DMD #5249

Hepatic, extrahepatic, microsomal, and mitochondrial activation

of the *N*-hydroxylated prodrugs

benzamidoxime, guanoxabenz, and Ro 48-3656

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1

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Running title: Activation of *N*-hydroxylated prodrugs

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Number of text pages: 25

Number of tables: 6

Number of figures: 6

Number of references: 42

Number of words in the abstract: 234

Number of words in the Introduction: 654

Number of words in the discussion: 1418

Non-standard Abbreviations:

p-HMB   *p*-hydroxymercuribenzoic acid

OMV   outer membrane vesicles

P450   Cytochrome P450

CR   rotenone-insensitive cytochrome c reductase

SuccCytc   succinate cytochrome c reductase

BAORed   benzamidoxime reductase

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 amidinohydrazones of various drugs and model compounds (Clement, 2002). One responsible enzyme system, the microsomal benzamidoxime reductase, consisting of cytochrome b_5, its reductase, and a P450 isoenzyme, was isolated from pig liver microsomes (Clement et al., 1997) . 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 also kidney preparations were studied. The reductase activities were measured by following the reduction of benzamidoxime to benzamidine, of guanoxabenz to guanabenz, and of Ro 48-3656 to Ro 44-3888. 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, amidinohydrazones, 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 antagonist, 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 non-marketed 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_2-adrenoreceptor agonist guanoxabenz (Benzérial). The reduction of guanoxabenz to its

amidinohydrazone guanabenz (Wytensin®, Hipten®, 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 b_5, NADH cytochrome b_5 reductase, and a 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 insensibility 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 can 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 protecting humans and other mammals against accumulation of noxious metabolites.

In this study the results of the investigations with liver, kidney, brain, lung, and intestine microsomes using the reduction of benzamidoxime to benzamidine (Clement et al., 1997), of 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 report demonstrates that porcine and human microsomes and mitochondria of all tested organs are able to reduce *N*-hydroxylated structures.

Furthermore, characteristics and intramitochondrial localisation of the mitochondrial benzamidoxime reductase were investigated.

Materials and Methods

Benzamidoxime (N-hydroxy-benzenecarboximidamide) was synthesized from benzonitrile, and hydroxylamine as described (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. Hydro-chloride salt.) by Laboratoires Houdé (Paris, France), and Ro 48-3656 ([[1-[(2S)-2-[[4-[(hydroxyamino)iminomethyl]benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy-acetic acid), and Ro 44-3888 ([[1-[(2S)-2-[[4-(aminoiminomethyl)benzoyl]amino]-1-oxopropyl]-4-piperidinyl]oxy]-acetic acid) 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 from J.T. Baker (Derventer, Holland), Hepes from Biochrom AG (Berlin, Germany). Acetonitrile was obtained from Promochem (Wesel, Germany), Triton X-100 from Serva Feinbiochemica (Heidelberg, Germany), acetic acid, BSA, DTT, EDTA (disodium salt), EGTA, potassium cyanide, potassium dihydrogen phosphate, NADH (disodium salt), NADPH (tetrasodium salt), sucrose, Tris-HCl, potassium hexacyanoferrate (III), and all other chemicals from Merck KGaA (Darmstadt, Germany) unless otherwise stated. Acetonitrile and methanol were of HPLC grade, while other chemicals and solvents were of analytical grade.

Preparation of subcellular fractions:

Preparation of microsomes. Microsomes were prepared by differential centrifugation. All steps were performed in standard phosphate buffer pH 7.4 (4.8 mM KH_2PO_4, 15.2 mM K_2HPO_4, 0.25 M sucrose, 1mM 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 Austin, Texas, USA), and centrifuged at 9000g for 30 minutes and then twice at 100000g for 60 minutes. 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 0-4°C.

Preparation of 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, 1 mM DTT, and perfused with the same buffer. All operations were performed at 0-4°C. The livers were homogenized. The homogenate was centrifuged at 750g for 20 minutes. The supernatant was collected and the pellet was resuspended and centrifuged again. The supernatants were combined and crude mitochondria were sedimented by centrifugation at 7710g for 20 minutes. The 7710g sediment was washed four times by careful resuspending in buffer and following centrifugation. In order to minimize microsomal contamination, the centrifugal force was gradually diminished at each centrifugation step: the suspended mitochondria were centrifuged at the first sedimentation step at 7350g for 20 minutes, and then at 6450g for 20 minutes, 5580g for 20 minutes, and finally at 5050g for 20 minutes.

The final pellet was resuspended in buffer and stored at –80°C.

In other cases and for 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 KH_2PO_4, 1 mM DTT, 0.1% (w/v) BSA, pH 7.4). The homogenate was centrifuged at 600g for 15 minutes. 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 10300g for 20 minutes. 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, 0.1% (w/v) BSA, pH 7.4), and centrifuged at 95000g for 40 minutes. The mitochondrial fraction was collected and washed twice by centrifugation at 6300g for 20 minutes. The final mitochondrial pellet was resuspended in isolation buffer and stored at −80°C. All operations were performed at 0-4°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 vesicles (OMV-fraction) from pig liver: The OMV was 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 10000g for 20 minutes und resuspended in hypotonic buffer (2.5 M KH_2PO_4, 2.5 mM K_2HPO_4, 5 mM EDTA, pH 7.2). After 20 minutes 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, 2.5 mM EDTA, pH 7.2): 15 ml 1.1 M sucrose, 25 ml 0.25 M sucrose.

After centrifugation at 141000g for 70 minutes the OMV band was collected at the interface between the 1.1 M and 0.25 M sucrose layers and suspended in 1.1 M sucrose buffer. 40 ml of this suspension was loaded on the bottom of a second discontinuous gradient, consisting of the following layers (all containing basic buffer): 10 ml 1.065 M sucrose and enough basic buffer to fill the tube. After centrifugation at 141000g for 16 hours the OMV-fraction was collected, suspended in basic buffer and washed twice by centrifugation at 235000g for 70 minutes. The final OMV pellet was resuspended in storage buffer (220 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4), and stored at –80°C. All operations were performed at 0-4°C.

Enzyme purification. Cytochrome b_5 was purified from pig liver microsomes according to a published method (Taniguchi et al., 1984; Clement et al., 1997).

Protein determination. Protein was assayed using bicinchonic acid (Smith et al., 1985), according to the manufactor's directions (BCA protein assay kit, Pierce, Rockford, USA)

Enzyme assays. *Cytochrome b_5* was estimated from difference spectra between the oxidized and the NADH reduced preparation (Estabrook and Werringloer, 1978). *Cytochrome P450* was determined by carbon monxide 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 b_5 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 K_m and V_{max} were estimated using nonlinear regression analysis (Sigma Plot 5.0; SPSS Science, Chicago, IL).

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

Incubation with guanoxabenz: Incubations were performed aerobically at 37°C in a shaking water bath with an incubation mixture consisting of 56-600 µg protein of various origin, 1.0-3.0 mM guanoxabenz and 1.0-1.5 mM NADH in a total volume of 300 µl (microsomal preparations) or 150 µl (mitochondrial preparations) 100 mM potassium phosphate buffer pH 6.0 (all mitochondrial preparations, lung microsomes), pH 6.3 (pig kidney microsomes, pig and human liver microsomes), pH

11

6.5 (brain microsomes, human kidney microsomes), or 7.0 (intestine microsomes). After a preincubation time of 3-8 minutes at 37°C the reaction was started by the addition of NADH and terminated after 20-30 minutes by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation and the supernatant was analysed 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 56-1000 µg protein of various origins, 1.0-2.0 mM Ro 48-3656 and 0.8-1.0 mM NADH in a total volume of 300 µl (microsomal preparations) or 150 µl (mitochondrial preparations) 100 mM potassium phosphate buffer pH 6.0 (mitochondrial preparations) or 6.3 (microsomal preparations). After 3 minutes of preincubation at 37°C the reaction was started by the addition of NADH. The samples were incubated for 20-30 minutes and stopped by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation and the supernatant was analysed by HPLC.

Inhibition studies. Incubations with benzamidoxime were performed as described above with slight modifications. After preincubation (5-30 minutes) of protein with inhibitor and NADH the reaction was started by the addition of benzamidoxime. Studies were performed with 0-100 µM p-HMB, 0-2000 µM potassium cyanide and 0-1M hydroxylamine-hydrochloride.

HPLC analysis. For HPLC analysis, the following system was used: Waters[TM] 600 S controller with pump 616, 486 TAD UV detector, autosampler 717 plus and EZ Chrom chromatographie software Version 6.7 or EZ Chrom Client chromatography software Version 2.8.3. (Scientific 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% acetonitrile (v/v) (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 KgaA, Darmstadt, Germany). 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 enzymes 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 conc. NH$_3$) by a LiChroCART$^®$ 125-4 HPLC-Cartridge with LiChrospher$^®$ 60 RP-select B (5 μm) and a LiChroCART$^®$ 4-4 guard column (Merck KGaA, Darmstadt, Germany). 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 enzymes 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 LC-MS 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 with 10 mM tetramethylammonium chloride (pH 4.5 with conc. H_3PO_4) and 4% (v/v) acetonitrile by a LiChroCART® 250-4 HPLC-Cartidge with LiChrospher® 60 RP-select B (5 µm) and a LiChroCART® 4-4 guard column (Merck KGaA, Darmstadt, Germany). 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 enzymes 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 utilising HPLC-column switching combined with turbo ion spray single quadrupole mass spectrometry (Timm et al., 1997).

Results

Characterisation 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 acitivity of succinate cytochrome c reductase in mitochondria the corresponding microsome fraction preparations usually contain < 10% mitochondria, while based on the specific acitivity of enzymes NADPH cytochrome c reductase in microsomes the corresponding mitochondria preparations contain usually about 20% microsomal impurities.

Concentrations and activities of cytochrome b_5, NADH cytochrome b_5 reductase, and cytochrome P450 in investigated subcellular fractions are listed in Table 1 and 2.

NADH cytochrome b_5 reductase activity measured in the OMV-fraction was (21.1 ± 0.25) U/mg (n=3), cytochrome b_5 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).

Characterisation of the mitochondrial benzamidoxime reductase.

An outer membrane enriched fraction (OMV-fraction) obtained from pig liver mitochondria was analysed for marker enzymes of the outer and the 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 the OMV fraction was obtained. The distribution of the benzamidoxime reductase was close to that of the outer membrane marker, showing an 11-fold

enrichment of rotenone-insensitive cytochrome c reductase, and 14-fold enrichment of benzamidoxime reductase in the OMV-fraction over the starting swell-disruption homogenate (see *Materials and Methods*) and indicates its outer membrane localisation.

As shown in Table 3 the OMV-fraction is a very potent preparation for reducing the model substrate benzamidoxime. The preferred cosubstrate of the reduction was NADH and the pH optimum was pH 6.0. The reduction also occured at pH 7.4 (data not shown). The catalytic efficiency was increased about a hundredfold in comparison to the unfractioned pig liver mitochondria (Table 6).

The NADH-dependent benzamidoxime reductase was completely inhibited by the NADH b_5-reductase inhibitor *p*-HMB (Shimada et al., 1998) at concentrations of 80 μM in mitochondria and 20 μM in the OMV-fraction. A 50% inhibition of mitochondrial benzamidoxime reduction was observed at concentrations of > 400 μM potassium cyanide (Fig. 2).

However, the benzamidoxime reductase activity of the OMV-fraction was insensitive and in contrast even enhanced by cyanide (Fig. 2).

25 μM hydroxylamine-HCl almost completely inhibited the benzamidoxime reductase activity in mitochondria and the OMV-fraction (Fig. 3). However, increasing concentrations of benzamidoxime in the incubation mixture reversed the inhibitory effect of hydroxylamine-HCl (Fig. 4). The rate of the benzamidoxime reduction in the OMV-fraction could be doubled by adding microsomal purified cytochrome b_5 (Fig. 5). The mitochondrial reduction of benzamidoxime was oxygen-insensitive as well as the microsomal hydroxylamine reductase (Kadlubar and Ziegler, 1974) (Fig. 6).

Discussion

Extrahepatic metabolism for the activation of *N*-hydroxylated prodrugs.

The reduction of benzamidoxime was described using liver microsomes as 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 in addition by cytosolic fractions (Dambrova et al., 1998).

The objective of these investigations was to demonstrate the reduction of several amidoximes to amidines by microsomes and also by 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 (Table 3, 4, 5). The only exception of mitochondrial superiority was the conversion of guanoxabenz (Table 4): in this case the microsomal kidney preparations showed reduction rates which were three times higher than those of the mitochondrial preparations of liver and kidney.

The specific microsomal reduction rates were always high in kidney, followed by liver, brain, lung, and intestine demonstrating different concentrations of the responsible enzymes (Table 3, 4, 5).

The mitochondrial superiority and the extrahepatic benzamidoxime reduction was similar to the recent findings of Andersson et al. (2005) using subcellular preparations of rats.

The gastrointestinal route is the most acceptable way of drug administration. Therefore the principle of amidoximes as prodrugs for amidines was developed (Clement, 1993). In contrast to the active amidines amidoximes are less basic, not protonated under physiological conditions and are absorbed from the gastrointestinal

19

tract. In this context, it is disadvantageous if the *N*-hydroxylated structures are already reduced by microsomal enzymes present in the intestine. This was demonstrated by this study and might be one reason why the bioavailability of amidoximes is comparably high but does not reach 100% (Clement, 1993).

The occurrence of a reducing system in the lung (Table 3, 4) offers an alternative application way for amidoximes as prodrugs for amidines by inhalation.

In order to be able to cross the blood-brain barrier by diffusion xenobiotics have to be lipophilic. This study demonstrates that enzymes which are able to reduce amidoximes to amidines are present in the brain (Table 3, 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 into the CNS 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 the capacity of reducing *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 b_5, 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).

In order 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 b_5 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 measured by its 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). Especially the lung 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. As we also discovered the importance

of a mitochondrial reduction further characterisation 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-dependet *N*-oxide reductase (Sugiura and Kato, 1977) were documented as well as oxygen – insensitive NADH-dependet 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 at pH 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 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 b_5 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 b_5 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 b_5 have been shown to exist in rat liver (Lederer et al., 1983). Cytochrome b_5 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 towards chemical and thermal denaturation (Altuve et al., 2001). We assume that similarly to the microsomal benzamidoxime reductase again cytochrome b_5 and its reductase are components of the mitochondrial enzyme system. This assumption could be verified by studies with p-hydroxymercuribenzoate, a known inhibitor of NADH cytochrome b_5 reductase (Shimada et al., 1988). Experiments with purified microsomal cytochrome b_5 enhanced the activity in the OMV-fraction. The activity was doubled. This raise was already saturated at ~10 pmol cytochrome b_5 (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.

In order to investigate if again a P450 enzyme is again 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 benzamideoxime reductase activity of the OMV-fraction was insensitive and in contrast even enhanced by cyanide (Fig. 2). This is an indication of a cyanide sensitive and insensitive reduction system.

Purification studies are in process 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.

Acknowledgement

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Footnotes

Preliminary results: Mau S, Clement B (1999) *Arch. Pharm.* 332, 31; Deters S,

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Legends for Figures

FIG. 1. Reductions of *N*-hydroxylated compounds

FIG. 2. Influence of potassium cyanide on the mitochondrial *N*-reduction of benzamidoxime.

Benzamidoxime reductase activity was determinated by HPLC. A complete incubation mixture consisted of 6-56 µg protein, 0.5 mM benzamidoxime, and 0.4-1.0 mM NADH in a total volume of 150 µl 100 mM phosphate buffer pH 6.0. After preincubation of protein and NADH with KCN for 30 minutes the reaction was started by adding benzamidoxime. Sample preparation and HPLC analysis were described in *Materials and Methods.* Data are means ± SD of four determinations.

FIG. 3. Influence of hydroxylamine-HCl on the mitochondrial *N*-reduction of benzamidoxime.

Benzamidoxime reductase activity was determinated by HPLC. A complete incubation mixture consisted of 6-56 µg protein, 0.5 mM benzamidoxime, and 0.4-1.0 mM NADH in a total volume of 150 µl 100 mM phosphate buffer pH 6.0. After preincubation of protein and NADH with hydroxylamine-HCl for 5 minutes the reaction was started by adding benzamidoxime. Sample preparation and HPLC analysis were performed as described in *Materials and Methods.* Data are means ± SD of four determinations.

FIG. 4. Inhibition of the mitochondrial benzamidoxime reduction by 0.25 mM hydroxylamine-HCl at various benzamidoxime concentrations.

Benzamidoxime reductase activity was determinated by HPLC. A complete incubation mixture consisted of 56 μg protein (pig liver mitochondria), benzamidoxime, and 1.0 mM NADH in a total volume of 150 μl 100 mM phosphate buffer pH 6.0. After preincubation of protein with benzamidoxime for 5 minutes 0.25 mM hydroxylamine-HCl was added, and the reaction was started with NADH. A control incubation with 0.5 mM benzamidoxime and without hydroxylamine-HCl was performed. Sample preparation and HPLC analysis were performed as described in *Materials and Methods*. Data are means ± SD of four determinations.

FIG. 5. Influence of microsomal cytochrome b_5 on the reduction of benzamidoxime by the OMV-fraction

Benzamidoxime reductase activity was determinated by HPLC. A complete incubation mixture consisted of 6 μg protein (OMV), 1-300 pmol cytochrome b_5, 0.5 mM benzamidoxime, and 0.4 mM NADH in a total volume of 150 μl 100 mM phosphate buffer pH 6.0. Sample preparation and HPLC analysis were performed as described in *Materials and Methods*. Data are means ± SD of four determinations.

FIG. 6. Influence of oxygen on the reduction of benzamidoxime by pig liver mitochondria

Benzamidoxime reductase activity was determinated by HPLC. A complete incubation mixture consisted of 0.056 mg protein, 0.5 mM benzamidoxime, and 1.0 mM NADH in a total volume of 150 μl 100 mM phosphate buffer pH 6.0. Aerobic incubations were exposed to laboratory air while anaerobic incubations were performed in argon-degassed buffers, were gassed with argon and the reaction tubes

were closed during incubation. Sample preparation and HPLC analysis were performed as described in *Materials and Methods*. Data are means ± SD of four determinations.

TABLE 1

Concentrations and activities of cytochrome b_5, NADH cytochrome b_5 reductase, and cytochrome P450 in investigated microsomes of various organs and species

Origin of microsomes	cytochrome b_5 [nmol/mg]	b_5 reductase [U/mg]	cytochrome P450 [nmol/mg]
kidney / pig	0.14	3.27	0.22
kidney / human	0.07	3.08	0.04
liver / pig	0.38	3.22	0.44
liver / human	0.21	2.17	0.63
brain / pig	0.06	1.43	ND[a]
lung / pig	0.08	2.72	ND[a]
intestine / pig	0.03	1.30	0.05

[a] Not detectable

Enzyme activities were measured spectrophotometrically. *Cytochrome b_5* was estimated from difference spectra between the oxidized and the NADH reduced preparation (Estabrook and Werringloer, 1978). *NADH cytochrome b_5 reductase* was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978). *Cytochrome P450* was determined by carbon monoxide (Omura and Sato, 1964).

TABLE 2

Concentrations and activities of cytochrome b_5, NADH cytochrome b_5 reductase, and

cytochrome P450 in investigated mitochondria of various organs and species

Origin of mitochondria	cytochrome b_5 [nmol/mg]	b_5 reductase [U/mg]	cytochrome P450 [nmol/mg]
kidney / pig	0.08	1.04	0.36
liver / pig	0.03	1.84	0.19
kidney / human	0.02	1.75	ND[a]
liver / human	0.04	1.38	0.31

[a] Not detectable

Enzyme activities were measured spectrophotometrically. *Cytochrome b_5* was estimated from difference spectra between the oxidized and the NADH reduced preparation (Estabrook and Werringloer, 1978). For determination the cytochrome b_5 content in pig kidney mitochondria, the protein suspension was sonicated before measuring difference spectra. *NADH cytochrome b_5 reductase* was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978). *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 3

Kinetic parameters of the reduction of benzamidoxime to benzamidine

	$K_m{}^a$	$V_{max}{}^b$	$V_{max}/K_m{}^c$
kidney			
microsomes (pig)	0.03	166	5.0×10^{-1}
mitochondria (pig)	12.8	1082	8.5×10^{-5}
mitochondria (human)	3.2	7.8	2.4×10^{-6}
liver			
microsomes (pig)	0.06	5.3	8.8×10^{-5}
mitochondria (pig)	0.89	84	9.4×10^{-5}
mitochondria (human)	0.71	7.2	1.0×10^{-5}
OMV (pig)	0.08	314	3.9×10^{-3}
brain			
microsomes (pig)	0.05	18.1	3.7×10^{-4}
lung			
microsomes (pig)	0.20	1.70	7.4×10^{-6}
intestine			
microsomes (pig)	0.10	0.10	1.0×10^{-6}

[a] mM

[b] nmol/min/mg protein

[c] min/mg protein

TABLE 4

Kinetic parameters of the reduction of guanoxabenz to guanabenz

	$K_m{}^a$	$V_{max}{}^b$	$V_{max}/K_m{}^c$
kidney			
microsomes (pig)	0.51	181	3.6×10^{-4}
mitochondria (pig)	1.04	53.2	5.1×10^{-5}
mitochondria (human)	0.43	7.10	1.7×10^{-5}
liver			
microsomes (pig)	0.19	8.64	4.6×10^{-5}
mitochondria (pig)	0.15	19.3	1.3×10^{-4}
mitochondria (human)	0.19	7.10	3.7×10^{-5}
brain			
microsomes (pig)	0.25	0.24	9.0×10^{-6}
lung			
microsomes (pig)	0.15	1.46	9.7×10^{-6}
intestine			
microsomes (pig)	12.1	1.40	1.2×10^{-7}

[a] mM

[b] nmol/min/mg protein

[c] min/mg protein

TABLE 5

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

	$K_m{}^a$	$V_{max}{}^b$	$V_{max}/K_m{}^c$
kidney			
microsomes (pig)	4.03	8.0	2.0×10^{-6}
mitochondria (pig)	0.30	9.2	1.8×10^{-6}
mitochondria (human)	0.51	0.3	5.5×10^{-7}
liver			
mitochondria (pig)	0.31	7.3	2.4×10^{-5}
mitochondria (human)	0.44	1.5	3.4×10^{-6}

[a] mM

[b] nmol/min/mg protein

[c] min/mg protein

TABLE 6

Enzyme activity of benzamidoxime reductase and marker enzymes in mitochondria

and purified OMV fraction from pig liver

	BAORed	CR	SuccCytc	CR/SuccCytc[c]
Homogenate	18.0 ± 1.7[a]	170 ± 17[a]	497 ± 23[a]	
	100[b]	100[b]	100[b]	1
OMV	256 ± 10[a]	1978 ± 161[a]	64 ± 3[a]	
	1430[b]	1170[b]	13[b]	90

[a] specific activity [nmol/min/mg protein] are means $\pm$ SD of at least seven determinations

[b] enrichment [%]

[c] quotient of the enrichment of the enzyme markers

SD-Homogenate and OMV fraction (see *Materials and Methods*) were assayed for the enzyme markers rotenone-insensitive cytochrome c reductase (CR), and succinate cytochrome c reductase (SuccCytc) spectrophotometrically. The enrichment of CR over SuccCytc (CR/SuccCytc) is the quotient of the enrichment of the two enzyme markers, respectively.

Benzamidoxime reductase activity (BAORed) was determined by HPLC. A complete incubation mixture consisted of 6-56 µg mitochondrial protein, 0.5 mM benzamidoxime, and 0.4–1.0 mM NADH in a total volume of 150 µl 100 mM phosphate buffer pH 6.0. Control incubations were run with denatured enzyme. Sample preparation and HPLC analysis were described in Materials and Methods. Data are means $\pm$ SD of four determinations.
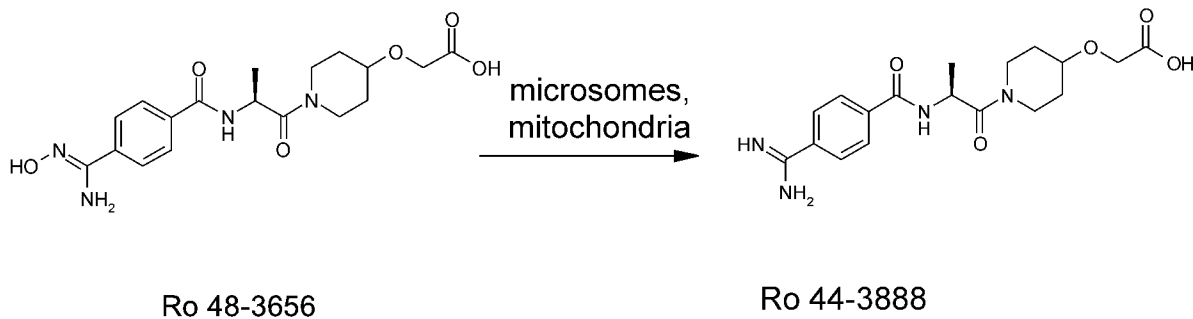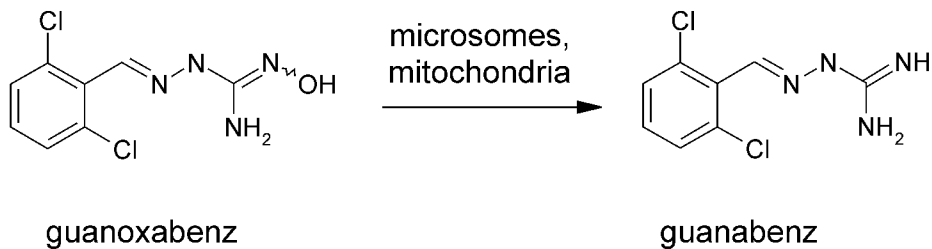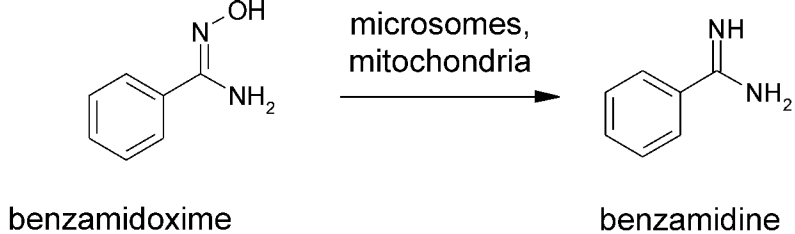
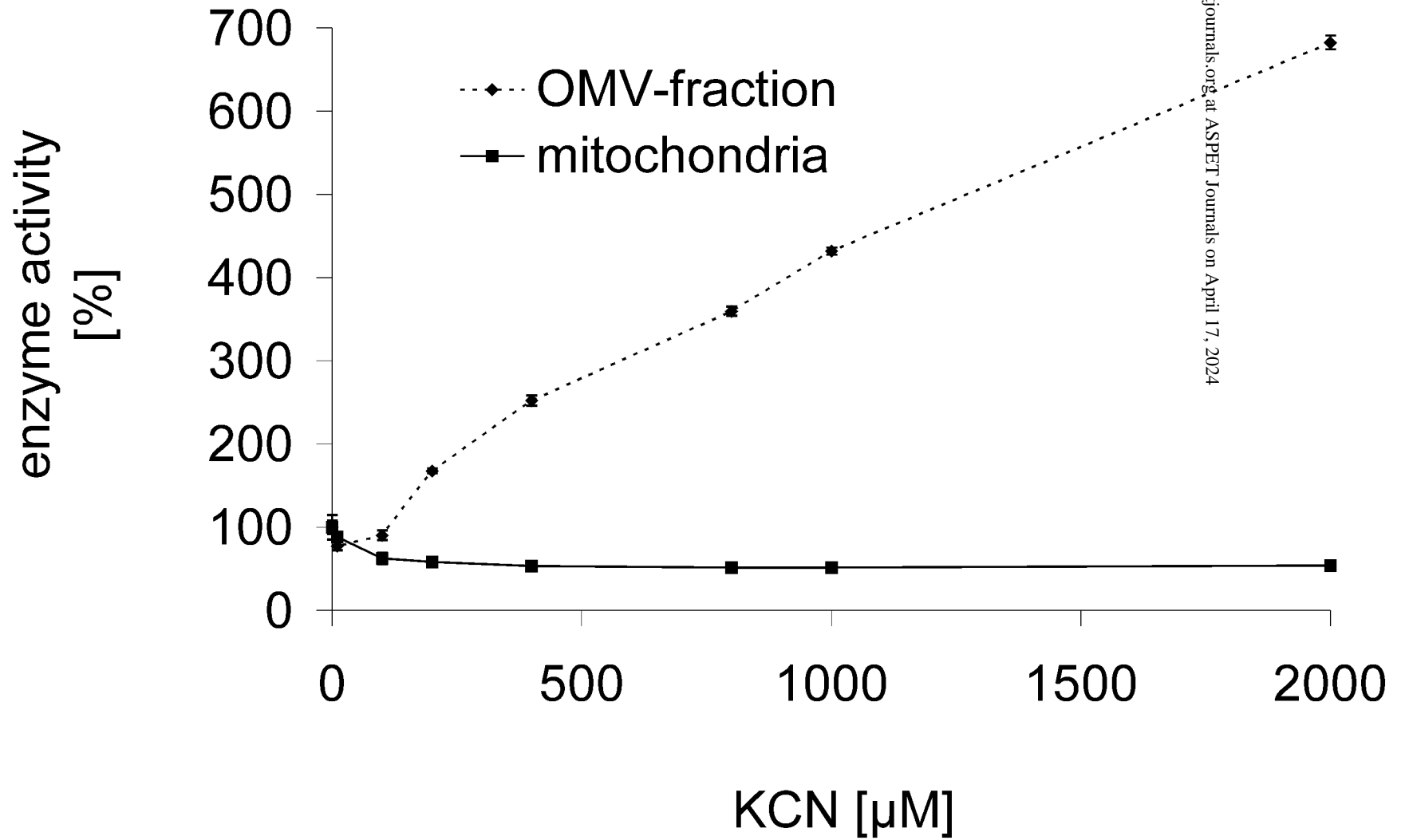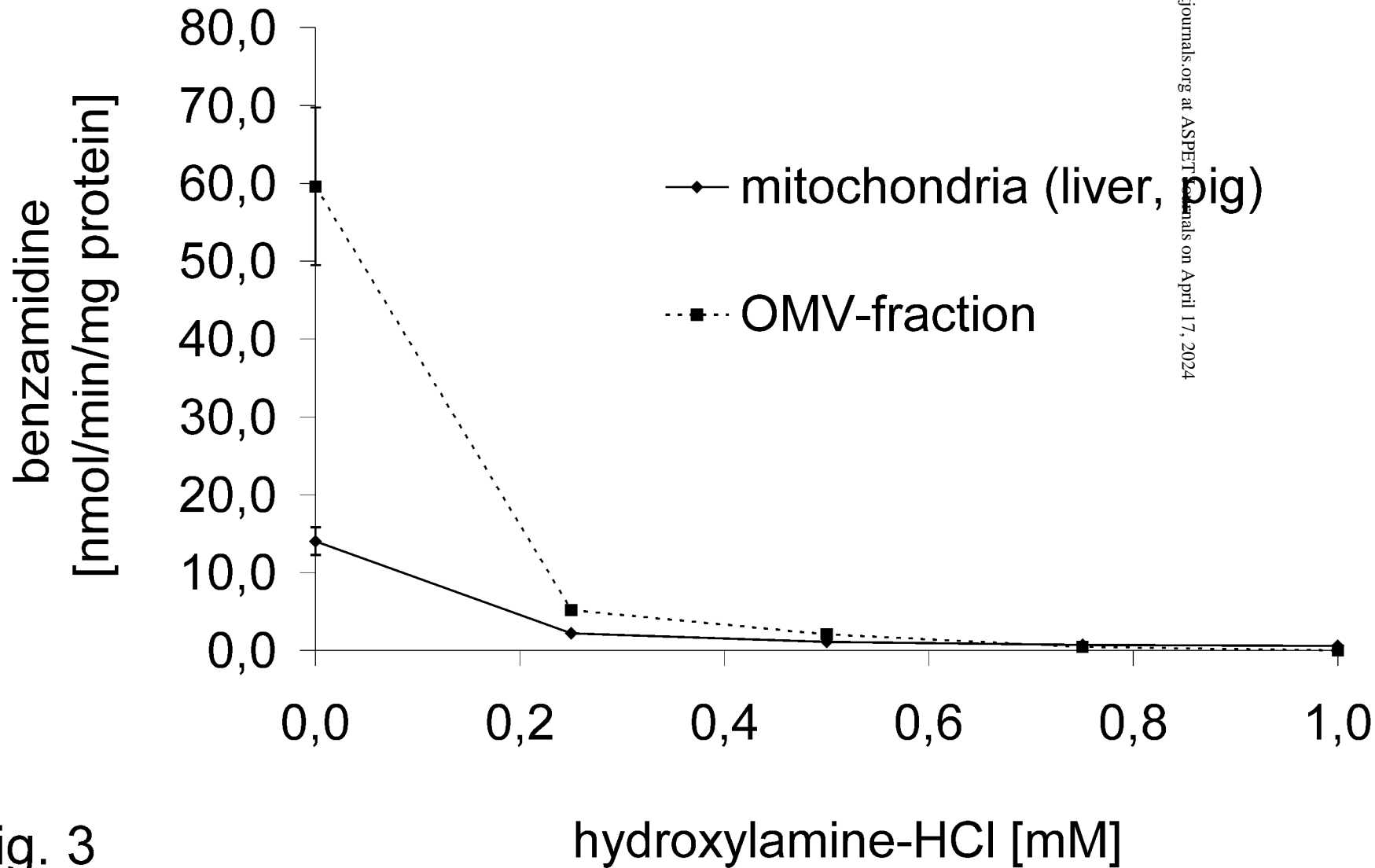benzamidoxime → benzamidine

guanoxabenz → guanabenz

Ro 48-3656 → Ro 44-3888

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6