



Synthesis and biological evaluation of benzimidazole-5-carbohydrazide derivatives as antimalarial, cytotoxic and antitubercular agents

José Camacho^a, Arthur Barazarte^a, Neira Gamboa^b, Juan Rodrigues^b, Rosario Rojas^c, Abraham Vaisberg^c, Robert Gilman^c, Jaime Charris^{a,*}

^a Laboratorio de Síntesis Orgánica, Universidad Central de Venezuela, Aptdo. 47206, Los Chaguaramos, 1041-A Caracas, Venezuela

^b Unidad de Bioquímica, Facultad de Farmacia, Universidad Central de Venezuela, Aptdo. 47206, Los Chaguaramos, 1041-A Caracas, Venezuela

^c Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú

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ABSTRACT

A series of N'-substituted-2-(5-nitrofuran or 5-nitrothiophen-2-yl)-3H-benzimidazole-5-carbohydrazide derivatives were synthesized and investigated for their abilities to inhibit β -hematin formation, hemoglobin hydrolysis and in vivo for their antimalarial efficacy in rodent *Plasmodium berghei*. Selected analogues were screened for their antitubercular activity against sensitive MTB H₃₇Rv and multidrug-resistant MDR-MTB strains, and cytotoxic activity against a panel of human tumor cell lines and two non-tumorigenic cell lines. Compounds **3a**, **5a**, **f**, **6g** were the most promising as inhibitors of β -hematin formation, however, their effect as inhibitors of hemoglobin hydrolysis were marginal. The most active compounds to emerge from the in vitro and in vivo murine studies were **3a** and **6i**, suggesting an antimalarial activity via inhibition of β -hematin formation and are as efficient as chloroquine. The cytotoxic and antitubercular activities of the present compounds were not comparable with those of the standard drugs employed. But, however, compound **5b** showed better antitubercular activity compared to rifampin against multidrug-resistant MDR-MTB strains. Compounds **3a**, **6i** and **5b** showed a good safety index.

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1. Introduction

Malaria caused 350–500 million clinical episodes annually and result in over one million deaths, most of which affect children under 5 years old in sub-Saharan Africa. Malaria is the fifth cause of death from infectious diseases worldwide (after respiratory infections, HIV/AIDS, diarrhoeal diseases and tuberculosis). Recent estimates show that as many as 3.3 billion people live in areas at risk of malaria in 109 countries. In addition to its health toll, malaria puts a heavy economic burden on endemic countries and contributes to the cycle of poverty people face in many countries.¹ Malaria mortality and morbidity began to increase in the 1980s due to a combination of factors such as the increase in parasite and vector resistance to the current antimalarial drugs and insecticides, the weakening of traditional malaria control programs, rapid decentralization and integration into deteriorating primary health service, and the development of humanitarian crisis situations in many malaria-endemic areas. This dramatic increase led to a compelling and urgent necessity for new antimalarials, with mechanisms of action different from the existing ones, and to identify new drug targets.² Chloroquine has recently been shown to inhibit hemozoin formation within the parasite food vacuole.³ This

process is also thought to be the molecular target of other quinoline antimalarials.⁴ Hemozoin was originally considered to be formed by the polymerization of heme, but it has now been demonstrated to be a crystalline cyclic dimer of ferriprotoporphyrin IX.^{5–8} Thus, hemozoin synthesis, a process unique to the malaria parasite, offers a logical and valuable potential target for new antimalarial drug development. New drugs that attack the same vital target of chloroquine but that are not subject to the same resistance mechanism would be highly desirable.

Despite the availability of highly potential antitubercular agents, tuberculosis (TB) remains primary cause of comparatively high mortality worldwide. The statistics shows that around 32% of the world's population is infected by *Mycobacterium tuberculosis*, the main causal agent of TB and today more people die from tuberculosis than ever before.^{9–11} Problems in the chemotherapy of TB arise when patients develop bacterial resistance to the first-line drugs. Therefore, the development of new drugs with activity against multi drug-resistant TB, extensively drugs-resistant TB and latent TB is a priority task.

Benzimidazole scaffolds is one of the privileged structures in medicinal chemistry. Indeed, various examples featuring these particular scaffolds have been prepared, many exhibiting remarkable biological activities.^{12–18}

On the other hand, nitroaromatic compounds are very important group, which have been used extensively in the treatment of

* Corresponding author. Fax: +58 2126052707.

E-mail addresses: jaimecharris11@cantv.net, jaime.charris@ucv.ve (J. Charris).

anaerobic infections, and are under continuum investigation. There is a direct proof that free-radical metabolites are involved in many applications including important antitumor, antiparasitic and antibacterial agents.^{19–23} After extensive literature search, it was observed that, till date enough effort has not been made to combine these moieties as a single molecular scaffold and identify new candidates that may be value in designing new, potent, selective and less toxic antimalarial and antitubercular agents. The choice of the carbohydrazide moiety was motive to the relation with the structure of the isoniazid and pyrazinamide agents used for tuberculosis chemotherapy. In view of this data, we reported the synthesis of nitrofuran and nitrothiophene incorporated with bezimidazole and carbohydrazide which possessed wide variety of biological activity encouraging antimalarial activity against *Plasmodium berghei* in vivo and antitubercular activity against *M. tuberculosis* H₃₇Rv and MDR. Additionally, we also report the cytotoxic activity against several cancer cell lines.

2. Results and discussion

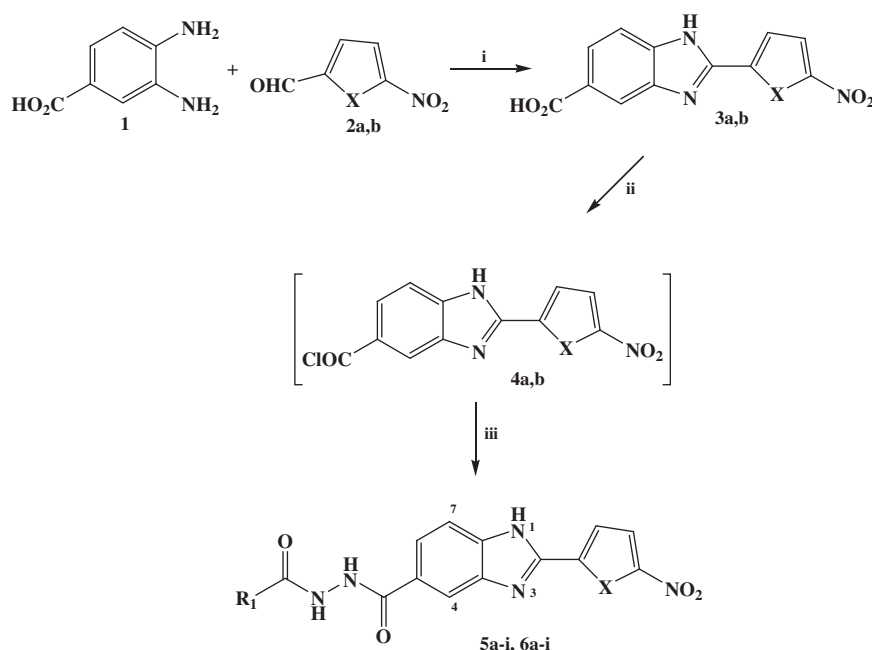
2.1. Synthesis

Our strategy for synthesis of **5a–i** and **6a–i**, is a very simple one as illustrated in Scheme 1. In the procedure here described **3a,b** were obtained by the reaction of 3,4-diaminobenzoic acid **1** with 5-nitro-2-furaldehyde **2a** and 5-nitro-2-thiophenecarbaldehyde **2b**, to obtain the title compounds in good yields (Scheme 1). In this case nitrobenzene was used as an oxidant following an analogous procedure.²⁴ Compounds **3a–b** were allowed to react with thionyl chloride to obtain the corresponding chloro derivatives, compounds **4a–b** were not isolated from the reaction medium, so were condensed with the appropriately substituted aryl acid hydrazides in DCM and DMAP to afford the target molecules. The final compounds were purified by recrystallization from ethanol, and the structure of the compounds was confirmed by IR, ¹H NMR and elemental analysis. The IR spectra of target compounds **5a–i** and **6a–i** showed the broad stretching band around 3400 and 3200 cm^{−1} due to (NH) and around 1530–1510 and 1350–1340 cm^{−1} due

(NO₂) with ¹H NMR a singlet around 10.5 and 10.6 ppm accounted for NH vanished on D₂O exchange, doublets around 7.5 and 7.9 ppm *J*: 3–4 Hz assigned to protons H₃ and H₄, respectively and benzimidazole moiety protons as doublets around 7.7 ppm *J*: 8.5 Hz assigned to proton H₇, doublet of doublets around 7.9 ppm *J*: 8.5 and 2.0 Hz assigned to proton H₆, and doublets around 8.3 ppm *J*: 2.0 Hz assigned to proton H₄. The synthetic route leading to the title compounds is summarized in Scheme 1.

2.2. Biological

All analogs of those derivatives were tested in vitro for their effects as inhibitors of β-hematin formation, inhibition of hemoglobin hydrolysis, and in vivo for their efficacy in a murine model (Table 1). The first mentioned in vitro assay was used to assess the abilities of the derivatives N'-substituted-2-(5-nitrofuran or 5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide derivatives **5a–i**, **6a–i** to inhibit β-hematin formation. To evaluate the potential antimalarial activity of compounds, we tested the ability of these compounds to inhibit heme crystallization, considering that heme can crystallize spontaneously under acid and low oxygen condition found in the vacuole of the parasite.²⁵ Results that showed more than 90% of inhibition of heme crystallization were considered significant (compounds **3a**, **5a**, **f**, **6g** Table 1), results reveal that the compounds were as active as chloroquine (94.19 ± 0.36%) inducing the inhibition of heme crystallization. The 5-nitrofuran moiety appeared to be favourable for the potential antimalarial activity, since most of the compounds possessing showed measurable levels of inhibition of β-hematin formation, except compound **6g** having 5-nitrothiophene-2-yl showed good activity. All compounds were tested for their capacity of inhibiting haemoglobin proteolysis, in an in vitro assay which uses trophozoite-rich extract to digest the native haemoglobin of mice. The electrophoretic analysis indicated that only compound **6c** was partially effective inhibiting the proteolysis of haemoglobin 44.93 ± 1.12% compared with leupeptin (LEU) and pepstatin (PEP) 91.62 ± 0.69 and 95.45 ± 0.66, respectively Table 1.



Scheme 1. Synthesis of N'-substituted-2-(5-nitrofuran or 5-nitrothiophene-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide derivatives **5a–i**, **6a–i**. (i) nitrobenzene, Δ. (ii) SOCl₂, benzene, DMF, Δ. (iii) CH₂Cl₂, DMAP, 0 °C → rt, X: O, S. R₁: H, phenyl, naphthyl.

Table 1

Inhibition of β -hematin synthesis (IHS), globin proteolysis (IGP) (%), effect of benzimidazole derivatives (20 mg/kg) on parasitemia at fourth day post-infection (%P) and survival days (SD) of *P. berghei* infected-mice by benzimidazole derivatives

No.	R ₁	I β HS	IC ₅₀ (μ M)	IGP	%P	SD
3a		95.43 \pm 0.58*	8.43	0	4.02 \pm 0.45†	17 \pm 1.26†
3b		57.63 \pm 1.96	21.09	0	11.5 \pm 2.06	12.2 \pm 1.15
5a	H	94.05 \pm 1.9*	8.63	24.24 \pm 1.14	25.8 \pm 0.86	8.2 \pm 0.86
5b	Ph	79.21 \pm 0.63	10.92	0	10.14 \pm 3.35	13.6 \pm 1.28
5c	3-ClPh	73.43 \pm 5.88	10.30	38.98 \pm 0.92	3.38 \pm 1.17†	12.4 \pm 2.29
5d	4-ClPh	59.62 \pm 3.61	23.35	33.75 \pm 1.74	27.4 \pm 1.08	9.8 \pm 1.07
5e	3-MeOPh	72.44 \pm 1.38	13.13	2.82 \pm 0.85	5.98 \pm 1.56†	11.8 \pm 1.98†
5f	3,4-MeOPh	94.76 \pm 0.49*	8.71	34.53 \pm 1.16	4.8 \pm 2.22†	12.2 \pm 1.65†
5g	3,4,5-MeOPh	69.38 \pm 2.45	14.40	0	9.4 \pm 0.6	12.2 \pm 0.58
5h	3-PhOPh	84.63 \pm 1.75	14.06	ND	12.95 \pm 3.8	10.6 \pm 0.81
5i	3-OH-2-Naphtyl	31.74 \pm 4.89	ND	12.87 \pm 0.43	ND	ND
6a	H	87.39 \pm 0.97	9.88	36.16 \pm 1.12	18.72 \pm 3	10.04 \pm 1.53
6b	Ph	86.18 \pm 0.73	10.78	39.19 \pm 0.45	10.12 \pm 2.13	12 \pm 1.41
6c	3-ClPh	78.59 \pm 0.74	15.96	44.93 \pm 1.10**	27.2 \pm 0.91	7.4 \pm 0.4
6d	4-ClPh	25.48 \pm 1.91	ND	12.72 \pm 1.21	13.52 \pm 3.71	10.8 \pm 1.59
6e	3-MeOPh	61.64 \pm 4.48	24.50	13.36 \pm 0.98	24.8 \pm 1.32	10.6 \pm 1.57
6f	3,4-MeOPh	70.77 \pm 6.95	21.36	38.18 \pm 0.87	16.06 \pm 2.64	13.8 \pm 2.35
6g	3,4,5-MeOPh	92.36 \pm 0.48*	9.56	ND	9.36 \pm 3.59	10 \pm 1.3
6h	3-PhOPh	76.49 \pm 2.7	19.12	0	7.28 \pm 1.67	11 \pm 2.4
6i	3-OH-2-naphtyl	75.76 \pm 0.99	11.10	14.08 \pm 0.88	1.8 \pm 0.49††	18.8 \pm 2.05††
Leu	—	—	—	91.62 \pm 0.69	—	—
Pep	—	—	—	95.45 \pm 0.66	—	—
CQ	—	94.19 \pm 0.36	—	24.12 \pm 1.16	1.3 \pm 0.3	>30
SS	—	—	—	—	21.8 \pm 2.31	11.66 \pm 1.66

X **3a**, (**5a–i**) = O; X **3b** (**6a–i**) = S; Leu = leupeptin; Pep = pepstatin; CQ = chloroquine; SS = saline solution.

The results are expressed by the mean \pm standard error of the mean.

* $p > 0.05$ compared to chloroquine.

** $p > 0.05$ compared to leupeptin (LEP) and pepstatin (PEP).

† $p < 0.05$.

†† $p < 0.001$ compared to saline solution and chloroquine. $n = 6$.

Compounds **5a–i** and **6a–i** were tested in infected mice with *P. berghei* ANKA, a chloroquine-susceptible strain of murine malaria. Compounds were given to mice (chloroquine or **5a–i**, **6a–i** in 20 mg kg^{−1}, ip once daily) for four consecutive days (days 1–4 post-infection). At day fourth post-infection, the parasitemia was determined; the survival days were monitored and compared with control mice receiving a saline solution (untreated mice). Control mice died within 8.2 \pm 0.37 days post-infection, compounds **3a**, **5f** and **6i** increased the survival time for 17 \pm 1.26, 12.2 \pm 1.65 and 18.8 \pm 2.05 days, respectively, while chloroquine prolonged the survival time of the infected mice to 30 days. Compounds **3a**, **5f** and **6i** were able to reduce and delay the progression of malaria (4.02 \pm 0.45, 4.8 \pm 2.22 and 1.8 \pm 0.49%), respectively but did not eradicate the infection compared with chloroquine 1.3 \pm 0.3 (Table 1).

On the other hand, due to the urgent need to discover new drugs for the treatment of TB, several colorimetric methods for rapid screening of pure compounds or crude natural product extracts have been developed.^{26–28} The microplate Alamar blue assay (MABA) is a rapid, nonradioactive, and inexpensive method that is based on the color change of the redox dye Alamar blue for the detection of viable *M. tuberculosis*.^{29,30} Members of our group have developed a variant of this assay that uses tetrazolium bromide instead of the MABA.³¹ This tetrazolium microplate assay (TEMA) determines MIC values as quickly (6–7 days) and accurately as the MABA procedure. Compounds **3a**, **5a,b,e–h** and **6e,g,i** were tested in vitro for their antimycobacterial activity against sensitive *M. tuberculosis* H₃₇Rv strain and a multidrug-resistant (MDR) clinical isolate. Results revealed that compounds **5b** exhibited a moderate antimycobacterial activity, with a MIC value of 12.5 μ g/mL

Table 2

Cytotoxicity and antimycobacterial activity of benzimidazole derivatives against 3T3, BALB/3T3 clone A31 embryonic mouse fibroblast cells; Vero, normal African green monkey kidney epithelial cells; H460, human large cell lung cancer; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human melanoma; HT-29, human colon adenocarcinoma; K562, human chronic myelogenous leukemia cells; sensitive MTB H₃₇Rv strain; multidrug-resistant MDR strain of MTB

No.	GI ₅₀ (μ g/mL)								MIC (μ g/mL)	
	3T3	VERO	H460	DU145	MCF-7	M-14	HT-29	K562	H ₃₇ Rv	MDR
3a	62.5	>250	125	125	>250	250	250	250	>25	>25
5a	62.5	62.5	31.25	15.63	62.5	31.25	62.5	31.25	>25	>25
5b	31.25	31.25	62.5	15.63	62.5	15.63	31.25	15.63	12.5	6.25
5e	15.63	31.25	62.5	15.63	15.63	7.81	31.25	15.63	25	25
5f	31.25	62.5	15.63	15.63	31.25	15.63	15.63	15.63	>25	25
5g	31.25	31.25	31.25	15.63	31.25	15.63	31.25	31.25	>25	25
5h	>7.8	>7.8	>7.8	>7.8	>7.8	>7.8	>7.8	>7.8	>25	25
6e	15.63	>250	62.5	62.5	15.63	15.63	31.25	15.63	>25	25
6g	31.25	31.25	62.5	62.5	31.25	62.5	62.5	31.25	>25	>25
6i	31.25	31.25	62.5	62.5	62.5	62.5	62.5	31.25	>25	>25
C-P	0.7	0.8	0.3	0.6	>5	3.3	5.3	0.3	—	—
INH									0.125	4 \rightarrow 32
RIF									0.063	2 \rightarrow 16

C-P, Cisplatin; INH, isoniazid; RIF, rifampin.

against sensitive *M. tuberculosis* H₃₇Rv strain and 6.25 µg/mL against the multidrug-resistant (MDR) clinical isolates, compared with reference drugs isoniazid (INH) and rifampin (RIF) MIC 0.063 and 32 µg/mL (Table 2).

Cytotoxic activity of compounds **3a**, **5a,b,e–h** and **6e,g,i** have been assayed using a murine fibroblast nontransformed cell line as control, one normal African green monkey kidney epithelial cells and seven human cancerous cell lines. The results of cytotoxic activity are summarized in Table 2. The results showed that compounds **3a**, **5a,b,e–h** and **6e,g,i** displayed moderate to weak activity (7.81–250 µg/mL) against control and cancerous cell lines.

2.3. Structure–activity relationship study

Having confirmed the activity of compound **3a**, we embarked on a hit-to-lead exploration program focusing on CO₂H of benzimidazole nucleus. The first step toward lead optimization was incorporation of formylhydrazido group. The inhibition of heme polymerization study data of this compound showed the same activity that **3a**, but the activity in vivo against *P. berghei* infected mice was marginal, the cytotoxic and antimicrobial activities also were marginal. One significant problem with accurate measurements of kinetic constant and other activities for this compound has been solubility. Hence we planned to introduce the aromatic group with electron withdrawing and donating groups at different position to study its influence on activity. Different analogs with H, chlorine, hydroxyl, aryloxy, and methoxy groups were synthesized. In majority of the cases, compounds having H, chlorine, phenoxy and monomethoxy groups exhibited marginal activity as antimalarial, antimicrobial and cytotoxic. However, compounds with di- or tri-methoxy substituents and a 5-nitrofuranyl or 5-nitrothiophene-2-yl moiety in position 2 of benzimidazole exhibited good activity in vitro as inhibitors against heme polymerization study. In exceptional case, *N*-(3-hydroxy-2-naphthyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide **6i** was found better in vivo as antimalarial.

3. Conclusion

The present study describes the synthesis and the in vitro, and in vivo antimalarial, antimicrobial and tumor cells activity of tripartite hybrids from pharmacophores benzimidazole, 5-nitrofuran or 5-nitrothiophene-2-yl and substituted aryl acid hydrazides. In summary, some of them showed good selectivity index between the parasite, antimicrobial and tumor cells. Compounds **3a**, **5a,f**, **6g** exhibited potential effects as inhibitors of β-hematin formation, **5b** against sensitive *M. tuberculosis* strain and multidrug-resistant (MDR) clinical isolate. The study confirms that antimalarial mechanism of action could be similar to that of chloroquine, as most of the compounds form an association complex with β-hematin and thereby inhibit hemozoin formation. The results provide basic information to establish that a 5-nitrothiophene-2-yl moiety on position two of the benzimidazole is not very essential for an inhibitory activity of heme dimerization, however, a 5-nitrofuranyl moiety is essential and opens new vistas for design of new antimalarial agents. Rationally, such a combination of antitropozal pharmacophores and other functionalities offers many attractive features for accelerating antimalarial, antimicrobial. Due to the better activity as inhibitors of β-hematin formation, compounds **3a**, **5a,f**, **6g**, 4-day suppressive test compound **6i**, and against tested MT-MDR clinical isolates, compound **5b** have been selected for further development and studies to acquire more information about structure–activity relationships and pharmacokinetic are in progress in our laboratories.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Thomas micro hot stage apparatus and are uncorrected. Infrared spectra were determined as KBr pellets on a Shimadzu model 470 spectrophotometer. The ¹H NMR spectra were recorded using a Jeol Eclipse 270 (270 MHz) spectrometer using DMSO-*d*₆, and are reported in ppm downfield from the residual DMSO. Elemental analyses were performed on a Perkin–Elmer 2400 CHN analyser, results were within ±0.4% of the predicted values for all compounds. Chemical reagents were obtained from Aldrich Chemical Co., USA. All solvents were distilled and dried in the usual manner.

4.1.1. General procedure for the synthesis of 2-(5-nitrofuran or 5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carboxylic acid **3a–b**

A mixture of the 5-nitro-2-furaldehyde/5-nitro-2-thiophen-carbaldehyde **2a–b** (5 mmol), 3,4-diaminobenzoic acid **1** (5 mmol), in nitrobenzene (25 mL) was refluxed for 8 h. The solvent was evaporated to dryness under reduced pressure, *n*-heptane was added (10 mL) and the solid thus obtained was collected by filtration, washed with methanol and ethyl ether. Further purification was accomplished by recrystallization from ethanol.

4.1.1.1. 2-(5-Nitrofuran-2-yl)-3H-benzo[d]imidazole-5-carboxylic acid **3a³².** Green solid. Yield 85%. mp 294–296 °C; IR (KBr) cm^{−1}: 3632, 3152, 1692, 1626 (CO₂H, CH=CH), 1546 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm, 80 °C: 7.53 (d, 1H, H₄, *J* = 3.9 Hz); 7.70 (d, 1H, H₇, *J* = 8.6 Hz); 7.83 (d, 1H, H₃, *J* = 3.9 Hz); 7.92 (dd, 1H, H₆, *J* = 1.3, 8.6 Hz); 8.24 (s, 1H, H₄); 13.25 (br s, 1H, OH). Anal. Calcd for C₁₂H₇N₃O₅: C, 52.76; H, 2.58; N, 15.38. Found: C, 52.80; H, 2.56; N, 15.41.

4.1.1.2. 2-(5-Nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carboxylic acid **3b.** Brown solid. Yield 89%. mp > 300 °C; IR (KBr) cm^{−1}: 3280, 1696, 1619 (CO₂H, CH=CH), 1516 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm, 80 °C: 7.68 (d, 1H, H₇, *J* = 8.7 Hz); 7.87 (d, 1H, H₄, *J* = 4.3 Hz); 7.90 (dd, 1H, H₆, *J* = 1.2, 8.7 Hz); 8.19 (d, 1H, H₃, *J* = 4.3 Hz); 8.21 (s, 1H, H₄); 13.40 (br s, 1H, OH). Anal. Calcd for C₁₂H₇N₃O₄S: C, 49.83; H, 2.44; N, 14.53. Found: C, 49.79; H, 2.46; N, 14.54.

4.1.2. General procedure for the synthesis of *N*-substituted-2-(5-nitrofuran or 5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide derivatives **5a–i**, **6a–i**

A mixture of **3a,b** (0.3 mmol), thionyl chloride (1.5 mmol), and benzene 25 mL was gradually heated to boiling, whereupon the acid chloride dissolved. After refluxing for 12 h, excess of thionyl chloride and solvent were removed in vacuo. The acid chloride was not characterized, then to a solution of acid chloride, DMAP (1.5 mmol) in dry CH₂Cl₂ (10 mL) was slowly added a dry CH₂Cl₂ (10 mL) solution of carbohydrazide respective (0.4 mmol) (30 min) at 0 °C, and the mixture was stirred for 36 h at rt. The solvent was removed in vacuo and the residue was slowly added water 5% KOH at 0 °C and the mixture stirred for 30 min. The solid was washed with water, methanol and ethyl ether, recrystallized from a mixture of ethanol and water (1:1) to give **5a–i**, **6a–i**.

4.1.2.1. *N*-Formyl-2-(5-nitrofuran-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide **5a.** Yield 66%. mp 250 °C (dec). IR (KBr), cm^{−1}: 3132 (NH), 1650, 1604 (Ar) 1344 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.57 (t, 1H, H₃, *J* = 4.0 Hz); 7.77 (d, 1H, H₇, *J* = 8.8 Hz); 7.90 (d, 1H, H₄, *J* = 4.0 Hz); 7.92 (dd, 1H, H₆, *J* = 1.5, 8.8 Hz); 8.27 (s, 1H, H₄);

9.03 (s, 1H, CHO); 10.68 (s, 1H, NH); 10.80 (s, 1H, NH). Anal. Calcd for $C_{13}H_9N_5O_5$: C, 49.53; H, 2.88; N, 22.21. Found: C, 49.50; H, 2.92; N, 22.30.

4.1.2.2. *N'*-Benzoyl-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5b. Yield 55%. mp 220–222 °C. IR (KBr), cm^{-1} : 3133 (NH), 1602 (Ar) 1342 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.54 (t, 2H, $H_{3',5'}$, $J = 7.7$ Hz); 7.57 (d, 1H, H_3 , $J = 3.6$ Hz); 7.61 (t, 1H, $H_{4'}$, $J = 7.3$ Hz); 7.75 (d, 1H, H_7 , $J = 8.4$ Hz); 7.88 (d, 1H, H_4 , $J = 3.6$ Hz); 7.92 (dd, 1H, H_6 , $J = 1.6, 8.6$ Hz); 7.94 (d, 2H, $H_{2',6'}$, $J = 7.4$ Hz); 8.28 (d, 1H, H_4 , $J = 1.6$ Hz); 10.64 (s, 1H, NH); 10.74 (s, 1H, NH). Anal. Calcd for $C_{19}H_{13}N_5O_5$: C, 58.32; H, 3.45; N, 17.90. Found: C, 58.37; H, 3.48; N, 18.01.

4.1.2.3. *N'*-(3-Chlorobenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5c. Yield 73%. mp 190 °C. IR (KBr), cm^{-1} : 3408, 3232 (NH), 1683, 1645 (Ar), 1520, 1344 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.58 (t, 1H, $H_{5'}$, $J = 7.5$ Hz); 7.61–7.65 (m, 2H, Ar); 7.65 (d, 1H, H_3 , $J = 3.0$ Hz); 7.76 (d, 1H, H_7 , $J = 9.0$ Hz); 7.89–7.92 (m, 2H, Ar); 7.97 (s, 1H, $H_{2'}$); 8.27 (s, 1H, H_4); 10.66 (s, 1H, NH); 10.68 (s, 1H, NH). Anal. Calcd for $C_{21}H_{19}N_5O_5Cl$: C, 59.24; H, 2.84; N, 16.45. Found: C, 58.97; H, 3.03; N, 16.70.

4.1.2.4. *N'*-(4-Chlorobenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5d. Yield 68%. mp 178–180 °C. IR (KBr), cm^{-1} : 3269 (NH), 1699, 1683, 1594 (Ar), 1542, 1347 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.60–7.62 (m, 3H, Ar); 7.75 (d, 1H, H_7 , $J = 8.4$ Hz); 7.88 (dd, 1H, H_6 , $J = 2.0, 8.4$ Hz); 7.92 (d, 1H, $H_{4'}$, $J = 4.0$ Hz); 7.96 (d, 2H, $H_{2',6'}$, $J = 8.4$ Hz); 8.25 (d, 1H, H_4 , $J = 2.0$ Hz); 10.53 (s, 1H, NH); 10.57 (s, 1H, NH). Anal. Calcd for $C_{21}H_{19}N_5O_5Cl$: C, 59.24; H, 2.84; N, 16.45. Found: C, 59.26; H, 2.89; N, 16.37.

4.1.2.5. *N'*-(3-Methoxybenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5e. Yield 73%. mp 182–184 °C. IR (KBr), cm^{-1} : 3296 (NH), 1686, 1603 (Ar), 1350 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.83 (s, 3H, OCH₃); 7.18 (dd, 1H, Ar, $J = 2.7, 8.3$ Hz); 7.38–7.54 (m, 3H, Ar); 7.65 (d, 1H, H_3 , $J = 3.5$ Hz); 7.77 (d, 1H, H_7 , $J = 9.0$ Hz); 7.91 (dd, 1H, H_6 , $J = 1.8, 9.0$ Hz); 7.94 (d, 1H, H_4 , $J = 3.5$ Hz); 8.28 (d, 1H, H_4 , $J = 1.8$ Hz); 10.54 (s, 1H, NH); 10.61 (s, 1H, NH). Anal. Calcd for $C_{20}H_{15}N_5O_6$: C, 57.01; H, 3.59; N, 16.62. Found: C, 57.15; H, 3.58; N, 16.78.

4.1.2.6. *N'*-(3,4-Dimethoxybenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5f. Yield 87%. mp 208 °C (dec). IR (KBr), cm^{-1} : 3392, 3232 (NH), 1683, 1651 (Ar), 1507, 1341 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.83 (s, 3H, OCH₃); 3.84 (s, 3H, OCH₃); 7.08 (d, 1H, Ar, $J = 6.3$ Hz); 7.55–7.60 (m, 2H, Ar); 7.57 (d, 1H, H_3 , $J = 3.7$ Hz); 7.76 (d, 1H, H_7 , $J = 8.3$ Hz); 7.88 (d, 1H, $H_{4'}$, $J = 3.6$ Hz); 7.90 (dd, 1H, H_6 , $J = 1.2, 8.3$ Hz); 8.27 (s, 1H, H_4); 10.29 (br s, 1H, NH); 10.42 (bs, 1H, NH). Anal. Calcd for $C_{21}H_{17}N_5O_7$: C, 55.88; H, 3.79; N, 15.51. Found: C, 56.02; H, 3.68; N, 15.70.

4.1.2.7. *N'*-(3,4,5-Trimethoxybenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5g. Yield 81%. mp 160 °C (dec). IR (KBr), cm^{-1} : 3472 (NH), 1694, 1649 (Ar), 1580, 1341 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.86 (s, 3H, OCH₃); 3.91 (s, 6H, OCH₃); 7.30 (s, 2H, $H_{2',6'}$); 7.58 (d, 1H, H_3 , $J = 3.8$ Hz); 7.76 (d, 1H, H_7 , $J = 8.6$ Hz); 7.90 (d, 1H, $H_{4'}$, $J = 3.8$ Hz); 7.91 (dd, 1H, H_6 , $J = 1.5, 8.6$ Hz); 8.28 (s, 1H, H_4); 10.38 (br s, 1H, NH); 10.47 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{19}N_5O_8$: C, 54.89; H, 3.98; N, 14.55. Found: C, 55.09; H, 3.83; N, 14.68.

4.1.2.8. *N'*-(3-Phenoxybenzoyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5h. Yield 52%. mp 210–212 °C. IR (KBr), cm^{-1} : 3472 (NH), 1654, 1649 (Ar), 1580, 1341 (NO₂). ¹H

RMN, DMSO-*d*₆, δ ppm: 7.08 (d, 1H, Ar, $J = 7.68$ Hz); 7.20 (t, 1H, $H_{5'}$, $J = 7.32$ Hz); 7.25 (dd, 1H, Ar, $J = 2.20, 8.08$ Hz); 7.44 (t, 2H, $H_{3',5'}$, $J = 7.3$ Hz); 7.54–7.57 (m, 3H, Ar); 7.60 (d, 1H, H_3 , $J = 3.5$ Hz); 7.73–7.76 (m, 2H, Ar); 7.89 (d, 1H, $H_{4'}$, $J = 3.5$ Hz); 7.92 (dd, 1H, H_6 , $J = 1.2, 8.6$ Hz); 8.29 (s, 1H, H_4); 10.56 (s, 1H, NH); 10.61 (s, 1H, NH). Anal. Calcd for $C_{25}H_{17}N_5O_6$: C, 63.11; H, 3.54; N, 14.49. Found: C, 63.19; H, 3.60; N, 14.40.

4.1.2.9. *N'*-(3-Hydroxy-2-naphthyl)-2-(5-nitrofur-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 5i. Yield 60%. mp >260 °C. IR (KBr), cm^{-1} : 3424 (NH), 1651, 1625 (Ar), 1507, 1344 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 5.6 (br s, 1H, OH); 7.36 (s, 1H, Ar); 7.52 (t, 1H, Ar, $J = 7.4$ Hz); 7.60 (d, 1H, H_3 , $J = 3.3$ Hz); 7.66 (t, 1H, Ar, $J = 6.3$ Hz); 7.75–7.78 (m, 2H, Ar); 7.89 (d, 1H, H_4 , $J = 3.5$ Hz); 7.91–7.93 (m, 2H, Ar); 8.29 (s, 1H, H_4); 8.57 (s, 1H, Ar); 10.74 (s, 1H, NH); 10.79 (s, 1H, NH). Anal. Calcd for $C_{23}H_{15}N_5O_6$: C, 60.40; H, 3.31; N, 15.31. Found: C, 60.42; H, 3.37; N, 15.49.

4.1.2.10. *N'*-Formyl-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6a. Yield 77%. mp 220 °C (dec). IR (KBr) cm^{-1} : 3424, 3248 (NH), 1686, 1651 (Ar), 1523, 1334 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.57 (d, 1H, H_3 , $J = 4.2$ Hz); 7.90–8.08 (m, 2H, Ar); 8.14–8.24 (m, 3H, Ar); 9.16 (s, 1H, CHO); 10.46 (s, 1H, NH); 10.68 (s, 1H, NH). Anal. Calcd for $C_{13}H_9N_5O_4S$: C, 47.13; H, 2.74; N, 21.14. Found: C, 47.10; H, 2.85; N, 21.32.

4.1.2.11. *N'*-Benzoyl-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6b. Yield 51%. mp >300 °C. IR (KBr), cm^{-1} : 3312 (NH), 1683 (Ar), 1520, 1342 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.54 (t, 2H, $H_{3',5'}$, $J = 7.7$ Hz); 7.58 (d, 1H, H_3 , $J = 4.3$ Hz); 7.61 (t, 1H, $H_{4'}$, $J = 7.3$ Hz); 7.74 (d, 1H, H_7 , $J = 8.3$ Hz); 7.88 (dd, 1H, H_6 , $J = 1.3, 8.3$ Hz); 7.92 (d, 1H, H_4 , $J = 4.3$ Hz); 7.94 (d, 2H, $H_{2',6'}$, $J = 7.4$ Hz); 8.27 (d, 1H, H_4 , $J = 1.3$ Hz); 10.63 (s, 1H, NH); 10.74 (s, 1H, NH). Anal. Calcd for $C_{19}H_{13}N_5O_4S$: C, 56.02; H, 3.22; N, 17.19. Found: C, 55.79; H, 3.23; N, 17.46.

4.1.2.12. *N'*-(3-Chlorobenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6c. Yield 54%. mp 190 °C (dec). IR (KBr), cm^{-1} : 3472 (Ar), 1700 (Ar), 1521, 1336 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.58 (t, 1H, $H_{5'}$, $J = 7.8$ Hz); 7.68 (d, 1H, Ar, $J = 8.1$ Hz); 7.74 (d, 1H, H_7 , $J = 8.4$ Hz); 7.88 (dd, 1H, H_6 , $J = 1.7, 8.4$ Hz); 7.91 (d, 1H, $H_{6'}$, $J = 8.1$ Hz); 7.95 (d, 1H, H_3 , $J = 4.8$ Hz); 7.97 (s, 1H, $H_{2'}$); 7.98 (d, 1H, H_4 , $J = 4.8$ Hz); 8.25 (d, 1H, H_4 , $J = 1.7$ Hz); 10.65 (s, 1H, NH); 10.68 (s, 1H, NH). Anal. Calcd for $C_{19}H_{12}N_5O_4ClS$: C, 51.65; H, 2.74; N, 15.85. Found: C, 51.82; H, 2.83; N, 15.97.

4.1.2.13. *N'*-(4-Chlorobenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6d. Yield 48%. mp 185 °C (dec). IR (KBr) cm^{-1} : 3471 (NH), 1699, 1635 (Ar), 1518, 1336 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.57 (d, 1H, H_3 , $J = 3.7$ Hz); 7.61 (d, 2H, $H_{3',5'}$, $J = 8.4$ Hz); 7.76 (d, 1H, H_7 , $J = 8.4$ Hz); 7.90 (dd, 1H, H_6 , $J = 1.5, 8.4$ Hz); 7.91 (d, 1H, $H_{4'}$, $J = 3.7$ Hz); 7.96 (d, 2H, $H_{2',6'}$, $J = 8.4$ Hz); 8.27 (d, 1H, H_4 , $J = 1.5$ Hz); 10.55 (s, 1H, NH); 10.57 (s, 1H, NH). Anal. Calcd for $C_{19}H_{12}N_5O_4ClS$: C, 51.65; H, 2.74; N, 15.85. Found: C, 51.63; H, 2.77; N, 16.13.

4.1.2.14. *N'*-(3-Methoxybenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6e. Yield 68%. mp 147–149 °C. IR (KBr) cm^{-1} : 3472 (NH), 1683 (Ar), 1578, 1337 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.84 (s, 3H, OCH₃); 7.16 (dd, 1H, Ar, $J = 2.2, 8.0$ Hz); 7.45 (t, 1H, $H_{5'}$, $J = 7.7$ Hz); 7.49 (s, 1H, $H_{2'}$); 7.53 (d, 1H, Ar, $J = 7.7$ Hz); 7.75 (d, 1H, H_7 , $J = 8.4$ Hz); 7.88–7.92 (m, 2H, Ar); 8.24–8.25 (m, 2H, Ar); 10.46 (s, 1H, NH); 10.50 (s, 1H,

NH). Anal. Calcd for $C_{20}H_{15}N_5O_5S$: C, 54.92; H, 3.46; N, 16.01. Found: C, 55.03; H, 3.52; N, 16.25.

4.1.2.15. *N'*-(3,4-Dimethoxybenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6f. Yield 48%. mp 198 °C (dec). IR (KBr), cm^{-1} : 3568, 3296 (NH), 1670, 1598 (Ar), 1518, 1337 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.75 (s, 3H, OCH₃); 3.80 (s, 3H, OCH₃); 7.08 (d, 1H, H_{5'}, *J* = 8.4 Hz); 7.53 (d, 1H, H₃, *J* = 3.3 Hz); 7.58 (dd, 1H, H_{6'}, *J* = 1.5, 8.4 Hz); 7.76 (d, 1H, H₇, *J* = 8.6 Hz); 7.88 (dd, 1H, H₆, *J* = 2.3, 8.6 Hz); 7.92 (d, 1H, H₄, *J* = 3.1 Hz); 8.24–8.26 (m, 2H, Ar); 10.39 (s, 1H, NH); 10.51 (s, 1H, NH). Anal. Calcd for $C_{21}H_{17}N_5O_6S$: C, 53.96; H, 3.67; N, 14.98. Found: C, 54.12; H, 3.69; N, 14.90.

4.1.2.16. *N'*-(3,4,5-Trimethoxybenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6g. Yield 76%. mp 285 °C (dec). IR (KBr), cm^{-1} : 3348 (NH), 1644 (Ar), 1584, 1520, 1334 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 3.73 (s, 3H, OCH₃); 3.85 (s, 6H, OCH₃); 7.29 (s, 2H, H_{2',6'}); 7.50 (d, 1H, H₃, *J* = 4.5 Hz); 7.74 (d, 1H, H₇, *J* = 8.4 Hz); 7.88 (dd, 1H, H₆, *J* = 2.1, 8.4 Hz); 7.94 (d, 1H, H₄, *J* = 4.5 Hz); 8.22 (d, 1H, H₄, *J* = 2.1 Hz); 10.48 (s, 1H, NH); 10.56 (s, 1H, NH). Anal. Calcd for $C_{22}H_{19}N_5O_7S$: C, 53.12; H, 3.85; N, 14.08. Found: C, 53.16; H, 3.79; N, 14.09.

4.1.2.17. *N'*-(3-Phenoxybenzoyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6h. Yield 82%. mp 200 °C(dec). IR (KBr), cm^{-1} : 3472 (NH), 1683 (Ar), 1578, 1337 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 7.08 (d, 2H, Ar, *J* = 7.7 Hz); 7.23 (t, 1H, Ar, *J* = 7.3 Hz); 7.25 (dd, 1H, Ar, *J* = 2.2, 8.1 Hz); 7.44 (t, 2H, H_{3',5'}, *J* = 7.3 Hz); 7.54–7.57 (m, 3H, Ar); 7.74–7.76 (m, 2H, Ar); 7.88 (dd, 1H, H₆, *J* = 1.7, 8.6 Hz); 7.93 (d, 1H, H₄, *J* = 4.6 Hz); 8.26 (d, 1H, H₄, *J* = 1.7); 10.56 (s, 1H, NH); 10.61 (s, 1H, NH). Anal. Calcd for $C_{25}H_{17}N_5O_5S$: C, 60.12; H, 3.43, N, 14.02. Found: C, 60.13; H, 3.47; N, 14.17.

4.1.2.18. *N'*-(3-Hydroxy-2-naphthyl)-2-(5-nitrothiophen-2-yl)-3H-benzo[d]imidazole-5-carbohydrazide 6i. Yield 89%. mp 230 °C (dec). IR (KBr), cm^{-1} : 3472 (NH), 1651, 1650 (Ar), 1521, 1336 (NO₂). ¹H RMN, DMSO-*d*₆, δ ppm: 5.4 (br s, 1H, OH); 7.35 (s, 1H, Ar); 7.52 (t, 1H, Ar, *J* = 6.9 Hz); 7.57 (d, 1H, H₃, *J* = 4.5 Hz); 7.66 (t, 1H, Ar, *J* = 6.3 Hz); 7.76–7.77 (m, 1H, Ar); 7.88 (dd, 1H, H₆, *J* = 1.6, 8.4 Hz); 7.91 (d, 1H, Ar, *J* = 8.4 Hz); 7.94 (d, 1H, H₄, *J* = 4.5 Hz); 8.26 (d, 1H, H₄, *J* = 1.6 Hz); 8.58 (s, 1H, Ar); 10.73 (s, 1H, NH); 10.79 (s, 1H, NH). Anal. Calcd for $C_{23}H_{15}N_5O_5S$: C, 58.35; H, 3.19; N, 14.79. Found: C, 58.37; H, 3.23; N, 14.67.

4.2. Biological assays

4.2.1. Inhibition of heme polymerization

The heme polymerization assay was performed according to an already published protocol,²⁵ briefly, a solution of hemin chloride (50 μ L, 4 mM), dissolved in DMSO (5.2 mg/mL), was distributed in 96-well micro plates. Different concentrations (100–5 mM) of the compounds dissolved in DMSO, were added in triplicate in test wells (50 μ L). Controls contained either water (50 μ L) or DMSO (50 μ L). β -Hematin formation was initiated by the addition of acetate buffer (100 μ L 0.2 M, pH 4.4). The plates were incubated at 37 °C for 48 h to allow for completion of the reaction and centrifuged (4000 rpm \times 15 min, IEC-CENTRA, MP4R). After discarding the supernatant, the pellet was washed twice with DMSO (200 μ L) and finally, dissolved in NaOH (200 μ L, 0.2 N). The solubilized aggregates were further diluted 1:2 with NaOH (0.1 N) and absorbance recorded at 405 nm (Microplate Reader, BIORAD-550). The results were expressed as a percentage of inhibition of flavoprotein (FP) polymerization.

4.2.2. Parasite, experimental host and strain maintenance

Male Balb-C mice, weighing 18–22 g were maintained on a commercial pellet diet and housed under conditions approved by Ethics Committee. *P. berghei* (ANKA strain), a rodent malaria parasite, was used for infection. Mice were infected by ip injection with 1×10^6 infected erythrocytes diluted in phosphate buffered saline solution (PBS, 10 mM, pH 7.4, 0.1 mL). Parasitemia was monitored by microscopic examination of Giemsa stained smears.³³

4.2.3. Parasite extracts

Blood of infected animals, at a high level of parasitemia (30–50%), was collected by cardiac puncture with an heparinized syringe and the blood pool was centrifuged (500g \times 10 min, 4 °C). Plasma and buffy coat were removed and the red blood cells (RBC) pellet was washed twice with chilled PBS-Glucose (5.4 %). The washed RBC pellet was centrifuged on a discontinuous percoll gradient (80–70% percoll in PBS-Glucose, 20,000g \times 30 min \times 4 °C).³⁴ The upper band (mature forms) was removed by aspiration, collected in eppendorf tubes and washed twice with chilled PBS-Glucose and the infected erythrocytes were lysed with the non-ionic detergent saponin (0.1% in PBS \times 10 min). 1 mL of cold PBS was added and the samples were centrifuged (13,000g \times 5 min, 4 °C) to remove erythrocyte cytoplasm content (including erythrocyte haemoglobin). The free parasites were mixed PBS-Glucose (5.4 %), and subjected to three freeze–thaw cycles (–70 °C/+37 °C). The final homogenate was used in the haemoglobin hydrolysis inhibition assay.³⁵

4.2.4. Mice native hemoglobin

Native hemoglobin from non-infected mice was obtained by treating one volume of pellet erythrocytes with two volumes of water. The resulting solution was used as the substrate in the inhibition of the hemoglobin hydrolysis assay.

4.2.5. Inhibition of hemoglobin hydrolysis

The proteolytic effect of the parasite extract on the native mice hemoglobin was assayed using 96-wells tissue culture plate (Greiner Bio-One). The assay mixture contained: mice native hemoglobin (10 μ L), parasite extract (50 μ L), GSH (10 μ L, 10 μ M), and acetate buffer (0.2 M, pH 5.4) to a final volume of 100 μ L. The compounds (10 μ M) were incorporated in the incubation mixture dissolved in DMSO. The incubations were carried out at 37 °C for 18 h and the reactions were stopped by addition of reduced sample buffer. The degree of digestion was evaluated electrophoretically by SDS–PAGE by visual comparison of the globin bands (14 kDa). A DMSO control was electrophoresed at the same time. Once the bands were obtained, the densitometer registered the band densities reported as intensity/mm² \pm SD, so we proceeded to check the densities in order to have a percentage of inhibition of hemoglobin hydrolysis.

4.2.6. 4-Day suppressive test

Balb-C mice (18–23 g) were infected iv (using caudal vein) with 10^6 infected red blood cells with *P. berghei* (*n* = 6). Two hours after infection, treatment began with the best compounds tested in the in vitro assays. These were dissolved in DMSO (0.1 M), diluted with Saline-Tween 20 solution (2%). Each compound (20 mg/kg) was administered once by ip for 4 days. At day four, the parasitemia was counted by examination of Giemsa stained smears. Chloroquine (25 mg/kg) was used as a positive control. The survival time beyond the control group (without drug treatment) was recorded. The results were expressed as percentage of parasitemia (% of parasitemia) and survival days of each compound-treated group over the control (non-treated group).³⁶

4.2.7. Anti-*Mycobacterium tuberculosis* assay

A suspension of MTB was made by mixing growth from slants (20–30 days old) with 100 μ L of Tween 80 into 0.2% bovine serum albumin. Turbidity of the suspension was then adjusted to a McFarland standard No. 1 (3×10^7 CFU/mL) by adding Tween 80 and bovine serum albumin. The bacterial suspension (300 μ L) was further transferred to 7.2 mL of 7H9GC broth (4.7 g of Middlebrook 7H9 broth base, 20 mL of 10% glycerol, 1 g of Bacto Casitone, 880 mL of distilled water, 100 mL of OADC (oleic acid, albumin, dextrose, catalase). For the bioassay, the compounds were resuspended in DMSO at a concentration of 4 mg/mL (stock solution). These stock solutions were further diluted with appropriate volumes of 7H9GC broth to yield final concentration of 0.4–25 μ g/mL. Final drug concentrations ranges of standard antibiotics used as positive controls were 0.125–32 μ g/mL for isoniazid and 0.063–16 μ g/mL for rifampin. The drug (100 μ L) was mixed in the wells with 100 μ L of bacterial inoculums, resulting in a final bacterial concentration of approximately 1.2×10^6 CFU/mL. The wells in column 11 served as inoculum-only controls. Solvent (DMSO) was included in every experiment as a negative control. Plants were sealed in plastic bags and then incubated at 37 °C for 5 days. On day 5, 50 μ L of tetrazolium-Tween 80 mixture {1.5 mL of tetrazolium[3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] at a dilution of 1 mg/mL, in absolute ethanol and 1.5 mL of 10% Tween 80} was added to the wells, and the plate was incubated at 37 °C for 24 h. After this period, the growth of the microorganism was visualized by the change in color of the dye from yellow to purple. The tests were carried out in triplicate. MIC is defined as the lowest drug concentration that prevents the aforementioned change in color.³⁷

4.2.8. Cell growth inhibition bioassay

The compounds were screened for cytotoxicity against a panel of human tumor cell lines and two nontumorigenic cell lines, using previously reported methodologies³⁸ at a maximum concentration of 250 μ g/mL. Briefly, growth inhibition was evaluated by preparing serial dilutions of each compound and incubating the cells in 96-well plates for 48 h at 37 °C. Appropriate solvents controls were tested for comparison. The percent inhibition of cell growth relative to the control was evaluated colorimetrically using a sulforhodamine B dye according to a published procedure³⁹ by comparison to the control. The GI₅₀ value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and was determined by linear regression analysis.

4.3. Data analysis

Data were statistically analyzed using one-way ANOVA and t-tests for specific group comparisons; assuming 95% of confidence according GRAPHPAD Prism 3.02.⁴⁰

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