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Peptidomimetic nitrile inhibitors of malarial protease falcipain-2 with high selectivity against human cathepsins

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ABSTRACT

Falcipain-2 (FP2) is an essential enzyme in the lifecycle of malaria parasites such as Plasmodium falciparum, and its inhibition is viewed as an attractive mechanism of action for new anti-malarial agents. Selective inhibition of FP2 with respect to a family of human cysteine proteases (that include cathepsins B, K, L and S) is likely to be required for the development of agents targeting FP2. Here we describe a series of *P2*-modified aminonitrile based inhibitors of FP2 that provide a clear strategy toward addressing selectivity for the P. falciparum and show that it can provide potent FP2 inhibitors with strong selectivity against all four of these human cathepsin isoforms.

Malarial infections are caused by blood-borne parasites from the genus *Plasmodium* that are transmitted to humans by bites from >30 species of female anopheles mosquitoes. The most deadly and prevalent *Plasmodium* species *P. falciparum* contributed to over 216 million cases of malaria in 2016 according to the WHO.[1] The worldwide health problem is further underlined by the susceptibility of children under five years old to malaria, with over 300,000 deaths having been recorded over the same time period. In light of the extent and severity of the malaria problem there remains a clear need for the discovery of new anti-infective agents to combat the disease, and new mechanisms of action are of especial interest as a means to combat increasingly prevalent drug resistance.[2,3]

The inability of *P. falciparum* parasites to biosynthesize some amino acids necessitates that they obtain these essential building blocks from host erythrocytes during the blood-phase of the parasite life-cycle.[4] Parasite survival ultimately depends on the ability to generate free amino acids by degradation of human hemoglobin. Numerous proteases are involved in this degradation cascade, including both host and parasite encoded enzymes. A parasite encoded family of papain-like cysteine proteases known as clan CA or falcipain plays a key role in hemoglobin degradation during the erythrocyte phase of the malaria cycle. Falcipain-2 (FP2) has been shown to be indispensable for parasite growth[2] and inhibitors of FP2 have thus emerged as a promising approach for the development antimalarial drugs.[5] The role of the related parasite falcipain-3 (FP3) enzyme is less well demonstrated but while its endogenous concentration in trophozoites is around 2-fold lower than FP2 its hemoglobin cleavage is around twice as rapid, suggesting that its overall contribution in the digestion process may be similar.[4]

Although targeting FP2 (and FP3) is an attractive therapeutic strategy, a concern is the potential for off-target effects on homologous cysteine protease enzymes in the mammalian host. Drug discovery efforts focused on vacuolar FP's should therefore incorporate measures to address the selectivity of inhibitors for parasite cysteine proteases over human homologues such as cathepsin B, K, S and L. The recent demonstration that cruzain inhibitors with unique *in vitro* selectivity for parasite *vs*. mammalian cysteine proteases offer new opportunities for the treatment of Chagas disease underlines the value of such an approach.[6] Our interest in the development of new antimalarials fostered the initiation of a research program targeting FP2 inhibitors as a complement to work ongoing in our laboratories toward *P. falciparum* histone deacetylase (HDAC) inhibitors.[7] The generation of a compound series with promising selectivity over human cathepsin enzymes was a stated goal at the outset of this work, and we report herein a series of potent and highly selective FP2 inhibitors.

Our studies initiated from two known inhibitors **1** and **2** (Fig. 1)[8,9] that were synthesized as biological tools and that were profiled in our in-house assay panel. Compound **1** inhibited the isolated FP2 enzyme with IC_{50} 8 nM but in our hands was essentially

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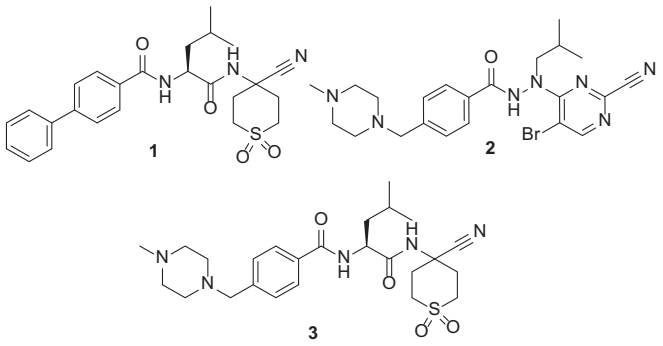

Fig. 1. Literature peptidomimetic aminonitrile based inhibitors of falcipain-2 (**1** and **2**) and a hybrid structure (**3**).

inactive (EC_{50} >25 μM) in a parasite growth assay in human erythrocytes. Compound **2** showed strong FP2 enzyme inhibition (0.6 nM) and its activity in our parasite growth assay proved analogous to the literature reported value.[9] To investigate the weak activity of **1** on parasites, hybrid structures such as compound **3** were prepared and despite somewhat lower FP2 enzyme inhibition were found to restore activity in the *P. falciparum* growth assay (EC_{50} 2.7 μM). Compounds **1**–**3** are peptidic in nature with an electrophilic "warhead" in position P1[8] designed to engage the active-site thiol of the target enzyme in a reversible covalent carbon–sulfur bond. However, as the panel of *in vitro* activities for these compounds shown in Table 1 illustrates, all three of these inhibitors have rather limited selectivity against human cysteine proteases from the cathepsin family. The weak selectivity of compounds such as **1** and **3** (that derive from counter-screening human cathepsin inhibitor libraries[8]) is perhaps unsurprising, and clearly efforts to develop more selective peptidomimetic nitriles are required.

While the S1 and S3 subsite residues are fairly well conserved between *Plasmodium* and human cysteine proteases, the S2 site is more varied. The S2 pocket is known to be an important determinant of specificity for cysteine proteases[10] and although S2 is predominantly hydrophobic in character, a striking difference is the presence of an acidic amino acid residue in S2 for the falcipain enzymes (Asp234 and Glu243 in FP2 and FP3, respectively) in place of a neutral amino acid in Cat-K (Leu209), Cat-L (Ala215) and Cat-S (Phe211). Although cathepsin B also contains an acidic residue in *P*2 (Glu245) the presence of a proline residue (Pro76) in place of Ile85 in FP2 results in a substantially changed shape for the *P*2 pocket of cathepsin B (suggesting that selectivity ought to be more readily achievable against this isoform). A degree of success toward achieving selectivity (c. 5-fold) for FP2 against cathepsins B and L has been achieved through changes at *P*2 in a related series of peptidomimetic nitriles[11] and based on the structural considerations and precedents described above our efforts to identify FP2 inhibitors structurally related to compound **3** but with improved selectivity over human cathepsins focused on changes at *P*2. An initial library of around 35 peptidomimetic nitrile inhibitors was prepared to explore both basic and hydrophobic amino acids in place of the leucine residue (the preferred *P*2 residue in FP2 substrates) that is present in **3**. The structure activity relationship (SAR) trends that emerged from this work, which was performed using compound **4** as reference, are summarized in Table 2. Compound **4** itself has a similar activity profile as its direct analog **1** and the presence of the 1-aminocyclopropane-1-carbonitrile based *P*1 residue[8] (for which the synthetic precursor is commercially available) simplified the synthetic work.

Despite the presence of an acidic residue in the S2 pocket of falcipain little success was achieved with aliphatic basic *P*2 residues (e.g. lysine analog **5**). It has been proposed[11] that the slightly deeper S2 pocket in falcipain may more readily accommodate salt-bridging interactions from longer (basic) *P*2 side chains, but disappointingly both the homo-lysine analog **6** and a number of structurally diverse analogs containing basic amines (such as compound **7**) showed weak activity across the full panel of enzymes. Phe analog **8** was (as expected based on known falcipain substrate specificity) less active than the leucine analog **4** against FP2, and approaches to introduce substituents on the phenyl ring (e.g. the weakly basic 4-dimethylamino analog **9** or the ether **10**) consistently worsened FP2 activity rather than improving it. However replacement of the phenyl ring with heterocyclic groups led to the best results from this compound library. In particular the 3-pyridine analog **11** stood out with comparable FP2 inhibition to the original lead **4**, strong selectivity over cathepsins B and L and around 10-fold selectivity against cathepsins K and S. Remarkably neither the 2- nor the 4-pyridine isomers (**12** and **13** respectively) showed a comparable profile, with both of these compounds proving inactive at the highest concentration at which they were tested (5 μM) against FP2. A selective profile was also identified for the thiazole analog **14**, though activity for this compound against FP2 was somewhat weaker (6-fold) than pyridine **11**. The trend toward a selective profile for compounds containing 3-pyridine substituents in the S2 pocket was confirmed by data for analogs **15** and **16**. Although these compounds were 10-fold weaker FP2 inhibitors than **11** they again displayed strong selectivity against cathepsin isoforms B, K and L. In line with the expected canonical binding of the *P*2 residue in the FP2 S2 pocket the d-enantiomer of **11** (compound **17**) was completely inactive. Around 20% of the compounds made in this initial array aimed to position a bicyclic heterocycle in the *P*2 pocket. Compounds of this type were generally very weak inhibitors of the FP2 enzyme but tended to show rather selective behavior for the human cathepsin S isoform (e.g. **18**). In only one case was sub-micromolar FP2 enzyme inhibition observed when a bicyclic heterocyclic substituent was incorporated at *P*2 (the hydroxytryptophan analog **19**). Further profiling of compound **11** demonstrated that it retained nanomolar inhibition of the falcipain-3 enzyme (IC_{50} 117 nM) but that it had weak anti-parasitic activity (EC_{50} > 25 μM) in our erythrocyte-based plasmodium growth assay. The lower cell based potency for compound **11** with respect to **3** likely reflects its increased lipophilicity and the absence of a basic group that may favor activity in the acidic environment of the parasite food vacuole. A combination of structural changes at *P*3 and *P*1 was explored to improve the potency and selectivity of compound **11** and the results from this work are presented in Table 3. Although the direct *P*2-pyridine analog of compound **3** was not made, a related analog (thiophene **20**) was found to show improved selectivity against cathepsins K and S. While the lead compound **11** was 9-fold selective against cathepsin K and 11-fold selective against cathepsin S, compound **20** was > 30-fold selective against both isoforms. The strong selectivity over cathepsin isoforms B and L that had been observed for the lead compound **11** was retained in **20** and cell based potency was restored to low micromolar levels (EC_{50} 7 μM). A geminally-disubstituted aminonitrile at *P*1 (that is known[8] to favor FP2 activ-

Table 1
Activity[a] against human and parasite cysteine proteases for compounds 1–3.

Assay	Cmpd **1**	Cmpd **2**	Cmpd **3**
FP2 IC_{50} (nM)	8	0.6	57
Cat B IC_{50} (nM)	2500	987	2320
Cat K IC_{50} (nM)	0.11	0.2	1
Cat L IC_{50} (nM)	99	0.7	4
Cat S IC_{50} (nM)	1.1	30	1
Pf growth EC_{50} (nM)	>25000	61	2700

[a] All values are the arithmetic mean of at least two independent determinations. Standard deviations are within 10% of the mean.