Malaria and African sleeping sickness (human African trypano-
somiasis), caused by the protozoa Plasmodium falcipa-
rum and Trypanosoma brucei, respectively, are among the most
severe tropical diseases and represent major health issues in
the developing world.[1,2] Approximately 250 million malaria in-
fecions and 1 million deaths are registered annually, with up
to 70% of the clinical cases attributed to P. falciparum concen-
trated within the African region.[1,3] Human African trypanoso-
miasis threatens millions of people in about 20 sub-Saharan
countries in Africa, with an estimated annual number of cases
between 50000 and 70000 and an annual mortality close to
25000.[4] The emergence of multidrug-resistant parasite strains,
in addition to limited available chemotherapies, demand the
urgent development of new and effective drugs with novel
mechanisms of actions. P. falciparum and T. brucei offer several
potential target enzymes that are implicated in pathogenesis
and host cell invasion, including a number of essential and
closely related cysteine proteases.[5] The largest subfamily
among them are the papain-like cysteine proteases (clan CA,
family C1).

In P. falciparum, various proteases catalyze the degradation
of human hemoglobin to provide nutrients for exponential
growth and maturation of the pathogen.[6] Hemoglobin cata-
bolism is initiated by a series of aspartic proteases, the plas-
mpsins, and a histo-aspartic protease to perform the first pro-
teolytic cleavage. Further degradation into smaller peptidic
fragments is achieved by unique food-vacuole cysteine pro-
teases, the falcipains.[7] Biological studies revealed overlapping
activity profiles of plasmepsins and falcipains, and it was fur-
ther concluded that plasmpsins are processed and activated
by falcipains.[8,9] This catalytic process is both vital and specific
for parasitic survival. Inhibition of falcipains has proven indis-

Other work suggested that the S2 pocket is the key determinant
of substrate specificity in papain-like cysteine proteases.[24] The
general structure of cysteine protease inhibitors con-
tains prevalently an electrophilic moiety to form a reversible,
covalent thioimidate intermediate with the catalytic cysteine.
We opted, specifically, for inhibitors featuring a nitrile residue
as the electrophilic head group. More than 30 nitrile-contain-
ing pharmaceuticals are prescribed for a variety of medicinal
indications, and several are in clinical development.[25,26] Unsur-
prisingly, nitriles are a well established class of cysteine pro-
tease inhibitors.[27,28] Oballa et al. hypothesized that the in-
creased electrophilicity of the nitrile moiety could impact the
reversibility of enzyme–inhibitor complex formation.[29] Accord-
ing to their calculated reactivities, aryl nitriles, particularly pyri-
midine and triazine nitriles, should possess the most reactive nitrile moieties.

Herein, we describe the structure-based design, efficient synthesis, and biological evaluation of a new series of triazine nitrile inhibitors to explore the binding properties of falcipain-2 and rhodesain. Guided by molecular modeling, we propose a binding model showing the accommodation of the different vectors in the apolar pockets of the active site. The inhibitors were tested against closely related human and viral cysteine proteases, as well as a serine protease, to investigate their general selectivity. Additionally, in vitro activity against \textit{P. falciparum} and \textit{T. brucei rhodesiense} parasites and cytotoxicity was studied.

Computer-aided modeling using the MAB force field within MOLOC\textsuperscript{(10)} was applied to design small drug-like molecules to occupy the active site. We identified a diamino-substituted triazine as suitable central scaffold to position vectors for the S1, S2, and S3 binding pockets and direct the thioimidate adduct into the stabilizing oxyanion hole (Figure 2a). Occupancy of the various pockets (Figure 2b) was subsequently optimized to gain high binding potency.

Active site analysis and 3D modeling revealed that a morpholine residue could act as suitable substituent to address the flat, predominantly solvent-exposed S1 pocket in falcipain-2. For occupancy of the large and mainly hydrophobic S2 pocket, we identified a 4-(\textit{n}-propyl)cyclohexyl substituent as optimal vector, undergoing several hydrophobic interactions with the side chains of Leu84, Ile85, and Ser149. To reach the wide S3 pocket, a 1,3-benzodioxol-5-yl moiety was chosen to stack on the amide backbone of Gly82 and Gly83. Figure 3 shows the proposed binding mode for lead compound 1 in the active site of falcipain-2.

We prepared a series of functionalized triazine nitrile inhibitors by varying the S1, S2, and S3 substituents. The synthesis of inhibitor 1 is shown in Scheme 1. Reductive amination of ketone 2 and amine 3 gave secondary amine 4 in good yield. Amine 4 was subsequently transformed into 4,6-dichlorotriazine derivative 5 by reaction with equimolar amounts of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and \textit{N,N}-diisopropylethylamine (\textit{iPr}_{2}\text{NEt}) at 0°C. Introduction of the second vector was achieved by subsequent nucleophilic aromatic substitution with morpholine under basic conditions to give 6. Cyanation of 6 using potassium cyanide in dimethyl sulfoxide at high temperature afforded ligand 1. Compounds 7-17 were accessed by similar routes. In addition, an efficient
one-pot procedure for the two nucleophilic chlorine substitution steps was developed for compounds 13, 14, 16, and 17 (for detailed experimental procedures, see the Supporting Information). Single crystals, suitable for X-ray analysis, were obtained for inhibitors 7, 13, and 15 (figures 1SI–3SI in the Supporting Information) confirming the constitution and favorable structural organization of the 4,6-diamino-1,3,5-triazine-2-carbonitrile compounds.

All compounds were tested against falcipain-2 from *P. falciparum* and rhodesain from *T. brucei rhodesiense* (Table 1), respectively, in standard fluorescence-based assays (see the Supporting Information). For falcipain-2, investigation of substituents for the mostly solvent-exposed S1 pocket revealed a

Table 1. Inhibition of falcipain-2 and rhodesain by compounds 1 and 7–17.

| Compd | R<sup>1</sup> | R<sup>2</sup> | K<sub>i</sub> [nM] falcipain-2 | rhodesain |
|-------|-------------|-------------|------------------------------|-----------|
| 1<sup>11</sup> | | | 1030 ± 120 | 2 ± 1 |
| 7<sup>11</sup> | | | 35 ± 3 | 490 ± 190 |
| 8 | | | n.d.<sup>x</sup> | 970 ± 200 |
| 9<sup>11</sup> | | | 20 ± 7 | 8 ± 1 |
| 10 | | | n.d. | n.d. |
| 11<sup>11</sup> | | | 12900 ± 100 | n.d. |
| 12<sup>11</sup> | | | 9800 ± 500 | n.d. |
| 13<sup>11</sup> | | | 36 ± 7 | 34 ± 3 |

Figure 3. Binding mode of inhibitor 1 in the active site of falcipain-2 (PDB code: 2GHU) as proposed by docking and energy minimization using MOLOC. Color code: C skeleton of enzyme: grey, C skeleton of 1: green, O atoms: red, N atoms: blue, S atom: yellow. H-bond distance is given in Å.

Scheme 1. Synthesis of inhibitor 1. a) 1) CH<sub>2</sub>Cl<sub>2</sub>, molecular sieves (4 Å), 25 °C, 1.5 h, 2) NaBH(OAc)<sub>2</sub>, 25 °C, 15 h, 71%; b) cyanuric chloride, iPr<sub>2</sub>NET, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 4 h, 79%; c) morpholine, iPr<sub>2</sub>NET, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C → 25 °C, 3.5 h, 84%; d) KCN, Me<sub>2</sub>SO, 120 °C, 4.5 h, 27%.
Table 1. (Continued)

| Compd | R¹ | R² | Kᵢ [nm] | falcipain-2 | rhodesain |
|-------|----|----|---------|-------------|-----------|
| 14⁰⁶ |   |    | 25 ± 5  | 11 ± 1      |           |
| 15⁰⁶ |   |    | 42 ± 7  | 36 ± 7      |           |
| 16⁰⁶ |   |    | 29 ± 5  | 74 ± 2      |           |
| 17⁰⁶ |   |    | 80 ± 1  | 100 ± 40    |           |

[a] All results are the mean of at least two independent measurements, each performed in duplicate. [b] Time-dependent inhibition. [c] n.d. = not determined; less than 35% inhibition in initial screen at 20 μM. [d] ad = 2-adamantyl.

Preference for the initially designed morpholine group, whereas cyclopropylamine derivative 8 proved to be inactive. Replacement of the morpholine with a 2-methoxethylamine vector resulted in no significant change in inhibitory constants (11: Kᵢ = 12900 ± 100 nm; 12: Kᵢ = 9800 ± 500 nm). The affinity of initially designed active compound 1 (Kᵢ = 1030 ± 120 nm) was considerably enhanced by omitting the n-propyl substituent. Compound 9, with an unsubstituted cyclohexyl moiety, was significantly more potent against falcipain-2 (Kᵢ = 20 ± 7 nm). Introduction of a bulkier 2-adamantyl group resulted in a loss of affinity by three orders of magnitude (11: Kᵢ = 12900 ± 100 nm). Binding affinity for falcipain-2 is greatly enhanced by decreasing the size of the S2 pocket vector in the order adamantyl (11: Kᵢ = 12900 ± 100 nm) > 4-(n-propyl)cyclohexyl (1: Kᵢ = 1030 ± 120 nm) > cyclohexyl (9: Kᵢ = 20 ± 7 nm). Furthermore, a phenyl ring was also found to be a suitable substituent for the S2 pocket; phenyl-substituted ligands yielded activities in the low nanomolar range (13: Kᵢ = 36 ± 7 nm; 14: Kᵢ = 25 ± 5 nm; 15: Kᵢ = 42 ± 7 nm). These data emphasize the importance of proper occupancy of the S2 pocket, as previously reported.[28] Variation of substituents for the wide S3 pocket revealed a preference for 1,3-benzodioxol-5-yl and cyclohexyl substituents (7: Kᵢ = 35 ± 3 nm; 9: Kᵢ = 20 ± 7 nm). Replacement of the 1,3-benzodioxol-5-yl moiety with a 3-(trifluoromethyl)benzyl residue resulted in complete loss of activity for compound 10. Introduction of smaller ethyl or isopropyl substituents again led to inhibitory constants in the double-digit nanomolar range (16: Kᵢ = 29 ± 5 nm; 17: Kᵢ = 80 ± 1 nm).

Inhibitory constants for the compounds were also assessed against rhodesain from T. brucei rhodesiense. Lead compound 1 and unsubstituted cyclohexyl analogue 9 exhibited the highest affinities in this series, with Kᵢ values of 2 ± 1 nm and 8 ± 1 nm, respectively. In general, activities against falcipain-2 and rhodesain correlate well; however, notable differences were observed regarding the occupancy of the S2 pocket. The 2-adamantyl-substituted triazines 11 and 12 were moderately active against falcipain-2 but inactive against rhodesain, suggesting a less extended S2 pocket within the latter enzyme. Most importantly, the two enzymes differ at the bottom of this pocket; while falcipain-2 has a strong preference for a shorter cyclohexyl substituent (9: Kᵢ = 20 ± 7 nm) over 4-(n-propyl)cyclohexyl (1: Kᵢ = 1030 ± 120 nm), both 1 and 9 display Kᵢ values against rhodesain in the single-digit nanomolar range (1: Kᵢ = 2 ± 1 nm; 9: 8 ± 1 nm). Modeling suggests that the bottom of the pocket in falcipain-2, lined by Ile85 and Asp234, is narrower and more polar than the comparable region in rhodesain, lined by Met68 and Ala208 (see figures 4SI and 5SI in the Supporting Information).

Competitive inhibition was confirmed by determination of apparent dissociation constants for rhodesain inhibition by compound 1 at various substrate concentrations, revealing a linear relationship between apparent dissociation constant and substrate concentration.[34] These results can presumably be assigned to the whole series.

In order to study the general selectivity of the synthesized inhibitors against related cysteine proteases, the compounds were tested against human cathepsin B and cathepsin L,[17] and against the severe acute respiratory syndrome–coronavirus (SARS-CoV) papain-like protease and main protease (table 1SI in the Supporting Information).[35] Superimposition of selected amino acids in the active sites of falcipain-2, cathepsin B, and cathepsin L emphasizes their structural similarity (see figure S6 in the Supporting Information). Only compounds 9, 14, 15, and 17 showed affinity for human cathepsin L in the low nanomolar range, whereas moderate to good selectivity against this enzyme was observed for derivatives 1, 7, 11, 12, 13, 16, and 17. Moreover, all compounds were highly selective against human cathepsin B and the viral cysteine proteases. Furthermore, all compounds were inactive against α-chymotrypsin,[18] revealing selectivity for cysteine over serine proteases.

The newly synthesized compounds were tested for their ability to inhibit growth of the malaria parasite P. falciparum and the trypanosomatid T. brucei rhodesiense in vitro (Table 2). Nitriles 1, 7, 9, 10, 11, 12, and 16 exhibited moderate activities against P. falciparum with IC₅₀ values between 0.6 and 3.7 μM. IC₅₀ values against T. brucei rhodesiense trypomastigotes ranged from 15.0 μM to 44.1 μM. Comparable data were obtained from assays against T. brucei brucei (strain TC224; data not shown). A correlation between inhibition of the parasitic cysteine proteases and in vitro activity could not be established. This might be due to off-target effects, physical chemical prop-
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Keywords: African trypanosomiasis · cysteine proteases · inhibitors · malaria · structure-based design

Table 2. In vitro activities of compounds 1 and 7–17 against P. falciparum and T. brucei rhodesiense.[a]

| Compd | IC50 [µM] P. falciparum[b] | IC50 [µM] T. b. rhodesiense[c] | IC50 [µM] L-6 cells[d] |
|-------|---------------------------|-------------------------------|-----------------------|
| 1     | 2.4                       | 31.2                          | 5.9                   |
| 7     | 3.7                       | 31.9                          | 25.1                  |
| 8     | >5.0                      | 25.8                          | 23.5                  |
| 9     | 2.9                       | 30.1                          | 11.1                  |
| 10    | 0.6                       | 34.2                          | 4.5                   |
| 11    | 1.1                       | 27.5                          | 23.2                  |
| 12    | 1.8                       | 19.6                          | 8.1                   |
| 13    | >5.0                      | 16.7                          | 17.9                  |
| 14    | >5.0                      | 15.0                          | 11.4                  |
| 15    | >5.0                      | 18.9                          | 22.0                  |
| 16    | 3.4                       | 30.9                          | 25.9                  |
| 17    | >5.0                      | 44.1                          | 45.5                  |

[a] All results are the mean of at least two independent measurements, each performed in duplicate. [b] P. falciparum strain NF54. [c] T. brucei rhodesiense strain STIB 900, trypanomastigote stage. [d] Rat skeletal myoblasts were used to assess cytotoxicity.

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