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Synthesis and biological evaluation of L-valine-amidoximeesters as double prodrugs of amidines

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ABSTRACT

In general, drugs containing amidines suffer from poor oral bioavailability and are often converted into amidoxime prodrugs to overcome low uptake from the gastrointestinal tract. The esterification of amidoximes with amino acids represents a newly developed double prodrug principle creating derivatives of amidines with both improved oral availability and water solubility. *N*-valoxybenzamidine (**1**) is a model compound for this principle, which has been transferred to the antiprotozoic drug pentamidine (**8**). Prodrug activation depends on esterases and mARC and is thus independent from activation by P450 enzymes. Therefore, drug-drug interactions or side effects will be minimized. The synthesis of these two compounds was established, and their biotransformation was studied in vitro and in vivo. Bioactivation of *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**) via hydrolysis and reduction has been demonstrated in vitro with porcine and human subcellular enzyme preparations and the mitochondrial Amidoxime Reducing Component (mARC). Moreover, activation of *N*-valoxybenzamidine (**1**) by porcine hepatocytes was studied. In vivo, the bioavailability in rats after oral application of *N*-valoxybenzamidine (**1**) was about 88%. Similarly, *N,N′*-bis(valoxy)pentamidine (**7**) showed oral bioavailability. Analysis of tissue samples revealed high concentrations of pentamidine (**8**) in liver and kidney.

1. Introduction

Prodrug principles are needed to overcome pharmaceutical, pharmacokinetic, and pharmacodynamic barriers such as poor solubility, insufficient oral absorption, inadequate blood–brain barrier permeability, or presystemic metabolism. Numerous drugs and drug candidates contain strong basic moieties, such as guanidines or amidines.[1] Due to their strong basicity,[2] amidines are protonated under physiological conditions forming highly mesomerically stabilized amidinium-ions. Since they predominantly exist in their protonated form in the gastrointestinal tract, they are not significantly absorbed via passive transport mechanisms. In most cases this results in extremely low bioavailability after oral application.[3,4] Amidoximes can be used as bioprecursors of amidines. Hydroxylation of an amidine to the corresponding amidoxime leads to a considerably decreased basicity and to an increase in lipophilicity.[5] Amidoximes are uncharged at physiological pH, in general pharmacologically inactive, and their absorbance from the gastrointestinal tract is significantly improved.[4] The extensive reduction of benzamidoxime (**2**) to the pharmacologically active benzamidine (**3**) has already been demonstrated in 1988[6,7] and has been studied in greater detail afterwards.[8,9] So far, no drug–drug interactions or toxic effects related to the reduction of amidoximes to amidines have been identified.[10] In mitochondria the *N*-reduction of amidoximes is mediated by a newly discovered molybdenum containing enzyme, the mitochondrial Amidoxime Reducing Component (mARC).[11,12] The amidoxime prodrug principle was first applied to pentamidine (**8**) by our laboratory in 1985.[13] In the meantime, it has been transferred to several other amidines in order to increase oral bioavailability.[4]

Pentamidine (**8**) is effective in therapy for the hemolymphatic stage of trypanosomiasis and antimony-resistant leishmaniasis.[14–16] Furthermore, it is established in the treatment of Pneumocystis carinii Pneumonia (PcP).[17] As an aromatic diamidine, pentamidine requires intravenous or inhalative application. Unfortunately, most of the infections mentioned above occur in tropical or subtropical countries that usually have poor medical care systems. Consequently, this way of application limits the medicinal use of pentamidine **1** in most regions and shows the need for a pentamidine derivative that can be administered orally. Moreover, intravenous application is associated with toxic side effects including neutropenia, abnormal function, rash, nausea, and vomiting, which may seriously limit the ability of patients to continue their treatment.[18] In order to reduce toxicity other research groups developed pentamidine analogues with modified linkers between

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the benzamidoxime moieties.[19,20] The most established compounds are DB75 (Furamidine) and its prodrug DB289 (Pafuramidine), which showed both oral bioavailability and efficacy in in vivo experiments.[21] However, development was discontinued in 2008 due to unexplained idiosyncratic drug induced organ toxicity.

The dicationic structure of pentamidine is essential for selective and rapid accumulation in the parasite and for its pharmacological effect against trypanosomes.[22–26] Owing to the highly ionic nature of the diamidines, these drugs are not absorbed from the gastrointestinal tract and do not readily pass the blood–brain barrier to achieve therapeutic levels in the brain. In contrast to the active principle pentamidine (**8**), the pentamidine diamidoxime (**5**) shows oral bioavailablility.[5,27] However, for therapy of late-stage sleeping sickness, where the infection is established in the central nervous system, only eflornithine and the arsenic containing drug melarsoprol are effective.[28] Arsenical drugs are highly toxic and increasing occurrence of resistance is becoming a major problem. Therefore, it is essential to develop drugs that can penetrate the blood–brain barrier and thus replace those highly toxic drugs.[29] In this respect

Previously, our group improved the lipophilicity and oral bioavailability of the pentamidine diamidoxime prodrug (**5**) by O-acetylation forming *N,N′*-bis(acetoxy)pentamidine. It was anticipated that *N,N′*-bis(acetoxy)pentamidine would also be able to pass the blood–brain barrier via passive diffusion.[30] However, a major problem of *N,N′*-bis(acetoxy)pentamidine is its low water solubility, which limits its usefulness for therapeutic application and has to be considered critically regarding absorption mechanisms in the gastrointestinal tract.

To date, several prodrug approaches to improve water solubility have been described, including salt formation and incorporation of ionizable and polar groups into molecules.[31,32] Especially esters such as phosphates or hemisuccinates are extensively used to increase the water solubility of drugs.[1,33,34]

A new double prodrug principle was developed for amidines in order to maintain their water solubility and at the same time improve bioavailability. This will be achieved by O-substitution of the amidoxime prodrug with the amino acid L-valine. Activation of the double prodrug proceeds in two steps: first the prodrug is hydrolyzed to its amidoxime, and subsequent is reduced to the active compound (Scheme 1).

Another aim was enabling the drug to penetrate through the blood–brain barrier by peptide transporters. Hence, the prodrug contains an amino acid moiety that can potentially target carrier-mediated transporters and thus cross cell membranes. Based on the results of previous screening studies, esterification with the amino acid L-valine appeared to be the most promising candidate.[35] The transport of valaciclovir through membranes was mediated predominantly by the PEPT1 transporter although this prodrug did not possess a peptide bond and resulted in a higher bioavailability.[36] This novel double prodrug principle allows the development of prodrugs that can be injected in case of emergency or may be applied orally during long-term therapy. Furthermore, these prodrugs may enable penetration through the blood–brain barrier. In case of pentamidine (**8**), this new approach might improve therapy, eliminating toxic effects caused by injection. With the double prodrug principle, tissue targeting of the drug may be possible.

The first aim of the study was the synthesis of the model compound *N*-valoxybenzamidine (**1**) and the investigation of its biotransformation in vitro and in vivo. Of special interest were the oral bioavailability and the tissue distribution after application of *N*-valoxybenzamidine (**1**). Afterwards, the prodrug principle was transferred to *N,N′*-bis(valoxy)pentamidine (**7**) and we examined water solubility, stability, in vitro bioactivation, and organ distribution of **7** including postabsorptive conversion to the active metabolite pentamidine (**8**) in rats.

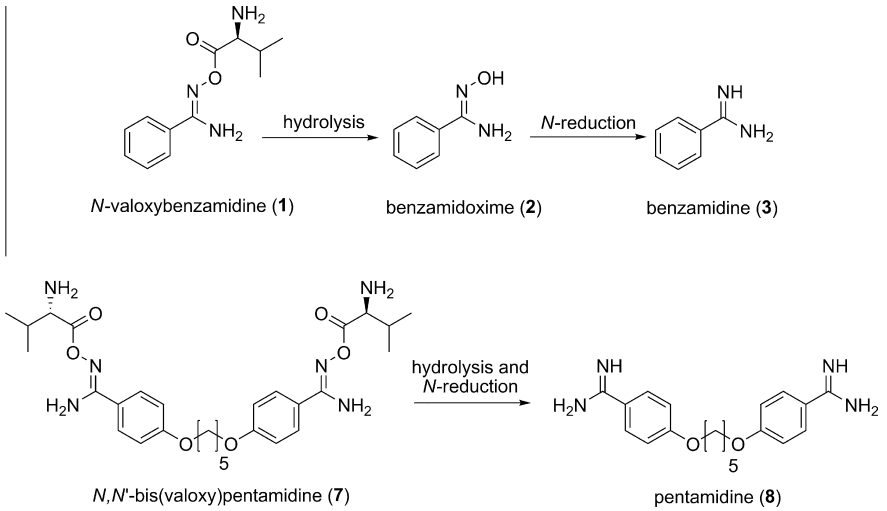
2. Materials and methods

2.1. Chemicals and reagents

Benzamidoxime (**2**) was synthesized according to a prior published method.[37] Pentamidine diamidoxime (**5**) was synthesized according to the method described by Clement and Raether.[13] Recombinant human mARC1 and 2 were obtained as described by Kotthaus et al.[38] All other chemicals were commercially available and of analytical grade, except acetonitrile and methanol, which were of HPLC grade.

2.2. Synthesis

Uncorrected melting points were measured on a Büchi 510 Melting Point apparatus. IR spectra were obtained on a Perkin Elmer FTIR 1600 PC spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 300 NMR spectrometer using the following frequencies: ^{1}H: 300.13 MHz and ^{13}C: 75.47 MHz. Chemical shifts (δ values) are reported in ppm relative to TMS as an internal standard. All coupling constants (*J* values) were obtained



Scheme 1. Metabolic activation of *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**).

by first order analysis of the multiplets and are quoted in Hertz. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), qn (quintet), m (unresolved multiplet). Low resolution electrospray ionisation mass spectra were recorded on a Bruker Esquire-LC mass spectrometer. The substances were dissolved in acetonitrile or methanol. Recording of the high mass resolution spectrum was on a Bruker FT-ICR APEX II spectrometer, electrospray ionization. The substance was dissolved in methanol. Elemental analysis was performed on a CHNS analysator (HEKAtech GmbH) at the department of inorganic chemistry (Christian-Albrechts-University of Kiel, Germany).

2.2.1. 2-(*N*-*tert*-Butoxycarbonylamino)-3-methyl-*O*-butyrylbenzamidoxime (4)

Benzamidoxime (**2**) (1.21 g, 8.9 mmol) was dissolved in anhydrous acetonitrile. After addition of *N*-*t*-butoxycarbonyl-L-valine (2.60 g, 12.0 mmol), 4-DMAP (0.15 g, 0.80 mmol) and DCC the solution was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was recrystallized from H_2O/EtOH (1/3, v/v). Yield 86%; mp: 147 °C; IR (KBr): *ν*~ 3496, 2928, 1744, 1686, 1612, 1392, 1374 cm^{-1}; ^1H NMR (DMSO-d_6): δ 0.83 (d, 6H, ^3J = 6.4, CH_3), 1.40 (s, 9H, CH_3), 2.08 (m, 1H, $CH(CH_3)_2$), 4.05 (t, 1H, ^3J = 8.0, CH–NH), 6.85 (s, 2H, NH_2), 7.31 (d, 1H, ^3J = 9.2, NH), 7.44 (m, 3H, Ar-H), 7.52 (m, 2H, Ar-H); ^{13}C NMR (DMSO-d_6): δ 18.3, 19.0, 28.2, 30.1, 59.1, 78.3, 126.7, 128.3, 130.5, 131.4, 155.7, 157.0, 168.9; LRMS (ESI) *m/z*: 693 $[2M+Na]^+$, 336 $[M+H]^+$, 280 $[M-(CH_3)_2C{=}CH_2+H]^+$, 137 $[BAO+H]^+$, 118 $[Val+H]^+$; Anal. Calcd for $C_{17}H_{25}N_3O_4$ (335.41): C, 60.88; H, 7.51; N, 12.53. Found: C, 60.96; H, 7.71; N, 12.63.

2.2.2. 2-Amino-3-methyl-*O*-butyrylbenzamidoxime dihydrochloride. *N*-valoxybenzamidine (1)

Boc-protected **4** was dissolved in anhydrous dioxane. Gaseous HCl was flushed through the solution for 5 min and the mixture was stirred overnight at room temperature. The product was precipitated by addition of EtOAc and afterwards washed several times with Et_2O. Yield 30%; IR (KBr): *ν*~ 3238, 3052, 2910, 2774, 1800, 1682, 1600, 1374 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.02 (d, 3H, ^3J = 4.7 Hz, CH_3), 1.04 (d, 3H, ^3J = 4.7 Hz, CH_3), 2.02 (m, 1H, $CH(CH_3)_2$), 3.94 (t, 1H, ^3J = 5.4, CH–NH_2), 7.22 (s, 2H, NH_2), 7.48 (m, 3H, Ar-H), 7.72 (m, 2H, Ar-H), 8.80 (s, 3H, NH_3), 9.03 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6): 18.1, 29.4, 57.2, 126.8, 128.3, 130.6, 130.1, 157.6, 165.40; LRMS (ESI) *m/z*: 493 $[2M+Na]^+$, 471 $[2M+H]^+$, 236 $[M+H]^+$, 137 $[BAO+H]^+$, 121 $[BA+H]^+$, 119 $[BAO-H_2O+H]^+$, 118 $[Val+H]^+$; Anal. Calcd for $C_{12}H_{19}N_3O_2Cl_2$ (308.21): C, 46.76; H, 6.21; N, 13.63. Found: C, 46.54; H, 6.25; N, 13.12.

2.2.3. 4,4′-(Pentamethylenedioxy)-*bis*-[2-(*N,N′*-*tert*-butoxycarbonyl-amino)-3-methyl-*O*-butyryl]benzamidoxime (6)

Pentamidine diamidoxime (**5**) (1.0 g, 2.7 mmol) was dissolved in anhydrous acetone. After addition of *N*-*t*-butoxycarbonyl-L-valine (1.77 g, 8.2 mmol), 4-DMAP (0.1 g, 0.8 mmol) and DCC (1.67 g, 8.1 mmol) the solution was stirred for 24 h at room temperature. The solvent was evaporated in vacuo and the crude product was recrystallized from H_2O/EtOH/acetone (1/3/1, v/v/v). Yield 35%; mp: 174 °C; IR (KBr): *ν*~ 3494, 2964, 1744, 1690, 1616, 1392, 1366 cm^{-1}; ^1H NMR (DMSO-d_6): δ 0.91 (d, 12H, ^3J = 6.6 Hz, CH_3), 1.40 (s, 18H, CH_3), 1.58 (qn, 2H, ^3J = 6.9 Hz, CH_2), 1.81 (qn, 4H, ^3J = 7.6 Hz, CH_2), 2.07 (m, 2H, $CH(CH_3)_2$), 4.06 (m, 6H, O–CH_2, CH–NH), 6.71 (s, 4H, NH_2), 7.00 (d, 4H, ^3J = 8.8 Hz, Ar-H), 7.30 (d, 2H, ^3J = 8.8 Hz, NH), 7.66 (d, 4H, ^3J = 8.7 Hz, Ar-H); ^{13}C NMR (DMSO-d_6): δ 18.2, 19.0, 22.1, 28.1, 28.3, 30.1, 59.1, 67.5, 78.2, 114.1, 123.3, 128.1, 155.7, 156.5, 160.4, 168.9; LRMS (ESI) *m/z*: 771 $[M+H]^+$, 572 $[M-C_{10}H_{17}NO_3+H]^+$, 373 $[DAO+H]^+$, 337 $[DAO-2H_2O+H]^+$; Anal. Calcd for $C_{39}H_{58}N_6O_{10}$ (770.93): C, 60.76; H, 7.58; N, 10.90. Found: C, 60.98; H, 7.87; N, 11.21.

2.2.4. 4,4′-(Pentamethylenedioxy)-bis-(2-amino-3-methyl-*O*-butyryl)benzamidoxime tetrahydrochloride. *N,N′*-bis(valoxy)pentamidine (7)

Boc-protected **6** was dissolved in anhydrous Et_2O and gaseous HCl was bubbled through the solution for 5 min. The mixture was stirred overnight at room temperature and the isolated precipitate washed several times with Et_2O. Yield 59%; IR (KBr): *ν*~ 3396, 2942, 1804, 1752, 1636, 1608, 1506, 1308, 1260 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.02 (d, 6H, ^3J = 6.8 Hz, CH_3), 1.03 (d, 6H, ^3J = 6.8 Hz, CH_3), 1.58 (qn, 2H, ^3J = 6.6 Hz, CH_2), 1.80 (qn, 4H, ^3J = 7.0 Hz, CH_2), 2.32 (m, 2H, $CH(CH_3)_2$), 3.92 (t, 2H, ^3J = 5.3 Hz, CH–NH), 4.05 (t, 4H, ^3J = 6.3 Hz, O–CH_2), 7.00 (d, ^3J = 8.9 Hz, 4H, Ar-H), 7.11 (s, 4H, NH_2), 7.67 (d, ^3J = 8.8 Hz, Ar-H), 8.34 (br s, 2H, NH), 8.81 (s, 6H, NH_3); ^{13}C NMR (DMSO-d_6): δ 17.9, 18.4, 22.1, 28.3, 29.4, 57.3, 67.6, 114.2, 122.9, 128.3, 157.2, 160.6, 165.5; HRMS (ESI) *m/z* calcd for $C_{29}H_{43}N_6O_6$ (MH^+) 571.32386, found 571.32385; Anal. Calcd for $C_{29}H_{46}N_6O_6Cl_4 \times 1.9H_2O$ (750.77): C, 46.40; H, 6.69; N, 11.19. Found: C, 46.52; H, 6.93; N, 10.98.

2.3. In vitro stability of *N*-valoxybenzamidine (1) and *N,N′*-bis(valoxy)pentamidine (7)

Both compounds were incubated in concentrations of 100 μM at 37 °C in 100 mM phosphate buffer pH 2.0, 6.3, and 7.4. Samples were taken at different timepoints between 0 and 120 min and analyzed by HPLC.

2.4. In vitro biotransformation of *N*-valoxybenzamidine (1) and *N,N′*-bis(valoxy)pentamidine (7) by subcellular enzyme preparations and mARC

In vitro assays were performed with human and porcine enzyme preparations, such as microsomes, mitochondria, 9000*g* supernatants, and cytosolic fractions. Human liver and kidney samples for enzyme preparations were obtained from the University Medical Center Schleswig-Holstein (Kiel, Germany). Donors were patients who were subjected to a partial hemi-hepatectomy because of secondary liver tumors. These studies were accredited from the local medical ethics committee and from the patients. A standard incubation mixture contained 0.3 mg protein and 1 mM prodrug (*N*-valoxybenzamidine (**1**) or *N,N′*-bis(valoxy)pentamidine (**7**)) in 100 mM potassium phosphate buffer pH 6.3. After preincubating the mixture for 5 min at 37 °C under aerobic conditions, the reaction was started by the addition of NADH (final concentration 1 mM) to a final volume of 250 μL. The mixture was incubated for 30 min at 37 °C under aerobic conditions. The reaction was terminated by the addition of 250 μL cold acetonitrile and vortexing. After centrifugation at 10000*g* (Mikrozentrifuge Hettich, Tuttlingen, Germany), 10 μL of the supernatant were analyzed by HPLC.

Additionally, incubations of both prodrugs with recombinant human mARC1 and mARC2 were performed according to a previously published method.[38] Those incubations contained 0.5 mM substrate (**1** or **7**), 1 mM NADH, 2 U carboxyl-esterases from pig liver (Sigma–Aldrich, Taufkirchen, Germany), 76 pmol cytochrome b_5, 7.6 pmol NADH cytochrome b_5 reductase, and 76 pmol recombinant hmARC1 or hmARC2 in 150 μL 20 mM MES buffer pH 6.0. After incubation at 37 °C for 30 min the reactions were terminated by the addition of 150 μL acetonitrile. Precipitated proteins were sedimented by centrifugation and the supernatant was analyzed by HPLC.

Further incubations were performed wit both prodrugs and 0.5 U carboxyl-esterases from pig liver (Sigma–Aldrich, Taufkirchen, Germany) in 100 mM potassium buffer pH 7.4.

2.5. In vitro biotransformation of *N*-valoxybenzamidine (1) by cultured pig hepatocytes

The isolation and cultivation of porcine hepatocytes was carried out according to the method published by Fröhlich et al.[39] Incubations were performed on day three after culturing. Hepatocytes were incubated with 1 mM substrate in Krebs–Henseleit buffer pH 7.4 for 1 h at 37 °C and 5% CO_2 in humidified air. The incubations were stopped by aspirating the supernatant from the culture dishes. The supernatant was frozen at −20 °C and freeze-dried. Afterwards, the residue was taken up in 150 μL of 100 mM phosphate buffer (pH 6.3)/methanol (90/10, v/v). 10 μL were analyzed by HPLC.

2.6. Animal study

Animal studies were approved by the department for agriculture, environment and rural areas of the german state Schleswig Holstein. Male Wistar rats ($n = 2$ or 3 for each substance, initial body weight 230 ± 10 g, Charles River Laboratories, Germany) were housed in cages with sawdust-covered solid flooring in a controlled environment (22 ± 2 °C, humidity 65%) with a 12-h light/dark cycle. Animals had free access to feed (maintenance laboratory chow Altromin, Lage) and tap water. The rats were anesthetized by intraperitoneal injection of a mixture of xylazine hydrochloride (10 mg kg^{-1}) and ketamine hydrochloride (75 mg kg^{-1}) and permanent canulas were surgically inserted into the carotid artery and the jugular vein. The catheters were externalised in the nape of the neck, and filled with physiological saline containing heparin (50 I.E. ml^{-1}). The animals were allowed to recover from surgery until complete compensation of body weight loss due to surgery. Bioavailability was tested in animals starved for 16 h and substances were applied either by oral gavage or intravenous injection. Blood withdrawal was performed via arterial catheter. Eight hours after prodrug application, animals were anesthetized by intraarterial pentobarbital injection and euthanized by complete blood withdrawal from the abdominal aorta. Tissues (lung, brain, heart, liver, spleen, and kidney) were removed and sampled for subsequent analyses. Principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) were followed and all animal experiments were approved by the Ethics Committee according to German Animal Protection Law. Each rat received an oral dose of *N*-valoxybenzamidine (**1**) of 50 mg kg^{-1} or an oral dose of *N,N′*-bis(valoxy)pentamidine (**7**) of 100 mg kg^{-1}. Compounds **1** and **7** were dissolved in 100 mM phosphate buffer pH 6.3. Benzamidine (**3**) was dissolved in water for injection and administered intravenously in a concentration of 10 mg kg^{-1}. Blood samples (300 μL) were taken at 30, 60, 90, 120, 240, and 480 min after oral application and at 10, 20, 40, 60, 120, and 360 min after injection. Due to the high tendency of pentamidine (**8**) to accumulate in tissues,[40] no plasma samples were taken after oral application of *N,N′*-bis(valoxy)pentamidine (**7**).

2.7. Processing of plasma samples

For the application of *N*-valoxybenzamidine (**1**) and benzamidine (**3**) blood samples were drawn into heparinized containers, immediately centrifuged (1500*g*, 10 min, 4 °C) and stored at −80 °C. For analysis of *N*-valoxybenzamidine (**1**) and benzamidine (**3**), samples were worked up by solid phase extraction (Strata X, 33 μm column, Phenomenex, Aschaffenburg, Germany). After equilibration and conditioning of the column, 150 μL plasma diluted with 450 μL aqua bidest were added. The column was washed with 600 μL water and the substances were eluted with 1000 μL water (pH 3)/methanol (40/60, v/v). The samples were concentrated to dryness. The residues were taken up in water/methanol (90/10, v/v) and concentrations of *N*-valoxybenzamidine (**1**), benzamidoxime (**2**), and benzamidine (**3**) were determined by HPLC analysis.

2.8. Tissue preparation

2.8.1. Application of *N*-valoxybenzamidine (1)

The organs were stored at −80 °C. After thawing they were washed and homogenized. The homogenate was resuspended in 600 μL water, centrifuged (5000*g*, 5 min), and precipitated by addition of 600 μL acetonitrile. After centrifugation (10000*g*, 10 min), the supernatant was concentrated to dryness with nitrogen, resuspended in 100 μL water/methanol (90/10, v/v), centrifuged (10000*g*, 10 min), and 10 μL were analyzed by HPLC.

2.8.2. Application of *N,N′*-bis(valoxy)pentamidine (7)

The work up procedure for the organs is similar to *N*-valoxybenzamidine (**1**). After concentration of the supernatant the residue was resuspended in 150 μL water/methanol (50/50, v/v).

2.9. HPLC methods

2.9.1. In vitro investigations

2.9.1.1. Analysis of *N*-valoxybenzamidine (1). Analysis were carried out on a Waters Alliance System consisting of a Waters e2695 XC separations module and a Waters 2995 photodiode array detector (260 nm) with column heater according to a prior published method.[41] Separations were carried out at room temperature on a LiChropsher 60 RP-select B column (125 × 4 mm; 5 μm, Merck, Darmstadt, Germany) with a RP-select B (4 × 4 mm) guard column. The mobile phase consisted of 0.1% trifluoroacetic acid with 20 mM potassium phosphate buffer (pH 6.5)/acetonitrile (65/35, v/v). The flow rate was set to 1 mL min^{-1} and the wavelength to 229 nm. Retention times were 1.9 ± 0.1 min for benzamidoxime (**2**), 3.5 ± 0.1 min for benzamidine (**3**), and 5.2 ± 0.2 min for *N*-valoxybenzamidine (**1**). The limit of quantification was 0.5 μM for all metabolites.

2.9.1.2. Analysis of *N,N′*-bis(valoxy)pentamidine (7). Analysis were performed on a LiChrospher 60 RP-select B (125 × 4 mm, 5 μm; Merck, Darmstadt, Germany) with a RP-select B (4 × 4 mm) guard column. The flow rate was set to 1 mL min^{-1}, the column temperature 25 °C, the wavelength to 260 nm, and the injection volume was 10 μL. The mobile phase for stability investigations consisted of 0.1% TFA pH 2.5 and methanol (42/58, v/v). *N,N′*-bis(valoxy)pentamidine (**7**) was eluted after 2.5 ± 0.1 min. The mobile phase for metabolism studies consisted of 30 mM octanesulfonic acid, 20 mM tetramethylammoniumchloride, pH 3.0 and methanol (48/52, v/v). The retention times were 7.1 ± 0.3 min for pentamidine diamidoxime (**5**), 8.3 ± 0.3 min for pentamidine monoamidoxime and 9.7 ± 0.4 min for pentamidine (**8**). The limit of quantification was 1 μM for all compounds.

2.9.2. In vivo investigations

2.9.2.1. Analysis of *N*-valoxybenzamidine (1). Separation was carried out at room temperature by isocratic elution on a Synergy Max RP 80A column (250 × 4.6 mm; 5 μm, Phenomenex, Aschaffenburg, Germany) with a C 12 (4 × 2 mm) guard column. The eluate was monitored at 229 nm. The mobile phase consisted of octanesulfonic acid 10 mM, pH 2.5 and acetonitrile (82.5/17.5, v/v). The retention times were 23.5 ± 0.5 min for benzamidoxime (**2**) and 25.6 ± 0.5 min for benzamidine (**3**). Standard curves for each metabolite were generated that were linear with correlation coefficients >0.99. Precision of the assays and accuracy were assessed by adding different concentrations 0–50 μM of benzamidoxime (**2**) and benzamidine (**3**) to plasma. Standard curves

were obtained by linear regression analysis. The recoveries were 100.8 ± 13.2% and 96.6 ± 9.3% for benzamidoxime (**2**) and benzamidine (**3**), respectively. The limit of quantification was about 0.25 μM for both, benzamidoxime (**2**) and benzamidine (**3**). For analysis of *N*-valoxybenzamidine (**1**) in different organs, the HPLC method described for in vitro investigations was used. The limit of quantification after working up the organs was about 0.375 μM for both, *N*-valoxybenzamidine (**1**) and benzamidine (**3**).

2.9.2.2. Analysis of *N,N′*-bis(valoxy)pentamidine (7). Separations were performed analogously to those described for the in vitro investigations with the slight modification that the concentration of octanesulfonic acid was increased to 30 mM. The retention times were 12.9 ± 0.6 min for pentamidine diamidoxime (**5**), 15.4 ± 0.5 min for pentamidine monoamidoxime and 18.5 ± 0.5 min for pentamidine (**8**). The limit of quantification was 1 μM for all compounds. Standard curves for each metabolite were generated and found to be linear with correlation coefficients >0.999. Precision of the assays and accuracy were assessed by adding various concentrations (i.e., 0–50 μM) of the diamidoxime (**5**), monoamidoxime**,** and pentamidine (**8**) to plasma. Recovery of pentamidine (**3**) from plasma and samples, which contained the same amount of each metabolite dissolved in water was 107.3 ± 9.8% and recovery of the monoamidoxime was 125.7 ± 11.2%. The limit of quantification was about 0.5 μM for all detected metabolites.

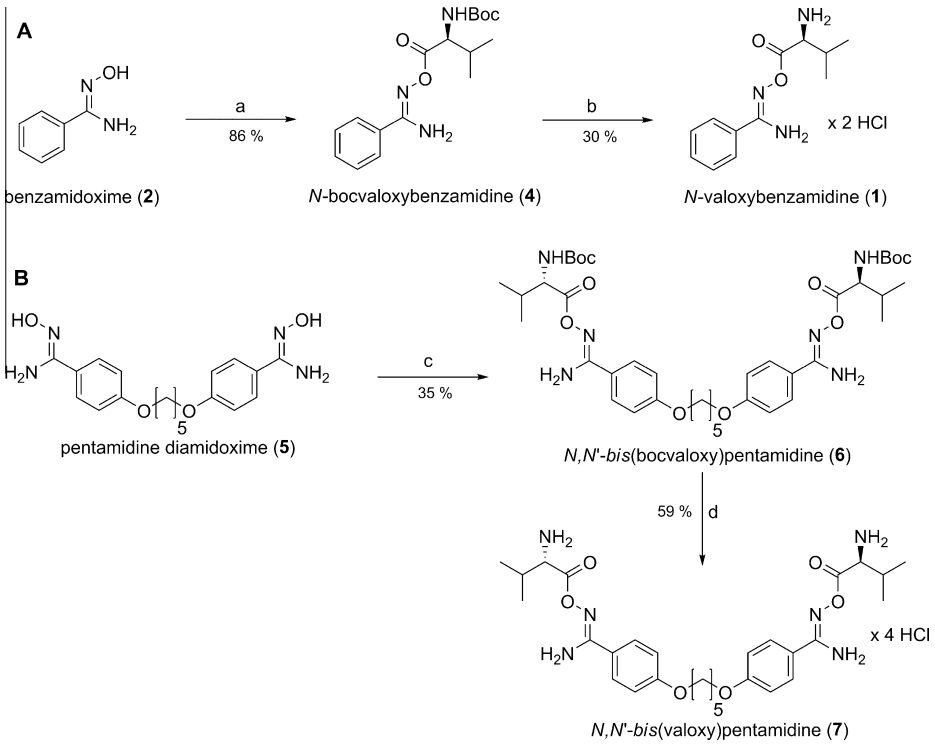
2.9.2.3. LC/MS method. An Agilent model 1100 HPLC system equipped with a binary pump G1312 A and a Hewlett Packard Degasser G1322 A was used for mass spectrometric analysis. Separations were carried out at room temperature on a LiChrospher 100 RP-18 column (250 × 4 mm; 5 μm, Merck, Darmstadt, Germany) and a RP-select B guard column (4 × 4 mm). The detection wavelength was 260 nm. The mobile phase consisted of 0.1% trifluoroacetic acid in aqua bidest and acetonitrile (76/24, v/v). The flow rate was 0.7 mL min^{-1}. The injection volume for both standards and samples was 50 μL. The retention times were 10.6 ± 0.3 min for pentamidine diamidoxime (**5**), 11.5 ± 0.3 min for pentamidine monoamidoxime**,** and 12.0 ± 0.4 min for pentamidine (**8**). The limit of quantification was 0.1 μM for all compounds. Electrospray ionization (ESI) mass spectra were recorded in the positive-ion mode on an Esquire-LC mass spectrometer (Bruker, Bremen, Germany). Synthetic standards were analyzed in comparison to metabolites from the in vivo samples. For diamidoxime, ions with *m/z* 373 $[M+H]^+$, 355 $[M-H_2O]^+$, and 187 $[M+2H]^{2+}$ were found. For the monoamidoxime, ions with *m/z* 357 $[M+H]^+$ and 179 $[M+2H]^{2+}$ were characteristic. Pentamidine (**8**) showed characteristic ions with *m/z* 341 $[M+H]^+$, 205 $[M-C_7H_8N_2O]^{\cdot+}$, 171 $[M+2H]^{2+}$, and 137 $[C_7H_8N_2O+H]^+$. The samples were assessed in full scan mode in the range of *m/z* 50–650 to detect other possible metabolites. Peak areas of ion chromatograms with the sum of ions at *m/z* were obtained by Data Analysis™ integration software (Data Analysis™, Version 3.0, Bruker Daltonics). Standard curves were linear over this range with correlation coefficients >0.995.

3. Results and discussion

3.1. Chemistry

Synthesis of the target compounds (i.e., *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**) was carried out as shown in Scheme 2.

In the first step, Boc-protected L-valine was esterified with benzamidoxime (**1**) using *N,N′*-dicyclohexylcarbodiimide (DCC) and 4-(*N,N*-dimethylamino)pyridine (4-DMAP) in anhydrous acetonitrile. Afterwards, the protecting group was cleaved with gaseous HCl in dry dioxane. *N,N′*-bis(valoxy)pentamidine (**7**) was synthesized analogously, only differing in the solvents used. The new prodrug of pentamidine possessed excellent solubility in aqueous media. 5 mM of **7** were soluble in 50 mM phosphate buffer pH 6.3. In



Scheme 2. (A) Synthesis of *N*-valoxybenzamidine (**1**) and (B) *N,N′*-bis(valoxy)pentamidine (**7**). Reagents: (a) *tert*-butoxycarbonyl-L-valine, DCC, 4-DMAP, acetonitrile; (b) $HCl_{(g)}$, dioxane; (c) *tert*-butoxycarbonyl-L-valine, DCC, 4-DMAP, acetone; (d) $HCl_{(g)}$, ether.

comparison, only 50 μM of *N,N′*-bis(acetoxy)pentamidine and 100 μM pentamidine diamidoxime (**5**) were soluble in that media.

3.2. In vitro stability of *N*-valoxybenzamidine (1) and *N,N′*-bis(valoxy)pentamidine (7)

Stability studies revealed that cleavage of the ester can occur by chemical hydrolysis (Fig. 1). *N*-valoxybenzamidine (**1**) was relatively stable at pH 6.3 and pH 7.4, whereas the degradation towards benzamidoxime (**2**) increased rigorously at pH 2.0 resulting in an immediate and complete hydrolysis to **2**. In contrast, hydrolysis of *N,N′*-bis(valoxy)pentamidine (**7**) is quite independent from pH-value. Interestingly, stability of compound **7** is increased compared to **1**, which might be a result from the substitution with the *O*-alkyl-residue. Consequently, in subsequent animal studies, prodrug **1** was administered in a buffered solution to prevent hydrolysis during gastrointestinal transit. Furthermore, complete hydrolysis of both prodrugs was observed in the presence of 0.5 U esterases/250 μL (data not shown).

3.3. In vitro metabolism of *N*-valoxybenzamidine (1) and *N,N′*-bis(valoxy)pentamidine (7)

In vitro metabolism of both *N*-valoxybenzamidine (**1**) into benzamidine (**3**) and *N,N′*-bis(valoxy)pentamidine (**7**) into pentamidine (**8**) was demonstrated by porcine and human microsomes, mitochondria, 9000*g* supernatants, and cytosolic fractions. The turnover rates determined are shown in Table 1. The highest amounts of benzamidine (**3**) and pentamidine (**8**) were detected when using microsomal or mitochondrial preparations. The cytosolic fractions were mainly able to cleave the ester but no further reduction of the amidoxime was observed. The enzyme responsible for reduction is bound to membranes and therefore not present in the cytosolic fractions. Human tissues were obtained from patients suffering from cancer or hepatitis, possibly resulting in lower or modified enzyme levels in comparison with healthy persons. Thus, a lack of quality of human tissues, from which microsomes and mitochondria were extracted, might be a reason for lower turnover rates in these experiments.

As expected, turnover rates of the pentamidine prodrug **7** are considerably lower than those of *N*-valoxybenzamidine (**1**). This observance can easily be explained by the more complex activation of **7** that requires hydrolysis and reduction of two *N*-valoxyamidine groups. mARC has recently been identified as a 35 kDa molybdoprotein and has been demonstrated to participate predominantly in the reduction of amidoximes or guanidines.[11,12,38] Our results

Table 1
In vitro activation of *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**) by human and porcine liver and kidney microsomes, mitochondria, 9000*g* supernatant, and cytosol[a]

Enzyme source	Activation of **1** to benzamidine (**3**)	Activation of **7** to pentamidine (**8**)
	[nmol min^{-1} (mg protein)$^{-1}$][b]	
Pig liver microsomes	7.0 ± 0.3	0.5 ± 0.2
Pig liver mitochondria	8.6 ± 0.3	1.0 ± 0.1
Pig liver cytosole	1.2 ± 0.2	ND
Pig liver 9000*g* supernatant	3.4 ± 0.5	0.4 ± 0.1
Pig kidney mitochondria	8.8 ± 0.9	0.8 ± 0.1
Pig kidney microsomes	10.4 ± 0.3	0.7 ± 0.2
Pig kidney 9000*g* supernatant	5.6 ± 1.0	0.2 ± 0.1
Human liver microsomes	0.9 ± 0.1	0.3 ± 0.1
Human liver mitochondria	1.8 ± 0.1	0.2 ± 0.1
Human liver cytosol	ND	ND
Human liver 9000*g* supernatant	0.2 ± 0.1	0.2 ± 0.1
Human kidney microsomes	1.3 ± 0.1	0.3 ± 0.1
Human kidney 9000*g* supernatant	ND	ND
Human kidney cytosol	ND	ND
Human kidney mitochondria	2.0 ± 0.1	0.4 ± 0.2

ND, not detectable <0.06 nmol min^{-1} (mg protein)$^{-1}$.
[a] For detailed protocols see Section 2.4. An incubation mixture consisted of 0.3 mg protein, 1 mM prodrug, 1 mM NADH as cosubstrate in 100 mM potassium phosphate buffer pH 6.3.
[b] Turnover rates are means ± SD of two independent experiments measured twice.

demonstrate an excellent activation of both *N*-valoxybenzamidine (**1**) and *N,Ń*-bis(valoxy)pentamidine (**7**) by hmARC1 and hmARC2 in the presence of cytochrome b_5 and NADH cytochrome b_5 reductase (Table 2).

Hepatocytes represent a well-accepted model for the simulation of in vivo biotransformation. In our laboratory, the biotransformation of several amidoximes has already been studied with human and porcine hepatocytes.[39,42] The present study demonstrates that *N*-valoxybenzamidine (**1**) was activated by porcine hepatocytes. Turnover rates of 650 ± 227 and 175 ± 39 pmol min^{-1} (mg protein)$^{-1}$ were obtained for the biotransformation of *N*-valoxybenzamidine (**1**) to benzamidoxime (**2**) and benzamidine (**3**), respectively, and indicate the perfect suitability of this prodrug principle.

3.4. In vivo metabolism of *N*-valoxybenzamidine (1)

N-Valoxybenzamidine was applied orally at 50 mg kg^{-1} body weight to three male Wistar rats in 100 mM phosphate buffer pH

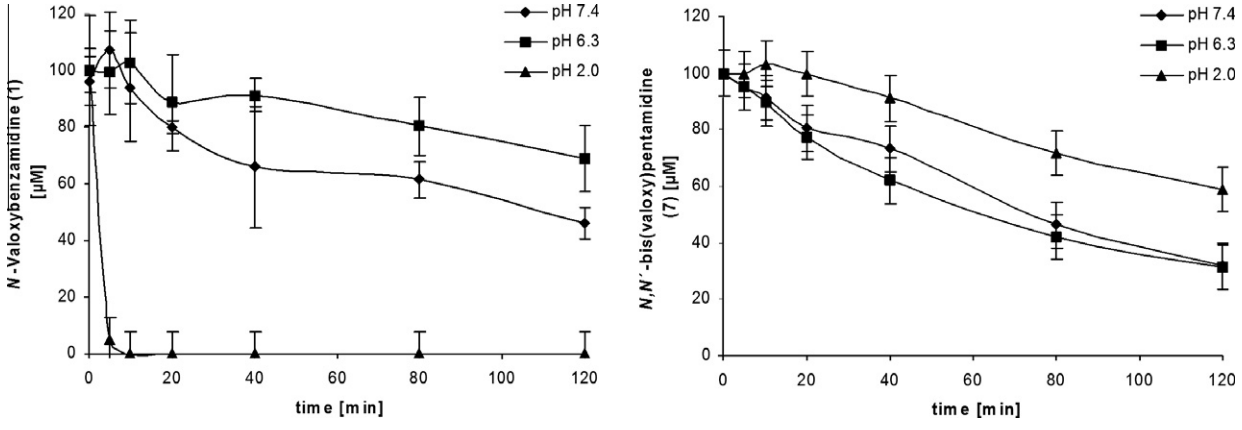


Figure 1. Effect of different pH values on the stability of *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**). The reaction contained 100 μM prodrug in 100 mM potassium phosphate buffer pH 2.0, pH 6.3, and pH 7.4.

Table 2
In vitro activation of *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**) by the recombinant human mitochondrial Amidoxime Reducing Component (hmARC)[a]

	Activation of **1** to benzamidine (**3**)	Activation of **7** to pentamidine (**8**)
	[nmol min^{-1} (mg protein)$^{-1}$][b]	
Standard incubation mixture		
With hmARC1	67.4 ± 14.7	48.8 ± 10.9
With hmARC2	43.7 ± 4.2	10.9 ± 0.9

[a] For detailed protocols see Section 2.4. An incubation mixture consisted of 0.5 mM prodrug, 1 mM NADH, 2 U carboxyl-esterase, 76 pmol cytochrome b_5, 7.6 pmol NADH cytochrom b_5 reductase, and 76 pmol of hmARC1 or 2 in 20 mM MES buffer pH 6.0.
[b] Turnover rates are means ± SD of three incubations.

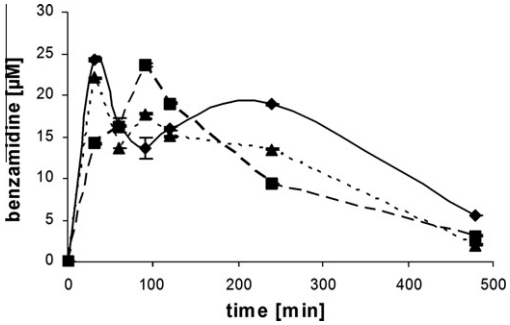


Figure 2. Plasma concentration–time curves of benzamidine (**3**) after oral application *N*-valoxybenzamidine (**1**) to three rats at 50 mg kg^{-1}.

Table 3
Pharmacokinetic parameters of benzamidine (**3**) after oral application of *N*-valoxybenzamidine (**1**) to three rats at 50 mg kg^{-1}

	Rat 1	Rat 2	Rat 3	Mean ± SD
t_{max} (min)	30.0	90.0	30.0	50 ± 35
c_{max} (µM)	24.2	23.6	22.1	23.3 ± 1.1
$t_{1/2}$ (min)	267.9	132.9	125.1	175.3 ± 80.3
MRT (min)	356.5	219.3	202.3	259.4 ± 84.5
V_d (dL kg^{-1})	154.5	122.8	107.9	128.4 ± 23.8
Cl (dL min^{-1} kg^{-1})	0.4	0.6	0.6	0.5 ± 0.1
Bioavailability (%)	118.8	74.5	69.8	87.7 ± 27.0

6.3 that should elevate the gastric pH and minimize hydrolysis of **1** prior to absorption. The corresponding plasma concentration–time curves of benzamidine (**3**) are shown in Figure 2.

In plasma, mainly benzamidine (**3**) was detected. Maximum plasma concentrations of up to 24 µM were observed (Table 3). In contrast, benzamidoxime (**2**) was found in only one sample, which was taken 30 min after oral application of **1**. The course of the plasma concentration–time curves shows that the prodrug was quickly absorbed from the gastrointestinal tract into the blood and subsequently activated to **3**. Maximum plasma concentration values of benzamidine (**3**) were already detected 30 min after oral application of the prodrug. Mean oral bioavailability of benzamidine (**3**) after application of *N*-valoxybenzamidine (**1**) was about 88% (Table 3). Previous studies revealed that oral bioavailability of benzamidine after application of benzamidoxime to rats was about 75%.[43]

In order to investigate distribution of benzamidine (**3**) we examined six organs (i.e., lung, brain, heart, liver, spleen, and kidney) that were removed 8 h after oral application of *N*-valoxybenzamidine **6** (Fig. 3).

No *N*-valoxybenzamidine (**1**) or benzamidoxime (**2**) was detected in any organ, whereas benzamidine (**3**) was detected in lung, heart, spleen, liver, and kidney with highest concentrations of **3** in the metabolically most active organs, liver and kidney. Unfortunately, no metabolite was found in the brain, probably due to extensive metabolism of *N*-valoxybenzamidine (**1**) to benzamidine (**3**) so that the prodrug did not reach the blood–brain barrier in sufficiently high concentrations (detection limit of benzamidine $\geqslant$ 0.375 µM). Furthermore, efflux mechanisms might have prevented accumulation in the brain.[44] Insufficient bleeding might be responsible for the high concentrations in spleen.

3.5. In vivo metabolism of *N,N′*-bis(valoxy)pentamidine (7)

N,N′-bis(valoxy)pentamidine (**7**) was given orally at 100 mg kg^{-1} to two rats. Analysis of tissue samples revealed a rapid and almost complete activation of the prodrug **7** into the drug pentamidine (**8**). Thus, no concentrations of the prodrug **7** and the pentamidine diamidoxime (**5**) were detected in the tissues examined, whereas pentamidine (**8**) and the intermediate pentamidine monoamidoxime were found in considerable concentrations. Pentamidine monoamidoxime was detected in the lung in concentrations of 200 ± 10 ng g^{-1} tissue and in the kidney in concentrations of 300 ± 20 ng g^{-1} tissue. The active form pentamidine (**8**) was found in every organ, but mainly in kidney and liver (Fig. 3). The identity of pentamidine monoamidoxime and pentamidine (**8**) in all organs was confirmed by HPLC and LC/MS.

Interestingly, pentamidine (**8**) was detected in the brain in concentrations of 200 ± 20 ng g^{-1} organ. The higher lipophilicity of the pentamidine-prodrug **7** compared to the benzamidine-prodrug **1** could be the reason pentamidine (**8**) being able to pass the

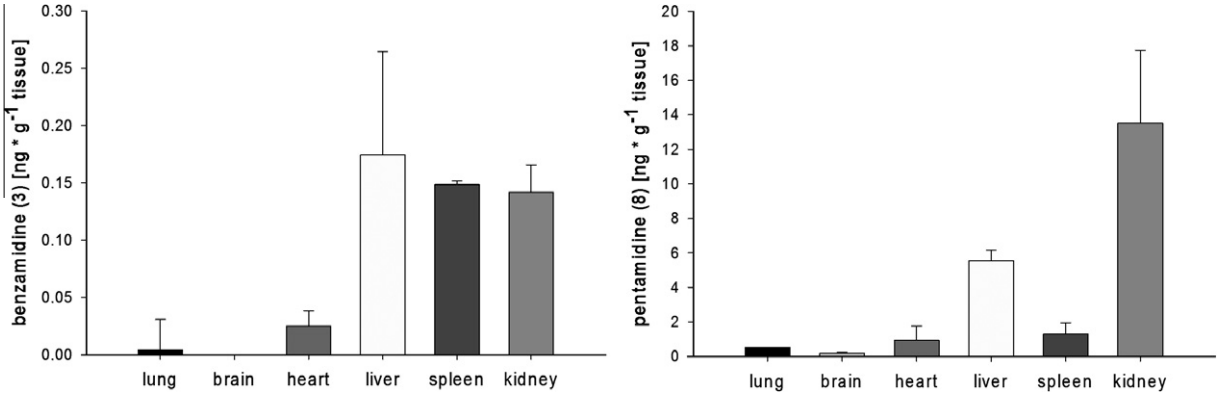


Figure 3. Detected concentrations of benzamidine (**3**) and pentamidine (**8**) in lung, brain, heart, liver, spleen, and kidney after oral application of *N*-valoxybenzamidine (**1**) (50 mg kg^{-1}, n = 3) and *N,N′*-bis(valoxy)pentamidine (**7**) (100 mg kg^{-1}, n = 2), respectively.

blood–brain barrier in small amounts. These results indicate that *N,N′*-bis(valoxy)pentamidine (**7**) might possibly be efficacious in the treatment of the late-stage African sleeping sickness. However, further studies are necessary to prove this assumption.

Due to the fact that pentamidine (**1**) is known to accumulate in tissues, we determined tissue contents and no plasma concentrations. Unfortunately, oral bioavailability is calculated by means of plasma concentrations. Thus we cannot declare a precise value for the oral bioavailability of *N,N′*-bis(valoxy)pentamidine (**7**). However, concentrations detected in the tissues indicate an adequate oral bioavailability the pentamidine prodrug **7**.

4. Conclusions

Conjugation of amidoximes with the amino acid L-valine represents a new double prodrug principle leading to compounds that are highly water soluble and have improved oral bioavailability compared to the unmodified amidoximes. Our in vitro and in vivo studies demonstrated the excellent suitability of this prodrug principle for amidines. Both *N*-valoxybenzamidine (**1**) and *N,N′*-bis(valoxy)pentamidine (**7**) were activated in vitro by all enzyme preparations investigated (i.e., porcine and human subcellular enzyme fractions, hmARC1, hmARC2, and hepatocytes). The activation relies on esterases and mARC and is thus independent from P450 enzymes minimizing the risk for drug-drug interactions and undesired side effects. Our results demonstrate the increase of solubility in comparison to the amidoxime prodrugs. Moreover, both prodrugs show excellent oral bioavailability. In addition to absorption by diffusion, the transport by amino acid and peptide transporters in the gastrointestinal tract is feasible and will be the subject of further investigations. In vivo, an oral bioavailability of benzamidine (**3**) of about 88% was observed after oral administration of prodrug **1** to rats. The high bioavailability also excludes the possibility of an ester hydrolysis prior to absorption. *N,N′*-bis(valoxy)pentamidine (**7**) entered the cells of all tissues investigated. This effect is essential for the antiprotozoic effect of pentamidine (**8**) considering that the parasites are spread throughout the body. Principally, **7** was activated completely to the drug pentamidine (**8**). Also, its solubility was improved over 100-fold in comparison to *N,N′*-bis(acetoxy)pentamidine and to pentamidine diamidoxime (**5**). These observations indicate that the development of this new prodrug principle may also considerably improve the treatment of the second stage African sleeping sickness. The drug can be applied intravenously in case of emergency as well as orally during long-term treatment with overall improved pharmacokinetic properties because of its excellent water solubility. Furthermore, the active drug pentamidine (**8**) was detected in the brain, although only in small amounts. Passing the blood–brain barrier is essential for curing the second stage of the African sleeping sickness. Consequently, further studies are necessary to investigate efficacy of the pentamidine prodrug **7**. In this respect, the development of pentamidine prodrugs based on other amino acids might be reasonable possibly resulting in prodrugs being better capable of crossing the blood–brain barrier.

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References and notes

1. Ettmayer, P.; Amidon, G. L.; Clement, B.; Testa, B. *J. Med. Chem.* **2004**, *47*, 2393–2404.
2. Albert, A. G.; Phillips, R. *J. Chem. Soc.* **1948**, 2240–2249.
3. Young, R. C.; Mitchell, R. C.; Brown, T. H.; Ganellin, C. R.; Griffiths, R.; Jones, M.; Rana, K.; Saunders, D.; Smith, I. R.; Sore, N. E. *J. Med. Chem.* **1988**, *31*, 656–671.
4. Peterlin-Masic, L.; Cesar, J.; Zega, A. *Curr. Pharm. Des.* **2006**, *12*, 73–91.
5. Clement, B. WO 9,501,168, 1995.
6. Clement, B.; Schmitt, S.; Zimmermann, M. *Arch. Pharm. (Weinheim, Ger.)* **1988**, *321*, 955–956.
7. Hauptmann, J.; Paintz, M.; Kaiser, B.; Richter, M. *Pharmazie* **1988**, *43*, 559–560.
8. Clement, B.; Mau, S.; Deters, S.; Havemeyer, A. *Drug Metab. Dispos.* **2005**, *33*, 1740–1747.
9. Clement, B. *Drug Metab. Rev.* **2002**, *34*, 565–579.
10. Bürenheide, A.; Kunze, T.; Clement, B. *Basic Clin. Pharmacol. Toxicol.* **2008**, *103*, 61–65.
11. Havemeyer, A.; Bittner, F.; Wollers, S.; Mendel, R.; Kunze, T.; Clement, B. *J. Biol. Chem.* **2006**, *281*, 34796–34802.
12. Grünewald, S.; Wahl, B.; Bittner, F.; Hungeling, H.; Kanzow, S.; Kotthaus, J.; Schwering, U.; Mendel, R. R.; Clement, B. *J. Med. Chem.* **2008**, *51*, 8173–8177.
13. Clement, B.; Raether, W. *Arzneim.-Forsch.* **1985**, *35*, 1009–1014.
14. Denise, H.; Barrett, M. P. *Biochem. Pharmacol.* **2001**, *61*, 1–5.
15. Apted, F. I. *Pharmacol. Ther.* **1980**, *11*, 391–413.
16. Bryceson, A. D.; Chulay, J. D.; Mugambi, M.; Were, J. B.; Gachihi, G.; Chunge, C. N.; Muigai, R.; Bhatt, S. M.; Ho, M.; Spencer, H. C. *Trans. R. Soc. Trop. Med. Hyg.* **1985**, *79*, 705–714.
17. Newman, S. P.; Simonds, A. K. *Lung* **1990**, *168*, 685–691.
18. Sands, M.; Kron, M. A.; Brown, R. B. *Rev. Infect. Dis.* **1985**, *7*, 625–634.
19. Jones, H. E.; Bundel, G. K.; Tidwell, R. R.; Hall, J. E.; Farr, S. J.; Richards, R. J. *Toxicology* **1993**, *80*, 1–12.
20. Boykin, D. W.; Kumar, A.; Spychala, J.; Zhou, M.; Lombardy, R. J.; Wilson, W. D.; Dykstra, C. C.; Jones, S. K.; Hall, J. E.; Tidwell, R. R. *J. Med. Chem.* **1995**, *38*, 912–916.
21. Mdachi, R. E.; Thuita, J. K.; Kagira, J. M.; Ngotho, J. M.; Murilla, G. A.; Ndung'u, J. M.; Tidwell, R. R.; Hall, J. E.; Brun, R. *Antimicrob. Agents Chemother.* **2009**, *53*, 953–957.
22. Matovu, E.; Seebeck, T.; Enyaru, J. C.; Kaminsky, R. *Microbes Infect.* **2001**, *3*, 763–770.
23. Bray, P. G.; Barrett, M. P.; Ward, S. A.; de Koning, H. P. *Trends Parasitol.* **2003**, *19*, 232–239.
24. Das, B. P.; Boykin, D. W. *J. Med. Chem.* **1977**, *20*, 1219–1221.
25. Wilson, B. N.; Tanious, F. A.; Mathis, A.; Hall, J. E.; Stephens, C. E.; Boykin, D. W. *Curr. Med. Chem.* **2005**, *5*, 389–408.
26. Neidle, S.; Pearl, L. H.; Skelly, J. V. *Biochem. J.* **1987**, *243*, 1–13.
27. Clement, B.; Immel, M.; Terlinden, R.; Wingen, F. J. *Arch. Pharm. (Weinheim, Ger.)* **1992**, *325*, 61–62.
28. Barrett, M. P.; Fairlamb, A. H. *Parasitol. Today* **1999**, *15*, 136–140.
29. Soeiro, M. N.; De Souza, E. M.; Stephens, C. E.; Boykin, D. W. *Expert Opin. Invest. Drugs* **2005**, *14*, 957–972.
30. Clement, B.; Bürenheide, A.; Rieckert, W.; Schwarz, J. *ChemMedChem* **2006**, *1*, 1260–1267.
31. Stella, V. J.; Martodihardjo, S.; Terada, K.; Rao, V. M. *J. Pharm. Sci.* **1998**, *87*, 1235–1241.
32. Varma, M. V.; Khandavilli, S.; Ashokraj, Y.; Jain, A.; Dhanikula, A.; Sood, A.; Thomas, N. S.; Pillai, O.; Sharma, P.; Gandhi, R.; Agrawal, S.; Nair, V.; Panchagnula, R. *Curr. Drug Metab.* **2004**, *5*, 375–388.
33. Huttunen, K. M.; Kumpulainen, H.; Leppanen, J.; Rautio, J.; Jarvinen, T.; Vepsalainen, J. *Synlett* **2006**, 701–704.
34. Mantyla, A.; Rautio, J.; Nevalainen, T.; Keski-Rahkonen, P.; Vepsalainen, J.; Jarvinen, T. *Eur. J. Pharm. Sci.* **2004**, *23*, 151–158.
35. Acosta, E. P.; Fletcher, C. V. *Ann. Pharmacother.* **1997**, *31*, 185–191.
36. Han, H.; de Vrueh, R. L.; Rhie, J. K.; Covitz, K. M.; Smith, P. L.; Lee, C. P.; Oh, D. M.; Sadee, W.; Amidon, G. L. *Pharm. Res.* **1998**, *15*, 1154–1159.
37. Krueger, P. *Ber. Dtsch. Chem. Ges.* **1885**, *18*, 1053–1060.
38. Kotthaus, J.; Wahl, B.; Havemeyer, H.; Kotthaus, J.; Schade, D.; Schönberg, D. G.; Mendel, R.; Bittner, F.; Clement, B. *Biochem. J* **2010**, *433*, 383–391.
39. Fröhlich, A. K.; Girreser, U.; Clement, B. *Drug Metab. Dispos.* **2005**, *33*, 1532–1537.
40. Wingen, F.; Brägas, B. *Arzneim.-Forsch.* **1991**, *41*, 937–945.
41. Kotthaus, J.; Steinmetzer, T.; van de Locht, A.; Clement, B. *J. Enzyme Inhib. Med. Chem* **2011**, *26*, 115–122.
42. Clement, B.; Christiansen, K.; Girreser, U. *Chem. Res. Toxicol.* **2001**, *14*, 319–326.
43. Liu, L.; Ling, Y.; Havel, C.; Bashnick, L.; Young, W.; Rai, R.; Vijaykumar, D.; Riggs, J. R.; Ton, T.; Shaghafi, M.; Graupe, D.; Mordenti, J.; Sukbuntherng, J. 13th North America ISSX Meeting; Maui, HI, 2005.
44. Chen, Y.; Dalwadi, G.; Benson, H. A. *Curr. Drug Delivery* **2004**, *1*, 361–376.