Natural Products

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Antiplasmodial Thiostrepton Derivatives: Proteasome Inhibitors with a Dual Mode of Action**

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Causing more than one million deaths annually, tropical malaria is a fundamental threat to human health worldwide. Successful chemotherapeutic treatment is increasingly complicated by frequent drug resistance of the malaria parasites *Plasmodium Tsp*.[1] To counter this, different antimalaria drugs like quinine, mefloquine, and artemisinine are administered increasingly in combination.[2] In addition, potent low-cost antibiotics such as doxycycline, clindamycin, and azithromycin are often applied.[2,3] The effectiveness of these inhibitors of bacterial protein biosynthesis against malaria is generally explained by the high similarity shared by 70S bacterial ribosomes and mitochondrial or apicoplast ribosomes from the eukaryotic parasite. Protein translation is then locally inhibited in these organelles.[3,4] A hallmark of this activity is the late onset of their antiplasmodial action, which typically occurs only four days after infection of the red blood cells.[4b,5] This so-called "delayed death effect" is ascribed to the distribution of defective apicoplasts into daughter merozoites during replication of the erythrocytic parasite.[6]

The readily accessible thiostrepton (**1**)[7] was identified early on as a very potent antibiotic with strong activity against many Gram-positive bacteria.[8] It belongs to the large family of thiopeptide antibiotics,[9] which are highly modified macrocyclic peptide natural products produced by ribosomal peptide biosynthesis.[10]

Thiostrepton blocks translation in bacteria by binding tightly to the GTPase-associated center of the 70S ribosome.[11] Studies on the action of **1** in eukaryotic cells reported activity associated with immunomodulation,[12] cancer cell proliferation,[13] and growth inhibition of *Plasmodium falciparum*.[2,14] Thiostrepton (**1**) was proven to suppress protein translation in the apicoplast.[4b,14b,c,15] However, immediate killing of the parasites led to the antimalarial effect,[4b] in contrast to the action of other ribosomal inhibitors. A delayed death effect was never observed for **1**, but the reasons for these deviating properties of thiostrepton remained unclear.[4b] Here, we report on semisynthetic thiostrepton derivatives with enhanced potency against *P. falciparum*, desribe initial structure–activity patterns, and show that the activity of these compounds is tightly linked to the inhibition of the 20S proteasome.[16]

To investigate the antimalarial profile of thiostrepton in detail and to elucidate its potential for possible applications, we needed synthetic access to a series of derivatives. Extending previous studies on thiostrepton semisynthesis,[11d] we found that the configurationally labile[17] thiazoline unit of **1**–**3** (ring C) can be selectively oxidized to the corresponding thiazole (Scheme 1). This modification conferred improved chemical stability to the compounds. We then tested a range of lipophilic and hydrophilic derivatives on the human malaria pathogen *P. falciparum*. This initial screening suggested that hydrophobic extensions at the dehydroamino acid terminus improved the antiplasmodial properties. A focused collection of candidate compounds was then synthesized based on combinations of tail truncation, oxidation, and addition of lipophilic thiols to the terminal dehydroamino acid (Scheme 1, Table 1). All compounds were obtained in good yield, purified by preparative HPLC, and characterized by NMR spectroscopy, HPLC, and HRMS (see the Supporting Information).

Compounds **1**–**14** were then tested for growth inhibition of *P. falciparum*. Synchronized ring stages were studied at a parasitemia of 1%, and parasite viability was monitored by measuring the activity of *Plasmodium*-specific lactate dehydrogenase (see the Supporting Information).[18] In line with previous reports,[4b,14a] we found that **1** suppressed parasite growth with an IC_{50} of 10 μM in our assay. No delayed death effect was observed (data not shown). Optimum substituents R* seemed to have medium chain lengths (**5** and **14**). Longer (**6**), sterically more-demanding (**10**, **13**), and polar (**11**) appendages were less effective. The B-ring-opened[17] com-

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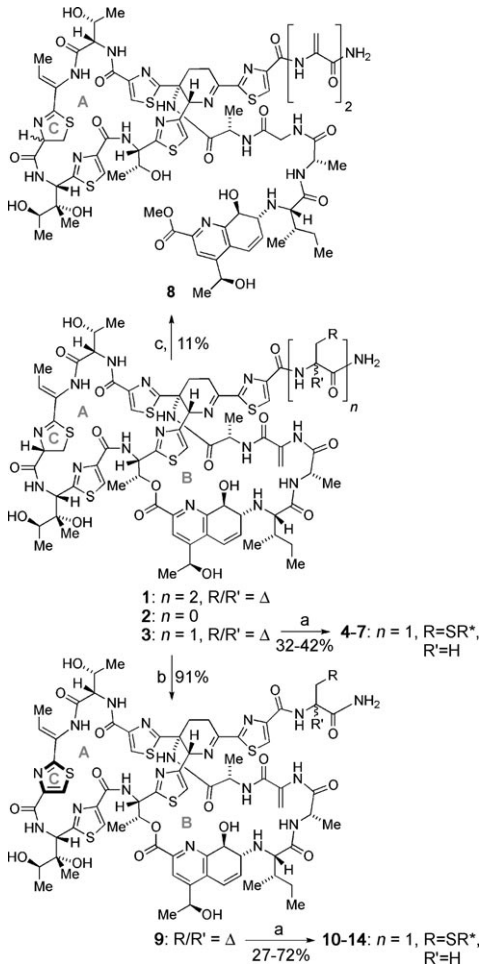

Scheme 1. Thiostrepton (**1**) and derivatives **2**–**14**. Key rings are indicated. Reagents and conditions: a) HSR* (1.2 equiv), NEt_3 (5 equiv), trifluoroethanol/H_2O (2:1), pH 9, 2–48 h; b) **3**, $CBrCl_3$ (2 equiv), DBU (1.1 equiv), THF, 0°C→20°C, 3 h; c) **1**, NaOMe (0.33 equiv), MeOH/$CHCl_3$ (2:1), 0°C→20°C, 6 h. Δ = methylidene, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, THF = tetrahydrofuran.

pound **8** (mixture of diastereomers in ring C) was inactive. Notably, compounds **5** and **14** were ten times more potent than the parent compound **1**. The activity pattern was observed to deviate from the antibacterial profile.[11d] Lipophilic side chains increased the activity against *P. falciparum*, whilst this modification reduced antibacterial potency (up to 500-fold).[11d] On the other hand, modifications that introduced polarity into the compound diminished antiplasmodial activity (e.g. **4**), while the antibacterial potential was not affected. If only the apicoplast ribosome was involved in the mode of action, one should expect the activity patterns to be more similar. Together with the absence of a delayed death effect, these data strongly indicated that targets beyond the ribosome contribute to the mode of action of thiopeptides in *P. falciparum*. In extension, this assumption should apply to other eukaryotic cells as well.[12,13]

Table 1: Synthesis of thiostrepton derivatives **2**–**14** and antiplasmodial activity (IC_{50} after monitoring for 72 h).

Cmpd	R*[a]	Ring C	Yield [%]	IC_{50} [μM]
1	n.p.	thiazoline	–	10 ± 2.0
2	n.p.	thiazoline	–[11d]	23 ± 1.1
3	n.p.	thiazoline	–[11d]	19 ± 4.1
4	$CH_2CHNAcCO_2Me$	thiazoline	–[11d]	inactive
5	$(CH_2)_4H$	thiazoline	42	1.3 ± 0.5
6	$(CH_2)_{16}H$	thiazoline	42	34 ± 10
7	$(CH_2)_8H$	thiazoline	–[11d]	4.3 ± 1.2
8	n.p.	thiazoline	11	inactive
9	n.p.	thiazole	91	3.1 ± 0.3
10	$CHMe_2$	thiazole	27	3.5 ± 0.4
11	$(CH_2)_4OH$	thiazole	54	7.5 ± 1.9
12	$(CH_2)_4H$	thiazole	51	2.5 ± 0.4
13	CH_2Ph	thiazole	71	2.6 ± 1.3
14	$(CH_2)_8H$	thiazole	35	1.2 ± 0.4

[a] n.p. = not present.

To gather information on additional targets, we performed cell microscopy studies with a fluorescently labeled thiostrepton probe.[11d] Labeled bioactive small molecules have been successfully used before to study the localization of target structures in cells.[19] Because of the difficult resolution of subcellular structures in the intraerythrocytic stages of the *Plasmodium* parasite (diameter of the parasites 1–4 μm), we used BSC-1 cells for this initial study. Water-soluble fluorescently labeled thiostrepton[11d] created characteristic staining patterns in fixed cells, which remained after washing (Figure 1). All staining could be suppressed dose-dependently by **1** as the competitor, clearly indicating specific binding. Among cellular substructures the mitochondria were prominently highlighted (Figure 1 a–c), as indicated by counterstaining with a mitochondrial marker. Therefore, within the eukaryotic cell, bicyclic thiopeptide antibiotics likely target the thiostrepton-sensitive 55S ribosomes in mitochondria,[20] which share similarities with bacterial 70S ribosomes and the ribosomes of the plasmodial apicoplast.[21] Binding of the probe to the 80S ribosomes of the endoplasmatic reticulum was not observed.

In addition, we noticed a slightly granular staining within the cytoplasm and the nucleus (without nucleoli) that did not match an organelle-associated distribution. After counterstaining experiments with antibodies targeting various cellular components, we were pleased to find that antibodies directed against the 20S proteasome displayed a highly similar staining pattern (Figure 1 d–f). Binding of the labeled probe was confirmed by fluorescence polarization measurements with isolated yeast 20S proteasomes, which gave an apparent dissociation constant of (1.75 ± 0.35) μM (see the Supporting Information).

20S proteasomes are highly conserved among eukaryotes and feature three proteolytic active sites which differ in their substrate specificity.[22] These sites show chymotryptic, tryptic, and caspase-like activity, and most of the known nonpeptidic inhibitors preferentially block the first.[23] The thiostrepton derivatives were hence further validated in a fluorogenic assay employing human erythrocyte 20S proteasomes and subsite-selective peptide substrates. Initial prescreenings of compounds **1**–**14** showed that only the caspase- and chymotrypsin-like activities were affected (Figure 2). Therefore, the trypsin-like activity was not tested further.

We then determined inhibition constants using optimized assay conditions (Figure 2 b, Table 2, Supporting Information) and found IC_{50} values in the low μM to nM range. Remarkably,

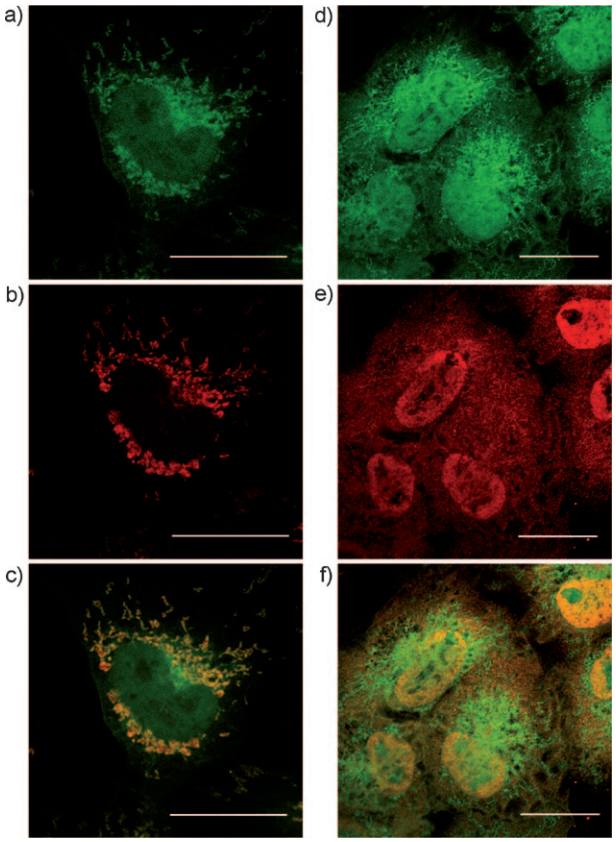

Figure 1. Localization of thiostrepton in fixed BSC-1 cells by immunofluorescence microscopy. a) Cells labeled with fluoresceinisothiocyanate(FITC)–thiostrepton (green). b) Cells labeled with MitoTracker red (Invitrogen) (red). c) Overlay of (a) and (b) confirms that **1** binds to mitochondria (yellow areas). d) Cells labeled with FITC–thiostrepton (green). e) Cells immunostained with an anti-20S-proteasome antibody (red). f) Overlay of (d) and (e), indicates strong colocalization of **1** with the 20S proteasome antibodies (yellow areas). Scale bar: 10 μm.

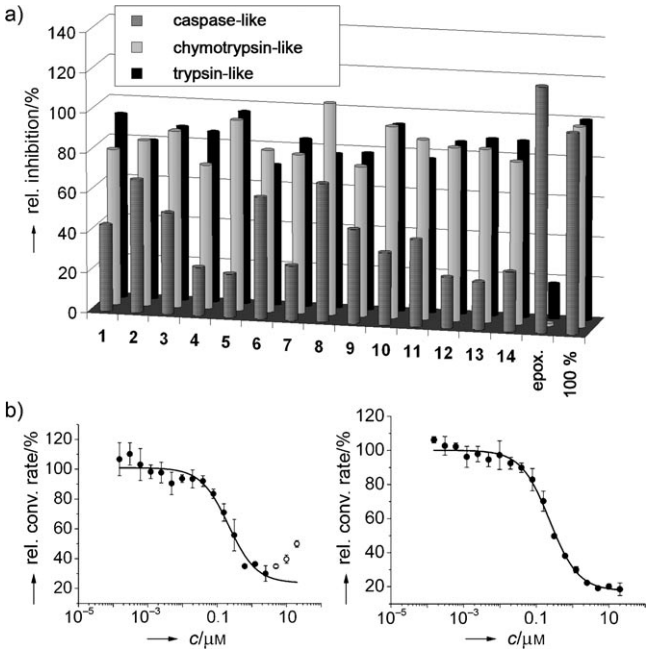

Figure 2. Inhibition of 20S proteasomes from human erythrocytes. a) Screening of compounds **1**–**14** for the chymotrypsin-, caspase-, and tryptic-like activities at $c = 1$ μM (epox. = epoxomycin, 0.1 μM); b) Inhibition curves for compound **14** (left: chymotrypsin-, right: caspase-like activity).

Table 2: Inhibition of chymotrypsin- and caspase-like activity.

Compound	IC_{50}(chym.) [μM]	IC_{50}(casp.) [μM]
1	5.2 ± 1.0	3.8 ± 2.4
2	1.9 ± 0.1	inactive
3	4.5 ± 1.1	inactive
4	1.1 ± 0.1	0.4 ± 0.1
5	0.32 ± 0.0	0.1 ± 0.0
7	0.2 ± 0.0	0.2 ± 0.0
8	15 ± 1.6	48 ± 38
9	3.7 ± 0.9	1.2 ± 0.1
12	1.2 ± 0.3	0.4 ± 0.1
14	0.2 ± 0.0	0.2 ± 0.1
MG132	0.02 ± 0.01	1.3 ± 0.2

several derivatives were more active than **1**. Adducts of lipophilic alkyl chains of four to eight methylene groups proved most favorable for the inhibitory activity (**5**/**12**, **7**/**14**); compound **5** was 40 times more potent than thiostrepton (0.1 μM caspase; 0.3 μM chymotrypsin). The ring-opened compound **8** was only weakly active, substantiating that an intact A/B-ring system is important. Interestingly, in many cases (e. g. **4**, **5**, **9**, **12**) we observed a stronger inhibition of the caspase-like than of the chymotrypsin-like activity (Figure 2 and Table 2). This profile is rare among known small-molecule inhibitors.[23] For the chymotryptic activity, the residual activity at full dose was 10–20%, whereas the caspase activity was reduced only to 40–50%. This suggests that a partial antagonistic or allosteric mode of action could be involved.

Oxidizing the thiazoline ring C of the thiostrepton scaffold in general resulted only in minor changes of inhibitory potency in vitro. The higher antiplasmodial activity of oxidized derivatives in live cells (cf. **3**/**9**, **7**/**14**) might reflect increased compound stability. Likewise, we determined different inhibitory activity for the hydrophilic compound **4**. While this compound was quite potent in the enzymatic assay (Table 2), it showed no effect in plasmodial growth inhibition. This difference might be explained by insufficient cell uptake. Overall, the enzyme inhibition data clearly go in line with the activity against *P. falciparum* in cell experiments, indicating a causal connection of compound activity and proteasome inhibition.

The proteasome executes the regulated degradation of all proteins in eukaryotic cells, and is present in all lifecycle stages of malaria parasites.[24] Proteasome inhibitors have been investigated as antimalaria agents before,[25,26] and it was shown that epoxomycin and bortezomib inhibit growth of *P. falciparum* by immediate killing, thus during the first replication cycle.[27] In vitro and in vivo data on **1**[4b,14,15] imply that thiostrepton derivatives enter the parasite and are directly effective on the growing pathogen.

In humans, proteasome inhibitors[16] have been explored as candidate anti-inflammatory[28] and antitumor drugs.[29] They have also been implicated in the treatment of stroke,[30] bone formation,[31] and neurotrophic activity.[32] But although bortezomib is approved as an antitumor drug for human use,[29b,33] high toxicity is often observed for proteasome inhibitors. In contrast to this, all of the compounds we tested (**1**–**14**) showed no apparent toxicity in WST assays, and did not affect erythrocyte cell integrity (data not shown).[14b]

In summary, we have shown that oxidation of a single thiazoline ring and extension of the terminus of thiostrepton (**1**) leads to potent agents against *P. falciparum*. We found that the antiplasmodial activity clearly correlates with the inhibition of the 20S proteasome. Notably, the new inhibitors were nontoxic in human cells and preferentially inhibited the caspase-like activity of the proteasome β1 subunit, a less-common mode of action for nonpeptide-based proteasome inhibitors.[22,34] These data indicate that in *P. falciparum* the apicoplast ribosomes[4b,14b,c,15] and the proteasome are targeted in parallel, which explains why a delayed death effect is not observed. Such a dual mode of action should render thiostrepton derivatives intrinsically less prone to resistance development than single-target based inhibitors. In contrast to human cells and yeast, the explicit functioning of the ubiquitin-proteasome system in *Plasmodium* currently not well characterized and clearly necessitates future studies. Its similarity to that of other eukaryotes[24–27] suggests, however, that it must be essential for parasite viability and development. Our thiopeptides are now clearly established as promising nontoxic scaffolds for proteasome inhibitor discovery,[35] and these results indicate new directions for developing antimalaria agents.

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