Novel Peptidomimetic Cysteine Protease Inhibitors as Potential Antimalarial Agents

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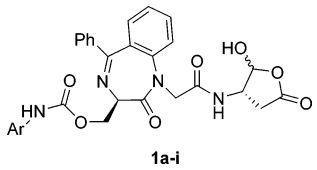
Abstract: The synthesis of a new class of peptidomimetics **1a**−**j**, based on a 1,4-benzodiazepine scaffold and on a C-terminal aspartyl aldehyde building block, is described. Compounds **1a**−**j** provided significant inhibitory activity against falcipains 2A and 2B (FP-2A and FP-2B), two cysteine proteases from *Plasmodium falciparum*.

Malaria, in particular that caused by *Plasmodium falciparum*, remains a serious health problem in Africa, South America, and many parts of Asia where it afflicts about 500 million people and is responsible for the death of two million children each year.[1] The main reasons for the persistence of malaria are the emergence of resistance to common antimalarial drugs,[2] cross-resistance to structurally unrelated drugs, inadequate control of mosquito vectors, and the lack of effective vaccines. Therefore, the identification and characterization of new targets for antimalarial chemotherapy are of urgent priority.

Several proteases are involved in the life cycle of malaria parasites,[3] including aspartic proteases named plasmepsins, cysteine proteases named falcipains, and a metalloprotease named falcilysin. In the erythrocytic stage of infection, these proteases participate in the hemoglobin hydrolysis within the parasite food vacuole to provide amino acids required for parasite survival. Among them, a cysteine protease from *P. falciparum*, falcipain-2 (FP-2), has been perceived as one of the most promising targets for antimalarial drug design because of its dual function: (i) it degrades hemoglobin at the early trophozoite stage; (ii) it cleaves ankyrin and protein 4.1, the cytoskeletal elements vital to the stability of the red cell membrane at the schizont stage.[4]

Recently, it has been demonstrated that *P. falciparum* contains two nearly identical copies of the FP-2 gene located on chromosome 11, encoding two distinct enzymes, namely, FP-2A and FP-2B.[5] The FP-2A corresponds to the previously known enzyme designated as FP-2. The FP-2A and FP-2B enzymes are 97% identical at the amino acid level. Their amino acid sequences differ at seven positions within their mature forms, including three amino acid replacements localized close to residues that are predicted to interact with substrate, and consequently may affect its affinity or specificity. The physiological role of FP-2B and the functional significance of the amino acid variations between FP-2 enzymes remain unclear. Like FP-2A, the recombinant FP-2B also functions as a hemoglobinase.

Chart 1



1a-i

Several peptide-based FP-2 inhibitors have been identified. These derivatives bear the most popular pharmacophores of cysteine protease inhibitors such as vinylsulfones,[6] ketones,[7] and aldehydes.[8] The activity of these electrophilic moieties is dependent on their capability to form a covalent bond with a cysteine residue present in the active site of the enzyme. Generally, peptidyl inhibitors have poor pharmacokinetic profiles because their amide bonds are susceptible to cleavage by other proteases.[9]

In the field of peptidomimetics, incorporation of a nonpeptidic scaffold into an amino acidic sequence and of unnatural amino acids has potential advantages that include increased potency and selectivity by stabilizing a biologically active conformation, increased membrane permeability, and enhanced oral bioavailability and stability toward degradation by enzymes.[10] Moreover, it is desirable to design reversible inhibitors (e.g., aldehydes) to minimize the potential toxicity that can be observed with irreversible inhibitors (e.g., vinylsulfones).

Taking into account these factors, we combined these observations and designed new FP-2 inhibitors based on a 1,4-benzodiazepine (BDZ) scaffold introduced internally to a peptide sequence, which mimics the fragment D-Ser-Gly, and on a C-terminal aspartyl aldehyde building block, which inhibits the enzyme by forming a reversible covalent bond at the active site.[11] This approach, which employs the BDZ scaffold, has already been successfully applied in the peptidomimetic field. Furthermore, BDZs represent a well-known class of drugs that are well tolerated and exhibit good oral bioavailability.[12]

We report herein the synthesis of a series of arylcarbamic acid 1-[(2-hydroxy-5-oxotetrahydrofuran-3(*S*)-ylcarbamoyl)-methyl]-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3(*R*)-yl methyl esters (**1a**−**i**, Chart 1) and their activity as FP-2 inhibitors.

As described in Scheme 1, the preparation of oxazolidine derivative **4**, the key intermediate for the construction of the 3-hydroxymethyl-1,4-benzodiazepine nucleus, was easily achieved starting from commercially available D-serine methyl ester hydrochloride (**2**), according to the procedure developed by McKillop et al.[13]

The acid **4**, obtained from the hydrolysis of intermediate **3**, was then converted into a mixed anhydride with isobutyl chloroformate in the presence of *N*-methylmorpholine and condensed in situ with 2-aminobenzophenone to afford derivative **5**. Intermediate **5** was refluxed under acidic conditions to induce the Boc deprotection of the nitrogen and the cleavage of the oxazolidine ring to afford the 3-hydroxymethyl substituted 1,4-benzodiazepine **6**. The protection of the hydroxy group with *tert*-butyldimethylsilyl chloride gave **7**, which was subsequently reacted with sodium hydride and ethyl bromoacetate in DMF to introduce the side chain at N-1 of the BDZ nucleus (**8**).

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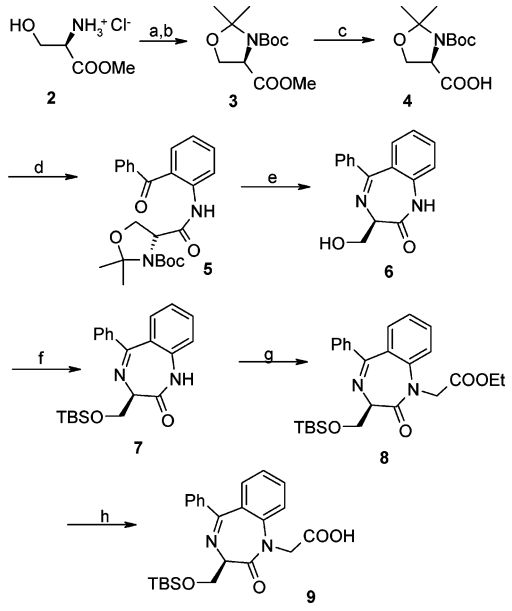
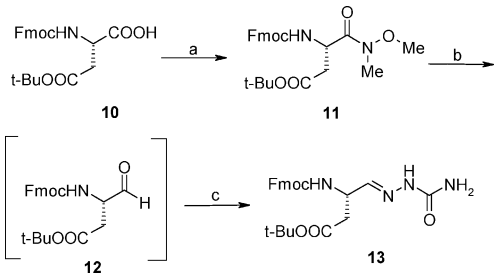
Scheme 1[a]



[a] Reagents: (a) aqueous $NaHCO_3$/THF (2/8), $(Boc)_2O$, room temp, 12 h (96%); (b) 2,2-DMP, $BF_3 \cdot Et_2O$, acetone, room temp, 2 h (95%); (c) 1 N LiOH, MeOH, 0 °C to room temp, 6 h (86%); (d) *i*-BuOCOCl, *N*-methylmorpholine, CH_2Cl_2, 0 °C to room temp, 30 min, then 2-aminobenzophenone, reflux, 20 min, then room temp, 13 h (79%); (e) 5% HCl/MeOH (1/5), reflux, 7 h (85%); (f) TBS-Cl, imidazole, CH_2Cl_2, 0 °C to room temp, 12 h (87%); (g) NaH, DMF, 0 °C to room temp, 30 min, then $BrCH_2COOEt$, 0 °C to room temp, 5 h (78%); (h) 1 N LiOH, MeOH, 0 °C to room temp, 6 h (82%).

Scheme 2[a]



[a] Reagents: (a) *i*-BuOCOCl, *N*-methylmorpholine, CH_2Cl_2, 0 °C to room temp, 30 min, then MeONHMe·HCl, room temp, 15 h (86%); (b) LAH, THF, −65 °C to room temp, 2.5 h; (c) $NH_2NHCONH_2 \cdot HCl$, CH_3COONa, EtOH/H_2O (7/3), 0 °C to room temp, 15 h (55%).

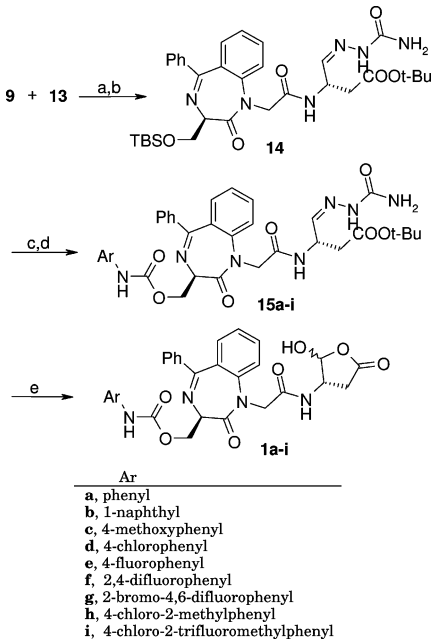
Finally, the ethyl ester group was hydrolyzed with 1 N LiOH to afford derivative **9**, which represents the constrained form of the dipeptidic fragment Ser-Gly.

To synthesize the aspartyl aldehyde building block, we chose a method that entailed the use of the stereochemically stable semicarbazone derivative **13** as a masked aldehyde equivalent.[14,15] This general strategy has been used fruitfully to prepare other peptide aldehydes in solution[14,16] and on a solid support.[17,18]

The reaction sequence used to construct semicarbazone **13** is shown in Scheme 2. Commercially available Fmoc-*β*-(*tert*-butyl ester)-L-aspartic acid (**10**) was converted into its Weinreb amide **11**, followed by selective reduction of the amide moiety with lithium aluminum hydride.[19] The obtained crude aldehyde **12** was immediately reacted with semicarbazide hydrochloride to afford semicarbazone **13**.

This aspartyl aldehyde synthon **13**, after removal of the Fmoc protecting group by diethylamine, was coupled to the carboxylic acid **9** by using ethyl(dimethylaminopropyl)carbodiimide (EDCI)/

Scheme 3[a]



Ar
a, phenyl
b, 1-naphthyl
c, 4-methoxyphenyl
d, 4-chlorophenyl
e, 4-fluorophenyl
f, 2,4-difluorophenyl
g, 2-bromo-4,6-difluorophenyl
h, 4-chloro-2-methylphenyl
i, 4-chloro-2-trifluoromethylphenyl

[a] Reagents: (a) CH_3CN/Et_2NH (5/1), room temp, 2 h; (b) EDCI, HOBt, DMF/CH_2Cl_2, (1/1), room temp, 15 h (65%); (c) TBAF, THF, room temp, 5 h (84%); (d) ArNCO, CH_2Cl_2, room temp, 12 h (66−80%); (e) TFA/CH_2Cl_2 (1/3), room temp, 6 h, then 37% HCHO/AcOH/MeOH (1/1/3), room temp, 20 h (19−42%).

Table 1

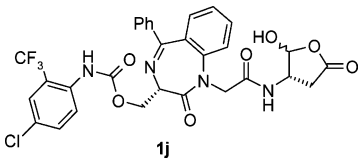
compd	FP-2A IC_{50}, μM	FP-2B IC_{50}, μM
1a	21.54 (15.97−29.06)	19.79 (15.49−25.29)
1b	11.19 (6.23−20.07)	17.94 (12.08−26.66)
1c	8.79 (5.97−12.95)	14.01 (8.70−22.57)
1d	15.04 (11.60−19.49)	9.53 (6.48−14.01)
1e	16.34 (12.29−21.71)	11.95 (8.80−16.24)
1f	13.51 (10.27−17.77)	14.70 (10.68−20.25)
1g	19.15 (15.20−24.12)	21.39 (16.50−27.72)
1h	8.25 (4.47−15.21)	11.19 (6.23−20.07)
1i	8.68 (5.86−12.84)	25.90 (11.27−59.52)
1j	10.29 (6.46−16.39)	19.31 (11.91−31.30)
MDL 28170	5.0	5.0

1-hydroxybenzotriazole (HOBt) to give **14** (Scheme 3). Desilylation of **14** followed by treatment with various aryl isocyanates afforded intermediates **15a**−**i** in high yield (70−80%). Finally, removal of *tert*-butyl ester and semicarbazone protecting groups by treatment with TFA and formaldehyde/acetic acid/methanol provided target compounds **1a**−**i**.

All compounds were tested on recombinant FP-2A and FP-2B expressed in *E. coli* as a fusion with maltose binding protein (MBP), in a fluorescence-based assay, and the results, expressed as IC_{50} values, are reported in Table 1.

The rate of hydrolysis of the fluorogenic substrate Z-Leu-Arg-7-amino-4-methylcoumarin (Z-Leu-Arg-AMC) in the presence of inhibitors at various concentrations was compared with the rate of hydrolysis in negative controls incubated with an equivalent volume of DMSO and with positive controls incubated with MDL 28170 (Z-Val-Phe-CHO), a standard inhibitor of papain-like family cysteine proteases.[20] As can be seen from the data reported in Table 1, in general, all our new peptidomimetic derivatives provided significant inhibition of FP-2A and FP-2B with IC_{50} in the range 8−26 μM. It is not known what effect, if any, the presence of the MBP tag has on the IC_{50} of various inhibitors. Experimentally, the isolation and biochemical separation of native FP-2A and FP-2B enzymes from *Plasmodium falciparum* are not feasible at this stage.

Chart 2



1j

In this test, phenyl derivative **1a** is the least potent inhibitor ($IC_{50} \approx 20$ μM on both the enzymes). The insertion of an electron-donating or electron-withdrawing group at position 4 of the phenyl ring of the carbamoyl moiety increased the inhibitory activity (e.g., **1c**).

By introduction of a methyl (**1h**) or a trifluoromethyl (**1i**) group at position 2 of 4-chloroderivative **1d**, an enhancement of the inhibitory properties was observed. Also, the extension of the aromatic area (**1b**) seems to be fruitful.

It is noteworthy that our compounds inhibit FP-2A and FP-2B at a similar level. This is of utmost importance, since a recent gene disruption study on FP-2A knockout parasites revealed that the loss of FP-2A seems to be compensated by the increased expression of FP-2B.[5b] This implies that the drug discovery process should be focused on the identification of compounds capable of inhibiting all the essential FPs.[5c]

To check the selectivity toward other cysteine proteases, these compounds were tested against a panel of active recombinant human caspases (i.e., caspases 1−9) according to the published procedure.[21] These compounds did not show any inhibitory activity up to 50 μM.

Another experimental focus was to evaluate the impact of chirality at the P_3 site on the inhibitory activity against FPs. In **1a**−**i**, an unnatural serine is incorporated in the peptide sequence. Thereby, in one of the most interesting compounds (**1i**), we replaced this amino acid with its natural counterpart. Compound **1j** (Chart 2) was synthesized following the same procedure described above but with L-serine methyl ester hydrochloride used as the starting material.

A comparison of the IC_{50} of diastereomers **1i** and **1j** indicates that the stereochemistry at the P_3 site does not significantly affect the inhibitory potency of this class of derivatives against FPs.

In summary, we synthesized a new class of peptidomimetics, incorporating a 1,4-benzodiazepin-2-one framework, which proved to inhibit FP-2A and FP-2B enzymes. Further biological experiments will be required to assess the significance of these findings to malaria infection in red blood cells both in vitro and in vivo. These results will form the basis for the development of novel antimalarial drugs.

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Supporting Information Available: Experimental procedures, characterization of new compounds, and references to known procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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