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Article

Old Dogs with New Tricks: Antiparasitic Potential of Structurally Diverse 5-Nitrofuran and 5-Nitrothiophene Imines and Acyl Hydrazones

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Abstract: Miscellaneous imines and acyl hydrazones were prepared from 5-nitrofuraldehyde and 5-nitrothiophene-2-carboxaldehyde. Their activities against *Toxoplasma gondii* and *Leishmania major* parasites were evaluated. Promising antiparasitic effects and selectivities were observed for certain acyl hydrazones and imines. Cobalt(II) and copper(II) complexes conserved the high anti-*Toxoplasma* activities of 3-hydroxy-2-naphthoic carboxyl hydrazone (**2a**). In addition, sound activities against *L. major* promastigotes were observed for various analogs of **2a** (**2b** and **2i**) and pyrid-2-ylpyrazole-based imines (**3g** and **3h**). Relatively low toxicities to kidney cells and macrophages indicate promising selectivity profiles for these compounds.

Keywords: nitroarene drugs; neglected tropical diseases; leishmaniasis; toxoplasmosis; antiparasitic drugs

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1. Introduction

Nitro-functionalized heterocycles play a vital role in the daily fight against various life-threatening infectious diseases. Nifurtimox and benznidazole are currently the therapeutics of choice for patients suffering from Chagas disease [1]. In 2009, nifurtimox–eflornithine combination therapy (NECT) was introduced for the treatment of second-stage gambiense Human African Trypanosomiasis and proved to be an applicable and safe therapy, even for patients in remote African regions [2,3]. Further structurally related and clinically applied antibiotics are the semicarbazone nitrofural, the 4-hydroxybenzhydrazide nifuroxazide, and the 5-nitrothienyl derivative nifurzide (Figure 1) [4,5]. Nifuroxazide has been repurposed for cancer treatment due to its STAT3-inhibitory activity [6]. It also showed considerable in vitro activity against *Leishmania donovani* promastigotes; however, its 5-nitrothiophene analog was slightly more active [7]. Notably, 3,4-dihalophenyl analogs of nifuroxazide showed distinct antiparasitic effects [7]. Thus, the structure of the aroyl hydrazide contributes significantly to the antiparasitic activity of 5-nitrofuran- and 5-nitrothiophene-based hydrazones and enables a chemical fine-tuning of the nitroarene drug candidates. In addition to halogenated aroyl moieties, hydroxy-substituted compounds (e.g., 3-hydroxy-2-naphthoyl and salicyl derivatives) and nicotinoyl analogs appear to be promising based on the outcomes of previous biological studies [7–9].

Toxoplasmosis is caused by infection with the protozoal apicomplexan parasite *Toxoplasma gondii* and is considered one of the most common zoonoses, affecting about one third of the world population [10,11]. While immune-competent infected persons usually

do not develop symptoms, immune-compromised individuals, such as HIV patients and transplant patients as well as newborn children, can suffer from severe complications, indicating the necessity of efficient medical management of the infection in vulnerable people. Clinically applied anti-*Toxoplasma* drugs include sulfonamide-based dihydropteroate synthase (DHPS) inhibitors, the dihydrofolate reductase (DHFR) inhibitors pyrimethamine and trimethoprim, as well as the redox-active naphthoquinone atovaquone, and the combination of the sulfonamide sulfadiazine with pyrimethamine is currently the standard therapy for toxoplasmosis [12]. Valuable targets for toxoplasmosis treatment include the apicoplast, the rhoptry organelles and mitochondria, the invasion complex, and tachyzoite–brachyzoite interconversion [13]. Resistance to sulfadiazine, pyrimethamine, and atovaquone treatment was observed in *T. gondii* strains and clinical isolates, which could pose a severe problem in the future and requires the development of new drugs [14]. Since sulfadiazine treatment is accompanied by nephrotoxicity, new drug candidates should also cause minimal renal damage [15].

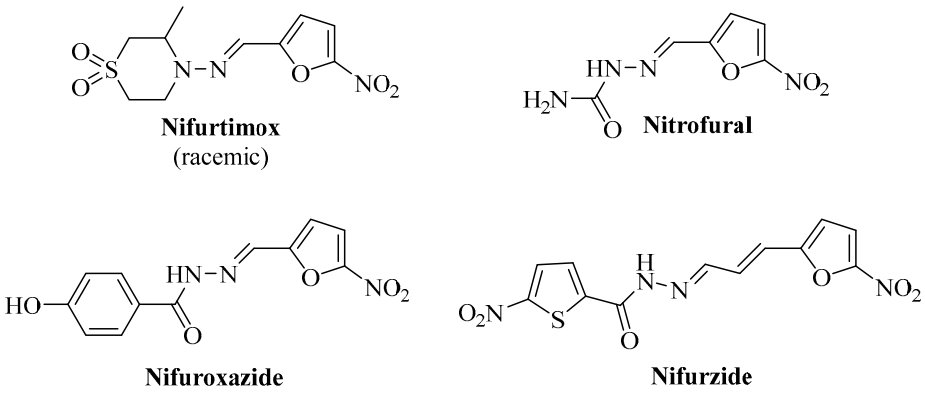


Figure 1. Clinically applied antiparasitic and antibiotic 5-nitrofuran drugs.

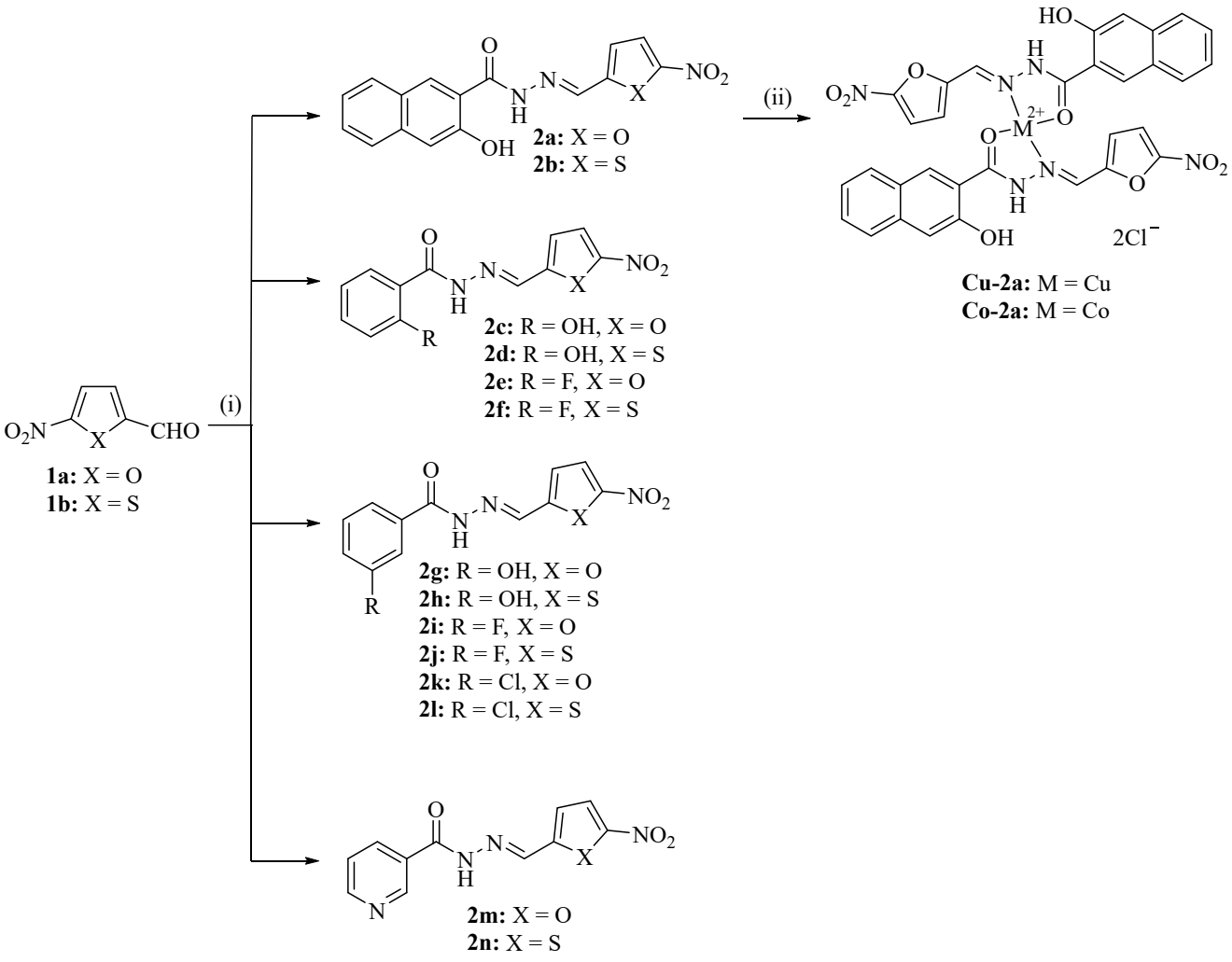
Leishmaniasis is another serious infectious disease, occurring clinically as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). CL is the most abundant form (ca. 1 million cases per year) and develops painful and disfiguring skin lesions upon infection with *L. major*, *L. tropica*, *L. mexicana*, or *L. amazonensis* [16,17]. Leishmaniasis is classified as a neglected tropical disease (NTD) lacking inexpensive and safe treatment options. CL patients are currently treated with pentavalent antimonials, miltefosine, amphotericin B, or pentamidine [16]. Emerging drug resistance and severe side effects limit the application of these drugs, and the identification and development of new drugs is required [18,19].

In this study, several known and new hydrazones and imines of 5-nitrofuraldehyde and 5-nitrothiophene-2-carboxaldehyde were prepared or repurposed and tested for their activities against *T. gondii* and *L. major* parasites in order to find new clues in the search for possible antiparasitic compounds suitable for further advanced preclinical and clinical investigations. In addition to the aforementioned aroyl hydrazide compounds, various new imines/Schiff bases of nitrofurans/-thiophenes with halogen-substituted anilines, semisynthetic 28-aminobetulin, and a pyridyl-substituted aminopyrazole were investigated. Betulins and betulinic acids are prominent anticancer and antiparasitic compounds, and, thus, imines of 28-aminobetulin can also become valuable antiparasitic drugs [20,21]. Derivatives of aminopyrazoles, including ureas and amides, were described as promising antiparasitic compounds by research groups associated with the Drugs for Neglected Diseases initiative (DNDi), and new related imines were designed and investigated in this study [17,22,23].

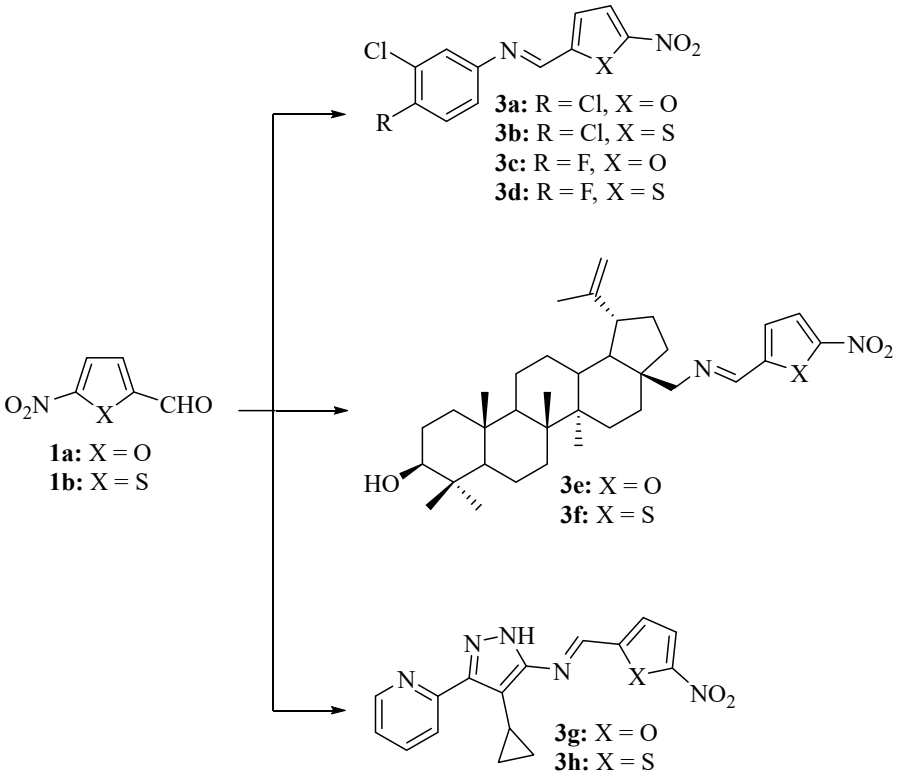
2. Results

2.1. Chemistry

N-Acyl hydrazones **2a–n** were prepared from 5-nitrofuraldehyde (**1a**) or 5-nitrothiophene-2-carboxaldehyde (**1b**) and the corresponding aroyl hydrazides in hot ethanol (Scheme 1). The 2-fluorophenyl derivatives **2e** and **2f** are new, while the other compounds (**2a–d** and **2 g–n**) were described before. However, within this compound series, the antileishmanial activities relevant for this study were only reported for compounds **2g** and **2h** [7]. The new Cu(II) and Co(II) complexes of **2a**, which were dubbed **Cu-2a** and **Co-2a**, were prepared from the reaction of **2a** with the metal chloride salts $CuCl_2$ and $CoCl_2$, respectively [24]. The new imines/Schiff bases **3a–h** were synthesized analogously to compounds **2a–n** from **1a** or **1b** and the corresponding amine precursor (Scheme 2). 3,4-Dihalogenated anilines were chosen because this structural motif had previously shown distinct antiparasitic effects [7]. In addition, 28-aminobetulin was selected for condensation with **1a** and **1b** because of the well-documented antiparasitic activities of betulin derivatives [21,25]. Recently, the globally acting open synthesis network of the DNDi identified 3-amino-4-cyclopropyl-5-(2-pyridyl)-pyrazole as a promising scaffold for the development of antitrypanosomal drug candidates, and it was applied in this study to prepare the new imines **3g** and **3h** [26].



Scheme 1. Reagents and conditions: (**i**) aroyl hydrazide, EtOH, reflux, 2 h; (**ii**) MCl_2, 1,4-dioxane, reflux, 1 h.



Scheme 2. Reagents and conditions: amine, EtOH, reflux, 2 h, 42–93%.

2.2. Antiparasitic Activity

Compounds **2a–n**, **Cu-2a**, **Co-2a**, and **3a–h** were tested for their activity against *Toxoplasma gondii* parasites (Table 1). 28-Aminobetulin was added to the panel of test compounds because there are no data for this betulin derivative in terms of antiparasitic activity. Vero kidney epithelial cells and macrophages served as non-malignant cells to evaluate the parasite selectivity and toxic side effects of the test compounds. The 3-hydroxy-2-naphthoic hydrazide derivatives **2a** and **2b** were the most active compounds, with excellent IC_{50} values almost reaching the activity of the positive control atovaquone. Compound **2a** displayed considerable selectivity for *T. gondii* when compared with Vero cells, with a selectivity index (SI) of 23. 3-Fluorobenzoic hydrazide **2i** also exhibited strong activity against *T. gondii* (IC_{50} below 1 μM). However, the selectivity of **2i** was lower when compared with the selectivities of **2a** and **2b**. The salicyl derivative **2c** also showed an amenable selectivity profile (SI = 17.7), while its thiophene analog (**2d**) was only moderately active without any selectivity for *T. gondii*. However, the other 5-nitrofuran-2-ylidene derivatives (**2e**, **2g**, **2k**, and **2m**) were much less active than their thiophene-based congeners (**2f**, **2h**, **2l**, and **2n**). The investigation of the toxicities of the test compounds to macrophages, and their correlation with the found antitoxoplasmal activities revealed an excellent SI value of over 100 (123) for **2a**, while **2b** reached an SI value of almost 60 (Table 1).

In order to evaluate the scope of the metal compounds based on the highly active ligand **2a**, the Cu(II) and Co(II) complexes **Cu-2a** and **Co-2a** were prepared and tested for their activities against *T. gondii*. Both complexes conserved the high activity of their ligand (**2a**). Complex **Co-2a** also exhibited considerable selectivity for *T. gondii* and a toxicity to Vero kidney cells and macrophages similar to ligand **2a**. The Cu(II) complex **Cu-2a** was more toxic to Vero cells than **Co-2a**, while both complexes showed similar toxicities to macrophages.

Table 1. Cytotoxic concentrations (CC_{50}, μM) of test compounds **2a–n**, **Cu-2a**, **Co-2a**, **3a–h**, and 28-aminobetulin (28-AB) when applied to cells of the Vero (African green monkey kidney epithelial) cell line and inhibitory concentrations (IC_{50}) when applied to macrophages and cells of *Toxoplasma gondii*. [1] Atovaquone (ATO) and amphotericin B (AmB) were applied as positive controls.

Compd.	IC_{50} (*T. gondii*)	CC_{50} (Vero)	IC_{50} (Macrophages)	SI (Vero/*T. gondii*) [2]	SI (Macrophages/*T. gondii*) [2]
2a	0.12	2.77	14.8	23.1	123
2b	0.35	3.28	20.3	9.4	58.0
2c	1.16	20.5	28.8	17.7	24.8
2d	12.7	11.2	24.7	0.88	2.0
2e	20.2	40.0	24.5	2.0	1.2
2f	3.07	23.2	16.4	7.6	5.3
2g	22.5	47.6	18.9	2.1	0.84
2h	3.78	30.6	9.27	8.1	2.5
2i	0.90	2.71	7.72	3.0	8.6
2j	1.30	10.1	7.23	7.8	5.6
2k	19.8	31.7	15.0	1.6	0.76
2l	1.61	14.2	9.36	8.8	5.8
2m	12.9	14.2	28.4	1.1	2.2
2n	1.27	4.53	10.5	3.6	8.3
Cu-2a	0.28	1.78	11.2	6.4	40.0
Co-2a	0.15	2.70	11.0	17.9	73.3
3a	13.7	12.1	9.96	0.88	0.73
3b	4.15	12.6	12.3	3.0	3.0
3c	4.77	13.2	15.8	2.8	3.3
3d	4.92	11.2	15.8	2.3	3.2
3e	7.97	5.67	13.5	0.71	1.7
3f	15.8	17.7	7.51	1.1	0.48
3g	9.90	24.4	17.9	2.5	1.8
3h	14.1	15.6	26.2	1.1	1.9
28-AB	0.16	0.34	10.5	2.1	65.6
ATO [3]	0.07	9.5	-	136	-
AmB [3]	-	-	8.1	-	-

[1] Values are means of at least three independent experiments (SD ± 15%) from concentration–response curves (percentage of treated cells compared to untreated controls) after 72 h. [2] Selectivity index (SI) was calculated from the corresponding CC_{50} (Vero) and IC_{50} values (*T. gondii*) or the corresponding IC_{50} values of macrophages and *T. gondii*. [3] Values were taken from ref. [27].

Among the new imines **3a–h**, derivatives **3b–d** were active against *T. gondii* at concentrations below 5 μM, while the other compounds showed rather moderate activities (IC_{50} values of 8–16 μM). The SI values calculated with Vero cells were generally low, with a value of three for **3b** being the best SI value found in this compound series. Most strikingly, 28-aminobetulin was much more active against *T. gondii* and toxic to Vero cells than its imines (**3e** and **3f**), indicating that other strategies of 28-aminobetulin modification should be explored in order to conserve its very promising anti-*Toxoplasma* activity while reducing its kidney cell toxicity. However, 28-aminobetulin revealed a low toxicity to macrophages, and, thus, a considerable SI value of 65.6 was determined with macrophages.

Next, the antileishmanial effects of the test compounds were studied using *L. major* promastigotes and amastigotes and were compared with their toxic effects on macrophages (Table 2). Compound **2i** showed by far the highest activity against *L. major* promastigotes, distinctly exceeding the activity of amphotericin B by a factor of nearly 10. Moreover, the toxicity of **2i** to macrophages was 89 times lower than to promastigotes (SI = 88.7). Compounds **2b**, **2j**, and **2k** also revealed promising activities against *L. major* promastigotes, while of these compounds only **2j** was likewise active against the amastigotes. Compounds **2b** and **2i** were the only compounds with sound activities against *L. major* promastigotes and *T. gondii* parasites, which were accompanied by distinctly lower toxic effects on Vero cells and macrophages. Compound **2k** was particularly selective for promastigotes because amastigotes, *T. gondii* cells, Vero cells, and macrophages were much less sensitive to this compound. In contrast, **2h** and **2l** displayed reasonable activity against amastigotes (IC_{50} = 2.75 and 2.91 μM, respectively) while being less active against the promastigotes.

Compounds **2h** and **2l** were less than one third as toxic to macrophages compared to amastigotes. Compound **2a** and its metal complexes were only moderately active against *L. major* promastigotes and amastigotes. Only the cobalt complex **Co-2a** was slightly more active against promastigotes than ligand **2a**. It is noteworthy that the imines **3g** and **3h** exhibited considerable activities against *L. major* promastigotes, which were significantly higher than against *L. major* amastigotes, *T. gondii* parasites, Vero cells, and macrophages. The betulin derivatives **3e** and **3f** were also more active against promastigotes than against amastigotes and *T. gondii*. *L. major* parasites were generally much less sensitive to their precursor (28-aminobetulin) than *T. gondii* parasites. However, 5-nitrofuran **3e** revealed the highest activity against amastigotes among the imine series of compounds, which was 2.5 times higher than against macrophages. Only 28-aminobetulin and the positive control (amphotericin B) showed higher selectivities for the *L. major* amastigotes (SI = 6.6 and 17.2, respectively). The chlorophenyl derivatives **3a**, **3b**, and **3d** were active against promastigotes, with IC_{50} values comparable to the activities of **3e** and **3f**. Only **3c** was distinctly less active.

Table 2. Inhibitory concentrations (IC_{50}, μM) of test compounds **2a–n**, **Cu-2a**, **Co-2a**, **3a–h**, and 28-aminobetulin (28-AB) when applied to promastigotes and amastigotes of *Leishmania major*. [1] Amphotericin B (AmB) was applied as a positive control.

Compd.	IC_{50} Promastigotes	IC_{50} Amastigotes	SI (Macrophages/ Promastigotes) [2]	SI (Macrophages/ Amastigotes) [2]
2a	5.84	5.53	2.53	2.68
2b	0.88	24.0	23.1	0.85
2c	4.83	32.0	5.96	0.90
2d	3.78	28.8	6.53	0.86
2e	6.49	22.7	3.78	1.08
2f	5.11	17.7	3.21	0.93
2g	8.36	21.1	2.26	0.90
2h	17.9	2.75	0.52	3.37
2i	0.087	9.01	88.7	0.86
2j	1.36	3.75	5.32	1.93
2k	1.02	16.4	14.7	0.92
2l	4.84	2.91	1.93	3.22
2m	5.23	32.7	5.43	0.87
2n	3.26	11.2	3.22	0.94
Cu-2a	7.52	8.15	1.49	1.37
Co-2a	4.74	6.66	2.32	1.65
3a	4.63	11.6	2.15	0.86
3b	4.25	9.30	2.89	1.32
3c	15.7	11.5	1.01	1.37
3d	4.92	9.48	3.21	1.67
3e	3.01	5.49	4.49	2.46
3f	5.51	14.1	1.36	0.53
3g	1.11	14.2	16.1	1.26
3h	1.00	29.8	26.2	0.88
28-AB	2.94	1.59	3.57	6.60
AmB [3]	0.83	0.47	9.76	17.2

[1] Values are the means of three experiments (SD ± 15%). They were obtained from concentration–response curves by calculating the percentage of treated cells compared to untreated controls after 72 h. [2] Selectivity index (SI) was calculated from the corresponding IC_{50} values of macrophages (see Table 1) and promastigotes or amastigotes. [3] Values were taken from ref. [27].

3. Discussion

The testing of various 5-nitrofuran and 5-nitrothiophene derivatives for their activities against *T. gondii* and *L. major* parasites revealed encouraging results. But in addition to antiparasitic activity, the selectivity of test compounds for parasites is a crucial factor for the identification of new drug candidates. The 3-hydroxy-2-naphthoic hydrazide **2a** was identified as a promising anti-*Toxoplasma* compound in terms of activity and selectivity. Nitroaromatic compounds are prodrugs that are activated by cellular nitroreductases, leading to toxic radicals and reactive oxygen species in trypanosomatids [28]. This is an efficient way to kill *T. gondii* parasites, and nitrofurantoin, for example, exhibited strong in vitro

and in vivo anti-toxoplasmal activities [29]. Moreover, the immunomodulatory activities of nitrofuran drugs might be relevant for future applications of **2a** and its analogs [30]. The benefits and problems of nitro compounds for the treatment of protozoal parasites were reviewed recently [31]. Aside from toxic effects based on nitroaryl-correlated oxidation mechanisms, additional structure-dependent mechanisms of action likely play a role in the observed differences in the activity of compounds **2a–n**. For instance, compound **2j** was identified as a protein import blocker in the mitochondria of yeast and mammalian cells that targets a Tim44 interaction with Hsp70 [32]. In addition, dynasore, which is a catechol-based analog of **2a** and **2b**, is a well-established endocytosis inhibitor that blocks the guanosine triphosphatase (GTPase) dynamin [33]. Dynasore also regulates cellular cholesterol homeostasis and induces actin remodeling. More recently, it was shown that dynasore and its derivatives are strong inhibitors of viral ribonucleases necessary for the processing of RNA molecules [33,34]. Dynamin proteins play crucial roles in the life cycle of *T. gondii* parasites [35–37]. Thus, it is not surprising that dynasore and its analogs exert eminent antiparasitic effects in *T. gondii* infection models, which include drug-related influences on invasion, migration, and interleukin-10 (IL-10) formation [8,38,39]. The extent that compounds **2a** and **2b** also act in a dynasore-like manner against protozoal parasites remains to be clarified. However, the 3-hydroxy-2-naphthoic acid moieties of **2a** and **2b** obviously play a significant role in their activity and might also have a positive impact on the activities of other antiparasitic drugs as well as on the circumvention of drug resistance mechanisms [14,18]. In particular, atovaquone resistance was observed in apicomplexan parasites including *T. gondii* and *Plasmodium falciparum*, which necessitates the development of new drugs [14,40]. Moreover, cases of *T. gondii* infection prophylaxis failure in hematopoietic cell transplant recipients were reported upon treatment with atovaquone [41].

In addition, metal complexes of **2a** conserved its activity against *T. gondii* and, thus, are valuable alternatives to **2a** for the treatment of toxoplasmosis that warrant advanced investigations. For instance, the activity of these compounds against other *T. gondii*-related apicomplexan parasites such as *Plasmodium* might turn out to be equally promising, and new drugs against malaria are required in order to overcome the shortcomings of currently available therapies. The copper complex **Cu-2a** was distinctly more toxic to Vero cells than **2a**, which might be attributed to the well-documented cytotoxic effects of copper ions and complexes [42,43]. Only a few Cu(II) complexes with considerable anti-*Toxoplasma* activity were described, while no Co(II) complexes have been published until now showing activity against *T. gondii* parasites [44]. Antileishmanial Co(II) complexes with comparable activities were recently disclosed, and their mechanism of action involved mitochondrial damage associated with the induction of apoptosis-like parasite death [45]. Both Cu(II) and Co(II) complexes were also reported to target DNA, while Co(II) salts such as $CoCl_2$ are well-established hypoxia inducers [46–48].

This study shows that there is no general advantage of 5-nitrofurans over 5-nitrothiophenes. Although the most active compounds **2a** and **2i** were 5-nitrofurans, the NH_2-substituted starting compound and binding partner (i.e., the hydrazide or amine starting compound) often played a crucial role in the antiparasitic effects of the target compounds. 5-Nitrothiophenes were casually more active than their 5-nitrofuran congeners, which matched the results of a published study with compound **2** analogs that were presumably based on a higher reduction rate of nitrothiophenes by nitroreductases because of unoccupied sulfur d orbitals [7]. However, differences sometimes also depended on the tested parasite species and even on certain cell forms within one species, such as the promastigotes and amastigotes of *L. major*. These differences were clearly less pronounced among the new imine compound series (**3a–h**). However, the elucidation of the underlying mechanisms is a challenging task, and the observed activities can be explained only in part by the peculiar chemical and physical properties of the described structural scaffolds. The activity of 28-aminobetulin against *T. gondii* and *L. major* was remarkable. Its new analog **3e** also displayed considerable activity against *L. major* amastigotes and promastigotes, and, thus, the preparation and investigation of more 28-aminobetulin derivatives appears

reasonable in the future. Strikingly, pyrazoles **3g** and **3h** were among the more active antileishmanial imine derivatives, and various compounds based on pyrid-2-ylpyrazole scaffolds have already been identified as antiparasitic drugs with significant selectivity for certain kinetoplastid parasites [23,25]. The mechanism of action of this class of antiparasitic aminopyrazoles is still not clearly understood, but it was shown that potent examples were not substrates for drug efflux pumps associated with multi-drug resistance [22]. Moreover, it has to be mentioned that the imines **3a–d** appeared to be less antileishmanial than previously published analogous 3,4-dichlorobenzoic hydrazide derivatives [7].

4. Materials and Methods

4.1. Chemistry

The known compounds **2a–d** and **2g–n** were prepared and analyzed in accordance with reports in the literature [7,32]. Starting compounds were purchased from common providers. 28-Aminobetulin was obtained from Nature Science Technologies (Olaine, Latvia), and 3-amino-4-cyclopropyl-5-(pyrid-2-yl)-pyrazole was obtained from TCG Lifesciences Pvt. Ltd. (Kolkata, India). Melting points were determined with an Electrothermal 9100 and were not corrected. NMR spectra were measured with a Bruker Avance 300 spectrometer, and chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as an internal standard. High-resolution mass spectra were measured with a UPLC/Orbitrap (ESI-HRMS).

2-Fluorobenzoic (5-nitrofuran-2-ylidene)hydrazide (**2e**)

2-Fluorobenzoic hydrazide (154 mg, 1.0 mmol) and 5-nitrofuraldehyde (141 mg, 1.0 mmol) were dissolved in EtOH (15 mL). The reaction mixture was stirred under reflux for 2 h. The formed precipitate was collected, washed with EtOH, and dried in a vacuum. Yield: 213 mg (0.77 mmol, 77%); orange solid of m.p. 217–218 °C; υ_{max}(ATR)/cm^{-1} 3358, 3141, 1668, 1611, 1550, 1514, 1471, 1393, 1351, 1335, 1285, 1253, 1239, 1211, 1172, 1143, 1097, 1056, 1013, 958, 906, 880, 825, 809, 784, 754, 737, 685, 654; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 7.2–7.4 (3 H, m), 7.5–7.7 (2 H, m), 7.8–7.9 (1 H, m), 8.29 (1 H, s), 12.25 (1 H, s); ^{13}C NMR (125.8 MHz, DMSO-d$_6$) δ 115.2–116.4 (m), 122.5–122.7 (m), 124.3, 124.8, 129.2, 129.8, 130.0, 130.2, 130.4, 132.4, 133.2–133.3 (m), 137.3, 141.5, 146.3, 150.5, 151.0, 157.5, 160.7, 160.8, 167.4; HRMS for $C_{12}H_9O_3N_3FS$ [M$^+$ + H]: calcd. 294.03432, found 294.03386.

2-Fluorobenzoic (5-nitrothien-2-ylidene)hydrazide (**2f**)

2-Fluorobenzoic hydrazide (154 mg, 1.0 mmol) and 5-nitrothiophene-2-carboxaldehyde (157 mg, 1.0 mmol) were dissolved in EtOH (15 mL). The reaction mixture was stirred under reflux for 2 h. The formed precipitate was collected, washed with EtOH, and dried in a vacuum. Yield: 165 mg (0.52 mmol, 52%); brown solid of m.p. 204–205 °C; υ_{max}(ATR)/cm^{-1} 3320, 3124, 1658, 1614, 1547, 1530, 1501, 1482, 1456, 1438, 1359, 1336, 1311, 1284, 1242, 1209, 1158, 1143, 1099, 1073, 1032, 965, 928, 907, 879, 822, 814, 784, 752, 731, 701, 682; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 7.3–7.4 (2 H, m), 7.5–7.7 (3 H, m), 8.1–8.2 (1 H, m), 8.56 (1 H, s), 12.24 (1 H, s); ^{13}C NMR (125.8 MHz, DMSO-d$_6$) δ 115.2–116.4 (m), 122.5–122.7 (m), 124.3, 124.8, 129.2, 129.8, 130.0, 130.2, 130.4, 132.4, 133.2–133.3 (m), 137.3, 141.5, 146.3, 150.5, 151.0, 157.5, 160.7, 160.8, 167.4; HRMS for $C_{12}H_9O_3N_3FS$ [M$^+$ + H]: calcd. 294.03432, found 294.03386.

Copper(II) complex (**Cu-2a**)

Compound **2a** (100 mg, 0.306 mmol) was dissolved in 1,4-dioxane (10 mL), and $CuCl_2$ x $2H_2O$ (26 mg, 0.153 mmol) was added. The reaction mixture was stirred under reflux for 1 h. After cooling down, the formed precipitate was collected, washed with ethanol, and dried in a vacuum. Yield: 35 mg (0.045 mmol, 29%); brown solid of m.p. 206–208 °C (dec.); υ_{max}(ATR)/cm^{-1} 3250, 3129, 3079, 2965, 2920, 2869, 1634, 1591, 1560, 1535, 1503, 1461, 1396, 1341, 1254, 1221, 1148, 1113, 1080, 1068, 1055, 1026, 969, 961, 931, 895, 862, 812, 801, 768, 754, 734, 669; HRMS for $C_{32}H_{21}O_{10}N_6Cu$ [M$^+$ - H]: calcd. 712.06097, found 712.06004.

Cobalt(II) complex (**Co-2a**)

Compound **2a** (100 mg, 0.306 mmol) was dissolved in 1,4-dioxane (5 mL), and $CoCl_2$ x $6H_2O$ (36 mg, 0.153 mmol) was added. The reaction mixture was stirred under reflux for 1 h. After cooling down, the formed precipitate was collected, washed with *n*-hexane, and

dried in a vacuum. Yield: 110 mg (0.141 mmol, 92%); yellow solid of m.p. 235–237 °C (dec.); υ_{max}(ATR)/cm^{-1} 3257, 3136, 3047, 2909, 2861, 1637, 1607, 1563, 1527, 1475, 1395, 1346, 1254, 1220, 1150, 1120, 1080, 1025, 970, 956, 923, 887, 871, 810,771, 760, 744, 735, 696; HRMS for $C_{32}H_{21}O_{10}N_6Co$ [M$^+$ - H]: calcd. 708.06457, found 708.06317.

(E)-N-(3,4-Dichlorophenyl)-1-(5-nitrofuran-2-ylidene)imine (**3a**)

3,4-Dichloroaniline (162 mg, 1.0 mmol) and 5-nitrofuraldehyde (141 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 3 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 264 mg (0.93 mmol, 93%); yellow solid of m.p. 123–125 °C; υ_{max}(ATR)/cm^{-1} 3140, 3079, 3013, 1563, 1514, 1484, 1458, 13973 1352, 1333, 1247, 1198, 1170, 1121, 1029, 963, 901, 875, 820, 810, 799, 739, 691, 669, 659, 599, 584; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.11 (1 H, dd, J = 8.5 Hz, 2.5 Hz), 7.20 (1 H, d, J = 3.9 Hz), 7.36 (1 H, d, J = 2.5 Hz), 7.41 (1 H, d, J = 3.9 Hz), 7.47 (1 H, d, J = 8.5 Hz), 8.34 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 112.6, 115.4, 120.7, 123.0, 131.1, 131.6, 133.4, 147.4, 149.3, 152.6; HRMS for $C_{11}H_7O_3N_2Cl_2$ [M$^+$ + H]: calcd. 284.98282, found 284.98233.

(E)-N-(3,4-Dichlorophenyl)-1-(5-nitrothien-2-yl)methanimine (**3b**)

3,4-Dichloroaniline (162 mg, 1.0 mmol) and 5-nitrothiophene-2-carboxaldehyde (157 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 3 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 134 mg (0.45 mmol, 45%); yellow solid of m.p. 154–155 °C; υ_{max}(ATR)/cm^{-1} 3093, 1605, 1558, 1529, 1494, 1464, 1435, 1394, 1332, 1314, 1272, 1220, 1190, 1137, 1041, 1026, 954, 913, 868, 828, 816, 733, 706, 693, 679, 640, 600; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.10 (1 H, dd, J = 8.5 Hz, 2.5 Hz), 7.35 (1 H, d, J = 2.5 Hz), 7.39 (1 H, d, J = 4.5 Hz), 7.46 (1 H, d, J = 8.5 Hz), 7.90 (1 H, d, J = 4.5 Hz), 8.50 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 120.9, 122.9, 128.4, 130.3, 131.0, 131.3, 133.3, 147.6, 149.0, 152.3, 154.4; HRMS for $C_{11}H_7O_2N_2Cl_2S$ [M$^+$ + H]: calcd. 300.95998, found 300.95939.

(E)-N-(3-Chloro-4-fluorophenyl)-1-(5-nitrofuran-2-yl)methanimine (**3c**)

3-Chloro-4-fluoroaniline (146 mg, 1.0 mmol) and 5-nitrofuraldehyde (141 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 3 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 195 mg (0.73 mmol, 73%); yellow solid of m.p. 154–156 °C; υ_{max}(ATR)/cm^{-1} 3147, 3122, 1624, 1567, 1519, 1490, 1400, 1356, 1335, 1255, 1233, 1197, 1163, 1122, 1051, 1026, 961, 917, 868, 823, 812, 807, 761, 737, 701, 690, 666, 591; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.1–7.2 (3 H, m), 7.3–7.4 (1 H, m), 7.41 (1 H, d, J = 3.8 Hz), 8.34 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 112.7, 115.1, 117.1–117.4 (m), 121.2–121.3 (m), 123.2, 146.5–146.8 (m), 152.8, 155.9–159.2 (m); HRMS for $C_{11}H_7O_3N_2ClF$ [M$^+$ + H]: calcd. 269.01237, found 269.01196.

(E)-N-(3-Chloro-4-fluorophenyl)-1-(5-nitrothien-2-yl)methanimine (**3d**)

3-Chloro-4-fluoroaniline (146 mg, 1.0 mmol) and 5-nitrothiophene-2-carboxaldehyde (157 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 3 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 120 mg (0.42 mmol, 42%); yellow solid of m.p. 152–153 °C; υ_{max}(ATR)/cm^{-1} 3108, 1609, 1586, 1536, 1489, 1438, 1389, 1366, 1350, 1333, 1311, 1253, 1222, 1208, 1187, 1122, 1052, 1039, 965, 937, 914, 870, 814, 797, 761, 732, 712, 702, 661, 600, 565; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.1–7.2 (2 H, m), 7.3–7.4 (2 H, m), 7.90 (1 H, d, J = 4.2 Hz), 8.50 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 117.0–117.3 (m), 121.4–122.0 (m), 123.1, 128.4, 130.0, 146.2–146.3 (m), 147.8, 151.7, 155.7–159.0; HRMS for $C_{11}H_7O_2N_2ClFS$ [M$^+$ + H]: calcd. 284.98953, found 284.98892.

(E)-N-Betulin-28-yl-1-(5-nitrofuran-2-yl)methanimine (**3e**)

28-Aminobetulin (250 mg, 0.57 mmol) and 5-nitrofuraldehyde (80 mg, 0.57 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 5 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 205 mg (0.36 mmol, 63%); off-white solid of m.p. 249–250 °C; υ_{max}(ATR)/cm^{-1} 3389, 3084, 2982, 2938, 2867, 1642, 1572, 1528, 1485, 1454, 1402, 1389, 1375, 1349, 1323, 1294, 1259, 1183, 1131, 1105, 1082, 1043, 1016, 972, 963, 945, 881, 825, 810, 739, 642, 583; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.6–0.7 (1 H, m), 0.75 (3 H, s), 0.82 (3 H, s), 1.0–1.1 (10 H, m), 1.2–1.3 (4 H, m), 1.4–1.5 (5 H, m), 1.6–2.0 (15 H, m), 2.4–2.5 (1 H, m), 3.1–3.2 (1 H, m), 3.30 (1 H, d, J = 12.0 Hz), 3.80 (1 H, d,

J = 12.0 Hz), 4.58 (1 H, s), 4.69 (1 H, s), 6.99 (1 H, d, J = 3.8 Hz), 7.34 (1 H, d, J = 3.8 Hz), 8.16 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.8, 15.4, 16.1, 18.3, 19.2, 20.8, 25.2, 26.8, 27.4, 28.0, 29.6, 30.3, 34.2, 34.9, 37.2, 37.4, 38.7, 38.9, 41.0, 42.7, 47.8, 47.9, 49.0, 50.4, 55.3, 60.8, 78.9, 109.8, 112.5, 112.8, 148.8, 150.4, 153.1; HRMS for $C_{35}H_{53}O_4N_2$ [M^+ + H]: calcd. 565.39998, found 565.39888.

(E)-N-Betulin-28-yl-1-(5-nitrothien-2-yl)methanimine (**3f**)

28-Aminobetulin (240 mg, 0.54 mmol) and 5-nitrothiophene-2-carboxaldehyde (85 mg, 0.54 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred under reflux for 5 h. The solvent was evaporated, and the residue was recrystallized from CH_2Cl_2/*n*-hexane. Yield: 150 mg (0.26 mmol, 48%); off-white solid of m.p. 229–230 °C; υ_{max}(ATR)/cm^{-1} 3364, 2933, 2868, 1622, 1533, 1505, 1456, 1438, 1388, 1376, 1336, 1227, 1212, 1106, 1043, 1027, 983, 965, 946, 883, 815, 749, 733, 685, 609, 575; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.6–0.7 (1 H, m), 0.75 (3 H, s), 0.8–0.9 (4 H, m), 0.9–1.1 (13 H, m), 1.2–2.0 (29 H, m), 2.4–2.5 (1 H, m), 3.1–3.2 (1 H, m), 3.25 (1 H, d, J = 12.2 Hz), 3.76 (1 H, d, J = 12.2 Hz), 4.58 (1 H, s), 4.69 (1 H, s), 7.16 (1 H, d, J = 4.2 Hz), 7.84 (1 H, d, J = 4.2 Hz), 8.28 (1 H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.9, 15.4, 16.1, 18.3, 19.2, 20.9, 25.2, 26.8, 27.4, 28.0, 29.7, 30.4, 34.2, 34.9, 37.2, 37.4, 38.7, 38.9, 41.0, 42.7, 47.9, 49.0, 50.4, 55.3, 59.7, 79.0, 109.7, 127.3, 128.4, 149.1, 150.5, 152.7; HRMS for $C_{35}H_{53}O_3N_2S$ [M^+ + H]: calcd. 581.37714, found 581.37587.

(E)-N-(4-Cyclopropyl-3-(pyridin-2-yl)-1H-pyrazol-5-yl)-1-(5-nitrofuran-2-yl)methanimine (**3g**)

3-Amino-4-cyclopropyl-5-(pyrid-2-yl)-pyrazole (200 mg, 1.0 mmol) and 5-nitrofuraldehyde (141 mg, 1.0 mmol) were dissolved in EtOH (15 mL). The reaction mixture was stirred under reflux for 2 h. The formed precipitate was collected, washed with EtOH, and dried in a vacuum. Yield: 272 mg (0.84 mmol, 84%); yellow solid of m.p. >240 °C (dec.); υ_{max}(ATR)/cm^{-1} 3273, 3132, 3009, 1590, 1568, 1523, 1504, 1485, 1465, 1432, 1382, 1348, 1302, 1252, 1230, 1209, 1181, 1153, 1126, 1093, 1055, 1015, 1000, 983, 962, 904, 840, 808, 789, 759, 747, 734, 673; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.8–1.0 (4 H, m), 2.1–2.3 (1 H, m), 7.3–7.4 (1 H, m), 7.44 (1 H, d, J = 3.9 Hz), 7.82 (1 H, d, J = 3.9 Hz), 7.9–8.0 (1 H, m), 8.6–8.7 (1 H, m), 8.87 (1 H, s), 13.39 (1 H, s); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 5.83, 7.79, 114.3, 115.3, 117.9, 121.4, 122.8, 137.1, 140.8, 144.8, 148.4, 149.4, 152.4, 153.2, 155.4; HRMS for $C_{16}H_{14}O_3N_5$ [M^+ + H]: calcd. 324.10912, found 324.10777.

(E)-N-(4-Cyclopropyl-3-(pyridin-2-yl)-1H-pyrazol-5-yl)-1-(5-nitrothien-2-yl)methanimine (**3h**)

3-Amino-4-cyclopropyl-5-(pyridyl-2y)-pyrazole (200 mg, 1.0 mmol) and 5-nitrothiophene-2-carboxaldehyde (157 mg, 1.0 mmol) were dissolved in EtOH (15 mL). The reaction mixture was stirred under reflux for 2 h. The formed precipitate was collected, washed with EtOH, and dried in a vacuum. Yield: 277 mg (0.86 mmol, 86%); yellow-brown solid of m.p. >230 °C (dec.); υ_{max}(ATR)/cm^{-1} 3203, 3095, 3005, 1592, 1571, 1534, 1495, 1466, 1447, 1408, 1336, 1297, 1236, 1212, 1186, 1140, 1094, 1054, 1041, 1001, 980, 960, 914, 887, 827, 810, 789, 762, 745, 730, 697, 675; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.8–1.0 (4 H, m), 2.1–2.3 (1 H, m), 7.3–7.5 (1 H, m), 7.76 (1 H, d, J = 4.4 Hz), 7.9–8.0 (1 H, m), 8.18 (1 H, d, J = 4.4 Hz), 8.6–8.7 (1 H, m), 9.14 (1 H, s), 13.39 (1 H, s); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 5.91, 7.80, 115.4, 121.2, 122.2, 122.8, 130.6, 131.5, 137.2, 140.6, 148.4, 149.5, 149.6, 149.9, 152.2, 154.4; HRMS for $C_{16}H_{14}O_2N_5S$ [M^+ + H]: calcd. 340.08627, found 340.08521.

4.2. Toxoplasma gondii Cell Line, Culture Conditions, and Assay

Serial passages of Vero cells (ATCC® CCL81™, Manassas, VA, USA) were applied for the cultivation of *T. gondii* tachyzoites (RH strain, a gift from Dr. Saeed El-Ashram, State Key Laboratory for Agrobiotechnology, China Agricultural University, Bejing, China). Vero cells were cultured using complete RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with heat-inactivated 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2 atmosphere at 37 °C. In addition, 96-well plates (5×10^3 cells/well in 200 µL of RPMI 1640 medium) were used for the cultivation of the Vero cells, which were incubated at 37 °C and 5% CO_2 for one day, followed by the removal of the medium and washing the cells with phosphate-buffered saline (PBS). Then, RPMI 1640 medium containing 2% FBS and *T. gondii* tachyzoites (RH strain) was added to the cells at a ratio of

5 (parasites) : 1 (Vero cells). The tests were performed in the following way: Control: RPMI 1640 medium containing DMSO (1%). Experimental: medium + compounds (from stock solutions in DMSO; final concentrations of 50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg mL^{-1}).

After incubation at 37 °C and 5% CO_2 for 72 h, the cells were washed with PBS, fixed in 10% formalin, and stained with 1% toluidine blue. An examination of the cells and a determination of the infection index (number of cells infected from 200 cells tested) of *T. gondii* were carried out with an inverted photomicroscope. The following equation was used for the calculation of the inhibition in %:

$$\text{Inhibition (\%)} = (\text{I Control}) - (\text{I Experimental})/(\text{I Control}) \times 100$$

where "I Control" refers to the infection index of untreated cells and "I Experimental" refers to the infection index of cells treated with test compounds. The effects of the test compounds on parasite growth were expressed as IC_{50} (inhibitory concentration at 50%) values. IC_{50} values were obtained from three independent experiments [27,49].

4.3. Leishmania Major Cell Isolation, Culture Conditions, and Assays

Promastigotes of *L. major* were isolated from a Saudi patient (February 2016) and maintained in a tissue culture flask at 26 °C in Schneider's Drosophila medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated FBS and antibiotics with weekly transfers. Promastigotes were cryopreserved in liquid nitrogen at concentrations of 3×10^6 parasites/mL. Virulent *L. major* parasites were maintained in female BALB/c mice by injecting their hind footpads with 1×10^6 stationary-phase promastigotes. *L. major* amastigotes were isolated from the mice after 8 weeks. The isolated amastigotes were converted to promastigotes by cultivation in Schneider's medium supplemented with antibiotics and 10% FBS at 26 °C. Amastigote-derived promastigotes, which had undergone less than five in vitro passages, were used for infection.

The BALB/c mice (male and female individuals) for these experiments were obtained from the Pharmaceutical College of King Saud University, Kingdom of Saudi Arabia, and maintained in specific pathogen-free facilities. The handling of the laboratory animals followed the instructions and rules of the committee of research ethics, Deanship of Scientific Research, Qassim University (permission number 20-03-20).

Logarithmic-phase *L. major* promastigotes were cultured in phenol-red-free RPMI 1640 medium containing 10% FBS and suspended on 96-well plates (10^6 cells mL^{-1}, 200 μL/well) after counting with a hemocytometer. Compounds were added to the cell cultures, reaching final concentrations of 50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg mL^{-1}. The negative controls were cultures containing DMSO (1%) without a test compound, and positive control wells contained cultures with decreasing concentrations of amphotericin B (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg mL^{-1}) as an antileishmanial reference drug. After incubation at 26 °C for 72 h, the number of viable promastigotes was assessed using a colorimetric method (tetrazolium salt colorimetric assay, MTT). The formed colored formazan was isolated and solubilized by the addition of a detergent solution. The samples were analyzed using an ELISA reader at 570 nm. IC_{50} values were calculated from three independent experiments [27].

To evaluate the activity against amastigotes in macrophages, peritoneal macrophages were collected from female BALB/c mice (6–8 weeks of age) via aspiration. First, 5×10^4 cells/well were placed into 96-well plates containing phenol-red-free RPMI 1640 medium with 10% FBS and were incubated to promote cell adhesion at 37 °C for 4 h in a 5% CO_2 atmosphere. Thereafter, the medium was discarded, and the cells were washed with PBS. An *L. major* promastigotes solution (200 μL at a ratio of 10 promastigotes : 1 macrophage in RPMI 1640 medium with 10% FBS) was added to each well, and the plates were incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere to enable macrophage infection and differentiation to amastigotes. The infected macrophages were washed three times with PBS to remove the free promastigotes and overlaid with fresh phenol-red-free RPMI 1640 medium containing test compounds (50, 25, 12.5, 6.25, 3.13, 1.65,

and 0.75 μg mL^{-1}). Then, the cells were incubated at 37 °C for 72 h in a humidified 5% CO_2 atmosphere. Cultures solely containing DMSO (1%) were used as negative controls, while wells containing cultures with decreasing concentrations of amphotericin B (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg mL^{-1}) were used as positive controls. The percentage of infected macrophages was evaluated microscopically after the removal of the medium, washing, fixation, and the Giemsa staining of the cells. Calculated IC_{50} values were obtained from three independent experiments [27,50].

4.4. In Vitro Cytotoxicity Assay

MTT assays were carried out to evaluate the cytotoxicity of the test compounds. Briefly, both Vero and macrophage cells were cultured in 96-well plates (5×10^3 cells/well/200 μL) in RPMI 1640 medium with 10% FBS and 5% CO_2 at 37 °C for 24 h. Cells were washed with PBS, followed by treatment with test compounds for 72 h at varying concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg mL^{-1}) in a 10% FBS medium. Cells treated solely with medium in 2% FBS were used as a negative control. The supernatant was discarded, and 50 μL of RPMI 1640 medium containing 14 μL of MTT (5 mg mL^{-1}) was added. Then, the cells were incubated for 4 h. The supernatant was removed, and DMSO (150 μL) was added in order to dissolve the formed formazan. A FLUOstar OPTIMA spectrophotometer was applied for the colorimetric analysis (λ = 540 nm) of the experiments. The cytotoxicity was expressed by CC_{50} values (cytotoxic concentration that caused a 50% reduction in viable cells). CC_{50} values were calculated from three independent experiments [27,51].

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