Synergistic blending of high-valued heterocycles inhibits growth of *Plasmodium falciparum* in culture and *P. berghei* infection in mouse model

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A series of phthalimide analogues, novelized with high-valued bioactive scaffolds was synthesized by means of click-chemistry under non-conventional microwave heating and evaluated as noteworthy growth inhibitors of *Plasmodium falciparum* (3D7 and W2) in culture. Analogues 6a, 6h and 6u showed highest activity to inhibit the growth of the parasite with IC50 values in submicromolar range. Structure-activity correlation indicated the necessity of unsubstituted triazoles and leucine linker to obtain maximal growth inhibition of the parasite. Notably, phthalimide 6a and 6u selectively inhibited the ring-stage growth and parasite maturation. On other hand, phthalimide 6h displayed selective schizonticidal activity. Besides, they displayed synergistic interactions with chloroquine and dihydroartemisinin against parasite. Additional *in vivo* experiments using *P. berghei* infected mice showed that administration of 6h and 6u alone, as well as in combination with dihydroartemisinin, substantially reduced the parasite load. The high antimalarial activity of 6h and 6u, coupled with low toxicity advocate their potential role as novel antimalarial agents, either as standalone or combination therapies.

Malaria is a devastating infectious disease in humans, causing ~214 million clinical cases globally with 438,000 deaths per annum1. Severe complications and mortality results primarily from infection with *Plasmodium falciparum*, which predominates in Africa. Over the last 15 years, several initiatives, including insecticide-treated bed nets, insecticide sprays and artemisinin-based combination therapies (ACT) led to reduced lethality of malaria (~4% per year) with a 40% reduction in clinical malaria cases between 2000 and 20152–4. However, significant challenges remain including drug resistance, prolonged duration of infection in the human host5, high cost of anti-malarial drugs and lack of development for novel antimalarial drugs with potent activity6. Current frontline treatments for *P. falciparum* are based on ACT, which involve administration of artemisinin derivatives in combination with effective secondary agents, such as mefloquine, lumefantrine and piperaquine. The emergence of drug-resistance to malaria drugs, including the most reliable artemisinin-based therapies, has become a major global concern for controlling malaria, particularly in several countries of Southeast Asia7–13. The drug resistance coupled with the demand of a newly accepted set of antimalarial target product profiles has prompted the search for new inexpensive and stable antimalarials with novel modes of action that can be implemented for the treatment of all malaria species.

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Phtalimide (Pht) skeleton is an imperative nucleus for various bioactive molecules\textsuperscript{14–17}, starting material for alkaloids, pharmacophores\textsuperscript{18, 19} and antimalarials\textsuperscript{20}. We also recently reported Pht analogues tailored with cyclic amines as moderate inhibitors of \textit{P. falciparum}\textsuperscript{21, 22}. The presence of additional high-valued bioactive heterocycles may intensify the efficacy of the Phts. The broad spectrum pharmacological properties\textsuperscript{23–25} and antimalarial activities\textsuperscript{26–29} of benzimidazole and triazole heterocycles created the interest in the unification of these examined scaffolds into a single molecule. As a part of our ongoing efforts and diverse therapeutic efficiency of these heterocycles, we report here the design of synergistic association of Pht, benzimidazole and flexible triazoles anticipating new analogues as new entry for antimalarial chemotherapy. Click reactions under non-conventional microwave heating created new 31 Pht analogues (6a–6e') and one representative analogue was characterized by single crystal X-ray crystallography. All the listed analogues were screened against chloroquine sensitive (3D7) and resistant (W2) strains of \textit{P. falciparum}. The top three Pht analogues 6a, 6h and 6u were also examined as combination regimens with CQ and DHA. In vivo experiments carried out for 6h and 6u on a murine model of malaria (\textit{P. berghei}) also suggested their candidature as antimalarial agents. In summary, efforts at generating new antimalarial entries based on Phts was achieved through key structural variations that included the addition of benzimidazole and flexible triazoles.

Results and Discussion

Compound Design and Synthesis. We devised a chemical strategy that promoted the valuable fusion of Pht, benzimidazole and triazole. Numerous alterations on triazole scaffold were attempted, including substituted aromatic rings, alkyl chains and hydrophilic substituents to improve the activity profile. Amino acids with aliphatic chains i.e. valine, leucine and isoleucine were used as linkers. A simple and rationally compatible synthetic route was designed (Fig. 1) to build the library of new Pht analogues. Synthesis of the benzimidazole linked Pht, a key synthon began with the coupling between N-phenylenediamine, \textit{o}-phenylenediamine, 2 followed by acetic acid catalyzed cyclization at refluxed temperature to furnish intermediates 3a–c. The alkyne intermediate 5a was prepared by the alkylation of 3a with propargylic bromide (4). Initially, compound 5a showed only 20% yield, however, efforts to standardize the reaction conditions resulted in a 62% yield. Likewise, compounds 5b and 5c were isolated in 47% and 72% yields, respectively.

Subsequently, the alkylated products 5a–c were treated with various azides (click reactions) under microwave irradiations to acquire the final products 6a–6e'. The scope of various azides for synthesis of new Pht analogues is shown in Table S1. All the synthesized Pht analogues were characterized with various spectroscopic techniques (\textit{1}H NMR, \textit{13}C NMR, HRMS and IR spectroscopy, etc.). In addition, single crystals were grown for one representative analogue 6a and subjected to single crystal X-ray diffraction. The molecular diagram of 6a is depicted in Fig. 2.

Compound 6a crystallized in the monoclinic system with a \textit{C2/c} space group. The details of data collection, structure solution and refinement are listed in Table S2.

Biological Studies and Structure-Activity relationships (SARs) Analysis. Antimalarial activity of all the listed Pht analogues was initially assessed on asynchronous cultures of \textit{P. falciparum} 3D7 (Pf3D7) clone using the SYBR Green assay. The most active compounds were those with a mean 50% growth inhibitory concentration (IC\textsubscript{50}) < 5.14 \textmu M, set as reference cut-off IC\textsubscript{50} value based on the Pht (I) reference molecule mean IC\textsubscript{50}. The mean IC\textsubscript{50} values are based on three separate experiments (Table 1). Four new analogues 6a, 6h, 6m and 6u displayed antiplasmodial activity against the Pf3D7 strain with IC\textsubscript{50} < 5.14 \textmu M. In addition, all four compounds inhibited the growth of CQ resistant PfW2 strain with IC\textsubscript{50} concentrations below the reference cut-off IC\textsubscript{50}. 6a IC\textsubscript{50} = 0.7 (±0.01) \textmu M, 6h IC\textsubscript{50} = 3.8 (±0.34) \textmu M and 6u IC\textsubscript{50} = 0.9 (±0.6) \textmu M. Twelve out of the 31 Pht analogues lacked a dose-dependent effect on parasite growth, hence the IC\textsubscript{50} was unattainable (no dose response, NDR). CQ and DHA were tested alongside the Phts as quality assurance and control of the assay, and SYBR Green derived IC\textsubscript{50} values for both standard antimalarials were as recommended by literature\textsuperscript{30}.

**Figure 1.** Synthesis of Pht analogues 6a–e'.
The structure-activity relationship is centred at the various substitutions of triazole ring and amino acids (Tables 1 and 2). Functionalized Phts were synthesized and screened for antimalarial activity against cultured \textit{Pf}3D7 and compared with unsubstituted Pht I (IC$_{50}$ = 5.14 ± 1.67 \(\mu\)M), CQ (IC$_{50}$ = 0.03 ± 0.67 \(\mu\)M) and DHA (IC$_{50}$ = 0.003 ± 0.3 \(\mu\)M). During the design of the new molecules Pht, benzimidazole scaffolds were kept constant and its flanking sides, triazoles were diversified. It is evident from the screening results (Tables 1 and 2) that the variations of R or R’ substituents play an important role in the potency of the compounds against growth of the parasite.

The antimalarial data demonstrates that the introduction of larger R groups (isobutyl or sec-butyl) increases the potency as noticed in case of Phts 6a, 6h and 6u whereas relatively smaller groups lower the activity (i.e. 6m). As shown in Table 1, the insertion of a phenyl substituent also influences the potency of the molecules. Pht analogues possessing 4-fluorophenyl ring on the triazoles moiety with R represented as butyl group (6h) was noticed as more active over the analogues containing substituted phenyl group at triazole moiety. This result, at least in part, appears to be due to the high electronegativity of fluoro group. Analogues with an unsubstituted aromatic ring also exhibited significant inhibition of the parasite growth, but only when R was replaced with an isobutyl group (e.g. 6a). In the absence of a functional group on the triazole ring with R represented as an isobutyl group, we observed the highest potency against the \textit{P. falciparum} 3D7 strain (i.e. 6u).

\textbf{Stage-Specificity and Effects on Parasitemia Titres.} Next, we sought to determine stage-specificity of the antiplasmodial activity of the four active analogues (6a, 6h, 6m and 6u) on synchronized \textit{Pf}3D7 strain cultures at 2% hematocrit and 1% parasitemia, with concentrations corresponding to individual drug IC$_{50}$. To determine the effect of the compounds on both early and late ring stage parasites, the treatment was conducted on newly synchronized rings for 12 hours, and drug effect on parasite growth and morphology was monitored at 6 and 12 hours after exposure. Similarly, early trophozoites were exposed to each compound and incubated for 16 hours, and monitored at 6 and 16 hours post-exposure. The effect of the compounds on parasite morphology and development was compared in exposed and unexposed drug wells (Figs 3 and 4). Compounds 6a and 6u were active against ring-stage forms as indicated by a marked reduction in parasite density and abnormal ring stage morphology at 6 hour and 12 hour post-exposure. However, compounds 6a and 6u did not interfere with development of parasite progression from trophozoites to schizonts when they were exposed to a separate culture of trophozoites. Treatment with 6h and 6m did not arrest ring-stage maturation as indicated by the marked increase in ring-stage growth at 12 hours, like the no treatment group. However, upon examination of both 6h and 6m treatment on mature blood-stages, 6h resulted in complete destruction of trophozoites at 6 hours after drug exposure of early-stage trophozoites. Further, monitoring the effect of 6h at 16 hours showed the presence of schizonts with abnormal morphology.

Although, trophozoite growth did not appear to be affected by treatment with 6m at 6 hours, the resultant schizonts appeared less granular and lacked distinguishable merozoites upon 16 hours exposure. The effect on the analogues on parasitemia counts was correlated with their stage-specificity. Analogues 6a and 6u caused a reduction in ring stage parasitemia at 6 hrs post exposure, while their effect on mature blood stage parasite titres at 16 hours was negligible (Fig. 5). Although, 6h and 6m did not affect parasite growth at 6 hours post exposure (i.e., ring-stage), both analogues caused a reduction in parasitemia at 16 hours (i.e., trophozoite stage, Fig. 5).
Drug-Drug Interaction Assays. We then explored the synergistic drug inhibitory activities between the Pht analogues and CQ or DHA. Synergistic inhibitory activities were observed between three analogues (6a,

| Entry | Compound | R          | R'          | IC50 (SE) µM | IC50 (SE) µg/mL |
|-------|----------|------------|------------|--------------|----------------|
| 1     | 6a       | CH2CH(CH3)2 |            | 0.9 (±0.14)  | 0.7 (±0.03)    |
| 2     | 6b       | CH2CH(CH3)2 | Cl         | NDR          | NDR            |
| 3     | 6c       | CH2CH(CH3)2 | F          | 17.3 (±0.0)  | 8.8 (±0.0)     |
| 4     | 6d       | CH2CH(CH3)2 | H3CO       | NDR          | NDR            |
| 5     | 6e       | CH2CH(CH3)2 |            | 25 (± 1.5)   | 13 (± 0.8)     |
| 6     | 6f       | CH(CH3)CH2CH3|            | NDR          | NDR            |
| 7     | 6g       | CH(CH3)CH2CH3| Cl         | NDR          | NDR            |
| 8     | 6h       | CH(CH3)CH2CH3| F          | 0.9 (±0.0)   | 0.7 (±0.0)     |
| 9     | 6i       | CH(CH3)CH2CH3| H3CO       | 40 (± 6.2)   | 22.5 (± 2.9)   |
| 10    | 6j       | CH(CH3)CH2CH3|            | 73 (± 0.0)   | 33.4 (± 0.0)   |
| 11    | 6k       | CH(CH3)2    |            | 30.86 (±0.0) | 16 (± 0.0)     |
| 12    | 6l       | CH(CH3)2    | Cl         | NDR          | NDR            |
| 13    | 6m       | CH(CH3)2    | F          | 3.5 (± 2.9)  | 1.8 (± 1.3)    |
| 14    | 6n       | CH(CH3)2    | H3CO       | 9.6 (± 0.0)  | 5.5 (±0.0)     |
| 15    | 6o       | CH(CH3)2    |            | 23.5 (±11.9) | 17.5 (±5.7)    |
| 16    |          |            |            | 5.14 (±1.67) | 0.8 (±0.25)    |
| 17    | CQ       |            |            | 0.03 (±0.67) | 0.015 (±0.33)  |
| 18    | DHA      |            |            | 0.003 (±0.3) | 0.0017 (±0.16) |

Table 1. SAR Study of R' Substituent: Aromatic Rings. Note: Illustration of Pht analogues, Pht (I), and chloroquine and dihydroartemisinin IC50 values. IC50 value < 5.14 µM: active and IC50 value > 5.14 µM: inactive.
| Entry | Compound | R         | R’         | IC50 (SE) µM | IC50 (SE) µg/mL |
|-------|----------|-----------|------------|--------------|----------------|
| 1     | 6p       | CH₂CH(CH₃)₂ | bond      | 62 (±2.5)    | 31 (±1.2)      |
| 2     | 6q       | CH₂CH(CH₃)₂ | bond      | 8.4 (±0.5)   | 4.1 (±0.3)     |
| 3     | 6r       | CH₂CH(CH₃)₂ | bond      | 7 (±0.7)     | 3.5 (±0.34)    |
| 4     | 6s       | CH₂CH(CH₃)₂ | bond      | No IC50      | No IC50        |
| 5     | 6t       | CH₂CH(CH₃)₂ | bond      | 28 (±0.7)    | 14 (±0.3)      |
| 6     | 6u       | CH₂CH(CH₃)₂ | H         | 0.7 (±0.0)   | 0.3 (±0.0)     |
| 7     | 6v       | CH(CH₃)CH₂CH₁ | bond      | No IC50      | No IC50        |
| 8     | 6w       | CH(CH₃)CH₂CH₁ | bond      | 15.6 (±0.0)  | 11.6 (±0.0)    |
| 9     | 6x       | CH(CH₃)CH₂CH₁ | bond      | No IC50      | No IC50        |
| 10    | 6y       | CH(CH₃)CH₂CH₁ | bond      | No IC50      | No IC50        |
| 11    | 6z       | CH(CH₃)CH₂CH₁ | H         | No IC50      | No IC50        |
| 12    | 6a’      | CH(CH₃)₂ | bond      | No IC50      | No IC50        |
| 13    | 6b’      | CH(CH₃)₂ | bond      | 7.2 (±0.0)   | 3.8 (±0.0)     |
| 14    | 6c’      | CH(CH₃)₂ | bond      | No IC50      | No IC50        |
| 15    | 6d’      | CH(CH₃)₂ | bond      | 48.3 (±0.6)  | 20 (±0.29)     |
| 16    | 6e’      | CH(CH₃)₂ | bond      | 16.7 (±2.1)  | 6.9 (±10.1)    |

Table 2. SAR of R’ Substituent Antimalarial Activity: Aliphatic and Glycoside Groups.
6h and 6u in combination with both CQ and DHA against the 3D7 and W2 strains (Table S3). The determination of drug interactions between the analogues with CQ and DHA is necessary for identifying possible partner drugs to combat resistance to current antimalarial therapies. Recent reports on the emergence of CQ sensitive \(P. falciparum\) strains achieved in some malaria endemic regions is attributed to replacement with sulfadoxine-pyrimethamine (SP) and ACTs\(^{31,32}\). CQ was an ideal
Figure 5. Effect of Pht analogues on parasite growth titres (Note: Graphical description of the inhibitory effect of select analogues on parasite growth titres 6 hours after incubation with ring-stages and 16 hours after drug incubation with trophozoite stage. Parasitemia percentage was derived by counting the number of infected erythrocytes from a total of 2,000 erythrocytes on Giemsa stained thin smears from each experiment. Bar diagrams represent the average of three different experiments).

Figure 6. Antimalarial effect of Pht analogs (6h and 6u) alone and in combination with Artemisinin (6u and ART) on parasitemia and survival in mice infected with P. berghei NK65. Mice were injected with 1 × 10⁷ P. berghei infected RBCs. After 48 hours mice were treated with control (PBS: untreated), 6h and 6u (alone), and 6u in combination with artemisinin (all injected subcutaneously) for four consecutive days. The % of parasitemia was determined for the groups on days 5, 8 and 15 by randomly selecting 10 different optical fields on blood smears. (A) Administration of 6h and 6u (alone), and 6u in combination with ART. Data are the mean ± SEM from six animals per treatment group. (B) Survival in the treatment groups (n = 6/group). (C) Photomicrograph of blood smears of untreated versus treatment groups at day 15 post infection (100x magnification). ART, Artemisinin; *P < 0.05.
antimalarial due to its pharmacokinetics, safety profile and low cost. Since \textit{P. falciparum} resistance developed primarily because of administration as a monotherapy, identifying potential CQ partner drugs can benefit in reducing development of historical parasite resistance to CQ monotherapy. Artemisinin (ART) and its derivatives are the current front line of defense against uncomplicated \textit{P. falciparum} malaria and are administered as ACTs. Emergence of resistance to both ACT drug components warrants identification of ART replacement and possible combination chemotypes\textsuperscript{33–35}. Notably, three Pht analogues (6a, 6h and 6u) showed synergistic activity when combined with CQ and DHA against both \textit{Pf}3D7 and \textit{Pf}W2, suggesting their potential for use in combination therapies.

**Antimalarial Effect of Pht Analogs Alone and in Combination with Artemisinin in Plasmodium berghei Infected Mice.** The antimalarial effect of two active analogues, 6h and 6u (administered at 50 mg/kg of body weight), was determined in mice infected with \textit{P. berghei} NK65, a strain, which results in high levels of blood-stage parasitemia. Administration of either 6h or 6u alone for four consecutive days caused suppression of the parasite load on days 5 and 8 of infection and improved survival as compared to untreated (Fig. 6A and B). However, the 6u analogue had better antimalarial efficacy than the 6h analogue (Fig. 6A). As such, we then evaluated the efficacy of 6u (50 mg/kg) in combination with artemisinin (5 mg/kg of body weight) at reduced dosage in the murine malaria model. As shown in Fig. 6, neither of the compounds delivered as monotherapy conferred clearance of parasitemia, however, co-administration of the 6u analogue with ART considerably enhanced the antimalarial efficacy by reducing the parasite load and extending survival (\textit{P} < 0.05, Fig. 6A and B). The median survival times of animals treated with Pht 6u alone, as well in combination with ART were 23 and 27.5 days, respectively (\textit{P}<0.001). These results demonstrate the compound 6u in combination with ART has the greatest therapeutic efficacy in the murine model of malaria.

**Cytotoxicity Evaluation.** As a final step in the investigative pipeline, we determined the cytotoxic effect of the four active analogues (6a, 6h, 6m and 6u) in U937 cell lines by measuring 50% cytotoxic concentrations (CC\textsubscript{50}) and calculating selectivity indices (SIs) as a measure of toxicity in human cells.

Compounds 6a and 6m displayed potent CC\textsubscript{50} values <1 \textmu M of 0.91 ± 0.32 \textmu M and 0.78 ± 0.32 \textmu M, respectively, and were considered to possess higher selectivity for \textit{U937} cell lines vs. \textit{P. falciparum} (SI values of 1.01 ± 1.5 \textmu M and 1.11 ± 1.5 \textmu M, respectively). Analogues 6h and 6u possessed less selectivity for \textit{U937} cell lines with CC\textsubscript{50} values of 28.82 ± 0.67 \textmu M and 2.08 ± 1.6 \textmu M, respectively, and SI values of 41.2 ± 1.7 \textmu M and 2.31 ± 0.76 \textmu M, respectively, and were, therefore, considered less toxic to human cells. As such, additional chemical modifications of 6a and 6m may be required to produce analogues with less selectivity and toxicity for human cell lines in the context of retaining their antiplasmodial activity.

**Conclusion**

In summary, we present simple and inexpensive chemical procedures with readily available starting materials considering the need for low-cost, novel antimalarial agents for use in malaria endemic areas. Results presented here investigated \textit{in vitro} and \textit{in vivo} antimalarial activity of novel Pht analogues blended with benzimidazole and triazoles, which have been synthesized by means of click reactions under microwave conditions. Amongst all active members, eight analogues displayed growth inhibition of \textit{P. falciparum} in culture. The synergy of the lead compounds 6h and 6u with standard antimalarial drugs such as ART, demonstrate their suitability as combination regimens. Future studies will focus on lead optimization, pharmacokinetics and parasite target site(s) to advance Pht analogues as potential antimalarial candidates for clinical use.

**Methods**

**Chemistry.** Solvents and reagents were purchased from commercial sources and used without purification for the experiments. Homogeneity/purity of all the products was assayed by thin-layer chromatography (TLC) on alumina-coated plates (Merck). Product samples in chloroform (CH\textsubscript{3}Cl) were loaded on TLC plates and developed in Ethyl acetate/Petroleum ether (1:1, v/v). When slight impurities were detected by iodine vapour/UV light visualization, compounds were further purified by chromatography on silica gel columns (100–200 mesh size, CDH). Reactions using microwave were run in a closed vial applying a dedicated CEM-Discover monomode microwave apparatus operating at a frequency of 2.45 GHz with continuous irradiation power from 0–300 W (CEM Corporation, P.O. Box 200, Matthews, NC 28106). Melting points were determined on Melting point machine M-560 (Buchi). Infrared (IR) spectra were recorded in KBr medium using a Perkin-Elmer Fourier Transform-IR spectrometer, whereas \textsuperscript{1}H and \textsuperscript{13}C nuclear magnetic resonance (NMR) spectra were recorded in CDCl\textsubscript{3} DMSO and D\textsubscript{2}O medium on a JEOL ECX-400P NMR at 400 MHz and 100 MHz, respectively at USIC, University of Delhi. Compounds 1a–1c were prepared following literature procedures\textsuperscript{36}. General Procedure for Synthesis of Compounds 3a–3c. In first step, respective compounds 1a–1c (38 mmol) were dissolved in 250 mL of DMF and DIPEA (45 mmol) was added drop-wise at 0–5°C. After 10 minutes the essential amount of TBTU (45 mmol) was added slowly and the reaction contents were stirred for 30 minutes at the same temperature. Thereafter, o-phenylenediamine (38 mmol) was added and the resulting mixture was stirred at 0–5 °C for 6 hours. After completion of the reaction as confirmed by TLC, the reaction mixture was quenched with ice cold water, which resulted in precipitate formation. The precipitate was filtered off, washed...
with excess of ice cold water and dissolved in appropriate amount of ethyl acetate. The resulting organic phase was washed with 1 N HCl followed by saturated solution of NaHCO₃ and at last with water. The separated ethyl acetate layer was dried over Na₂SO₄ and concentrated under reduced pressure to acquire the crude product. Next, the crude product was dissolved in 150 mL glacial acetic acid and the resulting suspension was refluxed for 6 hours. After completion of the reaction as confirmed by TLC, the reaction mixture was cooled to room temperature, concentrated under reduced pressure and diluted with ice cooled water. The resulting solid was filtered off and washed thoroughly with ice cold water and saturated NaHCO₃ solution to obtain the desired products. The products were purified by silica gel column chromatography eluting with 20% mixture of ethyl acetate in n-hexane and the final compounds 3a–3c were isolated.

**General Procedure for Synthesis of Compounds 5a–5c.** In a RB flask, respective compounds 3a–3c (15 mmol), Cs₂CO₃ (45 mmol) and appropriate amount of DMF were mixed and the contents were heated at 100 °C for 20 minutes and subsequently, propargyl bromide (4) (22 mmol) was added drop wise. This resulted in a turbid reaction mixture, which was stirred at 100 °C for next 8 hours. After completion of the reaction as indicated by TLC, the reaction mixture was cooled to attain room temperature and concentrated under vacuo to give residue. Thereafter, ice cooled water was added to the residue and the resulting precipitate was filtered and dried. The residue was purified by silica gel column chromatography eluting with 10% mixture of ethyl acetate in n-hexane to afford the titled compounds 5a–5c.

**General Synthetic Procedure of New Analogues 6a–6e.** A microwave vial was charged with respective compounds 5a–5c (1.34 mmol) and azide (2.02 mmol) and THF:H₂O (4:1, v/v, 5 mL). The required amount of CuSO₄.5H₂O (0.27 mmol) and sodium ascorbate (0.54 mmol) was added and the vial was sealed tightly, which was heated at 70 °C under microwave irradiation of 70 W for 20 minutes. The progress of the reaction was confirmed by TLC. After completion of the reaction, the reaction mixture was transferred into a RF flask and concentrated under vacuo to give a residue that was quenched with ammonia solution and filtered off. Thus, obtained residue was purified by silica gel column chromatography eluting with appropriate mixture (~25%) of ethyl acetate in petroleum ether to afford the titled compounds 6a–6e. The spectral data of all new compounds is described in supporting information.

**Biological Evaluation.** In vitro cultivation of P. falciparum. The laboratory-adapted P. falciparum strains 3D7 (Africa; CQ sensitive) and W2 (Africa; CQ resistant), were acquired from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA, USA) and maintained with type O +ve erythrocytes suspended in continuous complete culture medium as described. Complete culture media consisted of 10.43 g/litre of RPMI 1640-HEPES supplemented with 10% (vol/vol) human AB serum, 92.6 mg/litre L-glutamine, 50 mg/liter hypoxanthine, 2 g/litre sodium bicarbonate (Sigma-Aldrich, St. Louis, MO). Incubation of cultures was at 37 °C and maintained in a low oxygen atmosphere (5% O₂, 5% CO₂, 90% N). The levels of parasitemia in the cultures were maintained at between 2 and 10%, with 5% hematocrit. Media was changed after every 24 hours and parasitemia monitored every 48 hours. Synchronous cultures were prepared by sorbitol lysis. Stock solutions of the Pht, Pht analogues, and DHA were prepared in absolute dimethyl sulfoxide (DMSO) while CQ was prepared in distilled water. The prepared drug stock solutions were used immediately or stored at −80 °C for not longer than one month before use. Stock solutions were further diluted in serum free RPMI 1640 media (stock solution solvent final conc. 0.05%) before performing a 2-fold serial dilution to achieve dose ranges of 0.2 µM to 100 µM (Pht and derivatives), 7.8 to 2,000 nM (CQ) and 0.17 to 87.5 nM (DHA). 25 µL of the drug diluents were aliquoted into 96 well plates and used immediately. Alternatively, the stock solutions were diluted in distilled water, 2-fold serial dilution performed on a 96 well plate and allowed to air dry in a laminar flow hood after which dried plates were stored at 4 °C until use. SYBR Green I assay technique with additional modification was used for drug susceptibility testing of the parasites. Parasite cultures of >1% parasitemia were diluted to 1% parasitemia and 2% hematocrit and 200 µL were transferred onto drug pre-dosed plates and incubated at 37 °C for 90 hours. Drug exposure was terminated by freezing the drug plates at −80 °C for 24 hours after which lysis buffer containing (per liter) 100 mM Tris-HCl, 10 mM EDTA, 0.016% Saponin, 1.6% triton X–100 and 20X SYBR Green I dye was added and the sample incubated for 2 hours in the dark. Relative fluorescence units (RFU) were read using the Perkin Elmer Wallac 1420 fluorescence plate reader, with excitation and emission wavelengths of 485 nm and 535 nm respectively. The readouts values were aligned with corresponding drug doses and analysis performed by Graph-pad Prism software. The drug concentrations (x value) were transformed using X = Log[X] and plotted against the counts (y values) and the data analysed by non-linear regression (sigmoid dose-response/variable slope equation) to yield the IC₅₀.

**Drug interaction combination assays and analysis.** A modified fixed-ratio isobologram method was used to assess interaction between CQ/DHA and Pht analogues. Briefly, a total of 5 solutions containing fixed-ratio mixtures of Pht derivatives with either CQ or DHA were prepared in the following ratios: 1:1, 2:1, 3:2, 2:3, and 1:4. The starting concentrations for ten 2 fold serial dilutions across the microtiter plate were assigned so that the IC₅₀ of each drug would be in the 5th serial dilution of the plate. Each drug was tested alone and at fixed ratios of its IC₅₀. The assessment of drug interaction was based on the calculation of the fractional inhibitory concentration (FIC) of two drugs in the 4 combination ratios. FIC was calculated for each association by dividing the IC₅₀ of the drug in the combination by the IC₅₀ of the drug alone. The sum of these two FIC (ΣFIC); (ΣFIC = 1) indicates an additive effect between drug A and drug B, (ΣFIC < 1), suggesting a synergistic effect and (ΣFIC > 1) indicates antagonism.
Measurement of cytotoxic activity on U937 cells. Cytotoxicity of the 4 select Pht compounds on human cells was evaluated by assessing cell viability of the U937, a human acute monocytic leukemia cell line by use of the MTT assay. U937 was acquired from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and Penicillin-streptomycin (1% v/v) (Gibco, UK) at 37 °C. Robust U937 cells at a concentration of 80,000 cells/ml were plated into 96-well plates and incubated for 24 hours. Six concentrations of the Pht compounds were added in a two-fold dilution from starting concentration of 50 to 1.56µM in triplicate and incubated with the cells for 24 hours. This was followed by addition of 10 µL MTT solution (5mg/mL) into each well, incubated for 4 hour at 37 °C followed by addition of 50 µL DMSO to dissolve the formazan precipitate according to manufacturer’s protocol. Aliquots were drawn from each well and color intensity was measured spectrophotometrically in an ELISA plate reader (Biotek, ELx800) at 540 nm. The cell viability ratio was calculated by the following formula: % cytotoxicity = [Mean OD of test cells] – [Mean OD of control cells]/[Mean OD of control cells] × 100. Viability counts were then plotted against corresponding drug concentrations to yield cytotoxicity (CC50) through non-linear regression (sigmoid dose-response/variable slope equation). Drug CC50 values were then used to define selectivity indices, which is a measure of drug safety in human relative to parasitic cells and was calculated as the ratio of the CC50 value determined on the U937 cells (cytotoxicity) and the IC50 value determined on parasite growth inhibition 3D7 (anti-plasmodial activity). In this study, we set SI > 2 as cut-off as an indicative of low drug cytotoxicity.

In vivo experiments. All animal experiments were performed in female Swiss albino mice (4 to 5 weeks old, weighing 25 to 30 g). The animals were housed under standard controlled conditions at 25 °C with a 12-h light-dark cycle and access to sterilized food pellets and water. All experiments were carried out in accordance with the standard procedures approved by the Animal Ethics Committee of the University of Delhi South Campus, under the Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. To examine the therapeutic efficacy of most potent Pht analogues 6h and 6u, a murine model of malaria was developed by intraperitoneal i.p. administration of standard inoculum of rodent strain of Plasmodium berghei NK65 carrying 1 × 107 parasitized erythrocytes per 200 µL volume to each experimental Swiss albino mouse. The antimalarial activity was carried out in accordance with the slightly modified version of the Peter’s 4-day suppressive test. The animals were assigned to each group (n = 6). Subsequently, after 48 hour of postinfection, the parasitemia level reached 1 to 2%, and all the groups of mice were treated by subcutaneous (s.c.) injection with compounds 6h and 6u alone as well in combination with artesinin solubilized in DMSO. One group was kept as a control and treated with physiological saline. The efficacy of the treatment was monitored by measuring the parasitemia and survival on days 5, 8 and 15 posttreatments by obtaining thin smears of blood withdrawn from the tail vein of infected mice and staining with 10% Giemsa. The level of parasitemia was determined by counting infected and noninfected erythrocytes from 10 to 15 randomly selected optical fields at 100x magnification and expressed as the number of infected erythrocytes per 100 erythrocytes. The survival of mice was recorded and observed for external symptoms, such as change in body weight, ruffled fur, lethargy and paralysis, until 30 or 40 days posttreatment. The reduction in the level of parasitemia was taken as the index for the curative activities of the drugs. The percentage of parasitemia was calculated manually with the Cell Counting Aid software using the formula (total no. of parasitized RBCs)/ (total no. of RBCs) × 100.

Statistical analysis. For in-vivo experiments, statistical differences between two groups were determined by Student’s t test and between multiple groups using one-way analysis of variance (ANOVA), with P values of <0.05, by GraphPad Prism (version 5.01; GraphPad Software, Inc., CA). The survival of the mice was followed up to day 30 or 40 postinfection using Kaplan–Meier survival analysis, and statistical differences in animal survival were analyzed by a log rank test.

References
1. World Malaria Report 2015; World Health Organization (2015).
2. Farnery, E. L., Chatterjee, A. K. & Winzeler, E. A. Antimalarial drug discovery [mdash] approaches and progress towards new medicines. Nat Rev Microbiol. 11, 849 (2013).
3. Bhatt, S. et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature 526, 207–211 (2015).
4. Galatas, B., Bassat, Q. & Mayor, A. Malaria Parasites in the Asymptomatic: Looking for the Hay in the Haystack. Trends Parasitol. 32, 296 (2016).
5. Bretscher, M. T. et al. The distribution of Plasmodium falciparum infection durations. Epidemics 3, 109–118 (2011).
6. Gamo, F. J. et al. Thousands of chemical starting points for antimalarial lead identification. Nature 465, 305–310 (2010).
7. Hanboonkunupakarn, B. & White, N. J. The threat of antimalarial drug resistance. Tropical Diseases, Travel Medicine and Vaccines. 2, 1–5 (2016).
8. Corey, V. C. et al. A broad analysis of resistance development in the malaria parasite. Nature Commun. 15, 11901 (2016).
9. Dondorp, A. M. et al. Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 361, 455–467 (2009).
10. Tun, K. M. et al. Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect. Dis. 15, 415–421 (2015).
11. Phyo, A. P. et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet 379, 1960–1966 (2012).
12. Takala-Harrison, S. et al. Independent Emergence of Artemisinin Resistance Mutations Among Plasmodium falciparum in Southeast Asia. J. Infect. Dis. 211, 670–679 (2015).
13. Wells, T. N., Van Huisjduijnen, R. H. & Van Voorhis, W. C. Malaria medicines: a glass half full? Nat Rev Drug Discov. 14, 424–442 (2015).
14. Vennerstrom, J. L. et al. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430, 900–904 (2004).
15. Shihiedo, H. et al. A phthalimide derivative that inhibits centrosomal clustering is effective on multiple myeloma. PLoS ONE 7, e38878 (2009).

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16. Coelho, L. C. D. et al. Novel phthalimide derivatives with TNF-α and IL-1β expression inhibitory and apoptotic inducing properties. Med. Chem. Commun. 5, 758–765 (2014).
17. Sharma, U., Kumar, P., Kumar, N. & Singh, B. Recent advances in the chemistry of phthalimide analogues and their therapeutic potential. Mini Rev. Med. Chem. 10, 678–704 (2010).
18. Horvat, M. et al. Evaluation of antiproliferative effect of N-(alkyladamantyl)phthalimides in vitro. Chem. Biol. Drug. Des. 79, 497–506 (2012).
19. Papp, K. et al. Efficacy of aminopropyl in the treatment of moderate to severe psoriasis: a randomised controlled trial. Lancet 380, 738–46 (2012).
20. González, M. A., Clark, J., Connelly, M. & Rivas, F. Antimalarial activity of abietane furruginol analogues possessing a phthalimide group. Bioorg. Med. Chem. Lett. 24, 5234–5237 (2014).
21. Singh, A. K. et al. Design, synthesis and biological evaluation of functionalized phthalimides: a new class of antimalarials and inhibitors of falcipain-2, a major hemoglobinase of malaria parasite. Bioorg. Med. Chem. 23, 1817–1827 (2015).
22. Singh, A. K. et al. Hydroxethylamine Based Phthalimides as New Class of Plasmspin Hits: Design, Synthesis and Antimalarial Evaluation. PLoS ONE 10, e0139347 (2015).
23. Camacho, J. et al. Synthesis and biological evaluation of benzimidazole-5-carboxylic acid derivatives as antimalarial, cytotoxic and antitubercular agents. Bioorg. Med. Chem. 19, 2023–2029 (2011).
24. Ndukula, A. J. et al. Antimalarial Pyrido[1,2-β]benzimidazoles. J. Med. Chem. 54, 4581–4589 (2011).
25. Patil, V. et al. Antimalarial and antileishmanial activities of histone deacetylase inhibitors with triazole-linked cap group. Bioorg. Med. Chem. 18, 415–425 (2010).
26. Lentz, C. S. et al. In Vitro Activity of WALADin Benzimidazoles against Different Life Cycle Stages of Plasmodium Parasites. Antimicrob. Agents Chemother. 59, 654–663 (2015).
27. Magistraldo, P. A. et al. Plasmodium falciparum Cyclic Amine Resistance Locus (PfCARL), a Resistance Mechanism for Two Distinct Compound Classes. ACS Infect. Dis. 2, 816–826 (2016).
28. Devender, N. et al. Identification of 3-Amino alcohol grafted 1,4,5 trisubstituted 1,2,3-triazoles as potent antimalarial agents. Eur. J. Med. Chem. 109, 187–198 (2016).
29. Adimulam, C. S. et al. Design, Synthesis and Biological Evaluation of Novel Fluorinated Heterocyclic Hybrid Molecules Based on Triazole & Quinoxaline Scaffolds Lead to Highly Potent Antimalarials and Antibacterials. Lett. Drug Des. Discov. 12, 393–407 (2015).
30. Traore, K. K. et al. Drying anti-malarial drugs in vitro tests to outsource SYBR green assays. Malaria J. 14, 90 (2015).
31. Mekonnen, S. K. et al. Return of chloroquine-sensitive Plasmodium falciparum parasites and emergence of chloroquine-resistant Plasmodium vivax in Ethiopia. Malar. J. 13, 244 (2014).
32. Kiarie, W. C., Wangai, L., Agola, E., Kimani, F. T. & Hungu, C. Chloroquine sensitivity: diminished prevalence of chloroquine-resistant gene marker pfcrt-76 13 years after cessation of chloroquine use in Msambweni, Kenya. Malar. J. 14, 328 (2015).
33. Dondorp, A. M. et al. Artemisinin resistance: current status and scenarios for containment. Nat. Rev. Microbiol. 8, 272–280 (2010).
34. Yeung, S., Socheat, D., Moorhy, V. S. & Mills, A. J. Artemisinin resistance on the Thai-Cambodian border. Lancet 374, 1418–1419 (2009).
35. Leang, R. et al. Efficacy of Dihydroartemisinin-Piperaquine for Treatment of Uncomplicated Plasmodium falciparum and Plasmodium vivax in Cambodia, 2008 to 2010. Antimicrob Agents Chemother. 57, 818–826 (2013).
36. Furniss, B. S., Hannaford, A. J., Smith, P. W. G., Tatchell, A. R. Vogel's Textbook of Practical Organic Chemistry, 5th ed.; Longman Scientific and Technical: London (1989).
37. Trager, W. & Jensen, J. B. Continuous culture of Plasmodium falciparum: its impact on malaria research. Int. J. Parasitol. 27, 989–1006 (1997).
38. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418–20 (1979).
39. Smilkstein, M., Srivilaijaroen, N., Kelly, J. X., Wilairat, P. & Rissoe, M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob. Agents Chemother. 48, 1803–1806 (2004).
40. Johnson, J. D. et al. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrob. Agents Chemother. 51, 1926–1933 (2007).
41. Fiveland, Q. L., Adagu, I. S. & Warhurst, D. C. Modified Fixed-Ratio Isobologram Method for Studying In Vitro Interactions between Atovaquone and Proguanil or Dihydroartemisinin against Drug-Resistant Strains of Plasmodium falciparum. Antimicrob. Agents Chemother. 48, 4097–4102 (2004).
42. Loosdrecht, A. A., Beelen, R. H. J., Ossenkoppel, G. J., Broekhoven, M. G. & Langenhuijsen, M. M. A. C. A tetrazolium-based colorimetric MTT assay to quantify human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J. Immunol. Methods 174, 311–320 (1994).
43. Mcgimpsey, M., Papp, K., Schubert, M. & Perlman, P. The four-day suppressive in vivo antimalarial test. Annu. Trop. Med. Parasitol. 69, 155–171 (1995).
44. Ma, C., Harrison, P., Wang, L. & Cuppel, R. L. Automated estimation of parasitemia of Plasmodium yoelii-infected mice by digital image analysis of Giemsa-stained thin blood smears. Malar. J. 9, 348 (2010).

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