N,*N*′-Dihydroxyamidines: A New Prodrug Principle To Improve the Oral Bioavailability of Amidines

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N,*N*′-Dihydroxybenzamdine represents a model compound for a new prodrug principle to improve the oral bioavailability of drugs containing amidine functions. The activation of the prodrug could be demonstrated in vitro by porcine and human subcellular enzyme fractions, the mitochondrial benzamidoxime reducing system, and porcine hepatocytes. In vivo, the bioavailability of benzamidine after oral application of *N*,*N*′-dihydroxybenzamidine was about 91% and exceeded that of benzamidine after oral application of benzamidoxime, being about 74% (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. Species comparison of in vitro and in vivo conversion of five *N*-hydroxyamidine prodrugs of fVIIA inhibitors to their corresponding active amidines. Presented at the 13th North America ISSX Meeting, Maui, HI, 2005).

Introduction

Insufficient pharmacokinetic characteristics often terminate clinical development of drug candidates. Possibilities exist in order to improve the bioavailability of a drug after oral application. Prodrugs are an established concept to overcome barriers to a drug's usefulness. Prodrug design could lead to improved aqueous solubility, permeability, transporter mediated absorption, or prevention of presystemic degradation.[2]

Since the amidine moiety is necessary for the pharmacological effect of several active forms, there is an eminent demand for prodrugs masking this moiety and enabling the absorption after oral application. Amidines are very hydrophilic, stable toward hydrolysis, and very strong bases that form highly mesomerically stabilized cations after protonation at the double bonded nitrogen.[3] Because of their charge, they are usually unable to pass mucosal membranes and cannot be absorbed from the gastrointestinal tract after oral application. Several prodrug principles were applied to the amidine moiety of drugs;[4] however, the amidoxime function has been the most promising alternative.[5]

The hydroxylation of the amidine function to amidoximes leads to a vast decrease in basicity and to an increase in lipophilicity.[5] The extensive reduction of benzamidoxime to the active amidines has already been demonstrated in 1988[6,7] and has been studied in greater detail afterward.[8,9] The prodrug principle for amidoximes instead of amidines was first applied to pentamidine by our laboratory.[5] In the meantime, it has been transferred to several other amidines in order to achieve sufficient oral absorption and improved bioavailability.[4]

Nevertheless, clinical studies have shown that amidoximes undergo enzymatic degradation to the corresponding amidines after oral application prior to absorption from the gastrointestinal tract into blood. Ximelagatran, the first oral thrombin inhibitor, was rapidly absorbed after oral administration and converted to melagatran, which represents the active form of ximelagatran and the predominant compound in plasma.[10] However, the bioavailability of melagatran in humans was about 20%. The low blood concentrations were derived from incomplete oral absorption and first pass metabolism of ximelagatran.[10]

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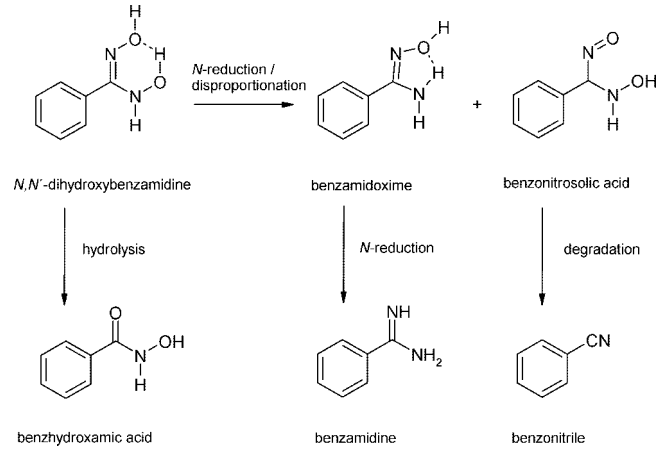
Scheme 1. In Vitro Biotransformation of *N*,*N*′-Dihydroxybenzamidine



To prevent presystemic reduction of amidoximes to amidines leading to a decrease in absorption and therefore to a reduced oral bioavailability, a new prodrug principle was investigated. *N*,*N*′-Dihydroxybenzamidine represents a model compound for this strategy. The substance is activated by reduction in two steps via benzamidoxime toward benzamidine (Scheme 1). Because of the prolonged method of activation in comparison to benzamidoxime, the unwanted reduction toward benzamidine already in the gastrointestinal tract prior to absorption into blood might be prevented. With a pK_a of 3.8,[11] *N*,*N*′-dihydroxybenzamidine possesses an even lower pK_a than benzamidoxime (pK_a = 4.82)[3] and is therefore completely unprotonated under physiological conditions.

The aim of the studies presented here was to examine the biotransformation of *N*,*N*′-dihydroxybenzamidine in vitro and in vivo, the extent of the reduction, the oral bioavailability, and thus the usefulness of this prodrug approach.

Results and Discussion

Chemistry. *N*,*N*′-Dihydroxybenzamidine can be synthesized according to the following reaction routes. With the addition of hydroxylamine hydrochloride to a solution of benzaldehyde

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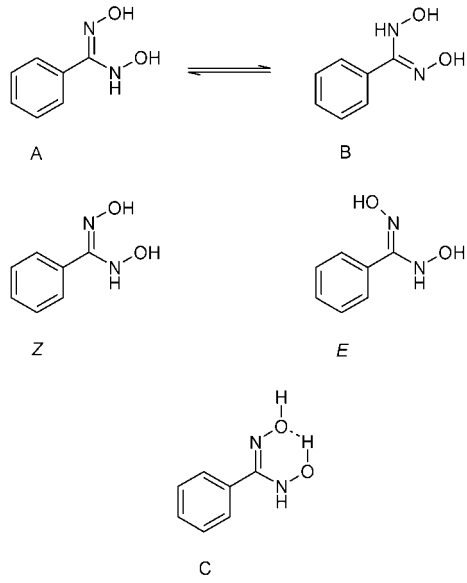
Scheme 2. Tautomeric Forms (A and B), Configuration (*Z* and *E*), and Conformation (C) of *N*,*N*′-Dihydroxybenzamidine



in water/ethanol (1/1, v/v), benzaldoxime was obtained. The addition of *N*-chlorosuccinimide in dimethylformamide rendered α-chlorobenzaldoxime.[12] Consecutively, the reaction with hydroxylamine led to the formation of *N*,*N*′-dihydroxybenzamidine.[13]

Another procedure for the synthesis of *N*,*N*′-dihydroxybenzamidine is described by Van't Riet et al.[14] The addition of hydroxylamine hydrochloride to a solution of 3,4-dihydroxybenzamidoxime in methanol led to the formation of 3,4-dihydroxybenzhydroxyamidoxime, which was precipitated in the form of a hydrochloride. In our laboratory, the method for the synthesis of *N*,*N*′-dihydroxybenzamidine according to Liu et al.[12] and Ley[13] has been more convenient. The method described by Van't Riet et al. rendered a mixture of *N*,*N*′-dihydroxybenzamidine and benzamidoxime, which had to be purified via chromatography.

Configurational Analysis of *N*,*N*′-Dihydroxybenzamidine. *N*,*N*′-Dihydroxyamidines can exist in the two tautomeric forms A and B (Scheme 2). The ^{15}N NMR analysis revealed that, as expected, a rapid equilibrium between A and B exists. Two signals were observed in accordance with an oxime type nitrogen and a hydroxylamine type nitrogen. No NH coupling could be detected as a result of the rapid tautomerisation. In contrast to *N*,*N*′-dihydroxybenzamidine, a stable tautomeric form could be determined for benzamidoxime.[15] This prodrug only exists in the form of an oxime; the tautomeric form of the *N*-hydroxyamidine was not observed in the ^{15}N NMR analysis. The spectrum of benzamidoxime is characterized by a singlet at 287.7 ppm and a triplet at 63.2 ppm.[15] The oxime type nitrogens of *N*,*N*′-dihydroxybenzamidine and benzamidoxime nearly exhibit identical chemical shifts, whereas as expected a low-field shift of the hydroxylamine type nitrogen of *N*,*N*′-dihydroxybenzamidine was observed because of the reduced electron density caused by the adjacent hydroxyl group.

Barassin et al.[16] studied the conformation and the configuration of *N*,*N*′-dihydroxybenzamidine and demonstrated that the *Z*-configuration is energetically favored. Furthermore, they showed that conformation C (Scheme 2) is the predominant form, which is stabilized by hydrogen bond formation.

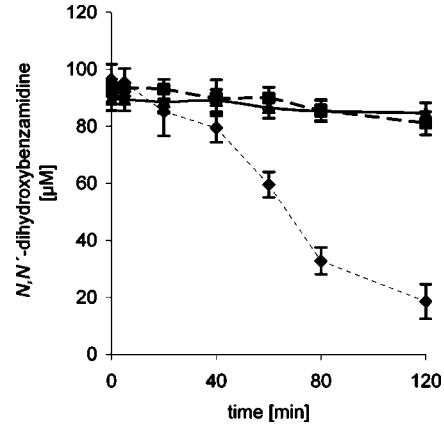


Figure 1. Effect of different pH values on the stability of *N*,*N*′-dihydroxybenzamidine. The reaction vessel consisted of *N*,*N*′-dihydroxybenzamidine in a concentration of 100 μM in 100 mM potassium phosphate buffer pH 2.0, pH 6.3, and pH 7.4. For procedure and HPLC analysis, see Experimental Section: pH 2 (▲), pH 6.3 (■), pH 7.4 (◆).

In Vitro Metabolism of *N*,*N*′-Dihydroxybenzamidine. *N*,*N*′-Dihydroxybenzamidine is rapidly reduced in two steps to the corresponding amidine (Scheme 1). Whereas the reduction toward benzamidoxime occurs enzymatically as well as nonenzymatically, the second step of the reduction toward benzamidine is purely dependent on the presence of reductases. The substance is quite stable in acidic media; the degradation toward benzamidoxime accelerates with an increase of the pH value (Figure 1). In alkaline media *N*,*N*′-dihydroxybenzamidine is reduced nonenzymatically to benzamidoxime. A small amount of the prodrug disproportionates to benzamidoxime and benzonitrosolic acid. The latter compound is not stable, and benzonitrile was reported to be one of the degradation products.[17] Our studies revealed that the formation of benzonitrile could be observed solely after an incubation time of 80 min in phosphate buffer, pH 7.4, at 1.96 μM. After 120 min the metabolite was detected at 3.39 μM. Wieland et al. postulated that the transformation to benzonitrile proceeds via the formation of benzaldoxime;[17] however, this intermediate could not be detected (limit of quantification, 1 μM). In acidic media, *N*,*N*′-dihydroxybenzamidine is slowly hydrolyzed toward benzhydroxamic acid. These results correspond to the investigations of Wieland et al. and Armand et al.[17,18] Benzhydroxamic acid can be further metabolized toward benzamide and benzoic acid.[19,20] The hydroxamic moiety is a suitable ligand for zinc and iron atoms of prosthetic groups of several enzymes.[21] Therefore, a vast range of interactions with several drugs can be expected and the formation of benzhydroxamic acid was analyzed carefully.

The characterization of the in vitro biotransformation of *N*,*N*′-dihydroxybenzamidine by porcine liver microsomes (Table 1) demonstrated that the preferred cosubstrate for the reduction to benzamidine was NADH. The substitution with NADPH led to reduction rates that were reduced by 80%, compared to the standard incubation mixture. Similarly the incubation of *N*,*N*′-dihydroxybenzamidine in phosphate buffer, pH 7.4, led to a 5-fold decrease in the reduction rates to benzamidine. Furthermore, the enzymatic formation of benzonitrile and benzhydroxamic acid could be excluded.

The activation of *N*,*N*′-dihydroxybenzamidine via benzamidoxime toward benzamidine by porcine and human liver and kidney microsomes, mitochondria, 9000 g supernatant, and cytosol could be demonstrated for the first time (Table 2). Only

Table 1. Characterization of the Biotransformation of *N*,*N*′-Dihydroxybenzamidine in Vitro[a]

	nmol·min^{-1}·(mg protein)$^{-1}$[b]			
	benzhydroxamic acid	benzamidoxime	benzamidine	benzonitrile
standard incubation mixture	ND	3.66 ± 0.47	2.25 ± 0.48	ND
without NADH	ND	ND	ND	ND
without NADH/ with NADPH	ND	2.36 ± 0.16	0.43 ± 0.03	ND
standard incubation mixture, pH 7.4	ND	4.35 ± 0.22	0.40 ± 0.06	ND
without protein	ND	ND	ND	ND

[a] For incubation conditions and HPLC analysis, see Experimental Section. An incubation mixture consisted of 0.3 mg of protein, 1 mM *N*,*N*′-dihydroxybenzamidine, 1 mM NADH as cosubstrate in 100 mM potassium phosphate buffer, pH 6.3. In the other experiments, NADPH was added instead of NADH and the pH value of the 100 mM phosphate buffer was changed from 6.3 to 7.4. ND, not detectable. [b] The conversion rates are the mean ± SD of four determinations.

Table 2. In Vitro Activation of *N*,*N*′-Dihydroxybenzamidine via Benzamidoxime toward Benzamidine by Human and Porcine Liver and Kidney Microsomes and Mitochondria, Porcine 9000 g Supernatant, and Porcine Cytosol[a]

	nmol·min^{-1}·(mg protein)$^{-1}$[b]	
enzyme source	benzamidoxime product	benzamidine product
pig liver microsomes	3.8 ± 0.05	2.19 ± 0.1
pig liver 9000 g supernatant	0.6 ± 0.26	ND
pig kidney mitochondria	2.88 ± 0.14	5.29 ± 0.36
pig kidney microsomes	3.17 ± 0.33	3.73 ± 0.37
pig kidney 9000 g supernatant	2.27 ± 0.20	1.56 ± 0.25
pig liver mitochondria	2.53 ± 0.17	3.38 ± 0.05
pig liver cytosole	1.30 ± 0.13	0.06 ± 0.03
human liver microsomes	1.41 ± 0.17	0.54 ± 0.02
human liver mitochondria	1.80 ± 0.09	0.23 ± 0.01
human liver 9000 g supernatant	1.84 ± 0.11	ND
human kidney microsomes	0.82 ± 0.04	0.10 ± 0.01
human kidney mitochondria	1.60 ± 0.08	0.43 ± 0.04
human kidney 9000 g supernatant	1.55 ± 0.13	ND

[a] For incubation conditions and HPLC analysis, see Experimental Section. An incubation mixture consisted of 0.3 mg of protein, 1 mM *N*,*N*′-dihydroxybenzamidine, 1 mM NADH as cosubstrate in 100 mM potassium phosphate buffer, pH 6.3. ND, not detectable. [b] The conversion rates are the mean ± SD of four determinations.

the 9000 g supernatant fractions were not always capable of forming benzamidine.

The mitochondrial benzamidoxime reducing component (mARC) has been recently identified as a 35 kDa molybdo protein. It has been demonstrated that this protein participates predominantly in the mitochondrial N-reductive enzyme system.[22] The reduction of *N*,*N*′-dihydroxybenzamidine to benzamidoxime and benzamidine could be demonstrated by the mitochondrial benzamidoxime reducing component in the presence of cytochrome b_5 and NADH cytochrome b_5 reductase and will be the subject of further publications.

Porcine hepatocytes represent an accredited model for the simulation of in vivo biotransformation. Because of their ability to conduct N-reductions as well as phase 2 metabolism, the metabolism of amidoximes has been studied with hepatocytes.[23,24] In the present work, it could be demonstrated that *N*,*N*′-dihydroxybenzamidine is metabolized by porcine hepatocytes. Reduction rates of 1557.58 ± 536.91 and 336.93 ± 68.27 pmol·min^{-1}·(mg of protein)$^{-1}$ were obtained for the reduction of *N*,*N*′-dihydroxybenzamidine to benzamidoxime and benzamidine, respectively. After the cleavage of glucuronides, a not significant increase of 3.6% in the amount of benzamidoxime could be detected. The N-reduction represents the main pathway in porcine hepatocytes, whereas the formation of glucuronides in terms of phase 2 metabolism only occurs to a minor extent. These results correspond to the observations described for benzamidoxime.[23,24]

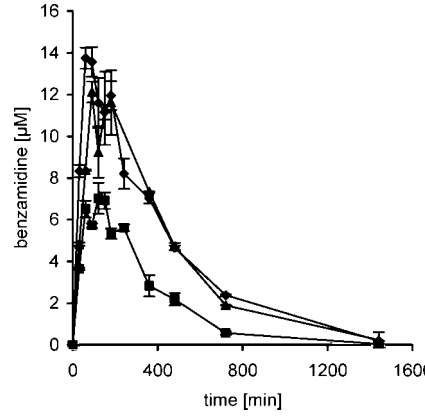


Figure 2. Plasma concentration−time curves of benzamidine following oral administration of *N*,*N*′-dihydroxybenzamidine to three pigs at 10 mg/kg body weight. For workup procedure, see Experimental Section: pig 1 (▲), pig 2 (◆), pig 3 (■).

Overall the characteristics of the reduction of *N*,*N*′-dihydroxybenzamidine were similar to those of benzamidoxime. Thus, it can be speculated that the same enzyme systems[9] are involved.

In Vivo Biotransformation of *N*,*N*′-Dihydroxybenzamidine. *N*,*N*′-Dihydroxybenzamidine was applied orally at 10 mg/kg body weight to three pigs. Some of the plasma samples were worked up directly and others after enzymatic pretreatment with β-glucuronidase/sulfatase. The prodrug was rapidly metabolized so that *N*,*N*′-dihydroxybenzamidine could not be detected in the plasma samples. Benzamidoxime could only be found 30 min after the application of *N*,*N*′-dihydroxybenzamidine in one pig at 30.2 nM. Figure 2 shows the plasma concentration−time curves of benzamidine after the oral application of *N*,*N*′-dihydroxybenzamidine. Benzamidine could be detected in concentrations up to 13.7 μM. Benzhydroxamic acid and benzamide have not been found probablybecause of the fast and extensive reduction of *N*,*N*′-dihydroxybenzamidine to benzamidine. Indications for sulfatation and glucuronidation were observed in the plasma samples. After deconjugation free benzamidoxime could be detected at up to 0.4 μM (Figure 3). These results show that conjugations to a glucuronide or sulfate prevent the complete reduction toward benzamidine. This corresponds to previous work demonstrating indirect evidence of in vivo glucuronidation of benzamidoxime.[25] Furthermore, the study of the biotransformation of benzamidoxime by human and pig hepathocytes revealed that the N-reduction of benzamidoxime represents the dominant reaction, but to a much smaller extent glucuronides and sulfates could also be found.[23,24] Both phase 2 metabolites exhibit no mutagenicity in the Ames test.[23] To investigate the formation of benzamidoxime *O*-acetate, the plasma samples were analyzed by LC/MS. The determination limit was 100 nM. This metabolite could not be detected,

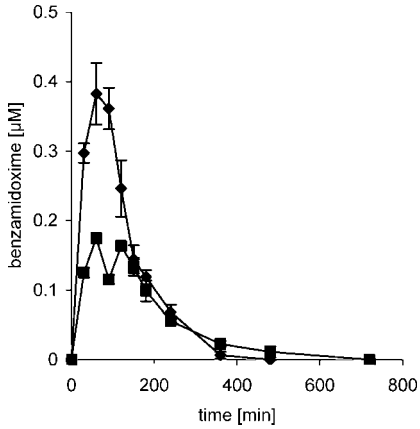


Figure 3. Plasma concentration−time curves of benzamidoxime after enzymatic cleavage of conjugates after oral administration of *N*,*N*′-dihydroxybenzamidine to two pigs at 10 mg/kg body weight. For the procedure, see Experimental Section: pig 2 (◆), pig 3 (■). The same pigs as for the experiments in Figure 2 were used. Pig 1 could not be included in the experiments shown in this figure. Due to problems with the catheter of pig 1, we were not able to take enough blood.

Table 3. Pharmacokinetic Parameters of Benzamidine after the Oral Application of *N*,*N*′-Dihydroxybenzamidine to Three Pigs[a]

	pig 1	pig 2	pig 3
t_{max} [min]	90.0	60.0	120.0
c_{max} [μM]	12.1	13.7	7.0
$t_{1/2}$ [min]	222.0	207.8	177.0
MRT [min]	369.4	361.8	300.0
AUC [min·μmol]	5549.5	5923.7	2665.4
V_d [dl/kg]	47.0	41.4	79.1
Cl [dL·min^{-1}·kg^{-1}]	0.1	0.1	0.3

[a] Abbreviations: t_{max}, time at maximal plasma concentration; c_{max}, maximal plasma concentration value; $t_{1/2}$, terminal half-life; MRT, mean plasma residence time; AUC, area under the curve, V_d: volume of distribution; Cl, total body clearance.

Table 4. Oral Bioavailability of Benzamidine after the Oral Application of *N*,*N*′-Dihydroxybenzamidine to Three Pigs

	bioavailability [%]	mean ± SD [%]
pig 1	107	
pig 2	114	91 ± 34
pig 3	51	

so phase 2 conjugation of benzamidoxime can be considered as a detoxification reaction in the metabolism of *N*,*N*′-dihydroxybenzamidine.

The pharmacokinetic parameters following oral application of *N*,*N*′-dihydroxybenzamidine were evaluated (Table 3). The resorption and elimination of the prodrug are characterized by a short application phase and a long elimination phase.

After the oral administration of *N*,*N*′-dihydroxybenzamidine, an average bioavailability of benzamidine in three pigs of 91% ± 34% could be estimated (Table 4). A bioavailability of benzamidine of 74% after the oral application of benzamidoxime to rats has been reported by Lui et al.[1] Furthermore, species differences in the in vitro and in vivo conversion of *N*-hydroxyamidine prodrugs to parent amidines have been demonstrated to be insignificant.[1] Therefore, the comparison of the bioavailabilities leads to the assumption that the preactivation in the gastrointestinal tract might be decreased by using dihydroxylated amidines instead of the monohydroxylated species. The stability profile of *N*,*N*′-dihydroxybenzamidine (Figure 1) suggests that the prodrug is relatively stable in the upper sections of the gastrointestinal tract after oral application because of the prevailing pH of approximately 4–5.[26] The minor stability at pH 7.4 leads to a fast conversion to benzamidoxime after the uptake into blood. The short application phase after oral administration demonstrates that the prodrug is rapidly absorbed into blood and afterward efficiently reduced to the corresponding amidine.

Conclusion

In conclusion, N-reduction of *N*,*N*′-dihydroxybenzamidine is the predominant reaction in vitro and in vivo. In vivo, the prodrug was rapidly absorbed and converted to benzamidine. Benzamidine has been found to be the predominant metabolite in plasma after oral application. The bioavailability of benzamidine after oral administration of *N*,*N*′-dihydroxybenzamidine was about 91% and exceeded those of benzamidine after the oral application of benzamidoxime (74%),[1] presumably because of a decreased presystemic reduction to the free amidine. We have shown that the dihydroxylation of amidines represents a suitable prodrug principle leading to a higher bioavailability than the concepts used before, in order to improve the oral bioavailability of drug candidates containing the amidine functional group.

Experimental Section

Chemicals and Reagents. Benzamidine, benzamide, benzonitrile, and benzhydroxamic acid were obtained from Sigma Chemie (Deisenhofen, Germany). All other chemicals were commercially available and of analytical grade except acetonitrile and methanol, which were of HPLC grade.

Synthesis. Uncorrected melting points were measured on a Büchi 510 melting point apparatus (Büchi Labortechnik AG, Flawil, Switzerland). IR spectra were obtained by an FTIR 1600 PC spectrophotometer (Perkin-Elmer, Überlingen, Germany). ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded on an AM 400 spectrometer (Bruker Biospin, Rheinstetten, Germany) using the following frequencies: ^{1}H, 300.13 MHz; ^{13}C, 75.47 MHz; ^{15}N, 40.51 MHz. Chemical shifts (δ) are in ppm relative to the internal standard TMS (^{1}H, ^{13}C NMR) and to ammoniac as an external standard (^{15}N NMR).

Benzamidoxime. Benzamidoxime was synthesized according to a published method.[27]

Benzamidoxime *O*-Acetate. Benzamidoxime *O*-acetate was synthesized according to the methods published by Eloy and Leaners[28] and Schulz.[29]

***N*,*N*′-Dihydroxybenzamidine.** *N*,*N*′-Dihydroxybenzamidine was synthesized according to the methods described by Liu et al.[12] and Ley.[13] Yield 64%. Mp: 116 °C (lit.[13] 115 °C). IR (KBr): 3400m, 3300w, 3150m, 1620s, cm^{-1}. ^{1}H NMR (DMSO-d_6): in accordance with results of Barassin et al.[16] ^{13}C NMR ($CDCl_3$): δ 156.88, 131.91, 128.74, 127.72, 127.69. ^{15}N NMR ($CDCl_3$): δ 129.51, 299.72.

Assay for the Activation of *N*,*N*′-Dihydroxybenzamidine. A standard incubation mixture contained 0.3 mg of protein and 1 mM *N*,*N*′-dihydroxybenzamidine in 100 mM potassium phosphate buffer, pH 6.3. After preincubation for 5 min at 37 °C under aerobic conditions, the reaction was started by the addition of NADH (final concentration, 1 mM) to a total volume of 250 μL. The mixture was incubated for 30 min at 37 °C under aerobic conditions. The reaction was terminated by the addition of 250 μL of cold acetonitrile/formic acid so that the resulting pH was 3.0 and by vortexing. After centrifugation at 10 000 U/min (Mikrozentrifuge Hettich, Tuttlingen, Germany), 10 μL of the supernatant was analyzed by HPLC.

Processing of Plasma Samples. Blood samples were drawn into heparinized containers, immediately centrifuged (1500*g*, 10 min, 4 °C), and stored at −80 °C. The samples were worked up by solid-phase extraction (Strata X, 10 μm column, Phenomenex, Aschaffenburg, Germany). After equilibration and condition of the column, an amount of 1000 μL of plasma was added, the column was

washed with 500 μL of methanol and aqua bidest, pH 3 (20/80, v/v), and the substances were eluted with 1000 μL of the same solvent. The samples were concentrated to dryness. The residues were taken up in the eluents to be used for HPLC analysis of *N*,*N*′-dihydroxybenzamidine, benzamidoxime, benzamidine, benzhydroxamic acid, and benzamide. The plasma samples of *N*,*N*′-dihydroxybenzamidine were worked up directly after the blood withdrawal.

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Supporting Information Available: A figure illustrating the examples of amidine prodrugs discussed in the introductory paragraphs, two schemes showing the synthesis of *N*,*N*′-dihydroxybenzamidine, experimental procedures concerning the in vitro and in vivo studies, HPLC and LC/MS data for all compounds, and description of the estimation of the pharmacokinetic parameter. This material is available free of charge via the Internet at http://pubs.acs.org.

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