three compounds. The highest specific rates were found in kidney followed by liver, brain, lung, and intestine, and usually the mitochondrial reduction rates were superior. From the determined characteristics, similarities between the enzyme systems in the different organs and organelles were detected. Furthermore, properties of the benzamidoxime reductase located in the outer membrane of pig liver mitochondria were studied. In summary, these results demonstrate that in addition to the microsomal reduction, mitochondria are involved to a great extent in the activation of amidoxime prodrugs. The importance of extrahepatic metabolism in the reduction of N-hydroxylated prodrugs is demonstrated.

Numerous drugs and drug candidates contain strongly basic functional groups, such as guanidines, amidinohydrzones, and amidines. Because of their strong basicity, they are protonated under physiological conditions and usually not absorbed in the gastrointestinal tract. In our laboratory, a prodrug principle was developed in particular for amidines by N-hydroxylating this functional group to the corresponding amidoximes, substances with a less basic functional group. Consequently, they are not protonated under physiological conditions and can easily be absorbed as free bases. By creating these prodrugs of the active principle, the oral bioavailability of amidines can be increased. Thus amidoximes and similar functional groups can be used as prodrugs for amidines and related groups.

The prodrug principle was developed for pentamidine (Clement, 1993) and was then applied to other amidines such as trypanocidal compounds (Zhou et al., 2004), glycoprotein IIIa/IIb receptor antagonists, such as sibrafiban (Weller et al., 1996), and the thrombin inhibitor ximelagatran, which was recently approved and is the first orally available direct thrombin inhibitor on the market (Gustafsson et al., 2001).

Benzamidoxime is a model compound for such amidoxime prodrugs, which are known to be reduced by liver microsomes from different species as well as by the purified enzyme system from pig liver (Clement et al., 1997). Therefore, the reduction of this substrate and its HPLC analysis can be taken as a reliable activity assay (Fig. 1).

Sibrafiban is a nonmarketed oral platelet aggregation inhibitor and a double prodrug. After oral administration, the amidoxime ethylester sibrafiban is adsorbed and hydrolyzed into Ro 48-5656 (amidoxime and free acid) and by reduction of the N-hydroxylated structure Ro 48-3656 into the active metabolite Ro 44-3888 (Timm et al., 1997) (Fig. 1).

The third substrate under investigation was the centrally acting α,β-adrenoceptor agonist guanoxabenz (Benzbéral). The reduction of

ABBREVIATIONS: HPLC, high-performance liquid chromatography; Ro 48-5656, amidoxime (free acid); Ro 48-3656, [1-[2S]-2-[4-[N-hydroxyaminoiminomethyl]benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy]-acetic acid; Ro 48-3888, [1-[2S]-2-[4-[aminoiminomethyl]benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy]-acetic acid; P450, cytochrome P450; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol; OMV, outer membrane vesicle(s); p-HMB p-hydroxymercuribenzoic acid.
guanoxabenz to its amidinohydrazine guanabenz (Wytensin, Hipten, and Rexitene) has been described in previous studies with pig, rabbit, and human liver microsomes (Clement et al., 1996). This compound was chosen as a model compound for N-hydroxyamidinohydrazones as a prodrug of an amidinohydrazone. Furthermore, guanoxabenz has been shown to be mutagenic (Clement et al., 1996). Thus, the reduction is of high toxicological interest as a detoxification reaction.

Previous investigations demonstrated that microsomal enzymes from rabbit, rat, pig, and human liver are involved in the reduction of N-hydroxylated xenobiotics (Clement, 2002). In addition, one enzyme system capable of reducing N-hydroxylated derivatives of strongly basic functional groups has been identified so far in pig liver microsomes consisting of cytochrome b₅, NADH cytochrome b₅ reductase, and a cytochrome P450 (P450) isoenzyme of the subfamily 2D (Clement et al., 1997). The microsomal enzyme system showed similarities with the microsomal hydroxylamine reductase, a porcine liver enzyme with insensitivity to oxygen and highest activity in acid medium (Kadlubar and Ziegler, 1974), and the mitochondrial reduction of benzamidoxime showed common characteristics to the mitochondrial hydroxylamine reductase, a cyanide and mercury-sensitive NADH-dependent enzyme system (Bernheim and Hochstein, 1968).

The aim of this study was to investigate in which organs the N-reduction to the active amidine forms can take place and consequently take effect. In addition, the presence of these reducing systems in the gastrointestinal tract can decrease the bioavailability, because the amidoximes might already be reduced to their amidines before absorption. On the other hand, the absence of such enzyme systems might be correlated with organ-typical genotoxic properties of several N-hydroxylated structures. Thus, this reductase may play a vital role in protecting humans and other mammals against the accumulation of noxious metabolites.

In this study, the results of the investigations with liver, kidney, brain, lung, and intestinal microsomes using the reduction of benzamidoxime to benzamidine (Clement et al., 1997), guanoxabenz to guanabenz (Clement et al., 1996), and the sibrafiban metabolite Ro 48-3656 to the active form of sibrafiban Ro 44-3888 are summarized. This study demonstrates that porcine and human microsomes and mitochondria of all tested organs can reduce N-hydroxylated structures. Furthermore, characteristics and intramitochondrial localization of the mitochondrial benzamidoxime reductase were investigated.

Materials and Methods

Benzamidoxime (N-hydroxy-benzenecarboximidamide) was synthesized from benzonitrile and hydroxylamine as described previously (Krüger, 1885). Guanabenz acetate (2-[(2,6-dichlorophenyl)methylene]-hydrazinecarboximidamide, acetate salt) was kindly supplied by Wyeth-Pharma GmbH (Münster, Germany), guanoxabenz-HCl (2-[(2,6-dichlorophenyl)methylene]-N-hydroxy-hydrazinecarboximidamide, hydrochloride salt) by Laboratoires Houde (Paris, France), and Ro 48-3656 and Ro 44-3888 by Hoffmann-La Roche (Basel, Switzerland). All other chemicals were commercially available. Hydroxylamine-HCl, dipotassium hydrogen phosphate, phosphoric acid, tetramethylammonium chloride, cytochrome c (from horse heart), mannitol, 1-octanesulfonic acid (sodium salt), Percoll, p-hydroxymercuribenzoic acid (sodium salt), rotenone, succinic acid (disodium salt), MOPS, and benzamidine were purchased from Sigma-Aldrich (Taufkirchen, Germany). Methanol was from J. T. Baker (Derventer, Holland), and HEPES was from Biochrom AG (Berlin, Germany). Acetonitrile and methanol were of HPLC-grade, whereas other chemicals and solvents were of analytical grade.

Preparation of Subcellular Fractions: Microsomes.

Microsomes were prepared by differential centrifugation. All steps were performed in standard
phosphate buffer, pH 7.4 (4.8 mM KH₂PO₄, 15.2 mM K₂HPO₄, 0.25 M sucrose, and 1 mM EDTA). Centrifugation steps and storage were carried out in standard phosphate buffer without EDTA.

Briefly, the tissues were excised, washed, homogenized with a homogenizer (developed from University of Texas at Austin, Austin, Texas), and centrifuged at 9000×g for 30 min and then twice at 100,000×g for 60 min. The resulting pellet was suspended in phosphate buffer with a motorized Teflon pestle glass tube homogenizer, adjusted to a pH of 7.4, and frozen at −80°C. All organs except intestine and lung were pooled. All operations were performed at 0 to 4°C.

Preparation of Subcellular Fractions: Mitochondria. Pig liver mitochondria. Pig livers (slaughterhouse) were placed in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, and 1 mM DTT and perfused with the same buffer. All operations were performed at 0 to 4°C. The livers were homogenized. The homogenate was centrifuged at 750×g for 20 min. The supernatant was collected, and the pellet was resuspended and centrifuged again. The supernatants were combined, and crude mitochondria were sedimented by centrifugation at 7710×g for 20 min. The 7710×g sediment was washed four times by careful resuspending in buffer and following centrifugation. To minimize micromosal contamination, the centrifugal force was gradually diminished at each centrifugation step: the suspended mitochondria were centrifuged at the first sedimentation step at 7350×g for 20 min, then at 6450×g for 20 min, 5580×g for 20 min, and finally at 5050×g for 20 min. The final pellet was resuspended in buffer and stored at −80°C.

In other cases, and for the isolation of the outer mitochondrial membrane, pig liver mitochondria were obtained by differential centrifugation and isotonic Percoll gradient (Hovius et al., 1990). The livers were excised, perfused, and homogenized in isolation buffer [0.25 M sucrose, 1 mM EDTA, 10 mM KH₂PO₄, 1 mM DTT, and 0.1% (w/v) BSA, pH 7.4]. The homogenate was centrifuged at 600×g for 15 min.

The pellet was discarded, and this centrifugation step was repeated four times. The crude mitochondrial fraction was then sedimented from the supernatant by centrifugation at 10,300×g for 20 min. The pellet was resuspended in isolation buffer, the suspension was layered on top of Percoll buffer [30% (v/v) Percoll in 225 mM mannitol, 1 mM EGTA, 25 mM HEPES, and 0.1% (w/v) BSA, pH 7.4] and centrifuged at 95,000×g for 40 min. The mitochondrial fraction was collected and washed twice by centrifugation at 6300×g for 20 min. The final mitochondrial pellet was resuspended in isolation buffer and stored at −80°C. All operations were performed at 0 to 4°C.

Pig kidney mitochondria. Kidney cortex was prepared as described above for the preparation of pig liver mitochondria with slight modifications.

Human liver and kidney mitochondria. Human liver and kidney mitochondria were prepared from pooled liver/kidney samples of cancer patients. Only morphologically intact tissue was used. The separation was performed as described above for the preparation of pig liver mitochondria with slight modifications. Prior consent of the local medical ethics committee and from the donors were obtained for these studies.

Isolation of mitochondrial outer membrane vesicle fractions from pig liver. The outer membrane vesicles (OMV) were purified using the swell-disruption method followed by two steps of sucrose density gradient centrifugation (de Kroon et al., 1997). Purified mitochondria were sedimented by centrifugation at 10,000×g for 20 min and resuspended in hypotonic buffer (2.5 M KH₂PO₄, 2.5 mM K₂HPO₄, and 5 mM EDTA, pH 7.2). After 20 min of stirring on ice, the suspension was homogenized, and the swell-disruption homogenate was loaded on a discontinuous sucrose gradient in tubes (70 ml) of the following layers (all containing basic buffer 10 mM MOPS and 2.5 mM EDTA, pH 7.2): 15 ml of 1.1 M sucrose and 25 ml of 0.25 M sucrose.

After centrifugation at 141,000×g for 70 min, the OMV band was collected at the interface between the 1.1 M and 0.25 M sucrose layers and sedimented in 1.1 M sucrose buffer. Forty milliliters of this suspension was loaded on the bottom of a second discontinuous gradient consisting of the following layers (all containing basic buffer): 10 ml of 1.065 M sucrose and enough basic buffer to fill the tube. After centrifugation at 141,000×g for 16 h, the OMV fraction was collected, suspended in basic buffer, and washed twice by centrifugation at 235,000×g for 70 min. The final OMV pellet was resuspended in storage buffer (220 mM mannitol, 70 mM sucrose, and 2 mM HEPES, pH 7.4) and stored at −80°C. All operations were performed at 0 to 4°C.

Enzyme Purification. Cytochrome b₅ was purified from pig liver microsomes according to published methods (Taniguchi et al., 1984; Clement et al., 1997).

Protein Determination. Protein was assayed using bichinchoninic acid (Smith et al., 1985) according to the manufacturer’s directions (BCA Protein Assay Kit; Pierce, Rockford, IL).

Enzyme Assays. Cytochrome b₅ was estimated from difference spectra between the oxidized and NADH-reduced preparation (Estabrook and Werringloer, 1978). P450 was determined by carbon monoxide difference spectra (Omura and Sato, 1964). In the case of mitochondria, it was necessary to preincubate the enzyme preparation with 0.05% Triton X-100. NADH cytochrome b₅ reductase was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978). Rotenone-insensitive NADH cytochrome c reductase (marker of mitochondrial outer membrane) and succinate cytochrome c reductase (marker of mitochondrial inner membrane) were determined according to the method of Sottocasa et al. (1967). NADPH cytochrome c reductase (microsomal marker) was measured spectrophotometrically (Yasukochi and Masters, 1976). Mitochondria were examined for microsomal impurities by assaying NADPH cytochrome c reductase; microsomes were examined for mitochondrial impurities by assaying succinate cytochrome c reductase.

Calculation of Apparent Kinetic Parameters. To determine reduction kinetics, activities were measured at a minimum of 0.5 mM substrate concentrations with two replications at each concentration level. Apparent kinetic parameters Kₘ and Vₘₐₓ were estimated using nonlinear regression analysis (Sigma Plot 5.0, SPSS Inc., Chicago, IL).

Incubation with Benzamidoxime. Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. Incubation mixtures contained 6 to 600 μg of protein of various origins, 0.8 to 1.5 mM benzamidoxime, and 0.4 to 1.0 mM NADH in a total volume of 300 μl (microsomal preparations) or 150 μl (mitochondrial preparations) of potassium phosphate buffer, pH 5.5 (brain microsomes), 6.3 (human liver microsomes), or 7.0 (intestinal microsomes). For all other preparations, pH 6.0 was used. After a preincubation time of 3 min at 37°C, the reaction was initiated by the addition of NADH and terminated after 15 to 40 min by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation, and the supernatant was analyzed by HPLC.

Incubation with Guanoxabenz. Incubations were performed aerobically at 37°C in a shaking water bath with an incubation mixture consisting of 56 to 600 μg of protein of various origins, 1.0 to 3.0 mM guanoxabenz, and 1.0 to 1.5 mM NADH in a total volume of 300 μl (microsomal preparations) or 150 μl (mitochondrial preparations) of potassium phosphate buffer, pH 6.0 (all mitochondrial preparations and lung microsomes), 6.3 (pig kidney microsomes and pig and human liver microsomes), 6.5 (brain and human kidney microsomes), or 7.0 (intestinal microsomes). After a preincubation time of 3 to 8 min at 37°C, the reaction was started by the addition of NADH and terminated after 20 to 30 min by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation, and the supernatant was analyzed by HPLC.

Incubation with Ro 48-3656. Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. The usual incubation mixture contained 56 to 1000 μg of protein of various origins, 1.0 to 2.0 mM Ro 48-3656, and 0.8 to 1.0 mM NADH in a total volume of 300 μl (microsomal preparations) or 150 μl (mitochondrial preparations) of 100 mM potassium phosphate buffer, pH 6.0 (mitochondrial preparations) or 6.3 (microsomal preparations). After 3 min of preincubation at 37°C, the reaction was started by the addition of NADH. The samples were incubated for 20 to 30 min and stopped by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation, and the supernatant was analyzed by HPLC.

Inhibition Studies. Incubations with benzamidoxime were performed as described above with slight modifications. After preincubation (5–30 min) of protein with inhibitor and NADH, the reaction was started by the addition of benzamidoxime. Studies were performed with 0 to 100 μM p-hydroxymercuribenzoic acid (p-HMB), 0 to 2000 μM potassium cyanide, and 0 to 1 M hydroxyamine-HCl.

HPLC Analysis. For HPLC analysis, the following system was used: Waters 600S Controller with 616 Pump, 486 TAD UV Detector, 717-plus Autosampler (Waters, Milford, MA), and EZChrom chromatography software version 6.7 or EZChrom Client chromatography software version 2.8.3 (Sci-
entific Software Inc., San Ramon, CA). Solvents used in the analysis were filtered through a 0.45-μm Sartolon membrane filter (Sartorius AG, Göttingen, Germany) and degassed with helium or by sonication.

**HPLC method for the reduction of benzamidoxime to benzamidine.** The separation was carried out isocratically by 10 mM 1-octylsulfonate sodium salt and 17% (v/v) acetonitrile (pH not adjusted) by a LiChroCART 250-4 HPLC Cartridge with LiChrospher 60 RP-select B (5 μm) and a LiChroCART 4-4 guard column (Merck). The mobile phase was passed through the column at a rate of 1.0 ml/min. The effluent was monitored at 229 nm. For the determination of the recovery rate, incubation mixtures with defined concentrations of synthetic reference substance (5–200 μM) were incubated and worked up under the same conditions as the experimental samples but without adding cofactor. The standard curves were linear over this range with correlation coefficients of 0.999 (n = 48). The signals (peak areas) obtained were compared with those of the same amount of benzamidine dissolved in the mobile phase. The recovery rate from pig liver mitochondria amounted to 105% (r = 0.9993). Similar values were obtained from the other enzyme sources. The retention times were 7.9 ± 0.1 min (benzamidoxime) and 26.7 ± 0.1 min (benzamidine). The formation of the reductive metabolite benzamidine was also already identified by comparison of its HPLC, TLC and mass spectral characteristics with the data of the reference compound (Clement et al., 1988).

**HPLC method for the reduction of guanoxabenz to guanabenz.** The separation was carried out isocratically by 30% (v/v) methanol, 0.5% (v/v) acetic acid, and 69.5% (v/v) water (pH 4.0 with concentrated NH₃) by a LiChroCART 125-4 HPLC Cartridge with LiChrospher 60 RP-select B (5 μm) and a LiChroCART 4-4 guard column (Merck). The mobile phase was passed through the column at a rate of 1.0 ml/min. The effluent was monitored at 272 nm. For the determination of the recovery rate, incubation mixtures with defined concentrations of synthetic reference substance (1–100 μM) were incubated and worked up under the same conditions as the experimental samples but without adding cofactor. The standard curves were linear over this range with correlation coefficients of 0.9986 (n = 40). The signals (peak areas) obtained were compared with those of the same amount of guanoxabenz dissolved in the mobile phase. The recovery rate from pig liver microsomes amounted to 107% (r = 0.9993). Similar values were obtained from the other enzyme sources. The retention times were 14.2 ± 0.4 min (guanoxabenz) and 19.4 ± 0.5 min (guanabenz). The formation of guanabenz as the reductive metabolite of guanoxabenz was already confirmed by liquid chromatography-mass spectrometry analysis (Clement et al., 1996).

**HPLC method for the reduction of Ro 48-3656 to Ro 44-3888.** The separation was carried out isocratically by 96% (v/v) 100 mM phosphate buffer pH 4.5 and 4% (v/v) acetonitrile by a LiChroCART 250-4 HPLC Cartridge with LiChrospher 60 RP-select B (5 μm) and a LiChroCART 4-4 guard column (Merck). The mobile phase was passed through the column at a rate of 1.0 ml/min. The effluent was monitored at 240 nm. For the determination of the recovery rate, incubation mixtures with defined concentrations of reference substance (10–400 μM) were incubated and performed under the same conditions as the experimental samples but without adding cofactor. The standard curves were linear over this range with correlation coefficients of 0.9986 (n = 44). The signals (peak areas) obtained were compared with those of the same amount of Ro 44-3888 dissolved in the mobile phase. The recovery rate from pig kidney microsomes amounted to 104% (r = 0.9941). Similar values were obtained from the other enzyme sources. The retention times were 6.5 ± 1.1 min (Ro 44-3888) and 11.4 ± 2.0 min (Ro 48-3656). The retention times for the metabolite agreed with those of the reference substance. The active metabolite Ro 44-3888 was also already determined using HPLC column-switching combined with turbo ion spray single quadrupole mass spectrometry (Timm et al., 1997).

**Results**

**Characterization of Subcellular Fractions.** The comparison of the activities of the two marker enzymes NADPH cytochrome c reductase (microsomal impurities) and succinate cytochrome c reductase (mitochondrial impurities) indicates that the microsomal preparations are usually less contaminated than the mitochondrial preparations. Based on the specific activity of succinate cytochrome c reductase in mitochondria, the corresponding microsome fraction preparations usually contain <10% mitochondrial, whereas based on the specific activity of enzymatic NADPH cytochrome c reductase in microsomes, the corresponding mitochondria preparations contain usually about 20% microsomal impurities. Concentrations and activities of cytochrome bs, NADH cytochrome bs reductase, and cytochrome P450 in investigated subcellular fractions are listed in Tables 1 and 2.

NADH cytochrome bs reductase activity measured in the OMV fraction was 21.1 ± 0.25 U/mg (n = 3); cytochrome bs content measured in the OMV fraction was 0.06 ± 0.01 nmol/mg (n = 3). Low contents of cytochrome P450 were detectable in the OMV fraction but could not be quantified.

**Microsomal and Mitochondrial Reduction of N-Hydroxylated Structures. Reduction of benzamidoxime to benzamidine.** The reduction of the model substrate benzamidoxime to benzamidine was detected at physiological pH in all microsomal and mitochondrial preparations (Table 3). The rate of the mitochondrial benzamidoxime reduction was higher than in microsomes, and it could be shown that the specific rate of reduction depended on the origin of the organelles. The specific rates were highest in kidney, followed by liver, brain, lung, and intestine except for human kidney mitochondria (Table 3). The preferred cosubstrate of the reduction is NADH, and the pH optimum is usually at weak acid pH (data not shown).

**Reduction of guanoxabenz to guanabenz.** Guanoxabenz, which is known to be reduced by liver microsomes (Clement et al., 1996), was also transformed by all preparations (Table 4). Replacing NADH by NADPH decreased the reduction rates (data not shown). These results demonstrate again that NADH was the preferred cosubstrate and that the reduction rate was higher in mitochondria than in microsomes. The reduction in microsomes was again more pronounced in kidney than in liver, followed by lung, intestine, and brain (Table 4).

**Reduction of Ro 48-3656 to Ro 44-3888.** Ro 48-3656 was reduced to its metabolite Ro 44-3888 (Table 5). In mitochondria of pig organs, NADH was the preferred cosubstrate (data not shown). In contrast to the other two drugs, the kidney and liver rates were very similar (pig preparations), or the reduction exhibits greater activity in liver mitochondria (human preparations) (Table 5).

**Characterization of the Mitochondrial Benzamidoxime Reductase.** An outer membrane-enriched fraction (OMV fraction) obtained from pig liver mitochondria was analyzed for marker enzymes of the outer and inner mitochondrial membrane, rotenone-insensitive cytochrome c reductase, and succinate cytochrome c reductase, respectively, as summarized in Table 6. The quotient of the enrichment of the two enzyme markers indicates that a high degree of purification of

**TABLE 1**

Concentrations and activities of cytochrome bs, NADH cytochrome bs reductase, and cytochrome P450 in investigated microsomes of various organs and species

<table>
<thead>
<tr>
<th>Origin of Microsomes</th>
<th>Cytochrome bs</th>
<th>bs Reductase</th>
<th>Cytochrome P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol/mg</td>
<td>U/mg</td>
<td>mmol/mg</td>
</tr>
<tr>
<td>Kidney/pig</td>
<td>0.14</td>
<td>3.27</td>
<td>0.22</td>
</tr>
<tr>
<td>Kidney/human</td>
<td>0.07</td>
<td>3.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Liver/pig</td>
<td>0.38</td>
<td>3.22</td>
<td>0.44</td>
</tr>
<tr>
<td>Liver/human</td>
<td>0.21</td>
<td>2.17</td>
<td>0.63</td>
</tr>
<tr>
<td>Brain/pig</td>
<td>0.06</td>
<td>1.43</td>
<td>N.D.</td>
</tr>
<tr>
<td>Lung/pig</td>
<td>0.08</td>
<td>2.72</td>
<td>N.D.</td>
</tr>
<tr>
<td>Intestine/pig</td>
<td>0.03</td>
<td>1.30</td>
<td>0.05</td>
</tr>
</tbody>
</table>

N.D., not detectable.
To determine the cytochrome content in pig kidney mitochondria, the protein suspension was sonicated before measuring difference spectra. NADH cytochrome b reductase was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978) and indicating its outer membrane localization.

Materials and Methods

Enzyme activities were measured spectrophotometrically. Cytochrome P450 was determined by carbon monoxide difference spectra after preincubation of the enzyme preparation with 0.05% Triton X-100 (Omura and Sato, 1964).

TABLE 2
Concentrations and activities of cytochrome b5, NADH cytochrome b5 reductase, and cytochrome P450 in investigated mitochondria of various organs and species

<table>
<thead>
<tr>
<th>Origin of Mitochondria</th>
<th>Cytochrome b5</th>
<th>NADH reductase</th>
<th>Cytochrome P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney/pig</td>
<td>0.08</td>
<td>1.04</td>
<td>0.36</td>
</tr>
<tr>
<td>Liver/pig</td>
<td>0.03</td>
<td>1.84</td>
<td>0.19</td>
</tr>
<tr>
<td>Kidney/human</td>
<td>0.02</td>
<td>1.75</td>
<td>N.D.</td>
</tr>
<tr>
<td>Liver/human</td>
<td>0.04</td>
<td>1.38</td>
<td>0.31</td>
</tr>
</tbody>
</table>

N.D., not detectable.

TABLE 3
Kinetic parameters of the reduction of benzamidoxime to benzoamide

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg of protein)</th>
<th>$V_{max}/k_m$ (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Microsomes (pig)</td>
<td>0.03</td>
<td>166</td>
<td>$5.0 \times 10^{-1}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (pig)</td>
<td>12.8</td>
<td>1082</td>
<td>$8.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (human)</td>
<td>3.2</td>
<td>7.8</td>
<td>$2.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>Liver Microsomes (pig)</td>
<td>0.06</td>
<td>5.3</td>
<td>$8.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>Liver Mitochondria (pig)</td>
<td>0.89</td>
<td>84</td>
<td>$9.4 \times 10^{-4}$</td>
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<tr>
<td>Liver Mitochondria (human)</td>
<td>0.71</td>
<td>7.2</td>
<td>$1.0 \times 10^{-5}$</td>
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<tr>
<td>Liver OMV (pig)</td>
<td>0.08</td>
<td>314</td>
<td>$3.9 \times 10^{-3}$</td>
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<tr>
<td>Brain Microsomes (pig)</td>
<td>0.05</td>
<td>18.1</td>
<td>$3.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>Brain Microsomes (pig)</td>
<td>0.20</td>
<td>1.70</td>
<td>$7.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>Intestine Microsomes (pig)</td>
<td>0.10</td>
<td>0.10</td>
<td>$1.0 \times 10^{-6}$</td>
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</table>

TABLE 4
Kinetic parameters of the reduction of guanoxabenz to guanabenz

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg of protein)</th>
<th>$V_{max}/k_m$ (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Microsomes (pig)</td>
<td>0.51</td>
<td>181</td>
<td>$3.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (pig)</td>
<td>1.04</td>
<td>53.2</td>
<td>$5.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (human)</td>
<td>0.43</td>
<td>7.10</td>
<td>$1.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>Liver Microsomes (pig)</td>
<td>0.19</td>
<td>8.64</td>
<td>$4.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>Liver Mitochondria (pig)</td>
<td>0.15</td>
<td>19.3</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Liver Mitochondria (human)</td>
<td>0.19</td>
<td>7.10</td>
<td>$3.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>Brain Microsomes (pig)</td>
<td>0.25</td>
<td>0.24</td>
<td>$9.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Brain Microsomes (pig)</td>
<td>0.15</td>
<td>1.46</td>
<td>$9.7 \times 10^{-6}$</td>
</tr>
<tr>
<td>Intestine Microsomes (pig)</td>
<td>12.1</td>
<td>1.40</td>
<td>$1.2 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

TABLE 5
Kinetic parameters of the reduction of Ro 48-3656 to Ro 44-3888

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg of protein)</th>
<th>$V_{max}/k_m$ (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Microsomes (pig)</td>
<td>4.03</td>
<td>8.0</td>
<td>$2.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (pig)</td>
<td>0.30</td>
<td>9.2</td>
<td>$1.8 \times 10^{-6}$</td>
</tr>
<tr>
<td>Kidney Mitochondria (human)</td>
<td>0.51</td>
<td>0.3</td>
<td>$5.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>Liver Microsomes (pig)</td>
<td>0.31</td>
<td>7.3</td>
<td>$2.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>Liver Mitochondria (human)</td>
<td>0.44</td>
<td>1.5</td>
<td>$3.4 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

TABLE 6
Enzyme activity of benzamidoxime reductase and marker enzymes in mitochondria and purified OMV fraction from pig liver

<table>
<thead>
<tr>
<th>Origin of Mitochondria</th>
<th>$k_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg of protein)</th>
<th>$V_{max}/k_m$ (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Microsomes (pig)</td>
<td>18.0</td>
<td>$170 \pm 170$</td>
<td>$947 \pm 230$</td>
</tr>
<tr>
<td>Kidney Mitochondria (pig)</td>
<td>100</td>
<td>$100^\circ$</td>
<td>$100^\circ$</td>
</tr>
<tr>
<td>Kidney Mitochondria (human)</td>
<td>1430</td>
<td>$1170^\circ$</td>
<td>$13^\circ$</td>
</tr>
</tbody>
</table>

Discussion

Extrahepatic Metabolism for the Activation of N-Hydroxylated Prodrugs.

The reduction of benzamidoxime was described using liver microsomes as the enzyme source (Clement et al., 1997; Andersson et al., 2005), and the reduction of guanoxabenz was also investigated.
with liver microsomes (Clement et al., 1996) and cytosolic fractions (Dambrova et al., 1998).

The objective of these investigations was to demonstrate the reduction of several amidoximes to amidines by microsomes and mitochondria of different organs (Fig. 1). Although the liver is usually the main organ for drug metabolism, the conversion rates in porcine kidney preparations were usually higher than in liver preparations, and mitochondrial reduction rates were higher than microsomal ones (Tables 3, 4, and 5). The only exception of mitochondrial superiority was the conversion of guanoxabenz (Table 4); in this case, the microsomal reduction rates were higher than microsomal ones (Tables 3 and 4) offers an alternative application way for amidoximes as produgs for amidines by inhalation.

To be able to cross the blood-brain barrier by diffusion, xenobiotics have to be lipophilic. This study demonstrates that enzymes that can reduce amidoximes to amidines are present in the brain (Tables 3 and 4). So the concept of amidoximes as prodrugs of amidines might also be used to overcome the blood-brain barrier for hydrophilic amidines. Of course, the amount of amidoximes penetrating the central nervous system will be limited by the reduction of amidoximes in other organs, but it might still be high enough to achieve a desired pharmacodynamic effect.

For our studies, pig organs were chosen because of their easy availability and the similarities between human and pig liver in their capacity to reduce N-hydroxylated compounds (Clement et al., 1997).
These observations suggest that reductase activities could also be present in different human organs. In all human preparations, the detected conversion rates were lower than in the porcine preparations but still very high for foreign compound metabolism. All investigated human organs reduced the three substrates.

Because of the similar characteristics, it can be speculated that one enzyme system is responsible for these reductions. Obviously, these enzymes reduce N-hydroxylated structures as part of different types of compounds (Fig. 1). This discovery is of great importance for drug research and development with regard to the development of prodrugs (Ettmayer et al., 2004).

An enzyme system, consisting of cytochrome b5, its reductase, and a cytochrome P450 isoenzyme from the subfamily 2D was isolated from pig liver and called the microsomal benzamidoxime reductase (Clement et al., 1997). To investigate the potential presence of the same enzyme system in other organs, the presence of the three components of the microsomal benzamidoxime reductase was analyzed. Cytochrome b5 and its reductase could be detected in all other investigated microsomal preparations (Table 1) and in all mitochondrial preparations (Table 2). It is known that cytochrome P450 genes are expressed in many extrahepatic tissues, even in all investigated organs (Guengerich, 1995; Pelkonen and Raunio, 1997). In brain and lung microsomes, cytochrome P450 was not detectable by carbon monoxide difference spectra. It has been described before that P450 concentrations are too low to be analyzed by this assay but can be measured by their activity (Ghersi-Egea et al., 1994) or by immunohistochemical techniques (Krishna and Klotz, 1994). Brain cytochrome P450 could only measured spectrally after extraction by hydrophobic chromatography and not in microsomal fractions (Warner and Gustafsson, 1994). Other studies have demonstrated the expression of xenobiotic-metabolizing cytochrome P450 forms in porcine tissues (Nissen et al., 1998; Skaanild and Friis, 1999). The lung especially has an extensive range of these xenobiotic-metabolizing enzymes, including 1A1, 2B, 2E1, 2F1, 3A, and 4B1 (Pelkonen and Raunio, 1997). It can be suggested that in kidney, brain, lung, and intestine, the same enzyme system as in the liver is responsible for the observed conversion rates. However, the presence and participation of other enzyme systems cannot be excluded. This will be the subject of further studies.

In summary, these results demonstrate the importance of extrahepatic metabolism for the activation of N-hydroxylated prodrugs. Because we also discovered the importance of a mitochondrial reduction, further characterization of the mitochondrial enzyme systems was undertaken. So far, anaerobic reduction in rat liver mitochondria of a quinone-dependent N-oxide reductase (Kitamura et al., 1999) and NADPH-dependent N-oxide reductase (Sugiura and Kato, 1977) have been documented as well as an oxygen-insensitive NADH-dependent reduction of hydroxylamines and aryl hydroxamates (Bernheim and Hochstein, 1968).

The mitochondrial reduction exhibited similar properties to the microsomal reduction. The reduction is oxygen-insensitive (Fig. 6). NADH is the preferred cosubstrate, and its pH optimum is 6.0. Reduction could also be observed at physiological pH (data not shown). These properties are also similar to the characteristics of the mitochondrial hydroxylamine reductase (Bernheim and Hochstein, 1968). Thus, inhibition studies showed that the reduction of benzamidoxime could be inhibited by hydroxylamine (Fig. 3) and that the inhibition could be reversed by enhanced concentrations of benzamidoxime (Fig. 4).

Studies with a membrane fraction of pig liver indicate that the benzamidoxime reductase activity is located in the outer membrane. Cytochrome b5 and its reductase are compounds of the outer mitochondrial membrane (Sottocasa and Sandri, 1970; Taniguchi et al., 1973) and could also be detected in this study in the OMV fraction of pig liver mitochondria.

NADH cytochrome b5 reductase is an integral membrane protein essential for microsomes and the outer mitochondrial membrane (Borgese and Pietrini, 1986). In mammals, the reductase in its various locations is molecularly identical (Meldolesi et al., 1980).

Two distinct forms of cytochrome b5 have been shown to exist in rat liver (Lederer et al., 1983). Cytochrome b5 localized in the outer mitochondrial membrane is distinguishable from the microsomal form in spectral and immunological properties as well as in primary structure. Furthermore, the mitochondrial isoform has a more negative reduction potential and is more stable toward chemical and thermal denaturation (Altuve et al., 2001). We assume that—again like the microsomal benzamidoxime reductase—cytochrome b5 and its reductase are components of the mitochondrial enzyme system. This assumption could be verified by studies with p-hydroxymercurobenzoic acid, a known inhibitor of NADH cytochrome b5 reductase (Shimada et al., 1998). Experiments with purified microsomal cytochrome b5 enhanced the activity in the OMV fraction. The activity was doubled. This raise was already saturated at ~10 pmol of cytochrome b5 (Fig. 5). It cannot be excluded that similarly to the microsomal benzamidoxime reductase a third protein is a component of the benzamidoxime reductase in the outer mitochondrial membrane.

To investigate again whether a P450 enzyme is included in the benzamidoxime reduction, its content was analyzed. Only low amounts of P450 could be detected in the OMV fraction by carbon monoxide difference spectra (data not shown). The location of P450 isoenzymes in mitochondria is usually the inner mitochondrial membrane (Sottocasa and Sandri, 1970; Taniguchi et al., 1973; della-Cioppa et al., 1986). However, a report about a cyanide-insensitive enzyme system containing P450 in the purified outer membrane was published (Uemura and Chiesara, 1976). A partial inhibition of mitochondrial benzamidoxime reduction was observed at concentrations >400 μM potassium cyanide. However, the benzamidoxime reductase activity of the OMV fraction was insensitive and in contrast even enhanced by cyanide (Fig. 2). This indicates a cyanide-sensitive and -insensitive reduction system.

Purification studies are underway to clarify the participation and identification of a potential third component of the mitochondrial benzamidoxime reductase, which shares common characteristics with...
the microsomal protein described by Kadlubar and Ziegler (1974). The presence of additional N-reductive enzyme systems in other submitochondrial compartments cannot be excluded.

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References


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