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Identification and Mechanistic Evaluation of Hemozoin-Inhibiting Triarylimidazoles Active against *Plasmodium falciparum*

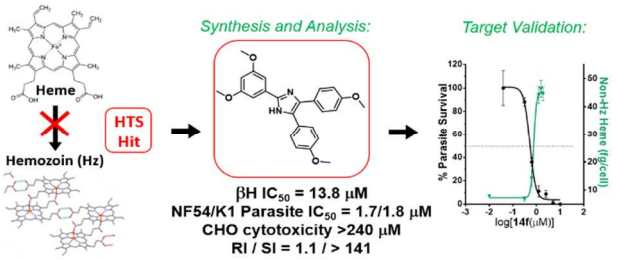
Kathryn J. Wicht[a], Jill M. Combrinck[b], Peter J. Smith[b], Roger Hunter[a], Timothy J. Egan[a,*]

[a]Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa.

[b]Division of Pharmacology, Department of Medicine, Faculty of Health Sciences, University of Cape Town, Observatory 7925, South Africa.

KEYWORDS: Antimalarial, Plasmodium falciparum, triarylimidazole, hemozoin

ABSTRACT: In a previous study, target based screening was carried out for inhibitors of β-hematin (synthetic hemozoin) formation and a series of triarylimidazoles were identified as active against *Plasmodium falciparum*. Here we report the subsequent synthesis and testing of derivatives with varying substituents on the three phenyl rings for this series. The results indicated that a 2-hydroxy-1,3-dimethoxy substitution pattern on ring A is required for submicromolar parasite activity. In addition, cell-fractionation studies revealed uncommonly large, dose-dependent increases of *P. falciparum* intracellular exchangeable (free) heme, correlating with decreased parasite survival for β-hematin inhibiting derivatives.



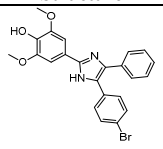
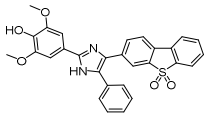
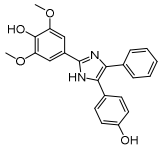
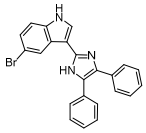
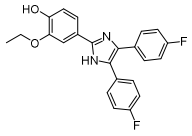
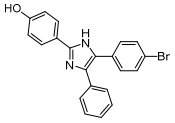
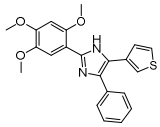
No new chemical classes of antimalarials have been introduced into clinical practice since 1996 and the shortage of novel, publicly accessible antimalarial scaffolds has had a negative impact on drug discovery efforts.[1,2] Past and present therapies are based on a limited number of chemotypes (e.g. quinolines, cycloguanils, tetracylines and sesquiterpene lactones) which often leads to cross-resistance.[3] Target-based high throughput screening (HTS) of diverse libraries is a popular approach to identifying novel drug scaffolds and requires assays for inhibition of specific *P. falciparum* pathways. Hemozoin (Hz) inhibition is one such valid pathway, since this process is not directly affected by the structure-specific efflux mechanism which causes chloroquine resistance in the parasite.[4] Synthetic Hz, β-hematin (βH), can be efficiently and reliably synthesized in the laboratory with minimal equipment using a variety of methods.[5-8] Furthermore, detecting the formation of βH is easily achieved through a number of spectroscopic techniques including IR,[9] UV-vis of the dissolved crystals,[10] or UV-vis of the ferrihemochrome complex formed between "free" Fe(III)PPIX and pyridine (free heme refers to non-Hz/βH, non-hemoglobin heme that coordinates to pyridine at pH ≈7).[11,12] Several groups have endeavored to find novel βH inhibitors via target-based HTS projects.[13,14,15, 16] In the most recent, a total of 144,330 compounds from the Vanderbilt University (VU) chemical compound library were screened for their ability to inhibit βH formation using an NP-40 based assay described previously and the ferrihemochrome method of detection.[8,15,16] Of the 530 confirmed βH inhibitors, 171 inhibited parasite growth by ≥90% at 23 μM, (32.3% of the 530 βH inhibitors) and 25 compounds exhibited favorable IC_{50} values below 1 μM. Furthermore, cell fractionation studies successfully validated Hz formation as the mechanism of action in parasites for a subset of tested compounds, which represented twelve of the fourteen novel scaffolds identified. Dose-dependent measurements of intracellular free heme and Hz revealed that different levels of free heme were attained in the parasite, depending on compound type. Three compounds caused extraordinarily high levels of non-Hz free heme (30-50% of total heme) at 2.5 times their respective IC_{50} when compared to those seen with CQ and other quinoline-containing scaffolds (8-14%).[17,18] Two of these compounds were benzamides, reported elsewhere,[19] and the other, a triarylimidazole, which attained a free heme content of 43%. The triarylimidazole class also displayed a high hit rate for parasite activity, as six of seven βH inhibitors were active against *P. falciparum* (*Table 1*).

Recently, in phenotypic screens carried out by GSK and Novartis several triarylimidazoles were discovered to have activity against the 3D7 strain of *P. falciparum*[1, 20] and three triarylimidazoles in the MMV Malaria Box were reported as inhibitors of βH formation.[21] Consequently, this scaffold was selected as interesting for investigating Hz inhibition of this class owing to the novelty of the chemotype. Furthermore, additional analysis of the HTS hits and their derivatives with potent βH inhibition activity, but with a range of parasite activities and intracellular free heme levels was required for an

ongoing study into the mechanistic aspects of non-quinoline Hz inhibitors. Therefore, the aim of this study was to investigate a range of triarylimidazoles as probe compounds to gain a better understanding of the relationships between activity and levels of non-Hz free heme in the parasite. This involved identifying the pharmacophore and preliminary SARs for this series as a step towards exploring their mechanism of action and antimalarial potential.

Seven triarylimidazoles were found to inhibit parasite growth in the HTS, with three showing IC_{50} values below 1 μM.[16] Interestingly all seven were very good βH inhibitors, at least twice as active as CQ, but parasite activity decreased significantly upon variation of the 3,5-dimethoxy-4-hydroxy substituents on ring A. The identified compounds showed D6 IC_{50}s from 0.3 to 11.7 μM (*Table 1*), with small structural changes having significant influence on activity as evident when comparing **1** (0.3 μM), **3** (0.7 μM), **5** (3.4 μM) and **6** (9.0 μM).

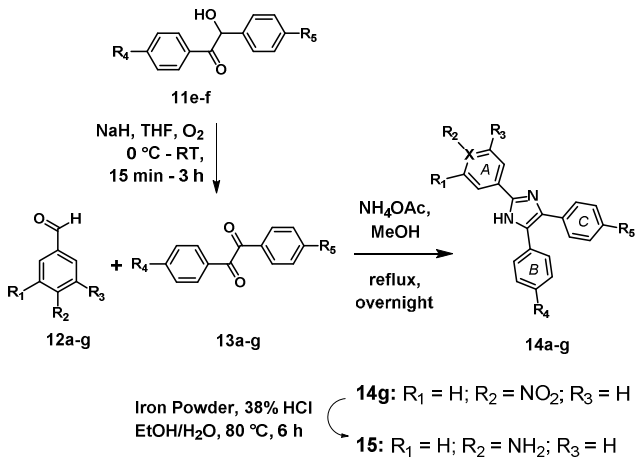
Table 1. The seven βH inhibiting triarylimidazoles from the HTS active against *P. falciparum*.[16]

	Structure	βH IC_{50} (μM)	D6 IC_{50} (μM)[a]
1	 VU0132562	12.6	0.31
2	 VU0065892	6.2	0.61
3	 VU0094619	2.4	0.70
4	 VU0123773	12.7	2.24
5	 VU0129734	18.7	2.40
6	 VU0099289	5.6	9.06 **(43% Δ free heme)**
7	 VU0099279	10.0	11.7

[a] Compounds are listed in order of decreasing D6 activity. The intracellular % free heme increase is shown in bold for **6**.

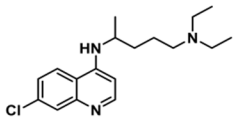
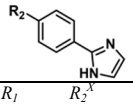
The triarylimidazole scaffold was initially deconstructed in order to determine the minimum structural features for βH inhibition activity (*Table 2*). Ring B and C in the HTS hits appeared to have a less important influence on βH activity than ring A. As a result, rings B and C were removed to evaluate whether they are required. The monoarylimidazoles 2-phenylimidazole (**8a**) and 4-(1H-imidazol-2-yl)phenol (**8b**) were obtained commercially and tested in the NP-40 assay. Compound **8a** was found to be entirely inactive against βH formation, while **8b** showed only very weak activity, more than ten-fold less active than those possessing rings B and C, confirming that they are essential for potent activity. Additional data on the triarylimidazole chemotype, which was made available via a collaboration between UCT and Vanderbilt University (VU), indicated that the unsubstituted 2,4,5-triphenylimidazole (**9**) also did not inhibit βH formation.

The next evaluated derivative was the purchased compound 4-(4,5-diphenyl-1H-imidazol-2-yl)phenol (**10**), which was identified as the simplest triphenylimidazole to strongly inhibit βH formation (IC_{50} of 17.7 μM). In order to further investigate the SARs for this scaffold, derivatives **14a-d** were synthesized using the standard method available in the literature by refluxing the appropriate aldehyde, benzil and sodium acetate in MeOH, according to *Scheme 1*. Target compounds **14e** and **14f** containing substituents on the B and C rings required 4-chlorobenzil (**13e**) and 4,4'-dimethoxybenzil (**13f**) as starting materials respectively. Since these compounds were not commercially available at the time of synthesis, they were synthesized from the corresponding benzoins, **11e** and **11f** via oxidation of the hydroxyl to the carbonyl with sodium hydride and O_2. Attempts to synthesize target compound **15** containing a *para*-amino group on the A-ring from *p*-aminobenzaldehyde were unsuccessful. It is likely that the aldehyde preferentially self-condenses under these conditions to give oligomeric products, however this was not further investigated. An alternative route was followed whereby the nitro derivative (**14g**) was formed and then reduced to the amine using iron powder and a catalytic amount of HCl. The conditions reported by Wan *et al.*[22] were followed for the formation of both **14g** and **15**, which involved a 4 h glacial acetic acid reflux at 118 °C for **14g** and then a 6 h reflux at 80 °C with Fe powder in an EtOH/water (2:1) mixture to afford **15**.



Scheme 1. Preparation of the triarylimidazole derivatives **14a-g** and **15**.

Table 2. βH and D6 or NF54 and K1 parasite IC_{50} values.

Code	Structure				βH inhibition IC_{50} μM	D6[d] or NF54 IC_{50} μM	K1 IC_{50} μM (RI)
CQ[a]					31.5 ± 0.5	0.010	0.140 (14)
8a				R_2 = H	>1000	ND	ND
8b				R_2 = OH	347 ± 12	>20	ND
	R_1	R_2[X]	R_3	R_4/R_5			
9	H	H[a]	H	H	>1000	>20[d]	ND
10	H	OH[a]	H	H	17.7 ± 0.5	>32	ND
14a	OMe	OH[a]	OMe	H	10.2 ± ND	1.3 ± 0.5	1.9 ± 0.8 (1.5)
14b	OMe	OMe[a]	OMe	H	10.9 ± 0.3	3 ± 2	ND
14c[c]	OMe	H[a]	OMe	H	14.6 ± 0.2	7.0 ± 0.1	4.7 ± 0.1 (0.7)
14d[c]	H	OCF_3[a]	H	H	>1000	4.2 ± 0.1	ND
14e	OMe	H[a]	OMe	Cl/H	16.6 ± 0.6	1.7 ± 0.5	2.7 ± 0.4 (1.6)
14f[c]	OMe	H[a]	OMe	OMe/ OMe	13.8 ± 0.4	1.7 ± 0.4	1.8 ± 0.5 (1.1)
14g[c]	H	NO_2[a]	H	H	19 ± 1	15 ± 4	ND
15	H	NH_2[a]	H	H	16.1 ± 0.5	42 ± 16	ND
16	H	-[b]	H	H	25.4 ± ND	10.8[d]	ND

[a] X = C, see *Scheme 1*
[b] X = N, see *Scheme 1*
[c] Cytotoxicity against CHO cells. **14c**: >269 μM (SI > 206); **14d**: 29 μM (SI = 6.9); **14f**: > 240 μM (SI > 141); **14g**: 149 μM (SI = 7.8).
[d] CQ-sensitive D6 parasite strain from VU for **9** and **16**

Compound **14a** resembles the parent compound, **1**, but lacks the bromo substituent on ring B. This derivative maintained potent activity against βH formation, confirming that the substituents on ring B and C are not essential. The substituents on ring A were varied by replacing the 4-hydroxy group with a methoxy group (**14b**) or a hydrogen atom (**14c**). These analogues also maintained activity against βH formation, demonstrating that with $R_1 = R_3 =$ OMe, substituents at R_2 are not required. By contrast, βH inhibition IC_{50} data for derivatives with $R_1 = R_3 =$H suggested that a substituent at R_2 is necessary but not sufficient for activity. Specifically, analogues with $R_2 =$ OH (**10**), NO_2 (**14g**) and NH_2 (**15**) were βH inhibitors while those with $R_2 =$ H (**9**) or OCF_3 (**14d**) showed no inhibition up to 1000 μM. This indicated that compounds with both electron withdrawing and releasing groups as well as both planar and non-planar substituents on ring A are tolerated. Finally, analogues of **14c**, which contained either a 4-chloro atom on ring B (**14e**) or 4-methoxy groups on both rings B and C (**14f**) were evaluated as an indication of the influence of substituents on these rings. These compounds displayed similar activity to **14c**, with IC_{50} values of 16.6 μM and 13.8 μM for the chloro and dimethoxy analogues respectively. This also demonstrated that both electron withdrawing and releasing as well as planar and non-planar substituents are tolerated on rings B and C. Replacing phenyl ring A with pyridyl, which corresponds to the active compound **16** from VU, gave a βH inhibition IC_{50} of 25.4 μM. The βH activity data for the triarylimidazoles are shown in *Table 2*.

Derivatives **8b** and **9**, which were not βH inhibitors below 100 μM, also showed no parasite activity up to 20 μM. Surprisingly, the other non-inhibitor, **14d**, showed a moderate activity of 4.2 μM. Derivative **16**, which was a potent βH inhibitor, showed weak activity of 10.8 μM in the CQ-sensitive D6 strain. The remaining purchased and synthesized triarylimidazoles were tested in the LDH parasite growth inhibition assay using the CQ sensitive NF54 strain (*Table 2*). Compound **10** was found to be inactive at concentrations up to 32 μM, despite displaying potent βH activity. Other derivatives which performed poorly in the assay were the 4-nitro (**14g**) and 4-amino (**15**), analogues of **10**. This showed that having substituents only in the *para* position of ring A was not sufficient for parasite activity. For the βH inhibiting compounds containing 3,5-methoxy groups on ring A, **14a-c**, the parasite activity improved from 7.0 to 1.3 μM with variation of the *para* R_2 substituent H < OMe < OH, illustrating that the ring A substitution pattern of the parent compound (**1,** *Table 1*) was superior for activity. The ability of βH inhibiting derivatives with a greater number of hydroxyl and methoxy substituents to inhibit parasite growth can be seen graphically in *Figure 1*. This had a much greater impact on parasite activity than did βH inhibition IC_{50}. Indeed, the relationship with βH activity was not statistically significant.

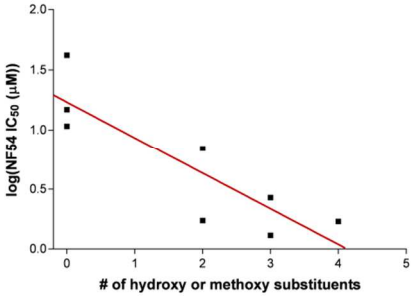


Figure 1. A linear correlation between the log of the parasite activity with the number of hydroxyl or methoxy substituents on the phenyl rings in a triarylimidazole given by log(NF54 IC_{50}) = -0.30(# of OH or OMe) + 1.23; ($r^2 = 0.76$, P = 0.0048).

The most active triarylimidazoles in the HTS, with submicromolar activity, contained a *para* Br or OH substituent on ring B (*Table 1*), suggesting that the effect of these groups, while not a significant factor for βH inhibition, is to increase parasite activity. The preparation of **14e** and **14f** was carried out in order to determine whether the activity of triarylimidazoles with other ring A substituents could also be improved by attaching ring B and ring C substituents. The 4-chloro and 4,4'-dimethoxy analogues were tested and found to improve activity from 7.0 μM for **14c** to ≈1.7 μM for both **14e** and **14f**. This >4-fold activity improvement upon the introduction of ring B and/or C substituents was almost identical to that of **14c** vs **1** (*Table 1*). This trend was also observed when comparing **10** with poor activity (>32 μM) to its 4-bromo derivative **6** from the HTS, with IC_{50} of 9.1 μM. Compounds **14a**, **14c**, **14e** and **14f**, were selected for testing in the CQ-resistant K1 strain of *P. falciparum* and were found to have low resistance indices (RIs), indicating no significant CQ cross resistance for this series. Furthermore, compounds **14c**, **14f** and **14g** showed low cytotoxicity against Chinese hamster ovarian (CHO) cells (>100 μM), demonstrating specific activity against the malaria parasite (*Table 2* footnote). By contrast, **14d** was an order of magnitude more cytotoxic, with a selectivity index of only 7, suggesting that its activity against

Plasmodium is likely based on its cytotoxicity. The SAR is summarized in *Figure 2*.

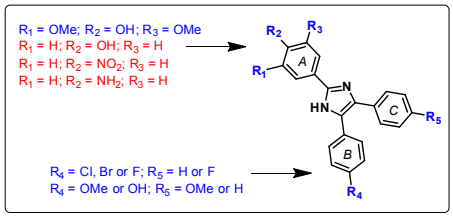


Figure 2. Favorable (blue) and unfavorable (red) substituents for parasite activity within the triarylimidazole HTS hits and synthesized analogues.

The triarylimidazole **6** (*Table 1*) from the HTS was shown to cause an increase in non-Hz, non-hemoglobin "free" heme to 43% of total heme.[16] Nevertheless, data for additional derivatives with a range of potencies, were deemed necessary in order to support the biological mechanism of action for this series. Compounds **14c**, **14f** and **14g**, which had different ring A substituents from the HTS hits and showed a marked difference in activity, were subjected to cell fractionation studies. **14c** and **14f** were shown to cause a very large increase in free heme in the cell (>40 fg/cell which corresponds to >50% of total cellular heme) at 2.5 times the corresponding IC_{50} of the compound (*Figure 3a* and *S4.2a*). **14g** also caused a large increase in free heme (>20 fg/cell, see *S4.2b*), smaller than **14c** and **14f**, but larger than CQ.[18] This study confirmed that these triarylimidazoles inhibit Hz formation, causing an increase in free heme in the malaria parasite in a dose dependent manner that coincides with decreased parasite survival. This strongly suggests that inhibition of Hz formation is the mechanism of action of these compounds. By contrast, **14d**, which exhibited parasite activity despite showing no βH inhibition activity, brought about no increase in free heme with decreasing parasite survival (*Figure 3b*). Thus, **14d** appears to exhibit an entirely different mechanism of action. This is reinforced by flow cytometry data, which showed that parasite cells treated with **14d** contained less DNA and were considerably smaller and less complex than those treated with **14c**, **14f** and **14g** (see *S4.2c-f*). They also appeared to be pyknotic by light microscopy (see *S4.2g*). It is noteworthy that this compound is much more cytotoxic than the others are and this may be the basis of its parasite activity.

In this study, purchased or synthesized triarylimidazoles were evaluated for their ability to inhibit βH formation and prevent parasite growth. Furthermore, the RIs and SIs were assessed for selected analogues, which showed low CQ cross-resistance and high selectivity for *P. falciparum* over mammalian cells, supporting the Hz-inhibition pathway as an effective and unique drug target. Parasite activity relied on very specific substituents, showing that the scaffold was not amenable to structural changes on ring A. There did not appear to be a direct correlation between βH inhibition and parasite activity. This is not surprising given that access to the digestive vacuole, including pH trapping, is likely to play a crucial role in the relative potency of a compounds ability to inhibit cellular hemozoin formation. Indeed, for this series there is clear evidence of hemozoin inhibition in the cell. For the three βH inhibitors selected for testing, intracellular free heme levels correlated to parasite growth inhibition. These derivatives are extraordinary probe molecules for investigating the properties and effects of free heme in the malaria parasite in view of the unusually high levels achieved. Investigations of the relationship between cellular accumulation, free heme levels and activity for this and other series are currently ongoing.[19] Such data are crucial for gaining a detailed understanding of the mechanism of action of Hz inhibitors.

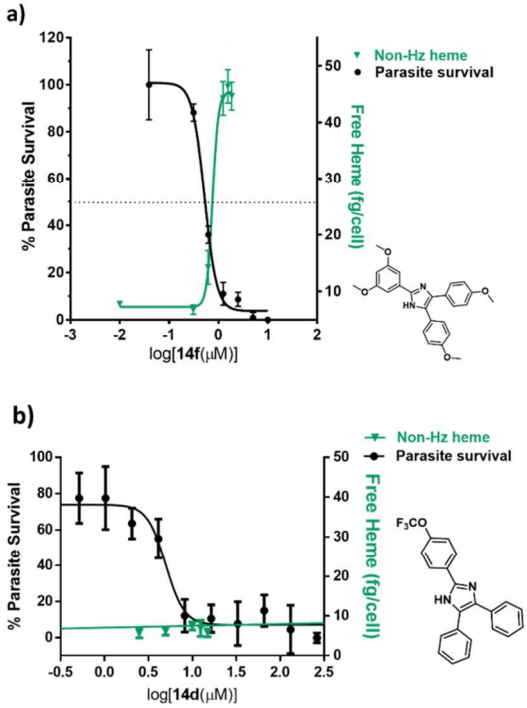


Figure 3. Dose response curves for amount of free non-Hz heme (fg/cell) and the percent parasite survival for a) **14f**, showing a cross over near the IC_{50} and b) **14d**, showing no increase in free heme with parasite growth inhibition.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge on the ACS Publications website at DOI: XXX

Experimental methods including chemistry, detergent mediated assay for β-hematin inhibition, LDH malaria parasite survival assay, cell fractionation and cytotoxicity (PDF). Additional cell fractionation and flow cytometry data.

AUTHOR INFORMATION

Corresponding Author

* Email: timothy.egan@uct.ac.za

Author Contributions

The manuscript was written by K.J.W with guidance and editing from T.J.E. and R.H. High throughput screening, scaffold analysis, synthesis and SAR analysis was carried out by K.J.W under the supervision of T.J.E and R.H. Parasite assays and cell fractionation studies were conducted and analyzed by J.M.C under the supervision of P.J.S and T.J.E. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

βH, β-hematin; Hz, hemozoin; LDH, lactate dehydrogenase; CQ, chloroquine; RI, resistance index; SI, selectivity index; CHO, Chinese hamster ovarian; VU, Vanderbilt University; UCT, University of Cape Town.

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