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Evaluation of bisbenzamidines as inhibitors for matriptase-2

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ABSTRACT

The serine protease matriptase-2 has attracted much attention as a potential target for the treatment of iron overload diseases. In this study, a series of 27 symmetric, achiral bisbenzamidines was evaluated for inhibitory activity against human matriptase-2, against the closely related enzyme human matriptase, as well as against human thrombin, bovine factor Xa and human trypsin. The conformationally restricted piperazine derivative **19** and the oxamide-derived bisbenzamidine **1** were identified as the most potent inhibitors of this series for matriptase-2 and matriptase, respectively.

Matriptase-2 is a multi-domain enzyme, first described in 2002 as a type II transmembrane serine protease and predominantly expressed in the liver. Matriptase-2 shares the common features of type II transmembrane serine proteases, a cytoplasmic N-terminal domain, a transmembrane domain, a stem region consisting of various domains, in this case a SEA domain, two CUB domains, three LDLRA domains and a C-terminal serine protease domain.[1] Physiologically, matriptase-2 was identified to be a key regulator of iron homeostasis since mutations in the corresponding *TMPRSS6* gene have been shown to cause iron refractory iron deficiency anemia.[2] It has been demonstrated that matriptase-2 suppresses hepcidin and thereby increases plasma iron levels via BMP/SMAD signaling, by cleaving the BMP co-receptor hemojuvelin.[3] Hepcidin reduces ferroportin, which facilitates iron export from macrophages and enterocytes. If matriptase-2 is inhibited and thus hemojuvelin cleavage is abrogated, the BMP/SMAD cascade is activated, hepcidin is induced and, as a consequence, plasma iron levels are down-regulated. Therefore, matriptase-2 becomes an attractive new target to correct a lack in hepcidin which is manifested in iron-related disorders, for example in hemochromatosis or β-thalassemia. While primary iron overload can be treated with phlebotomy as standard therapy, treatment of secondary iron overload such as in case of β-thalassemia is more challenging. β-Thalassemia is a genetic disorder with an increased but ineffective erythropoiesis resulting in iron overload, either from the disease itself or from blood transfusions that are necessary to balance the abnormal formation of hemoglobin.[4]

In the current therapy of β-thalassemia, iron chelators are applied to remove excess iron. However, their use is limited by compliance issues; the well-established drug deferoxamine, for example, needs to be administered by subcutaneous injection over several hours almost daily.[5] For the treatment of secondary iron overload, inhibition of matriptase-2 could be an alternative approach, for which support has been provided by mouse studies when crossing *Tmprss6*$^{-/-}$ mice with models of HFE-related hemochromatosis,[6] or β-thalassemia.[7] The usage of *Tmprss6* small interfering RNA,[8] or *Tmprss6* specific antisense oligonucleotides in these mouse models also provided evidence that targeting matriptase-2 decreases iron overload.[9]

Synthetic inhibitors of matriptase-2 should be useful for the future treatment of iron overload and, moreover, constitute valuable tool compounds for pharmacological and biochemical investigations. A comparative three-dimensional model of the catalytic domain of matriptase-2 was used previously for the identification of the first small molecule inhibitors of matriptase-2,[10] and representatives of different chemotypes have been reported as matriptase-2 inhibitors.[11–14] Interaction of matriptase-2 with proteinaceous inhibitors has also been examined, such as with sunflower trypsin inhibitor analogues and Kunitz-type inhibitors HAI-1 and HAI-2.[15]

Bisbenzamidines exhibit a broad spectrum of antiprotozoal and antifungal activities. Pentamidine, a *para*-oriented bisbenzamidine with a $O(CH_2)_5O$ linker was introduced as a trypanocidal agent and is still in clinical use for the treatment of sleeping sickness, visceral

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leishmaniasis and *Pneumocystis* pneumonia. The activity of pentamidine and several related linker-connected bisbenzamidines is basically attributed to their strong affinity for adenine/thymine-rich sites of the double-strand DNA by insertion into the minor groove.[16] The DNA-dependent fluorescent properties of certain bisbenzamidines have also been discovered.[17] Besides their importance for the treatment of parasitic infections, bisbenzamidines have gained attraction as enzyme inhibitors for trypsin-like proteases.[12,18–22]

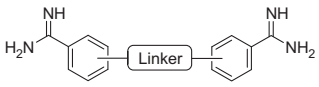
In the present study, we have generated a focused library of bisbenzamidines for an evaluation of their matriptase-2 inhibiting activities.

Compounds **1**–**12** (Table 1) contain different linkers all of which are connected to the terminal benzamidine moieties via an amide bond. Several of these derivatives have been investigated for their anti-trypanosomal and anti-*Pneumocystis* activities. In particular, the adipic diamide **4** has been found to be exceptionally potent in vitro against *Pneumocystis carinii* and *Trypanosoma brucei*.[23,24] In compounds **11**–**16** (Table 1), a central phenylene core is connected via amide or oxymethylene groups with both benzamidine moieties.

To evaluate the inhibitory activity against human matriptase-2, a screening of the bisbenzamidines at a single inhibitor concentration was carried out and a fluorogenic peptide substrate was applied.[27] The medium of transfected HEK cells was used as the source of human matriptase-2.[12–14,28] Most of the compounds listed in Table 1 are only moderately active. The insertion of alkylidene chains into the oxamide substructure of **1** led to a loss of activity in compounds **2**–**10**. Besides **1**, bisbenzamidines **14**–**16** showed more than 80% inhibition at 40 μM. An inversion of the oxymethylene units (**14** vs **13**) reduced the inhibitory ability.

Compounds with α,ω-diamine linker structures are listed in Table 2. Such derivatives have also been described as trypanocidal agents. The conformationally restricted piperazine and homopiperazine derivatives **19** and **20** displayed particularly strong activity against different trypanosome isolates.[29] Moreover, relatives of **19** with *N*-(cyclo)alkyl substituents at both amidino moieties were potent against *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense*.[30] Besides their antiparasitic properties, most of the compounds of our study have already been evaluated as inhibitors of the NMDA receptor.[26]

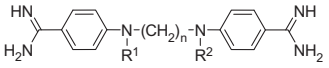
Table 1
Matriptase-2 inhibition by compounds **1**–**16**



Compd[a]	Linker	Position[b]	Residual activity @ 40 μM[c] (%)
1	NHCOCONH	*para*	17
2	$NHCO(CH_2)_2CONH$	*para*	40
3	$NHCO(CH_2)_3CONH$	*para*	68
4	$NHCO(CH_2)_4CONH$	*para*	57
5	$NHCO(CH_2)_4CONH$	*meta/para*	78
6	$NHCO(CH_2)_5CONH$	*para*	63
7	$NHCO(CH_2)_6CONH$	*para*	62
8	$NHCO(CH_2)_7CONH$	*para*	49
9	$NHCO(CH_2)_8CONH$	*para*	33
10	$NHCO(CH_2)_{10}CONH$	*para*	49
11	NHCO-*m*-C_6H_4-CONH	*para*	32
12	NHCO-*p*-C_6H_4-CONH	*para*	>50[d]
13	CH_2O-*o*-C_6H_4-OCH_2	*para*	46
14	OCH_2-*o*-C_6H_4-CH_2O	*para*	19
15	OCH_2-*m*-C_6H_4-CH_2O	*para*	17
16	OCH_2-*p*-C_6H_4-CH_2O	*para*	16

[a] Compounds were available from previous studies.[23–26]
[b] Position of the linker relative to the amidino group.
[c] Inhibitor stock solutions were prepared in DMSO. Reactions were monitored in 96 well plates on a FLUOstar Optima fluorimeter with an excitation wavelength of 340 nm and emission wavelength of 460 nm.
[d] Limited solubility. At a concentration of 5 μM, 93% residual activity was observed.

Table 2
Matriptase-2 inhibition by compounds **17**–**27**



Compd	R^1	R^2	*n*	Residual activity @ 40 μM[a] (%)
17	H	H	2	23
18	H	Me	2	21
19	$(CH_2)_2$		2	5.3
20	$(CH_2)_3$		2	15
21[b]	$(CH_2)_3$		2	45
22	H	H	3	55
23	Me	Me	3	23
24	H	H	4	27
25	H	H	6	21
26	H	H	7	16
27	H	H	8	8.6

[a] Inhibitor stock solutions were prepared in DMSO. Reactions were monitored in 96 well plates on a FLUOstar Optima fluorimeter with an excitation wavelength of 340 nm and emission wavelength of 460 nm.
[b] Compound **21** contains a *N*-cyclopropyl residue at both amidine moieties.

In general, this subseries of bisbenzamidines (**17**–**27**) exhibited a somewhat stronger inhibition of human matriptase-2. The conformationally restricted derivatives **19** and **20** were potent inhibitors, whereas the introduction of a *N*-cyclopropyl substituent at both amidino moieties diminished the inhibitory activity (**20** vs **21**). Increasing lengths of the alkylidene chains beyond $n = 2$ improved the protease inhibition (compare **22**, **24**, **25**, **26** and **27**). We selected the oxamide derivative **1**, the phenylene core-containing compounds **14** and **16**, the (homo)piperazine derivatives **19** and **20**, as well as **26** and **27** with a hepta- and octamethylene chain for further investigations.

For the seven selected bisbenzamidines, K_i values of matriptase-2 inhibition were determined (Table 3). We confirmed the screening results and identified bisbenzamidines **19** and **27** as the most potent inhibitors of human matriptase-2 exhibiting K_i values of less than 2 μM. Activity against related serine proteases was also examined and inhibitory profiles were determined. The progress curves were linear in all cases, indicating that inhibition was not time-dependent, in accordance with the expected reversible mode of interaction of the bisbenzamidines with trypsin-like enzymes.

The eponymous enzyme of the matriptase subfamily is an epithelia-specific, membrane-anchored type II serine protease. Due to its critical involvement in tumorigenesis, matriptase is considered a potential therapeutic target.[31,32] As in case of matriptase-2, we have used Boc-Gln-Ala-Arg-AMC as fluorogenic substrate for human matriptase.[33] The K_m value was determined to be 32.3 ± 3.5 μM. Noteworthy, the seven compounds inhibited matriptase to a greater extent. The oxamide derivative **1** showed a K_i value of around 500 nM and certain selectivity for matriptase over matriptase-2, thrombin and factor Xa.

With **14** and **16**, we also identified potent inhibitors of the coagulation proteases human thrombin and bovine factor Xa, the former being moderately selective for thrombin over matriptase-2, matriptase and factor Xa.[34,35] All of the bisbenzamidines listed in Table 3 also inhibited human trypsin.[36] However, when used as pharmacological tools, the accompanying trypsin inhibition might not be a critical feature because of the different tissue distribution.

The potency of dibasic compounds, in particular bisbenzamidines, against trypsin-like serine proteases reflects the substrate