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A Critical Evaluation of Pyrrolo[2,3-*d*]pyrimidine-4-amines as *Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) Inhibitors

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We have determined that a previously reported class of pyrrolo[2,3-*d*]pyrimidine-4-amines exhibit low-binding to apical membrane antigen 1 (AMA1) and suffer from unattractive qualities, such as aggregation. We attempted to remove these traits by generating molecules with improved solubility, however this did not translate into enhanced binding affinity or inhibition of erythrocytic growth. These results indicate that anti-malarial activity is not primarily due to inhibition of AMA1 function, but mediated by an alternate or additional mechanism of action.

Introduction

Malaria is a major health concern for many of the world's most vulnerable and impoverished societies, affecting peoples throughout sub-Saharan Africa and Southeast Asia, in particular. This infectious disease continues to threaten a large number of people, with more than 40% of the global population at risk of infection and 2% of human mortalities worldwide.[1] Malaria is caused by intracellular parasites of the genus *Plasmodium*, which are transmitted to humans when a female *Anopheles* mosquito takes a blood meal. All of the symptoms of malaria arise as a result of the parasite's asexual reproductive cycle, that occurs within erythrocytes of the human host.[2] For this reason existing treatments target this blood-stage infection.

Treatment strategies for malaria have changed markedly over recent decades, as the parasite has developed resistance to previously effective drugs. The current frontline approach is that of the artemisinin-combination therapies (ACTs).[1] However, recent evidence shows that resistance to artemisinin is emerging in Southeast Asia, particularly along the Thailand–Myanmar border.[3] Although there are other potential therapeutics in the pipeline, these are not sufficiently advanced to be therapeutically relevant at this stage. Moreover, the current portfolio of candidates in development, although improving, is still lacking in diversity.[4] Therefore, there is a clear and present need for new targets for drugs to combat malaria.[5]

Mature apical membrane antigen 1 (AMA1), a 66-kDa type 1 integral membrane protein with a short well-conserved cytoplasmic region, forms a complex with parasite rhoptry neck (RON) proteins as part of a moving junction that forms between the invading parasite and the host cell.[6,7] Crystal structures of the ectodomain of AMA1 from *Plasmodium falciparum* and related species reveal three domains, including two closely-packed PAN domains.[8,9] From these PAN domains, seven flexible loops extend to surround a large hydrophobic cleft consisting of 12 well-defined and conserved residues.[8] This cleft is the site of interaction between AMA1 and RON2,[6,10] and numerous inhibitory peptides and antibodies have also been shown to target this site.[11-13] Although recent studies have used genetic knockouts to challenge previous evidence that the AMA1–RON2 interaction is essential to host cell invasion by *P. falciparum* and related parasites in cell culture,[14,15] it remains clear that diverse inhibitors of this interaction do inhibit invasion.[6,11,12,16-19] Moreover, the strong conservation of the AMA1–RON2 interaction in the Apicomplexa phylum,[7,20] even in the face of strong selective pressure from host immune systems,[21,22] implies an important functional role *in vivo*.

On this basis, we and others have proposed AMA1 as a potential drug target against malaria.[13,23,24] Several additional factors contribute to its attractiveness in this regard. Firstly, there are no human homologues of AMA1 or the RON proteins. Secondly, inhibitors of the AMA1–RON2 interaction will have their site of action in the bloodstream, thereby avoiding the difficulties associated with targeting the intracellular stages of the malaria parasite. This point is particularly relevant in light of the role of drug transporters in mediating resistance to known anti-malarials.[25]

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Recently, Srinivasan *et al*. reported the first example of small-molecule inhibitors of the AMA1–RON2 complex, identified via an AlphaScreen assay of a ~21,000 member library, utilising a truncated RON2 peptide.[26] This screen identified seven molecules, three of which blocked merozoite invasion *in vitro* with IC_{50} values in the range 21 – 29 μM (Figure 1).

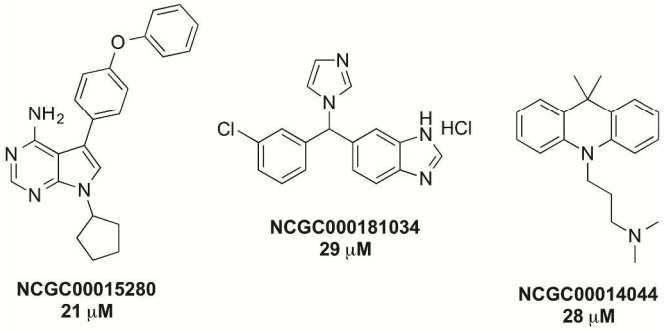


Figure 1. Putative AMA1 inhibitors identified by Srinivasan *et al*.[26]

Re-synthesis of NCGC00015280 and chemical elaboration of this scaffold identified two related molecules NCGC00262650 and NCGC00262654 that showed enhanced inhibition with reported IC_{50} values of 9.8 μM and 6 μM, respectively, compared to the re-synthesised NCGC00015280's activity of 30 μM (Figure 2).

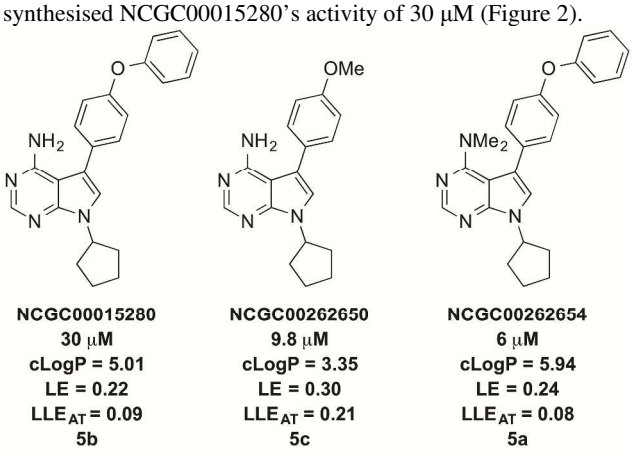


Figure 2. Elaboration of NCGC00015280 by Srinivasan *et al*.[26]

Srinivasan *et al*. present an array of cell-based assays to support their proposed mode of action for these compounds. They also attempted to demonstrate a direct interaction with AMA1 by surface plasmon resonance (SPR).[26] However, these experiments were compromised by the poor solubility of the compounds, and the shape and concentration dependence of the observed sensorgrams are consistent with low-affinity super-stoichiometric interaction with the SPR biosensor surface, rather than the high-affinity stoichiometric interaction expected of a specific inhibitor. Two of the identified compounds have calculated partition co-efficients (cLogP) that fall outside the traditional Lipinski 'rule of 5' upper limit of cLogP = 5.[27] Another useful metric of inhibitor quality is the lipophilic ligand efficiency (LLE_{AT}), as described by Astex, which incorporates the number of heavy atoms as an indicator of lipophilicity.[28] By this metric as well, these molecules are far from the attractive value of ≥ 0.3 kcal mol^{-1} per heavy atom (Figure 2). To assess the suitability of this class of compounds for further development as AMA1 inhibitors, we synthesised a panel of analogues and studied their solution behaviour and interactions with AMA1.

Results

We endeavoured to evaluate the pyrrolo[2,3-*d*]pyrimidine scaffold by using our methods for monitoring AMA1 ligand binding[29] and to ameliorate the unfavourable traits, such as the high cLogP and low LLE_{AT} values, whilst maintaining activity. Although compound lipophilicity is frequently an important factor for inhibitor potency, it can also contribute to off-target effects, leading to unwanted toxicity.[30] Small molecules that exhibit poor solubilities in aqueous buffers are often identified as promiscuous hits in a wide range of assays, complicating the interpretation of binding and/or activity data.[30-33] Understanding the physicochemical properties of a compound in an aqueous environment is therefore essential in evaluating chemical entities as lead candidates. To assess these issues for the class of compounds reported by Srinivasan *et al*.,[26] **5a–5c** were synthesised (see SI for details). They were then assessed at a range of concentrations (5, 10, 20, 40 and 80 μM) using 1D ^{1}H NMR spectroscopy, following the methods outlined by LaPlante *et al*.[31,32] This allowed direct observations of the aggregation propensities of these molecules in aqueous buffers.

The ^{1}H NMR spectra of **5a** and **5b** (Figure 3A & B) show clear evidence of extensive aggregation. Both compounds gave very weak NMR signals that did not increase with concentration. As there were no visible precipitates in these samples, these observations suggest that the compounds were self-associating to form colloidal aggregates over the entire concentration range tested.[31] These large aggregates tumble more slowly than non-aggregating compounds and therefore exhibit faster NMR relaxation and signals that are broadened beyond detection.

To confirm this interpretation of these data, surfactant (Tween 20) was added into these samples. The NMR signals were consistent with those expected for these molecules, albeit with significant residual line broadening (top panel, Figure 3A & B). These observations may be explained by the fact that surfactant is capable of dissociating large assemblies into smaller entities with better relaxation properties for NMR detection.[32] In contrast to **5a** and **5b**, **5c** gave sharp proton signals with peak intensities that increased with concentration over the range tested (Figure 3C).

However, the concentration dependence of the peak intensities is not linear (Figure 3D), while chemical shifts for a number of resonances showed a weak concentration dependence (Figure 3E), indicating that compound **5c** also aggregates in aqueous solution, albeit to a much lesser extent than do **5a** and **5b**. The signals of compound **5c** were shifted and broadened in the presence of Tween 20, presumably as a result of the interactions between the compound and surfactant. Furthermore, peak intensities increased in the presence of the surfactant (Figure 3D), again indicating the presence of large aggregates that are disrupted by the addition of Tween 20 into the sample.

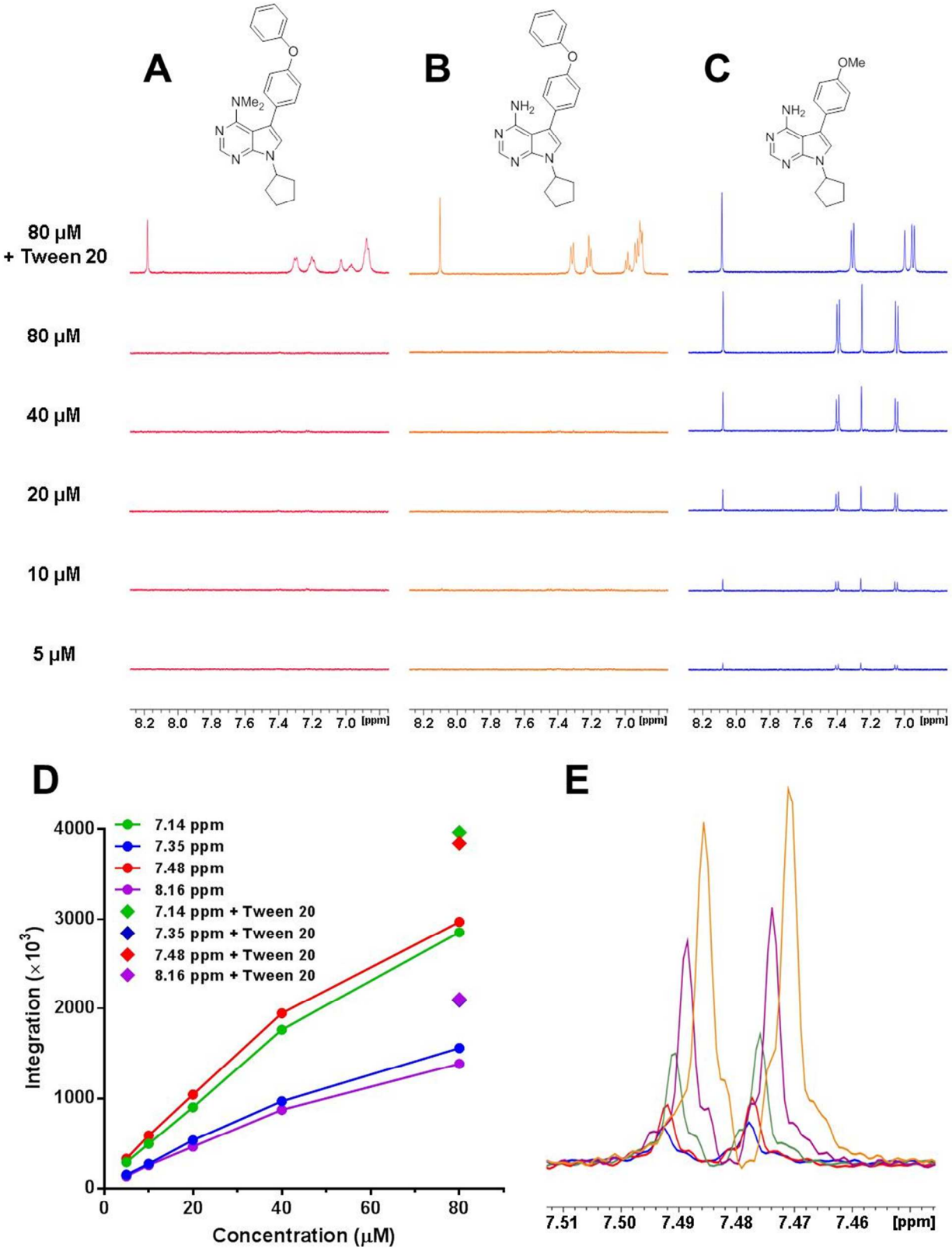


Fig 3. 1D ^{1}H NMR spectra of (A) **5a**, (B) **5b** and (C) **5c** at different concentrations and in the presence of 0.05% Tween 20. (D) Peak intensities of **5c** proton signals (7.14, 7.35, 7.48 and 8.16 ppm) at increasing compound concentrations and with the addition of surfactant. (E) Concentration dependence of the chemical shifts of **5c** at 5, 10, 20, 40 and 80 µM, respectively.

We then assessed the interaction of these compounds with AMA1 using techniques developed in our own search for inhibitors of the AMA1–RON2 interaction.[29] The aggregation behaviour of **5a** and **5b**, described above, precluded the use of compound-detected NMR strategies, so only **5c** was evaluated in the Carr–Purcell–Meiboom–Gill (CPMG) binding assay (Figure 4). This method utilises the CPMG spin-lock filter to eliminate signals of the rapidly relaxing AMA1 protein and bound ligands, with the signals of free compounds less affected.[34] CPMG spectra for detergent-free samples containing 80 µM **5c** in the presence and absence of 10 µM 3D7 *Pf*AMA1 were first acquired with 0 and 200 ms spin-relaxation filters. Thereafter, R1 and RON2 peptides were added into the samples and the same set of CPMG spectra was acquired to evaluate the binding activities of **5c** at the AMA1 hydrophobic cleft. The signal intensities and chemical shifts of a number of peaks for **5c** were affected by the presence of AMA1 (Figure 4A). These effects are exemplified using the proton resonance of **5c** at around 7.35 ppm in Figure 4B. The signals were broadened and shifted slightly upfield in the presence of AMA1 and, when a 200 ms spin-lock filter was applied, the signal in the presence of AMA1 relaxed significantly faster than the signal in the absence of protein (R_2 relaxation rates of 8.2 and 1.4 s^{-1}, respectively). These results are indicative of **5c** binding to AMA1.

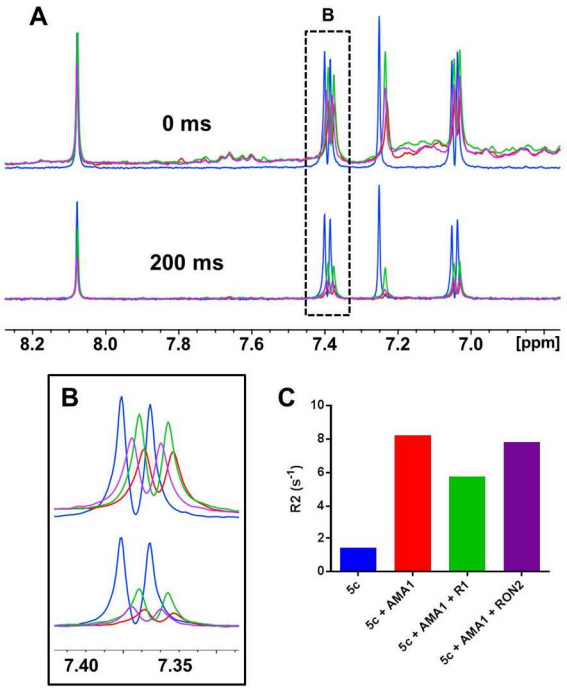
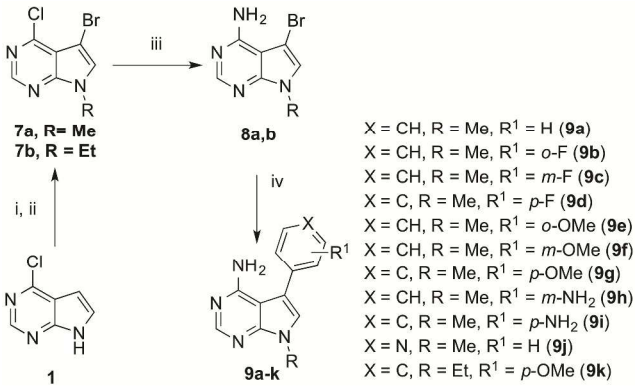


Fig 4. CPMG binding assay. (A and B) All CPMG spectra were acquired with both 0 (top spectra) and 200 ms (bottom spectra) spin-relaxation filters in the absence of detergent. Blue and red spectra were results for samples containing **5c** in the presence or absence of 3D7 *Pf*AMA1, respectively. Green and purple spectra correspond to samples containing **5c** and 3D7 *Pf*AMA1 with the additions of R1 and RON2 peptides, respectively. (C) Transverse relaxation rate (R2) for different samples used in the binding studies.

When R1 and RON2 peptides were added to the samples containing both AMA1 and **5c**, the compound peaks shifted slightly downfield towards the chemical shift of the free compound. Also, partial restoration of the free compound line shape and relaxation rate was observed in both samples with the peptides added. These observations are consistent with competition between **5c** and R1 and RON2 peptides for binding sites on AMA1. The presence of the competing peptides reduced the fraction of bound **5c**, which in turn decreased the average transverse relaxation rate of the compound, and thus increased **5c** signals after the CPMG period (Figure 4B and C). Similar results were observed when the same set of experiments was conducted in the presence of Tween 20 (Figure S1). This result confirms that **5c** in its monomeric form is able to bind AMA1.

Finally, we interrogated these compounds, including their synthetic precursors, by SPR, to estimate the binding affinities to AMA1 using methods described previously.[29] Not unexpectedly, the precursor molecule fragments **1**–**3**, showed minimal interaction with AMA1 at concentrations up to 200 µM. Once the 4-amino or 4-dimethylamino group was introduced, we saw evidence of super-stoichiometric binding, in the form of responses that exceeded the maximal response expected for these compounds and that failed to saturate, even at the highest concentrations studied. These issues were particularly acute in the case of **5a** and **5b**, and precluded any estimate of the affinity of these molecules with AMA1. For **5c**, we observed unambiguous over-binding only at higher concentrations. However, the response observed at lower concentrations is inconsistent with an affinity for AMA1 tighter than ~ 1mM, while the IC_{50} for this compound reported by Srinivasan *et al.* is 9.8 µM.[26] This discrepancy suggests that this series of compounds exert their effects on host cell invasion by *P. falciparum* merozoites by some mechanism other than direct inhibition of AMA1, or by an additional mechanism of action.

To address the shortcomings of the three molecules, as outlined above, a series of pyrrolo[2,3-*d*]pyrimidine-4-amines was synthesised in an attempt to improve solubility whilst maintaining or improving affinity for AMA1 (Scheme 1). This was achieved by alkylation, bromination, amination and Suzuki coupling. We envisaged that replacement of the 7-cyclopentyl with a methyl group should aid in solubility, and we also explored substitutions on the 5-aryl group while retaining the 4-amino group. This scaffold featuring the *N*-methyl substitution is the core of a known protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) inhibitor that has been selected as a preclinical candidate for tumour inhibition.[35,36] The compounds produced had cLogP values of 0.93–2.35, which, in contrast to the cLogP value of 3.35 for **5c**, provided soluble compounds for study. Aggregation propensities of **9a–k** were evaluated using 1D ^{1}H NMR spectroscopy (Figure S2) as described earlier for **5a–c**. The 7-methyl and 7-ethyl series' exhibited substantially reduced aggregation behaviour, when compared to the 7-cyclopentyl series (**5a–c**), with or without the addition of Tween 20, demonstrating our success in eliminating some of the unwanted behaviour of **5a–c**.



Scheme 1. Reagents and conditions: (i) NaH, DMF, 0 °C, 30 min, b) for **6a**, MeI and for **6b**, EtI, 60 °C, 4 h; (ii) NBS, DCM, 25 °C; (iii) NH_4OH, *i*-PrOH, 100 °C, 40 h; (iv) R^1-$B(OH)_2$, $PdCl_2(PPh_3)_2$, THF/1M Na_2CO_3, 100 °C, 2 h.

All pyrrolo[2,3-*d*]pyrimidines (**5a**–**5c** and **9a**–**9k**) were analysed for binding to AMA1 using SPR, utilising similar conditions to those employed by Srinivasan *et al*.[26] In general, the sensorgrams showed weak binding affinities, with K_D values ≥1 mM. There were no visible precipitates in the samples and all compounds were soluble in the buffer conditions employed. From this small library of compounds, however, no meaningful structure-activity relationships (SAR) could be inferred and no increased affinity for AMA1 was observed, suggesting that these compounds are not potent inhibitors of AMA1 interactions. Finally, **9a–k** were tested to ascertain their ability to inhibit the erythrocytic growth of *P. falciparum* (Figure S3).[37] Compounds **5c** and **9a–k** showed substantial inhibitory activity in this assay, although this activity did not correlate with their affinity for AMA1 by SPR. **5a** and **5b** demonstrated IC_{50} values > 1 mM, compared to the reported values of 6 and 30 μM, respectively.[26] The IC_{50} value for **5c** was 63 μM, in comparison to the reported value of 9.8 μM and the best analogue had an IC_{50} value < 31 μM for **9c**. This represents still further evidence in support of an alternative mechanism of anti-malarial action by these compounds.

Discussion

Our results suggest that these compounds in their current form are not suitable for development as AMA1 inhibitors given their apparent low binding affinity to AMA1, sub-optimal potency, relative insolubility and tendency to form aggregates. Our findings suggest that these compounds may exhibit an alternative or additional mechanism of anti-malarial action. Compound **5b** was identified initially as a Src-family kinase inhibitor[38] and molecules containing the pyrrolo[2,3-*d*]pyrimidine scaffold have been shown to be involved in a number of therapeutically relevant areas such as Huntington's disease[39] and acute myeloid leukaemia,[40] where they are believed to act via a Src-family kinase mechanism. Srinivasan *et al*. demonstrated that a structurally different Src-inhibitor did not block invasion.[26] However, this does not preclude the possibility of the pyrrolo[2,3-*d*]pyrimidine inhibitors acting as kinase inhibitors in this context. A structurally similar framework, incorporating an extra nitrogen at the 6-position, namely the pyrazolo[3,4-*d*]pyrimidin-4-amine scaffold with comparable N1– and 3–position modifications, has been implicated in a related apicomplexan species, *Toxoplasma gondii*, as a calcium-dependent protein kinase 1 (CDPK1) inhibitor.[41,42] CDPK is known to control microneme secretion and consequently block invasion in *T. gondii*, as well as *Plasmodium falciparum*.[43] Therefore, it is conceivable that these pyrrolo[2,3-*d*]pyrimidines could be acting via a CDPK mechanism, or in addition to, AMA1-mediated inhibition. Colloidal aggregation of compounds is well known to contribute to promiscuity in high-throughput screens and in a range of other assays.[30-33,44] The observed aggregation properties of **5a** and **5b**, may be responsible for their inhibitory effects,[26] rather than being specific inhibitors of the AMA1–RON2 interaction.[44] Compound **5c** seems to be genuinely binding AMA1, but with insufficient affinity (using recombinant AMA1) to explain its reported activity. It remains possible that the binding affinity to native AMA1 is different from that measured using recombinant AMA1, although we note that these two preparations afford essentially identical results for genuine AMA1 inhibitors such as the peptide R1. Our attempts to produce more soluble analogues (**9a–k**) with increased activity did not generate any meaningful SAR.

Conclusion

Candidate pyrrolo[2,3-*d*]pyrimidine-4-amines (**5a–c**) appear to be moderately potent inhibitors of erythrocyte invasion by *P. falciparum* merozoites, that prevent the formation of the moving junction and the formation of the AMA1–RON complex.[26] These effects may not be mediated primarily by direct stoichiometric interaction with AMA1; inhibitory activity could include off-target mechanisms, which may be related to their tendency to form colloidal aggregates in aqueous solution, and/or their activity as kinase inhibitors. Molecules with lower cLogP values were synthesised (**9a–k**), but failed to achieve greater inhibitory activity. Furthermore, this series of compounds carries significant physicochemical liabilities that are likely to impede their further development.

Acknowledgments

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A Critical Evaluation of Pyrrolo[2,3-*d*]pyrimidine-4-amines as *Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) Inhibitors

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Graphical Abstract