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# Synthesis and in vitro evaluation of hydrazinyl phthalazines against malaria parasite, *Plasmodium falciparum*

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ABSTRACT

In this report, we describe the synthesis of 1-(Phthalazin-4-yl)-hydrazine using bronsted acidic ionic liquids and demonstrate their ability to inhibit asexual stage development of human malaria parasite, *Plasmodium falciparum*. Through computational studies, we short-listed chemical scaffolds with potential binding affinity to an essential parasite protein, dihydroorotate dehydrogenase (DHODH). Further, these compounds were synthesized in the lab and tested against *P. falciparum*. Several compounds from our library showed inhibitory activity at low micro-molar concentrations with minimal cytotoxic effects. These results indicate the potential of hydralazine derivatives as reference scaffolds to develop novel antimalarials.



Malaria remains a major health burden to the developing world, with an estimated death toll of half a million people every year.[1] Despite concerted efforts, a malaria vaccine is not yet available. Therefore, chemotherapy remains the primary mode of treatment.[2–5] However, rapidly increasing resistance against conventional drugs such as chloroquine, artemisinin, mefloquine and sulfadoxine/pyrimethamine is contributing to the decline in prognosis and survival rate from malaria.[6,7]

Recent research highlights the significance of plasmodial dihydroorotate dehydrogenase (DHODH), a critical enzyme involved in pyrimidine biosynthesis, as a therapeutic target.[8] Generally, pyrimidine biosynthesis occurs either de novo or by salvage pathway. In contrast, *Plasmodium* species avail pyrimidines solely through de novo synthesis which involves DHODH activity.[9] Thus, we set out to design and analyze potent small molecules that can manipulate DHODH function and thereby survival and replication of the parasites within human red blood cells.

In search of better antimalarials, various heterocycles with structural similarity with quinolines have been studied extensively including phthalazine derivatives. Several studies have demonstrated the antimalarial potential of various phthalazine analogues.[10–14] Herein, we designed phthalazine based small molecules, which are isomeric with quinazoline ring and tested them for potential antimalarial activity. Piperidinyl phthalazine derivatives have shown antimalarial properties in prior studies, which reflect the possible use of these nuclei as antimalarials. Thus, in continuation of our research to synthesize and explore the pharmacological properties of various heterocycles[15–24], we designed and evaluated novel phthalazines against malaria parasites. Our results revealed that, some of the compounds exhibited inhibition of parasite development at concentrations comparable to previously tested DHODH inhibitors.

*General synthesis of phthalazine derivatives:* Initially, we synthesized 1-(Phthalazin-4-yl) hydrazine (**4**) by a two-step simple and efficient synthetic route shown in Scheme 1A. The phthalazine

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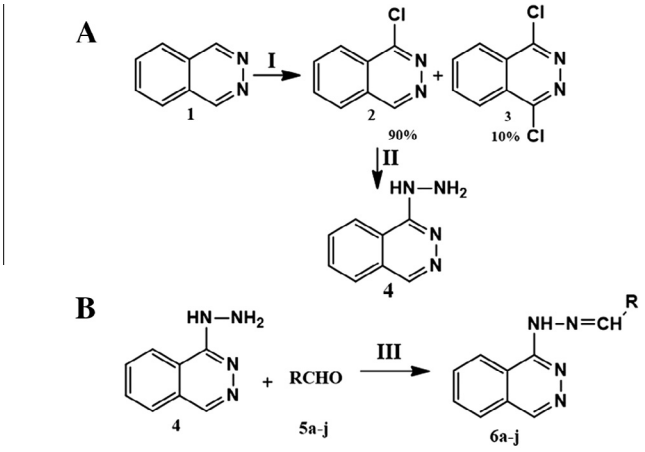



I. DMF, triisochlorocynuricacid, 40-50°C, 4h; II. Ethanol, hydrazine hydrate99%, reflux, 5h; III. 1,2,3-Trimethylimidazoliummethylsulphate, 50°C, 1h.

**Scheme 1.** (A and B) Schematic representation for the synthesis of the novel hydralazine reported here.

**Table 1**
Physical data of the newly prepared hydrazinyl phthalazines

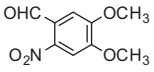
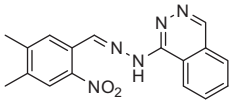
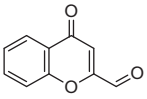
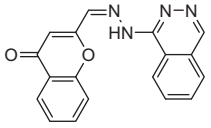
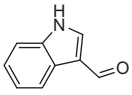
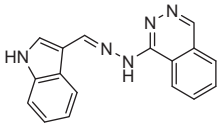
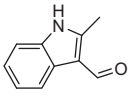
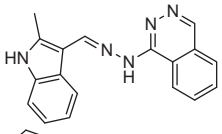
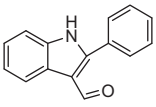
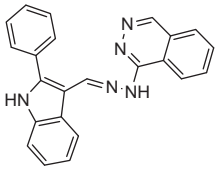
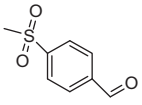
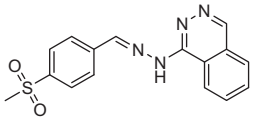
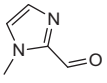
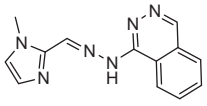
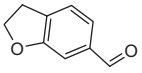
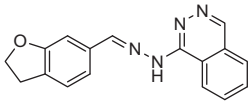
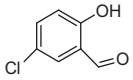
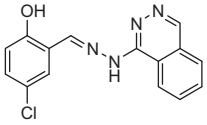
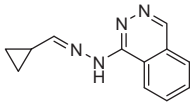
| Entry | Aldehyde (**5a–l**) | Product | Yield |
|---|---|---|---|
| **6a** |  |  | 87[a] |
| **6b** |  |  | 86[a] |
| **6c** |  |  | 91[b] |
| **6d** |  |  | 90[b] |
| **6e** |  |  | 88[b] |
| **6f** |  |  | 81[a] |
| **6g** |  |  | 88[b] |
| **6h** |  |  | 83[a] |

**Table 1** (*continued*)

| Entry | Aldehyde (**5a**–**l**) | Product | Yield |
|---|---|---|---|
| **6i** |  |  | 91[a] |
| **6j** |  |  | 77[a] |

[a] Novel compounds.
[b] Literature reported compounds.

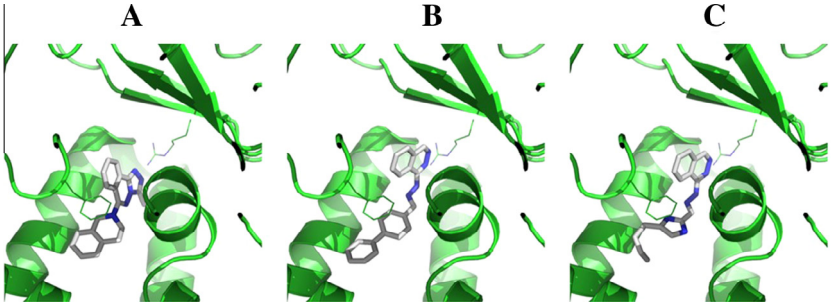



**Figure 1.** Predicted protein-ligand interactions of DHODH and hydralazine-derived compounds: A cut through the binding site of DHODH is shown as green cartoon (PDB: 4CQ9), interaction centers Arg-265 and Phe-227 are highlighted as lines. (A) Co-crystallized ligand IDI-6253 is shown as sticks in atomic coloring (white: carbon, blue: nitrogen). (B) The predicted docking pose of compound **6b** shows high similarity in orientation and anchor points in the binding pocket.

(**1**) was selectively chlorinated by trichloroisocyanuric acid at 40 °C–50 °C in DMF to get 1-chloro phthalazine (**2**) as product and 1,4-dichlorophthalazine (**3**) as byproduct. The 1-chlorophthalazine was allowed to react with excess of 99% hydrazine hydrate in ethanol to get the required product (**4**) and it was recrystallized in ethanol.

The use of ionic liquids as a green solvent in organic transformations has gained significant attention due to their wide liquid range, essentially limited vapor pressure and good solvating ability which contributes to the replacement of organic solvents.[25,26] In the second step, we condensed compound **4** with various substituted aldehydes to prepare Schiff base derivatives of 1-(Phthalazin-4-yl)-hydrazine. The using of bronsted acids ($H_2SO_4$, HCl, PPA, AcOH) as dehydrating agents resulted in the formation of impure products with poor yield. Therefore, we used bronsted acidic ionic liquids (1,2,3-trimethylimidazoliummethylsulphate) as a dehydrating agent in the new approach to get desired product with high purity and good yield (Table 1). The reaction is outlined in Scheme 1B. The obtained Schiff base derivatives of 1-(Phthalazin-4-yl)-hydrazine (**6a**–**j**) of hydralazine were characterized by LC–MS, $^1$H NMR, $^{13}$C NMR, IR and elemental analysis and details are provided as Supplementary information.

*In silico analysis to identify possible hydralazines with binding affinity to Plasmodium falciparum dihydroorotate dehydrogenase (DHODH):* Initially, we searched the Protein Data Bank to identify protein-ligand co-crystal structures involving *Pf*DHODH with known chemical scaffolds and detailed methodology is provided in Supplementary information. From these analyses, we observed that our hydrophobic ligands show similar chemical scaffolds to the co-crystallized ligand IDI-6253 involving several nitrogen-containing aromatic rings.[27] Average calculated log*P*s for our compounds is 3.38 (standard deviation SD = 0.72) and therefore very similar to log*P* = 3.00 for the template ligand IDI-6253. Moreover, the number of hydrogen bond acceptors is very similar with on average 4.94 (SD = 1.08) versus 5 in IDI-6253.

After successfully redocking the co-crystallized ligand to the binding site with an all atom RMSD of 0.25 Å, we docked the full series of hydralazine compounds to DHODH. We found a consistent binding mode for the set of compounds. The phthalazine anchor group is involved in hydrogen bonding with Arg-265, thereby replacing the triazole of IDI-6523 (see Fig. 1). The apolar tail groups form several hydrophobic and π-contacts involving Phe-227, thus occupying a similar region as the tetrahydro-isoquinoline of IDI-6523.

*In vitro validation of new hydralazines against P. falciparum:* First, we evaluated the effect of newly synthesized hydralazines against common laboratory strains (3D7) of *P. falciparum* at trophozoite stage (~30 h post-invasion (hpi)) at three different concentrations (1, 20, and 100 μM) and detailed methodology is provided in Supplementary information. DMSO-treated parasites were included as negative control. After 48 h, parasitemia was estimated by flow cytometry.[28] Five of the compounds tested, namely, **6c**, **6d**, **6e**, **6g**, and **6i** displayed significant inhibitory potential against *P. falciparum* (Fig. 2A) with estimated $IC_{50}$ less than 20 μM, which is comparable to previously reported *Pf*DHODH inhibitors synthesized by Pavadai et al.[29] These compounds were selected for further experiments.

To document the precise inhibitory activity of these compounds, trophozoite stage parasites (3D7, ~24–26 hpi) were incubated at concentrations ranging from 1.6 μM to 100 μM for 48 h. Parasites treated with DMSO and chloroquine were included in the assay as negative and positive controls, respectively. After the assay was completed, parasites were harvested and counted through flow cytometry. Compounds **6c**, **6d**, **6e**, **6g**, and **6i** had $IC_{50}$ values of 13.7, 12.1, 3.4, 3.4, and 1.6 μM, respectively against 3D7 parasites (Fig. 2B). In a parallel experiment performed using

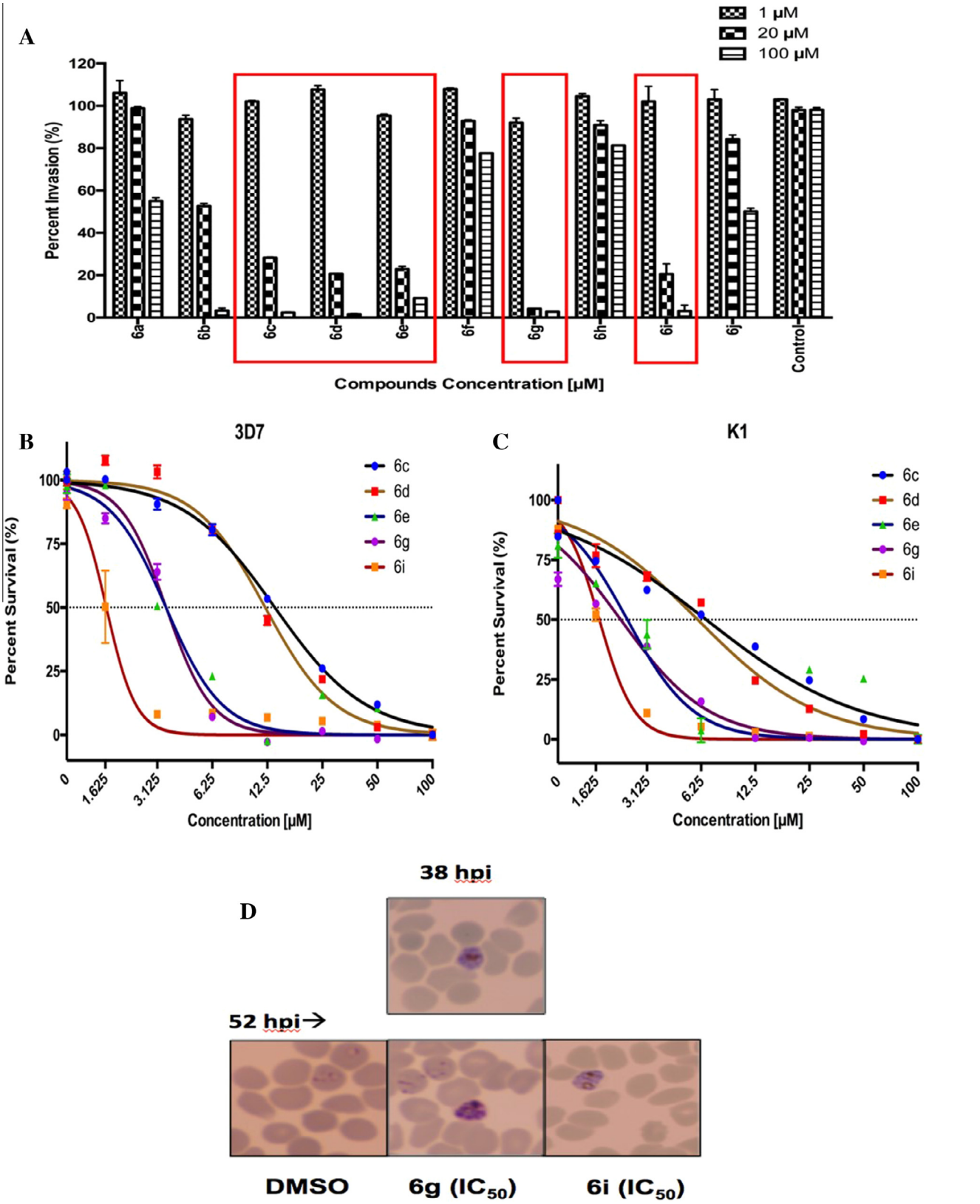



**Figure 2.** Inhibitory activity of hydrazinyl phthalazine compounds used in this study against *P. falciparum* (A). Dose response curve for the 5 most effective compounds are shown in the figure. These compounds were screened against laboratory strain of malaria parasite, 3D7 (B) and chloroquine resistant parasite variant, K1 (C). Screening was performed at concentrations ranging from 0 µM to 100 µM. Both parasite strains showed comparable sensitivity to most inhibitors (all except **6c** and **6d**). Microscopic examination of Giemsa-stained smears indicated a general inhibition of parasite maturation at schizont stage. Representative phenotypes observed for compounds **6g** and **6i** are shown in D.

**Table 2**
Overall susceptibility to each molecules of *P. falciparum* strains 3D7 and K1. Sensitivity of parasites (3D7 and K1) within a 0–100 μM compound concentration range was evaluated. Further, cytotoxicity of the same compounds was tested on the MDCK cell line, and the resulting selectivity is evaluated as 3D7 $IC_{50}$/MDCK $IC_{50}$

| Entry | $IC_{50}$ 3D7 (μM) | 95% Confidence interval | $IC_{50}$ K1 (μM) | 95% Confidence interval | Cytotoxicity $IC_{50}$ MDCK (μM) | Selectivity index |
|---|---|---|---|---|---|---|
| **6c** | 13.74 | 12.91–14.63 | 6.66 | 5.51–8.05 | >200 | 14.56 |
| **6d** | 12.18 | 11.00–13.48 | 5.98 | 5.08–7.04 | >200 | 16.42 |
| **6e** | 3.49 | 3.002–4.058 | 2.43 | 1.77–3.34 | >200 | 57.31 |
| **6g** | 3.495 | 3.246–3.764 | 2.23 | 1.85–2.69 | >200 | 57.22 |
| **6i** | 1.646 | 1.513–1.791 | 1.69 | 1.60–1.80 | >100 | 60.75 |

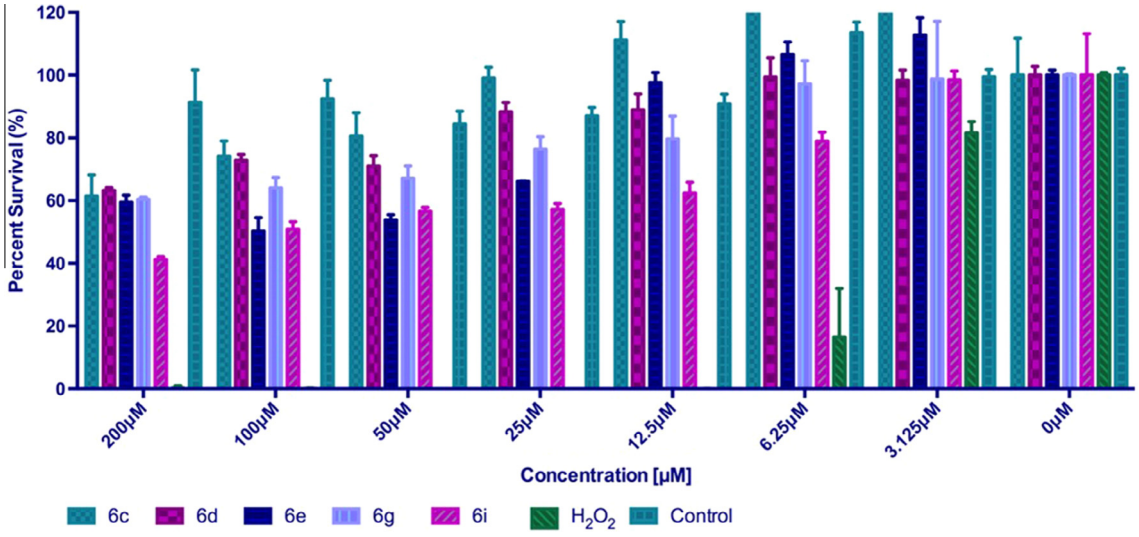



**Figure 3.** Cytotoxicity assays (MTT) was performed against Madin-Darby Canine Kidney (MDCK) cell lines, using the compounds **6c**, **6d**, **6e**, **6g** and **6i**, together with appropriate controls. None of the compounds showed significant cytotoxicity up to 200 μM.

chloroquine-resistant K1 strain, $IC_{50}$ values of 6.6, 5.9, 2.4, 2.2 and 1.6 μM were obtained for the same compounds (Fig. 2C). Giemsa-stained images prepared from representative experiments were also tested to examine possible phenotypes (Fig. 2D). These images demonstrated a general inhibition of merozoite maturation at schizont stage.

To evaluate the toxic effect of the lead compounds against mammalian cells and thereby to estimate selectivity, we performed cytotoxicity assay (MTT) using Madin-Darby Canine Kidney (MDCK) cell line.[30,31] None of the compounds induced detectable cytotoxicity at concentrations up to 200 μM (Table 2 and Fig. 3). From these observations, it was apparently clear that most compounds are capable of selectively inhibiting the malaria parasite and possibly not the host cells.

We further performed stage specific assays on *P. falciparum*. To do this, compounds were introduced at distinct time points/life stages (ring, trophozoite and schizont) and general growth/maturation and morphological characteristics were assessed. In brief, compounds were added to rings (2–4 h)/trophozoite (24–26 h)/schizonts (36–38 h) at a range of concentrations (4 * $IC_{50}$, 2 * $IC_{50}$, $IC_{50}$ and 0.25 * $IC_{50}$—estimated from Table 2). Roughly 12 h later, samples were harvested for each treatment. Flow cytometry and microscopy-based examination of parasites was conducted to monitor any stage specific inhibitory effect induced by the compounds. All compounds showed a potential inhibitory effect on schizont to ring stage transition (Fig. 4A–C), which indicates that these compounds act by interfering with late stage schizont development (Table 3). This is consistent with prior studies where inhibitors of *Pf*DHODH were tested against malaria parasites. Furthermore, the observed phenotypes are similar to the antimalarial activities of DSM265, the first DHODH inhibitor to reach clinical development for malaria treatment, arresting the parasite growth and development before the formation of multinucleated schizont stage.[32]

Structural activity relationship analyses of the tested compounds revealed that the presence of electron donating hydroxyl or amino groups attached to gamma position to the phthalazine ring like phthalazine—NH—N=CH—C=C—OH CH—CH—OH (or NH) was responsible for profound inhibitory activity of the compounds. On the other hand, the presence of electron withdrawing groups like nitro, carbonyl, or sulphonyl moiety at the same position reduced the inhibitory potential.

In conclusion, herein we report the synthesis and antimalarial activity determination of novel hydralazine derivatives. The antimalarial activity of new compounds is likely to be mediated through the inhibition of DHODH, an important enzyme in de novo biosynthesis of pyrimidines and therefore nuclear division in *P. falciparum*.

## Acknowledgments

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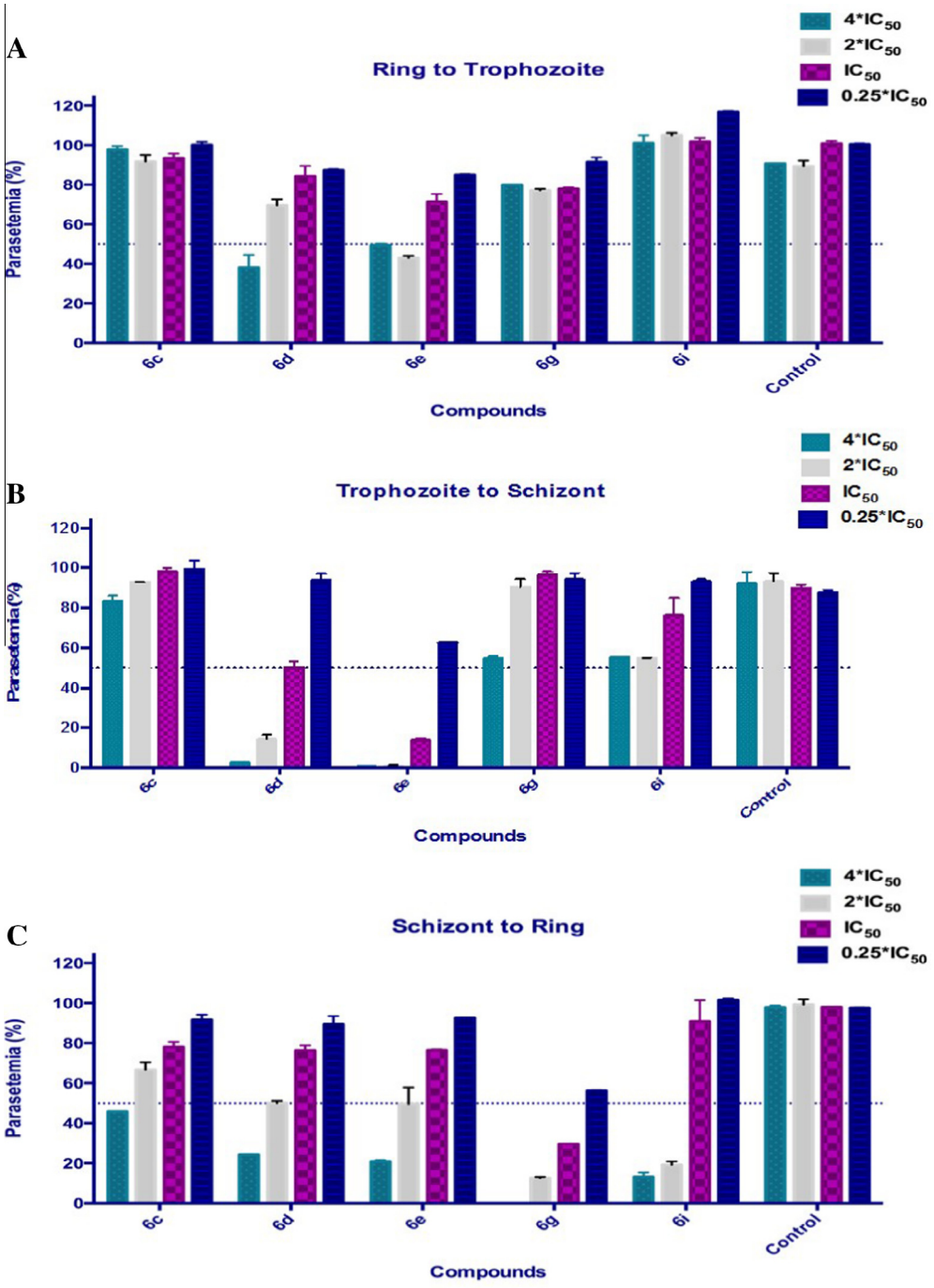


**Figure 4.** Effect of the selected compounds on distinct stages of parasite development. To document stage-specificity of the compounds tested, experiments were carried out using parasites at early stage during ring-trophozoite transition (A), trophozoite-schizont transition (B) and schizont-ring transition (C), at four different compound concentrations (4 * $IC_{50}$, 2 * $IC_{50}$, $IC_{50}$ and 0.25 * $IC_{50}$). Majority of the compounds showed an effect during the later stages of parasite development, and appeared to inhibit merozoite formation.

**Table 3**
Stage-specific effects of the compounds presented here on *Plasmodium falciparum*. '–' represents no effect; '+' indicates effect at 4 * $IC_{50}$; '++' indicates effect at 4 * $IC_{50}$ and 2 * $IC_{50}$; '+++' indicates effect at 4 * $IC_{50}$, 2 * $IC_{50}$ and $IC_{50}$. It can be seen that most compound are active on the later stages of parasite development

| Entry | Ring to trophozoite | Trophozoite to schizont | Schizont to ring |
|---|---|---|---|
| **6c** | – | – | + |
| **6d** | + | ++ | ++ |
| **6e** | – | ++ | ++ |
| **6g** | – | – | +++ |
| **6i** | – | – | ++ |
| Control | – | – | – |

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.049.

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