



Antimalarial activity of thiosemicarbazones and purine derived nitriles

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ABSTRACT

Malaria is a devastating illness caused by multiple species of the *Plasmodium* genus. The parasite's falcipain proteases have been extensively studied as potential drug targets. Here we report the testing of two established cysteine protease inhibitor scaffolds against both chloroquine sensitive and chloroquine resistant parasites. A subset of purine derived nitriles killed the parasite with moderate potency, and these inhibitors do not seem to exert their antiproliferative effects as cysteine protease inhibitors. Compound potency was determined to be similar against both parasite strains, indicating a low probability of cross resistance with chloroquine. These compounds represent a novel antimalarial scaffold, and a potential starting point for the development new inhibitors.

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Malaria infects 250 million and kills nearly one million people every year, with most fatalities occurring in children.¹ The protozoan parasite *Plasmodium falciparum* causes the most deadly form of malaria, and the cost of and developing resistance to currently available drugs requires the development of new therapies. Hemoglobin degradation in the parasite's food vacuole is an essential function for parasite survival, and *P. falciparum* depends on hemoglobin degradation by the partially redundant proteolytic activities of the falcipains^{2,3} and plasmepsins.^{4,5} Three members of the falcipain family of cysteine proteases (FP-2, FP-2', and FP-3) are known to be involved in hemoglobin catabolism.^{6–8} Significant effort has been directed towards developing inhibitors of these proteases to discover new antimalarials. These studies have resulted in potent falcipain inhibitors with promising antimalarial activity both in vitro and in vivo.^{9–17} The thiosemicarbazone^{18–20} and purine nitrile^{21,22} scaffolds have well established activity against both mammalian and protozoan cathepsins. In addition, the thiosemicarbazones have been reported to have antimalarial activity^{19,23–26}, though they may act through alternative targets. We have previously developed series of both classes for use as anti-trypanosomal agents.^{20,22,27,28}

It is known that cysteine protease inhibitors display marked synergy with pepstatin A, a potent inhibitor of plasmepsin activity, in inhibiting parasite growth.^{29–31} Although pepstatin A is only weakly potent in inhibiting parasite growth, it does produce a shift of approximately 200-fold in the EC₅₀ of the broad spectrum cysteine protease inhibitor e64D. We sought to determine whether

antimalarial activity of these inhibitors was due to cysteine protease inhibition. To explore this hypothesis, all inhibitors were screened in both the presence and absence of 10 μM pepstatin A against cultured parasites.

Chloroquine has had a central role as both a frontline treatment and prophylactic since the mid-1940s. Its heavy usage lead to the development of resistance starting in the 1960s,^{32–34} presumably due to decreased drug accumulation in the parasite food vacuole.^{35–37} Increased clinical resistance to this drug has contributed to the need for the development of new antimalarials that are not cross resistant with chloroquine. To identify inhibitors with this activity profile, all compounds in this study were tested against both chloroquine sensitive (3D7) and resistant (K1) parasites.

Parasites were grown in presence of fresh group O-positive erythrocytes in Petri dishes at a hematocrit of 4–6% in RPMI 1640 (pH 7.3) supplemented with 0.5% AlbuMAX II, 25 mM HEPES, 25 mM NaHCO₃, 100 μg/mL hypoxanthine, and 5 μg/mL gentamy-

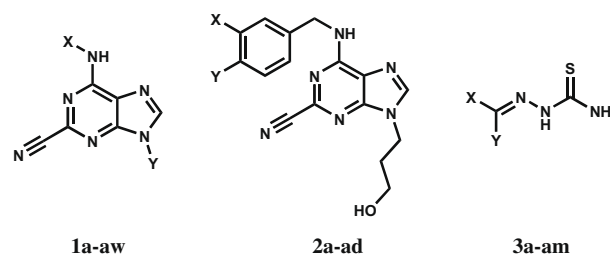


Figure 1. Inhibitor scaffolds.

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Table 1
Inhibitor structures

Compound	X	Y
1a	Hexyl	Methyl cyclohexyl
1b	Hexyl	Cyclopentyl
1c	Hexyl	Benzyl
1d	α -Methyl benzyl	Butyl
1e	α -Methyl benzyl	Cyclopentyl
1f	α -Methyl benzyl	Benzyl
1g	4-Cl benzyl	Butyl
1h	4-Cl benzyl	Mnethyl cyclohexyl
1i	4-Cl benzyl	Cyclopentyl
1j	4-Cl benzyl	Benzyl
1k	3-Me benzyl	Butyl
1l	3-Me benzyl	Cyclopentyl
1m	4-MeO benzyl	Cyclopentyl
1n	2-Methyl butyl	Cyclopentyl
1o	2-Methyl butyl	Benzyl
1p	Benzyl	Benzyl
1q	Benzyl	Butyl
1r	Phenylethyl	Butyl
1s	Phenylethyl	Benzyl
1t	Phenylethyl	3-Pyridinyl
1u	Benzyl	3-Hydroxypropyl
1v	Benzyl	4-Methoxy benzyl
1w	Benzyl	2-Tetrahydropyran
1x	4-Me benzyl	3-Hydroxypropyl
1y	4-Me benzyl	Butyl
1z	4-Me benzyl	Benzyl
1aa	4-MeO benzyl	Butyl
1ab	4-MeO benzyl	Benzyl
1ac	4-F benzyl	Butyl
1ad	4-F benzyl	4-Hydroxy butyl
1ae	4-F benzyl	Benzyl
1af	Benzyl	3-CF ₃ benzyl
1ag	4-Cl benzyl	4-Hydroxy butyl
1ah	4-Cl benzyl	3-CF ₃ benzyl
1ai	4-Cl benzyl	4-Methoxy benzyl
1aj	4-Br benzyl	Butyl
1ak	4-Br benzyl	Benzyl
1al	4-Br benzyl	Phenylpropyl
1am	3,4-Cl ₂ benzyl	Butyl
1an	3,4-Cl ₂ benzyl	3-Hydroxypropyl
1ao	3,4-Cl ₂ benzyl	Benzyl
1ap	3,4-Cl ₂ benzyl	2-Hydroxypropyl
1aq	3,4-Cl ₂ benzyl	5-Hydroxypropyl
1ar	3,4-Cl ₂ benzyl	2,3-Dihydroxypropyl
1as	3,4-Cl ₂ benzyl	2-Methoxymethoxy ethyl
1at	3,4-Cl ₂ benzyl	3-Methoxy propyl
1au	3,4-Cl ₂ benzyl	3,3-Dimethoxy propyl
1av	3,4-Cl ₂ benzyl	3-Carboxy propyl
1aw	3,4-Cl ₂ benzyl	3-CF ₃ benzyl
2a	3,5-Cl ₂ phenyl	H
2b	3,4-Cl ₂ phenyl	H
2c	3-Cl phenyl	H
2d	3-Dimethylamino phenyl	H
2e	4-Cl phenyl	H
2f	3-Methoxycarbonyl phenyl	H
2g	4-Methoxy phenyl	H
2h	3-Vinyl phenyl	H
2i	4-Vinyl phenyl	H
2j	4-Acetyl amino	H
2k	Naphthyl-2-yl	H
2l	Furan-2-yl	H
2m	Furan-3-yl	H
2n	Thiophen-2-yl	H
2o	Thiophen-3-yl	H
2p	Pyridin-3-yl	H
2q	2,4-(MeO) pyridin-5-yl	H
2r	Indol-6-yl	H
2s	Phenylsulfonyl-1-indol-2-yl	H
2t	Phenylsulfonyl-1-indol-3-yl	H
2u	2-Isopropoxy, 3-Br phenyl	H
2v	Phenyl	H
2w	3-Acetyl amino	H
2x	2-Me phenyl	H
2y	2-Isopropoxy phenyl	H
2z	H	Furan-2-yl

Table 1 (continued)

Compound	X	Y
2aa	H	3-Cl phenyl
2ab	H	Thiophen-2-yl
2ac	H	Pyridin-3-yl
2ad	H	Thiophen-3-yl
3a	3,4 Cl ₂ phenyl	Butyl
3b	4-Me phenyl	Phenyl
3c	3-Me phenyl	3-F phenyl
3d	4-Me phenyl	3-CF ₃ phenyl
3e	Phenylethyl	Phenyl
3f	Phenylethyl	3-Cl phenyl
3h	Phenylethylene	3-CF ₃ phenyl
3i	4-Me phenyl	3,5 Cl ₂ phenyl
3j	Phenyl	3,5 Cl ₂ phenyl
3k	Thiophen-2-yl	3,5 Cl ₂ phenyl
3l	4-Me Phenyl	3,5 CF ₃ phenyl
3m	Phenylethyl	3,5 CF ₃ phenyl

cin. Cultures were incubated at 37 °C with 5% O₂ and 5% CO₂. For EC₅₀ determinations, 20 μ L of RPMI 1640 was plated per well in a 384 well assay plate. Compounds were prepared as either 5 mM or 10 mM stock solutions in DMSO and transferred to the assay plate by a 384 head pintool. 20 μ L of a synchronized culture suspension (1% rings, 10% hematocrit) was added to each well to make a final hematocrit and parasitemia of 5% and 1%, respectively. The final DMSO concentration was 0.65%. For synergy experiments, pepstatin A was added to the cell suspension prior to plating. Assay plates were incubated for 72 h and parasitemia was determined using a previously reported DNA stain based assay.³⁸

A series of nearly 100 inhibitors from the three scaffolds was tested for antimalarial activity in both parasite strains in the presence and absence of 10 μ M pepstatin A (Fig. 1, Table 1). Predicted cell permeability of a subset of these compounds was previously tested in a parallel artificial membrane permeability assay (PAMPA) and most are predicted to have good cellular availability.^{20,28} Additionally, the inhibitors in this test set have also been previously shown to be relatively non-toxic in a panel of mammalian cell lines.^{20,22,28}

Although the majority of the inhibitors tested did not have significant activity at the highest activity tested (32.5 μ M), these studies identified a subset of inhibitors with good predicted membrane permeability as well as modest growth inhibitory potency against cultured parasites (Fig. 2). Weakly active inhibitors were identified from all three scaffolds, with scaffold 2 giving the most active compounds. Scaffolds 1 and 3 did not yield any inhibitors with potencies below 25 μ M against either strain, and there is no clearly discernable SAR for either scaffold.

The three most active inhibitors (**2w**, **2z**, and **2aa**) displayed potencies in the 10 μ M range. Inhibitor **2w** was the most active against both parasite strains with EC₅₀ values of 6 ± 3 μ M and 15 ± 3 μ M against K1 and 3D7 in the presence of 10 μ M pepstatin. Moving the acetylamino substituent of **2w** from the 3' to the 4' position (**2j**) on the distal phenyl ring resulted in a 3–5-fold decrease in potency against the K1 parasite. It is notable that for scaffold 2, aryl substitutions at the Y-position appear to be more effective than at the X-position. In the cases of **2z** and **2aa**, moving the aryl substituent from the Y-position to the X-position resulted in a 2–3-fold decrease in potency.

Only minor shifts in antimalarial potency were observed in the presence of pepstatin A for any of the active inhibitors against either the K1 or 3D7 parasites. In contrast, the EC₅₀ of the falcipain inhibitor e64D shifted 200–300-fold when combined with pepstatin A. These data suggest that cysteine protease inhibition is not likely to be a significant mediator of antimalarial activity for this inhibitor series.

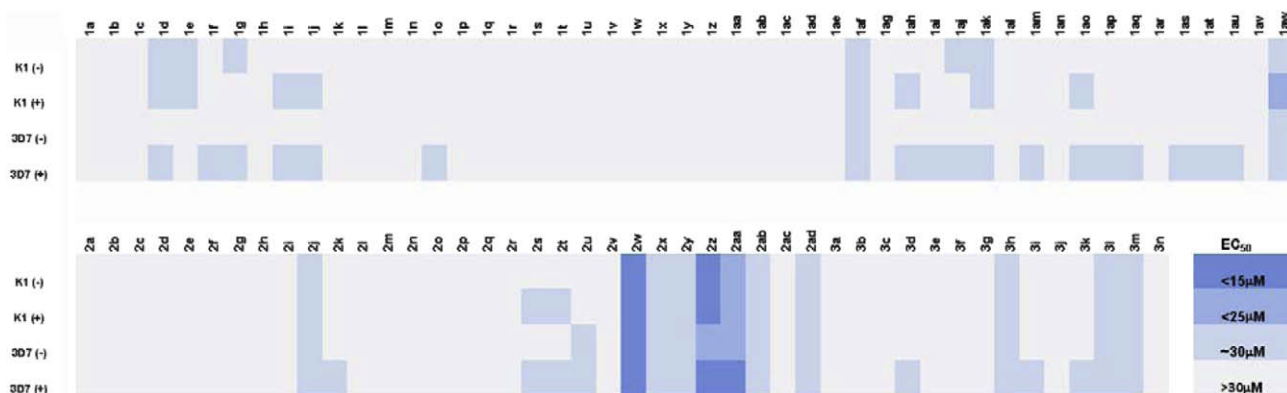


Figure 2. Antimalarial activity.

There does not seem to be a significant difference in inhibitor potency between the K1 and 3D7 parasites. Some inhibitors show minor differences in potency between strains, although overall there is a similar activity profile across all three scaffolds, indicating a low chance of chloroquine cross resistance.

The expected membrane permeability of a subset of inhibitors from all three scaffolds was previously tested by PAMPA. Although a wide range of permeability values was observed, there was no clear structure–property relationship between permeability and antimalarial activity.

We have reported the evaluation of a series of inhibitors based on the purine nitrile and thiosemicarbazone scaffolds for activity against two strains of cultured *P. falciparum*. There were no significant differences in potency between the two strains, indicating that chloroquine cross resistance is unlikely. Antimalarial activity of the series was modest, although inhibitors were identified with moderate potency against both K1 and 3D7 parasites. Active compounds did not act synergistically with the aspartyl protease pepstatin A, suggesting that they do not exert their antiproliferative effects by cysteine protease inhibition. Previous experiments with other cysteine protease inhibitors including peptidyl aziridines, phenyl urenyl chalcones, and vinyl sulfone derivatives suggest these scaffolds also act at targets other than the falcipains.^{15–17} This is in contrast to studies of this inhibitor series with *Trypanosoma brucei*, in which a clear relationship between trypanocidal activity and potency against the target protease was observed.^{22,28}

Although the mechanism of action of these compounds in *P. falciparum* is unclear, the combination of low cytotoxicity, good membrane permeability, and lack of chloroquine cross resistance suggest the purine nitriles may be a useful starting point for the development of new antimalarials. The thiosemicarbazones tested were generally inactive against the parasite, and do not represent promising antimalarial leads.

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Supplementary data

Full data set of antimalarial inhibitor potencies. Experimental details of malaria assay. Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2009.04.142](https://doi.org/10.1016/j.bmcl.2009.04.142).

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