DOI: 10.1002/cmdc.201100422

New Prodrugs of the Antiprotozoal Drug Pentamidine

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Pentamidine is an effective antimicrobial agent that is approved for the treatment of African trypanosomiasis but suffers from poor oral bioavailability and central nervous system (CNS) penetration. This work deals with the development and systematic characterisation of new prodrugs of pentamidine. For this reason, numerous prodrugs that use different prodrug principles were synthesised and examined in vitro and in vivo. Another objective of the study was the determination of permeability of the different pentamidine prodrugs. While some of the prodrug principles applied in this study are known, such as the conversion of the amidine functions into amidoximes or the O-alkylation of amidoximes with a carboxymethyl residue, others were developed more recently and are described here for the first time. These newly developed methods aim to increase the affinity of the prodrug for the transporters and mediate an active uptake via carrier systems by conjugation of amidoximes with compounds that improve the overall solubility of the prodrug. The different principles chosen resulted in several pentamidine prodrugs with various advantages. The objective of this investigation was the systematic characterisation and evaluation of eight pentamidine prodrugs in order to identify the most appropriate strategy to improve the properties of the parent drug. For this reason, all prodrugs were examined with respect to their solubility, stability, enzymatic activation, distribution, CNS delivery, and oral bioavailability. The results of this work have allowed reliable conclusions to be drawn regarding the best prodrug principle for the antiprotozoal drug pentamidine.

Introduction

Pentamidine (**1**) is an antimicrobial drug used to treat trypanosomiasis, leishmaniasis, and *Pneumocystis carinii* pneumonia (PcP). Due to two strongly basic amidine moieties, pentamidine suffers from poor oral bioavailability and thus has to be given by intramuscular (i.m.) or intravenous (i.v.) administration. Unfortunately, most of the infections mentioned above occur in tropical or subtropical countries that usually have poor medical care systems. Consequently, the need for i.m. or i.v. administration limits the clinical use of pentamidine (**1**) in most regions and shows the need for a pentamidine derivative that can be administered orally. Furthermore, pentamidine (**1**) lacks sufficient central nervous system (CNS) penetration and so is only effective in the treatment of early stages of African trypanosomiasis, and not in the meningoencephalitic state, where the pathogens have infiltrated the CNS. Consequently, several attempts have been made to improve both the oral bioavailability and CNS penetration of pentamidine (**1**). Some research efforts involved altering the structure of pentamidine (**1**) by modifying different residues or by cyclising the amidine function.[1–3] The most successful derivatives that arose from these efforts were DB75 (furamidine) and its prodrug DB285 (pafuramidine), which showed good efficacy both in vitro and in vivo.[4] However, clinical development of DB289 was ceased in 2008 due to unexplained idiosyncratic drug-induced organ toxicity.

Further approaches have dealt with the development of pentamidine prodrugs. In order to overcome both poor oral bioavailability and insufficient CNS penetration, we previously synthesised several prodrugs that rely on different prodrug principles.[5–7] Earlier studies dealt with the conversion of the amidine moieties into less basic amidoximes resulting in more lipophilic molecules. Since amidoximes are uncharged at physiological pH, the gastrointestinal absorption rates of these pharmacologically inactive amidoximes are significantly improved.[5] The extensive reduction of amidoximes into their pharmacologically active amidines was first demonstrated in 1988 for the model compound benzamidoxime and was later shown in vivo for pentamidine–monoamidoxime **2** and pentamidine–diamidoxime **3**.[6,7] Recently, the enzyme system responsible for this reduction was identified as a previously unknown molybdenum-containing system, which was named mitochondrial amidoxime reducing component (mARC).[8–10] However, studies in rats revealed that both pentamidine–monoamidoxime **2** and -diamidoxime **3** have low oral bioavailability.[6]

In order to optimise the pharmacokinetic properties and CNS delivery of these compounds, further modifications were made that resulted in *N*,*N*′-bis(acetoxy)pentamidine **4**, which has greatly improved lipophilicity compared with the amidoxime prodrugs **2** and **3**, and showed oral bioavailability in studies with rats and pigs. Unfortunately, this drug has very limited solubility, the bioavailability detected was very low, and no

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CNS penetration was demonstrated in pigs.[11] Further attempts dealing with the development of prodrugs with improved solubility led to the identification of *N*,*N*′-bis(valoxy)pentamidine **9**. This prodrug was designed in analogy to the valin conjugate valacyclovir that is absorbed by peptide transporter 1 (PepT1).[12] However, prodrug **9** showed adequate oral bioavailability in rats, but only small amounts were detected in the brain.[13]

Taken together, none of these previously described prodrugs fulfill all or at least most of the favoured criteria, such as high oral bioavailability, CNS penetration, and good solubility. Consequently, this study aimed to develop pentamidine prodrugs with significantly improved solubility, oral absorption, blood–brain barrier permeability, and presystemic metabolism. Thus, we successfully designed and examined four new pentamidine prodrugs, which are based on different prodrug principles. Some prodrug principles are described within this work for the first time; others were originally established for the model compound benzamidoxime and have now been applied to pentamidine (**1**).[14,15]

There are several rationales for choosing the different prodrug principles applied here. Conjugation with polar moieties resulted in significantly improved solubility (prodrugs **5** and **8**), which is an essential advantage compared with previously described pentamidine prodrugs. Prodrugs **6** and **7** show elevated metabolic stability due to the more complex mode of activation requiring O-dealkylation and/or multiple reduction steps. This mode of activation contributes to a reduced presystemic activation and as a consequence might increase oral bioavailability and CNS penetration. However, both solubility and presystemic activation are known to limit the oral bioavailability of the pentamidine prodrugs developed previously.[11] Moreover, the activation of prodrug **5** depends on peptidylglycine α-amidating monooxygenase (PAM), an enzyme with highest activities in the CNS.[14] Thus, this mode of activation could contribute to brain targeting.[16] Prodrug **8** is assumed to be a substrate for transporters resulting in an increased oral bioavailability by active uptake mechanisms. Despite many efforts to understand the exact substrate specificity of human transport systems, the knowledge is still very restricted and does not allow reliable predictions as to whether a newly discovered compound is a substrate or not. Thus, on the basis of knowledge we currently have, we speculate that prodrug **8** might be a substrate of human organic anion transporters (OATs) or monocarboxylate transporters (MCTs). Those transporters are known to possess broad substrate specificity and to be involved in the uptake of several drugs, such as methotrexate and pravastatin.[17,18]

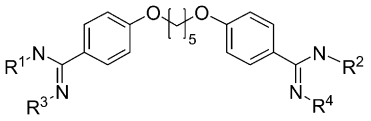
Within this work, we synthesised eight prodrugs for systematic characterisation and evaluation with respect to their solubility, stability, bioactivation, permeability, and bioavailability to draw a reliable conclusion on the most appropriate prodrug of pentamidine.

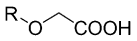
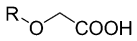
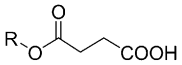
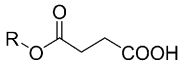
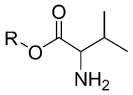
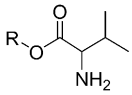
Results and Discussion

Synthesis of pentamidine prodrugs

In this work, we successfully synthesised eight prodrugs of the antiprotozoal drug pentamidine that rely on different prodrug principles. *N*,*N*′-Bis(methoxy)pentamidine **6** was obtained by alkylation of pentamidine–diamidoxime **3** with dimethylsulfate as described in the Experimental Section. Formation of *N*,*N*′-bis(dihydroxy)pentamidine **7** was achieved by addition of 4,4′-pentamethylendioxy-bis-(*N*-hydroxybenzencarboximidoylchloride) to hydroxylamine and subsequent stirring overnight according to the conditions described in the Experimental Section. *N*,*N*′-Bis(succinyloxy)pentamidine **8** was prepared by treating pentamidine–diamidoxime **3** with succinic anhydride and subsequent recrystallisation from toluene. All other pentamidine prodrugs were synthesised according to the literature.[11,13,14,19] The chemical structures are summarised in Table 1.

Table 1. Structures of all pentamidine derivatives examined.



Compd[a]	R^1,R^2	R^3	R^4
Pentamidine (**1**)	–H	–H	–H
Pentamidine–monoamidoxime (**2**)	–H	–OH	–H
Pentamidine–diamidoxime (**3**)	–H	–OH	–OH
N,*N*′-Bis(acetoxy)pentamidine (**4**)	–H		
N,*N*′-Bis(carboxymethoxy)pentamidine (**5**)	–H		
N,*N*′-Bis(methoxy)pentamidine (**6**)	–H	$-OCH_3$	$-OCH_3$
N,*N*′-Bis(dihydroxy)pentamidine (**7**)	–OH	–OH	–OH
N,*N*′-Bis(succinyloxy)pentamidine (**8**)	–H		
N,*N*′-Bis(valoxy)pentamidine (**9**)	–H		

Evaluation of their drug-likeness in silico

The drug-likeness of all prodrugs was evaluated according to Lipinski's rule of five to get an initial idea of their potential oral bioavailability via passive absorption from the intestine.[20] All essential parameters are summarised in Table 2, and the results

Table 2. Evaluation of all compounds according to Lipinski's rule of five.[a]

Compd	MW [g mol^{-1}]	*n* [O/N]	*n* [OH/NH]	clog *P*[b]	Violations
1	340.6	6	**6**	2.31	1
2	356.4	7	**6**	2.54	1
3	372.4	8	**6**	2.78	1
4	456.5	10	4	3.87	0
5	488.5	**12**	5	3.10	1
6	400.5	8	4	4.29	0
7	404.4	10	**6**	1.92	1
8	**572.6**	**14**	**6**	3.22	3
9	**570.7**	**12**	**8**	4.26	3

[a] The rule of five predicts good oral bioavailability if not more than one of the following criteria is violated: not more than 10 hydrogen-bond acceptors; not more than 5 hydrogen-bond donors; a molecular weight not greater than 500 Da; a clog *P* value not greater than 5.[20] Violations of the rule of five are illustrated in bold style. [b] Lipophilicity (clog *P*) was calculated using the software ChemBioDraw Ultra (version 11).

Table 3. Solubility of pentamidine derivatives **1**–**9** at different pH levels and their protein binding.[a]

Compd	Solubility [μM]			Protein
	pH 2.0	pH 7.4	pH 9.0	binding [%]
1	> 35 000	> 35 000	> 35 000	54.6 ± 5.3
2	22 285 ± 1244	1370 ± 291	1257 ± 40	74.4 ± 2.6
3	4211 ± 231	12 ± 1	4 ± 1	92.8 ± 1.6
4	14 ± 8	2 ± 1	3 ± 2	n.d.
5	924 ± 152	11 626 ± 71	10 530 ± 1572	98.2 ± 1.2
6	1304 ± 28	8 ± 1	10 ± 2	n.d.
7	> 35 000	95 ± 8	21 ± 3	96.5 ± 2.1
8	unstable	7500 ± 340	10 780 ± 70	97.1 ± 1.2
9	> 35 000	157 ± 19	84 ± 18	90.6 ± 4.5

[a] Solubility and protein binding were determined as described in the Experimental Section. n.d. = not determined due to solubility issues.

show that, with the exception of *N*,*N*′-bis(succinyloxy)pentamidine **8** and *N*,*N*′-bis(valoxy)pentamidine **9**, all prodrugs are assumed to possess adequate oral bioavailability according to the rule of five (i.e., one or less violations). In contrast to the other prodrugs examined, prodrugs **8** and **9** are designed to target transporters and as such their availability would not be dependent on passive uptake. Consequently, the negative estimations for these prodrugs predicted by the rule of five (three violations) can be disregarded. These results prove the necessity to further investigate all prodrugs to identify the most appropriate application for pentamidine (**1**).

Evaluation of the drug-likeness in vitro

Solubility and protein binding

Solubility and protein binding are important properties that have to be considered when developing new drugs. Both can limit the clinical use by causing difficulties concerning bioavailability or drug–drug interactions. In general, good solubility is considered to have a positive influence on oral bioavailability. Thus, we investigated the solubility at three essential pH levels simulating conditions in the stomach (pH 2.0), intestine (pH 9.0), and blood circulation (pH 7.4). Solubility data are summarised in Table 3 and demonstrate that most prodrugs have good or fair solubility, with the exception of prodrug **4** at pH 2.0. At pH 7.4 and 9.0, only prodrugs **2**, **5** and **8** show good solubility, whereas prodrugs **3**, **4** and **6** are nearly insoluble.

The protein binding of all prodrugs was determined by ultrafiltration. As can be seen in Table 3, all prodrugs possess protein bindings of at least 74%. In general, high protein binding of more than 90% is considered to be critical in regard to drug–drug interactions.[21] Most prodrugs examined have protein binding levels within this critical range and thus, might cause drug–drug interactions, when co-administered with other drugs that possess high protein binding. However, rapid activation of the prodrug into the active form considerably lowers the risk of those side effects. In summary, none of the prodrugs show significant advantages or disadvantages regarding protein binding.

Stability

Incubations in phosphate buffer at pH 2.0, 9.0 and 7.4 simulate conditions in the stomach, intestine and blood circulation, respectively. They were performed to provide evidence of possible chemical instability that might limit the development of these prodrugs as successful drug. Particular interest was set on the behaviour of the prodrugs at pH 2.0 and 9.0 to assess the possible extent of hydrolysis or inactivation in the stomach and intestine. Our experiments demonstrated that most prodrugs are highly stable in the different media tested. However, O-acylated derivatives **4**, **8** and **9** were hydrolysed—as expected—at basic and acidic pH (Figure 1). In particular, the hydrolysis of prodrug **8** in acidic media is very pronounced. Thus, a gastro-resistant formulation would be required to prevent prodrugs **4** and **8** from undergoing extensive hydrolysis prior to absorption. Interestingly, valine derivative **9** is rather stable at pH 2.0 and can be administered orally without the need for a gastro-resistant formulation. Similar findings were obtained from incubations in murine and human plasma, in which rapid hydrolysis of prodrugs **4** and **9** was observed. Interestingly, in comparison to these derivatives, prodrug **8** is rather slowly activated by plasma enzymes. Moreover, there were no further metabolites detectable except those resulting from ester hydrolysis. Prodrug **6** showed a slight degradation at pH 7.4 and 9.0. All other prodrugs showed no degradation (data not shown). Hydrolysis of O-acylated prodrugs **4**, **8**, and **9** in plasma is desired because ester cleavage is required for activation. Further incubations performed with prodrugs **4**, **8**, and **9** and unspecific carboxylic esterase proved good cleavage of the ester and thus activation of these prodrugs (data not shown).

Activation

The pentamidine prodrugs described in this work are based on diverse prodrug principles that require different enzyme sys-

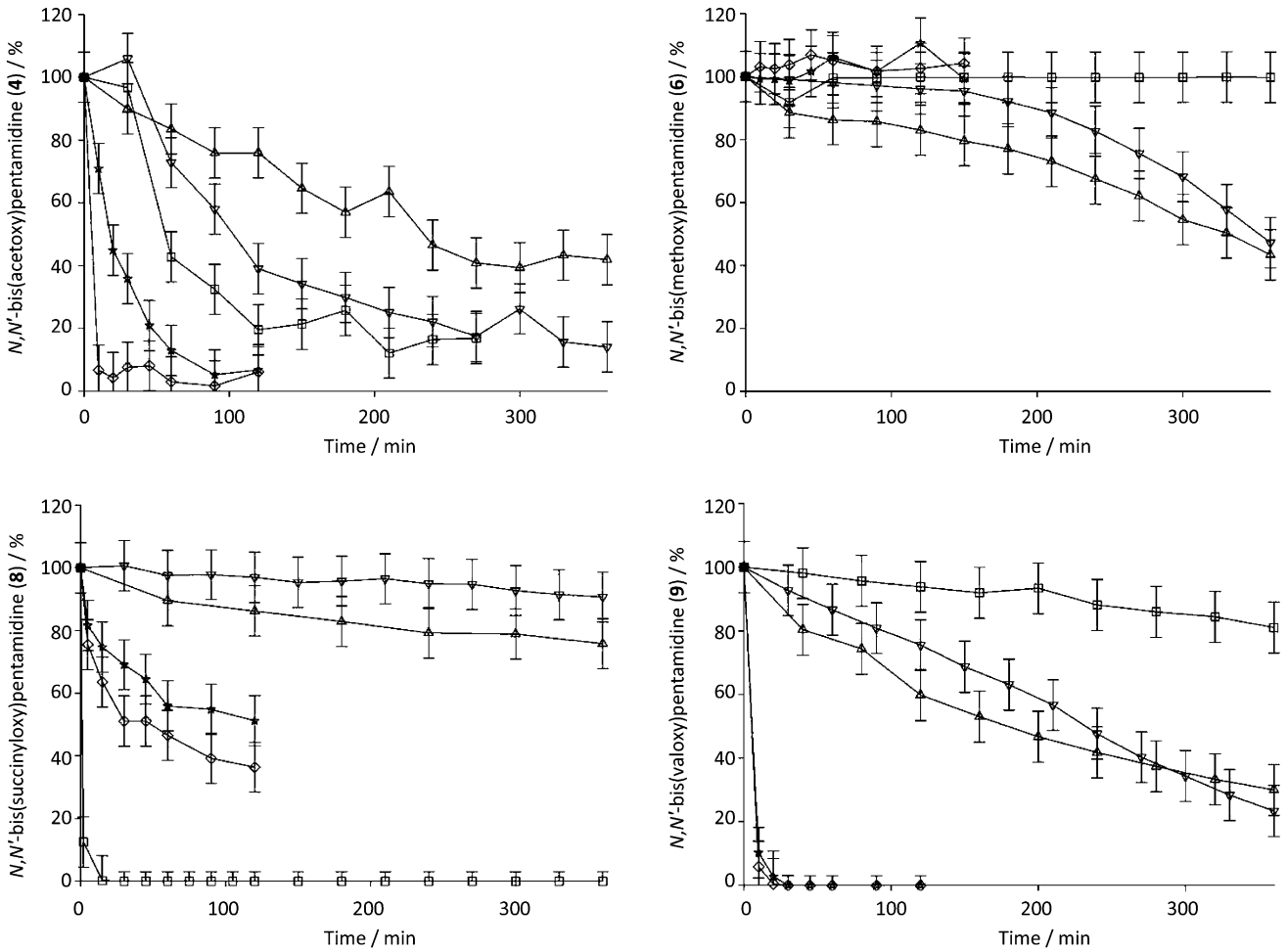
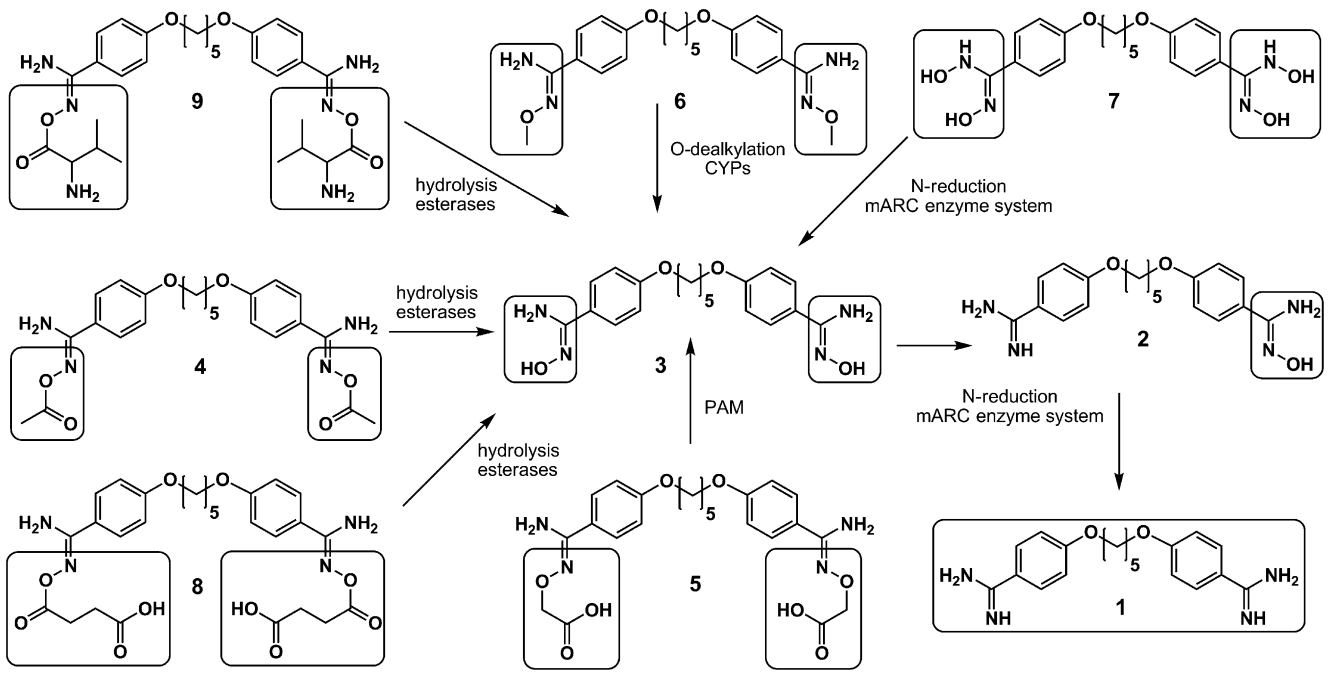


Figure 1. Stability of prodrugs **4**, **6**, **8**, and **9** in diverse media. pH 2.0: □; pH 7.4: △; pH 9.0: ▽; human plasma: ★; rat plasma: ◇.

tems, such as mARC, cytochrome P450s (CYPs) and PAM for activation (Scheme 1).[8,14,22,23] For that reason, the incubation mixtures varied in their compositions (see the Experimental Section). Thus, a direct comparison of activation rates cannot be made. However, we investigated the activation of each prodrug by human and porcine enzyme preparations in an optimised assay. Porcine enzyme preparations were used because of their suitability for the prediction of physiological conditions in human.[24,25] The results of our experiments demonstrate considerable rates of conversion from the prodrug into the active drug pentamidine (**1**) for all prodrugs examined, proving the suitability of the principles used for the development of our prodrugs (Table 4). Incubations with human enzyme preparations resulted in slightly lower conversion rates compared with those obtained with porcine enzyme sources (data not shown). Human tissues, from which microsomes and mitochondria were extracted, were obtained from patients suffering from cancer or hepatitis, possibly resulting in lower or modified enzyme levels when compared with healthy individuals; this might be the reason for lower turnover rates in these experiments. Due to difficulties simulating the complex activation of prodrug **5** in a single in vitro assay containing PAM and reductive enzyme preparations, we examined the activation of **5** by conversion into **3**, which is intensively investigated and known to be excellently activated.

Prodrugs **6** and **7** were designed to increase the metabolic stability of pentamidine derivatives and consequently reduce their presystemic activation. As can be seen in Table 4, both prodrugs possess relatively low turnover rates and thus demonstrate improved metabolic stability compared with other prodrugs examined.

The different principles result in several advantages and disadvantages when considering drug–drug-interactions and tissue distribution of activating enzymes. For example, *N*,*N*′-bis-(methoxy)pentamidine **6** is activated by oxidative dealkylation by CYPs to give pentamidine–diamidoxime **3**, and therefore it bears the risk of mediating drug–drug interactions. Similar observations were made for the pentamidine analogue DB289 (pafuramidine), which relies on the same prodrug principle and is O-demethylated by CYP1A2, CYP3A4, and CYP4F.[23,26] All other pentamidine derivatives are activated independently of CYP enzymes by esterases, PAM or the mARC enzyme system.[5,8,9,11,14] Interactions mediated by these enzymes have not yet been described in the literature. PAM shows the high-



Scheme 1. Activation of pentamidine prodrugs. Compound names: pentamidine (**1**); pentamidine–monoamidoxime (**2**); pentamidine–diamidoxime (**3**); *N*,*N*′-bis(acetoxy)pentamidine (**4**); *N*,*N*′-bis(carboxymethoxy)pentamidine (**5**); *N*,*N*′-bis(methoxy)pentamidine (**6**); *N*,*N*′-bis(dihydroxy)pentamidine (**7**); *N*,*N*′-bis(succinyloxy)pentamidine (**8**); *N*,*N*′-bis(valoxy)pentamidine (**9**). Abbreviations: mitochondrial amidoxime reducing component (mARC); peptidylglycine α-amidating monooxygenase (PAM).

Table 4. Prodrug activation by different enzyme preparations.[a]

Prodrug	Conversion rates [nmol pentamidine (**1**) $min^{-1} mg^{-1}$ protein]			
	PL Ms	PL Mt	PK Ms	PK Mt
2	6.67 ± 0.48	13.57 ± 1.81	20.24 ± 3.04	22.86 ± 4.92
3	0.97 ± 0.07	5.08 ± 0.17	6.43 ± 0.40	4.78 ± 0.16
4	1.16 ± 0.01	0.54 ± 0.10	2.05 ± 0.13	0.10 ± 0.02
6	0.12 ± 0.07	0.20 ± 0.11	0.15 ± 0.05	0.24 ± 0.14
7	0.14 ± 0.01	0.25 ± 0.01	0.34 ± 0.06	0.27 ± 0.02
8	0.79 ± 0.24	1.63 ± 0.03	1.51 ± 0.46	0.41 ± 0.09
9	0.47 ± 0.19	0.97 ± 0.08	0.65 ± 0.22	0.81 ± 0.05
	[nmol pentamidine–diamidoxime (**3**) $min^{-1} mg^{-1}$ protein] PAM			
5	16.63 ± 0.87			

[a] Pig liver microsomes (PL Ms); pig liver mitochondria (PL Mt); pig kidney microsomes (PK Ms); pig kidney mitochondria (PK Mt); peptidylglycine α-amidating monooxygenase (PAM).

est expression level in the CNS and is the enzyme responsible for the activation of prodrug **5**.[14,16] This selective activation pathway is assumed to contribute to a brain targeting.

In summary, all pentamidine derivatives except *N*,*N*′-bis(methoxy)pentamidine **6** appear to be appropriate candidates for use as prodrugs of pentamidine.

Permeability and transport studies

Determination of permeability is important for the prediction of oral bioavailability of potential drug candidates. Permeability studies are often carried out before performing in vivo experiments. Thus, we evaluated our pentamidine prodrugs and compared their permeability with that of pentamidine (**1**). Pentamidine (**1**) and *N*,*N*′-bis(carboxymethoxy)pentamidine **5** show only low permeability. For prodrug **5**, this might be caused by high efflux rates at the apical membrane due to active transport mechanisms, since we observed increased transport rates from basolateral to apical media for **5** in comparison to the rates observed for apical to basolateral. All other prodrugs showed permeability values greater than the parent drug pentamidine (**1**) by factor two to three (Figure 2).

In summary, we were able to show increased permeability for our prodrugs in comparison to the active drug pentamidine (**1**). The highest permeability increase was observed for *N*,*N*′-bis(succinyloxy)pentamidine **8**, which could be seen as the most suitable prodrug of pentamidine so far (Figure 2).

Evaluation of the drug-likeness in vivo

Initial bioavailability studies with **3**, **4**, **5**, **6**, **7**, **8** and **9** in 2 rats showed that *N*,*N*′-bis(dihydroxy)pentamidine **7** and *N*,*N*′-bis(succinyloxy)pentamidine **8** are the most suitable prodrug candidates for pentamidine (data not shown). Thus, we focused on these derivatives in an enlarged study with 33 rats. For details see the Experimental Section.

Oral bioavailability and distribution

Analysis of plasma samples obtained after i.v. administration of pentamidine (**1**) resulted in plasma profiles with a rapid initial

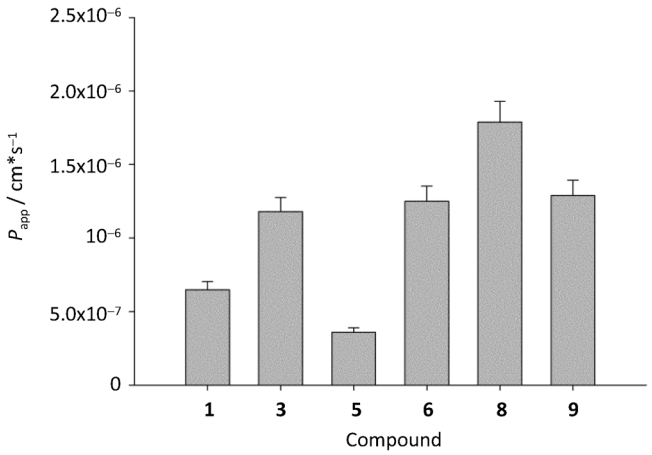


Figure 2. Caco-2 permeability of all compounds examined.

distribution phase and a long terminal half-life of approximately 300 min, which is in accordance with literature data.[27] Unfortunately, analysis of plasma samples after oral administration of either pentamidine prodrugs **7** or **8** at a dose of 50 mg kg^{-1} revealed no detectable concentrations of pentamidine (**1**). Within this study, prodrug **8** was administered in 100 mM phosphate buffer (pH 9.0) to limit the hydrolysis of **8** prior to absorption in the acidic environment of the stomach.

Pentamidine (**1**) is known to accumulate in tissues—predominantly in the liver and kidney.[28] For this reason, we harvested six organs (liver, kidney, spleen, lung, heart, and brain) and analysed them to determine the tissue content of **1**. We were able to detect considerable concentrations of **1** in every tissue indicating an adequate oral bioavailability of both **7** and **8**. As expected, the highest concentrations were found in the liver and kidney, whereas concentrations in spleen, lung, heart, and brain were significantly lower (Figure 3). Unfortunately, the concentrations of **1** in the brain were quite low, ranging from 62 ± 34 ng g^{-1} (prodrug **7**) to 25 ± 14 ng g^{-1} (prodrug **8**). Control studies with **1** given orally were performed, and the results confirmed the poor oral bioavailability of the unmodified drug as detectable amounts of **1** were only found in the liver and kidney. Table 5 shows the calculated relative bioavailability of all compounds tested.

Table 5. Relative bioavailability of both pentamidine prodrugs examined.

Tissue	Bioavailability[a] [%] Compd **1** 10 mg kg^{-1} (i.v.)	Compd **1** 50 mg kg^{-1} (p.o.)	Prodrug **7** 50 mg kg^{-1} (p.o.)	Prodrug **8** 50 mg kg^{-1} (p.o.)
Liver	100	4.5 ± 011	71.7 ± 60.1	97.8 ± 73.7
Kidney	100	1.1 ± 0.9	3.8 ± 2.2	6.2 ± 2.8
Lung	100	n.d.	1.4 ± 1.2	4.9 ± 2.7
Spleen	100	n.d.	3.1 ± 2.2	1.0 ± 1.6
Heart	100	n.d.	5.0 ± 2.3	3.5 ± 1.3
Brain	100	n.d.	1.2 ± 2.5	5.3 ± 4.4

[a] The relative bioavailability was calculated as described in the Experimental Section. n.d. = not detected (< 0.2 %).

While we were able to demonstrate oral bioavailability, due to our study design, we have not examined efficacy so far. Thus, to gain more data, we compared the IC_{50} values of **1** against trypanosomes (0.8–3.2 nM), leishmania (820–2590 nM) and plasmodia (35–129 nM) with the concentrations of **1** detected in the tissues examined.[29–32] To compare our data (ng g^{-1}) with the IC_{50} values from the literature (nM), we converted tissue concentrations into micromolar (μM) values using a hypothetical water content in the tissue of 70 %. In doing so, we obtained tissue concentrations in the micromolar range except in the brain. Thus, oral application of both prodrugs resulted in efficacious concentrations of **1** in all tissues.

In summary, we were able to demonstrate that the prodrug principles used are suitable to increase the oral bioavailability of pentamidine (**1**). Both *N*,*N*′-bis(dihydroxy)pentamidine **7** and *N*,*N*′-bis(succinyloxy)pentamidine **8** are absorbed and activated, yielding efficacious concentrations of pentamidine (**1**).

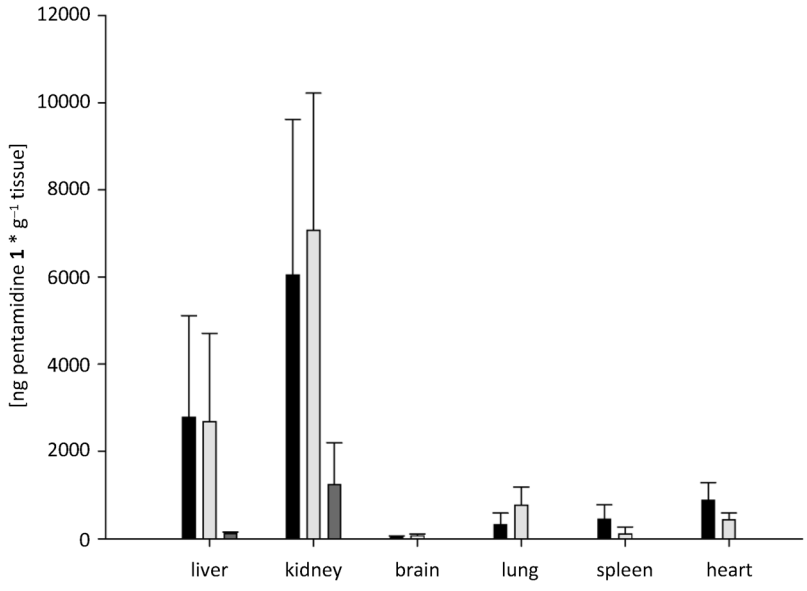


Figure 3. Distribution of pentamidine **1** after oral administration of prodrugs **7** (■), **8** (■) and pentamidine **1** (■). Dosage: prodrugs **7** and **8**: 50 mg kg^{-1}; pentamidine **1**: 10 mg kg^{-1}.

Conclusions

Based on the results obtained from the in silico, in vitro, and in vivo experiments, we were able to evaluate eight pentamidine prodrugs systematically. These results demonstrate that all prodrug principles evaluated

are suitable for application to pentamidine (**1**). Some prodrugs show certain drawbacks that might limit their therapeutic use. For example, prodrugs **3**, **4** and **6** suffer from poor solubility, whereas prodrug **5** shows only low permeability and did not show improved CNS delivery in preliminary tests in rats. In addition, prodrug **6** bears the risk that it may mediate drug–drug interactions due to its activation by cytochrome P450 enzymes.

The *N*,*N*′-bis(succinyloxy)pentamidine **8** was identified as the most appropriate prodrug. It displays the best characteristics with respect to solubility, activation, permeability, and consequently oral bioavailability. The instability at acidic pH is not considered to be a critical factor, because this hydrolysis does not limit the clinical use when administered in a gastro-resistant formulation. However, further studies concerning efficiency against trypanosomiasis and leishmaniasis are needed to obtain more detailed information about the suitability of this prodrug. In this respect, particular interest is set at the ability of *N*,*N*′-bis(succinyloxy)pentamidine **8** to cross the blood–brain barrier, a property that is needed to be efficacious against the second state of African trypanosomiasis. For this reason, efficacy studies of **8** are needed and will be performed in the future. Currently, in vivo imaging studies with radiolabelled pentamidine prodrugs and detection by means of single photon emission computed tomography (SPECT) are currently in progress.

Experimental Section

Synthesis

Reagents: Pentamidine–monoamidoxime **2**, pentamidine–diamidoxime **3**, *N*,*N*′-bis(acetoxy)pentamidine **4**, *N*,*N*′-bis(carboxymethoxy)pentamidine **5** were synthesised according to previously published procedures.[11,14,19] Pentamidine was purchased as the diisethionate salt Pentacarinat 300 (Sanofi–Aventis, Frankfurt, Germany). All other reagents used were obtained commercially in the highest purity available.

General: Melting points were measured on a Büchi 510 Melting Point apparatus and are uncorrected. Infrared (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) are reported in ppm relative to tetramethylsilane (TMS) as an internal standard. All coupling constants (J) were obtained by first-order analysis of the multiplets and are quoted in Hz. The following NMR abbreviations are used: broad (br), singlet (s), doublet (d), triplet (t), quintet (qn), unresolved multiplet (m). Low-resolution electrospray ionisation (ESI) mass spectra (MS) were recorded on a Bruker Esquire LC mass spectrometer. Compounds were dissolved in MeCN or MeOH. High-resolution mass spectrometry (HRMS) was performed on a Bruker FT-ICR APEX II spectrometer using electrospray ionisation. Here, the compounds were dissolved in MeOH. Elemental analyses were performed on a CHNS analyser (HEKAtech GmbH, Wegberg, Germany) at the Department of Inorganic Chemistry, University of Kiel, Germany.

4,4′-Pentamethylendioxy-bis-(*N*-methoxy)benzamidine (*N*,*N*′-bis-(methoxy)pentamidine) (6): Pentamidine–diamidoxime **2** (0.3 g, 0.8 mmol) was dissolved in dioxane (1 mL) and 2 N NaOH (10 mL). After addition of dimethyl sulfate (0.75 mL) in dioxane (1 mL) at 0–5 °C, the mixture was stirred for 2 h at RT. The compound was extracted with ethyl acetate (150 mL) and purified by column chromatography (SiO_2; hexane/ethyl acetate, 4:6) to give compound **6** as a white crystalline powder (96 mg, 30%); mp: 215 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.57 (m, 2H, CH_2), 1.78 (qn, 4H, ^3J = 6.7 Hz, CH_2), 3.72 (s, 6H, CH_3), 3.99 (t, 4H, ^3J = 6.4 Hz, O-CH_2), 5.93 (s, 4H, NH_2), 6.92 (m, 4H, AA′BB′, ArH), 7.58 ppm (m, 4H, AA′BB′, Ar-H); ^{13}C NMR ([D$_6$]DMSO): δ = 22.1 (CH_2), 28.3 (CH_2), 60.4 (CH_3), 67.4 (O-CH_2), 113.9 (ArCH), 124.6 (ArC), 127.0 (ArCH), 150.8 (ArC), 159.5 ppm (C=N); IR (KBr):$\tilde{\nu}$ = 3446, 2936, 1636, 1610, 1518, 1398, 1246, 1050, 836 cm^{-1}; MS (ESI) *m/z*: 401 $[M+H]^+$, 235 $[C_{13}H_{18}N_2O_2+H]^+$, 201 $[M+2H]^{2+}$, 185, 177, 136, 119 $[C_7H_7N_2]^+$; Anal. calcd for $C_{21}H_{28}N_4O_4\cdot1.1H_2O$ (MW = 420.3): C 60.01, H 7.24, N 13.33, found: C 60.02, H 6.99, N 13.14.

4,4′-Pentamethylendioxy-bis-(*N*,*N*′-dihydroxy)benzencarboximidamide (*N*,*N*′-bis(dihydroxy)pentamidine) (7): Hydroxylamine (0.2 g, 6.1 mmol) was dissolved in dry EtOH (15 mL) and treated dropwise with a solution of 4,4′-pentamethylendioxy-bis-(*N*-hydroxybenzencarboximidoylchloride) (0.25 g, 0.6 mmol) in dry Et_2O (15 mL). After stirring overnight at RT, the mixture was filtrated, and the filtrate was concentrated to approximately 5 mL and treated with petroleum ether (5 mL). The first precipitation was discarded. After storage in a refrigerator overnight, the desired compound was isolated as a white, crystalline solid (121 mg, 50%); mp: 129 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.57 (m, 2H, CH_2), 1.79 (qn, 4H, ^3J = 7.3 Hz, CH_2), 4.02 (t, 4H, ^3J = 6.4 Hz, CH_2), 6.91 (m, 4H, AA′BB′, Ar-H), 7.49 (m, 4H, AA′BB′, Ar-H), 8.09 (s, 2H, OH), 8.32 (s, 2H, OH), 10.16 ppm (s, 2H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 22.2 (CH_2), 28.3 (CH_2), 67.4 (O-CH_2), 113.7 (ArCH), 124.0 (ArC), 129.0 (ArCH), 156.6 (ArC), 159.1 (C=N); IR (KBr): $\tilde{\nu}$ = 3324, 2938, 1652, 1520, 1470, 1250, 1176, 986 cm^{-1}; MS (ESI) *m/z*: 405 $[M+H]^+$, 389 $[C_{19}H_{24}N_4O_5+H]^+$; Anal. calcd for $C_{19}H_{24}N_4O_6\cdot0.4H_2O$ (MW = 411.63): C 55.44, H 6.07, N 13.61, found: C 55.55, H 6.27, N 13.32.

4,4′-Pentamethylendioxy-bis-(*N*-carboxypropionyloxy)benzamidine (*N*,*N*′-bis(succinyloxy)pentamidine) (8): Pentamidine–diamidoxime **3** (1 g, 2.7 mmol) was dissolved in acetone (250 mL) and succinic anhydride (540 mg, 5.4 mmol) was added. The mixture was stirred under reflux for 4 h. The solvent was evaporated in vacuo and the precipitate was recrystallised from toluene to give the desired compound as a white crystalline powder (1 g, 68%); mp: 141 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.59 (m, 2H, CH_2), 1.79 (qn, 4H, ^3J = 6.7 Hz, CH_2), 2.52 (t, 4H, ^3J = 6.6 Hz, CH_2), 2.68 (t, 4H, ^3J = 6.6 Hz, CH_2), 4.04 (t, 4H, ^3J = 6.5 Hz, O-CH_2), 6.63 (s, 4H, NH_2), 6.99 (m, 4H, AA′BB′, Ar-H), 7.65 (m, 4H, AA′BB′, Ar-H), 12.18 ppm (br s, 2H, COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 22.1 (CH_2), 27.9 (CH_2), 28.3 (CH_2), 28.8 (CH_2), 67.5 (O-CH_2), 113.9 (ArCH), 123.5 (ArC), 128.1 (ArCH), 156.2 (ArC), 160.3 (C-NH_2), 170.2 (COOR), 173.5 ppm (COOH); IR (KBr): $\tilde{\nu}$ = 3478, 3348, 2940, 2870, 1732, 1698, 1612, 1472, 1250 cm^{-1}; MS (ESI) *m/z*: 573 $[M+H]^+$, 555 $[M-H_2O+H]^+$, 473 $[M-C_4H_4O_3+H]^+$, 455 $[M-C_4H_4O_3-H_2O+H]^+$, 373 $[C_{19}H_{24}N_4O_4+H]^+$, Anal. calcd for $C_{27}H_{32}N_4O_{10}$ (MW = 572.56): C 56.64, H 5.63, N 9.79, found: C 56.85, H 6.01, N 9.60.

Biological evaluation

Calculation of lipophilicity (clog P): The lipophilicity of all compounds was calculated for their uncharged state using ChemBioDraw Ultra (version 11) (CambridgeSoft, Cambridge, MA, USA).

Stability in diverse media: The stability of all prodrugs was tested at a concentration of 200 μM in 50 mM potassium phosphate buffer at pH 2.0, pH 7.4, and pH 9.0 at 20 °C over a time period of

360 min. Every 15 min, a sample (100 μL) was analysed by HPLC to quantify the concentrations of prodrug and degradation products. Additional incubations were performed with all prodrugs at a concentration of 200 μM in human and murine plasma for 150 min at 37 °C. Every 15 min, a sample (100 μL) was taken and treated with MeCN (100 μL). After centrifugation at 10000 *g* for 10 min, the supernatant was analysed by HPLC and the concentrations of the prodrug and metabolites were measured. Furthermore, prodrugs **4**, **8**, and **9** were incubated at a concentration of 100 μM with unspecific carboxylic esterase (1 U) from pig liver (Sigma–Aldrich, Taufkirchen, Germany) at 37 °C in 50 mM potassium phosphate buffer (pH 7.4) for 60 min. Every 15 min, samples (100 μL) were analysed by HPLC.

Determination of solubility: An insoluble amount (5 mg) of every prodrug was shaken in 50 mM potassium phosphate buffer (300 μL) at pH 7.4 or pH 9.0, or 0.01 N HCl (pH 2.0) for 60 min. After centrifugation at 13000 *g* for 10 min, the supernatant was analysed by HPLC to quantify the concentration of prodrug dissolved.

Incubations with liver enzyme preparations: Human and porcine mitochondria and microsomes from liver and kidney were incubated at 37 °C in a mixture containing prodrug (500 μM), NADH (1 mM) and protein (0.3 mg) in 50 mM potassium phosphate buffer (150 μL) at pH 6.3 over a period of 30 min. Moreover, incubations of prodrugs **4**, **8**, and **9** contained carboxylic esterase (1 U) from pig liver (Sigma–Aldrich, Taufkirchen, Germany), and those performed with prodrug **6** contained an addition amount of NADPH (1 mM). Afterwards, reactions were terminated by addition of MeCN (150 μL). Samples were shaken for 10 min, proteins were sedimented by centrifugation (10000 *g*, 10 min), and supernatants were analysed by HPLC.

Incubations with peptidylglycine α-amidating monooxygenase (PAM): Incubations were performed in analogy to those described previously by Schade et al.[14]

Determination of protein binding: The protein binding of each prodrug was examined at three different concentrations (10 μM, 25 μM, and 50 μM) by ultrafiltration. The prodrugs were solved in 50 mM potassium phosphate buffer (pH 7.4) with 4% albumin, incubated for 15 min and centrifuged using Microcon centrifugal filter units YM 30 (Millipore, Billerica, MA, USA) at 13000 *g* for 10 min. Control samples were treated analogously but contained no albumin. The filtrate was analysed by HPLC. Protein binding data reported in Table 3 represent the mean value of the determinations at the three different concentrations. Due to poor solubility, prodrug **3** was examined at a concentration of 10 μM only.

Cell culture and transport studies: The human colon adenocarcinoma cell line, Caco-2 was purchased at passage 40 from the European Collection of Cell Cultures (ECACC), Salisbury, UK. Cultured monolayers of Caco-2 cells were used in transport experiments between passages 45–60. The cells were cultured at 37 °C in 5% CO_2 and maintained in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™ (Life Technologies, Darmstadt, Germany), D-glucose (4500 mg L^{-1}), foetal calf serum (FCS; 10%), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and glutamine (1.5 mg mL^{-1}) but without sodium pyruvate. Stock cultures were grown in 75 cm^2 tissue culture flasks and were split 1:6 at 80–90% confluency using ethylenediaminetetraacetic acid (EDTA; 0.02%) and trypsin (0.05%). Cells were seeded at 200000 cells per cm^2 on polyethylene terephtalate membrane inserts (1.13 cm^2, 2×10^6 pores per cm^2, 0.4 μm pore size) and cultured for 21 days. The medium was changed every two to three days and on the day before the experiment.

The integrity of the monolayers was evaluated by means of transepithelial electrical resistance (TEER) measurements. Only monolayers with a TEER value of at least 250 $\Omega\times cm^{-2}$ were used for transport experiments. The transport of lucifer yellow (100 μg mL^{-1}) across the monolayers was measured after the experiment for 1 h. Only monolayers with lucifer yellow passage $\leq$1% were analysed. Transport experiments were carried out in apical medium (2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM; Hank's buffered salt solution (HBSS, 10×), 100 mL; twice-distilled H_2O, 900 mL) and basolateral medium (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM; D-(+)-glucose, 25 mM; HBSS (10×), 100 mL; twice-distilled H_2O, 900 mL L. The monolayers were washed twice and then preincubated for 15 min. After TEER measurement, the experiment was started by adding 600 μL of medium containing the test compound. The inserts were then transferred to a new cell culture plate containing 1500 μL medium per well. An aliquot of apical medium (100 μL) was removed immediately to determine the t_0 concentration. After 10, 25, 45, 70, 120 min, the inserts were moved to cell culture plates containing fresh medium. The medium was analysed by HPLC. Permeability was calculated by the following equation: $P_{app} = (dc/dt)/A\times c_{0(donor)}$. Compounds **2**, **4**, and **7** were not tested in these studies.

Animals: Male Sprague Dawley (SD) rats (Charles River Laboratories, Sulzfeld, Germany) were used. The study was conducted according to the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and authorised by the local regulatory authority (Ministerium für Landwirtschaft, Umwelt and ländliche Räume des Bundeslandes Schleswig–Holstein, Germany). The animals were kept at room temperature with a 12/12 h dark (8 AM–8 PM)/light (8 PM–8 AM) cycle. They received a standard diet and water ad libitum. Rats (398 ± 20 g) were habituated to research assistants and vice versa two weeks before drug treatment was initiated. Rats were randomised into groups receiving either pentamidine **1** i.v. ($n=10$), pentamidine **1** p.o. ($n=3$), prodrug **7** or **8** both p.o. ($n=10$).

Surgery: Two days before the pharmacokinetic study, chronic polyethylene catheters were inserted into the right femoral vein and artery while the animals were under pentobarbitone anaesthesia (67 mg kg^{-1}). Catheters were tunnelled under the back skin, exteriorised in the region of the cervical vertebra, and fixed at the skin. Thereafter, rats were housed individually in cages (height × width × length: 20 × 22 × 25 cm) until the end of the study.

Animal study: The oral bioavailability of prodrugs **7** and **8** was investigated in a study with 33 rats. Both compounds were suspended in a solution of gum arabic (10%) dissolved in 100 mM phosphate buffer and orally administered by gavage to rats at a dose of 50 mg kg^{-1} ($n=10$). Due to the rapid hydrolysis of compound **8**, the solution was adjusted to pH 9.0 to increase the gastric pH and minimise the fraction of hydrolysed prodrug. Moreover, pentamidine **1** (50 mg kg^{-1}) was given orally by gavage to a control group of rats ($n=3$). Blood samples were taken with microvettes (Sarstedt, Nümbrecht, Germany) after 20, 40, 60, 90, 120, 240 and 360 min via an arterial implanted catheter. Pentamidine **1** was given intravenously to rats at a dose of 10 mg kg^{-1} ($n=10$). Blood samples of approximately 300 μL were collected 5, 10, 20, 40, 75, 150, 300 min after intravenous dosing. Plasma samples were obtained by centrifugation at 10000 *g* for 5 min and were immediately frozen at −80 °C. Animals were euthanised by decapitation 360 min after application of the test compounds, and the following organs were harvested: liver, kidney, lung, spleen, heart, and brain. The tissues were frozen immediately and stored at −80 °C.

Preparation of plasma and tissue samples: Plasma samples were treated with equal volumes of MeCN. Samples were then shaken for 45 min and centrifuged at 10000 *g* for 15 min. The supernatant was analysed by HPLC. Tissue samples were thawed to room temperature and cut into pieces weighing approximately 1000 mg (liver) or 500 mg (other tissues). The tissue samples were homogenised in water (2 mL) and then treated with MeCN (2 mL) to precipitate proteins. After shaking for 45 min, the samples were centrifuged at 12000 *g* for 15 min. The supernatants were dried with compressed air and lyophilised overnight. The residues were resuspended in MeOH/water (50:50, 400 μL), shaken for 1.5 h, and centrifuged at 12000 *g* for 15 min. The supernatant was analysed by HPLC.

Determination of the relative bioavailability: In general, oral bioavailability is calculated from the plasma concentration versus time plot for a drug after both intravenous and oral administration. Due to the high protein binding of pentamidine **1** and its tendency to accumulate in tissues, no plasma concentrations of pentamidine **1** were detectable after oral administration of either prodrug examined. In order to compare the prodrugs, we used the pentamidine concentrations detected in the different tissues and defined the concentration obtained after intravenous application of pentamidine as 100%. The relative bioavailability was calculated by means of the pentamidine concentrations measured after oral treatment with prodrug and that obtained after intravenous application of pentamidine.

HPLC methods

General: Columns were purchased from Merck KGaA, Darmstadt, Germany. Analyses 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.

Stability and solubility: Separations were performed on a LiChroCart LiChrospher 60 RP-select B column (125×4 mm, 5 μm) with a mobile phase of H_2O + 0.1% trifluoroacetic acid (TFA) (pH 2.5) and MeCN. The flow rate was set to 1 mL min^{-1}, the column temperature was kept at 25 °C, and the injection volume was 10 μL. The percentage MeCN varied depending on the particular derivative: (**1**) 20% MeCN, $t_R = 3.4 \pm 0.2$ min; (**2**) 20% MeCN, $t_R = 3.2 \pm 0.2$ min; (**3**) 40% MeCN, $t_R = 3.0 \pm 0.2$ min; (**4**) 40% MeCN, $t_R = 5.1 \pm 0.2$ min; (**5**) 25% MeCN, $t_R = 5.8 \pm 0.2$ min; (**6**) 25% MeCN, $t_R = 3.6 \pm 0.2$ min; (**7**) 30% MeCN, $t_R = 3.4 \pm 0.2$ min; (**8**) 20% MeCN, $t_R = 4.4 \pm 0.2$ min; (**9**) 30% MeCN, $t_R = 7.1 \pm 0.3$ min.

Activation: Separations were performed on a LiChroCart LiCHrospher 60 Rp-select B column (125×4 mm, 5 μm) with a mobile phase of H_2O + octylsulfonate (10 mM) + tetramethylammonium chloride (20 mM) (pH 3.0) and MeOH (52:48). The flow rate was set to 1 mL min^{-1}, the column temperature was maintained at 25 °C, and the injection volume was 20 μL. Pentamidine **1** eluted at $t_R = 10.7 \pm 0.4$ min.

In vivo study: Pentamidine was detected by RP-HPLC. Separations were performed on a Superspher 60 RP-select B column (250× 3 mm, 5 μm) with a mixture of H_2O + 0.1% TFA (pH 2.5) and MeOH (60:40). The flow rate was set to 0.32 mL min^{-1}, and the injection volume was 35 μL. Pentamidine **1** eluted at $t_R = 24.7 \pm 0.5$ min.

Acknowledgements

We thank Franziska Hohm (Pharmaceutical Institute, University of Kiel) for excellent technical assistance. The study described here arose from a larger project within the scope of a patent value fund. In this context, we acknowledge the financial support provided by the Dritte Patentportfolio Beteiligungsgesellschaft GmbH & Co. KG.

Keywords: amidoximes · oral bioavailability · pentamidines · prodrugs · trypanosomiasis

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Received: September 6, 2011
Published online on October 7, 2011

