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Identification of β-hematin inhibitors in a high-throughput screening effort reveals scaffolds with *in vitro* antimalarial activity

Rebecca D. Sandlin [a], Kim Y. Fong [a], Kathryn J. Wicht [b], Holly M. Carrell [a], Timothy J. Egan [b], David W. Wright [a,*]

[a] Department of Chemistry, Vanderbilt University, Station B 351822, Nashville, TN 37235, USA
[b] Department of Chemistry, University of Cape Town, Rondebosch 7700, South Africa

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ABSTRACT

The emergence of drug resistant strains of *Plasmodium* spp. creates a critical need for the development of novel antimalarials. Formation of hemozoin, a crystalline heme detoxification process vital to parasite survival serves as an important drug target. The quinoline antimalarials including chloroquine and amodiaquine owe their antimalarial activity to inhibition of hemozoin formation. Though *in vivo* formation of hemozoin occurs within the presence of neutral lipids, the lipophilic detergent NP-40 was previously shown to serve as a surrogate in the β-hematin (synthetic hemozoin) formation process. Consequently, an NP-40 mediated β-hematin formation assay was developed for use in high-throughput screening. Here, the assay was utilized to screen 144,330 compounds for the identification of inhibitors of crystallization, resulting in 530 hits. To establish the effectiveness of these target-based β-hematin inhibitors against *Plasmodium falciparum*, each hit was further tested in cultures of parasitized red blood cells. This effort revealed that 171 of the β-hematin inhibitors are also active against the parasite. Dose–response data identified 73 of these β-hematin inhibitors have IC_{50} values $\leqslant 5$ μM, including 25 compounds with nanomolar activity against *P. falciparum*. A scaffold-based analysis of this data identified 14 primary scaffolds that represent 46% of the 530 total hits. Representative compounds from each of the classes were further assessed for hemozoin inhibitory activity in *P. falciparum* infected human erythrocytes. Each of the hit compounds tested were found to be positive inhibitors, while a negative control did not perturb this biological pathway in culture.

1. Introduction

Plasmodium falciparum, the most virulent species of the malaria parasite, is responsible for nearly one million deaths each year (2013 World Malaria Report). The overwhelming majority of these deaths occur among young children residing in sub-Saharan Africa. Alarmingly, the parasite has developed resistance mechanisms to nearly all-affordable, widely available antimalarials (Noedl et al., 2008; Cheeseman et al., 2012). It is therefore critical to enrich the antimalarial drug discovery pipeline with new chemotypes. Fortunately, several unique pathways have been identified that serve as drug targets including inhibition of DNA synthesis, de novo heme biosynthesis, glycolysis and hemozoin formation (Surolia and Padmanaban, 1992; Subbayya et al., 1997; Egan, 2003; Mitra et al., 2012).

During the intraerythrocytic stages of infection, the malaria parasite consumes more than 80% of the infected red blood cell's hemoglobin to serve as a source of amino acids (Francis et al., 1997). This process of hemoglobin degradation occurs within the parasite's digestive food vacuole, an acidic organelle (pH ~4.8–5.2). As a consequence of hemoglobin degradation, toxic free heme is liberated. Lacking an enzymatic method of heme detoxification, the malaria parasite has evolved a method by which it converts soluble free heme into an insoluble, nontoxic biomineral called hemozoin, the malarial pigment (Egan, 2008). Though hemozoin has been studied since the 18th century, it was not until 1999 that XRD revealed hemozoin consists of a centrosymmetric triclinic unit cell comprised of reciprocal head-to-tail dimeric units of heme bound through propionate O-Fe(III) (Pagola et al., 2000). These heme dimers form an extended crystal structure through hydrogen bonding of acidic propionic groups from adjacent dimers. The *in vivo* mechanism of hemozoin formation has been contested for many years, though emerging evidence implicates the involvement of neutral lipids present within the digestive food vacuole as the

* Corresponding author. Tel.: +1 (615) 322 2636; fax: +1 (615) 343 1234.
E-mail address: David.Wright@vanderbilt.edu (D.W. Wright).

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site of crystal nucleation and growth (Egan et al., 2006; Kapishnikov et al., 2012a,b). Mass spectrometric analysis of the neutral lipid particles concentrated in the digestive food vacuole identified a specific blend of neutral lipids in a 4:2:1:1:1 ratio of monostearic, monopalmitic, dipalmitic, dioleic and dilinoleic glycerols (Jackson et al., 2004; Pisciotta et al., 2007). Synthetic neutral lipid droplets (SNLDs) that consist of this biologically relevant blend of neutral lipids successfully promote the rapid formation of β-hematin (half-life of 1.9 ± 0.01 min) under physiological pH and temperature conditions. Further, soluble free heme was shown to rapidly partition in the SNLDs in a pH-dependent manner. The pH profile of heme partitioning resembles that of β-hematin formation (Hoang et al., 2010a,b; Ambele and Egan, 2012). Collectively, this evidence substantiates a role for neutral lipid particles in the process of hemozoin formation.

As perturbations to hemozoin formation lead to parasite death, understanding the mechanism of hemozoin formation is useful. Chloroquine (CQ), one of the most successful antimalarials ever developed, owes its activity to inhibition of hemozoin formation (Egan and Marques, 1999; Combrinck et al., 2013). Unfortunately, the parasite has developed resistance to the use of this quinoline-based antimalarial. However, this resistance is not due to changes in the hemozoin formation pathway, but rather arises from mutations in *Pf*CRT, a membrane protein localized to the digestive food vacuole (Fidock et al., 2000; Johnson et al., 2004; Martin et al., 2009). Mutations are thought to be responsible for reduced accumulation of CQ within the digestive food vacuole, thereby preventing CQ-heme interactions from occurring. This efflux mechanism is specific for quinoline-based antimalarials (Lehane et al., 2008). Consequently, hemozoin formation remains an important drug target for the development of new non-quinoline antimalarials.

Recently, the NP-40 β-hematin formation assay was validated for use in high-throughput screening (HTS). This assay utilizes the lipophilic detergent, Nonidet P-40 (NP-40), to serve as a surrogate for *in vitro* β-hematin formation under physiologically relevant assay conditions (Fig. 1) (Carter et al., 2010). This detergent mediator is low cost and requires no special handling steps in assay setup (Sandlin et al., 2011). Here, the β-hematin formation assay has been utilized to screen a library of 144,330 commercially available compounds in the Vanderbilt University Institute of Chemical Biology (VICB) library (Fig. 2). Each of the target-specific hits was analyzed for *in vitro* antimalarial activity in cultures of *P. falciparum*. Follow-up dose–response data was collected for each *in vitro* antimalarial compound. Those that exhibited nanomolar activity against the parasite were further examined in a multi-drug resistant strain of *P. falciparum*. The drug target pathway of hemozoin inhibition was subsequently validated within a chloroquine-sensitive strain of *P. falciparum* to emphasize the biological relevance of our *in vitro* target based screen. Scaffold representatives were screened in a culture of *P. falciparum* infected erythrocytes to assess the biological inhibition of heme detoxification.

2. Materials and methods

2.1. Materials

Nonidet P-40 (NP-40, Shell Chemical Co.) originated from Pierce Biotechnology, Rockford, Il (not to be confused with different detergents also referred to as NP-40). Flat bottom, 384-well plates (3680, Corning) and optical bottom plates (142761, Nunc) were purchased from Fisher. Hemin (⩾98%, Fluka), amodiaquine, sodium acetate trihydrate, saponin, and pyridine were obtained from Sigma–Aldrich, St. Louis, MO. A (+) human plasma and erythrocytes were purchased from Valley Biomedical, Winchester, VA. SYBR Green-I nucleic acid gel stain (10,000×) was supplied by Invitrogen. The screening library consisted of compounds originating from ChemBridge and ChemDiv.

2.2. Detergent-mediated NP-40 β-hematin formation assay

The β-hematin formation assay was adapted for use in a 384-well microtiter plate as previously described (Sandlin et al., 2011). Solutions were added to the microtiter plate in the order of water (20 μL), NP-40 stock solution (5 μL), acetone (7 μL), and heme suspension (25 μL). The NP-40 stock solution (348 μM) was prepared in water. A 25 mM stock solution of hematin was prepared by dissolving hemin chloride in DMSO followed by one minute of sonication. The heme solution was then filtered through a 0.22 μm PVDF membrane filter unit. From this solution, the heme suspension (228 μM) was added to a 2 M acetate buffer at pH 4.9 and vortexed for ~5 s. The plate was then incubated for six hours in a shaking water bath at 45 rpm and 37 °C. Following incubation, the microtiter plate was removed from the water bath and the assay was analyzed using the pyridine-ferrochrome method (Ncokazi and Egan, 2005). Following the addition of 15 μL of acetone to each well of the plate, 8 μL of a pyridine solution was added (50% pyridine, 20% acetone, water and 200 mM HEPES, pH 7.4) so that the final concentration of pyridine was 5% (v/v). Following 30 min of shaking to facilitate the solubilization of free heme, the absorbance of the resulting complex was measured at 405 nm on a SpectraMax M5 plate reader.

2.3. Identification of β-hematin inhibitors

Test compounds in the Vanderbilt University High-Throughput Screening Facility originated from ChemBridge and ChemDiv. A Labcyte Echo 550 non-contact acoustic liquid delivery system was used to deliver all control and test compounds to the 384-well assay plate. Positive controls consisted of a 100 μM final concentration of amodiaquine (dissolved in DMSO) and negative controls consisted of DMSO only (0.19%). Controls were added to the first and last two columns of each plate in an alternating, checkerboard pattern. All test compounds (10 mM in DMSO) were added so that the final test concentration was 19.3 μM (320 total compounds tested per plate). Following addition of controls and test compounds, the reagents of the β-hematin formation assay described above were added using a Thermo Scientific Multidrop Combi Bulk Reagent Dispenser. Inhibition of β-hematin was assessed relative to the positive and negative controls on each plate. Compounds inhibiting ⩾80% β-hematin formation were considered hits. This stringent threshold for identifying β-hematin inhibitors facilitated the identification of a set of potent inhibitors of crystallization. Each hit was then tested in duplicate in a dose–response assay to identify false-positives and establish IC_{50} values using a range of concentrations of test compound from 0.5–110 μM. The test compounds were delivered from a 10 mM stock solution in DMSO and DMSO was backfilled to maintain a uniform percentage (0.1%) throughout the plate. Sigmoidal dose–response curves were generated using GraphPad Prism v5.0 (March 7, 2007).

2.4. P. falciparum culture conditions

P. falciparum strains D6 (Walter Reed Army Institute of Research [WRAIR]/Sierra Leone) and C235 (WRAIR/Thailand) were maintained using a modification of methods described by Trager and Jensen (1976). RPMI 1640 medium supplemented with 25 mM HEPES, 11 mM glucose, 0.24% sodium bicarbonate, 10% human A (+) plasma (heat-inactivated) and 29 μM hypoxanthine was prepared weekly. Cultures were maintained at 5% hematocrit in A (+) blood (washed two times with RPMI medium and used no longer

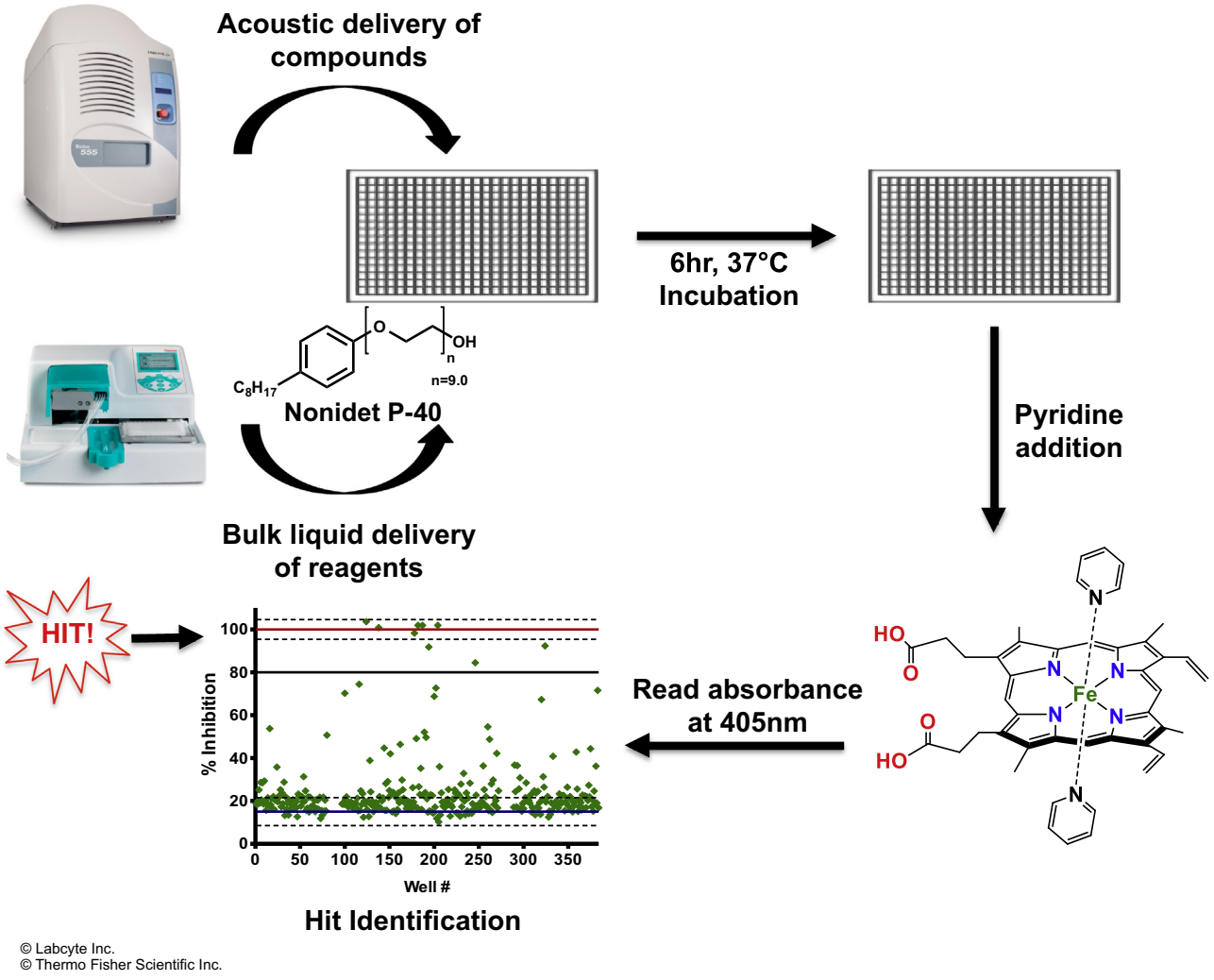


Fig. 1. Workflow of the high-throughput β-hematin assay. The 144,330 test compounds were delivered to 384-well microtiter plates using a noncontact liquid handler, followed the addition of buffer, hemin, and NP-40 detergent (shown) using a bulk liquid delivery system. The plates were incubated while shaking for six hours at 37 °C prior to the addition of pyridine. The absorbance values of the test compounds were read using a SpectraMax M5 plate reader to establish hits of ⩾80% inhibition.

than one week). Culture medium was routinely exchanged and subcultured upon reaching 5% parasitemia (every 3–4 days). Cultures were incubated at 37 °C in a gas mixture of 5% O_2, 5% CO_2 and N_2.

2.5. Malaria SYBR Green I fluorescence (MSF) assay

Inhibitors of β-hematin formation were tested in the CQ-sensitive, D6 strain of *P. falciparum* using a modification of literature methods (Johnson et al., 2007). Briefly, test compounds were prescreened at a concentration of 23 μM at 0.3% starting parasitemia (2% hematocrit) in duplicate in 384-well optical bottom microtiter plates. Positive and negative controls consisted of a kill concentration of CQ (400 nM in water) and DMSO (0.25%), respectively. Dose–response curves were established for each active compound from a concentration of 0–23 μM. Sigmoidal dose–response curves were generated using GraphPad Prism v5.0 (March 7, 2007). Compounds exhibiting nanomolar IC_{50} values were further tested in a dose–response format against the multidrug resistant C235 strain of *P. falciparum*.2.6 Heme speciation assay for target validation

Commercially available hit compounds active both in the target-based β-hematin assay and the phenotypic MSF assay were selected from each scaffold and further tested using a protocol previously described (Combrinck et al., 2013). In short, a culture of the CQ-sensitive strain D6 consisting of sorbitol synchronized early ring stage parasites at 5% parasitemia and 2% hematocrit was evenly divided into four culture flasks and treated with compound. The concentrations chosen were obtained from the results of the MSF assay and consisted of 0, 0.5, 1, 2, and 3 times the phenotypic IC_{50} value of the compound. The cultures were incubated under the conditions described in Section 2.4 for 32 h before isolating the trophozoites using saponin lysis (0.05%). The trophozoite pellet was lysed using the freeze-thaw method followed by addition of HEPES buffer (0.02 M, pH 7.5) and SDS (4%). After sonication and centrifugation, the supernatant was collected as Fraction 1, while pyridine (5%) was added to the pellet. A second centrifugation step resulted in collection of the supernatant as Fraction 2 and the pellet was solubilized with sodium hydroxide (0.3 M) as Fraction 3. Each fraction was analyzed by collecting the absorbance spectra between 300 and 800 nm. The peak maximum observed at 405 nm was used to calculate the ratios of heme species: *Pf* hemoglobin (Fraction 1), intercellular free heme (Fraction 2), and hemozoin (Fraction 3). Parasite survival was determined using SYBR Green I fluorescence and morphological changes were observed using microscopy analysis.

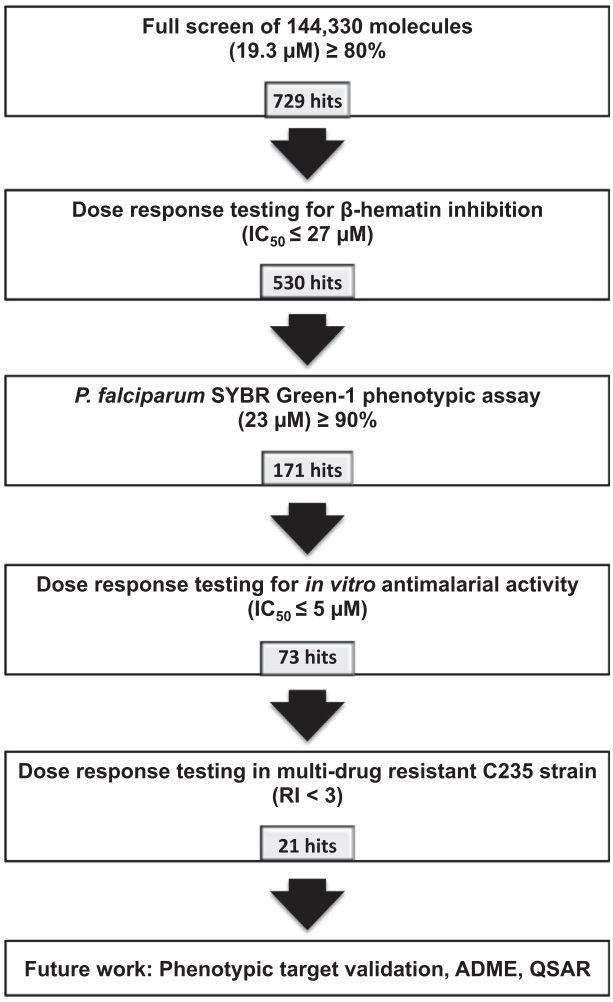


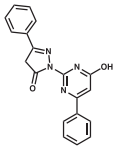
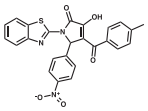
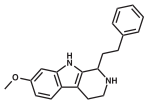
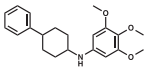
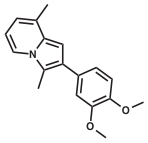
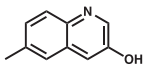
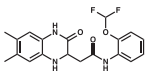
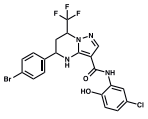
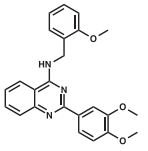
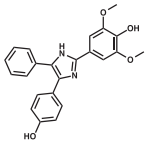
Fig. 2. Workflow of the Vanderbilt University Institute of Chemical Biology library screen. Test compounds were screened for β-hematin inhibition, followed by testing *in vitro* antimalarial activity in two strains for *Plasmodium falciparum*. Hits from both the primary and secondary screenings will be examined in target validation and pharmacokinetic studies.

3. Results

3.1. Identification of β-hematin inhibitors

144,330 compounds were tested in the NP-40 β-hematin formation assay. This screening effort resulted in the identification of 729 compounds exhibiting ⩾80% inhibitory activity relative to controls (0.5% hit rate). This activity threshold ensured that only potent β-hematin inhibitors were identified and provided a manageable number of compounds for follow-up dose response testing. Each preliminary hit was cherry-picked and tested in a dose–response assay (0.5–110 μM) to identify false-positives and to establish the potency of each hit against β-hematin formation. Compounds exhibiting IC_{50} values against hemozoin formation of ⩽27 μM were confirmed as hits. Using this data approach, a total of 530 hits were confirmed (0.14% false-positive hit rate), as shown in Supplementary Data, which will only be available to readers online. All had IC_{50} values more potent than CQ (53.0 μM) and 416 were more potent than AQ (21.0 μM) in this assay. The top ten most potent β-hematin inhibitors identified in this screen are shown in Table 1 and highlight the structural diversity resulting from this screening effort.

Table 1
The top ten most potent inhibitors of β-hematin formation in the VICB library.

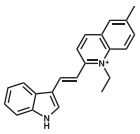
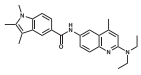
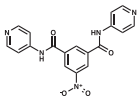
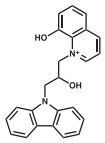
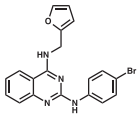
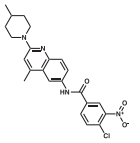
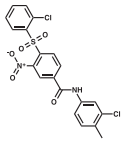
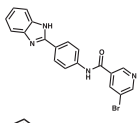
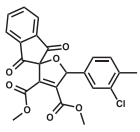
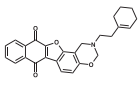
VU identifier	Structure	β-Hematin IC_{50} (μM)	D6 IC_{50} (μM)
VU0014981		0.4	Inactive
VU0063871		0.4	Inactive
VU0015078		1.1	0.81
VU0020967		1.5	Inactive
VU0042031		1.7	3.52
VU0000264		2.0	0.59
VU0099210		2.2	Inactive
VU0123869		2.4	Inactive
VU0358176		2.4	1.72
VU0094619		2.4	0.70

3.2. Activity of β-hematin inhibitors in cultures of P. falciparum

All confirmed β-hematin inhibitors were counter-screened to establish whether or not they retained activity in cultures of *P. falciparum* parasitized red blood cells, using the *in vitro* malaria SYBR Green I fluorescence-based assay (Johnson et al., 2007). Each β-hematin inhibitor was prescreened at a concentration of 23 μM. Percent inhibition was determined relative to positive (kill concentration of CQ) and negative (DMSO only) controls. Test compounds that exhibited ⩾90% inhibition of parasitemia were considered hits. Using these criteria, 171 of the β-hematin inhibitors identified in the primary screen were also active against the parasite cultures. This high hit rate (32%) was consistent with the pilot screen (Sandlin et al., 2011).

Follow-up dose–response analysis of the 171 *in vitro* antimalarial β-hematin inhibitors revealed that 73 compounds exhibited

Table 2
The top ten most potent *in vitro* antimalarial β-hematin inhibitors identified. RI = IC_{50} multidrug-resistant strain/IC_{50} drug sensitive strain.

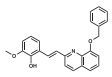
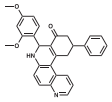
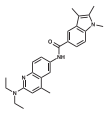
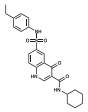
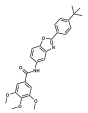
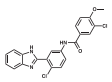
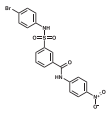
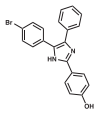
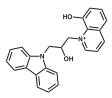
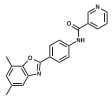
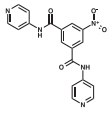
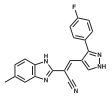
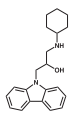
VU identifier	Structure	β-Hematin IC_{50} (μM)	D6 IC_{50} (μM)	C235 IC_{50} (μM)	RI
VU0098755		12.6	0.11	0.13	1.2
VU0073687		6.3	0.18	0.55	2.9
VU0001281		5.9	0.19	0.17	0.9
VU0065708		16.2	0.20	0.18	0.9
VU0096505		8.8	0.24	0.22	0.9
VU0107282		17.0	0.29	0.54	1.9
VU0114785		13.4	0.35	4.82	13.9
VU0002101		14.3	0.35	0.41	1.2
VU0028177		13.3	0.35	0.46	1.3
VU0063971		8.9	0.38	0.83	2.2

⩽5 μM activity against *P. falciparum*, including 25 compounds with nanomolar activity. The ten most potent compounds identified in this screen are shown in Table 2. Furthermore, each compound exhibiting nanomolar activity against the D6 strain was also tested against the multidrug-resistant C235 strain of *P. falciparum*. Following the determination of IC_{50} values in C235, the resistance index (RI = IC_{50} multidrug-resistant strain/IC_{50} drug sensitive strain) was calculated for each compound to identify those that are predisposed for resistance (Raynes et al., 1995). For 21 of the 25 nanomolar potent *in vitro* antimalarials identified, the calculated RI was <3, indicating that these compounds are approximately as effective against drug resistant strains as they are against sensitive strains. This suggests that the majority of these compounds can serve as valuable starting points for lead probe optimization.

3.3. Target validation of β-hematin inhibitors in cultures of *P. falciparum* through heme speciation

Several representative compounds from each of the scaffold classes were chosen as exemplars to validate the drug target pathway within a culture of *P. falciparum* infected erythrocytes (Table 3). These β-hematin and parasite inhibitors were tested in the CQ-sensitive D6 strain at 0, 0.5, 1, 2, and 3 times the IC_{50} values obtained in the MSF assay. Through the quantification of *Pf* hemoglobin, free heme, and hemozoin remaining in the parasite following drug treatment, each compound was probed for activity against the hemozoin formation biological pathway. An increase in parasitic free heme concentrations along with a decrease in hemozoin and parasite survival levels would result in an active inhibitor of hemozoin formation. Alternatively, if a dose responsive drug

Table 3
Representative hit compounds used for target validation in a parasite culture.

VU identifier	Structure	Scaffold	β-Hematin IC_{50} (μM)	D6 IC_{50} (μM)	C235 IC_{50} (μM)	Δ Free heme (%)
VU0358505		A	18.1	4.22	ND	10
VU0068286		G	19.1	4.09	ND	8
VU0073687		J	6.3	0.18	0.55	14
VU0077964		K	10.3	3.1	ND	6
VU0054902		B	17.9	4.57	ND	51
VU0122425		D	21.5	4.32	ND	28
VU0118993		E	15.9	9.88	ND	26
VU0099289		N	5.7	9.06	ND	43
VU0065708		H	16.2	0.2	0.18	14
VU0357882		M	8.9	17.83	ND	26
VU0001281		MISC	5.86	0.194	0.174	33
VU0194156		MISC	10.51	6.153	ND	23
Negative control		Not Active		0.99	ND	0.4

treatment results in parasite death, but free heme levels resemble those of an untreated culture, it is likely that the compound does not target the hemozoin formation pathway. Each of the hits examined resulted in a rise of free heme relative to hemozoin with decreased parasite survival, indicating a perturbation of the hemozoin formation pathway. To ensure that the assay is valid, a compound found to be active against the parasite, but did not inhibit β-hematin formation was used as a negative control. While still resulting in parasite death, did not show a significant difference of free heme from baseline levels, suggesting another biological drug

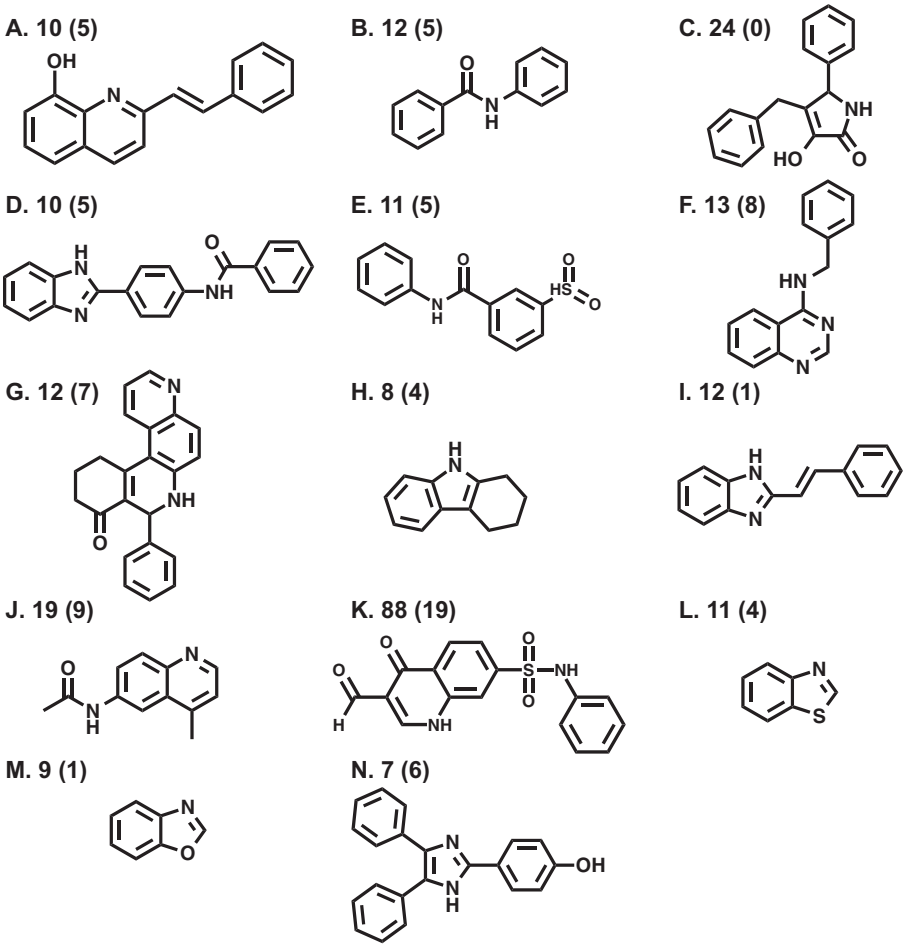


Fig. 3. The 14 primary scaffolds identified in the β-hematin inhibitory screen. Values shown indicate the number of β-hematin inhibitors associated with each scaffold and the number of those inhibitors that also possess *in vitro* antimalarial activity (in parentheses).

target, as expected. The high percentage of compounds shown to be hemozoin inhibitors is again indicative of our biologically relevant NP-40 β-hematin assay. Thus, the results of the heme speciation can give evidence for activity of the remaining hits of our β-hematin and antimalarial high-throughput screens.

3.4. Structural analysis of hits

In the initial pilot screen of the VICB HTS library, there were six unique scaffolds identified as β-hematin inhibitors (Sandlin et al., 2011). The completed HTS screen refines the original scaffold analysis and the 530 hits were binned into categories based upon a common scaffold. These compounds were identified from the dataset using ChemCart by Delta Soft version 5.3, resulting in 14 predominant scaffolds that represent 46% of the total number of β-hematin inhibitors identified (Fig. 3). The number of β-hematin inhibitors associated with each scaffold, as well as the number of those β-hematin inhibitors that also showed *in vitro* antimalarial activity, is shown above each scaffold. A subset of the full VICB chemical library was used in the pilot screen. The compounds in this subset were randomly selected and were found to represent 85% of the possible scaffolds available in the entire library. A complete examination of the library resulted in greater diversity in structures, as additional scaffolds were found present. The remaining compounds not falling into one of the 14 scaffolds consist of a variety of chemically distinct structures.

4. Discussion

Inhibition of hemozoin formation is an important parasite-specific target for the discovery of new antimalarials. Current evidence suggests neutral lipids as the mediator of hemozoin formation *in vivo* (Carter et al., 2010; Sandlin et al., 2011; Kapishnikov et al., 2012a,b). Recently, we have validated an HTS assay that substitutes the readily available lipophilic detergent, NP-40, to serve as a surrogate for expensive neutral lipids. Utilizing the biologically relevant conditions under which hemozoin formation occurs within the digestive food vacuole of the parasite, 144,330 compounds were screened using the NP-40 β-hematin formation assay. As a result of this effort, 530 β-hematin inhibitors were identified. Subsequent dose–response curves revealed that all of these inhibitors to be more potent than CQ in this assay. Each of the 530 β-hematin inhibitors was tested in the *P. falciparum* assay against the D6 (CQ-sensitive) strain of the parasite to identify those compounds possessing *in vitro* antimalarial activity. 32% of these hits were confirmed to inhibit $\geqslant$90% parasite growth, consistent with trends observed in the pilot screen (Sandlin et al., 2011). The 25 compounds with nanomolar dose–response activity in the D6 strain were also tested in the C235 (multidrug-resistant) strain. Twenty-one of these compounds were as effective against drug resistant strains as they were against sensitive strains.

Previous successful efforts to identify β-hematin inhibitors in HTS have been reported (Kurosawa et al., 2000; Rush et al.,

2009). In each instance, however, very few β-hematin inhibitors exhibiting *in vitro* antimalarial activity resulted from these efforts. In order to enrich the subset of β-hematin inhibitors that translate into *in vitro* antimalarial compounds, the assay utilized here was designed to mimic the biological conditions where hemozoin formation occurs. Using an assay design that more faithfully recapitulates the environment of hemozoin formation, 32% of the β-hematin inhibitors were found to also kill the parasite. A previously reported assay utilizing a mechanism dissimilar to the *in vivo* process of hemozoin formation resulted in only 3% of β-hematin inhibitors retaining phenotypic activity (Rush et al., 2009). Our findings demonstrate a greater correlation between β-hematin inhibitory and *in vitro* antimalarial activity since our assay more accurately mimics the natural environment of the parasite. This retention in activity suggests that higher quality leads could result from target-based assays if designed to effectively mimic biological conditions.

Examination of the structures that were identified as having activity against β-hematin formation reveals 14 distinct scaffolds that represent 246 of the total number of compounds identified in this screen (Fig. 3). The remaining unassociated 284 compounds encompass a wide range of structural diversity. While recent phenotypic screening campaigns have identified thousands of active compounds, their targets have remained largely unknown (Plouffe et al., 2008; Gamo et al., 2010; Guiguemde et al., 2010). In the approach taken here, 171 *in vitro* antimalarial compounds have been elucidated from an HTS library with their possible target identified through probing the hemozoin formation pathway in a parasite culture. Representatives of each scaffold class were subsequently tested in order to validate that they were active against the putative target, hemozoin. Twelve of the most active, commercially available compounds were chosen to represent the range of scaffold classes on the hit list. These scaffolds demonstrated a dose responsive decrease in the parasite's hemozoin formation with a concomitant rise in intraparasitic concentrations of free heme.

4.1. Quinoline-based (scaffolds A, G, J and K)

Quinoline-based compounds are among the most successful and widely used antimalarials developed to date. Though most strains of the parasite have developed resistance to CQ, several classes of quinoline derivatives that evade the mechanism of resistance have been developed including ferroquine which is currently in clinical development (De et al., 1996; Iwaniuk et al., 2009). The ability to overcome quinoline resistance suggests that creative medicinal chemistry could perpetuate quinolines as a valid scaffold. Of the 530 β-hematin inhibitors identified, several quinoline-based scaffolds were noted. Scaffolds A, G, J and K represented 10, 12, 19 and 88 of the total number of β-hematin inhibitors identified, respectively. Further, approximately one-third of these quinoline-based β-hematin inhibitors were active against the parasite. While scaffold A was previously reported in the results of the pilot screen (38,400 compounds were screened), scaffolds G, J and K were only identified after completion of screening the entire VICB HTS library (Sandlin et al., 2011). Derivatives of scaffold G have previously been reported in the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) as having activity against *P. falciparum* targeting M18 aspartyl aminopeptidase (AID 1822), plastid activity (AID 504834) and glucose-6-phosphate dehydrogenase activity (AID 504690). Scaffold G is of particular interest as it has been shown previously to possess activity against *P. falciparum* gametocytes (AID 743093), suggesting a possible therapeutic strategy that would target multiple stages of the parasite life cycle. Recently scaffold K was determined to have activity against multiple strains of *P. falciparum* (AID 743322, 743323, 743324,). Quinolines have previously been linked to cardiotoxicity (White, 2007). Therefore, it will be critical to evaluate the cardiotoxic potential of each quinoline scaffold prior to pursuing them as valuable starting points for lead optimization. When the quinoline scaffold was examined for biological target validation, each of the representative compounds chosen exhibited activity against the hemozoin formation pathway. Notably, each of the quinolines tested required only a slight increase in free heme concentration in order to cause a significant decrease in parasite survival. This low requirement compared to other structures may be indicative of the potency of the quinoline scaffold to create toxic effects. Mechanistic studies are ongoing to understand how quinoline compounds perturb the hemozoin formation pathway.

4.2. Phenyl benzamides (scaffolds B, D and E)

Phenyl benzamides were also identified from the results of the HTS effort, a scaffold not previously recognized in the pilot screen (Sandlin et al., 2011). Benzamides have previously been investigated for their activity against *P. falciparum* (AID 2306) and have also been identified as inhibitors of the *P. falciparum* pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase *Pf*DHODH (Heikkila et al., 2007; Jung et al., 2009; Guiguemde et al., 2010; Sandlin et al., 2011). Scaffolds B, D and E contained 12, 10 and 11 β-hematin inhibitors, respectively, with IC_{50}s ranging from 5.0 to 24.4 μM. Surprisingly, nearly half (45%) of these phenyl benzamide β-hematin inhibitors were also active in parasite cultures exhibiting IC_{50}s between 0.35 and 16.1 μM. Three compounds were used as exemplars for these scaffolds to test for perturbation of the hemozoin formation pathway within a parasite culture, each of which were found to be active. As opposed to the quinoline scaffold discussed in Section 4.1, these compounds resulted in drastic increases of intracellular free heme levels corresponding to parasite death. These differences may be due to the specific interactions of the compound with free heme or the hemozoin crystal and are currently being investigated.

4.3. Benzylethene (scaffolds A and I)

Scaffolds A and I contain an ethene-bridged phenyl group to either a quinoline or benzimidazole. Scaffolds A and I contained 10 and 12 compounds, respectively. 50% of scaffold A β-hematin inhibitors were active against the parasite while 8% of the β-hematin inhibitors from scaffold I were active against the parasite. Though A is an 8-hydroxyquinoline (and by structural definition is a quinoline), the quinoline portion of this scaffold does not explain its activity. Previous analysis of 8-hydroxyquinoline has demonstrated that this compound is not active against β-hematin formation (Sandlin et al., 2011). Therefore, the activity of scaffold A must be related to the addition of the ethene-bridged phenyl group. As such, it is more accurate to group scaffold A with scaffold I than to associate it strictly with the quinoline chemotype. While the two scaffolds, A and I, containing this structural motif have previously been reported to have *in vitro* antimalarial activity (AID 2306) (Guiguemde et al., 2010) and to inhibit both the M1 alanyl aminopeptidase (AID1445) and the M17 leucine aminopeptidase targets (AID 1619).

4.4. Triaryl imidizole (scaffold N)

The triaryl imidazole scaffold has been shown to exhibit antibacterial and analgesic activity and to have high *in vitro* antimalarial activity (Khan et al., 2008; Sandlin et al., 2011). However, prior to this study the mechanism of interaction with heme as a drug target has not been explored. The screening efforts yielded seven triaryl imidazoles with IC_{50} values ranging from 2.4 to 22.9 μM, six of these possessing *in vitro* antimalarial activity consisting of IC_{50} values between 0.61 and 11.7 μM. Recent phenotypic screens

by GlaxoSmithKline (GSK) and Novartis also identified numerous compounds of the triaryl imidazole scaffold to have IC_{50} values of less than 1 μM (Plouffe et al., 2008; Gamo et al., 2010). No previous reported target activity for scaffold N was found for *P. falciparum*.

4.5. Miscellaneous Compounds

In addition to the 14 scaffolds identified in this effort, a large number of interesting compounds were not associated with a specific scaffold type, or only accounted for a very small percentage of the overall hit rate. Specifically, 284 β-hematin inhibitors that did not fall into a specific scaffold were identified including 92 *in vitro* antimalarial compounds. The structural diversity of these compounds includes pyridines, amides, indoles, acridinediones, amides, ethers and thiols, among others. Importantly, the majority of these compounds have not previously been reported as inhibitors of β-hematin formation. A search through the PubChem database of previous screens revealed overlap in activity in multiple strains of *P. falciparum* not only within scaffolds, but also for unique compounds. Of particular interest is VU0015069, as it is not only an *in vitro* antimalarial and β-hematin inhibitor (IC_{50} = 1.90 μM and 10.4 μM, respectively), but in a previous screen was found to inhibit the development of the apicoplast in *P. falciparum* with a reported potency of 6.57 μM (AID 504832). Since this compound (CID 2842454) inhibits two separate *P. falciparum* target pathways, it is more likely to be a highly effective drug and should be made a priority for future target validation and pharmacokinetic studies.

5. Conclusion

HTS has become a valuable technique to rapidly identify *in vitro* antimalarial compounds. Notably, GSK, St. Jude Children's Research Hospital and Novartis have screened millions of compounds against *P. falciparum* (Plouffe et al., 2008; Gamo et al., 2010; Guiguemde et al., 2010). Despite these screens resulting in thousands of *in vitro* antimalarials, there is still a challenge in optimizing these lead compounds since their targets are unknown. The reductionist approach reported herein facilitates the prioritization of HTS *in vitro* antimalarial compounds based on their activity against a specific antimalarial target, hemozoin formation. This biological target pathway can be validated in *P. falciparum* through the quantitation of *Pf* hemoglobin, free heme, and hemozoin as previously described (Combrinck et al., 2013). Representative compounds from each of the scaffold classes were tested as a small subset of the entire hit list, resulting in the confirmation of the perturbation of the hemozoin formation pathway. Concurrently, all hit compounds are being examined for cytotoxicity in the WRAIR RAW 264.7 cytotoxicity screen. An initial examination of 35 hits active against both *in vitro* β-hematin inhibition and parasites showed that 33 of 35 were non-toxic up to 20 μM (Sandlin et al., 2011). Once compounds have been identified to inhibit the β-hematin pathway and pass the cytotoxicity screen, they will be tested further in terms of solubility, permeability and metabolic stability. With such a diverse library of compounds, the probability of identifying multiple lead candidates for further development is strong.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2014.08.002.

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