ACS Infectious Diseases

pubs.acs.org/journal/aidcbc

Article

Benzimidazole Derivatives Are Potent against Multiple Life Cycle Stages of *Plasmodium falciparum* Malaria Parasites

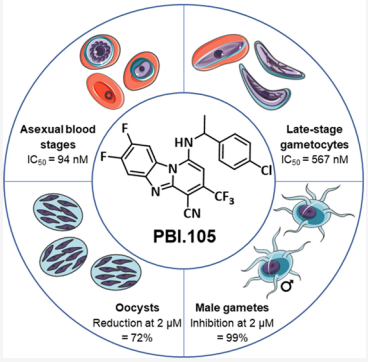
Meta Leshabane, Godwin Akpeko Dziwornu, Dina Coertzen, Janette Reader, Phanankosi Moyo, Mariëtte van der Watt, Kelly Chisanga, Consolata Nsanzubuhoro, Richard Ferger, Erica Erlank, Nelius Venter, Lizette Koekemoer, Kelly Chibale,* and Lyn-Marie Birkholtz*

Cite This: *ACS Infect. Dis.* 2021, 7, 1945−1955

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The continued emergence of resistance to front-line antimalarial treatments is of great concern. Therefore, new compounds that potentially have a novel target in various developmental stages of *Plasmodium* parasites are needed to treat patients and halt the spread of malaria. Here, several benzimidazole derivatives were screened for activity against the symptom-causing intraerythrocytic asexual blood stages and the transmissible gametocyte stages of *P. falciparum*. Submicromolar activity was obtained for 54 compounds against asexual blood stage parasites with 6 potent at IC_{50} < 100 nM while not displaying any marked toxicity against mammalian cells. Nanomolar potency was also observed against gametocytes with two compounds active against early stage gametocytes and two compounds active against late-stage gametocytes. The transmission-blocking potential of the latter was confirmed as they could prevent male gamete exflagellation and the lead compound reduced transmission by 72% in an *in vivo* mosquito feeding model. These compounds therefore have activity against multiple stages of *Plasmodium* parasites with potential for differential targets.



KEYWORDS: *malaria, benzimidazole, pyridobenzimidazole, Plasmodium falciparum, transmission-blocking, gametocytes*

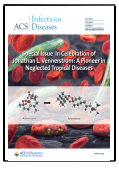
Despite well-established control strategies, over half of the global population remains at risk of malaria. However, the spread of resistance of malaria parasites to multiple drugs, including first-line therapy, threatens to slow down any progress made thus far toward malaria elimination.[1] Novel compounds that possess alternative modes of action or novel modes of inhibition of known targets are therefore urgently needed to continue treatment efficacy to circumvent antimalarial drug resistance.

Five species of *Plasmodium* parasites cause human malaria with *Plasmodium falciparum* resulting in the most severe cases and the highest global mortality associated with asexual blood stage (ABS) parasites. The ABS proliferate rapidly (~48 h replication time) during which parasites mature from ring stages to metabolically active trophozoites (including digestion of hemoglobin digestion) before schizogony occurs to produce up to 32 daughter merozoites. Clinical manifestation of the disease is associated with these proliferative stages. Disease transmission between the human host and the *Anopheles* mosquito vector occurs either through sporozoites or gametocytes, marking these stages to be targeted to block the transmission cycle. Gametocytes are formed from only a small proportion of ABS and differentiate over 10−14 days, characterized by at least five morphological stages (stages I−V) for *P. falciparum*. Immature gametocytes (stages I−IV) are sequestered particularly in bone marrow, and only mature stage V gametocytes circulate in the bloodstream and can be transmitted to a feeding mosquito. The majority of compounds targeting ABS are inactive against gametocytes and the latter therefore remain in the human host even after the asexual stages are cleared. Targeting this reservoir of transmissible gametocytes is essential to reach elimination goals.[2,3] Therefore, current drug discovery programmes aim to identify hits able to target multiple stages of malaria parasites, with specific target candidate profiles (TCPs) defined including ABS-targeted as TCP-1 and gametocyte-targeted, transmission-blocking compounds as TCP-5.[3]

The heterobicyclic benzimidazole scaffold has seen extensive use in medicinal chemistry due to the ability to interact with multiple biological systems.[4,5] Aside from the many benzimidazole-based drugs in current clinical use, antimalarial benzimidazole agents have been reported in the literature.[6−13] Relatedly, pyrido[1,2-*a*]benzimidazole (PBI) derivatives have demonstrated broad-spectrum biological properties[14−20] and thus are equally an important starting point in drug discovery. Recently, we demonstrated the antimalarial[13,21−24] and

Special Issue: In Celebration of Jonathan Venner-strom: A Pioneer in Neglected Tropical Diseases

Received: December 30, 2020
Published: March 5, 2021



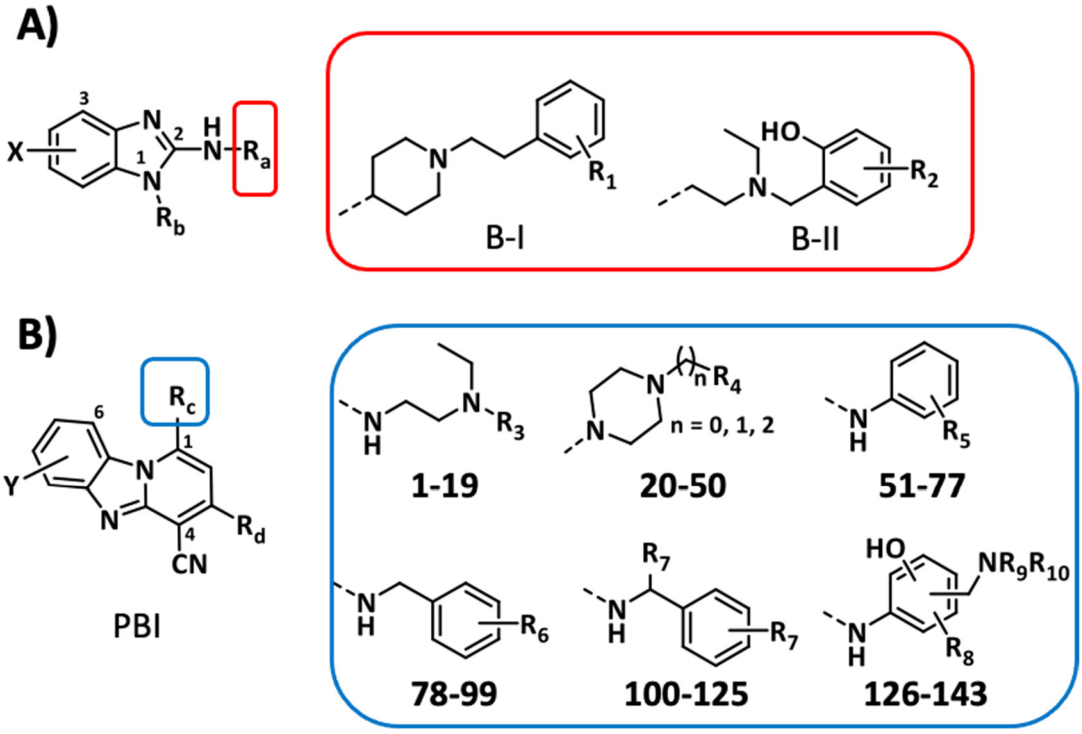


Figure 1. Summary of the two main chemical series studied in the current work. (A) The benzimidazole series with two diverse amine moieties (B-I, B-II) at the C2 position of the benzimidazole core scaffold. (B) The tricyclic pyrido[1,2-*a*]benzimidazole (PBI) series with key amine modifications at the C1 position of the PBI core scaffold. Compound SMILES are provided in Supporting Information 2.

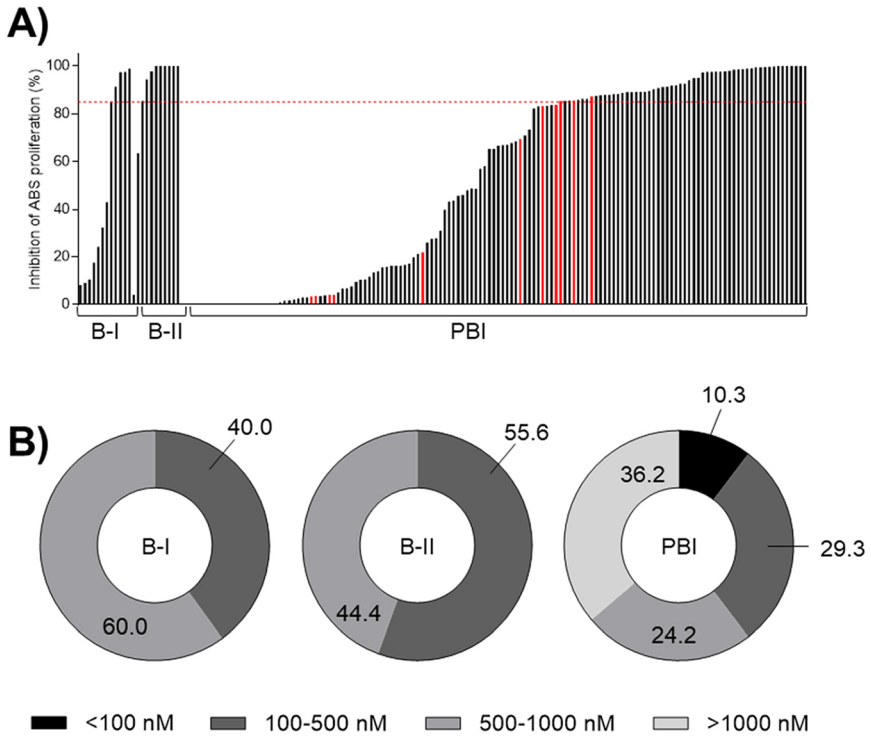


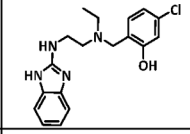
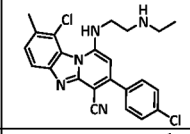
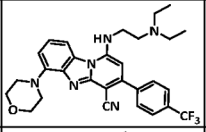
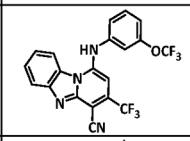
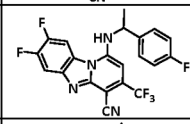
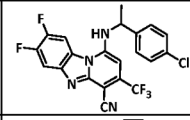
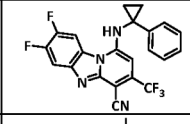
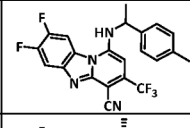
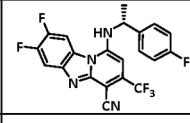
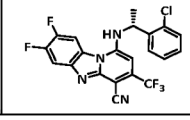
Figure 2. Activity of benzimidazole scaffolds against (ABS) parasites. (A) Inhibition (%) of ABS proliferation by 1 μM benzimidazoles after 96 h drug pressure, as measured with SYBR Green I fluorescence. Data are arranged by series in increasing order of inhibition at 1 μM treatment with red bars indicating compounds with an additional >50% HepG2 cell cytotoxicity. Data are from a single biological replicate in technical triplicates. The dashed horizontal red line indicates the hit selection criteria of >85% inhibition @ 1 μM. (B) Grouping of IC_{50} values of selected compounds with >85% inhibition at 1 μM against ABS. Individual values provided in Supporting Information.

antischistosomal[22,24,25] activities of these fused heterocyclic compounds.

In an effort to explore new chemotypes for both TCP-1 and TCP-5 antimalarial activity, we evaluated our in-house

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

Table 1. *In Vitro* Activity of Selected Benzimidazole Compounds from the B-II and PBI Scaffolds Active against Different Stages of *P. falciparum* Parasites[a]

Compound	Structure	*P. falciparum* (NF54) life cycle activities				HepG2 cells
		ABS	EG	LG	Male gamete	
		IC_{50} (nM)			% inhibition @ 2 μM	% viability @ 2 μM
B-II.4		130	382.1	>5 μM	ND	100
PBI.13		80.5	>5 μM	>5 μM	ND	100
PBI.19		195.0	>5 μM	1842.0	29.7	54.1
PBI.58		1276	>5 μM	3180	ND	83.7
PBI.103		97.7	>5 μM	1737.0	47	100
PBI.105		94.2	>5 μM	568.6	99.8 (IC_{50}=697.1 nM)	100
PBI.109		53.7	102.1	>5 μM	ND	100
PBI.111		94.0	>5 μM	>5 μM	ND	100
PBI.120		74.3	>5 μM	953.0	93.8 (IC_{50}=997.8 nM)	100
PBI.125		>5000	>5 μM	4113.0	ND	96.3

ABS = asexual blood stages, EG = early-stage gametocytes, LG = late-stage gametocytes
ND= not determined due to compounds not meeting selection criteria with LG IC_{50} > 2 μM

[a]Only compounds passing the specific activity thresholds were included in this table.

compound library of benzimidazole derivatives (Figure 1) further for multistage activity by investigating, in parallel, their ABS and transmission-blocking potentials. Briefly, the B-I analogues constitute analogues with changes at the C2 position designed to improve the antiplasmodial activity of the antihistaminic drug astemizole.[10] The B-II subseries incorporated an intramolecular hydrogen-bonding motif in the basic side chain of an ethyl ethylenediamino linker to the benzimidazole nucleus.[6] The PBI analogues vary predominantly in the amine side chain at the C1 position. Six different

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

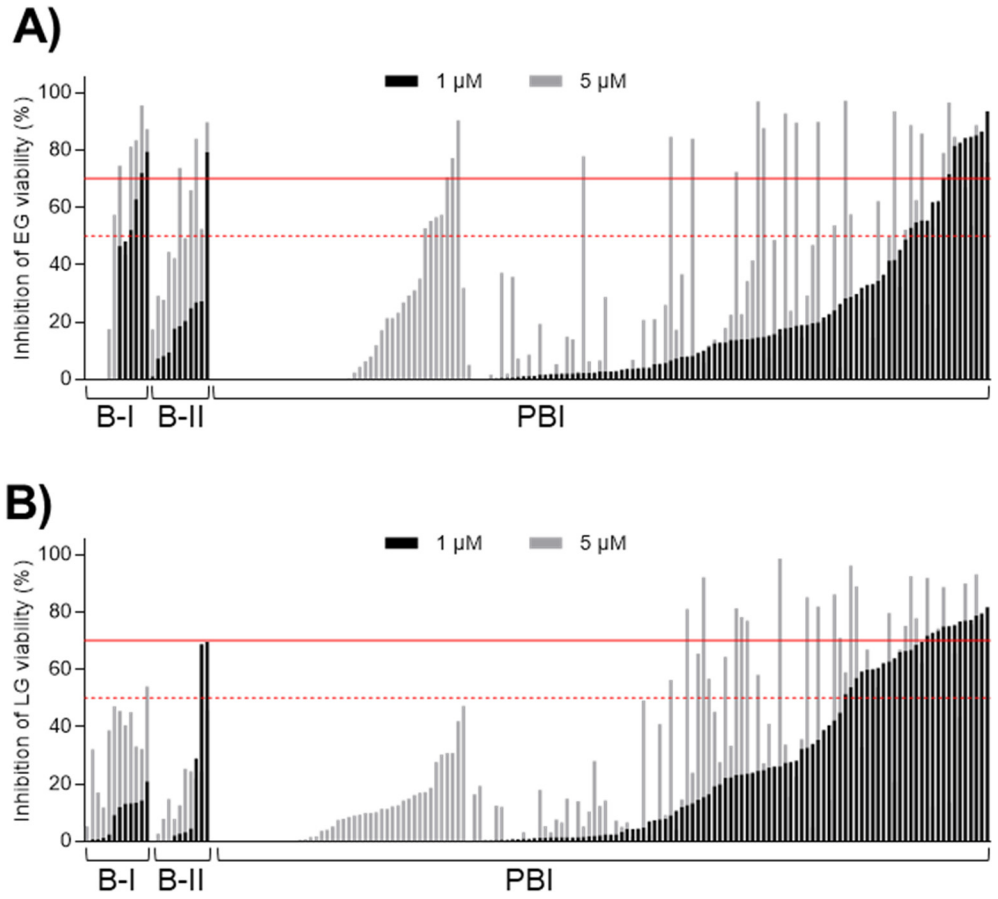


Figure 3. Antiplasmodial activity of benzimidazoles against early- (EG) and late-stage (LG) gametocytes. (A) Inhibition (%) of EG and (B) LG viability arranged by increasing order of inhibition at 1 μM treatment for 48 h. Horizontal red lines indicate hit selection criteria of >70% inhibition at 5 μM and >50% inhibition at 1 μM. Data are from a single biological repeat performed in technical triplicates.

amine side chains on the PBI scaffold were investigated for their contributions to multistage antiplasmodial activity (Figure 1B). The detailed structural modifications within this chemical space have been previously described.[13,22,23,25] Meanwhile, the chemical and spectroscopic information on three previously unreported PBI analogues are presented in Supporting Information 1.

Compounds were screened for activity against ABS, early stage and late-stage gametocytes, male gametes, and oocysts in mosquitoes with several compounds displaying activity across multiple stages of *P. falciparum* parasites *in vitro* (Figure S1). Furthermore, one PBI analogue was able to kill ABS and has 72% transmission-reducing activity, marking this compound for further development as a potential multistage active antimalarial candidate.

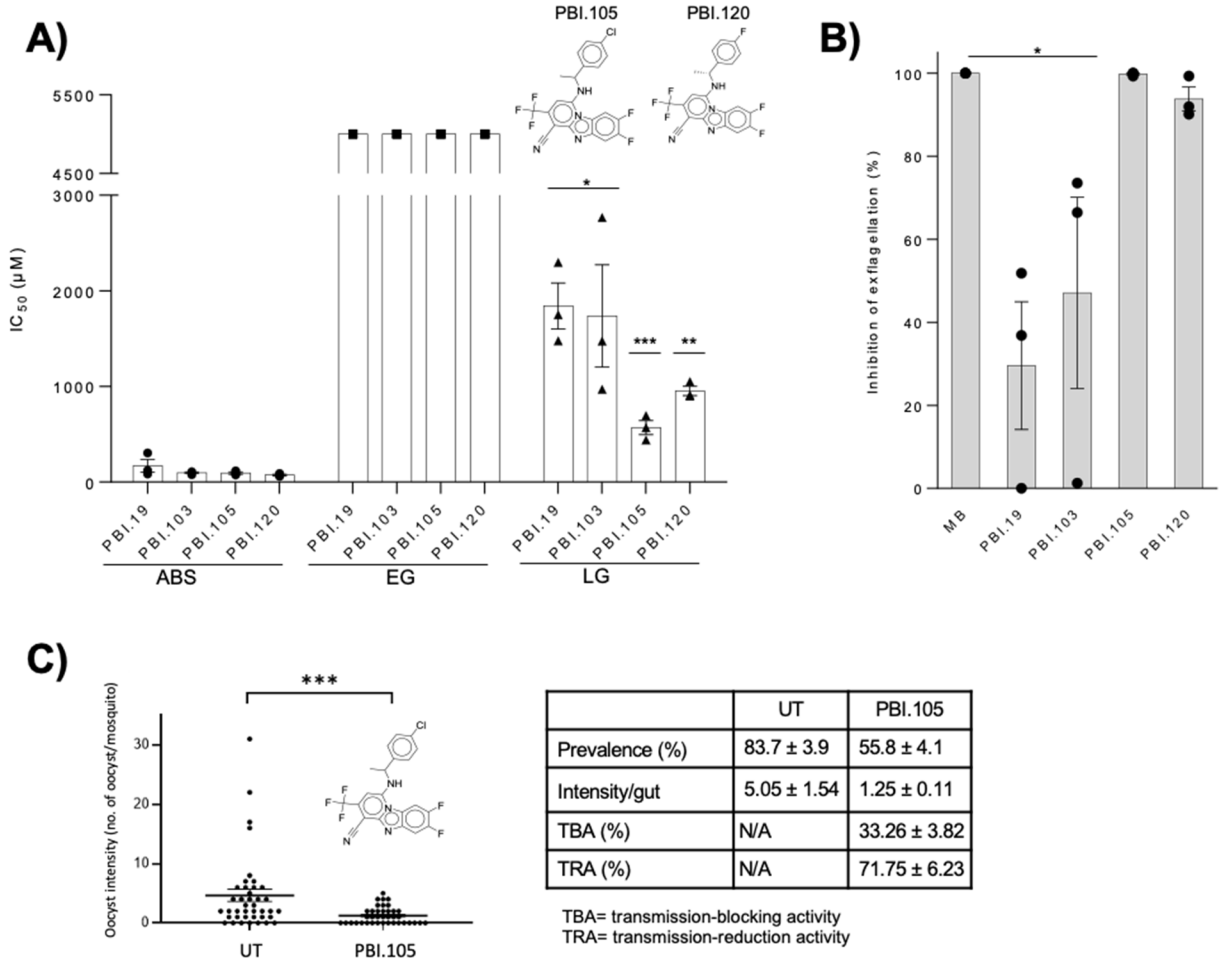
RESULTS AND DISCUSSION

Benzimidazole Scaffolds Inhibit Parasite Proliferation of Asexual Blood Stage *P. falciparum* Parasites. The 12 B-I, 11 B-II, and 143 PBIs were evaluated for their ABS activity and a total of 70 hits were identified at >85% inhibition of proliferation at 1 μM against asexual, drug sensitive *Pf*NF54 blood stage parasites (Figure S1, Supporting Information). Hit rates of 43%, 82%, and 39% were obtained for the B-I, B-II, and PBI series, respectively (Figure 2A). From the 70 hits, 67 did not affect human hepatocellular carcinoma cells (HepG2) with at least >50% viability remaining at 2 μM and 33 of the compounds not affecting human cells at all at this concentration (>95% viability remaining, Supporting Information). The potency of the compounds was confirmed in dose−response studies (Figure 2B). The B-II series had the highest hit rate and five of these compounds were active with IC_{50}s of <500 nM (Supporting Information) with only 2 B-Is active at this cutoff. Of the PBIs, 23 compounds were active with IC_{50} < 500 nM against ABS (Table 1) with 6 compounds even active at <100 nM, and the two most potent of which were **PBI.109** and **PBI.120** at 54 and 74 nM, respectively (Table 1).

Several Benzimidazole Analogues Are Active against Gametocytes with Some Transmission-Blocking Potential. The gametocytocidal activity of the benzimidazoles were evaluated in parallel to the ABS activity against both early- (>90% stage II/III) and late-stage (>95% stage IV/V) gametocytes. As expected, overall fewer compounds were active against the gametocytes compared to the ABS, similar to earlier reports.[26] Using our defined gametocyte hit criteria (>50% inhibition at 1 μM and >70% inhibition at 5 μM, Supporting Information), 13 hits each were identified against the early stage and late-stage gametocytes albeit not the same compounds involved in each instance (Figure 3, Supporting Information). Early stage hits are stratified by series as 33% B-I, 9% B-II, and 6% PBIs, whereas all the late-stage hits were PBIs (9% hit rate).

Interestingly, the hit rate profile between the ABS and early stage gametocytes differ with ABS hits ranked as B-II > B-I > PBI whereas the early stage gametocytes actives ranked as B-I > B-II > PBI. Submicromolar potency against early stage

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955



	UT	PBI.105
Prevalence (%)	83.7 ± 3.9	55.8 ± 4.1
Intensity/gut	5.05 ± 1.54	1.25 ± 0.11
TBA (%)	N/A	33.26 ± 3.82
TRA (%)	N/A	71.75 ± 6.23

Figure 4. Transmission-blocking ability selected compounds from the PBI class. (A) IC_{50} of PBI compounds against ABS, EG, and LG. IC_{50} data are from three independent biological repeats (n = 3), each performed in technical triplicates, mean ± S.E, individual data points indicated. (B) Transmission-blocking confirmation on male gamete exflagellation at 2 μM. Methylene blue (MB) was used as the drug control and data are from three independent biological repeats, mean ± S.E., individual data points indicated. An unpaired Student's t-test was used to determine significant differences for data in panels A and B with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (C) Effect of selected compound **PBI.105** on oocyst intensity for three independent biological replicates each performed in technical triplicates. The Mann−Whitney Test was used to compare the statistical significance between the drug treatment and untreated control (UT) with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

gametocytes was observed for only two compounds, **B-II.4** and **PBI.109** (Table 1). In both instances, these compounds had ABS activity as well but lost activity against late-stage gametocytes.

Two of the PBIs (**PBI.105** and **PBI.120**) showed submicromolar activity against late-stage gametocytes (Table 1). An interesting life cycle profile was observed for these two compounds as well as two other similar compounds (**PBI.19** and **PBI.103**), albeit the latter with slightly less activity against late-stage gametocytes at ∼1.8 μM. These compounds did not retain activity against early stage gametocytes (>3-fold loss in activity at >5 μM) yet were active against the late-stage gametocytes (at <1 μM), albeit with a significant loss in activity compared to ABS (**PBI.105**, 6-fold; **PBI.120**, 13-fold; $P < 0.01$ and $P < 0.001$ respectively, n = 3, unpaired Student's t-test, Figure 4A).

This observed activity against late-stage gametocytes translates to activity against male gametes for both **PBI.105** and **PBI.120**, showing 99.78% and 93.81% inhibition of male exflagellation at 2 μM, respectively (Figure 4B), compared to **PBI.19** and **PBI.103** that displayed <50% inhibition (Table 1). The IC_{50}s of **PBI.105** and **PBI.120** against male gametes were comparable to their activities on late-stage gametocytes: **PBI.105** at 697.1 nM versus 568.8 nM and **PBI.120** at 997.8 nM versus 953.0 nM for male gametes versus late-stage gametocytes, respectively (Table 1). The most potent compound on late-stage gametocytes and gametes, **PBI.105**, was also able to reduce transmission by 72% (Figure 4C) with a significant 5-fold reduction ($P < 0.05$, n = 3, unpaired Mann−Whitney t-test) in oocyst intensity/mosquito.

The life cycle profile for **PBI.105** and **PBI.120** is quite distinct and does not conform to the norm where ABS active compounds typically retain activity on early stage gametocytes but lose efficacy against late-stage gametocytes. Although they retain activity against late-stage gametocytes and male gametes, and this translates to transmission-blocking in mosquito stages thereby marking these compounds with transmission-blocking potential, their inactivity on early stage gametocytes could complicate their use, as any early stage gametocytes that escape drug pressure on ABS will not be targeted and will feed the pool of late-stage gametocytes, resulting in insufficient or ineffective potency.[26] This lack of activity against early stage gametocytes is not easily explained due to different biological targets since the mode(s) of action (MoA) of the PBIs in any

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

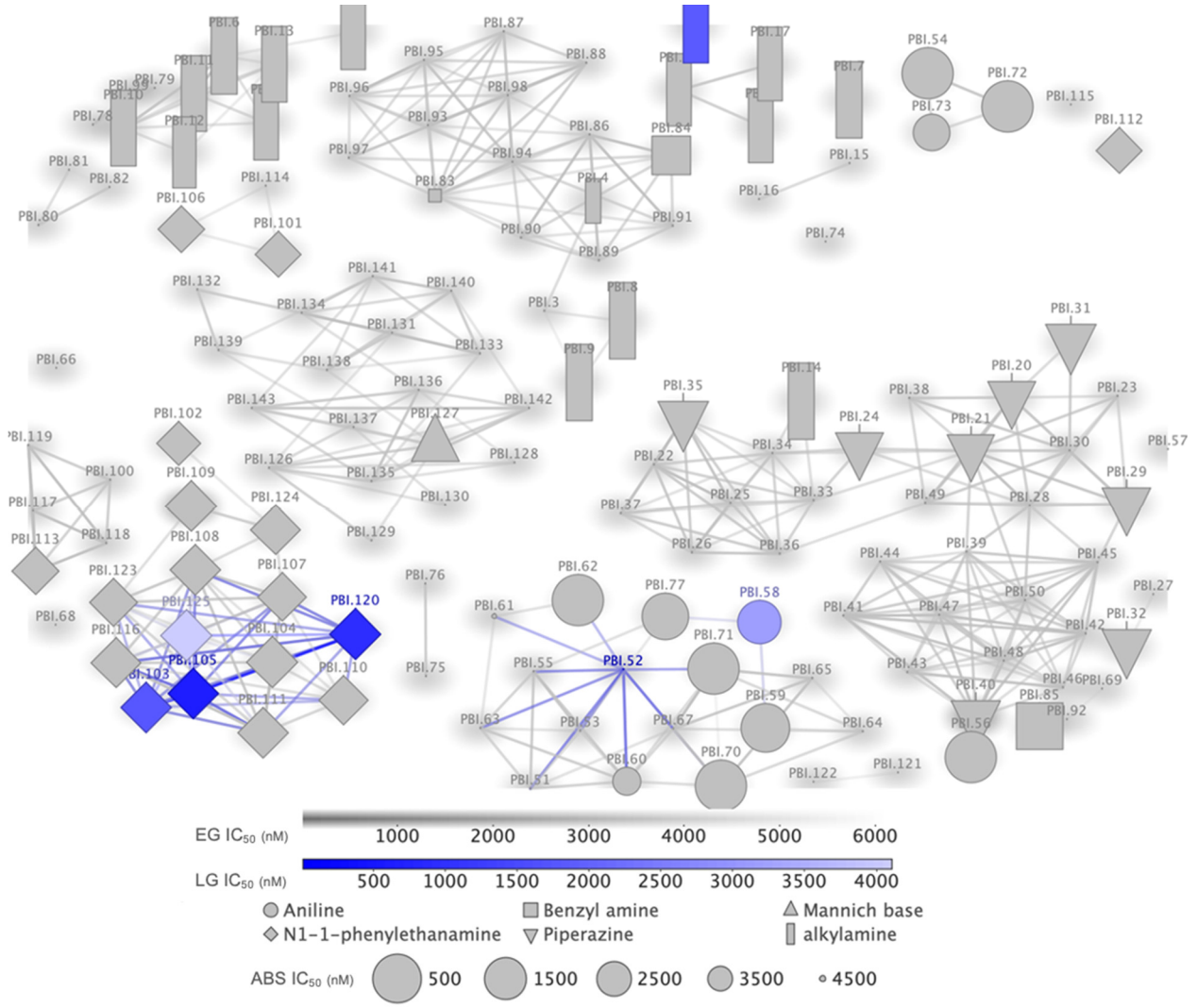


Figure 5. Structure−activity landscape index (SALI) similarity map of the PBI compounds. Pairwise structural feature (SkelSphere) and gametocyte data for both early- and late-stage gametocytes was superimposed with PfNF54 cliff analysis (Osiris DataWarrior) at a compound structural similarity threshold of 86%. For any pair of compounds, the SALI value is an indication in gain or loss of activity based on small changes in compound structure. Clusters indicate structurally related compounds with compounds indicated as nodes and structural connectivity indicated through the edges. Node size reflects IC_{50} values against ABS, node color indicates activity against late-stage (LG) gametocytes, and node shadow (clouds) shows activity against early stage gametocytes (EG).

Plasmodium life cycle stage remains uncertain. For example, intracellular colocalization studies performed on **PBI.1**, which has ABS activity (Supporting Information) showed cytoplasmic accumulation without any hemozoin inhibition, in contrast to the closely related congener **PBI.2**.[27] However, the possibility of a shared target between ABS and late-stage gametocytes cannot be excluded, but these results suggest that within the PBI chemical series different mechanistic actions are at play; thus, to a large extent analogues must be considered on their own merit. Additionally, it is unlikely that uptake differences can explain this phenomena since these two compounds do not show marked physical differences compared to other close analogues (e.g., **PBI.103**).

Structure−Activity Landscape and Mechanistic Implications. A global evaluation of the structure−activity landscape based on the analogues under investigation herein revealed a fingerprint for the activity of the benzimidazole chemotype, particularly for the PBIs, against multiple life cycle stages of *P. falciparum* (Figure 5). The N^1-1-phenylethanamine and aniline moieties confer strong ABS activity, while benzyl amine and Mannich bases are the least active. Among the anilines, only **PBI.58** had any appreciable gametocyte activity and could be associated with the *meta* position of the trifluoromethoxy substituent on the aniline, which, if moved to the ortho (**PBI.59**) or para (**PBI.77**) positions completely abolishes gametocyte activity while only retaining ABS activity. It is important to note, however, that the aniline moiety has previously been associated with genotoxicity, and subsequent studies must include such experiments.[28,29] Nonetheless, such compounds are great potential for TCP-1 activity.

A strong correlation is observed for the most gametocyte active PBI compounds that were highly active against both ABS and late-stage gametocyte *P. falciparum* stages. All four compounds, **PBI.103**, **PBI.105**, **PBI.120**, and **PBI.125**, with such dual activity have the N^1-1-phenylethanamine group at C1 and difluoro substituents on the benzene group (Table 1, Figure 5) with the most closely related compounds, **PBI.103**, **PBI.105**, and **PBI.125**, as also the most active against male

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

gametes (inhibition >65%). The activity of these compounds compared to **PBI.111**, which bears an N^1-1-4-methylphenylethanamine group, relative to the 4-fluorophenylethanamine (as in **PBI.103** and **PBI.120**) and 4-chlorophenylethanamine (as in **PBI.105**) suggests that the electron-withdrawing halogens rather than the electron-releasing methyl group potentiates activity against late-stage gametocytes and gametes, although there is no apparent discrimination against ABS activity (IC_{50} < 100 nM, Table 1). Like **PBI.111**, the lack of activity of **PBI.104** (Supporting Informatio), which bears the 4-mesylphenylethanamine group, suggests that electron-withdrawing hydrophilic substituents are also detrimental to activity. The better ABS activity of **PBI.105** relative to **PBI.125** suggests that para substitution on the phenylethanamine group is preferred over the ortho position since racemization (i.e., based on ABS activity of **PBI.103** and **PBI.120**, Table 1) has little effect on activity. Furthermore, **PBI.109** showed ABS and early stage gametocyte activity, that is, the only compound in this cluster with this profile. Although it belongs to the same N^1-1-phenylethanamine PBI chemical subclass, in **PBI.109** the 1-aminoethyl group is functionalized to a 1-aminocyclopropyl group. This modification could account for the loss in gamete activity although this is compensated by early stage gametocytocidal activity contrary to the congeners (**PBI.103**, **PBI.105**, and **PBI.120**) described above (Table 1). Perhaps, a combination of the structural characteristics of **PBI.109** (ABS and early stage gametocyte activity) as well as those of **PBI.103**, **PBI.105**, and **PBI.120**, which have ABS, late-stage gametocyte, and gamete activities, may be key to obtaining PBI compounds with activity against all stages of *P. falciparum* parasites. Interestingly, **PBI.19** with gametocyte activity is closely related to other alkylamine containing PBI compounds that only possess good ABS activity and all of which have a C1 amino phenolic Mannich base side chain substituent and a 4-trifluorophenyl side group at C3.[24]

From a mechanistic point of view, the compounds investigated in this study have shown *in vitro* inhibitory activity against β-hematin, which is the synthetic surrogate of hemozoin crystals. Heme detoxification is an essential process for parasite survival particularly during ABS, and its disruption leads to parasite death.[30] In fact, the first-generation B-I series[10] and some PBIs[13,27] are true inhibitors of hemozoin formation as demonstrated in a cell-based assay. Although not critical to early stage gametocytes, heme degradation is present in the earlier stages of development but not in late-stage gametocytes.[31] Notwithstanding, the lack of widespread inhibition of hemozoin formation among the PBIs despite their propensity through their flat tricyclic backbone to facilitate $\pi-\pi$ interactions with heme molecules, congruent to the aminoquinoline antimalarials and benzimidazoles,[11] clearly implies hemozoin inhibition is only a contributory MoA, but more importantly that other structural features significantly contribute to how these compounds affect their antiplasmodial activities.

It is noteworthy that not much is reported on other mechanisms by which antiplasmodial benzimidazoles act. However, anthelminthic benzimidazole-based drugs bind to the colchicine site of β-tubulin, thereby disrupting microtubule polymerization.[32−34] In fact, microtubule inhibitors have demonstrated varying ABS activity.[33] Microtubule formation is present in all specific stages of asexual parasites, that is, in early stage gametocyte development (II−III) and elongation (stage IV), but disassembled during gametocyte maturation (stage V),[31,35] and some key stages in the mosquito including sporozoites motility. Inhibition of microtubule formation has thus been described as a viable target and cannot be ruled out as a potential MoA contributing to the observed ABS, gametocytocidal, and male gamete activities of the compounds, especially the PBIs reported herein, given their close structural conformation to colchicine.[36] It is apparent, therefore, that even within the antimalarial drug discovery space, subject to structural modifications on the privileged benzimidazole scaffold, different stages of the parasite life cycle could be targeted, and yet within a specific life cycle stage diverse mechanisms of action could be triggered.

CONCLUSION

A strong asexual blood stage activity was observed for compounds across all the chemical series (B-I, B-II, PBI) without excessive toxicity toward the HepG2 mammalian cells, further substantiating the TCP1 potential of these compounds for treatment of malaria. Selected hit compounds are currently further characterized to establish their TCP1, including indications of rapid kill action through determining the parasite reduction ratio, to translate to fast parasite clearance *in vivo*. Overall, far less compounds were active on either the early stage and late-stage gametocyte stages with none of the compounds described here showing activity against both early stage and late-stage gametocytes. Furthermore, only the PBIs were active against either gametocyte stage, thus showing potential for TCP5 activity, particularly for compounds **PBI.105** and **PBI.120.** Although structure−activity analyses are able to describe some of the observed profiled, it is essentially important to determine the targets of these compounds as they may be different in the gametocyte stages or even present the same/multiple targets in the asexual blood stages, possibly independent of the inhibition of hemozoin formation. Overall, novel benzimidazole compounds were described here with TCP1 activity and potential for TCP5 dual activity, one of which (**PBI.105**) was active against asexual blood stages, late-stage gametocytes, male gametes, and oocyst stages of *P. falciparum* stages.

METHODS

Chemistry. The chemical and spectroscopic information on new compounds is presented in Supporting Information. All other compounds have been described before.[6,10,22,23,25]

Ethical Clearance. The *in vitro* work described holds ethics approval from the University of Pretoria (Health Sciences (506/2018) and Natural Sciences (180000094) Ethics Committee and the University of the Witwatersrand Human Research Ethics Committee (M130569) and Animal Ethics Committee (20190701−7O).

Cultivation of Asexual Blood Stage Parasites and Antiproliferative Activity of Benzimidazole Scaffolds. Asexual *Pf*NF54 (drug susceptible) parasite strains obtained from the Malaria Research and Reference Reagent Resource Center (MR4 BEI resources, Manassas, U.S.A.) were cultivated under previously described conditions.[37] Parasites were maintained at 5% hematocrit (A^+/O^+ human erythrocytes) in complete culture media [RPMI 1640 (Sigma-Aldrich) supplemented with 25 mM HEPES, 20 mM D-glucose, 200 μM hypoxanthine, 0.2% (w/v) sodium bicarbonate, 24 μg/mL gentamicin, and 0.5% (w/v) AlbuMAX II)] and kept under

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

hypoxic conditions (90% N_2, 5% O_2, and 5% CO_2) at 37 °C with agitation. D-sorbitol [5% (w/v)] was used to synchronize parasite cultures, resulting in predominantly (>95%) ring stages, when required. Parasite progression and morphology of asexual blood stages were monitored microscopically using Giemsa-stained thin smears.

The SYBR Green I assay enabled analysis of synchronized *in vitro* *Pf*NF54 parasites (1% parasitemia, 1% hematocrit) exposed to drug pressure for 96 h at 90% N_2, 5% O_2, and 5% CO_2 with chloroquine the positive control for inhibition of parasite proliferation. Parasite proliferation was determined once the 96 h incubation had elapsed by adding equal volumes (100 μL) of parasite suspension and SYBR Green I lysis buffer (0.2% μL/ml of 10 000x SYBR Green I, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.008% saponin (w/v) and 0.08% Triton x-100) and incubated for 1 h at room temperature in the dark. Fluorescence was quantified with the GloMaxR-Multi+ Detection System at 485/538 nm and used as a direct measure of proliferation.[38] Data for preliminary hit identification was performed for one biological replicate in technical triplicates whereas three independent biological replicates of technical triplicates were performed for dose−response evaluation.

Cultivation of Gametocytes and Antiplasmodial Activity of Benzimidazole Scaffolds. Gametocytogenesis was induced on highly synchronized (>95%) asexual ring stage parasites from the *Pf*NF54-pfS16-GFP-Luc line (gift from D. Fidock, Columbia University, U.S.A.) at 0.5% parasitemia and 6% hematocrit and thereafter maintained in glucose-deprived media as previously described.[39] After 3 days, the hematocrit was decreased to 4%. Parasites were maintained in complete culture media supplemented with 50 mM *N*-acetyl-glucosamine 5−10 days post induction to inhibit proliferation of residual asexual blood stages. Gametocyte progression and morphology was also monitored microscopically using Giemsa-stained thin smears.

Gametocyte viability of homogenized early stage (>85% stage II/III) and late-stage (>85% stage IV/V) gametocytes (2% gametocytemia, 1.5% hematocrit) was assessed using the luciferase-reporter assay for a 48 h (90% N_2, 5% O_2, and 5% CO_2) drug pressure at 1 and 5 μM with methylene blue used as the reference control[39] for a single biological experiment. The dose−response was then evaluated for selected hits each for three independent biological replicates performed in technical triplicates. Equal volumes (30 μL) of parasite lysate and luciferin substrate (Promega luciferase assay system) were added to white 96-well plates with bioluminescence detected at a 10 s integration constant (GloMaxR-Multi+ Detection System).[39] Data for preliminary hit identification was performed for one biological replicate in technical triplicates whereas three independent biological replicates with technical triplicates were used for dose−response evaluation.

Cultivation of Hepatocellular Carcinoma Cells and *In Vitro* Cytotoxicity Assays. Human Caucasian hepatocellular carcinoma cells of the HepG2 line (gifted by Duncan Cromarty, University of Pretoria, SA) were cultivated *in vitro* in complete DMEM media (Hyclone, U.S.A.) supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin.[38] Cells were detached with 0.25% Trypsin-EDTA once ∼80% confluency was observed,[40] stained with trypan blue to determine cell viability and counted using a hemocytometer. Cells were then added to 96-well plates (2×10^4 cells/well) and incubated overnight at 37 °C under 5% CO_2 and 95% humidity. Following incubation, cells were exposed to 2 μM of compounds [Final DMSO concentration <0.1% (v/v)] for 48 h at 37 °C under 5% CO_2 humid (95%) conditions. Cytotoxicity was then determined using the lactate dehydrogenase release assay (Cytoselect Inc.) as per manufacturer's instruction whereby 10 μL of Cytoselect reagent was added to 90 μL of supernatant and, thereafter, incubated for 30 min under 5% CO_2 humid (95%) conditions. Absorbance at 450 nm was used to quantify cytotoxicity with cytotoxic agent, emetine, as the reference control. Data were obtained for a single biological repeat in technical triplicates.

Male Gamete Exflagellation Inhibition Assays. Mature *Pf*NF54 gametocytes (>95% stage V) were treated with 2 μM of benzimidazole compounds in complete culture media and incubated for 48 h at 37 °C under hypoxic gas conditions (90% N_2, 5% O_2, and 5% CO_2). Gametogenesis was induced by exposing the mature gametocytes (>95% stage V) to ookinete media [RPMI-1640 media (Sigma-Aldrich) supplemented with 25 mM HEPES, 0.2% sodium bicarbonate, pH 8.0, 20% (v/v) human serum, and 100 μM xanthurenic acid] at room temperature for 15 min. The inhibition of exflagellation was measured through drug pressure carry over as previously described[41] with methylene blue used as the reference control for inhibition of male exflagellation. Cultures were settled in a Neubauer chamber at room temperature and exflagellating centers captured with video microscopy (Carl Zeiss NT 6 V/10W Stab480 microscope with a MicroCapture camera, 10× magnification). Exflagellating centers were semiautomatically identified for 15 randomly located fields with each video recorded for 8−10 at 30 s intervals. The total exflagellating centers per treatment were quantified using ICY (open source imaging software GPLv3) and normalized to an untreated control.[42] A minimum of 16 centers were counted for the untreated and methylene blue control experiments.

Standard Membrane Feeding Assay. The standard membrane feeding assay was conducted as previously described,[42] to confirm the transmission-blocking ability of selected benzimidazole compounds. Shortly, *Anopheles gambiae* mosquitoes *s.s.* (Colonised in 2009 from the Republic of the Congo, acronym COGS)[43] were reared at 80% humidity, 25 °C, 12 h day/night cycle with 45 min dusk/dawn transitions.[44] Mosquitoes were fed a solution of 10% sucrose supplemented with 0.05% (v/v) 4-aminobenzoic acid. Mature *Pf*NF54 gametocytes (>95% stage V, 1.5−2.5% gametocytaemia, 50% hematocrit) were exposed to 2 μM of selected compounds for 48 h with DMSO as a vehicle control. The SMFA was validated with epoxomicin as positive control, as reported before.[42] The gametocytes were tested for male gamete exflagellation and a male-to-female ratio of 1:3 was confirmed for use in the SMFA. At conclusion of the 48 h incubation, the treated gametocytes were fed to 25 unfed (2−3 h starvation) female *An. gambiae* mosquitoes (5−7 days old) for 40 min in the dark at room temperature using glass feeders covered with bovine intestine. Mosquitoes were individually processed whereby unfed/partially fed mosquitoes were then removed, housed for 8−10 days and then dissected to remove the midguts. The midguts were rinsed with PBS, incubated in 0.1% (v/v) mercurochrome for 8−10 min after which the oocysts were counted using brightfield microscope (20−40× magnification). The transmission-reduction activity (TRA) which measure the reduction in oocyst intensity, and the transmission-blocking activity (TBA) which measure the reduction in oocyst prevalence were determined (eqs 1 and 2 below). Data was collected for biological triplicates, each in duplicate.

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

Data were managed in Microsoft Excel and statistical analysis were done using GraphPad Prism version 9.0.0. All data were evaluated for normality using the Shapiro-Wilk test.[45] Data were not normally distributed, and the Mann−Whitney test was performed to determine statistical significance between treatment and control cohorts. Mann−Whitley test was used as the prevalence and intensity data are not normally distributed. Unpaired *t* test was used to determine if there was a statistically significant difference between the means in two unrelated groups, TBA from *An. arabiensis* versus TBA from *An. gambiae*. It was also used to evaluate the differences between the TRA of *An. arabiensis* and *An. gambiae*.

A *p*-value <0.05 was considered statistically significant.

$$\%\mathrm{TRA} = \frac{Ci - Ti}{Ci} \times 100 \quad (1)$$

$$\%\mathrm{TBA} = \frac{Cp - Tp}{Cp} \times 100 \quad (2)$$

Reduction in prevalence (transmission-blocking activity where *p* is oocyst prevalence, *C* is control, and *T* is treated) and reduction in number of oocysts intensity (transmission-reducing activity where *i* is oocyst number (intensity), *C* is control, and *T* is treated).

Structure Function Activity Analysis. Structure−activity landscape index (SALI) was performed using Osiris Datawarrior V 5.2.1 software (www.openmolecules.org). The compound similarity/activity cliff analyses was performed with similarity based on the SMILES structure taking stereochemistry into account and separated based on the compound neighbor with a Tanimoto similarity threshold at 86%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00910.

Figure S1: screening cascade; chemical and spectroscopic information on new compounds (PDF)

Summary of all data for all *in vitro* experiments (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

Kelly Chibale − *Department of Chemistry, Institute of Infectious Disease and Molecular Medicine, and South African Medical Research Council Drug Discovery and Development Research Unit, University of Cape Town, Rondebosch 7701, South Africa;* orcid.org/0000-0002-1327-4727; Email: Kelly.chibale@uct.ac.za

Lyn-Marie Birkholtz − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa;* orcid.org/0000-0001-5888-2905; Email: lynmarie.birkholtz@up.ac.za

Authors

Meta Leshabane − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa*

Godwin Akpeko Dziwornu − *Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa*

Dina Coertzen − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa*

Janette Reader − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa*

Phanankosi Moyo − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa*

Mariëtte van der Watt − *Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa*

Kelly Chisanga − *Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa*

Consolata Nsanzubuhoro − *Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa*

Richard Ferger − *Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa*

Erica Erlank − *Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, and Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg 2193, South Africa*

Nelius Venter − *Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, and Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg 2193, South Africa*

Lizette Koekemoer − *Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, and Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg 2193, South Africa*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.0c00910

Author Contributions

M.L., G.D., D.C., J.R., P.M., M.v.d.W., K. Chisanga, C.N., R.F., E.E., and N.V. performed the experiments. M.L., G.D., and D.C. performed data analyses with E.E., N.V., and L.K. analyzing data for SMFA. M.L., G.D., and D.C. wrote the manuscript with L.B., K. Chibale, and L.K. L.B., K. Chibale, and L.K. conceived and supervised the project.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was in part supported by the South African Medical Research Council, DSI/NRF South African Research Chairs Initiative Grants (L.B. UID: 84627; L.K. UID: 64763; K. Chibale UID: 64767) and a NRF Community of Practice Grant (UID: 110666 L.B., K. Chibale, and L.K.). The UP ISMC is a MRC Centre for Collaborative Research on Malaria. Dr. L. Nardini, Ms. A. Aswat, and Mnr J. W. Zawada are thanked for assistance during mosquito dissections.

■ ABBREVIATIONS:

ABS: asexual blood stages; EG: early stage gametocytes; HepG2: human hepatocellular carcinoma; LG: Late-stage

gametocytes; MoA: mode of action; PBI: pyrido[1,2-*a*]-benzimidazole; SALI: structure−activity landscape index; SMFA: standard membrane feeding assay; TBA: transmission-blocking activity; TRA: transmission-reducing activity

REFERENCES

(1) WHO. (2019) *World Malaria Report*, World Health Organization, Geneva, Switzerland..

(2) Birkholtz, L.-M., Coetzer, T. L., Mancama, D., Leroy, D., and Alano, P. (2016) Discovering New Transmission-Blocking Antimalarial Compounds: Challenges and Opportunities. *Trends Parasitol. 32*, 669−681.

(3) Burrows, J. N., Duparc, S., Gutteridge, W. E., Hooft van Huijsduijnen, R., Kaszubska, W., Macintyre, F., Mazzuri, S., Möhrle, J. J., and Wells, T. N. C. (2017) New developments in anti-malarial target candidate and product profiles. *Malar. J. 16*, 26.

(4) Horton, D. A., Bourne, G. T., and Smythe, M. L. (2003) The combinatorial synthesis of bicyclic privileged structures or privileged substructures. *Chem. Rev. 103*, 893−930.

(5) Keri, R. S., Hiremathad, A., Budagumpi, S., and Nagaraja, B. M. (2015) Comprehensive review in current developments of benzimidazole-based medicinal chemistry. *Chem. Biol. Drug Des. 86*, 19−65.

(6) Attram, H. D., Wittlin, S., and Chibale, K. (2019) Incorporation of an intramolecular hydrogen bonding motif in the side chain of antimalarial benzimidazoles. *MedChemComm 10*, 450−455.

(7) Camacho, J., Barazarte, A., Gamboa, N., Rodrigues, J., Rojas, R., Vaisberg, A., Gilman, R., and Charris, J. (2011) Synthesis and biological evaluation of benzimidazole-5-carbohydrazide derivatives as antimalarial, cytotoxic and antitubercular agents. *Bioorg. Med. Chem. 19*, 2023−2029.

(8) Chong, C. R., Chen, X., Shi, L., Liu, J. O., and Sullivan, D. J. (2006) A clinical drug library screen identifies astemizole as an antimalarial agent. *Nat. Chem. Biol. 2*, 415−416.

(9) Keurulainen, L., Vahermo, M., Puente-Felipe, M., Sandoval-Izquierdo, E., Crespo-Fernandez, B., Guijarro-Lopez, L., Huertas-Valentin, L., de las Heras-Duena, L., Leino, T. O., Siiskonen, A., Ballell-Pages, L., Sanz, L. M., Castaneda-Casado, P., Jimenez-Diaz, M. B., Martinez-Martinez, M. S., Viera, S., Kiuru, P., Calderon, F., and Yli-Kauhaluoma, J. (2015) A developability-focused optimization approach allows identification of in vivo fast-acting antimalarials: N-[3-[(benzimidazol-2-yl) amino] propyl] amides. *J. Med. Chem. 58*, 4573−4580.

(10) Kumar, M., Okombo, J., Mambwe, D., Taylor, D., Lawrence, N., Reader, J., van der Watt, M., Fontinha, D., Sanches-Vaz, M., Bezuidenhout, B. C., Lauterbach, S. B., Liebenberg, D., Birkholtz, L.-M., Coetzer, T. L., Prudencio, M., Egan, T. J., Wittlin, S., and Chibale, K. (2019) Multistage antiplasmodium activity of astemizole analogues and inhibition of hemozoin formation as a contributor to their mode of action. *ACS Infect. Dis. 5*, 303−315.

(11) L'abbate, F. P., Müller, R., Openshaw, R., Combrinck, J. M., de Villiers, K. A., Hunter, R., and Egan, T. J. (2018) Hemozoin inhibiting 2-phenylbenzimidazoles active against malaria parasites. *Eur. J. Med. Chem. 159*, 243−254.

(12) Ramachandran, S., Hameed, P. S., Srivastava, A., Shanbhag, G., Morayya, S., Rautela, N., Awasthy, D., Kavanagh, S., Bharath, S., Reddy, J., Panduga, V., Prabhakar, K. R., Saralaya, R., Nanduri, R., Raichurkar, A., Menasinakai, S., Achar, V., Jimenez-Diaz, M. B., Martinez, M. S., Angulo-Barturen, I., Ferrer, S., Sanz, L. M., Gamo, F. J., Duffy, S., Avery, V. M., Waterson, D., Lee, M. C. S., Coburn-Flynn, O., Fidock, D. A., Iyer, P. S., Narayanan, S., Hosagrahara, V., and Sambandamurthy, V. K. (2014) N-aryl-2-aminobenzimidazoles: novel, efficacious, antimalarial lead compounds. *J. Med. Chem. 57*, 6642−6652.

(13) Singh, K., Okombo, J., Brunschwig, C., Ndubi, F., Barnard, L., Wilkinson, C., Njogu, P. M., Njoroge, M., Laing, L., Machado, M., Prudencio, M., Reader, J., Botha, M., Nondaba, S., Birkholtz, L.-M., Lauterbach, S., Churchyard, A., Coetzer, T. L., Burrows, J. N., Yeates, C., Denti, P., Wiesner, L., Egan, T. J., Wittlin, S., and Chibale, K. (2017) Antimalarial pyrido [1, 2-a] benzimidazoles: Lead optimization, parasite life cycle stage profile, mechanistic evaluation, killing kinetics, and in vivo oral efficacy in a mouse model. *J. Med. Chem. 60*, 1432−1448.

(14) Badawey, E., and Gohar, Y. (1992) Benzimidazole condensed ring systems. 8 (1). Synthesis of some substituted 1-oxo-1H, 5H-pyrido [1, 2-a] benzimidazole-4-carbonitriles with anticipated antimicrobial activity. *Farmaco (Societa chimica italiana: 1989) 47*, 489.

(15) Badawey, E., and Kappe, T. (1995) Benzimidazole condensed ring system. IX. Potential antineoplastics. New synthesis of some pyrido [1, 2-α] benzimidazoles and related derivative. *Eur. J. Med. Chem. 30*, 327−332.

(16) El-Hawash, S., Badawey, A. e.-S., and Kappe, T. (1999) Benzimidazole condensed ring systems. XII. Synthesis and anticancer evaluation of certain pyrido [1, 2-a] benzimidazole derivatives. *Die Pharmazie 54*, 341−346.

(17) Pieroni, M., Tipparaju, S. K., Lun, S., Song, Y., Sturm, A. W., Bishai, W. R., and Kozikowski, A. P. (2011) Pyrido [1, 2-a] benzimidazole-based agents active against tuberculosis (TB), multi-drug-resistant (MDR) TB and extensively drug-resistant (XDR) TB. *ChemMedChem 6*, 334.

(18) Prostakov, N., Varlamov, A., Shendrik, I., Anisimov, B., Krapivko, A., Lavani-Edogiaverie, S., and Fomichev, A. (1983) New method for the synthesis of pyrido [1, 2-a] benzimidazole. *Chem. Heterocycl. Compd. 19*, 1102−1104.

(19) Rida, S. M., Soliman, F. S., Badawey, E. S. A., El-Ghazzawi, E., Kader, O., and Kappe, T. (1988) Benzimidazole condensed ring systems. 1. Syntheses and biological investigations of some substituted pyrido [1, 2-a] benzimidazoles. *J. Heterocycl. Chem. 25*, 1087−1093.

(20) Takeshita, H., Watanabe, J., Kimura, Y., Kawakami, K., Takahashi, H., Takemura, M., Kitamura, A., Someya, K., and Nakajima, R. (2010) Novel pyridobenzimidazole derivatives exhibiting antifungal activity by the inhibition of β-1, 6-glucan synthesis. *Bioorg. Med. Chem. Lett. 20*, 3893−3896.

(21) Churcher, T. S., Blagborough, A. M., Delves, M., Ramakrishnan, C., Kapulu, M. C., Williams, A. R., Biswas, S., Da, D. F., Cohuet, A., and Sinden, R. E. (2012) Measuring the blockade of malaria transmission−an analysis of the standard membrane feeding assay. *Int. J. Parasitol. 42*, 1037−1044.

(22) Mayoka, G., Njoroge, M., Okombo, J., Gibhard, L., Sanches-Vaz, M., Fontinha, D., Birkholtz, L. M., Reader, J., van der Watt, M., Coetzer, T. L., Lauterbach, S., Churchyard, A., Bezuidenhout, B., Egan, T. J., Yeates, C., Wittlin, S., Prudêncio, M., and Chibale, K. (2019) Structure-Activity Relationship Studies and Plasmodium Life Cycle Profiling Identifies Pan-Active N-Aryl-3-trifluoromethyl Pyrido-[1,2- a]benzimidazoles Which Are Efficacious in an in Vivo Mouse Model of Malaria. *J. Med. Chem. 62*, 1022−1035.

(23) Ndakala, A. J., Gessner, R. K., Gitari, P. W., October, N., White, K. L., Hudson, A., Fakorede, F., Shackleford, D. M., Kaiser, M., Yeates, C., Charman, S. A., and Chibale, K. (2011) Antimalarial pyrido [1, 2-a] benzimidazoles. *J. Med. Chem. 54*, 4581−4589.

(24) Okombo, J., Brunschwig, C., Singh, K., Dziwornu, G. A., Barnard, L., Njoroge, M., Wittlin, S., and Chibale, K. (2019) Antimalarial Pyrido [1, 2-a] benzimidazole Derivatives with Mannich Base Side Chains: Synthesis, Pharmacological Evaluation, and Reactive Metabolite Trapping Studies. *ACS Infect. Dis. 5*, 372−384.

(25) Probst, A., Chisanga, K., Dziwornu, G. A., Haeberli, C., Keiser, J., and Chibale, K. (2020) Expanding the Activity Profile of Pyrido [1, 2-a] benzimidazoles: Synthesis and Evaluation of Novel N 1−1-Phenylethanamine Derivatives against Schistosoma mansoni. *ACS Infect. Dis. 64*, 3907−3915.

(26) Van der Watt, M. E., Reader, J., Churchyard, A., Nondaba, S. H., Lauterbach, S. B., Niemand, J., Abayomi, S., van Biljon, R. A., Connacher, J. I., van Wyk, R. D., Le Manach, C., Paqut, T., Gonzales Cabrera, D., Brunschwig, C., Theron, A., Lozano-Arias, S., Rodrigues, J. F. I., Herreros, E., Leroy, D., Duffy, J., Street, L. J., Chibale, K., Mancama, D., Coetzer, T. L., and Birkholtz, L. M. (2018) Potent *Plasmodium falciparum* gametocytocidal compounds identified by

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955

exploring the kinase inhibitor chemical space for dual active antimalarials. *J. Antimicrob. Chemother. 73*, 1279−1290.
(27) Korkor, C. M., Garnie, L. F., Amod, L., Egan, T. J., and Chibale, K. (2020) Intrinsic fluorescence properties of antimalarial pyrido [1, 2-a] benzimidazoles facilitate subcellular accumulation and mechanistic studies in the human malaria parasite *Plasmodium falciparum*. *Org. Biomol. Chem. 18*, 8668−8676.
(28) Bomhard, E. M., and Herbold, B. A. (2005) Genotoxic activities of aniline and its metabolites and their relationship to the carcinogenicity of aniline in the spleen of rats. *Crit. Rev. Toxicol. 35*, 783−835.
(29) Kugler-Steigmeier, M. E., Friederich, U., Graf, U., Lutz, W. K., Maier, P., and Schlatter, C. (1989) Genotoxicity of aniline derivatives in various short-term tests. *Mutat. Res., Fundam. Mol. Mech. Mutagen. 211*, 279−289.
(30) Banerjee, R., and Goldberg, D. E. (2001) *The Plasmodium food vacuole* (Rosenthal, P. J., Ed.) p 43−63, Springer, New Jersey.
(31) van Biljon, R., van Wyk, R., Painter, H. J., Orchard, L., Reader, J., Niemand, J., Llinás, M., and Birkholtz, L.-M. (2019) Hierarchical transcriptional control regulates *Plasmodium falciparum* sexual differentiation. *BMC Genomics 20*, 920.
(32) Hadfield, J. A., Ducki, S., Hirst, N., and McGown, A. T. (2003) Tubulin and microtubules as targets for anticancer drugs. *Progr. Cell Cycl. Res. 5*, 309−326.
(33) Kappes, B., and Rohrbach, P. (2007) Microtubule inhibitors as a potential treatment for malaria. *Future Microbiol. 2*, 409−23.
(34) Mota, M. M., and Rodriguez, A. (2002) Invasion of mammalian host cells by *Plasmodium* sporozoites. *BioEssays 24*, 149−156.
(35) Dearnley, M. K., Yeoman, J. A., Hanssen, E., Kenny, S., Turnbull, L., Whitchurch, C. B., Tilley, L., and Dixon, M. W. (2012) Origin, composition, organization and function of the inner membrane complex of *Plasmodium falciparum* gametocytes. *J. Cell Sci. 125*, 2053−2063.
(36) Usanga, E., O'Brien, E., and Luzzato, L. (1986) Mitotic inhibitors arrest the growth of *Plasmodium falciparum*. *FEBS Lett. 209*, 23−27.
(37) Trager, W., and Jensen, J. B. (1976) Human malaria parasites in continuous culture. *Science 193*, 673−675.
(38) Verlinden, B. K., Niemand, J., Snyman, J., Sharma, S. K., Beattie, R. J., Woster, P. M., and Birkholtz, L.-M. (2011) Discovery of novel alkylated (bis)urea and (bis)thiourea polyamine analogues with potent antimalarial activities. *J. Med. Chem. 54*, 6624−6633.
(39) Reader, J., Botha, M., Theron, A., Lauterbach, S. B., Rossouw, C., Engelbrecht, D., Wepener, M., Smit, A., Leroy, D., Mancama, D., Coetzer, T. L., and Birkholtz, L.-M. (2015) Nowhere to hide: interrogating different metabolic parameters of *Plasmodium falciparum* gametocytes in a transmission blocking drug discovery pipeline towards malaria elimination. *Malar. J. 14*, 213.
(40) Coertzen, D., Reader, J., Van Der Watt, M., Nondaba, S. H., Gibhard, L., Wiesner, L., Smith, P., D'Alessandro, S., Taramelli, D., and Wong, H. N. (2018) Artemisone and artemiside are potent panreactive antimalarial agents that also synergize redox imbalance in Plasmodium falciparum transmissible gametocyte stages. *Antimicrob. Agents Chemother. 62*, 02214−17.
(41) Coetzee, N., von Grüning, H., Opperman, D., van der Watt, M., Reader, J., and Birkholtz, L.-M. (2020) Epigenetic inhibitors target multiple stages of *Plasmodium falciparum* parasites. *Sci. Rep. 10*, 2355.
(42) Reader, J., van der Watt, M. E., Taylor, D., Le Manach, C., Mittal, N., Ottilie, S., Theron, A., Moyo, P., Erlank, E., Nardini, L., Venter, N., Lauterbach, S., Bezuidenhout, B., Horatscheck, A., van Heerden, A., Spillman, N. J., Cowell, A. N., Connacher, J., Opperman, D., Orchard, L. M., Llinás, M., Istvan, E. S., Goldberg, D. E., Boyle, G. A., Calvo, D., Mancama, D., Coetzer, T. L., Winzeler, E. A., Duffy, J., Koekemoer, L. L., Basarab, G., Chibale, K., and Birkholtz, L.-M. (2021) Multistage and transmission-blocking targeted antimalarials discovered from the open-source MMV Pandemic Response Box. *Nat. Commun. 12*, 269.
(43) Koekemoer, L. L., Spillings, B. L., Christian, R. N., Lo, T. C., Kaiser, M. L., Norton, R. A., Oliver, S. V., Choi, K. S., Brooke, B. D., Hunt, R. H., and Coetzee, M. (2011) Multiple insecticide resistance in Anopheles gambiae (Diptera: Culicidae) from Pointe Noire, Republic of the Congo. *Vector-Borne Zoonotic Dis. 11*, 1193−200.
(44) Hunt, R. H., Brooke, B. D., Pillay, C., Koekemoer, L. L., and Coetzee, M. (2005) Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector Anopheles funestus. *Med. Vet. Entomol. 19*, 271−5.
(45) Shapiro, S. S., and Wilk, M. B. (1965) An analysis of variance test for normality (complete samples). *Biometrika 52*, 591−611.

https://dx.doi.org/10.1021/acsinfecdis.0c00910
ACS Infect. Dis. 2021, 7, 1945−1955