Chemical Target Validation Studies of Aminopeptidase in Malaria Parasites Using α -Aminoalkylphosphonate and Phosphonopeptide Inhibitors[∇]

Eithne Cunningham,¹ Marcin Drag,² Pawel Kafarski,² and Angus Bell^{1*}

Department of Microbiology, School of Genetics and Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland,¹ and Faculty of Chemistry, Biochemistry, and Biotechnology, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland²

Received 15 October 2007/Returned for modification 12 December 2007/Accepted 27 April 2008

During its intraerythrocytic phase, the most lethal human malarial parasite, *Plasmodium falciparum*, digests host cell hemoglobin as a source of some of the amino acids required for its own protein synthesis. A number of parasite endopeptidases (including plasmepsins and falcipains) process the globin into small peptides. These peptides appear to be further digested to free amino acids by aminopeptidases, enzymes that catalyze the sequential cleavage of N-terminal amino acids from peptides. Aminopeptidases are classified into different evolutionary families according to their sequence motifs and preferred substrates. The aminopeptidase inhibitor bestatin can disrupt parasite development, suggesting that this group of enzymes might be a chemotherapeutic target. Two bestatin-susceptible aminopeptidase activities, associated with gene products belonging to the M1 and M17 families, have been described in blood-stage *P. falciparum* parasites, but it is not known whether one or both are required for parasite development. To establish whether inhibition of the M17 aminopeptidase is sufficient to confer antimalarial activity, we evaluated 35 aminoalkylphosphonate and phosphonopeptide compounds designed to be specific inhibitors of M17 aminopeptidases. The compounds had a range of activities against cultured *P. falciparum* parasites with 50% inhibitory concentrations down to 14 μ M. Some of the compounds were also potent inhibitors of parasite aminopeptidase activity, though it appeared that many were capable of inhibiting the M1 as well as the M17 enzyme. There was a strong correlation between the potencies of the compounds against whole parasites and against the enzyme, suggesting that M17 and/or M1 aminopeptidases may be valid antimalarial drug targets.

Malaria remains a widespread and devastating disease, and new drugs effective against the most lethal human parasite, *Plasmodium falciparum*, are desperately needed. The spread of drug-resistant parasites makes it crucially important to identify chemical classes and parasite targets that have not previously been exploited in antimalarial chemotherapy (9). During its intraerythrocytic cycle, the parasite digests up to 75% of the host cell hemoglobin, incorporating some of the liberated amino acids into parasite protein (19). This pathway of hemoglobin degradation has been studied intensively, and recent work has indicated that the process is needed for parasite survival (2, 25, 33). Inhibitors of aspartyl endopeptidases (plasmepsins I, II, and IV and the closely related histo-aspartyl protease) and cysteinyl endopeptidases (falcipains 2, 2', and 3) active on globin are under investigation as potential antimalarial drugs (10, 31). The initial cleavages take place in a digestive vacuole to which erythrocyte cytosol is transported. The terminal stages of globin degradation are thought to be catalyzed by one or more aminopeptidases that have generally been believed to be cytosolic (16), although recent work suggests that some may be active in the digestive vacuole (6). Aminopeptidase inhibitors, including bestatin, nitrobestatin, and amastatin, have antimalarial activity in culture (16, 27),

and different combinations of endo- and aminopeptidase inhibitors show synergism (17).

Aminopeptidases catalyze the hydrolysis of amino acids from the N termini of proteins or peptides. Most are metalloproteases, and they are classified into families based on sequence similarity (30) (<http://merops.sanger.ac.uk>). Often, members of the same family will share substrate preferences; e.g., M17 aminopeptidases generally have preferences for leucine at the N terminus and have often been termed leucine aminopeptidases, although many will hydrolyze other similarly hydrophobic residues (29). (We therefore use the MEROPS family designation for the enzymes where possible in this paper and use terms such as "leucyl aminopeptidase" [LAP] to refer to a measured activity on a leucine-containing substrate.) Aminopeptidases are involved in a number of diverse processes, including protein maturation, activation, and stability, and have been found to be associated with many diseases. For example, altered LAP activity is seen in cancer, cataracts, inflammation, and human immunodeficiency virus infection (20).

A number of aminopeptidase genes have been identified in the *P. falciparum* genome (38). The best studied of these are the metalloaminopeptidases *P. falciparum* aminopeptidase M1 (PfAP-M1; also referred to as PfA-M1) (1, 14) and PfAP-M17 (also referred to as PfLAP) (35), which have been characterized in blood-stage parasites. Both PfAP-M1 and PfAP-M17 are inhibited by bestatin, but it is not clear whether one or both of these enzymes are the targets for the antimalarial activity of this compound (1, 35). Both were located in the parasite cytosol and were more active at near-neutral pHs. Partially purified PfAP-M1 has been shown to be processed from a 122-

* Corresponding author. Mailing address: Department of Microbiology, Moyne Institute, Trinity College, Dublin 2, Ireland. Phone: (353 1) 896 1414. Fax: (353 1) 679 9294. E-mail: abell@tcd.ie.

§ Present address: Burnham Institute for Medical Research, 10901 North Torrey Pines Rd., La Jolla, CA 92037.

[∇] Published ahead of print on 5 May 2008.

kDa protein to two smaller fragments of 96 and 68 kDa. Although M1 family enzymes are generally regarded as "alanine" aminopeptidases (AAPs), PfAP-M1 preferentially digested lysine substrates in vitro, followed closely by alanine, arginine, and leucine substrates. The AAP and LAP activities of the protein were very similar (1). The *PfAP-M17* gene encodes a protein of 68 kDa that appears, like most M17 aminopeptidases, to form catalytically active homohexamers. Recombinant protein had good LAP activity (but very poor AAP activity), and this LAP activity was greatly increased by incubation with divalent metal ions, particularly Co^{2+} or Mn^{2+} (35). As expected, a cytosolic extract from the parasites demonstrates AAP and LAP activities (16). A parasite extract incubated with CoCl_2 and separated by high-performance liquid chromatography showed two peaks of LAP activity at ~82 kDa and ~320 kDa, presumed to correspond to fractions containing the M1 and M17 enzymes, respectively. The 82-kDa peak also had AAP activity, but the 320-kDa peak did not. The other aminopeptidase genes identified in the *P. falciparum* genome are not expected to encode products susceptible to bestatin and are not considered further here. Methionine aminopeptidases (M24A family) have been proposed to be good drug targets (5, 39), but they are unlikely to be involved in globin digestion.

So far, no knockouts of either the PfAP-M1 or the PfAP-M17 aminopeptidase gene in *P. falciparum* have been reported. Transgenic parasites carrying the *PfAP-M17* gene on a multicopy plasmid demonstrated reduced susceptibility to bestatin, suggesting that PfAP-M17 might be the major target of this agent (15). The overexpression of *PfAP-M17* was not quantified, however, and an indirect effect on PfAP-M1 due to the increased *PfAP-M17* copy number could not be ruled out. No line overproducing PfAP-M1 was obtained. Therefore, it is not clear whether one or both of the two enzymes are the targets for the antimalarial activity of bestatin. Although bestatin is generally specific for M1 and M17 family aminopeptidases, the mammalian (M16) endopeptidase nardilysin is also susceptible (32).

The best inhibitors of M17 family aminopeptidases (based primarily on work with the M17 family aminopeptidase from porcine kidney [pkLAP]) are analogues of short peptides, e.g., the well-known bestatin. Amino acid analogues are also good inhibitors, e.g., L-leucinal and the phosphonic acid analogue of L-leucine (LeuP), with the latter group being more specific for metallopeptidases. Both groups of inhibitors act as transition state analogues, binding to the metal ions in the active site of the enzyme (18). In this study, we have assessed the antimalarial and antiaminopeptidase activities of a series of phosphonic derivatives designed against M17 aminopeptidase. In order to assess the validity of the PfAP-M17 enzyme as a target, we employed as chemical tools a structurally related series of α -aminoalkylphosphonates and phosphonopeptides designed to target the active site. We used knowledge of the differing substrate specificities and cation dependencies of the M1 and M17 enzymes to dissect the inhibition of the two activities in parasite cytosolic extracts. Our results support the contention that PfAP-M17 may be a valid antimalarial drug target amenable to the design of specific inhibitors. They also suggest that PfAP-M1 is a possible alternative or additional target.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Aldrich, Dublin, Ireland, unless otherwise stated. Stock solutions of inhibitors were prepared by dissolving them in phosphate-buffered saline to concentrations of 1 to 5 mM, followed by filter sterilization.

Synthesis of inhibitors. The compounds used in this study were synthesized as described previously. Racemic 1-aminoalkane phosphonic acids were obtained using either Oleksyszyn or modified Arbuzov methodologies (24, 28). Enantiomerically pure or diastereomer pairs of 1-aminoalkylphosphonates were synthesized using the Hamilton-Walker reaction (22). 2-Amino-1-hydroxyphosphonates were synthesized as described by Drag et al. (8). Dipeptidic analogues of the 1-aminoalkylphosphonates were synthesized as described by Kafarski and Lejczak (23). Compound D36 was obtained by the monoesterification of the diphenyl ester of the 1-aminoalkylphosphonate, as described by Szewczyk et al. (36).

Parasite culture and growth inhibition. *P. falciparum* clone 3D7 (obtained from M Grainger, National Institute of Medical Research, London, United Kingdom) was cultured in O^+ erythrocytes as described previously (11). Inhibitor susceptibility assays were carried out on asynchronous, asexual, blood-stage parasites in 96-well plates by using the spectrophotometric parasite lactate dehydrogenase assay described by Makler et al. (26). Dose-response curves were constructed from absorbance readings after 72 h of incubation, and the 50% inhibitory concentrations (IC_{50}) were determined graphically.

Preparation of material for analysis of aminopeptidase activity. Free parasites were harvested from infected erythrocytes according to the method of Zuckerman et al. (40), and cytosolic parasite extracts were prepared by three cycles of freeze-thawing followed by centrifugation at $12,000 \times g$ and 4°C for 20 min, as described previously (16). Recombinant tPfAP-M17, a truncated form of PfAP-M17 lacking the first 82 residues and with the asparagine residues at positions 152, 515, and 546 changed to glutamines, was provided by John P. Dalton, IBID, University of Technology, Sydney, Australia. The recombinant enzyme was produced in baculovirus-infected Sf9 cells and was purified by Ni^{2+} chelate affinity chromatography, as described previously (35). Protein concentrations were determined by the Bradford method (3).

Analysis of aminopeptidase activity and inhibition. Aminopeptidase activity was determined by measurement of the release of the fluorogenic leaving group 7-amino-4-methyl-coumarin (AMC) from peptide substrates (Leu-AMC and Ala-AMC, used to measure LAP and AAP activities, respectively) essentially as described previously (35). The parasite extract (final concentration, ~25 $\mu\text{g}/\text{ml}$) or recombinant protein (final concentration, ~2.7 $\mu\text{g}/\text{ml}$ or 0.05 μM) was incubated in 50 mM Tris-Cl, pH 8.0, with or without CoCl_2 (1 mM) and/or inhibitors (10 or 0.1 μM) as required, for 15 min at 37°C before addition of the substrate to a final concentration of 10 μM . Reactions were measured over a 30-min period and were read using a spectrofluorimeter (LS-50B; Perkin-Elmer) with excitation at 370 nm and emission at 440 nm. The 15-min preincubation was found to be sufficient for maximal inhibition of enzyme activity by the compounds used at the concentrations indicated here. For all compounds, the reactions were linear for the full duration of the experiment, and for a subset of the compounds tested, the reactions were linear for as long as 2 h (see Fig. 1 inset).

RESULTS

Antimalarial activities of α -aminoalkylphosphonates and phosphonopeptides. The series of α -aminoalkylphosphonate and phosphonopeptide compounds were tested against cultured *P. falciparum* parasites. They had various potencies against the parasites, ranging from inactivity at the highest concentrations possible to IC_{50} as low as 14 μM (Table 1). The two most active compounds were D14 and D12, alicyclic phosphonates (IC_{50} , 14 μM and 15 μM , respectively). These were closely followed by D7 and D17, phosphonic acid analogues of phenylalanine with extended aliphatic chains of one or three methylene groups, respectively (IC_{50} , 21 and 22 μM , respectively). D36, a monophenyl ester derivative of D7, was synthesized in the hope of improving uptake into parasites. It demonstrated similar but not improved activity. A number of the compounds had very low or negligible antimalarial activity,

TABLE 1. Activities of compounds on cultured *P. falciparum* parasites

Compound	Structure	Configuration	IC ₅₀ (μM) ^a	% Inhibition of parasite growth ^b
D14		RS	14	
D12		RS	15	
D17		RS	21	
D7		RS	22	
D36		RS	25	
D5		RS	53	
D21		R, >99%	54	
D3		RS	57	
D2		RS	65	
D13		RS	119	
D20		RS:SS, 1:1	135	
D38		R	>128	30 ± 13
D24		RS:SR, 1:1	>128	11 ± 5
D6		RS	>256	34 ± 6
D28		RR:RS, 1:1	>256	22 ± 9
D18		RS	>256	17 ± 0
D33		SS:SR, 1:1	>256	17 ± 6
D10		R, >99%	>256	15 ± 2

Continued on following page

TABLE 1—Continued

Compound	Structure	Configuration	IC ₅₀ (μM) ^a	% Inhibition of parasite growth ^b
D1		RS	>256	14 ± 4
D26		SS, >98%	>256	14 ± 4
D30		RR:RS	>256	14 ± 5
D19		RS:SS, 1:1	>256	10 ± 6
D8		RS	>256	8 ± 3
D34		RS:SS, 1:1	>256	7 ± 3
D22		S, >99%	>256	7 ± 2
D35		RS	>256	5 ± 4
D31		SS:SR, 1:1	>256	2 ± 2
D4		RS	>256	1 ± 1
D23		S, >99%	>256	1 ± 1
D25		SSS:RSS, 8:2	>256	1 ± 1
D9		RS	>256	0 ± 0
D11		S, >99%	>256	0 ± 0
D27		RS:SR, 1:1	>256	0 ± 0
D29		SS:SR, 1:1	>256	0 ± 0
D32		RR:RS, 1:1	>256	0 ± 0

^a Geometric mean IC₅₀ were calculated from six determinations from three separate experiments as described in Materials and Methods.^b Compounds for which no IC₅₀ could be calculated are shown in order of decreasing inhibition of parasite growth at a fixed, highest possible concentration. Each result represents the arithmetic mean ± standard error of the mean (*n* ≥ 4) of the percentage of inhibition of parasite growth at the highest concentration of the compound tested (i.e., 128 μM for D24 and D38 and 256 μM for all others).

TABLE 2. Inhibition of the aminopeptidase activities of the parasite cytosolic extract

Compound	% Inhibition ^a of aminopeptidase activity by the indicated concn of compound			
	LAP activity		AAP activity	
	10 μ M	0.1 μ M	10 μ M	0.1 μ M
Bestatin	98 $\pm$ 1	33 $\pm$ 2	97 $\pm$ 0	29 $\pm$ 2
D14	95 $\pm$ 1	17 $\pm$ 3	95 $\pm$ 1	15 $\pm$ 1
D12	94 $\pm$ 0	17 $\pm$ 3	94 $\pm$ 1	13 $\pm$ 1
D17	97 $\pm$ 1	28 $\pm$ 5	97 $\pm$ 1	28 $\pm$ 3
D7	93 $\pm$ 1	15 $\pm$ 2	95 $\pm$ 0	15 $\pm$ 3
D36	90 $\pm$ 0	8 $\pm$ 2	92 $\pm$ 1	12 $\pm$ 0
D5	71 $\pm$ 1	6 $\pm$ 3	76 $\pm$ 1	0 $\pm$ 4
D21	12 $\pm$ 4	0 $\pm$ 1	8 $\pm$ 3	2 $\pm$ 0
D3	43 $\pm$ 1	2 $\pm$ 1	52 $\pm$ 1	2 $\pm$ 0
D2	39 $\pm$ 0	5 $\pm$ 7	43 $\pm$ 0	0 $\pm$ 0
D13	27 $\pm$ 3	3 $\pm$ 7	20 $\pm$ 3	0 $\pm$ 0
D20	4 $\pm$ 2	0 $\pm$ 5	0 $\pm$ 5	0 $\pm$ 1
D38	15 $\pm$ 0	0 $\pm$ 3	24 $\pm$ 2	0 $\pm$ 1
D24	17 $\pm$ 1	—	—	—
D6	6 $\pm$ 2	—	—	—
D28	9 $\pm$ 0	—	—	—
D18	37 $\pm$ 1	—	—	—
D33	11 $\pm$ 2	—	—	—
D10	10 $\pm$ 6	—	—	—
D1	8 $\pm$ 2	—	—	—
D26	6 $\pm$ 1	—	—	—
D30	11 $\pm$ 1	—	—	—
D19	9 $\pm$ 2	—	—	—
D8	6 $\pm$ 3	—	—	—
D34	12 $\pm$ 1	—	—	—
D22	8 $\pm$ 3	—	—	—
D35	7 $\pm$ 4	—	—	—
D31	3 $\pm$ 3	—	—	—
D4	8 $\pm$ 1	—	—	—
D23	2 $\pm$ 6	—	—	—
D25	5 $\pm$ 0	—	—	—
D9	6 $\pm$ 4	—	—	—
D11	6 $\pm$ 2	—	—	—
D27	5 $\pm$ 2	—	—	—
D29	9 $\pm$ 1	—	—	—
D32	1 $\pm$ 3	—	—	—

^a Results are means $\pm$ standard errors of the means ($n \geq 4$) of the percentages of inhibition of the LAP and AAP activities of the parasite cytosolic extract after incubation with compounds at 10 μ M or 0.1 μ M. —, not determined.

inhibiting parasite growth by less than 10% at the highest concentration tested (256 μ M).

Inhibition of the LAP and AAP activities of the parasite extract. We first investigated the abilities of the series of compounds to inhibit the aminopeptidase activities of parasite cytosolic extracts (14, 27). The rationale for this approach was to assess whether the compounds were, like bestatin, capable of inhibiting the entire measurable aminopeptidase activity of asexual blood-stage parasites. The extracts were expected, on the basis of previous work (summarized above), to contain both PfAP-M1 and PfAP-M17 activities. Compounds were tested at two fixed concentrations, 10 μ M and 0.1 μ M, by incubating them with the cytosolic extract before the addition of the fluorogenic substrate, Leu-AMC or Ala-AMC. A range of activities against LAP activity was seen (Table 2), with the five most potent compounds, D14, D12, D17, D7, and D36, inhibiting LAP activity by $\geq 90\%$ when assayed at 10 μ M, while the least effective compounds could inhibit LAP activity only

by a few percentage points or not at all. The compounds are ordered by decreasing antimalarial activity (i.e., by increasing IC₅₀ [see Table 1]) in Table 2 and demonstrate a corresponding trend of a decreasing percentage of inhibition of enzyme activity, indicating the good correlation between antimalarial and anti-LAP activities. There were a couple of minor exceptions to the general trend of agreement, including D18, which might be expected to have better antimalarial activity (e.g., closer to that of D2 or D13) given its anti-aminopeptidase activity. Conversely, D21 might be expected to inhibit the parasite extract to a higher degree given its IC₅₀.

A similar pattern of inhibition of the AAP activity of the extract, and a similar correlation with antimalarial activity, was observed. This indicated that the compounds might be having some effect on the M1 aminopeptidase of *P. falciparum*, since previous work has indicated that PfAP-M17 has low AAP activity (35). Inhibition of PfAP-M1 was not entirely unexpected; although these compounds were designed as specific M17 aminopeptidase inhibitors, some of them demonstrated activity against the mammalian M1 family enzyme aminopeptidase N (7).

Contributions of PfAP-M17 and PfAP-M1 to the aminopeptidase activities of the parasite cytosolic extract. In order to investigate which aminopeptidase activity was being inhibited by the compounds, the parasite extract was tested for LAP and AAP activities in the presence of CoCl₂, which was expected, on the basis of previous results (35; also unpublished data), to boost PfAP-M17 activity but reduce PfAP-M1 activity. Following incubation with CoCl₂, the overall LAP activity of the extract was reduced markedly, to 21% of its original activity, but the AAP activity was virtually abolished, down to 4% (Fig. 1). Therefore, we deduced that, providing that the enzymes behave similarly in pure and impure preparations, (i) assaying the parasite extract without CoCl₂ and with Ala-AMC as the substrate should measure PfAP-M1 almost exclusively, while (ii) assaying the extract in the presence of CoCl₂ and with Leu-AMC as the substrate should measure predominantly PfAP-M17. Although the effect of Co²⁺ on the PfAP-M1 holoenzyme preparation of Allary et al. (1) was not measured, other reports of aminopeptidase-containing *Plasmodium* fractions with characteristics similar to those of the M1 enzyme indicated that their activities were substantially reduced by 1 mM Co²⁺ (4, 21, 37), though not much affected by 0.5 mM Co²⁺ (35).

Inhibition of the LAP activity of the parasite extract in the presence and absence of Co²⁺ ions. The same general trend of inhibition of LAP activity was seen for the parasite extract incubated with CoCl₂ as for the extract without CoCl₂ (Table 3). Overall, there was a strong trend for the compounds to show some increased inhibition of LAP activity in the presence of CoCl₂, i.e., under conditions favoring PfAP-M17 activity. This led us to consider the possibility that most of the inhibitors may (unlike bestatin) be better inhibitors of PfAP-M17 than of PfAP-M1. However, the percentages of inhibition are not comparable because of differences in the K_m between PfAP-M1 (60 μ M [1]) and PfAP-M17 (12 μ M [35]). Unfortunately, it was not possible to compare the susceptibilities of PfAP-M17 and PfAP-M1 directly, because the latter was not available in recombinant form.

FIG. 1. Aminopeptidase activities of the *P. falciparum* cytosolic extract. The extract was incubated in 50 mM Tris-Cl (pH 8.0) with or without CoCl₂ (1 mM) at 37°C for 15 min before the addition of the substrate, Ala-AMC or Leu-AMC, to a final concentration of 10 μ M. Activity is expressed as a percentage of the AAP activity of the extract in the absence of CoCl₂. (Inset) Linear reaction rate observed for the inhibition of the LAP activity of the parasite extract. The fluorescence produced by the cleavage of a Leu-AMC substrate by the parasite extract (preincubated in 1 mM CoCl₂, with or without an inhibitor) was observed over a 2-h period. The open circles and the continuous line indicate the LAP activity of the extract alone, while the filled triangles and the dotted line indicate the inhibition of the activity of the extract by D14. Similar profiles were observed for D7 and D12 (data not shown).

Correlation of the antimalarial and aminopeptidase activities of inhibitors. A clear linear relationship between the antimalarial activities of the compounds and their aminopeptidase activities was seen when the percentages of inhibition (at 10 μ M) of the LAP activity (in the presence of CoCl₂) and AAP activity of the extract were plotted against the log

IC₅₀ for growth in culture (Fig. 2). The data were analyzed using Spearman nonparametric correlation and were found to have *r* values of -0.8531 ($P = 0.0008$) and -0.8671 ($P = 0.0005$) for LAP and AAP activities, respectively. This suggests a good negative correlation between the log IC₅₀ of the compounds and their percentages of inhibition of the aminopepti-

TABLE 3. Inhibition of the LAP activity of the parasite extract in the absence and presence of CoCl₂

Compound	% Inhibition ^a by the indicated concn of compound			
	In the absence of CoCl ₂		In the presence of 1 mM CoCl ₂	
	10 μ M	0.1 μ M	10 μ M	0.1 μ M
Bestatin	98 $\pm$ 1	33 $\pm$ 2	93 $\pm$ 1	6 $\pm$ 2
D14	95 $\pm$ 1	17 $\pm$ 3	91 $\pm$ 0	51 $\pm$ 2
D12	94 $\pm$ 0	17 $\pm$ 3	91 $\pm$ 1	43 $\pm$ 5
D17	97 $\pm$ 1	28 $\pm$ 5	92 $\pm$ 0	25 $\pm$ 3
D7	93 $\pm$ 1	15 $\pm$ 2	92 $\pm$ 0	41 $\pm$ 2
D36	90 $\pm$ 0	8 $\pm$ 2	73 $\pm$ 3	19 $\pm$ 11
D5	71 $\pm$ 1	6 $\pm$ 3	70 $\pm$ 5	20 $\pm$ 9
D21	12 $\pm$ 4	0 $\pm$ 1	67 $\pm$ 3	50 $\pm$ 8
D3	43 $\pm$ 1	2 $\pm$ 1	73 $\pm$ 1	38 $\pm$ 1
D2	39 $\pm$ 0	5 $\pm$ 7	71 $\pm$ 1	46 $\pm$ 5
D13	27 $\pm$ 3	3 $\pm$ 7	69 $\pm$ 1	21 $\pm$ 9
D20	4 $\pm$ 2	0 $\pm$ 5	0 $\pm$ 6	1 $\pm$ 0
D38	15 $\pm$ 0	0 $\pm$ 3	52 $\pm$ 0	9 $\pm$ 3

^a Results are means $\pm$ standard errors of the means ($n \geq 4$) of the percentages of inhibition of the LAP activity of the parasite cytosolic extract after incubation with compounds (at 10 μ M or 0.1 μ M).

FIG. 2. Correlation of antimalarial activity and inhibition of the aminopeptidase activity of the parasite extract. The percentages of inhibition of the LAP and AAP activities of the parasite extract by the 12 most active compounds (at 10 μ M) were plotted against their log IC₅₀ for parasite growth. The open circles and the continuous line indicate the inhibition of the LAP activity of the parasite extract preincubated in CoCl₂ (1 mM) ($r = -0.8531$; $P = 0.0008$), while the filled triangles and the dotted line indicate the inhibition of the AAP activity ($r = -0.8671$; $P = 0.0005$). Spearman rank correlation was used to analyze the data.

TABLE 4. Inhibition of the LAP activities of the parasite extract and recombinant tPfAP-M17 incubated with CoCl_2

Compound	% Inhibition ^a by the indicated concn of compound				K_i (μM) ^b	
	Parasite extract		tPfAP-M17		pkLAP	APN
	10 μM	0.1 μM	10 μM	0.1 μM		
Bestatin		6 $\pm$ 2		34 $\pm$ 4		
D14		51 $\pm$ 2		67 $\pm$ 7	9.57	41.6
D12		43 $\pm$ 5		61 $\pm$ 1	0.21	37.1
D17		25 $\pm$ 3		27 $\pm$ 3	0.33	3.69
D7		41 $\pm$ 2		47 $\pm$ 1	0.14	15.9
D21	67 $\pm$ 3		94 $\pm$ 6		7.89	161
D13	69 $\pm$ 1		86 $\pm$ 6		0.75	54.7
D22	50 $\pm$ 1		61 $\pm$ 3		6.96	>2,000
D23	26 $\pm$ 3		9 $\pm$ 13		0.23	53
D25	0 $\pm$ 7		0 $\pm$ 4			

^a Results are mean percentages of inhibition ($\pm$ standard errors of the means) of the LAP activities of the parasite cytosolic extract ($n \geq 4$) and recombinant tPfAP-M17 ($n = 2$) after incubation with compounds (at 10 μM or 0.1 μM) and CoCl_2 (1 mM).

^b Results from Drag et al., 2005 (7). APN, porcine kidney microsomal aminopeptidase N.

dase activities of the extract; i.e., as the antimalarial activity decreases, so too does the inhibition of the aminopeptidase activity of the extract.

Inhibition of recombinant PfAP-M17 activity. A subset of the compounds was tested for their abilities to inhibit the LAP activity of the recombinant *P. falciparum* M17 aminopeptidase tPfAP-M17 (Table 4). This recombinant enzyme is a truncated (58-kDa) form of the protein produced in baculovirus-infected insect cells. Very similar profiles of LAP inhibition of the recombinant enzyme and the extract were seen. As a result, there is a correspondingly good correlation between the inhibition of the recombinant enzyme and antimalarial activity, although D17 is a slight exception to this. Considering its good inhibition of the mammalian M1 family enzyme aminopeptidase N relative to that by the other compounds, it is possible that this inhibitor has slightly more activity against PfAP-M1. D14 is the best inhibitor of recombinant tPfAP-M17, and it also has the best antimalarial activity of all the compounds tested. However, it is the poorest inhibitor of pkLAP, with a K_i of 9.57 μM , indicating that it may be somewhat selective for PfAP-M17 over the mammalian enzyme.

DISCUSSION

Comparison of the results of the antimalarial and antiaminopeptidase (whole-parasite extract and recombinant enzyme) activity assays with the data previously obtained for mammalian M1 and M17 aminopeptidase inhibition enables the identification of the properties likely to confer potency and selectivity against PfAP-M17. The inhibitors had a range of antimalarial potencies down to low micromolar IC_{50} . The most potent compounds were those with long, hydrophobic side chains (D14, D12, D17, and D7) that might occupy the S1 subsite of the enzyme. As can be seen from the structures of these compounds, replacement of the aromatic ring with the more flexible alicyclic ring does not alter the antimalarial activity greatly. By contrast, the least active group of compounds generally had shorter side chains. These results suggest that

the S1 pocket of PfAP-M17 is quite large and is thus able to accommodate compounds with bulky side chains. Also, the surface of the S1 cleft seems to be involved in multiple interactions with the bound inhibitors, especially deep inside the pocket, since effectors with extended side chains were exclusively the most active. Unfortunately, no crystal structure data of PfAP-M17 with which one could unambiguously judge this hypothesis are available. The data outlined here also suggest that the interactions are of a hydrophobic nature, since the presence of the hydroxy group on the compounds tested dramatically decreased their inhibitory potencies. Moreover, these observations correlate precisely with the results obtained in previous studies for mammalian (porcine kidney) LAP, where the inhibitors with bulky substituents were the best inactivators of the enzyme. The weak inhibitory potencies of the phosphonodipeptides equipped with the reactive group at the C terminus indicate that the enzyme is a classic monoexopeptidase that is able to cleave only one amino acid at a time and is not capable of binding large peptides effectively. The presence of the free amine at the α position is also indispensable for the effective interaction between inhibitors and the PfAP-M17 aminopeptidase, as observed by the very weak inactivation by compounds with a hydroxy group at this position (D24 to D27).

Within the group of compounds with IC_{50} in the 50 to 70 μM range (D5, D21, D3, and D2), it seems that the presence of the methoxy group in the *para* position is important, since increased inhibition of activity was seen with the inhibitors that contained this functional group. Alteration to a free hydroxyl group in this position (D1, D35, D4) and/or another (D6, D35) decreased the antimalarial activity dramatically.

Comparison of the antimalarial and antiaminopeptidase activities of the R enantiomers (corresponding to the natural L-amino acids) tested here (D21, D38, and D10) with those of their S configuration counterparts (D22, D23, and D11, respectively) shows a preference for the R enantiomers. This is in agreement with the expected chiral specificity of enzymes, especially aminopeptidases, which usually favor substrates with natural L chirality.

All of the active compounds appeared to inhibit both the PfAP-M17 and PfAP-M1 enzymes to some degree, inhibiting both the LAP and AAP activities of extracts. The compounds appeared to have slightly higher inhibitory effects against the aminopeptidase activities of extracts under conditions favoring PfAP-M17 over PfAP-M1 (i.e., assaying activity against a leucine substrate after preincubation with 1 mM CoCl_2). However, as already stated, the differences in the K_m values for the two aminopeptidases meant that it was not possible directly to compare the percentages of inhibition of extract aminopeptidase activities. It is possible that apparent differences in susceptibility may in fact be due to differing substrate affinities.

There was good agreement between M17 aminopeptidase inhibition in extracts and in recombinant tPfAP-M17 produced in insect cells. The fact that compound D14, which had marked selectivity for PfAP-M17 over pkLAP, was also the best inhibitor of parasite growth suggests that D14 should be further investigated in the hope of increasing its potency.

Owing to the lack of active recombinant PfAP-M1 and the difficulty and low yield of its purification (1, 14), we did not test this enzyme in complete isolation from PfAP-M17, so our data cannot be used to validate PfAP-M1 as an antimalarial target.

Quinoline (13)- and malonic hydroxamate (12)-based inhibitors of PfAP-M1 with antimalarial activity have nevertheless been described. It would be informative to test combinations of representatives of these groups with the best compounds described here so as to determine whether inhibition of both aminopeptidase classes might confer synergistic antimalarial action.

While this paper was under review, Skinner-Adams et al. published work on a much smaller group of phosphinate dipeptide M17 aminopeptidase inhibitors with a range of antimalarial activities similar to those of the compounds described here (34). Their results indicated that two compounds with relatively high affinities for the recombinant M17 aminopeptidase, hPheP(CH₂)Phe and hPheP(CH₂)Tyr, had IC₅₀ in the 13 to 75 μ M range and that the former compound was effective in a nonlethal *Plasmodium chabaudi chabaudi* model of murine malaria.

Taken together, the results indicate that the M17 and/or M1 aminopeptidase of *P. falciparum* may be a valid antimalarial target and that apparent differences in the S1 subsites of parasite and human M17 enzymes may be exploited to design more-potent and selective inhibitors.

ACKNOWLEDGMENTS

We thank John Dalton for the gift of recombinant tPfAP-M17.

This work was supported by grants from Enterprise Ireland (SC/2003/111/B) and the Health Research Board of Ireland (RP/2005/57) to A.B.

REFERENCES

- Allary, M., J. Schrével, and I. Florent. 2002. Properties, stage-dependent expression and localization of *Plasmodium falciparum* M1 family zinc-aminopeptidase. *Parasitology* **125**:1–10.
- Bonilla, J. A., T. D. Bonilla, C. A. Yowell, H. Fujioka, and J. B. Dame. 2007. Critical roles for the digestive vacuole plasmepsins of *Plasmodium falciparum* in vacuolar function. *Mol. Microbiol.* **65**:64–75.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Charet, P., E. Aissi, P. Maurois, S. Bouquelet, and J. Biguet. 1980. Aminopeptidase in rodent *Plasmodium*. *Comp. Biochem. Physiol. B* **65**:519–524.
- Chen, X., C. R. Chong, L. Shi, T. Yoshimoto, D. J. Sullivan, Jr., and J. O. Liu. 2006. Inhibitors of *Plasmodium falciparum* methionine aminopeptidase 1b possess antimalarial activity. *Proc. Natl. Acad. Sci. USA* **103**:14548–14553.
- Dalal, S., and M. Klemba. 2007. Roles for two aminopeptidases in vacuolar hemoglobin catabolism in *Plasmodium falciparum*. *J. Biol. Chem.* **282**:35978–35987.
- Drag, M., J. Grembecka, M. Pawelczak, and P. Kafarski. 2005. α -Aminoalkylphosphonates as a tool in experimental optimisation of P1 side chain shape of potential inhibitors in S1 pocket of leucine- and neutral aminopeptidases. *Eur. J. Med. Chem.* **40**:764–771.
- Drag, M., R. Latajka, E. Gumienna-Kontecka, H. Kozłowski, and P. Kafarski. 2003. Stereoselective synthesis, solution structure and metal complexes of (1S,2S)-2-amino-1-hydroxyalkylphosphonic acids. *Tetrahedron Asymmetry* **14**:1837–1845.
- Edwards, G., and G. A. Biagini. 2006. Resisting resistance: dealing with the irrepressible problem of malaria. *Br. J. Clin. Pharmacol.* **61**:690–693.
- Ersmark, K., B. Samuelsson, and A. Hallberg. 2006. Plasmepsins as potential targets for new antimalarial therapy. *Med. Res. Rev.* **26**:626–666.
- Fennell, B. J., J. A. Naughton, E. Dempsey, and A. Bell. 2006. Cellular and molecular actions of dinitroaniline and phosphorothioamide herbicides on *Plasmodium falciparum*: tubulin as a specific antimalarial target. *Mol. Biochem. Parasitol.* **145**:226–238.
- Flipo, M., T. Beghyn, V. Leroux, I. Florent, B. P. Deprez, and R. F. Deprez-Poulain. 2007. Novel selective inhibitors of the zinc plasmodial aminopeptidase PfA-M1 as potential antimalarial agents. *J. Med. Chem.* **50**:1322–1334.
- Flipo, M., I. Florent, P. Grellier, C. Sergheraert, and R. Deprez-Poulain. 2003. Design, synthesis and antimalarial activity of novel, quinoline-based, zinc metallo-aminopeptidase inhibitors. *Bioorg. Med. Chem. Lett.* **13**:2659–2662.
- Florent, I., Z. Derhy, M. Allary, M. Monsigny, R. Mayer, and J. Schrével. 1998. A *Plasmodium falciparum* aminopeptidase gene belonging to the M1 family of zinc-metalloproteases is expressed in erythrocytic stages. *Mol. Biochem. Parasitol.* **97**:149–160.
- Gardiner, D. L., K. R. Trenholme, T. S. Skinner-Adams, C. M. Stack, and J. P. Dalton. 2006. Overexpression of leucyl aminopeptidase in *Plasmodium falciparum* parasites. Target for the antimalarial activity of bestatin. *J. Biol. Chem.* **281**:1741–1745.
- Gavigan, C. S., J. P. Dalton, and A. Bell. 2001. The role of aminopeptidases in haemoglobin degradation in *Plasmodium falciparum*-infected erythrocytes. *Mol. Biochem. Parasitol.* **117**:37–48.
- Gavigan, C. S., S. G. Machado, J. P. Dalton, and A. Bell. 2001. Analysis of antimalarial synergy between bestatin and endoprotease inhibitors using statistical response-surface modelling. *Antimicrob. Agents Chemother.* **45**:3175–3181.
- Giannousis, P. P., and P. A. Bartlett. 1987. Phosphorus amino acid analogues as inhibitors of leucine aminopeptidase. *J. Med. Chem.* **30**:1603–1609.
- Goldberg, D. E. 2005. Hemoglobin degradation. *Curr. Top. Microbiol. Immunol.* **295**:275–291.
- Grembecka, J., and P. Kafarski. 2001. Leucine aminopeptidase as a target for inhibitor design. *Mini Rev. Med. Chem.* **1**:133–144.
- Gyng, F. N., B. Poole, and W. Trager. 1982. Peptidases from *Plasmodium falciparum* cultured in vitro. *Mol. Biochem. Parasitol.* **5**:263–273.
- Hamilton, R., B. Walker, and B. J. Walker. 1995. A highly convenient route to optically pure α -aminophosphonic acids. *Tetrahedron Lett.* **36**:4451–4454.
- Kafarski, P., and B. Lejczak. 1989. Mixed carboxylic-carbonic anhydride method in phosphonopeptide synthesis. *Tetrahedron* **45**:7387–7396.
- Kowalik, J., L. Kupczyk-Subotkowska, and P. Mastalerz. 1981. Preparation of dialkyl 1-aminoalkane phosphonates by reduction of dialkyl 1-hydroxyiminoalkane phosphonates with zinc in formic acid. *Synthesis* **1981**:57–58.
- Liu, J., E. S. Istvan, I. Y. Gluzman, J. Gross, and D. E. Goldberg. 2006. *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. *Proc. Natl. Acad. Sci. USA* **103**:8840–8845.
- Makler, M. T., J. M. Ries, J. A. Williams, J. E. Bancroft, R. C. Piper, B. L. Gibbins, and D. J. Hinrichs. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am. J. Trop. Med. Hyg.* **48**:739–741.
- Nankya-Kitaka, M. F., G. P. Curley, C. S. Gavigan, A. Bell, and J. P. Dalton. 1998. *Plasmodium chabaudi chabaudi* and *P. falciparum*: inhibition of aminopeptidase and parasite growth by bestatin and nitrobestatin. *Parasitol. Res.* **84**:552–558.
- Oleksyszyn, J., R. Tyka, and P. Mastalerz. 1978. Direct synthesis of 1-aminoalkane phosphonic and 1-aminoalkane phosphinic acids from phosphorus trichloride or dichlorophosphines. *Synthesis* **1978**:479–480.
- Rawlings, N. D., and A. J. Barrett. 1993. Evolutionary families of peptidases. *Biochem. J.* **290**:205–218.
- Rawlings, N. D., F. R. Morton, and A. J. Barrett. 2006. MEROPS: the peptidase database. *Nucleic Acids Res.* **34**:D270–D272.
- Rosenthal, P. J. 2004. Cysteine proteases of malaria parasites. *Int. J. Parasitol.* **34**:1489–1499.
- Scornik, O. A., and V. Botbol. 2001. Bestatin as an experimental tool in mammals. *Curr. Drug Metab.* **2**:67–85.
- Sijwali, P. S., J. Koo, N. Singh, and P. J. Rosenthal. 2006. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **150**:96–106.
- Skinner-Adams, T. S., J. Lowther, F. Teuscher, C. M. Stack, J. Grembecka, A. Mucha, P. Kafarski, K. R. Trenholme, J. P. Dalton, and D. L. Gardiner. 2007. Identification of phosphinate dipeptide analog inhibitors directed against the *Plasmodium falciparum* M17 leucine aminopeptidase as lead antimalarial compounds. *J. Med. Chem.* **50**:6024–6031.
- Stack, C. M., J. Lowther, E. Cunningham, S. Donnelly, D. L. Gardiner, K. R. Trenholme, T. S. Skinner-Adams, F. Teuscher, J. Grembecka, A. Mucha, P. Kafarski, L. Lua, A. Bell, and J. P. Dalton. 2007. Characterization of the *Plasmodium falciparum* M17 leucyl aminopeptidase. A protease involved in amino acid regulation with potential for antimalarial drug development. *J. Biol. Chem.* **282**:2069–2080.
- Szewczyk, J., B. Lejczak, and P. Kafarski. 1982. Transesterification of diphenyl phosphonates using the potassium fluoride/crown ether/alcohol system. Part 2. The use of diphenyl 1-aminoalkane phosphonates in phosphonopeptide synthesis. *Synthesis* **1982**:409–412.
- Vander Jagt, D. L., B. R. Baack, and L. A. Hunsaker. 1984. Purification and characterization of an aminopeptidase from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **10**:45–54.
- Wu, Y., X. Wang, X. Liu, and Y. Wang. 2003. Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. *Genome Res.* **13**:601–616.
- Zhang, P., D. E. Nicholson, J. M. Bujnicki, X. Su, J. J. Brendle, M. Ferdig, D. E. Kyle, W. K. Milhous, and P. K. Chiang. 2002. Angiogenesis inhibitors specific for methionine aminopeptidase 2 as drugs for malaria and leishmaniasis. *J. Biomed. Sci.* **9**:34–40.
- Zuckerman, A., D. Spira, and J. Hamburger. 1967. A procedure for the harvesting of mammalian plasmodia. *Bull. W. H. O.* **37**:431–436.