Dipeptidyl Nitroalkenes as Potent Reversible Inhibitors of Cysteine Proteases Rhodesain and Cruzain

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S Supporting Information

ABSTRACT: Dipeptidyl nitroalkenes are potent reversible inhibitors of cysteine proteases. Inhibitor **11** resulted to be the most potent one with K_i values of 0.49 and 0.44 nM against rhodesain and cruzain, respectively. According to enzymatic dilution and dialysis experiments, as well as computational and NMR studies, dipeptidyl nitroalkenes are tightly binding covalent reversible inhibitors.

KEYWORDS: Rhodesain, cruzain, inhibitors, Chagas' disease, sleeping sickness

The target proteases of the presented study, rhodesain and cruzain, are parasite proteases, which belong to the papain-family of cysteine proteases.^{1,2} They are related to human cathepsins.^{1,2} Rhodesain is expressed by the protozoa *Trypanosoma brucei rhodesiense*, which causes the African sleeping sickness.^{3,6,7} Cruzain is expressed by *T. cruzi*, the parasite causing Chagas' disease occurring in South and Central America.^{4,5} Both proteases are essential for the life cycles of the pathogens. Consequently, their inhibition is an important strategy for the treatment of these diseases.^{8,9}

K11777 (Figure 1a), a dipeptidyl vinyl sulfone, irreversibly inactivates cysteine proteases¹⁰ by conjugate addition of the thiolate of the cysteine at the active site to the double bond. The resulting carbanion is subsequently protonated driving the process thermodynamically to the more stable enzyme–inhibitor complex (Figure 1b).

Irreversible inhibitors can give rise to undesired side reactions. Turning dipeptidyl vinyl sulfones into compounds that reversibly react with thiols by introducing thioethers or halogen atoms into the structure has been reported.¹¹ In case of the halogenated vinylsulfones this leads to covalent reversible inhibition.¹²

We envisioned dipeptidyl nitroalkenes as inhibitors for cysteine proteases. Based upon the inhibition mechanism of

Figure 1. (a) Structures of vinyl sulfone K11777 and a dipeptidyl nitroalkene. (b) Inhibition mechanism by vinyl sulfones.

Received: July 15, 2016

Accepted: September 21, 2016

Published: September 21, 2016

vinyl sulfones, protonation of the carbanion might be less favored in the case of the arising nitroalkane carbanion since the acidity of the corresponding acid, namely, the nitroalkane, is higher, and thus, the basicity of the carbanion is lower (Figure 1b).¹³

Dipeptidyl nitroalkenes were prepared through a short and efficient synthetic route. The first step of the synthesis was a nitroaldol reaction between the corresponding N-protected amino aldehyde and nitromethane (or nitroethane) which afforded a mixture of nitroaldols 1–5. The next step was the deprotection and coupling with the corresponding N-protected amino acid giving rise to compounds 6–10. Finally dehydration of dipeptidyl nitroaldols through methanesulfonates yielded the desired dipeptidyl nitroalkenes 11–20 (Scheme 1).¹⁴

Scheme 1. Synthesis of Dipeptidyl Nitroalkenes

^aReagents and conditions: (a) nitromethane or nitroethane, triethylamine, CH₂Cl₂, 0 °C to rt, overnight, 85–95%; (b) TFA, CH₂Cl₂, 0 °C, 30 min then DIPEA, HOBT, EDC, CbzPhe or CbzLeu, 69–86%; (c) methanesulfonyl chloride, DIPEA, CH₂Cl₂, 0 °C to rt, overnight, 64–93%.

The dipeptidyl nitroalkenes 11–20 were screened against rhodesain and cruzain (Table 1), and K_i determinations showed

Table 1. Inhibition Data for Compounds 11–20 (K_i in nM)

inhibitor	rhodesain (RD)	cruzain (CRZ)	cathepsin B (CB)	cathepsin L (CL)
11	0.49	0.44	8	11
12	6.5	16	68	30
13	29	130	310	110
14	4.2	11	34	19.6
15	50	ND ^a	6300	280
16	30	130	310	200
17	18.4	25	210	46
18	18	28	710	49
19	14	17	72	27
20	544	ND	3200	2020
K11777 ^b	20	ND	390	80

^aND = Not determined. ^bk_{second} values [M⁻¹ s⁻¹] for inhibition by K11777: RD: 6.6 × 10⁵; CL: 9.2 × 10⁵; CB: 2.3 × 10⁴.

that the compounds are potent reversible inhibitors of these enzymes. Inhibitory activity was dependent on the peptidic framework. Compound 11 with L-alanine at the P1 position and L-phenylalanine at P2 proved to be the most potent inhibitor displaying a K_i in the picomolar range. Compounds 15 and 20, having a methyl group at the α-position of the double bond, were considerably less active. Compounds were also tested against human cysteine proteases cathepsins B and L, also

belonging to the papain-family (Table 1). In all cases K_i values were one or two orders of magnitude higher compared with the parasitic enzymes.

To confirm the reversibility of inhibition, dilution assays (see Supporting Information) with inhibitors 11, 12, 18, 17 (CL), 16, and 17 (CB), as well as dialysis assays (Figure 2) with

Figure 2. Experimental verification of the reversibility of the inhibition via dialysis assays. Enzyme and inhibitor (final concentration 1 μM) were incubated for 5 min, and the reaction mixture was subjected to dialysis with assay buffer using our published dialysis apparatus.¹⁵ The residual enzyme activity was measured after 30, 60, 90, and 120 min by adding the substrate (final concentration 10 μM).

compounds 12, 17, and 19 (RD) were performed. The assays revealed that the enzymes' activities recovered quite fast in the case of compounds 18 and 19, while slower regeneration of the enzyme activities was found with 11, 12, 16, and 17.

The fact that the dipeptidyl nitroalkenes are reversible inhibitors may have several reasons: first, it could be a consequence of the orientation of the warhead within the active site, which would prevent nucleophilic attack at the double bond. However, this stands in contrast to the high inhibition potencies of the compounds, which can hardly be explained by an only noncovalent binding of the recognition units to the binding pockets. Additionally, the docking experiments indicate that a reaction, i.e., a nucleophilic attack at the double bond, should be possible. The reversibility could also result from a weaker exothermicity of the covalent inhibition step enabling the back reaction. To test this possibility we computed the reaction energy of the addition reaction with the model warheads (CH₃)HCβ=Cα(NO₂)R. We computed both the reaction for R=H and R=CH₃ in order to investigate if the weaker inhibition potencies of the compounds 15 and 20 with R=CH₃ are due to differences in the reaction energies of the covalent inhibition step. Even if the docking investigations (see below) indicate that the addition of the thiolate group of Cys25 takes place at C_β while the proton is transferred to C_α, we computed both possibilities, i.e., attack of the thiolate at C_α and C_β (see Supporting Information). The reaction energies were obtained from B3LYP calculations^{16,17} in combination with the cc-pVTZ¹⁸ basis set. Possible influences of a polarizable environment were tested by additional COSMO computations employing ε = 78.39.¹⁹ The computations were performed with the TURBOMOLE program package.²⁰ The computations for a polar environment predict that the reaction energies for α- and β-attack are only about −12 kcal/mol for R=H. This indeed indicates that the addition reaction is reversible. The corresponding value for K11777 is far below −20 kcal/mol. For R=CH₃ the reaction is even less exothermic (−10 and −7

kcal/mol for the sulfur attack at C_β and C_ω respectively). This small difference compared to $R=H$ indicates that the weaker inhibition potencies of compounds **15** and **20** do not solely stem from the less exothermic reaction energy, but should mainly result from steric effects.

This is underlined by the docking studies that were performed with inhibitors **11**, **14**, and **15**. For the docking studies we used the X-ray structure of rhodesain in complex with K11777 as template (pdb 2P7U) and performed noncovalent (FlexX/LeadIT)²¹ as well as covalent (DOCK-TITE)²² docking. The results show that the dipeptidyl nitroalkenes should bind in a comparable manner as the corresponding vinylsulfones. Similar hydrogen bonds should be formed. Notably the key interactions of the warhead to the enzyme (Gln19, Trp184) are nearly identical (Figure 3). The

Figure 3. Proposed binding geometry and hydrogen bonding for inhibitor **11**: within the noncovalent enzyme–inhibitor complex (above) and within the covalent enzyme–inhibitor complex (below).

vinyl β -carbon atom comes in close proximity to the Cys25 sulfur (2.77–2.99 Å). The docking scores (Hyde score, kJ/mol/FlexX score: –22/–24.06 (**11**), –19/–21.39 (**14**), –11/–23.34 (**15**)) reveal that inhibitor **11** should form the most favorable noncovalent interactions with the enzyme, which is in accordance with the experimental results (Figure 3, see also Supporting Information).

¹⁵N-Rhodesain was expressed (see Supporting Information) and ¹H–¹⁵N-TROSY-HSQC spectra were recorded to measure the interaction of rhodesain with inhibitor **12**. First a 1:1 mol/mol mixture (protein/inhibitor) was incubated for 15 min and measured. Then the inhibitor **12** concentration was increased to a 1:3 molar ratio (rhodesain/inhibitor) by adding **12** in DMSO-*d*₆ and further incubation for 7 min before measurement. As observed in the spectra (Figure 4), full saturation of **12** binding to rhodesain is already achieved at a 1:1 molar ratio, which denotes inhibitor **12** to bind very tightly to rhodesain (Figure 4).

The activity of the most potent rhodesain inhibitors **11**, **12**, and **14** against *T. brucei brucei* was also determined (Table 2).^{23,24} All three compounds were found to exhibit good

Figure 4. ¹⁵N NMR spectra of rhodesain in complex with inhibitor (**12**); rhodesain (black), 1:1 molar ratio (orange), and 1:3 molar ratio (blue).

Table 2. Antitrypanosomal Activity against *Trypanosoma brucei brucei* (EC₅₀ in μ M)

inhibitor	EC ₅₀ (24 h)	EC ₅₀ (48 h)	EC ₅₀ (72 h)
11	5.64	7.17	10.82
12	0.99	1.31	2.06
14	1.62	2.10	3.45
chlorhexidine ^b	0.43	0.41	0.43

^aValues are mean values from two independent assays performed each in triplicate; the values of all experiments incl. standard deviations are given in the SI. ^bUsed as a control.

antitrypanosomal activity in the low micromolar range. The increasing EC₅₀ values during time may be a result of instability of the compounds in the medium after >24 h.

In summary, we report dipeptidyl nitroalkenes as a new type of highly potent covalent reversible inhibitors of cysteine proteases. The compounds exhibit certain selectivity for rhodesain and cruzain. According to the computations (Docking, QM), the reversibility of the inhibition, which is a difference to the related vinylsulfones, is a result of a weaker exothermicity and is not due to a different binding mode preventing nucleophilic attack of the active site Cys residue at the activated double bond.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00276.

Experimental procedures for the preparation of inhibitors, spectroscopic data, enzyme assays, antitrypanosomal activity, computational studies and protein NMR (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

We thank Fundación Española para la Ciencia y la Tecnología (Fecyt) and Generalitat Valenciana (AICO/2016/32) for financial support. T S. and B.E. thank the DFG (Deutsche Forschungsgemeinschaft) in the framework of the SFB630 for financial support. We thank Universitat Jaume I for technical support and funding. U.A.H. acknowledges support by the Carl-Zeiss foundation as well as the Center of Biomolecular Magnetic Resonance (BMRZ) funded by the state of Hesse.

Notes

The authors declare no competing financial interest.

■ DEDICATION

We dedicate this work to Prof. Dr. Gerhard Bringmann, Institute of Organic Chemistry, University of Würzburg, Germany, on the occasion of his 65th birthday.

■ ABBREVIATIONS

RD, rhodesain; CRZ, cruzain; CB, cathepsin B; CL, cathepsin L

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