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Development of Rhodesain Inhibitors with a 3-Bromoisoxazoline Warhead

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Novel rhodesain inhibitors were obtained by combining an enantiomerically pure 3-bromoisoxazoline warhead with a specific peptidomimetic recognition moiety. All derivatives behaved as inhibitors of rhodesain, with low micromolar K_i values. Their activity against the enzyme was found to be paralleled by an in vitro antitrypanosomal activity, with IC_{50} values in the mid-micromolar range. Notably, a preference for parasitic over human proteases, specifically cathepsins B and L, was observed.

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic disease transmitted by tsetse flies (genus *Glossina*) that afflicts millions of people in 36 countries in sub-Saharan Africa, with an estimated 30 000 cases each year.[1] HAT is caused by two subspecies of protozoa belonging to the genus *Trypanosoma*: *T. brucei* (*T. b.*) *gambiense* and *T. b. rhodesiense*.[2] While *T. b. gambiense* is widespread in west and central areas of Africa and causes a chronic form of the disease, *T. b. rhodesiense* is prevalent in the southeastern part of the continent and causes an acute form of HAT that develops within a few weeks of infection. The first stage of the disease—hemolymphatic—is characterized by fever, itching, headache, and joint pain. During the second neurological stage, the parasite invades the central nervous system (CNS), causing progressive mental deterioration, disorders of the sleep–wake cycle (from which the disease gets its common name), coma, and eventually death. Given the lack of an effective vaccine, chemotherapy is currently the only way to control the disease. The main drawbacks of currently available treatments are poor efficacy, poor pharmacokinetic properties, cost, and increasing drug resistance.[3,4] To overcome the difficulties encountered in the control of HAT, there is an urgent need to develop new drugs, and efforts have been made to identify new molecular targets.

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Cysteine proteases are very promising targets for the development of new antiparasitic agents. In particular, clan CA cysteine proteases play an important role in the life cycle of many parasites. During the hemolymphatic stage, *T. brucei* expresses two papain-like cysteine proteases: rhodesain (also known as brucipain or trypanopain) and TbCatB, which are respectively cathepsin L- and cathepsin B-like enzymes. Rhodesain is required by the parasite to cross the blood–brain barrier (BBB), leading to the second (and lethal) stage of HAT.[5] It is involved in the turnover of variant surface glycoproteins (VSG) of the *T. brucei* coat,[6] preventing effective intervention by the immune system; it also takes part in the degradation of host immunoglobulins. There is also a growing body of evidence that rhodesain plays a role as the major lysosomal protease.[7]

At present, the main class of rhodesain inhibitors is composed of a peptide backbone linked to a variety of warheads such as vinyl sulfones, aldehydes, diazomethyl ketones, α,β-epoxy esters, esters of aziridine-2,3-dicarboxylate, azadipeptide nitriles, and Michael acceptors (e.g., esters and amides of fumaric or crotonic acid).[8] Owing to the weaknesses of peptides as drug candidates, many other chemotypes have been identified as nonpeptidic inhibitors of rhodesain; these include thiosemicarbazones, isoquinolines, and triazine nitriles.[8,9]

In this area, our research group has been actively involved in the development of novel peptidomimetic cysteine protease inhibitors.[10–13] We successfully developed reversible inhibitors characterized by a recognition moiety formed by a 1,4-benzodiazepine scaffold, as a dipeptide mimic, properly functionalized at the 5-position with a phenyl ring and at the 3-position with a lipophilic carbamoyl moiety spanning into the P3 region, and by a reactive warhead at the P1 site that is a thioacylal or an acylal group (i.e., compounds **1** and **2**, Figure 1).[12] These inhibitors are endowed with rather good trypanocidal activity (IC_{50} = 14.2 μM for **1**, the most active compound), and display dissociation constants (a measure of enzyme affinity) in the sub-micromolar range (K_i = 0.7 and 0.8 μM, respectively) for rhodesain.

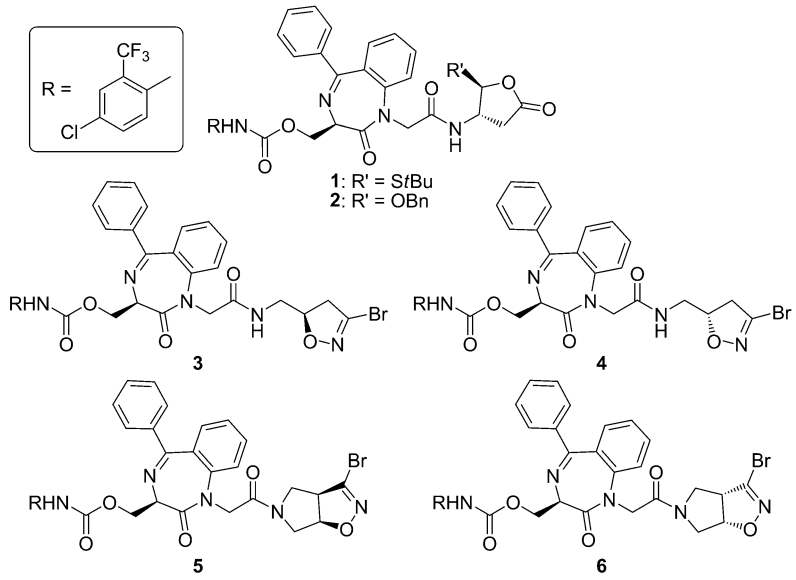


Figure 1. Model and target compounds.

More recently, we successfully developed covalent irreversible inhibitors of cytidine triphosphate synthetase (CTPS), a glutamine amidotransferase equipped with the catalytic triad Cys, His, Glu. The inhibitors are composed of an amino acid skeleton, mimicking the natural substrate glutamine, and a 3-bromoisoxazoline ring as the reactive warhead.[14,15]

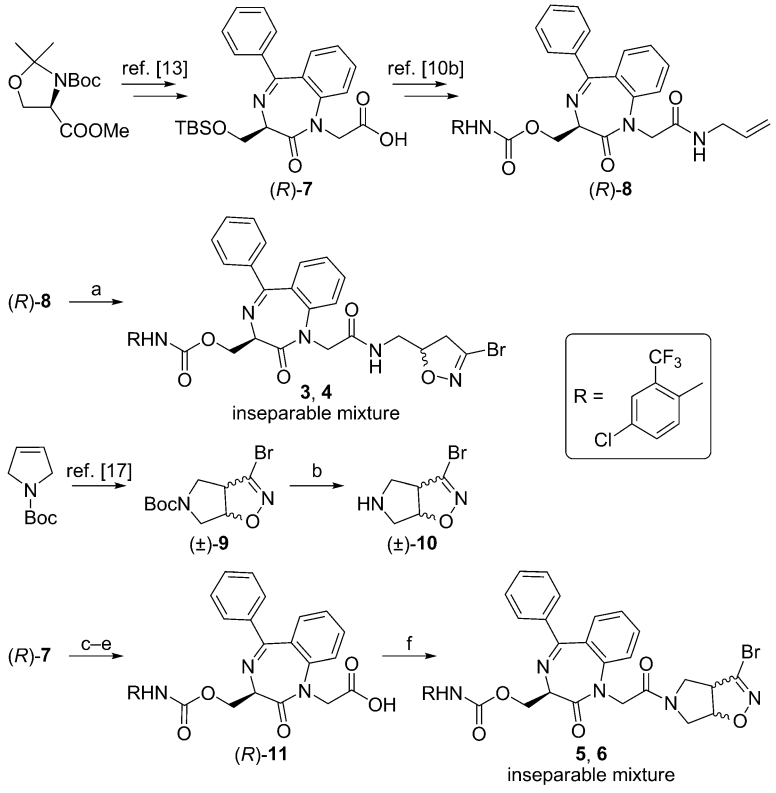
In the present project we combined the 3-bromoisoxazoline warhead with the recognition moiety of our lead compounds **1** and **2** (i.e., compounds **3** and **4**), in order to evaluate the efficacy of this novel warhead for rhodesain inhibition. In addition, we introduced molecular rigidification by incorporating the 3-bromoisoxazoline nucleus into a bicyclic system (compounds **5** and **6**). Herein we report the synthesis and biological evaluation of compounds **3**–**6** against rhodesain and falcipain-2 (FP-2), the structurally related cysteine protease of *P. falciparum*. The activity of these novel derivatives was evaluated against parasites in vitro. Their selectivity was checked by comparing their inhibitory activity against the target enzyme versus human cysteine proteases of the papain family, that is, cathepsins B and L.

Results and Discussion

Chemistry

The synthesis of compounds (*R*,*R*)-**3** and (*R*,*S*)-**4** (Scheme 1) was initially attempted by 1,3-dipolar cycloaddition between the dipolarophile (*R*)-**8**, prepared from suitably protected D-serine following the procedure previously described by us,[13,10b] and bromonitrile oxide, generated in situ by dehydrohalogenation of the stable precursor dibromoformaldoxime (DBF).[16] The cycloaddition produced a 1:1 mixture of diastereomeric isoxazoline derivatives (*R*,*R*)-**3** and (*R*,*S*)-**4**, which unfortunately turned out to be inseparable by flash chromatography (Scheme 1).

In parallel, for the synthesis of derivatives (*R*,*R*,*R*)-**5** and (*R*,*S*,*S*)-**6**, we initially condensed carboxylic acid (*R*)-**11** with racemic 3-bromo-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]-[1,2]oxazole (±)-**10**. Compound (*R*)-**11** was synthesized as shown in Scheme 1, starting from intermediate (*R*)-**7**,[13] where-



Scheme 1. *Reagents and conditions*: a) DBF, $NaHCO_3$, EtOAc, RT, 48 h; b) 30% TFA, CH_2Cl_2, 0 °C→RT, 4 h; c) conc. HCl/MeOH, RT, 12 h; d) 4-chloro-2-(trifluoromethyl)phenyl isocyanate, CH_2Cl_2, RT, 12 h; e) Na_2CO_3, H_2O/MeOH, 0 °C→RT, 24 h; f) (±)-**10**, EDCI, HOBt, DIPEA, CH_2Cl_2, 0 °C→RT, 12 h.

as the amine (±)-**10** was obtained after Boc cleavage from compound (±)-**9**.[17] However, also in this case, the obtained diastereomeric mixture of compounds (*R*,*R*,*R*)-**5** and (*R*,*S*,*S*)-**6** could not be separated by flash chromatography. Therefore, we decided to change strategy and synthesize each of the single isomers (*R*,*R*)-**3**, (*R*,*S*)-**4**, (*R*,*R*,*R*)-**5**, and (*R*,*S*,*S*)-**6** by condensing enantiomerically pure (*R*)-**11** with the enantiomerically pure amines (*R*)-**15**, (*S*)-**15**, (*R*,*R*)-**10**, and (*S*,*S*)-**10**, respectively.

To this aim we first had to establish a suitable method for the preparation of the required enantiomerically pure amines. The amine (*R*)-**15** was prepared by starting from enantiopure alcohol (*R*)-**12**, which was, in turn, obtained in 94% enantiomeric excess (*ee*) by following a previously reported methodology.[18] Alcohol (*R*)-**12** was converted into the corresponding mesylate (*R*)-**13**, which was, in turn, allowed to react with sodium azide in dry *N*,*N*-dimethylformamide. The obtained azide (*R*)-**14** was then converted into the corresponding amine (*R*)-**15** by reduction with triphenylphosphine and water (Scheme 2). Analogously, amine (*S*)-**15** was obtained from enantiopure alcohol (*S*)-**12** (>99% *ee*).[18]

The enantiopure amines (*R*,*R*)-**10** and (*S*,*S*)-**10** were obtained by resolving a racemic mixture of (±)-**9** by preparative chiral HPLC; an excellent enantiomeric separation was obtained with tris-(3,5-dimethylphenyl)carbamoyl amylose, allowing us to collect a substantial amount of the two enantiomers (−)-**9** and (+)-**9**, both in 99% *ee*. To establish their absolute configuration, we focused on the (+)-**9** enantiomer. We succeeded in growing diffraction-quality crystals directly from the reaction mother liquor (≈20:1 (*v*/*v*) hexane/EtOAc mixture). Due to an anomalous scattering by bromine, the absolute configuration was unequivocally determined as (+)-(*S*,*S*)-**9** (Figure 2).

Compounds (*S*,*S*)-**9** and (*R*,*R*)-**9** were treated with a 30% solution of trifluoroacetic acid in dichloromethane to afford (*S*,*S*)-**10** and (*R*,*R*)-**10**, respectively (Scheme 2). Carboxylic acid (*R*)-**11** was condensed with enantiopure amines (*R*)-**15**, (*S*)-**15**, (*R*,*R*)-**10**, and (*S*,*S*)-**10**, using the water-soluble carbodiimide EDCI and HOBt as coupling reagents, to afford the desired final derivatives (*R*,*R*)-**3**, (*R*,*S*)-**4**, (*R*,*R*,*R*)-**5**, and (*R*,*S*,*S*)-**6** (Scheme 3).

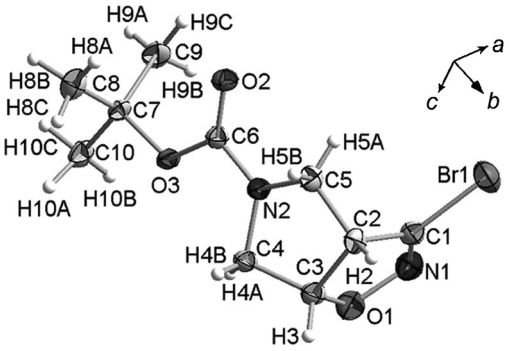


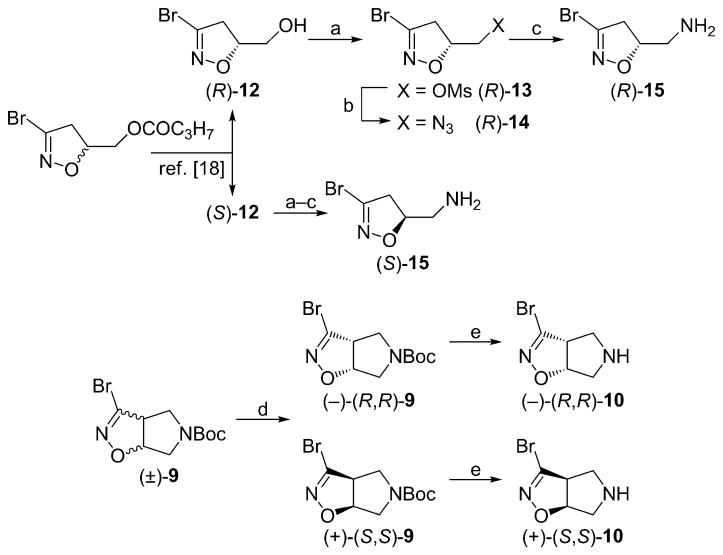
Figure 2. Asymmetric unit of the (+)-**9** enantiomer as retrieved from single-crystal X-ray diffraction at room temperature, with the atom numbering scheme shown. Thermal ellipsoids were plotted at the 30% probability level. The image was created with the Diamond v. 3.2i software package (© K. Brandenburg, 1997–2012, Crystal Impact GbR, Bonn, Germany).

Pharmacology

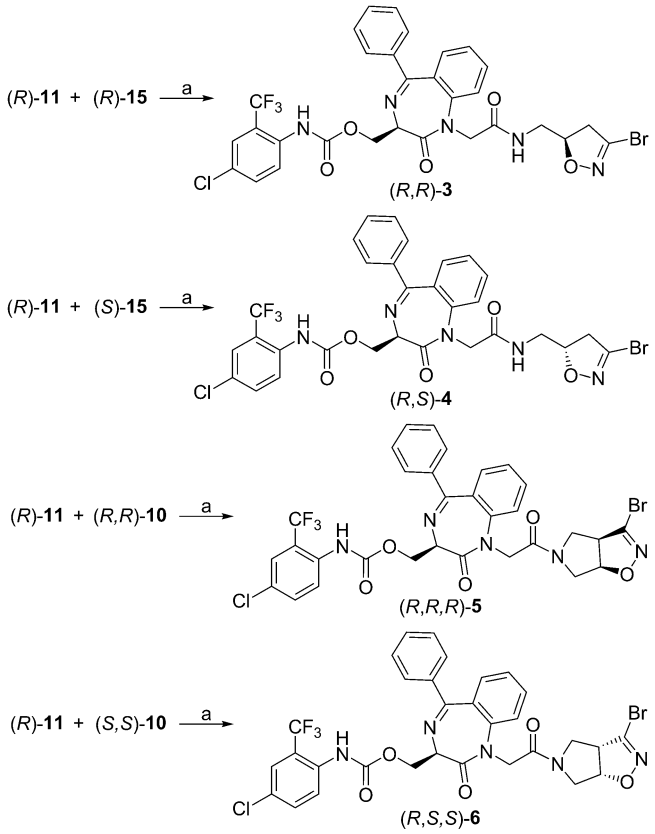
Compounds **3**–**6** were tested for their inhibitory activity against rhodesain, and the results are compared with those previously obtained with model compounds **1** and **2** (Table 1). Cbz-Phe-Arg-AMC was used as fluorogenic substrate. First, a preliminary screening with an inhibitor concentration of 20 μM was performed. An equivalent volume of DMSO was used as negative control. For compounds that passed the initial screening, continuous assays (progress curve method) were performed. Interestingly, the compounds showed time-independent inhibition, which is in contrast to the inhibition of CTPS by 3-bromoisoxazolines. Thus, only K_i values could be determined.

All peptidomimetics revealed to be active against rhodesain in the low micromolar/sub-micromolar range. In particular, compound **5**, bearing the 3-bromoisoxazoline ring incorporated into a bicyclic system, was found to be the most potent inhibitor, with a K_i value of 0.96 μM, which is similar to that obtained for model compounds **1** and **2**. These data indicate that the 3-bromoisoxazoline nucleus imparts compounds with inhibitory activities similar to those displayed by the acylal/thioacylal derivatives.

Compounds were also tested against FP-2, the papain-family cysteine protease of *P. falciparum*, which has a high degree of homology with rhodesain. As shown in Table 1, all the tested compounds displayed K_i values in the low micromolar range against FP-2 as well, with a slight preference for the *T. brucei* protease (e.g., compound **5** $K_i = 0.96$ and 6.00 μM against rhodesain and FP-2, respectively). A similar trend was also observed with model compounds **1** and **2**, and thus the preference for rhodesain over FP-2 more likely arises from the peptidomimetic portion rather than the warhead.



Scheme 2. *Reagents and conditions*: a) MsCl, TEA, CH_2Cl_2, −10 °C→RT, 16 h; b) NaN_3, DMSO, 60 °C, 4 h; c) Ph_3P, THF/H_2O, RT, 24 h; d) preparative chiral HPLC; e) 30% TFA, CH_2Cl_2, 0 °C→RT, 4 h.



Scheme 3. *Reagents and conditions*: a) EDCI, HOBt, DIPEA, CH_2Cl_2, 0 °C→RT, 12 h.

Table 1. Activity against rhodesain, FP-2, *T. b. brucei* and *P. falciparum*.[a]

Compd	Rhodesain K_i [μM]	FP-2 K_i [μM]	*T. b. brucei*[b] IC_{50} [μM] 48 h	IC_{50} [μM] 72 h	*P. falciparum* IC_{50} [μM]
3	3.52 ± 0.17	5.48 ± 2.80	14.85 ± 0.11	16.91 ± 0.46	27.75 ± 6.03
4	4.63 ± 0.57	8.96 ± 0.34	15.97 ± 0.79	17.64 ± 0.10	37.67 ± 2.93
5	0.96 ± 0.09	6.00 ± 0.20	12.48 ± 3.63	14.04 ± 1.02	> 100
6	2.22 ± 0.32	5.30 ± 0.20	16.42 ± 1.42	17.86 ± 0.05	> 100
1[12]	0.70 ± 0.02	8.2 ± 1.5	14.2 ± 1.0	ND	28.5 ± 0.8
2[12]	0.80 ± 0.04	8.9 ± 1.0	ND	ND	49.5 ± 4.9

[a] Values are the mean ± SD of $n = 2$ experiments performed in duplicate. [b] ND: not determined.

Selectivity assays were also performed, testing the inhibitors against two papain-family human cysteine proteases, cathepsins B and L. None of the tested compounds passed the initial screening at 20 μM against cathepsin L, whereas only compound **5** inhibited cathepsin B with a K_i value of 12.03 μM; this is, nevertheless, one order of magnitude higher than K_i values observed against the parasitic proteases.

The antitrypanosomal activity of rhodesain inhibitors **3**–**6** was also evaluated on *T. b. brucei* TC221 strains. In these assays the tested derivatives displayed IC_{50} values in the range of 12–18 μM. For all inhibitors an additional assay on *P. falciparum* 3D7 strains was also performed. Interestingly, only compounds **3** and **4** displayed antiplasmodial activity similar to model compounds **1** and **2** (Table 1), whereas compounds **5** and **6** were found to be inactive up to a concentration of 100 μM.

Conclusions

In summary, we have designed a series of new rhodesain inhibitors based on the 3-bromoisoxazoline nucleus as the warhead and a peptidomimetic portion as the recognition moiety. Their synthesis was based on the use of enantiomerically pure synthons, and to this aim we have developed two convenient methodologies to prepare the enantiomerically pure amines bearing the 3-bromoisoxazoline warhead (**10** and **15**). All the new derivatives were obtained in enantiomerically pure form.

All derivatives turned out to behave as inhibitors with low micromolar K_i values against both rhodesain and its closely related homologue FP-2, with a slight preference for the former. Interestingly, the activity against the enzyme was paralleled by an in vitro antitrypanosomal activity, with IC_{50} values in the mid-micromolar range. Notably, a preference for parasitic over human proteases, specifically cathepsins B and L, was observed. These data indicate that the 3-bromoisoxazoline nucleus is an efficient warhead which, when coupled to the appropriate recognition moiety, can be successfully used in the design of inhibitors of cysteine proteases such as rhodesain and falcipain-2.

Experimental Section

Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Elemental analyses were carried out on a Carlo Erba Model 1106 (Elemental Analyzer for C, H, and N), and the obtained results are within ±0.4% of theoretical values. Merck Silica gel 60 F_{254} plates were used for analytical TLC; flash column chromatography was performed on Merck Silica gel (200–400 mesh). ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^1H and ^{13}C chemical shifts (δ) are expressed in ppm, and coupling constants (J) in hertz (Hz). MS analyses were performed on a Varian 320-MS triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. Chiral HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV/Vis detector Jasco UV-975 (λ 220 nm) and a Kromasil 5-AmyCoat column (4.6×250 mm). Preparative chiral HPLC was performed with a 1525 Extended Flow Binary HPLC pump equipped with a Waters 2489 UV/Vis detector and a Kromasil 5-AmyCoat column (21.2×250 mm, AkzoNobel) at a flow rate of 15 mL min^{-1}.

Compounds (*R*)-**7**,[13] (*R*)-**8**,[10b] (±)-**9**,[17] (*R*)-**12**,[18] and (*S*)-**12**[18] were prepared as previously described.

(*R*)-2-(3-((4-Chloro-2-(trifluoromethyl)phenylcarbamoyloxy)methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-1-yl)-acetic acid [(*R*)-11]: A solution of the acid (*R*)-**7** (250 mg, 0.57 mmol) in a mixture of conc. HCl/MeOH (1:4 *v/v*, 50 mL) was stirred at room temperature for 12 h to promote, in a single step, the acid-catalyzed esterification of the carboxylic acid function and deprotection of the hydroxy group. The solvent was then evaporated, and the reaction mixture was neutralized with a saturated

solution of $NaHCO_3$ (100 mL) and extracted with EtOAc (3×50 mL). The organic layer was then dried over Na_2SO_4 and evaporated. The obtained crude material was purified by flash chromatography, eluting with $CHCl_3$/MeOH (9:1) to give the methyl 2-((*R*,*Z*)-2,3-dihydro-3-(hydroxymethyl)-2-oxo-5-phenylbenzo[*e*][1,4]diazepin-1-yl)acetate in high yield (189 mg, 0.56 mmol, 98%). R_f = 0.72 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$): δ = 2.83 (bs, 1H), 3.67 (s, 3H), 3.87 (t, J = 6.9 Hz, 1H), 4.18–4.28 (m, 1H), 4.33–4.46 (m, 1H), 4.58 (s, 2H), 7.18–7.67 ppm (m, 9H).

To a solution of the above 2-((*R*,*Z*)-2,3-dihydro-3-(hydroxymethyl)-2-oxo-5-phenylbenzo[*e*][1,4]diazepin-1-yl)acetate (189 mg, 0.56 mmol) in dry CH_2Cl_2 (5 mL) under N_2 atmosphere, 4-chloro-2-(trifluoromethyl)phenyl isocyanate (248 mg, 169 μL, 1.12 mmol) was added. The resulting mixture was then stirred for 12 h at room temperature, and after this time it was washed with H_2O (1×5 mL), dried over Na_2SO_4, and the crude material, obtained after evaporation of the solvent, was purified by flash chromatography, eluting with light petroleum/EtOAc (7:3) to give the (*R*)-ethyl 2-(3-((4-chloro-2-(trifluoromethyl)phenylcarbamoyloxy)methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-1-yl)acetate (270 mg, 0.47 mmol, 84%). R_f = 0.24 (light petroleum/EtOAc 7:3); $[\alpha]_{20}^{D}$ = −85.4 (c = 0.33, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ = 3.65 (s, 3H), 4.05 (t, J = 6.6 Hz, 1H), 4.65 (s, 2H), 4.90–5.05 (m, 2H), 6.90 (bs, 1H), 7.20–7.65 (m, 11H), 8.10 ppm (d, J = 8.9 Hz, 1H); MS: 560.0 $[M+H]^+$.

To a solution of the above (*R*)-ethyl 2-(3-((4-chloro-2-(trifluoromethyl)phenylcarbamoyloxy)methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-1-yl)acetate (270 mg, 0.47 mmol) in a mixture MeOH/H_2O 1:1 (20 mL) was added Na_2CO_3 (100 mg, 0.94 mmol), keeping the temperature at 0 °C for 1 h. The mixture was then allowed to warm to 25 °C and stirred until the disappearance of the starting material (TLC, $CHCl_3$/MeOH 9:1). The solvent was concentrated under reduced pressure, and the mixture was treated with 10% citric acid (5 mL) and extracted with ethyl acetate (2×50 mL), dried and concentrated to give the acid (*R*)-**11** (109 mg, 0.20 mmol, 43%). R_f = 0.45 (CH_2Cl_2/MeOH 8:2); $[\alpha]_{20}^{D}$ = +55.0 (c = 0.14, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ = 4.03 (t, J = 6.5 Hz, 1H), 4.55 (s, 2H), 4.87–5.05 (m, 2H), 7.02 (bs, 1H), 7.15–7.75 (m, 11H), 8.05 (d, J = 8.6 Hz, 1H); MS: 544.0 $[M+H]^+$.

(*R*)-(3-Bromo-4,5-dihydroisoxazol-5-yl)methanamine [(*R*)-15]: To a solution of (*R*)-(3-bromo-4,5-dihydroisoxazol-5-yl)methanol **12** (200 mg, 1.12 mmol) and Et_3N (0.468 μL, 3.36 mmol) in dry CH_2Cl_2 (6 mL), CH_3SO_2Cl (130 μL, 1.68 mmol) was added at −10 °C. The resulting mixture was stirred at room temperature for 16 h. The solution was diluted with Et_2O (40 mL) and washed with 0.5 N HCl (3× 10 mL) and brine (10 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was removed under reduced pressure. Mesylate (*R*)-**13** was obtained in quantitative yield (288 mg) and used without further purification in the subsequent reaction. R_f = 0.30 (cyclohexane/EtOAc 3:2); ^{1}H NMR ($CDCl_3$): δ = 3.07 (s, 1H), 3.15 (dd, J = 7.4, 17.6 Hz, 1H), 3.38 (dd, J = 11.0, 17.6 Hz, 1H), 4.30 (dd, J = 4.4, 11.6 Hz, 1H), 4.37 (dd, J = 3.8, 11.6 Hz, 1H), 4.94 ppm (dddd, J = 3.8, 4.4, 7.4, 11.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ = 38.0, 43.3, 69.0, 78.7, 137.6 ppm.

To a solution of mesylate (*R*)-**13** (210 mg, 0.81 mmol) in anhydrous DMSO (6 mL), NaN_3 (315 mg, 4.86 mmol) was added. The reaction mixture was warmed to 60 °C and stirred for 4 h under N_2 atmosphere. The mixture was cooled to room temperature, diluted with Et_2O (90 mL), and washed with brine (3×30 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was removed under reduced pressure. Purification by flash chromatography (cyclohexane/EtOAc 4:1) yielded the desired azide (*R*)-**14** as a pale-yellow oil (150 mg, 90%). R_f = 0.70 (cyclohexane/EtOAc 3:2); $[\alpha]_{20}^{D}$ = +214.2 (c = 1.10, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.10 (dd, J = 7.4, 17.3 Hz, 1H), 3.30 (dd, J = 10.7, 17.3 Hz, 1H), 3.42 (dd, J = 4.5, 13.2 Hz, 1H), 3.56 (dd, J = 4.5, 13.2 Hz, 1H), 4.85 ppm (dddd, J = 4.5, 4.5, 7.4, 10.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 44.3, 53.2, 80.0, 137.5 ppm; MS: 205.2 $[M+H]^+$.

A solution of (*R*)-**14** (140 mg, 0.68 mmol) and PPh_3 (205 mg, 0.78 mmol) in THF (3.3 mL) and H_2O (30 μL) was stirred at room temperature for 24 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (0–20% MeOH in EtOAc) to give (*R*)-**15** (90 mg, 64%) as a pale-yellow oil. R_f = 0.20 (EtOAc/MeOH 4:1); $[\alpha]_{20}^{D}$ = +119.2 (c = 0.52, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.35 (bs, 2H), 2.85 (dd, J = 6.0, 13.5 Hz, 1H), 3.02 (dd, J = 4.0, 13.5 Hz, 1H), 3.08 (dd, J = 7.0, 17.5 Hz, 1H), 3.23 (dd, J = 10.5, 17.5 Hz, 1H), 4.75 ppm (dddd, J = 4.0, 6.0, 7.0, 10.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 44.0, 45.0, 83.2, 137.6 ppm; MS: 179.2 $[M+H]^+$.

(*S*)-(3-Bromo-4,5-dihydroisoxazol-5-yl)methanamine [(*S*)-15]: Derivative (*S*)-**15** was prepared as described for its enantiomer (*R*)-**15**, starting from (*S*)-**12**.

(*S*)-14 $[\alpha]_{20}^{D}$ = +212.5 (c = 1.00, $CHCl_3$). All spectroscopic data are identical to those of the corresponding enantiomer.

(*S*)-15 $[\alpha]_{20}^{D}$ = +117.7 (c = 0.50, $CHCl_3$). All spectroscopic data are identical to those of the corresponding enantiomer.

((*R*)-1-(2-(((*R*)-3-Bromo-4,5-dihydroisoxazol-5-yl)methylamino)-2-oxoethyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)methyl-4-chloro-2-(trifluoromethyl) phenylcarbamate [(*R*,*R*)-3]: To a solution of (*R*)-**11** (272 mg, 0.50 mmol) in CH_2Cl_2 (10 mL) at 0 °C were added HOBt (74 mg, 0.55 mmol) and EDCI (198 mg, 0.55 mmol). After 5 min, the ice bath was removed and the amine (*R*)-**15** (90 mg, 0.5 mmol) was added together with DIPEA (94 μL, 0.55 mmol), and the obtained mixture was stirred at room temperature for 12 h. The organic layer was washed with H_2O (2×30 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 8:2) to give (*R*,*R*)-**3** (328 mg, 93%). R_f = 0.61 (cyclohexane/EtOAc, 6:4); $[\alpha]_{20}^{D}$ = +24.0 (c = 0.50, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.97 (dd, J = 7.9, 17.6 Hz, 1H), 3.20 (dd, J = 10.7, 17.6 Hz, 1H), 3.52–3.58 (m, 2H), 4.05 (t, J = 6.3 Hz, 1H), 4.28 (d, J = 15.7 Hz, 1H), 4.66 (d, J = 15.7 Hz, 1H), 4.70–4.82 (m, 1H), 4.95 (dd, J = 6.0, 11.3 Hz, 1H), 5.05 (dd, J = 7.4, 11.3 Hz, 1H), 6.54 (bt, 1H), 7.13–7.20 (bs, 1H), 7.25–7.64 (m, 11H), 8.06 ppm (d, J = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 42.01, 44.14, 52.68, 62.18, 65.54, 80.35, 121.08, 121.49, 121.53, 122.46, 124.89, 125.16, 125.35, 126.39, 128.60, 129.33, 129.46, 130.01, 130.86, 131.07, 132.48, 133.10, 134.48, 138.01, 138.41, 142.44, 153.47, 167.01, 169.05, 170.16 ppm; MS: 706.0 $[M+H]^+$; Anal. calcd for $C_{30}H_{24}BrClF_3N_5O_5$: C 50.97, H 3.42, N 9.91, found: C 51.12, H 3.28, N 9.98.

((*R*)-1-(2-(((*S*)-3-Bromo-4,5-dihydroisoxazol-5-yl)methylamino)-2-oxoethyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)methyl-4-chloro-2-(trifluoromethyl) phenylcarbamate [(*R*,*S*)-4]: Compound (*R*,*S*)-**4** was prepared as described for compound (*R*,*R*)-**3**, using amine (*S*)-**15**. R_f = 0.61 (cyclohexane/EtOAc, 6:4); $[\alpha]_{20}^{D}$ = −22.0 (c = 0.50, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.02 (dd, J = 7.4, 17.8 Hz, 1H), 3.25 (dd, J = 10.7, 17.8 Hz, 1H), 3.41 (bt, 1H), 3.70–3.79 (m, 1H), 3.98–4.06 (m, 1H), 4.11 (d, J = 15.1 Hz, 1H), 4.78 (d, J = 15.1 Hz, 1H), 4.79–4.85 (m, 1H), 4.90 (dd, J = 5.5, 11.0 Hz, 1H), 5.08 (dd, J = 7.9, 11.0 Hz, 1H), 6.94 (bt, 1H), 7.28–7.66 (m, 11H), 8.07 ppm (d, J = 9.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.98, 43.86, 52.66, 62.15, 65.56, 80.23, 121.09, 121.45, 121.56,

122.42, 124.87, 125.18, 125.34, 126.41, 128.62, 129.31, 129.47, 130.05, 130.91, 131.05, 132.49, 133.11, 134.49, 138.05, 138.39, 142.42, 153.43, 166.98, 169.02, 170.12 ppm; MS: 706.0 $[M+H]^+$; Anal. calcd for $C_{30}H_{24}BrClF_3N_5O_5$: C 50.97, H 3.42, N 9.91, found: C 51.20, H 3.22, N 9.96.

(3a*R*,6a*R*)-*tert*-Butyl 3-bromo-6,6a-dihydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-5(4*H*)-carboxylate [(*R*,*R*)-9] and (3a*S*,6a*S*)-*tert*-butyl 3-bromo-6,6a-dihydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-5(4*H*)-carboxylate [(*S*,*S*)-9]: Enantiomerically pure (*S*,*S*)-**9** and (*R*,*R*)-**9** were obtained from (±)-**9** by preparative chiral HPLC (λ 220 nm, eluent: *n*-hexane/iPrOH (9:1), flow rate: 15 mL min^{-1}, t_r (*R*,*R*)-**9**: 8.99 min; t_r (*S*,*S*)-**9**: 10.81 min). (*R*,*R*)-**9**: $[\alpha]_{20}^{D} = +100.0$ ($c = 0.60$, $CHCl_3$); crystallized from *n*-hexane/EtOAc as colorless prisms; mp: 80 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.43$ (s, 9H), 3.40 (dd, $J = 8.0$, 12.1 Hz, 1H), 3.51 (dd, $J = 5.8$, 13.1 Hz, 1H), 3.85–4.04 (m, 3H), 5.24 ppm (ddd, $J = 1.8$, 5.8, 9.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 28.54$, 48.57, 53.96, 57.43, 80.79, 85.01, 139.58, 154.23 ppm; MS: 291.03 $[M+H]^+$. (*S*,*S*)-**9**: $[\alpha]_{20}^{D} = +101.0$ ($c = 0.64$, $CHCl_3$); crystallized from *n*-hexane/EtOAc as colorless prisms; mp: 80 °C.

(3a*R*,6a*R*)-3-Bromo-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole [(*R*,*R*)-10]: Compound (*R*,*R*)-**9** (100 mg, 0.34 mmol) was treated with a 30% solution of TFA in CH_2Cl_2 (1 mL) at 0 °C. The solution was stirred at room temperature for 4 h until disappearance of the starting material. The volatiles were removed under vacuum, the residue was dissolved in a 5% $NaHCO_3$ solution (5 mL) and extracted with EtOAc (3×3 mL). The organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to afford the desired amine (*R*,*R*)-**10** (52 mg, 80%). $R_f = 0.20$ (EtOAc/MeOH 9:1); $[\alpha]_{20}^{D} = -181.0$ ($c = 0.50$, $CHCl_3$); crystallized from *n*-hexane/EtOAc as colorless needles; mp: 78 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.83$ (bs, 1H), 2.83 (dd, $J = 3.8$, 13.4 Hz, 1H), 2.87 (dd, $J = 7.4$, 12.3 Hz, 1H), 3.35 (d, $J = 12.3$ Hz, 1H), 3.50 (d, $J = 13.4$ Hz, 1H), 3.83 (dd, $J = 7.4$, 8.6 Hz, 1H), 5.25 ppm (dd, $J = 3.8$, 8.6 Hz, 1H); ^{13}C NMR (75 MHz,$CDCl_3$): $\delta =$ 52.3, 57.4, 60.1, 88.2, 139.5 ppm; MS: 191.1 $[M+H]^+$; Anal. calcd for $C_5H_7BrN_2O$: C 31.44, H 3.69, N 14.66, found: C 31.77, H 3.53, N 14.48.

(3a*S*,6a*S*)-3-Bromo-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole [(*S*,*S*)-10]: Compound (*S*,*S*)-**10** was obtained as described for its enantiomer (*R*,*R*)-**10** starting from derivative (*S*,*S*)-**9**. $[\alpha]_{20}^{D} = +180.0$ ($c = 0.60$, $CHCl_3$); crystallized from *n*-hexane/EtOAc as colorless needles; mp: 78 °C; Anal. calcd for $C_5H_7BrN_2O$: C 31.44, H 3.69, N 14.66, found: C 31.73, H 3.55, N 14.39. All spectroscopic data are identical to those of the corresponding enantiomer.

((*R*,4*Z*)-1-(2-((3a*R*,6a*R*)-3-Bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-2-oxoethyl)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-3-yl)methyl 4-chloro-2-(trifluoromethyl)-phenylcarbamate [(*R*,*R*,*R*)-5]: Compound (*R*,*R*,*R*)-**5** was prepared as described for compound (*R*,*R*)-**3**, using the amine (*R*,*R*)-**10**. $[\alpha]_{20}^{D} = -145.2$ ($c = 1.00$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.58$–3.63 (m, 1H), 3.72–3.79 (m, 1H), 3.89–4.10 (m, 4H), 4.18 (d, $J = 15.4$ Hz, 1H), 4.78 (d, $J = 15.4$ Hz, 1H), 4.86–5.08 (m, 2H), 5.38–5.46 (m, 1H), 6.90 (bs, 1H), 7.20–7.64 (m, 11H), 8.07 ppm (d, $J = 9.1$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 51.45$, 54.14, 56.27, 57.94, 61.97, 65.77, 85.33, 121.05, 121.56, 122.53, 124.97, 125.19, 126.30, 128.54, 128.81, 129.12, 129.21, 129.99, 130.56, 130.94, 132.10, 132.42, 133.15, 134.50, 138.61, 140.01, 142.80, 153.33, 166.82, 169.03, 170.53 ppm; MS: 718.1 $[M+H]^+$; Anal. calcd for $C_{31}H_{24}BrClF_3N_5O_5$: C 51.79, H 3.36, N 9.74, found: C 51.97, H 3.20, N 9.81.

((*R*,4*Z*)-1-(2-((3a*S*,6a*S*)-3-Bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-2-oxoethyl)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-3-yl)methyl 4-chloro-2-(trifluoromethyl)-phenylcarbamate [(*R*,*S*,*S*)-6]: Compound (*R*,*S*,*S*)-**6** was prepared as described for compound (*R*,*R*)-**3**, using the amine (*S*,*S*)-**10**. $[\alpha]_{20}^{D} = -38.3$ ($c = 1.00$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.35$–3.42 (m, 1H), 3.68–3.82 (m, 2H), 3.86–4.12 (m, 3H), 4.21 (d, $J = 15.4$ Hz, 1H), 4.91 (d, $J = 15.4$ Hz, 1H), 4.94–5.08 (m, 2H), 5.33–5.40 (m, 1H), 6.95 (bs, 1H), 7.28–7.68 (m, 11H), 8.09 ppm (d, $J = 9.1$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 51.40$, 54.11, 56.29, 57.65, 61.92, 65.75, 85.11, 121.06, 121.54, 122.51, 124.95, 125.20, 126.32, 128.53, 128.79, 129.10, 129.19, 129.96, 130.57, 130.93, 132.12, 132.39, 133.13, 134.51, 138.59, 140.00, 142.78, 153.28, 166.80, 169.08, 170.49 ppm; MS: 718.0 $[M+H]^+$; Anal. calcd for $C_{31}H_{24}BrClF_3N_5O_5$: C 51.79, H 3.36, N 9.74, found: C 51.92, H 3.24, N 9.90.

Single-crystal X-ray diffraction analysis of (+)-10

The single-crystal X-ray diffraction experiment was performed on a Bruker Smart Apex three-circle diffractometer equipped with an APEXII CCD area detector, employing graphite-monochromated $Mo_{K\alpha}$ radiation at a nominal source power of 50 kV×30 mA. Diffraction-quality crystals were grown directly from the reaction mother liquor (≈20:1 (*v*/*v*) hexane/EtOAc). Compound (+)-**9** was found to crystallize in the non-centrosymmetric orthorhombic $P2_12_12_1$ space group (no. 19), with cell parameters as large as $a = 6.3928(4)$ Å, $b =$ 9.8981(6) Å, $c = 19.8574(11)$ Å, $V = 1256.5(2)$ Å^3. The structural model was developed and refined within the independent atom approximation through the SHELX suite of programs.[19] Due to the presence of anomalous scattering by bromine, the absolute configuration was unequivocally determined as (+)-(*S*,*S*)-**9** with the refined Flack parameter being as low as 0.02(1). CCDC 963005 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Biology

Enzyme assays: The preliminary screening was performed with an inhibitor concentration of 20 μM using an equivalent amount of DMSO as negative control. The enzymes were recombinantly expressed as previously described.[7,20] Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC; 10 μM for rhodesain, and 40 μM for FP-2) was determined continuously over a period of 10 min. Compounds showing at least 40% inhibition at 20 μM were subjected to detailed assays. These were performed in a 50 mM sodium acetate buffer (pH 5.5) containing 10 mM DTT with Cbz-Phe-Arg-AMC (10 or 40 μM) as substrate.[20] The K_M values used to calculate K_i values from IC_{50} values were determined to be 0.9 μM (rhodesain)[21] and 21.5 μM (FP-2).[22] Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed twice in 96-well plates in a total volume of 200 μL. Fluorescence of the product AMC of substrate hydrolysis was measured with an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) at room temperature with a λ 380 nm excitation filter and a λ 460 nm emission filter. The dissociation constants K_i of the noncovalent complex E·I were obtained from progress curves (10 min) at various concentrations of inhibitor by fitting the progress curves to the four-parameter IC_{50} equation [Eq. (1)].

$$y = \frac{y_{max} - y_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^s} + y_{min} \quad (1)$$

for which y [dF min^{-1}] is the substrate hydrolysis rate, y_{max} is the maximum value of the dose–response curve observed at very low

inhibitor concentrations, y_{min} is the minimum value obtained at high inhibitor concentrations, and s denotes the Hill coefficient,[23] and correction to zero substrate concentration from $K_i = IC_{50}/(1+[S]K_M^{-1})$. Assays with cathepsins B and L were performed as described previously.[24] Cbz-Phe-Arg-AMC was used as substrate (80 μM for cathepsin B, 5 μM for cathepsin L). The K_M values used to calculate K_i values from IC_{50} values were 150 μM (cathepsin B) and 6.5 μM (cathepsin L).

Drug screening on P. falciparum cultures: Compounds were screened in quadruplicate against the human malaria pathogen *P. falciparum* at concentrations between 100 and 0.0488 μM. *P. falciparum* (strain FCBR) was maintained in continuous culture basically according to Trager and Jensen.[25] Parasites were cultured in human red blood cells (RBC; blood group A/Rh+) in RPMI 1640 medium supplemented with 25 mM HEPES (Molecular Probes, Invitrogen), 20 mM $NaHCO_3$, and 0.5% AlbuMAX I (Molecular Probes, Invitrogen) at 2.5% (*v*/*v*) hematocrit. Cultures were maintained at 37 °C under an atmosphere of 94% N_2, 1% O_2, and 5% CO_2. Synchronized ring stages of *P. falciparum* strain FCBR were plated in 96-well plates at a parasitemia of 1%, in the presence of test compounds (dissolved in DMSO and diluted 10-fold in 50% EtOH before addition to the cells). Incubation of parasites with an equal concentration of 50% EtOH alone was used as a negative control. To kill the plasmodia completely for the positive control, parasites were incubated in the presence of 2 μM chloroquine. Parasite viability was subsequently screened by using a previously published assay.[26]

Drug screening on T. b. brucei cultures: Trypomastigote forms of *T. b. brucei* laboratory strain TC221 were cultured in Baltz medium according to standard conditions.[27] Trypanocidal activity was determined using the Alamar Blue assay[29,29] in a 96-well plate format and a MR 700 Microplate ELISA Reader. Trypanosomes were added to culture medium containing various concentrations of test compound and 1% solvent to give a cell concentration of 10^4 cells mL^{-1} in a final volume of 200 μL. Positive and negative controls were wells containing medium, 1% solvent, and trypanosomes, and wells with test compounds but without trypanosomes, respectively. After 24 h, Alamar Blue (20 μL) was added to each well, and the plates were incubated again for a further 24 or 48 h. Absorbance was then measured at λ 550 nm with a reference wavelength of 630 nm. IC_{50} values were calculated by linear interpolation as described.[30] Experiments were repeated twice.

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