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Incorporation of an intramolecular hydrogen bonding motif in the side chain of antimalarial benzimidazoles†

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Analogues of a novel class of benzimidazoles with an intramolecular hydrogen bonding motif have been synthesized and evaluated *in vitro* for their antiplasmodium activity against chloroquine-sensitive (NF54) and multi-drug resistant (K1) strains of the human malaria parasite *Plasmodium falciparum*. Compounds were also screened for their cytotoxicity towards a mammalian Chinese hamster ovarian (CHO) cell line. Most of the compounds exhibited good antiplasmodium activity ($PfNF54$ $IC_{50} < 1 \mu M$) and were relatively noncytotoxic. Moreover, towards establishing the possible mode of action of these molecules, inhibition of beta-hematin formation was investigated and two compounds were found to be inhibitors. Single crystal X-ray data confirmed the existence of an intramolecular hydrogen bond.

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Introduction

Malaria, the disease caused by infection with protozoan parasites, primarily *Plasmodium falciparum* and *Plasmodium vivax*, continues to take an enormous toll on human health, particularly in tropical regions. This is despite the positive impact of interventions including medication, indoor spraying with insecticides to control the mosquito vector, and the use of insecticide-treated bed nets.¹ Resistance to treatment regimens, especially the emergence of resistance to artemisinin drugs currently used in combination therapies,^{2,3} poses a threat and highlights the importance of developing treatments containing new chemical classes or chemotypes with different modes of action.

Incorporation of an intramolecular hydrogen bond (IMHB) in drug molecules has been shown to have beneficial effects.⁴ The presence of an IMHB has been shown to significantly alter molecular properties due to the formation of various con-

formers that in turn influence solubility, permeability, pharmacokinetic and pharmacodynamic processes, as well as protein binding affinity.^{5–7} Molecules that can participate in IMHB shed water more readily when entering a low dielectric environment like a hydrophobic phospholipid bilayer. In this circumstance, IMHB results in lipophilic, less polar molecular conformations which are expected to have higher passive membrane permeability.⁸ This is due to the fact that a decrease in polarity is sometimes achieved through the formation of IMHBs, where the hydrogen bond donor (HBD) and acceptor (HBA) atoms are effectively shielded from water, thereby reducing the energetic penalty of desolvation required in moving from an aqueous environment through a phospholipid bilayer.⁹

Previous work by Burckhalter and co-workers on antimalarial α -aminocresols (A) led to the discovery of 4-*tert*-butyl-2-diethylaminomethyl phenol (B) (Fig. 1) as an active compound against trophozoite-induced avian malaria.¹⁰ This work on α -aminocresols showed the importance of having the hydroxyl group *ortho* to the α -amino group and the benefit of an additional hydrophobic substituent on the ring. This

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Fig. 1 Chemical structures of a generalised α -aminocresol (A) and 4-*tert*-butyl-2-diethylaminomethylphenol (B).

motif was later attached to the 7-chloroquinoline ring found in chloroquine to deliver the drug amodiaquine.¹⁰

Similar modifications were carried out on quinolinemethanols leading to the identification of mefloquine (Fig. 2).¹¹ Further work has been carried out on 4-aminoquinolines by incorporating an intramolecular hydrogen bonding motif in the side chain.¹² It was reported that most of the compounds within the series were active against both chloroquine-sensitive (3D7) and multidrug-resistant parasite (W2) strains.

Recently, structure–activity relationship studies of trifluoromethyl-substituted pyridine and pyrimidine analogues of 2-aminomethylphenols (JPC-2997, JPC-3186, and JPC-3210) were conducted.¹³ Of these compounds, JPC-3210 [4-(*tert*-butyl)-2-((*tert*-butylamino)methyl)-6-(5-fluoro-6-(trifluoromethyl)pyridin-3-yl)phenol] was selected as an optimized lead suitable for development as a potential preclinical candidate.¹³ The common structural feature shared by these compounds is a basic nitrogen within hydrogen-bonding proximity to a hydroxyl group.

In view of the above, and the reported^{14–18} antimalarial activity of various benzimidazoles, we hypothesized that compounds containing an intramolecular hydrogen-bonding motif in the basic side chain of a benzimidazole nucleus would represent a potential novel series of antimalarials (Fig. 2).

Here, we report the synthesis, antiplasmodium activity, cytotoxicity, solubility and beta-haematin inhibition data on this series along with the single crystal X-ray structure of a representative analogue.

Results and discussion

Chemistry

The compounds synthesized for this study are presented in Table 1 with the corresponding 2-step synthetic route outlined in Scheme 1. Briefly, target compounds were synthesized through a nucleophilic aromatic substitution reaction of commercially available 2-chlorobenzimidazole and *N*¹-ethylethane-1,2-diamine to give the desired intermediate I. Reductive amination of appropriate aldehydes using this intermediate was achieved in the presence of sodium borohydride

(NaBH₄) to afford the respective target compounds in moderate to good yields.¹⁹

Biological activity

All target compounds were evaluated for *in vitro* asexual blood stage activity against both the chloroquine-sensitive NF54 and multi-drug resistant K1 strains of *P. falciparum*. Compounds with (*Pf*NF54) IC₅₀ < 1 μM were also tested for cytotoxicity against the Chinese hamster ovarian (CHO) mammalian cell line and evaluated for their beta-hematin inhibition activity (BHIA) (Table 1).

***In vitro* antiplasmodium activity.** Using the modified [³H]-hypoxanthine incorporation assay,²⁰ all compounds were tested against both the chloroquine-sensitive (NF54) and the chloroquine-resistant (K1) strains of *P. falciparum* with chloroquine and artesunate used as reference drugs in both cases. All the compounds except A.8 and A.12 exhibited submicromolar potencies against the chloroquine-sensitive NF54 strain of *P. falciparum*, with IC₅₀ values between 0.079 μM and 0.968 μM. Against the chloroquine-resistant strain of *P. falciparum* (K1), these compounds displayed between 4 and 10-fold less activity, an observation that may suggest the possibility of cross-resistance with drugs like chloroquine. Considering structure–activity relationships, replacing the phenyl ring with a pyridine as in compound A.8 was detrimental to the potency. Overall, the most active compounds were those with electron withdrawing and lipophilic phenyl substituents with a *meta* substituted chloro, compound A.3 (IC₅₀ = 0.079 μM) portraying the best activity. A di-substituted chloro analogue (compound A.9, IC₅₀ = 0.521 μM) resulted in a decrease in potency compared to the mono-substituted chloro analogues A.3 and A.7 (IC₅₀ = 0.11 μM). Conversely, a di-substituted fluoro analogue (compound A.10, IC₅₀ = 0.222 μM) was well tolerated. Removal of the hydroxyl substituent as in compound A.12 (IC₅₀ = 3.604 μM) resulted in a loss of potency, indicating the importance of the hydroxyl substituent in these analogues.

***In vitro* cytotoxicity.** Cytotoxicity was determined against the Chinese hamster ovarian (CHO) cell lines (CHO-K1, ATCC, Manassas VA, USA) using the MTT assay^{21,22} (Table 1).

Fig. 2 Chemical structures of amodiaquine, mefloquine and a benzimidazole showing intramolecular hydrogen bonding.

Table 1 *In vitro* antiparasmodium activity, cytotoxicity and BHIA of benzimidazole analogues

Compound code	R	Antiplasmodium activity IC ₅₀ ^a (μM)		Cytotoxicity (CHO) IC ₅₀ (μM)	SI ^b	BHIA ^c IC ₅₀ (μM)	Solubility PBS pH 7.4 (μM)
		<i>Pf</i> NF54 strain	<i>Pf</i> K1 strain				
A.1		0.474	2.658	50	105	452	>200
A.2		0.299	1.487	50	167	161	160
A.3		0.079	0.335	21.58	273	32	160
A.4		0.32	3.042	50	156	489	160
A.5		0.4	1.397	50	125	30	160
A.6		0.408	2.425	24.50	60	4223	>200
A.7		0.11	0.762	25.10	228	125	160
A.8		>10	>10	50	ND	ND	>200
A.9		0.521	1.252	50	96	130	160
A.10		0.222	0.952	50	225	68	140
A.11		0.968	>10	50	52	250	160
A.12		3.604	1.879	ND	ND	ND	140
Chloroquine	—	12 nM	431 nM	—	—	53	—
Artesunate	—	7 nM	2.9 nM	—	—	—	—
Amodiaquine	—	—	—	—	—	13	—
Emetine	—	—	—	0.059	—	—	—

^a Determined from $n \geq 2$ independent [³H]-hypoxanthine incorporation experiments with multidrug resistant (K1) and chloroquine sensitive (NF54) strains of *P. falciparum*. ^b SI (selectivity index) = IC₅₀ CHO/IC₅₀ *Pf*NF54. ^c Determined from $n \geq 3$ independent experiments, individual values were within a 2-fold difference. ND – not determined.

Scheme 1 Synthetic protocol toward target compounds. Reagents and conditions: (a) Et₃N, reflux, 140 °C, 16 h (59%); (b) RCHO, MeOH, NaBH₄, 25 °C, 16 h (38–75%).

Cells were cultured in Hams/F12 media supplemented with 10% v/v fetal bovine serum (Thermo Fisher Scientific; South America). All the compounds showed a favourable cytotoxicity profile on account of their selectivity indices (SI > 10), with compound A.3 (SI = 273) exhibiting the highest SI.

Solubility. Solubility of all compounds synthesized was determined using the turbidimetric kinetic solubility assay. Hydrocortisone and reserpine were used as the respective positive and negative controls for this determination. From this assay, all the compounds were found to have high solubility in PBS at physiological pH 7.4 based on the general classification of turbidimetric solubility ranges for organic compounds.²²

Beta-hematin inhibition activity (BHIA). Towards assessing the putative mode of action of these compounds, the beta-hematin inhibition assay was carried out as previously described.²³ Evaluation of compounds in this assay was prompted by a publication by Wright and co-workers²³ who reported certain types of benzimidazoles as potent inhibitors of β -hematin formation. Using a discriminatory IC₅₀ cut-off of 100 μ M for good activity, compounds A.3 and A.5 inhibited this process potently with IC₅₀ values of 32 μ M and 30 μ M respectively, while chloroquine, used as a positive control, displayed BHIA with IC₅₀ of 53 μ M. It should be noted, however, that the beta hematin inhibition assay is a cell-free system, in which the test compound is in direct contact with

Fig. 3 Single crystal X-ray structure of compound A.2, where thermal ellipsoids are drawn at 40% probability level.

haemin. On the other hand, the antiplasmodium evaluation is a whole-cell assay. Therefore, it is noteworthy that the inhibition of beta-hematin formation in the cell-free assay is not a guarantee that this will occur in the parasite and would need to be validated in a cellular environment.

Single crystal X-ray analysis

To ascertain the existence of intramolecular hydrogen bond in these molecules, a single crystal X-ray structure of a representative compound was obtained (Fig. 3).

Single-crystal X-ray diffraction data were collected on a Bruker KAPPA APEX II DUO diffractometer using graphite-monochromated Mo-K α radiation ($\lambda = 0.71073$ Å). Data collection was carried out at 173 K. Temperature was controlled by

an Oxford Cryostream cooling system (Oxford Cryostat). Cell refinement and data reduction were performed using the program SAINT.²⁴ The data were scaled and absorption correction performed using SADABS.²⁵

The structure was solved by direct methods using SHELXS-97 and refined by full-matrix least-squares methods based on F^2 using SHELXL-2014 (ref. 25) and using the graphics interface program X-Seed.^{26,27} The programs X-Seed and POV-Ray²⁸ were used to prepare molecular graphic images.

All non-hydrogen atoms were refined anisotropically. All hydrogen atoms, except the functional hydrogen atoms, were placed in idealised positions and refined in riding models with U_{iso} assigned 1.2 or 1.5 times U_{eq} of their parent atoms and the bond distances were constrained from 0.95 to 0.99 Å. The hydrogen atoms H1N and H3N on N1 and N3 respectively were located in the difference density maps and refined independently without restraints. The hydroxyl hydrogen H1O and the water hydrogen atoms H2A and H2B were located in the difference density maps and refined with simple O–H bond length restraints. The structure was refined to R factor of 0.0432. The parameters for crystal data collection and structure refinements as well as some selected hydrogen bonding interactions are shown in Tables 2 and 3.

The distance between the nitrogen N4 and hydrogen H1O is 1.62 Å which is shorter than the sum of their Van der Waals radii (2.75 Å). Also, the bond angle between O1–H1O $\cdots$ N4 is 157.5°. This suggests that the intramolecular hydrogen bond that exists between O1–H1O $\cdots$ N4 is quite significant since the distance between the nitrogen and the hydrogen is short and the bond angle is also closer to 180° as expected.

Conclusions

In summary, a series of novel, potent and selective benzimidazole analogues with an intramolecular hydrogen bonding motif were designed and synthesized. These analogues exhibited promising *in vitro* antiplasmodium activity and showed an encouraging cytotoxicity profile. Inhibition of hemozoin formation was found to be a possible contributing mechanism by which these analogues exert their antiplasmodium activity. Moreover, the presence of intramolecular hydrogen bonds in these analogues was demonstrated based on a single crystal X-ray structure of a representative compound A.2.

Table 2 Crystallographic data and refinement parameters for compound A.2

Parameter	Value
Molecular formula	C ₁₈ H ₂₁ FN ₄ O·H ₂ O
Formula weight	346.40
Crystal system	Monoclinic
Space group	<i>P</i> 2 ₁ / <i>c</i>
<i>a</i> (Å)	13.6252(16)
<i>b</i> (Å)	10.5144(12)
<i>c</i> (Å)	12.6246(13)
α (°)	90
β (°)	96.075(2)
γ (°)	90
<i>V</i> (Å ³)	1798.5(3)
<i>Z</i>	4
<i>D</i> (calc) (g cm ^{−3})	1.279
μ (MoK α) (mm ^{−1})	0.093
<i>F</i> (000)	736
Crystal size (mm ³)	0.07 × 0.26 × 0.27
Temperature (K)	173
Radiation (Å)	MoK α 0.71073
θ min–max (°)	1.5–27.5
Index ranges $\pm h, \pm k, \pm l$	−17 : 17; −13 : 13; −8 : 16
Reflections (total)	13 245
Reflections (unique)	4132
<i>R</i> _{int}	0.045
Reflections with $I > 2\sigma(I)$	2914
<i>N</i> _{ref} , <i>N</i> _{par}	4132, 247
<i>R</i> ₁ , <i>wR</i> ₂ , <i>S</i>	0.0432, 0.1137, 1.02
Max. and Av. shift/error	0.00, 0.00
$\Delta\rho_{\text{min,max}}$ (e Å ^{−3})	−0.27, 0.18

Table 3 Hydrogen bonding interactions for compound A.2

D–H $\cdots$ A (interactions)	<i>d</i> (D–H)	<i>d</i> (H $\cdots$ A)	<i>d</i> (D $\cdots$ A)	$\angle$ D–H $\cdots$ A
N1–H1 $\cdots$ O2	0.875(18)	1.991(18)	2.8319(17)	160.6(17)
O1–H1O $\cdots$ N4	0.974(19)	1.616(18)	2.5435(16)	157.5(19)
O2–H2A $\cdots$ O1	0.954(14)	1.828(15)	2.7505(15)	161.8(13)
O2–H2B $\cdots$ N2	0.955(16)	1.809(15)	2.7627(16)	176(2)
N3–H3N $\cdots$ O2	0.868(18)	2.132(18)	2.9986(18)	176.5(14)
C12–H12A $\cdots$ F1	0.9900	2.4800	3.253(2)	135.00
C16–H16 $\cdots$ F1	0.9500	2.5500	3.439(2)	157.00

D = donor; A = acceptor; *d* = distance (Å); $\angle$ = angle (°).

Conflicts of interest

There are no conflicts to declare.

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