In Vitro Activity of wALADin Benzimidazoles against Different Life Cycle Stages of *Plasmodium* Parasites

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wALADin1 benzimidazoles are specific inhibitors of δ-aminolevulinic acid dehydratase from *Wolbachia* endobacteria of filarial nematodes. We report that wALADin1 and two derivatives killed blood stage *Plasmodium falciparum in vitro* (50% inhibitory concentrations, 39, 7.7, and 12.8 μM, respectively). One of these derivatives inhibited gliding motility of *Plasmodium berghei* ANKA infectious sporozoites with nanomolar affinity and blocked invasion into hepatocytes but did not affect intrahepatocytic replication. Hence, wALADin1 benzimidazoles are tools to study gliding motility and potential antiplasmodial drug candidates.

WALADin benzimidazoles are a recently described class of species-specific inhibitors of the heme biosynthesis enzyme δ-aminolevulinic acid dehydratase (ALAD; porphobilinogen synthase) of *Wolbachia* endobacteria of pathogenic filarial worms (1). The compounds killed filarial nematodes ex vivo in a *Wolbachia*-dependent manner with no cytotoxicity below 500 μM, thus representing drug lead candidates (1).

The malaria parasite *Plasmodium falciparum* is able to synthesize heme *de novo*, suggesting that it is a potential target pathway for new antimalarial drugs (2). *P. falciparum* uses two forms of ALAD for heme biosynthesis: one is host-derived ALAD (*Homo sapiens* ALAD [*Hs*ALAD]) imported from infected erythrocytes (3), and the second is an endogenous form (*Pf*ALAD) (4) targeted to the apicoplast (5). *Pf*ALAD accounts for ~10% of total parasite-derived ALAD activity but may be indispensable during certain life cycle stages or for apicoplast homeostasis (5). In contrast to *Hs*ALAD, *Pf*ALAD possesses a binding site for allosteric Mg^{2+} (4) similar to the *Wolbachia* protein, for which a functional competition with the activation by Mg^{2+} is part of the inhibitory mechanism (1). We therefore reasoned that wALADins might be effective inhibitors of *Pf*ALAD and hence possess antimalarial activity.

To determine the antiplasmodial activity of wALADins, *P. falciparum* strain 3D7 parasites were cultivated in human blood type A^+ erythrocytes under a defined gaseous atmosphere (92% CO_2, 3% O_2, 5% CO_2) at 37°C as published previously (6). Parasitemia was adjusted to 0.2 to 0.5% before the parasites were exposed to 62.5 μM wALADin1 (Fig. 1A, Table 1), the derivatives, or 0.625% dimethyl sulfoxide (DMSO) for 72 h. Compound 1 (wALADin1) and derivatives 4 and 7 reduced parasitemia to nearly 0%, while parasitemia of the DMSO control parasites increased to ~3% (Fig. 1C). None of the other derivatives had antiplasmodial activity (Fig. 1C). wALADin2, a nonbenzimidazole compound (Fig. 1B) with similar inhibitory activity and mechanism against *Wolbachia* ALAD (7), was inactive against blood stage *P. falciparum* (Fig. 1C). Dose-response curves for effective derivatives were very steep (Fig. 1D), and time curve experiments revealed similar progressions of the antiplasmodial effect for all active compounds (Fig. 1E to G). Parasites were killed within 24 to 72 h of exposure to >10 μM compound 4. At lower concentrations, parasite growth was retarded. The corresponding activity profile of

wALADin benzimidazoles is shown in Fig. 1H and Table 1. wALADin1 had the weakest activity (50% inhibitory concentration [IC₅₀], 39.3 ± 11.7 μM), while replacement of the R^1-3-CF_3-benzyl with 4-CF_3-benzyl in compound 7 led to an ~3-fold improvement (IC₅₀, 12.8 ± 0.02 μM). The most potent antiplasmodial compound was compound 4 (IC₅₀, 7.7 ± 1.7 μM), which differs from wALADin1 by repositioning of the R^3-COOH from position 5 to 6 of the benzimidazole core. In contrast, derivatives with R^3-COOH at position 4 or 7 were inactive. Replacement of either the R^1 or R^2 moiety with a hydrogen atom abrogated the antiplasmodial activity.

To elucidate whether the antiplasmodial effect was due to inhibition of *Pf*ALAD, we tested selected derivatives against recombinant *Pf*ALAD protein (5) (expression plasmid kindly provided by V. Arun Nagaraj). Enzymatic assays were performed as described previously for orthologs using 250 nM *Pf*ALAD in 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 200 μM 5-ALA for 60 min (1). wALADin1 and compound 9 showed marginal inhibitory activity at the highest concentration tested (533 μM), whereas neither compound 4 nor 7 was inhibitory (Table 1). Although it may not be ruled out that under physiological conditions the *Pf*ALAD may be more susceptible to inhibition, it is likely that the antiplasmodial activity elicited in the one-digit (compound 4) or low two-digit (wALADin1, compound 7) micromolar range is mediated via an alternative target than ALAD.

The specificity of the antiplasmodial effect was tested by determining the activity of wALADins against the related apicomplexan parasite *Toxoplasma gondii* for which neither wALADin1 nor
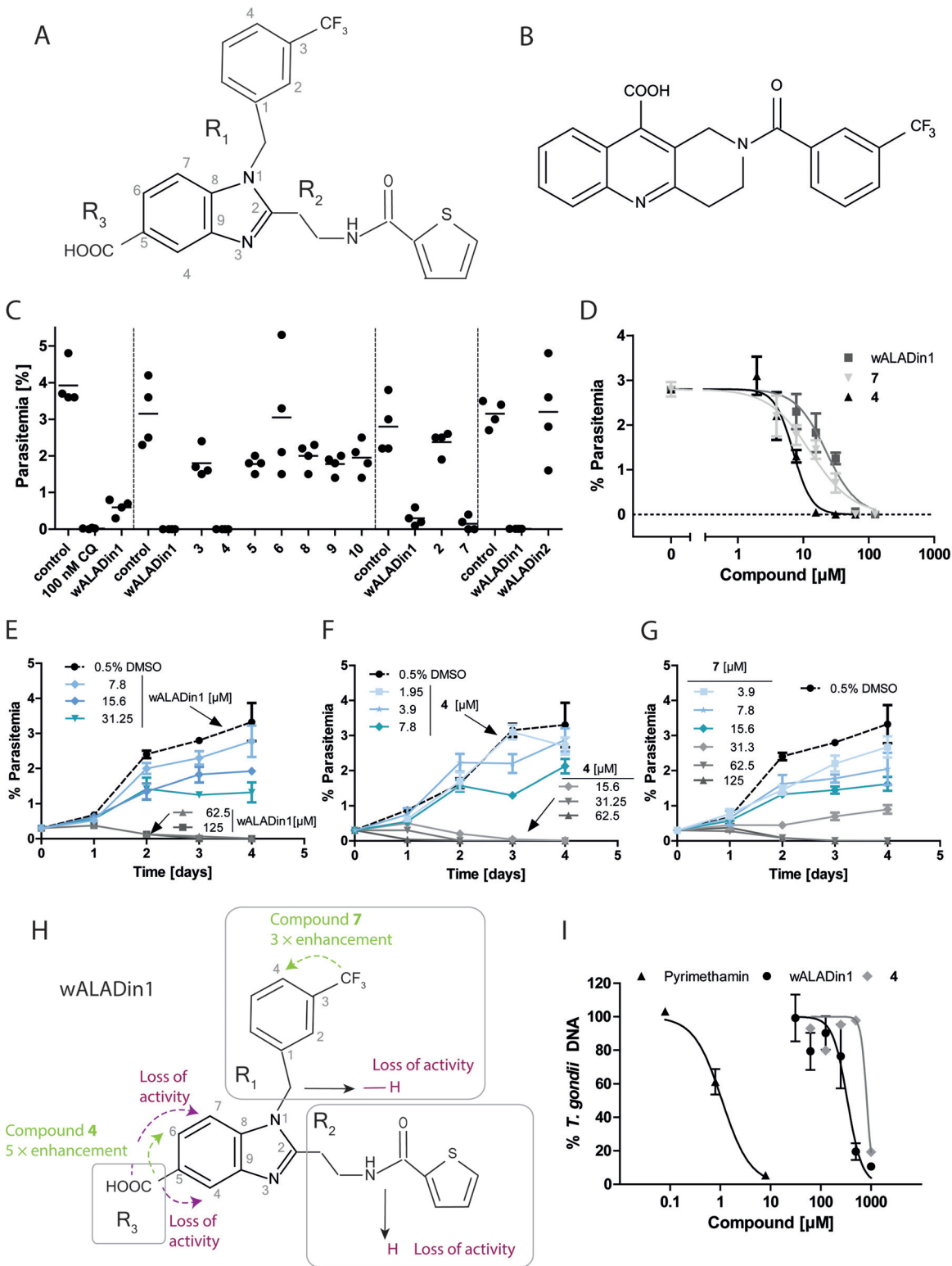
FIG 1 (A and B) Chemical structures of wALADin1 (A) and wALADin2 (B). (C) wALADin1 and derivatives were tested at 62.5 μM for 72 h in a *P. falciparum* erythrocyte culture. Parasitemia was determined by counting erythrocytes in blood smears. Chloroquine at 100 nM (CQ) was used as a positive control. Dashed vertical lines separate different experiments. (D) Dose-response curves for wALADin compounds 1, 4, and 7. Curves were fit to a sigmoidal (four-parameter) equation with GraphPad Prism 5.0 [log(inhibitor versus normalized response – variable slope); bottom = 0; top = shared by all data sets]. (E to G) Time course of parasitemia in *P. falciparum* culture in the presence of wALADin1 (E), compound 4 (F), and compound 7 (G) for 4 days. (H) Structure-activity profile of wALADin1 benzimidazoles in *P. falciparum* culture. The substituents R^1, R^2, and R^3 are highlighted in gray boxes. Dashed arrows indicate positional changes of substituent groups; continuous arrows indicate replacement with a different chemical group. Arrow or font color indicates an enhancement in antiplasmodial activity (green) or a loss of activity (purple). (I) wALADin1 and compound 4 inhibited replication of *T. gondii* in LLC-MK2 cells, as determined by real-time PCR, with IC_{50}s 1 order of magnitude (wALADin1) or several orders of magnitude (compound 4) higher than for *P. falciparum*, indicating specificity of the antiplasmodial effect (D). The graph shows means ± standard errors of the means of results for ≥2 experiments for pyrimethamine and wALADin1 and as means of results for a single experiment for compound 4.

TABLE 1 Antiplasmodial activity of wALADin1 benzimidazoles is not correlated with inhibitory activity against *Pf*ALAD

Compound	R¹ residue	R² residue	Position of R³ residue	*P. falciparum* erythrocyte culture LD₅₀ (μM)[a]	*Pf*ALAD IC₅₀ (μM)[b]
1 (wALADin1)	3-CF₃-benzyl	2-[(2-thienylcarbonyl)amino]ethyl	C-5	39.3 ± 11.7 ($n = 4$)	~568
2	H	2-[(2-thienylcarbonyl)amino]ethyl	C-5	NA	ND
3	3-CF₃-benzyl	H	C-5	NA	NI
4	3-CF₃-benzyl	2-[(2-thienylcarbonyl)amino]ethyl	C-6	7.7 ± 1.7 ($n = 3$)	NI
5	3-CF₃-benzyl	2-[(2-thienylcarbonyl)amino]ethyl	C-4	NA	ND
6	3-CF₃-benzyl	2-[(2-thienylcarbonyl)amino]ethyl	C-7	NA	~625
7 (ALPin1)	4-CF₃-benzyl	2-[(2-thienylcarbonyl)amino]ethyl	C-5	12.8 ± 0.02 ($n = 2$)	NI
8	4-CF₃-benzyl	H	C-5	NA	ND
9	CH₃	H	C-5	NA	ND
10	H	H	C-5	NA	ND
11 (wALADin2)	Tricyclic quinoline derivative			NA	NI

[a] NA, no antiplasmodial activity at 62.5 μM.
[b] IC₅₀ concentrations were determined by nonlinear regression analysis of data derived from several independent experiments. NI, no *in vitro* activity against *Pf*ALAD enzymatic activity (highest concentration tested, 533 μM). ND, not determined.

compound 4 has inhibitory activity against its ALAD ortholog (8). Briefly, LLC-MK2 cells were infected with *T. gondii* RH strain tachyzoites (multiplicity of infection [MOI], 5). After 2 h, nonadherent parasites were removed by washing and cells were exposed to wALADin1 or compound 4 for 48 h. After DNA extraction from infected cells, tachyzoite numbers were determined by real-time PCR of the *T. gondii* repeat DNA sequence (GenBank accession no. AF146527.1) normalized to the number of host cells determined by *Macaca mulatta* β-*actin* PCR (GenBank Gene ID 574285) (Fig. 1I; Table 2). wALADin1 inhibited *T. gondii* replication with an IC₅₀ of ~340 μM; compound 4 had an IC₅₀ between 500 and 1,000 μM. This minor anti-*Toxoplasma* activity is likely identical to the weak antiproliferative effect on eukaryotic cells previously reported for LLC-MK2 and HEK cells at concentrations of ≥500 μM (1). Thus, the selectivity window of the antiplasmodial effect of compound 4 (IC₅₀, 7.7 μM) over *T. gondii* and other mammalian cells is ~100-fold, and the antiplasmodial potency of this compound even exceeds the antifilarial potency of wALADin1 by >10-fold (7).

To delineate the activity of wALADins against other malarial parasite life cycle stages (sporozoites and liver stages), we used the rodent parasite *Plasmodium berghei* ANKA. To assess gliding motility, 10,000 infective sporozoites were preincubated with compounds for 15 min on ice. After 30 min of gliding on bovine serum albumin (BSA)-coated glass slides, samples were fixed and gliders and nongliders were differentiated according to the presence or absence of a trail of the major sporozoite surface protein, circumsporozoite protein (CSP), released from the apical end of gliding sporozoites (9). CSP was detected by immunofluorescence mi-

croscopy using a Zeiss Axiovert 100M microscope and Zeiss Image Examiner software. Compound 7 dramatically reduced the gliding motility of *P. berghei* ANKA sporozoites (75% reduction at 200 nM) and completely prevented sporozoites from adhering to glass slides at 40 μM. wALADin1 and compound 4 also prevented sporozoite adhesion at 40 μM but showed no effect on gliding motility at lower concentrations (Fig. 2A). Control compounds 2 and 3 had no effect on sporozoite motility. As gliding motility is required for successful invasion of hepatocytes, we tested whether compound 7 was able to inhibit this important infection process. Sporozoites were preincubated with compound 7 as described above and allowed to invade immortalized human hepatoma cells (Huh7) for 90 min before samples were fixed and stained according to the two-color host cell invasion method (10). Compound 7 blocked sporozoite invasion into hepatoma cells (Fig. 2 B), presumably as a result of suppressed gliding motility. In contrast to invasion into hepatocytes, intrahepatocytic replication was not affected by wALADins at concentrations up to 40 μM (Fig. 2 C and D). alamarBlue cytotoxicity assays with a concentration of 40 μM compound revealed a moderate antiproliferative effect of wALADin1 against Huh7 cells but not of compound 4 or 7, confirming the specificity of the latter compounds. Due to its potent effect on sporozoite gliding motility, we named compound 7 "wALADin-derived inhibitor of *Plasmodium* motility and invasion1 (ALPin1)" (the chemical structure is shown in Fig. 2E). As a small-molecule tool, ALPin1 may help identify unknown components of the actin-myosin motor-dependent gliding machinery (11, 12).

In addition to the specificity of the biological effect, the drug-

TABLE 2 PCR conditions for *T. gondii* repeat DNA and *M. mulatta* β-*actin* PCR[a]

Parameter	*T. gondii* repeat DNA PCR	*M. mulatta* β-*actin* PCR
Forward primer	5′-GATATCAGGACTGTAGATGAAGG-3′ (300 nM)	5′-GATGAGATTGGCTTTA-3′ (300 nM)
Reverse primer	5′-GCGTCGTCTCGTCTAGATC-3′ (300 nM)	5′-AACCGACTGCTGTCACCTTC-3′ (300 nM)
Hybridization probe	5′-6-FAM-AAGCGACGAGAGTCGGAGAGGGAG-3′-BHQ-1 (50 nM, TaqMan)	1× SYBR green dye
Conditions	0.5 U HotStar Taq; 50 μM dNTPs; 4 mM MgCl₂	0.5 U HotStar Taq; 50 μM dNTPs; 3 mM MgCl₂
Cycling protocol	15 min at 95°C followed by 45 cycles of 10 s at 95°C and 30 s at 60°C (fluorescence acquisition)	15 min at 95°C followed by 45 cycles of 15 s at 94°C, 20 s at 58°C, and 20 s at 72°C (fluorescence acquisition)

[a] 6-FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1; dNTPs, deoxynucleoside triphosphates.
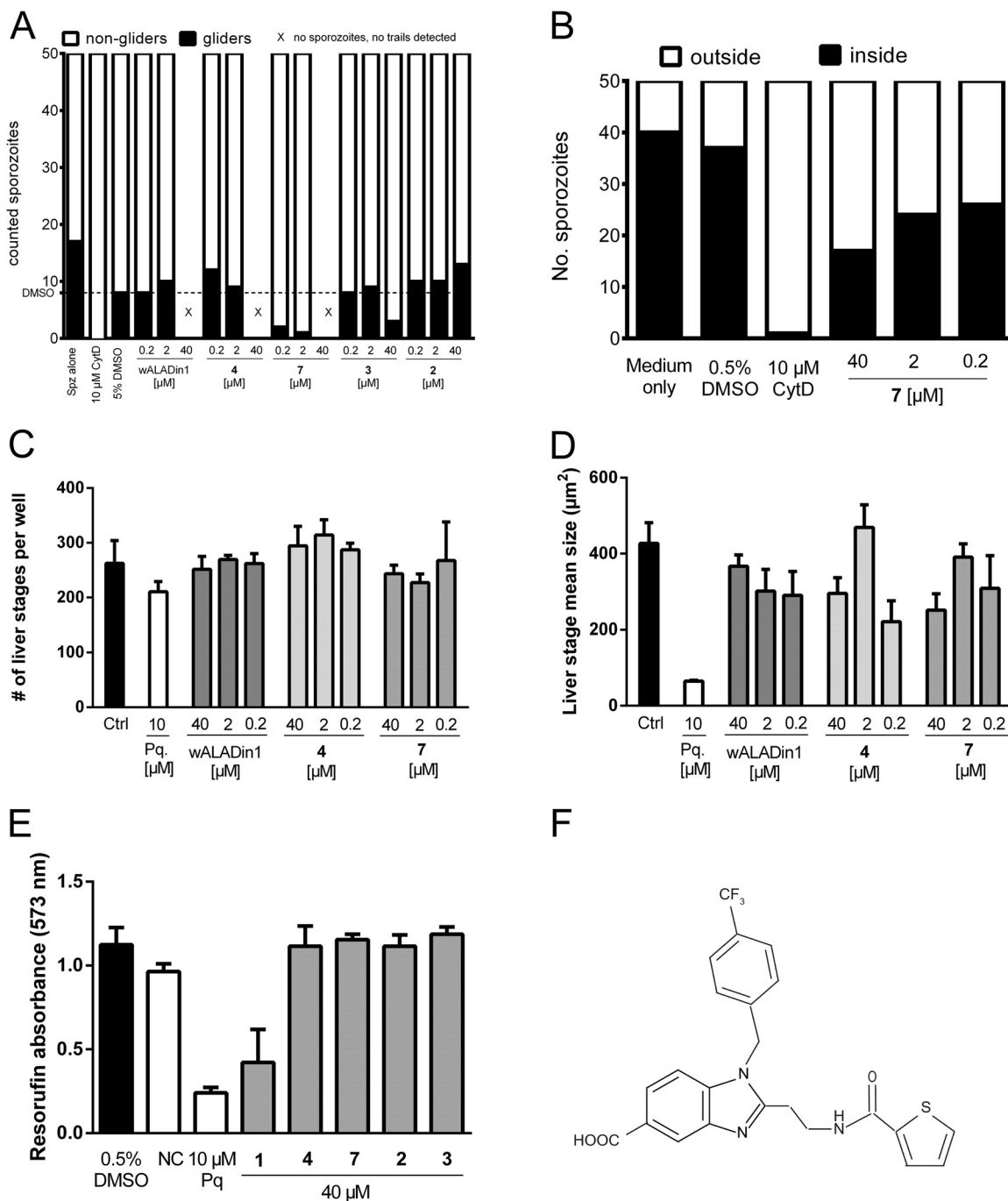
FIG 2 *P. berghei* ANKA sporozoites were extracted from the salivary glands of infected *Anopheles stephensi* mosquitoes on days 19 to 23 postinfection, taken up in RPMI medium supplemented with 3% BSA, and used on the same day. (A) Compound 7 potently inhibited the gliding motility of *P. berghei* ANKA sporozoites. A total of 10,000 sporozoites were added to glass slides precoated with 3% BSA–RPMI medium and allowed to glide for 30 min before fixation with 4% paraformaldehyde–phosphate-buffered saline. Gliding sporozoites were differentiated from adhesive but nongliding sporozoites by a trail of circumsporozoite protein (CSP) detected on the slide by immunofluorescence microsocopy after staining with monoclonal anti-CSP antibody and anti-mouse Alexa Fluor 488 (both at 1:300 in 10% fetal calf serum-PBS) (6). "X" indicates the absence of both sporozoites and CSP trails on the slide. (B) Compound 7 (ALPin1) inhibited the invasion of sporozoites into Huh7 cells. A total of 25,000 cells were seeded onto Lab-Tek 8-well chamber slides 1 day before infection with sporozoites for 90 min (MOI, 0.4). Parasite numbers were determined by fixation and staining using the two-color host cell invasion method. (C and D) For extraerythocytic stage development assays, Huh7 cells were infected with sporozoites for 90 min before free parasites were washed off and compounds were added. Cells were fixed after 24 h, 40 h, or 60 h with ice-cold methanol, followed by staining of liver stage parasites with monoclonal anti-*P. berghei* HSP70 antibody and goat anti-mouse Alexa Fluor 488 secondary antibody (13). None of the tested wALADins had any effect (up to 40 μM) on the number (C) or the size (D) of liver stage parasites. (E) A total of 6,000 Huh7 cells were seeded onto a 96-well plate before incubation with a 40 μM concentration of the indicated compound or with 0.5% DMSO for 72 h at 37°C and 5% CO$_2$ in triplicate experiments. Neither compound 4 nor compound 7 affected Huh7 cell viability or replication as assessed with alamarBlue reagent. The inhibitor of actin polymerization cytochalasin D (CytD) served as a positive-control inhibitor for motility and invasion studies; primaquine (Pq) was used for intrahepatic stages. NC, negative control. (F) Chemical structure of compound 7 (ALPin1).

like chemical properties of wALADin benzimidazoles, their easy synthetic accessibility, and their steep antiplasmodial activity profile (Fig. 1H) render a future detailed assessment of this compound class as antiplasmodial agents attractive. Validation of these compounds as new antimalarial agents will be completed by target identification using cross-linkable chemical probes based on the chemical scaffold of ALPin1 (compound 7) to identify the molecular target.

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