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Novel 2*H*-isoquinolin-3-ones as antiplasmodial falcipain-2 inhibitors

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ABSTRACT

A series of 1-aryl-6,7-disubstituted-2*H*-isoquinolin-3-ones (**2–10**) was synthesized and evaluated for their inhibition against *Plasmodium falciparum* cysteine protease falcipain-2, as well as against cultured *P. falciparum* strain FCBR parasites. All compounds displayed inhibitory activity against recombinant falcipain-2 and against in vitro cultured intraerythrocytic *P. falciparum*, with the exception of **9**. The new compounds exhibited no selectivity against human cysteine proteases such as cathepsins B and L. The inhibitory activity of the synthesized compounds was also evaluated against another protozoal cysteine protease, namely rhodesain of *Trypanosoma brucei rhodesiense*.

1. Introduction

Malaria remains to be one of the most deadly parasitic diseases affecting about 250 million people worldwide and leading to more than 1 million deaths per year.[1] The spread of multi-resistant *Plasmodium falciparum* strains combined with the lack of effective vaccines, represents therefore a serious concern for the population in malaria endemic regions and creates a great urge to the development of new and effective antimalarial agents.[2] Currently, artemisinin-based combination therapy seems to be the most safe and efficacious solution, thus strictly recommended from WHO for treatment of uncomplicated and severe malaria.[3] The recently discovered *Plasmodium* genome sequences have opened new perspectives for malaria research. Although no licensed therapies have yet resulted from genome-dependent experiments, it is likely that this discovery can lead to new therapeutic approaches.[4]

Cysteine protease falcipain-2 (FP-2) of *P. falciparum* emerged as a valid target for antimalarial drugs.[5] FP-2 plays an essential role for the parasite survival during the blood stage as a major peptide hydrolase within the hemoglobin degradation pathway along with other enzymes.[5,6] Moreover, it is responsible for the erythrocyte rupture by cleaving ankyrin and protein 4.1, the cytoskeletal elements vital to the stability of the red cell membrane.[7]

Plants are still important resources for the discovery of new drugs and are employed nowadays in folk medicine in poverty-stricken regions of Africa, Asia and South America against parasitic infections.[8] Several plants, traditionally used against malaria, contain alkaloids with an isoquinoline core which have a broad-spectrum antimicrobial activity and many other pharmacological effects.[9] Several of these isoquinoline alkaloids with remarkable antimalarial activity have been already identified and reported in literature, including nitidine, duguevalline, berberine and allied protoberberines such as palmatine and jatrorrhizine.[10]

Moreover, synthetic derivatives containing the isoquinoline core, for example, 1-aryl-6,7-disubstituted isoquinolines **1** (Fig. 1) designed on the basis of homology modeling studies,[11] were found to exhibit a promising inhibitory activity against FP-2 at micromolar level.[12]

On these basis and in connection with our ongoing efforts to develop FP-2 inhibitors for the treatment of malaria,[13] we sought to test 1-(4-aminophenyl)-3-methyl-6,7-methylenedioxy-isoquinoline **2**, previously synthesized in our research laboratories (Fig. 1) against FP-2 and cultured *P. falciparum*.[14]

The results of the biological evaluation prompted us to synthesize a series of analogues of compound **2** in order to identify new antimalarial lead compounds and find preliminary SAR trends.

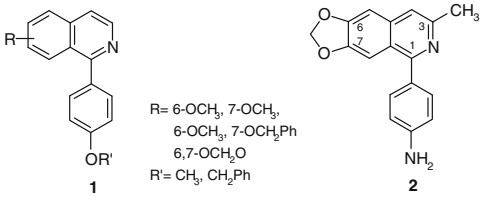

Figure 1. Structures of isoquinolines **1** and **2**.

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In particular, in order to explore the effects of structural variations at positions 2 and 3, the methyl group of **2** has been substituted with an ester moiety (**3**), a carbonyl group has been introduced at position 3 and an amine function at position 2 (**6**), which has been further functionalized (**4–5, 7–10**). In derivatives **4–10**, the 4′-NH_2 group has been replaced by alkoxy groups, according to the work of Batra et al., where it has been evidenced that the S_2 pocket of FP-2 prefers to accommodate hydrophobic groups.[12]

All compounds were tested against recombinant FP-2 as well as cultured *P. falciparum* strain FCBR parasites. Moreover, the inhibitory activity of the synthesized compounds was also evaluated against rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. Finally, selectivity against the target enzyme was estimated by testing the new compounds against human cysteine proteases of the papain-family, that is, cathepsins B and L.

2. Results and discussion

2.1. Chemistry

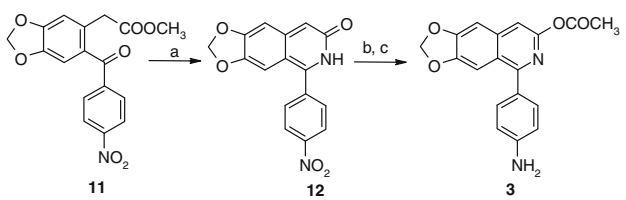
Target compounds were prepared following the synthetic strategies outlined in Schemes 1–4.

Methylester **11**,[14] was treated with formamide to give 2*H*-isoquinolin-3-one **12** which, by reaction with acetyl chloride and subsequent catalytic reduction of the nitro group afforded compound **3** (Scheme 1).

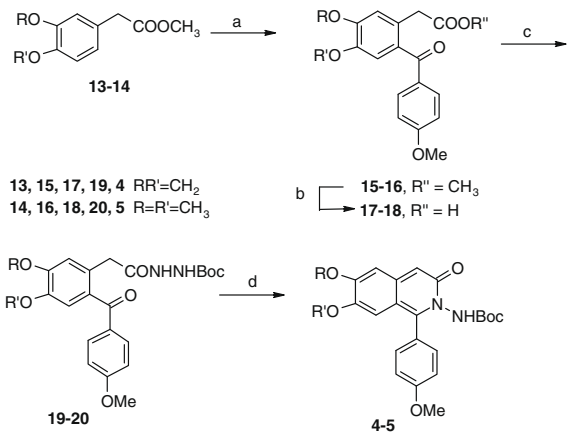
Ketoesters **15–16** were obtained via acylation of commercially available methyl phenylacetates **13–14** with 4-methoxybenzoic acid, in the presence of an excess of phosphorus pentoxide (Scheme 2).[15] The alkaline hydrolysis (LiOH) of **15–16** led the corresponding acids **17–18** which, by treatment with *N*-Boc-hydrazine, afforded the hydrazides **19–20**. The cyclization to N-substituted isoquinolin-3-ones **4–5** required the treatment with NaH in THF.

The removal of the *N*-Boc-protecting group of **4** was realized by treatment with trifluoroacetic acid (TFA) to afford **6** which was made to react with ethyl chloroformate in presence of triethylamine or with aryl isocyanates to give derivatives **7** and **8–9**, respectively (Scheme 3).

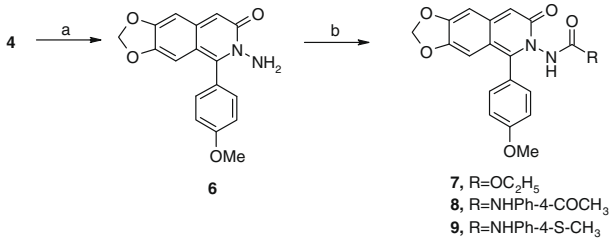
According to the previous work of Batra,[12] we decided to more closely investigate the modifications at position 4′ of the phenyl ring at C-1 by introducing a benzyloxy group in this position. Firstly, we established a convenient way to remove the *O*-methyl group of **15** with boron tribromide. However, the treatment of **15** with the harsh Lewis acid BBr_3 caused the methylenedioxy ring cleavage and the hydrolysis of the ester moiety, giving [4,5-dihydroxy-2-(4-methoxybenzoyl)-phenyl]-acetic acid **21** as the sole product. Direct and long-range heteronuclear chemical shift correlation experiments (HETCOR and LR-HETCOR) have been performed to confirm the structure of the obtained compound **21**. The key to the structure elucidation lies in a three-bond correlation of the MeO protons (3.88 ppm) with the carbon resonating at 164.97 ppm, assigned as C-4′ on the basis of long-range coupling



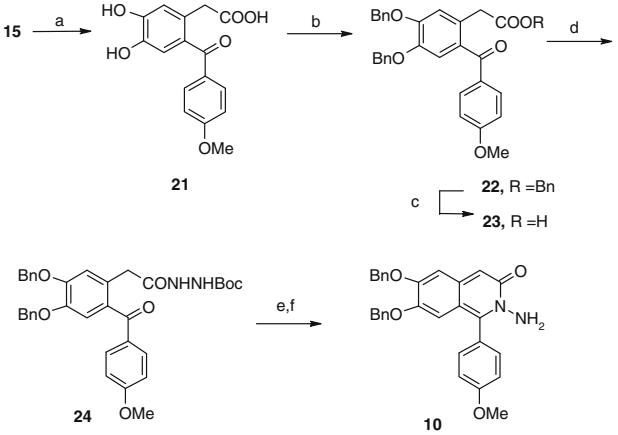
Scheme 1. Reagents and conditions: (a) $HCONH_2$, AcOH, reflux, 6 h; (b) AcCl, CH_2Cl_2, 2 h, room temperature; (c) H_2, 5% Pd–C, $CHCl_3$, 3 h, room temperature.



Scheme 2. Reagents and conditions: (a) anisic acid, P_2O_5, $(CH_2)_2Cl_2$, 16 h, room temperature; (b) LiOH 1 N, THF/CH_3OH (4/1), 4 h, room temperature; (c) $BocNHNH_2$, EDCI, CH_2Cl_2/DMF (1/1), 12 h, room temperature; (d) NaH, THF, 2 h, room temperature.



Scheme 3. Reagents and conditions: (a) TFA (25% in CH_2Cl_2), 5 h, room temperature; (b) ethyl chloroformate/Et_3N (for **7**), or ArNCO (for **8–9**), CH_2Cl_2, 3–6 h, room temperature.



Scheme 4. Reagents and conditions: (a) BBr_3, CH_2Cl_2, 0 °C, then 3 h, room temperature; (b) BnBr, K_2CO_3, acetone, 5 h, reflux; (c) LiOH 1 N, THF/CH_3OH (4/1), 8 h, room temperature; (d) $BocNHNH_2$, EDCI, CH_2Cl_2/DMF (1/1), 12 h, room temperature; (e) NaH, THF, 2 h, room temperature; (f) TFA (25% in CH_2Cl_2), 5 h, room temperature.

with H-2′,6′ (7.72 ppm), thus confirming that the O-demethylation failed. In spite of this result, we moved forward with the synthesis and derivative **21** was then reacted with benzyl bromide in alkaline medium to effect the O-benzylation of both hydroxy groups and esterification of carboxylic group to give intermediate **22** which was converted into the corresponding acid **23** by treatment with LiOH. With the same procedure depicted in Scheme 1, target

compound **10** was obtained by sequential treatment with *N*-Boc-hydrazine, NaH and TFA (Scheme 4).

2.2. Pharmacology

Compounds **2–10** were tested for their inhibitory activity against recombinant FP-2[16] using Cbz-Phe-Arg-AMC as fluorogenic substrate. First, a preliminary screening with inhibitor concentrations of 100 μM was performed. An equivalent volume of DMSO was used as negative control, and the irreversible standard inhibitor of clan CA family C1 cysteine proteases (papain-family), namely E-64,[17] was used as positive control. The screening showed all compounds to abolish enzyme activity in a reversible manner. Continuous assays (progress curve method) were then performed to determine the constants of inhibition $K_{i\ (\mu M)}$. Standard units of FP-2 were added to the reaction mixture containing the fluorogenic substrate and increasing from 0 to 100 μM concentrations of compounds. FP-2 activity results in the cleavage of Cbz-Phe-Arg-AMC, thus releasing the fluorescent AMC group. Hence, fluorescence of AMC in a sample reflects actual activity of the enzyme.

In parallel, all compounds were tested against *P. falciparum* strain FCBR. Dose-dependent effects of compounds on parasite development were quantified using a previously published assay.[18]

Data reported in Table 1 show that this structural class possesses an inhibitory activity towards the target enzyme in the micromolar range. Compounds **2** and **8–10** most potently inhibit FP-2. What does attract attention from a rough survey of these results is that the trend of the inhibitory potency does not match with the one of the antiplasmodial activity and in some cases it is even reversed. Compounds **4–5** and **7** possess an alkyloxycarbonylamino motif at position 2 of the isoquinoline core which seems to negatively affect the interaction with the enzyme differently to what observed for derivative **6** bearing a free amino group at the same position (i.e., **4** K_i = 24.4 μM, **5** K_i = 40.3 μM, **7** K_i = 62.7 μM vs **6** K_i = 15.0 μM). In compounds **4–5** and **7,** the functionalization of the amino group at C-2 reasonably increases the lipophilicity of these compounds which turn out to be the three most potent against cultured parasites (i.e., **4** IC_{50} = 7.7 μM, **5** IC_{50} = 9.3 μM, **7** IC_{50} = 8.4 μM). On the contrary, compounds with an arylureido motif at C-2 inhibit more efficiently FP-2 (i.e., **8** K_i = 5.9 μM and **9** K_i = 6.5 μM). This could be possibly due to the presence of an additional hydrogen donor group (NH) or of the phenyl ring which promotes hydrophobic interactions. Unexpectedly, compound **9** turned out to be inactive against the cultured *P. falciparum* whereas compound **8** displays a significant activity (IC_{50} = 11.8 μM) in the same assay.

The most potent compound in the enzymatic assay resulted to be **10** (K_i = 2.3 μM) underlying the importance of hydrophobic and bulky groups at positions 6 and 7 of the isoquinoline scaffold. The presence of an amino group at position 4′ (compounds **2–3**) seems to be basically favorable for FP-2 inhibition (K_i = 7.3 μM for **2** and K_i = 19.7 μM for **3**). It could be speculated that their fair antiplasmodial activity (IC_{50} = 38.8 μM for **2** and IC_{50} = 58.8 μM for **3**) could be explained in terms of the difficulty for this class of derivatives to cross the biological membranes of the parasites and reach the target enzyme.

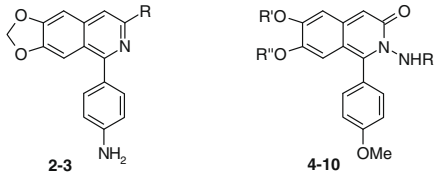
All the synthesized compounds were also tested against rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense* (Table 1). With the exception of compound **5**, all derivatives showed to inhibit this enzyme at micromolar level. The trend of the inhibition potency against rhodesain is somehow different from that against FP-2, however, also against rhodesain, the most potent inhibitor was again compound **10**.

Selectivity against the target enzyme was also estimated, testing inhibitors against papain-family human cysteine proteases such as cathepsins B and L (Table 1). Overall, these data demonstrate no selectivity towards FP-2 of the compounds under study: in general, this new class of cysteine protease inhibitors are slightly more potent towards human cathepsins with respect to parasitic FP-2 and rhodesain.

3. Conclusion

In conclusions, new synthesized 2*H*-isoquinolin-3-ones showed a good inhibitory activity against FP-2 as well as against cultured *P. falciparum* strain FCBR parasites. New synthesized compounds also inhibited rhodesain of *Trypanosoma brucei rhodesiense* in the micromolar range suggesting a potential use for treatment of sleeping sickness. Unfortunately, these isoquinoline derivatives did not show selectivity towards FP-2. Further work is required to improve the inhibitory properties as well as the antiplasmodial activity and to enhance selectivity towards the target enzyme.

Table 1
Inhibition of FP-2, antiplasmodial activity, inhibition of rhodesain, human cathepsins B and L of compounds **2–10**[a]



Compd	R	R′	R″	Falcipain-2 K_i (μM)	*P. falciparum* IC_{50} (μM)	Rhodesain K_i (μM)	Cathepsin B K_i (μM)	Cathepsin L K_i (μM)
2	CH_3			7.3 ± 0.3	38.8 ± 0.6	19.4 ± 3.8	6.9 ± 0.1	8.7 ± 0.9
3	$OCOCH_3$			19.7 ± 1.9	58.8 ± 5.8	38.3 ± 4.6	6.7 ± 0.1	13.6 ± 0.1
4	Boc	CH_2		24.4 ± 0.2	7.7 ± 0.4	19.4 ± 6.3	7.5 ± 1.5	4.1 ± 0.2
5	Boc	CH_3	CH_3	40.3 ± 4.9	9.3 ± 0.3	ni	ni	39.7 ± 9.5
6	H	CH_2		15.0 ± 0.6	25.5 ± 3.3	6.3 ± 0.6	16.3 ± 3	7.0 ± 0.5
7	$CO_2C_2H_5$	CH_2		62.7 ± 1.2	8.4 ± 0.8	9.4 ± 0.2	16.7 ± 0.1	7.7 ± 0.8
8	CONHPh-4-$COCH_3$	CH_2		5.9 ± 0.4	11.8 ± 1.5	14.0 ± 1.7	3.7 ± 0.6	2.1 ± 0.1
9	CONHPh-4-SCH_3	CH_2		6.5 ± 1.9	>100	18.7 ± 7.2	4.0 ± 0.3	0.85 ± 0.02
10	H	Bn	Bn	2.3 ± 0.5	16.9 ± 1.6	4.0 ± 0.2	3.3 ± 0.2	4.2 ± 1.2
E-64				0.29 ± 0.09	5.3 ± 1.05		16 (IC_{50}, nM)[22]	47 (IC_{50}, nM)[22]

[a] All results include standard deviations from two independent measurements, each performed in duplicate.

4. Experimental

4.1. Chemistry

4.1.1. Instruments and analyses

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental Analyzer for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F_{254} plates were used as analytical TLC; column chromatography was performed on Merck Silica Gel 60 (70–230 mesh). ^{1}H NMR spectra were recorded in $CDCl_3$, CD_3OD, or DMSO-d_6 by means of a Varian Gemini 300 spectrometer. Chemical shifts are expressed in δ (ppm) relative to TMS as internal standard and coupling constants (J) in hertz. For compounds **6** and **21**, complete ^{1}H and ^{13}C assignments were made by means of HETCOR and LR-HETCOR spectra, carried out by using the standard software package.

4.1.2. 6,7-Methylenedioxy-1-(4-nitrophenyl)-2*H*-isoquinoline-3-one (12)

Methyl 2-[4,5-methylenedioxyphenyl-4-(nitrobenzoyl)]acetate **11** (103 mg, 0.3 mmol), obtained as previously described,[14] was dissolved in AcOH (15 mL) and then formamide (3 mL) was added. The solution was stirred for 6 h at reflux, then cooled and water (50 mL) was added. The mixture was extracted with $CHCl_3$ (3 × 10 mL) and the combined organic layers washed with water (3 × 20 mL), aq Na_2CO_3 (2 × 10 mL), then dried (Na_2SO_4). After removal of the solvent, the product was purified by column chromatography ($CHCl_3$/EtOH 90:10), yellowish powder. Mp >300 °C (60 mg, 64%) R_f = 0.27 ($CHCl_3$/EtOH 90:10). ^{1}H NMR (300 MHz, DMSO-d_6), δ 5.99 (s, 2H, OCH_2O), 6.21 (s, 1H, H-4), 6.73 (s, 1H, H-5), 6.77 (s, 1H, H-8), 7.69 (d, J = 8.2 Hz, 2H, H-2′,6′), 8.52 (d, J = 8.2 Hz, 2H, H-3′,5′).

4.1.3. 3-Acetoxy-1-(4-aminophenyl)-6,7-methylenedioxy-isoquinoline (3)

To a stirred solution of **12** (60 mg, 0.19 mmol) in CH_2Cl_2 (10 mL), acetyl chloride (15 μl, 0.22 mmol) was added dropwise over a few min. The reaction mixture was stirred for further 2 h at room temperature, then was poured into aq $NaHCO_3$ (20 mL, 5%) and extracted with $CHCl_3$ (3 × 10 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated in vacuo. The crude product was purified by column chromatography (CH_2Cl_2/MeOH 95:5) to afford nitroderivative as a yellowish powder. Yield: 77% (52 mg). Mp: 262–266 °C. R_f = 0.71 (CH_2Cl_2/MeOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.06 (s, 3H, CH_3), 5.95 (s, 2H, OCH_2O), 6.30 (s, 1H, H-4), 6.72 (s, 1H, H-5), 6.79 (s, 1H, H-8), 7.74 (d, J = 8.5 Hz, 2H, H-2′,6′), 8.50 (d, J = 8.5 Hz, 2H, H-3′,5′). Anal. Calcd for $C_{18}H_{12}N_2O_6$: C, 61.37; H, 3.43; N, 7.95. Found: C, 61.29; H, 3.58; N, 8.07. A solution of nitroderivative intermediate (52 mg, 0.15 mmol) in $CHCl_3$ (10 mL) was stirred under H_2 (1 atm) for 3 h, in the presence of a catalytic amount of 5% Pd/C (10 mol %). The mixture was then filtered through Celite, and the solvent was evaporated under reduced pressure. Column chromatography (EtOAc/MeOH 98:2) afforded pure compound **3** as a brownish powder. Yield: 80% (38 mg). Mp >300 °C (dec.). R_f = 0.58 (EtOAc/MeOH, 98:2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.02 (s, 3H, CH_3), 5.98 (s, 2H, OCH_2O), 6.28 (s, 1H, H-4), 6.69 (s, 1H, H-5), 6.84 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.01 (s, 1H, H-8), 7.18 (d, J = 8.8 Hz, 2H, H-2′,6′). Anal. Calcd for $C_{18}H_{14}N_2O_4$: C, 67.07; H, 4.38; N, 8.69. Found: C, 67.22; H, 4.50; N, 8.47.

4.1.4. Methyl 2-[2-(4-methoxybenzoyl)-4,5-methylenedioxy-phenyl] acetate (15)

To a solution of **13** (1.056 g, 5.46 mmol) in CH_2Cl_2 (10 mL), were added 4-methoxybenzoic acid (1.081 g, 7.1 mmol) and phosphorous pentoxide (2.0 g). The mixture was stirred at room temperature for 16 h, then water (30 mL) was cautiously added and the mixture was extracted with $CHCl_3$ (2 × 30 mL). The organic layer was separated and sequentially treated with 10% NaOH (30 mL), brine (30 mL) and water (2 × 30 mL). The organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure to yield crude which was purified by column chromatography (Et_2O/light petroleum 60:40), white needles. Yield: 64% (1.15 g). Mp: 100–102 °C. R_f = 0.31 (Et_2O/light petroleum, 50:50). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.57 (s, 3H, $COOCH_3$), 3.74 (s, 2H, CH_2), 3.85 (s, 3H, OCH_3), 5.99 (s, 2H, OCH_2O), 6.82 (s, 1H, H-6), 6.86 (s, H-3), 6.92 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.77 (d, J = 8.8 Hz, 2H, H-2′,6′).

4.1.5. Methyl 2-[4,5-dimethoxy-2-(4-methoxybenzoyl)-phenyl]acetate (16)

With a similar procedure, **16** was prepared from **14** (1.072 g, 5.10 mmol) by treatment with *p*-methoxybenzoic acid (1.008 g, 6.63 mmol) and phosphorous pentoxide (2.0 g). Eluent: Et_2O/light petroleum (60:40), whitish prisms. Yield: 62% (1.09 g). Mp: 112–114 °C. R_f = 0.31 (Et_2O/light petroleum, 60:40). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.60 (s, 3H, $COOCH_3$), 3.79 (s, 2H, CH_2), 3.81 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 6.85 (s, 1H, H-6), 6.93 (s, 1H, H-3), 6.95 (d, J = 7.7 Hz, 2H, H-3′,5′), 7.80 (d, J = 7.7 Hz, 2H, H-2′,6′).

4.1.6. 2-[2-(4-Methoxybenzoyl)-4,5-methylenedioxyphenyl]-acetic acid (17)

Compound **15** (1.15 g, 3.5 mmol) was dissolved in a mixture of THF/MeOH (4:1, 25 mL), then 1 N LiOH (5 mL) was added and the solution was stirred at room temperature for 4 h. The mixture was diluted with H_2O and extracted with diethyl ether. The aqueous phase was acidified with 2 N HCl and extracted with EtOAc (2 × 50 mL). The combined organic layers were dried (Na_2SO_4) and the solvent removed under reduced pressure. The crude was triturated with diethyl ether to afford the acid **17** as a light-brown powder. Yield: 96% (1.06 g). Mp: 216–220 °C. R_f = 0.31 (Et_2O/light petroleum, 60:40). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.66 (s, 2H, CH_2), 3.92 (s, 3H, OCH_3), 6.08 (s, 2H, OCH_2O), 6.92 (s, 1H, H-6), 6.97 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.00 (s, 1H, H-3), 7.85 (d, J = 8.8 Hz, 2H, H-2′,6′).

4.1.7. 2-[4,5-Dimethoxyphenyl-2-(4-methoxybenzoyl)]acetic acid (18)

With a similar procedure, **18** was prepared from **16** (500 mg, 1.45 mmol) and 1 N LiOH (2.5 mL). Whitish powder. Yield: 87% (420 mg). Mp: 134–136 °C. R_f = 0.78 ($CHCl_3$/EtOH, 90:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.73 (s, 2H, CH_2), 3.80 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.95–6.99 (m, 4H, H-6, H-3, H-3′,5′), 7.83 (d, J = 8.5 Hz, 2H, H-2′,6′).

4.1.8. *N*′-{2-[4,5-Methylenedioxy-2-(4-methoxybenzoyl)-phenyl]acetyl}-hydrazine carboxylic acid *tert*-butyl ester (19)

Compound **17** (250 mg, 0.79 mmol) was dissolved in CH_2Cl_2/DMF (1:1, 10 mL), then *tert*-butyl carbazate (136 mg, 1.03 mmol), and EDCI (230 mg, 1.18 mmol) were added and the reaction mixture was stirred at room temperature. After being stirred for 12 h it was neutralized with 2% HCl and then extracted with chloroform (2 × 15 mL). The combined organic layers were treated with brine and water then dried over Na_2SO_4 and the solvent removed under reduced pressure to yield a crude that was purified by column chromatography ($CHCl_3$/EtOAc 80:20), clear solid residue. Yield: 82% (280 mg). Mp: 116–120 °C. R_f = 0.41 ($CHCl_3$/EtOAc, 80:20). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 9H, *t*-Bu), 3.56 (s, 2H, CH_2), 3.88 (s, 3H, OCH_3), 6.01 (s, 2H, OCH_2O), 6.42 (br s, 1H, NH), 6.82 (s, 1H, H-6), 6.94 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.01 (s, 1H, H-3), 7.79 (d, J = 8.8 Hz, 2H, H-2′,6′). 9.29 (br s, 1H, NH).

4.1.9. *N′*-{2-[4,5-Dimethoxy-2-(4-methoxybenzoyl)phenyl]-acetyl}-hydrazine carboxylic acid *tert*-butyl ester (20)

With a similar procedure, **20** was prepared from **18** (420 mg, 1.27 mmol) using *tert*-butyl carbazate (218 mg, 1.65 mmol), and EDCI (365 mg, 1.91 mmol). Eluent: $CHCl_3$/EtOAc (80:20), whitish solid residue. Yield: 89% (500 mg). Mp: 161–163 °C. R_f = 0.43 ($CHCl_3$/EtOAc, 80:20). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, *t*-Bu), 3.60 (s, 2H, CH_2), 3.79 (s, 3H, OCH_3-4′), 3.90 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 6.51 (br s, 1H, NH), 6.89 (s, 1H, H-6), 6.97 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.05 (s, 1H, H-3), 7.82 (d, J = 8.8 Hz, 2H, H-2′,6′), 9.38 (br s, 1H, NH).

4.1.10. 2-*tert*-Butyloxycarbonylamino-6,7-methylenedioxy-1-(4-methoxyphenyl)-2*H*-isoquinolin-3-one (4)

Compound **19** (280 mg, 0.65 mmol) dissolved in dry THF (20 mL) was added dropwise to a solution of NaH (18 mg, 0.75 mmol) in dry THF (10 mL). The mixture was stirred at room temperature for 2 h then the precipitate obtained was filtered off and dissolved in EtOAc and washed with brine. The organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure. The crude was purified by column chromatography ($CHCl_3$/EtOH 95:5), bright-yellow powder. Yield: 91% (243 mg). Mp: 208–210 °C. R_f = 0.32 ($CHCl_3$/EtOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H, *t*-Bu), 3.93 (s, 3H, OCH_3), 5.95 (s, 2H, OCH_2O), 6.33 (s, 1H, H-8), 6.59 (s, 1H, H-5), 6.72 (s, 1H, H-4), 7.00–7.47 (m, 4H, Ar-*H*) 7.76 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{22}N_2O_6$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.23; H, 5.48; N, 6.97.

4.1.11. 2-*tert*-Butyloxycarbonylamino-6,7-dimethoxy-1-(4-methoxyphenyl)-2*H*-isoquinolin-3-one (5)

With a similar procedure, **5** was prepared from **20** (500 mg, 1.12 mmol) and 30 mg (1.25 mmol) of NaH in dry THF (50 mL). Eluent: $CHCl_3$/EtOH (95:5), yellow soft powder. Yield: 88% (422 mg). Mp: 228–230 °C R_f = 0.32 (EtOAc/*c*-hexane/*i*-PrOH, 60:30:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9H, *t*-Bu), 3.64 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.24 (s, 1H, H-8), 6.52 (s, 1H, H-5), 6.71 (s, 1H, H-4), 6.96–7.04 (m, 2H, Ar-*H*), 7.25–7.53 (m, 3H, 2H Ar-*H* and NH). Anal. Calcd for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.15; N, 6.57. Found: C, 64.93; H, 6.08; N, 6.69.

4.1.12. 2-Amino-1-(4-methoxyphenyl)-6,7-methylenedioxy-2*H*-isoquinolin-3-one (6)

Compound **4** (243 mg, 0.59 mmol) was dissolved in a mixture of CH_2Cl_2 and TFA (3:1, 10 mL). The resulting solution was stirred at room temperature for 5 h, then diluted with $CHCl_3$ and washed with a saturated solution of $NaHCO_3$, dried over Na_2SO_4 and evaporated under reduced pressure to yield a crude which was purified by column chromatography ($CHCl_3$/EtOH, 90:10), bright-yellow powder. Yield: 87% (160 mg). Mp: 188–190 °C R_f = 0.31 (CH_2Cl_2/EtOH 95:5). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.91 (s, 3H, OCH_3), 5.79 (s, 2H, NH_2), 5.93 (s, 2H, OCH_2O), 6.34 (s, 1H, H-8), 6.64 (s, 1H, H-5), 6.71 (s, 1H, H-4), 7.11 (d, J = 8.5 Hz, 2H, H-3′,5′), 7.34 (d, J = 8.5 Hz, 2H, H-2′,6′). ^{13}C NMR (75 MHz, $CDCl_3$) δ 55.39 (OCH_3), 99.30 (C-5), 101.10 (C-8), 101.23 (OCH_2O), 106.31 (C-4), 113.88 (C-8a), 114.66 (C-3′,5′), 123.64 (C-1′), 130.46 (C-1), 130.69 (C-2′,6′), 140.10 (C-6), 145.54 (C-7), 151.81 (C-4a), 156.80 (CO), 160.69 (C-4′). Anal. Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.67; H, 4.64; N, 9.16.

4.1.13. 2-Ethyloxycarbonylamino-1-(4-methoxyphenyl)-6,7-methylene-dioxy-2*H*-isoquinolin-3-one (7)

Ethylchloroformate (0.64 mmol, 0.062 mL) was added to a stirred CH_2Cl_2 solution (10 mL) of **6** (100 mg, 0.32 mmol) in the presence of Et_3N (excess). The mixture was further stirred at room temperature for 3 h (TLC monitoring), then was diluted with $CHCl_3$ (10 mL) and washed with water. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude was purified by a chromatography column (EtOAc/*c*-hexane/*i*-PrOH 60:30:10) to afford desired compound **7** as a dark yellow powder. Yield: 72% (89 mg). Mp: 224–227 °C (dec.) R_f = 0.39 (EtOAc/*c*-hexane/*i*-PrOH, 60:30:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (t, 3H, J = 6.9 Hz, $COOCH_2CH_3$), 3.89 (s, 3H, OCH_3), 4.02 (q, 2H, J = 6.9 Hz, $COOCH_2CH_3$), 5.93 (s, 2H, OCH_2O), 6.30 (s, 1H, H-8), 6.57 (s, 1H, H-5), 6.69 (s, 1H, H-4), 6.97–7.05 (m, 2H, Ar-*H*), 7.28–7.45 (m, 2H, Ar-*H*), 8.15 (br s, NH). Anal. Calcd for $C_{20}H_{18}N_2O_6$: C, 62.82; H, 4.74; N, 7.33. Found: C, 62.96; H, 4.61; N, 7.12.

4.1.14. 2-(4-Acetylphenylureido)-1-(4-methoxyphenyl)-6,7-methylenedioxy-2*H*-isoquinolin-3-one (8)

4-Acetylphenylisocyanate (93.5 mg, 0.58 mmol) was added to a solution of **6** (90 mg, 0.29 mmol) in dry CH_2Cl_2 The mixture was stirred under nitrogen at room temperature until the disappearing of the starting material, then the solution was diluted with $CHCl_3$ and washed with water. The organic layer was dried over Na_2SO_4 and the solvent removed in vacuo. The crude was purified by a chromatographic column (EtOAc/*c*-hexane/*i*-PrOH, 60:30:10) to afford the desired compound **8** as a yellowish powder. Yield: 68% (93 mg). Mp: 211–215 °C. R_f = 0.40 (EtOAc/*c*-hexane/*i*-PrOH 60:30:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.39 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 5.98 (s, 2H, OCH_2O), 6.37 (s, 1H, H-8), 6.67 (s, 1H, H-5), 6.77 (s, 1H, H-4), 6.97–7.56 (m, 8H, Ar-*H*), 9.12 (br s, NH), 9.65 (br s, NH). Anal. Calcd for $C_{26}H_{21}N_3O_6$: C, 66.24; H, 4.49; N, 8.91. Found: C, 66.04; H, 4.66; N, 8.81.

4.1.15. 1-(4-Methoxyphenyl)-6,7-methylenedioxy-2-(4-methylthiophenyl-ureido)-2*H*-isoquinolin-3-one (9)

With the same procedure, **9** was obtained from **6** (113 mg, 0.36 mmol) and 4-methyltiophenylisocyanate (0.73 mmol, 0.1 mL). In this case, the solvent was removed in vacuo and the residue triturated with Et_2O. The obtained solid was filtered off to afford pure **9** as a yellow powder. Yield: 73% (126 mg). Mp: 228–232 °C. R_f = 0.72 (EtOAc/*c*-hexane/*i*-PrOH, 60:30:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.36 (s, 3H, SCH_3), 3.86 (s, 3H, OCH_3), 5.97 (s, 2H, OCH_2O), 6.37 (s, 1H, H-8), 6.67 (s, 1H, H-5), 6.81 (s, 1H, H-4), 6.98–7.59 (m, 8H, Ar-*H*), 8.62 (br s, 1H, NH), 9.43 (br s, 1H, NH). Anal. Calcd for $C_{25}H_{21}N_3O_5S$: C, 63.15; H, 4.45; N, 8.84. Found: C, 62.88; H, 4.28; N, 9.03.

4.1.16. [4,5-Dihydroxy-2-(4-methoxybenzoyl)-phenyl]acetic acid (21)

An ice-cold dichloromethane solution (15 mL) of **15** (250 mg, 0.76 mmol) was charged dropwise with BBr_3 (2.28 mL of 1 M solution in CH_2Cl_2), and equipped with a reflux condenser. The mixture was stirred at room temperature for 3 h, forming a viscous, deep-red solution; at this time, the reaction was cautiously quenched with MeOH (5 mL) and the solvent was removed in vacuo. This process was repeated until a clear red color solution was restored. The resulting sticky residue was purified by column chromatography (1% HCOOH in EtOAc/*c*-hexane (50:50), light-brown residue. Yield: 68% (156 mg). Mp: 167–170 °C R_f = 0.40 (1% HCOOH in $CHCl_3$/EtOAc, 50:50). ^{1}H NMR (300 MHz, CD_3OD) δ 3.71 (s, 2H, CH_2), 3.89 (s, 3H, OCH_3), 6.84 (s, 1H, H-6), 6.90 (s, 1H, H-3), 7.01 (d, J = 8.5 Hz, 2H, H-3′,5′), 7.76 (d, J = 8.5 Hz, 2H, H-2′,6′). ^{13}C NMR (75 MHz, CD_3OD) δ 39.35 (CH_2), 56.05 (OCH_3), 114.58 (C-3′,5′), 119.38 (C-3), 119.94 (C-6), 128.60 (C-1), 130.84 (C-2), 132.33 (C-1′), 133.72 (C-2′,6′), 144.31 (C-4), 149.37 (C-5), 165.03 (C-4′), 175.52 (COOH), 198.48 (CO).

4.1.17. Benzyl [4,5-dibenzyloxy-2-(4-methoxybenzoyl)-phenyl]acetate (22)

To a solution of **21** (156 mg, 0.52 mmol) in acetone (50 mL) was added K_2CO_3 (862 mg, 6.24 mmol) and benzyl bromide (0.2 mL,

1.98 mmol). The reaction mixture was heated at reflux for 5–6 h, then cooled to 0 °C to ensure complete precipitation of the base. The supernatant was isolated by filtration, and concentrated under reduced pressure. The residue was dissolved with ethyl acetate and washed with 10% HCl. The organic layer was dried (Na_2SO_4) and reduced under vacuum to a residue which furnished compound **22** by triturating with Et_2O. Light-yellow powder. Yield: 55% (164 mg). Mp: 185–187 °C. R_f = 0.36 (Et_2O/light petroleum, 80:20). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.81 (s, 2H, CH_2), 3.87 (s, 3H, OCH_3), 5.02 (s, 2H, OCH_2Ph), 5.09 (s, 2H, OCH_2Ph), 5.19 (s, 2H, OCH_2Ph), 6.82 (d, J = 8.5 Hz, 2H, H-3′,5′), 6.93 (s, 1H, H-6), 6.98 (s, 1H, H-3), 7.19–7.53 (m, 15H, Ar-*H*), 7.63 (d, J = 8.5 Hz, 2H, H-2′,6′). Anal. Calcd for $C_{37}H_{32}O_6$: C, 77.60; H, 5.63. Found: C, 77.42; H, 5.76.

4.1.18. [4,5-Dibenzyloxy-2-(4-methoxybenzoyl)-phenyl]acetic acid (23)

Following the same procedure employed for the synthesis of ketoacid **17**, **23** was obtained from **22** (164 mg, 0.29 mmol) and 1 N LiOH (2 mL). In this case, the reaction was prolonged for 8 h. Eluent: $CHCl_3$/MeOH (90:10), light-brown powder. Yield: 95% (131 mg). Mp: 226–229 °C. R_f = 0.34 ($CHCl_3$/MeOH, 90:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.65 (s, 2H, CH_2), 3.91 (s, 3H, OCH_3), 5.11 (s, 2H, OCH_2Ph), 5.25 (s, 2H, OCH_2Ph), 6.88 (d, J = 8.8 Hz, 2H, H-3′,5′), 6.99 (s, 1H, H-6), 7.10 (s, 1H, H-3), 7.27–7.55 (m, 10H, Ar-*H*), 7.67 (d, J = 8.8 Hz, 2H, H-2′,6′).

4.1.19. *N*′-{2-[4,5-Dibenzyloxy-2-(4-methoxybenzoyl)-phenyl]-acetyl}hydrazine carboxylic acid *tert*-butyl ester (24)

With the same procedure employed for the synthesis of intermediate **19, 24** was obtained from **23** (131 mg, 0.27 mmol) using *tert*-butyl carbazate (46 mg, 0.35 mmol), and EDCI (79 mg, 0.41 mmol). Eluent: $CHCl_3$/EtOAc (80:20), yellowish solid residue. Yield: 79% (128 mg). Mp: 189–193 °C. R_f = 0.29 ($CHCl_3$/EtOAc, 80:20). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 9H, *t*-Bu), 3.56 (s, 2H, CH_2), 3.88 (s, 3H, OCH_3), 5.07 (s, 2H, OCH_2Ph), 5.22 (s, 2H, OCH_2Ph), 6.44 (br s, 1H, NH), 6.85 (d, J = 9.0 Hz, 2H, H-3′,5′), 6.94 (s, 1H, H-6), 7.16 (s, 1H, H-3), 7.25–7.53 (m, 10H, Ar-*H*), 7.64 (d, J = 9.0 Hz, 2H, H-2′,6′), 9.31 (br s, 1H, NH).

4.1.20. 2-Amino-6,7-dibenzyloxy-1-(4-methoxyphenyl)-2*H*-isoquinolin-3-one (10)

With a similar procedure employed for the synthesis of compounds **4–5, 10** was prepared from **24** (128 mg, 0.21 mmol) and NaH (6 mg, 0.25 mmol) in dry THF (15 mL). Yellow powder. Yield: 82% (102 mg). Mp: 199–202 °C. R_f = 0.47 ($CHCl_3$/MeOH, 95:5). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, *t*-Bu), 3.90 (s, 3H, OCH_3), 5.09 (s, 2H, OCH_2Ph), 5.24 (s, 2H, OCH_2Ph), 6.05 (s, 1H, H-8), 6.41 (s, 1H, H-5), 6.70 (s, 1H, H-4), 6.94 (d, J = 8.8 Hz, 2H, H-3′,5′), 7.27–7.50 (m, 7H, Ar-*H*). The obtained intermediate was then deprotected with TFA as previously described, and purified by chromatographic column (EtOAc/*c*-hexane/*i*-PrOH 70:20:10) to afford the desired compound **10** as a dark yellow powder. Overall yield: 75% (75 mg). Mp: 175–177 °C. R_f = 0.32 (EtOAc/*c*-hexane/*i*-PrOH, 70:20:10). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.94 (s, 3H, OCH_3), 4.94 (s, 2H, OCH_2Ph), 5.23 (s, 2H, OCH_2Ph), 5.78 (s, 2H, NH_2), 6.35 (s, 1H, H-8), 6.66 (s, 1H, H-5), 6.67 (s, 1H, H-4), 7.07 (d, J = 8.5 Hz, 2H, H-3′,5′), 7.20–7.48 (m, 12H, Ar-*H*). Anal. Calcd for $C_{30}H_{26}N_2O_4$: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.16; H, 5.61; N, 5.91.

4.2. Pharmacology

4.2.1. Enzyme assays

The preliminary screening was performed with 100 μM inhibitor concentrations using an equivalent amount of DMSO as negative control. Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 40 μM for FP-2 and 10 μM for rhodesain) was determined continuously over a period of 10 min. Compounds showing at least 50% inhibition were subjected to detailed assays. These were performed in a 100 mM sodium acetate buffer, pH 5.5 containing 10 mM DTT with Cbz-Phe-Arg-AMC (40 or 10 μM) as substrate.[17] The K_m values used to correct K_{iapp} values was determined to 21.5 μM (FP-2) and 0,9 μM (rhodesain).[19] Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed twice in 96-well plates in a total volume of 300 μL. A Varian Cary Eclipse spectrofluorometer Varian, Darmstadt, Germany with a microplate reader (excitation 365 nm, emission 460 nm) was used. Standard units of FP-2 were added to the reaction mixture containing the fluorogenic substrate and increasing from 0 to 100 μM concentrations of compounds. K_{inac} was obtained by a Dixon plot[20] using equation $[E]_0/[E]_a = 1+[I]/K_{iapp}$ and correction to zero substrate concentration from $K_i = K_{iapp}/(1 + [S]K_m^{-1})$ with $[E]_0$ as enzyme activity in the absence, and $[E]_a$ as residual enzyme activities in the presence of the inhibitor. Assays with cathepsins B and L were performed as described previously.[21] Cbz-Phe-Arg-AMC was used as substrate (80 μM for cathepsin B, 5 μM for cathepsin L). The K_m values used to correct K_{iapp} values were 150 μM (cathepsin B) and 6.5 μM (cathepsin L).

4.2.2. Drug screening on *P. falciparum* cultures

The compounds were screened in quadruplicates 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.[23] Parasites were cultured in human red blood cells (RBC) (blood group ARh+25) in RPMI 1640 medium supplemented with 25 mM HEPES (Molecular Probes, Invitrogen), 20 mM sodium bicarbonate, and 0.5% AlbuMAX I (Molecular Probes, Invitrogen) at 2.5% (v/v) hematocrit. Cultures were maintained at 37 °C with a gaseous phase 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 the compounds (dissolved in DMSO and diluted 10-fold in 50% ethanol before added to the cells). Incubation of parasites with an equal concentration of 50% ethanol alone was used as a negative control. To kill the plasmodia completely for the positive control, the parasites were incubated in presence of 2 μM chloroquine. The viability of the parasites was screened subsequently using the assay according to Evers et al. (2008).[18]

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