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Novel catechol derivatives of arylimidamides as antileishmanial agents

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Two novel bis-arylimidamide derivatives with terminal catechol moieties (**9a** and **10a**) and two parent compounds with terminal phenyl groups (DB613 and DB884) were synthesized as dihydrobromide salts (**9b** and **10b**). The designed compounds were hybrid molecules consisting of a catechol functionality embedded in an arylimidamide moiety. All compounds were examined for *in vitro* antiparasitic activity upon promastigotes of *Leishmania major* and *L. infantum* as well as axenic amastigotes of *L. major*. It was shown that conversion of terminal phenyl groups into catechol moieties resulted in more than 10-fold improvement in potency, coupled with lower cytotoxicity against fibroblast cells, compared to the corresponding parent compounds. The furan-containing analog **9a** exhibited the highest activity with submicromolar IC_{50} values, ranging from 0.29 to 0.36 µM, which is comparable in efficacy to the reference drug amphotericin B (IC_{50} 0.28-0.33 µM). The results justify further study of this class of compounds. It seems that the combination of catechol chelating groups with potent antiparasitic agents could improve the efficacy by presenting novel hybrid compounds.

Keywords: Bis-arylimidamides; Catechol; Antileishmanial agents, Hybrid compounds

1. Introduction

According to the World Health Organization (WHO),[1] leishmaniases which are a group of diseases caused by protozoan parasites from more than 20 *Leishmania* species, have affected 12 million people worldwide. It is also estimated that 700,000–1,000,000 new cases and 20,000-30,000 deaths have been attributed annually to this neglected tropical disease. The bite of infected female phlebotomine sandflies transmits each of the three main forms of leishmaniasis. These forms are visceral (VL), also known as kala-azar which is the most serious form of the disease, cutaneous (CL), the most common form, and mucocutaneous leishmaniasis (MCL).

Since no effective vaccine has been approved yet,[2] and the medications used for leishmaniasis have different limitations such as drug resistance and toxicity, there is an urgent need to develop new efficient drugs.[3, 4] The diamidine pentamidine, as a first-line drug for CL and as a second-line agent for VL,[5, 6] is one of the most widely used antiparasitic agents to treat antimony-resistant leishmaniasis.[7, 8] Aromatic diamidines have also exhibited promising antiprotozoal activity. In the structure of these scaffolds, the alkyl chain between the two benzene rings bearing the amidine moieties in pentamidine is replaced with a five membered-ring heterocycle, furan in one of the strongest analogs (DB75).[9] The suggested mechanism of antiparasitic action for this class of compounds is binding to the kinetoplast DNA (kDNA) minor groove.[10] In another modification in the way of attachment of the amidine moieties to the aromatic ring, reverse amidines, later called arylimidamides (AIAs), were introduced. The mode of action of members of this scaffold, such as DB613[11] and DB884[12], which exhibited improved potency against several parasites seems to be more complex and requires more investigations.[13, 14] The structures of all above-mentioned compounds are illustrated in Figure 1.

Studies on the antileishmanial activity of a variety of natural products have proven some of them as appreciable antileishmanial agents.[15-19] Proposed mechanisms for these compounds include inhibition of kDNA synthesis, induction of topoisomerase-II mediated cleavage of kDNA minicircles by luteolin and quercetin,[16] contribution of quercetin as a chelating agent to interfere with metal-mediated proliferation of the parasites in leishmanial infection[20-22] and production of reactive oxygen species which leads to the leishmania parasite death.[23] A notable structural feature of many of the natural products is the presence of chelating catechol groups. The role of these groups in antileishmanial effects has been partly investigated.

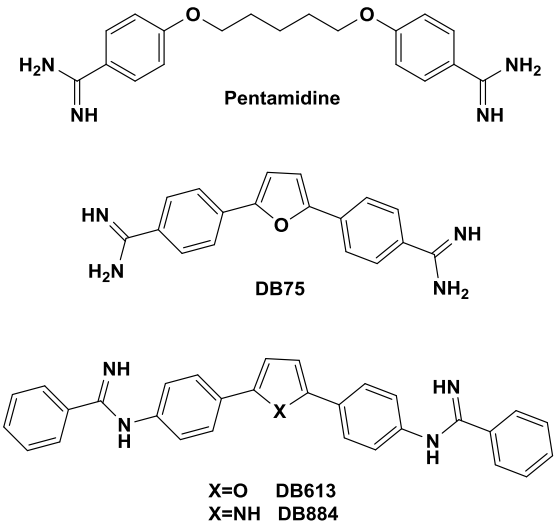


Figure 1. Chemical structures of pentamidine, DB75 and two arylimidamides

Given the essentiality of kinetoplast DNA and metal-mediated metabolism pathways in the proliferation of leishmania, and the highly probable interference of aromatic diamidines and catechol groups with the above-mentioned pathways, we decided to prepare novel catechol derivatives of AIAs (**9a** and **10a**). The designed compounds could be regarded as hybrid molecules

consisting of a catechol functionality which is embedded in an arylimidamide moiety. Thus, they are supposed to chelate metal cations such as iron which are essential for the parasite and also inhibit its growth by the mechanisms proposed for AIAs. The structures of these compounds are provided in Figure 2. For comparison purposes, the dihydrobromide salt of the previously reported arylimidamides DB613 and DB884, with a general structure close to the designed compounds were also prepared as **9b** and **10b**, respectively. The prepared compounds were evaluated for their *in vitro* antileishmanial activity against *L. major* and *L. infantum* promastigotes, and also *L. major* axenic amatigotes.

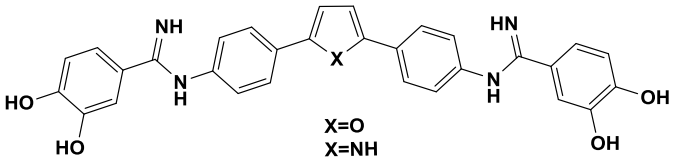
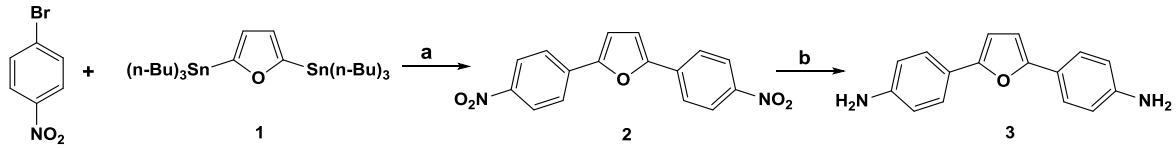


Figure 2. Chemical structures of the designed compounds

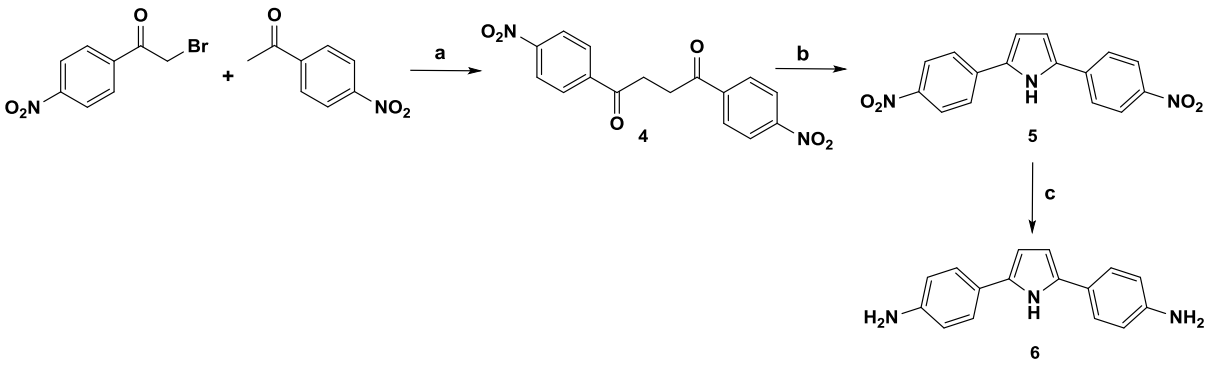
2. Results and Discussion

2.1. Chemistry

The desired bis-arylimidamides **9a**, **9b**, **10a** and **10b**, were prepared from their corresponding diamino intermediates **3** and **6** and S-(2-naphthylmethyl)-2-thioimidate hydrobromides **8a** and **8b**. The diamino compound **3**, with a furan ring in its structure**,** was obtained in two steps according to the method described previously.[11] This method starts with a Stille-coupling reaction between 1-bromo-4-nitrobenzene and 2,5-bis(tributylstannyl)furan (**1**) to furnish the corresponding 2,5-bis(4-nitrophenyl)furan (**2**). Subsequent reduction of the dinitro derivative **2** by catalytic hydrogenation produced the expected diamino compound **3** in 90% yield (Scheme 1). For the preparation of the diamino compound **6**, bearing a pyrrole ring in the structure, Paal-Knorr synthesis was applied.[24] The precursor 1,4-diketone was prepared using the aldol condensation of 2-bromo-4'-nitroacetophenone with 4'-nitroacetophenone in the presence of zinc chloride-diethylamine-*t*-butanol complex as the condensation agent. [25] The resulted diketone then yielded the 2,5-bis(4-nitrophenyl)pyrrole (**5**) in the Paal-Knorr reaction. The nitro groups in compound **5** were reduced to amino moieties using catalytic hydrogenation in the presence of Pd/C to give the desired 2,5-bis(4-aminophenyl)pyrrole (**6**) in 81% yield (Scheme 2).

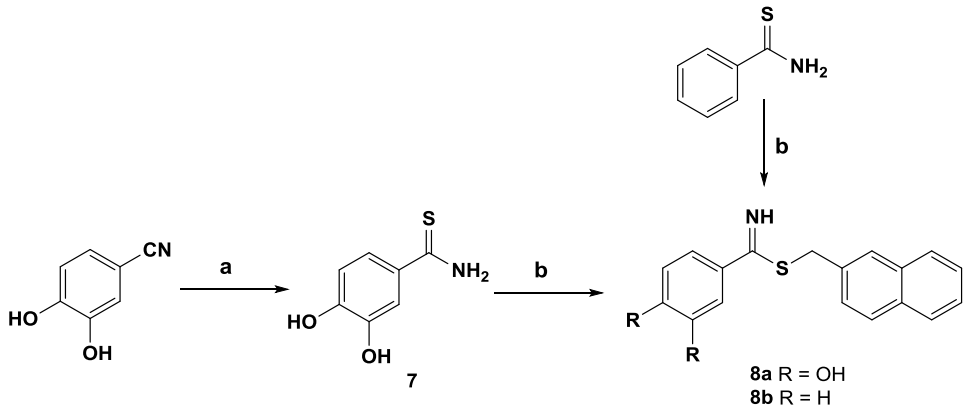


Scheme 1. Reagents and conditions: **a.** $Pd(PPh_3)_4$, reflux in 1,4-dioxane; **b.** Pd/C (10%), H_2, EtOH and EtOAc

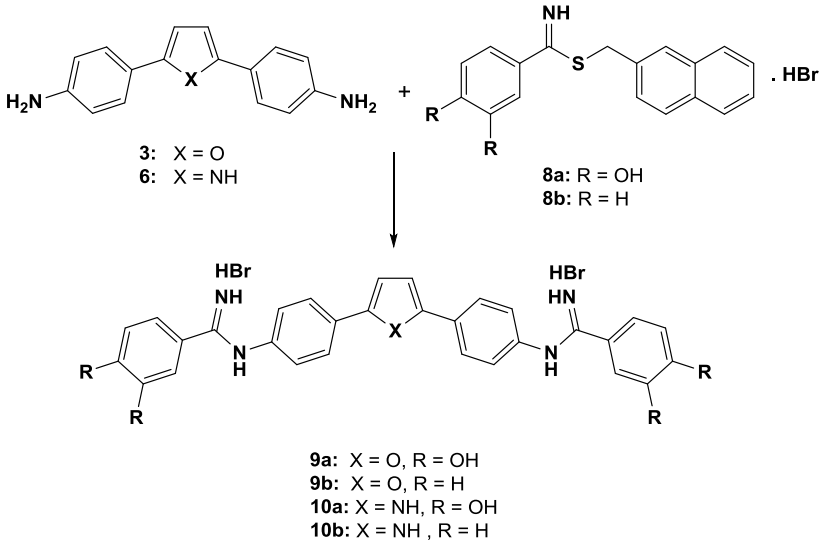


Scheme 2. Reagents and conditions: **a.** $ZnCl_2$, benzene, t-BuOH, diethylamine, room temperature; **b.** reflux in NH_4OAc, EtOH and HOAc; c. Pd/C (10%), H_2, EtOH and EtOAc

As shown in Scheme 3, the thioimidate reagent **8a** was prepared by two successive steps. The initial step was the conversion of 3,4-dihydroxybenzonitrile to the corresponding aryl thioamide **7** in the presence of sodium hydrosulfide hydrate and sulfuric acid.[26] The next reaction for the preparation of **8a** was the reaction between 3,4-dihydroxybenzothioamide (**7**) and 2-(bromomethyl)naphthalene in $CHCl_3$ which furnished the desired thioimidate reagent **8a**. S-(2-Naphthylmethyl)thiobenzimidate hydrobromide (**8b**) was prepared from commercially available thiobenzamide reacting with 2-(bromomethyl)naphthalene in refluxing $CHCl_3$ according to literature procedure.[11] A one-pot addition/elimination reaction[11, 27] between the diamino compounds **3**/**6** and **8a**/**8b** resulted in the desired compounds **9a**, **9b**, **10a** and **10b** in 62-89% yields (Scheme 4).



Scheme 3. Reagents and conditions:**a.** NaSH-xH_2O, H_2SO_4, triethylene glycol; **b.** 2-(bromomethyl)naphthalene, reflux in $CHCl_3$



Scheme 4. Reagents and conditions: MeCN, EtOH, room temperature

2.2. Biological evaluation

2.2.1. In vitro antileishmanial activities

The synthesized compounds **9a, b** and **10a, b** were tested for their ability to inhibit growth of *L. major* and *L. infantum* promastigotes and *L. major* axenic amastigotes. Amphotericin B was used as the reference drug, and the compounds concentrations causing 50% reduction in parasite viability (IC_{50}) are presented in Table 1. As can be seen in the table, the two new catechol derivatives **9a** and **10a** exhibited much more potent antileishmanial activity than their corresponding furan- and pyrrole-based analogs **9b** and **10b**, respectively. Of interest, the compound **9a** showed promising activity in all assays with submicromolar IC_{50} values (ranging from 0.29 to 0.36 μM), substantially possessing similar potency to amphotericin B (with IC_{50} values from 0.28 to 0.33 μM). The pyrrole-containing catecholic analog **10a** also showed good activity with IC_{50} values ranging from 4.54 to 7.45 μM which demonstrated more than 10-fold increase in inhibitory potency when compared to the non-catecholic parent compound **10b** (with IC_{50} values ranging from 64.44 to 81.46 μM). Notably, based on the comparison of parent compounds **9b** and **10b**, the pyrrole analog **10b** showed a dramatic reduction in antileishmanial activity. It should be noticed that furan analogs found to be more effective than pyrrole analogs of the same scaffold. However, the IC_{50} values of the pyrrole-containing catecholic analog **10a** and the furan-based non-catecholic analog **9b** showed similar levels of antileishmanial activities (IC_{50} values in the ranges of 4.54-7.45 μM and 4.85-5.56 μM for **10a** and **9b**, respectively).

2.2.2. Cytotoxicity

Cytotoxicity on human fibroblast cell line was evaluated for the synthetic compounds **9a, b** and **10a, b** (Table 1). The cytotoxic index (CI) was calculated as the ratio of cytotoxicity (TD_{50} value on fibroblast cells) to activity (IC_{50} value on leishmania promastigotes and axenic amastigotes). This *in vitro* therapeutic index showed that the furan-based catechol derivative **9a** possessed the best selectivity profile (CI of 33.03, 41.00 and 37.16 for *L. infantum* promastigotes, *L. major* promastigotes and axenic amastigotes, respectively). The least active compound **10b**, the pyrrole-based non-catecholic analog, exhibited the lowest CI (0.06 and 0.05). In general, the catechol derivatives **9a** and **10a**, demonstrated higher selective potency *in vitro* against leishmania cells than the non-catechol ones **9b** and **10b**, respectively, as evidenced by their cytotoxic indices.

Table 1. *In vitro* antileishmanial activities of bis-arylimidamide analogs

Compound	Cell toxicity TD_{50}[a] (μM)	*L. infantum* promastigotes		*L. major*			
				promastigotes		axenic amastigotes	
		IC_{50}[a] (μM)	Cytotoxic index TD_{50}/IC_{50}	IC_{50}[a] (μM)	Cytotoxic index TD_{50}/IC_{50}	IC_{50}[a] (μM)	Cytotoxic index TD_{50}/IC_{50}
9a	11.89 (±0.62)	0.36 (±0.05)	33.03	0.29 (±0.02)	41	0.32 (±0.04)	37.16
9b	4.76 (±0.31)	5.23 (±0.35)	0.91	4.85 (±0.25)	0.98	5.56 (±0.29)	0.86
10a	11.35 (±0.49)	5.72 (±0.24)	1.98	4.54 (±0.19)	2.50	7.45 (±0.38)	1.52
10b	3.97 (±0.26)	68.17 (±2.16)	0.06	64.44 (±3.81)	0.06	81.46 (±3.67)	0.05
Amphotericin B[b]	NT[c]	0.28 (±0.03)	-	0.33 (±0.02)	-	0.30 (±0.06)	-

[a] The values are means ± SD of three independent experiments.

[b] Amphotericin B was used as antileishmanial reference.

[c] NT = Not Tested

3. Conclusions

In the present report, two novel arylimidamides **9a** and **10a** bearing terminal catechol moieties have been synthesized and tested *in vitro* against *L. major* and *L. infantum* promastigotes along with *L. major* axenic amastigotes. Also, the dihydrobromide salts of the two previously reported AIAs, DB613 and DB884 with terminal unsubstituted phenyl groups were prepared as lead compounds **9b** and **10b**, respectively. Amphotericin B was used as the reference antileishmanial drug in biological evaluations. The IC_{50} values represented a significant improvement in antileishmanial activity of the structures bearing terminal catechol moieties over the non-catecholic derivatives. The catchol derivatives also exhibited higher therapeutic index than the non-catecholic ones as they were tested for toxicity against human fibroblast cells. Furthermore, it appears that furan analogs resulted in higher efficacy than pyrrole analogs. The furan-based catecholic analog **9a** possessed the highest activity upon both promastigotes and axenic amastigotes with submicromolar IC_{50} values and displayed activities as high as amphotericin B. It must be noted that although the pyrrole analog DB884 has demonstrated higher DNA binding affinity than the furan analog DB613 in the previous studies,[12] the pyrrole derivatives displayed lower efficacies than the furan ones in the present study. Accordingly, although the antileishmanial effect of AIAs is likely to be in part influenced by DNA affinity,[28] further mechanistic studies need to be done to explore additional modes of action.

As it was mentioned previously, AIAs as a promising class of molecules possess broad-spectrum antiprotozoal activities. In the current study, the *in vitro* antileishmanial effects of some novel derivatives of this chemical entity were evaluated. AIAs have shown considerable efficacies against other protozoan parasites such as *Trypanosoma cruzi* and *Plasmodium falciparum.* Furthermore, chelating agents have long been proven as effective antimalarial agents. Thus, complementary studies on the designed compounds to evaluate the possible broad range of antiparasitic activity of them and their *in vivo* efficacies is suggested. Since the newly synthesized compounds exhibited appreciable activity against both promastigotes and axenic amastigotes, they are likely to fulfill the first requirement of an ideal antiparasitic agent that is declared by WHO, namely combating different forms of a parasite in its life cycle. Finally, the approach of combining the chelating catechol moiety to arylimidamides appears to result in appreciable antileishmanial effects and this strategy deserves to be considered for further investigations.

4. Experimental Section

4.1. Chemistry

All of the reagents were purchased from commercial sources and freshly used after being purified by standard procedures. Melting points were determined on the Electrothermal 9200 Melting Point apparatus and were uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 on Bruker-Ultrashield 300MHz (for final compounds) and 400MHz (for intermediates) spectrometers (Germany). All chemical shifts are reported as (δ) values (ppm) against tetramethylsilane as an internal standard. Electrospray mass spectra (ESI-MS) were obtained in negative and positive ion mode on an AB SCIEX 3200 QTRAP spectrometer. FT-IR (KBr) spectra were recorded on JASCO 6300 (Japan) apparatus. All chemical reactions were monitored by analytical thin layer chromatography (TLC) on pre-coated silica gel 60 F254 aluminum plates (Merck, Germany). The purity of compounds was determined by thin layer chromatography using several solvent systems of different polarities. The structures of intermediates were confirmed by comparing their melting points with literature and ^{1}H-NMR spectra. Structural elucidation of all final compounds was performed using ^{1}H-NMR, ^{13}C-NMR, Mass and FT-IR spectroscopy.

*4.1.1. 2,5-Bis(4-nitrophenyl)furan (**2**)*

To a solution of 4-bromonitrobenzene (2.14 g, 10.6 mmol) and tetrakis-(triphenylphospine)palladium(0) (0.21 g) in anhydrous 1,4-dioxane (27 mL) was added 2,5-bis(tri-*n*-butylstannyl)furan[29, 30] (3.43 g, 5.3 mmol), and the mixture was heated overnight under nitrogen at 95-100 °C. The resulting orange suspension was diluted with hexane (8 mL), cooled down to room temperature, and filtered to give an orange solid (0.87 g, yield: 53%), mp 267-270 °C, lit.[11] mp 269-270 °C; ^{1}H-NMR (DMSO-d_6): δ 8.38 (d, *J*=8.8 Hz, 4H), 8.19 (d, *J*=8.8 Hz, 4H), 7.60 (s, 2H).

*4.1.2. 2,5-Bis(4-aminophenyl)furan (**3**)*

To a suspension of **2** (0.85 g, 2.7 mmol) in EtOAc (20 mL) and absolute EtOH (4 mL) was added Pd/C (10%) (0.12 g) and the mixture was shaken under hydrogen at the atmospheric pressure for 6 h, the resulting solution was filtered over celite and the filtrate was concentrated *in vacuo* to dryness to give the pure diamino as a pale yellow/tan solid (0.61 g, 90%), mp 217-219 °C, lit.[11] mp 218-221 °C.

*4.1.3. 1,4-Bis(4-nitrophenyl)butane-1,4-dione (**4**)*

Anhydrous $ZnCl_2$ (1.09 g, 8 mmol) was placed into a one-neck, 25-mL round bottomed flask and dried by melting under reduced pressure (1 torr) at 250-350 °C for 15 min. After cooling to room temperature under vacuum, dry benzene (4 mL), dry diethylamine (0.44 g, 6 mmol) and *t*-BuOH (0.56 mL, 6 mmol) were successively added. The mixture was stirred till $ZnCl_2$ was fully dissolved. Then, 4'-nitroacetophenone (0.99 g, 6 mmol) and 2-bromo-4'-nitroacetophenone (0.98 g, 4 mmol) were successively added. After stirring at room temperature for 4 days, the reaction mixture was quenched with 5% aq. H_2SO_4 whereupon a yellow solid was formed. The solid was filtered, washed successively with H_2O and MeOH. The 1,4-diketone was crystallized from THF to give shiny yellow crystals (1.1 g, 82%), mp 201-204 °C, lit.[25] mp 195-196 °C; ^{1}H-NMR (DMSO-d_6): δ 8.46 (d, *J*= 8.0 Hz, 4H), 8.34 (d, *J* = 8.0 Hz, 4H), 3.61 (s, 4H).

*4.1.4. 2,5-Bis(4-nitrophenyl)pyrrole (**5**)*

A solution of **4** (1.08 g, 3.3 mmol) and ammonium acetate (1.27 g, 16.5 mmol) in glacial acetic acid (5.5 mL) and EtOH (8 mL) was heated under reflux for 5 h. The reaction mixture was cooled down to room temperature, then poured into an ice-water mixture. The precipitated solid was collected by filtration, washed with water, and crystallized from THF to give dark red crystals (0.95 g, 93%), mp 290-292 °C, lit.[24] mp 295-297 °C; ^{1}H-NMR (DMSO-d_6): δ 11.98 (br s, 1H), 8.34 (d, *J* = 8 Hz, 4H), 8.15 (d, *J* = 8 Hz, 4H), 7.11 (s, 2H).

*4.1.5. 2,5-Bis(4-aminophenyl)pyrrole (**6**)*

To a solution of **5** (0.93 g, 3 mmol) in absolute EtOH (4.5 mL), and EtOAc (22 mL) was added 10% palladium on carbon (0.13 g). The mixture was shaken under hydrogen at atmospheric pressure for 6 h at room temperature. The reaction mixture was filtered through a pad of celite, and the solvent was evaporated to dryness under reduced pressure. A light brown solid was obtained, which was directly used in the next step without further purification (0.61 g), mp 216-218 °C decomposed, lit.[31] mp 200-201 °C.

*4.1.6. 3,4-Dihydroxybenzothioamide (**7**)*

To a thick-walled 15 mL tube with a resealable Teflon screw-cap was added 2.34 g (17.3 mmol) of 3,4-dihydroxbenzonitrile, 20 mL of triethylene glycol, 4.69 g of NaSH.xH_2O, and 1 mL of concentrated H_2SO_4. The tube was sealed with the cap, and the mixture was warmed to 110 °C and stirred for 3 days at this temperature. The reaction was quenched by pouring into 400 mL saturated aqueous NH_4Cl, and extracted twice with 200 mL of EtOAc. The combined organic phases were washed three times with 60 mL water, dried with $NaSO_4$, and concentrated to give 1.7 g (58% yield) of **7** as a yellow solid,[26] mp 151-153 °C, lit.[32] mp 153-154 °C.

*4.1.7. S-(2-Naphthylmethyl)-3,4-dihydroxybenzothioimidate hydrobromide (**8a**)*

To a stirring solution of 3,4-dihydroxybenzothioamide (10 mmol, 1.69 g) in $CHCl_3$ (20 mL) was added 2-(bromomethyl)naphthalene (10.5 mmol, 2.33 g). The mixture was heated up to reflux for 5 h, cooled down to room temperature and placed in an ice bath. The resulting off-white precipitate (2.62 g, 67%) was filtered off, dried *in vacuo* and used directly in the next step, mp 232-234 °C; FT-IR (KBr) v (cm^{-1}): 3000-3400 (O-H), 3487 (N-H), 3138 (Aromatic C-H), 1655 (C=N), 1604, 1494 (Aromatic C=C), 1305 (-S-CH_2). ^{1}H-NMR (DMSO-d_6): δ 11.54 (brs, 2H), 10.79 (s, 1H), 9.90 (s, 1H), 8.12 (s, 1H), 8.01 (m, 3H), 7.69 (d, *J* = 8 Hz, 1H), 7.62 (m, 2H), 7.45 (d, *J* = 8 Hz, 1H), 7.40 (s, 1H), 7.01 (d, 1H), 4.93 (s, 2H).

*4.1.8. S-(2-naphthylmethyl)thiobenzimidate hydrobromide (**8b**)*

The same procedure carried out for the preparation of **8a** was employed starting with thiobenzamide (10 mmol, 1.37 g) to give a white solid (2.8 g, 78%), mp 211-213 °C, lit.[11] mp 210-212 °C.

*4.1.9. 2,5-Bis[4-(3,4-dihydroxybenzimidoylamino)phenyl]furan dihydrobromide(**9a**)*

To a solution of 2,5-bis(4-aminophenyl)furan (1.2 mmol, 0.3 g) in dry MeCN (6 mL) was added dry EtOH (17 mL), and the solution was chilled briefly in an ice-water bath. S-(2-naphthylmethyl)-3,4-

dihydroxybenzothioimidate hydrobromide (0.98 g, 2.52 mmol) was then added, and the mixture was stirred overnight at room temperature. The resulting solution was concentrated to near dryness, which was triturated with ether to give a yellow/orange solid of the hydrobromide salt. The solid was washed with dichloromethane and dried *in vacuo* (1.2 g, 68%) mp 134-136 °C; FT-IR (KBr) v (cm^{-1}): 3000-3400 (O-H), 3320 (N-H), 3132 (Aromatic C-H), 1655 (C=N), 1510, 1607 (Aromatic C=C). ^{1}H-NMR (DMSO-d_6): δ 11.21 (s, 2H), 10.31 (br s, 2H), 9.61 (s, 4H), 8.86 (s, 2H), 7.96 – 8.04 (m, 4H), 7.81 (d, *J* = 8.1 Hz,1H), 7.54 (d, *J* = 8.1 Hz, 4H), 7.27 – 7.34 (m, 4H), 7.17-7.20 (m,1H), 7.03 (d, *J* = 8.1 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 163.19, 152.79, 151.80, 146.03, 134.75, 129.83, 126.38, 125.26, 121.81, 119.20, 116.49, 116.10, 109.76. Mass *m/z*: 519 (M-1).

*4.1.10. 2,5-Bis[4-(benzimidoylamino)phenyl]furan dihydrobromide (**9b**)*

The same procedure used for the synthesis of **9a** was exploited starting with diamine **3** and **8b** to give a yellow solid (79%), mp 232-234 °C, lit.[11] mp (dihydrochloride salt) 242-248 °C. ^{1}H-NMR (DMSO-d_6): δ 7.30 (s, 2H), 7.57 (d, *J* = 8, 4H), 7.84 (t, 4H), 7.81 (t, 2H), 7.95 (d, *J* = 8, 4H), 8.06 (d, *J* = 8, 4H), 9.16 (br s, 2H), 9.92 (br s, 2H), 11.51 (br s, 2H).

*4.1.11. 2,5-Bis[4-(3,4-dihydroxybenzimidoylamino)phenyl]pyrrole dihydrobromide (**10a**)*

The same procedure described for **9a** was adopted, using diamine **6** (1.2 mmol, 0.3 g) and S-(2-naphthylmethyl)-3,4-dihydroxybenzothioimidate hydrobromide (0.98 g, 2.52 mmol) as starting materials to yield a light brown/orange solid (1.3 g, 62%), mp 132-135 °C; FT-IR (KBr) v (cm^{-1}): 3000-3400 (O-H), 3411, 3313 (N-H), 3163 (Aromatic C-H), 1653 (C=N), 1608, 1508 (Aromatic C=C). ^{1}H-NMR (DMSO-d_6): δ (ppm) 11.52 (s, 1H), 11.11 (s, 2H), 10.29 (br s, 2H), 9.55 (s, 4H), 8.79 (s, 2H), 7.92-8.03 (m, 4H), 7.34-7.47 (m, 8H), 7.03(d, J = 8.1 Hz, 2H), 6.78 (s, 2H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 163.06, 151.64, 146.02, 133.20, 133.00, 132.41, 126.07, 125.63, 121.69, 119.29, 116.42, 116.09, 109.29. Mass m/z: 518 (M-1).

*4.1.12. 2,5-Bis[4-(benzimidoylamino)phenyl]pyrrole dihydrobromide (**10b**)*

A similar procedure to that outlined for the preparation of **9a** was followed, using diamine **6** and **8b** to yield **10b** as an orange solid (89%), mp 231-234 °C; ^{1}H-NMR (DMSO-d_6): δ 11.53 (s, 1H), 11.43(s, 2H), 9.86 (s, 2H), 9.09 (s, 2H), 8.04 (d, *J* = 8 Hz, 4H), 7.95 (d, *J*= 8 Hz, 4H), 7.81 (t, 2H), 7.70 (t , 4H), 7.52 (d, J = 8 Hz, 4H), 6.80 (s, 2H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 162.94, 133.95, 132.83, 132.55, 132.32, 129.21, 128.93, 125.84, 125.46, 109.15.

4.2. Biology

4.2.1. In vitro antileishmanial activity

Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO). 10-Fold serial dilutions of test compounds were prepared in culture media. The maximum concentration of DMSO in drug susceptibility assays was 1%, which was not hazardous to the parasites and amphotericin B was used as control.

4.2.1.1. Strains

Leishmania major (MRHO/IR/75/ER) and *L. infantum* (MCAN/IR/07/Moheb-gh) promastigotes were grown in 25 cm^3 plastic cell culture flasks in Brain Heart Infusion (BHI) medium at 37 g/L which was

sterilized by autoclave, and supplemented with 15% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL) at 26 °C. Promastigotes were maintained by subpassaging every 5-7 days into fresh culture media.

Leishmania major were cultured as axenic amastigotes using a modified method based on the previously reported approach.[33] Axenic amastigote forms were obtained in BHI medium supplemented with 25 % FBS at pH 5.5 (by titration of the medium with 10 mM succinate–TRIS) after 72 h of incubation at 37 °C in the presence of 5 % CO_2. The cellular morphology of the parasites was examined using both Giemsa-stained preparations and phase-contrast light microscopy.

4.2.1.2. Antileishmanial activity against promastigote stage

The activity of AIA compounds against the growth of *L. major* and *L. infantum* promasigotes was measured by the MTT colorimetric assay.[34] 45 µL of test compounds (10-fold serial dilutions) were added to each well, in triplicate, except three blank wells which only contained 90 µL of culture medium without any compounds or parasites. Late logarithmically growing promastigotes of *L. major* and *L. infantum* were seeded in sterile 96-well plates at a concentration of 10^6 parasites/mL, and they were dispensed in 45 µL aliquots (45×10^3 parasites/well). Negative controls consisted of promastigotes in culture media without testing compound, while the positive control contained varying concentrations of amphotericin B (reference drug purchased from Sigma Aldrich). The plates were incubated at 26°C. After 72 h of incubation, 10 µL of MTT solution (5 mg/mL) was added to each well followed by incubation for another 6 h. When purple precipitate was clearly visible under the microscope, 100 µL of DMSO was added to all wells, including control wells, and relative optical density (OD) was measured with a PolarStar plate reader at 570 nm. IC_{50}s were calculated by nonlinear regression analysis processed on dose–response curves, using Quest™ IC_{50} Calculator.[35]

4.2.1.3. Antileishmanial activity against axenic amastigote stage

The susceptibility of *L. major* axenic amastigotes to growth inhibition by tested compounds was also determined using MTT assay. Briefly, 45 µL/well of the culture which contained 10^6 cells/mL of *L. major* axenic amastigotes were placed into 96-well flat-bottom plates in the presence or absence of compounds. Plates were incubated at 37 °C in a humid 5% CO_2 atmosphere for 72 h. At the end of incubation, 10 µL of MTT solution was added to each well and plates were subsequently incubated for an additional 6 h. The enzyme reaction was then stopped by addition of 100 µL of DMSO and the absorbance of each well was measured at 570 nm using a PolarStar plate reader. The 4-parameter curve available in the program Quest™ IC_{50} Calculator was used to determine IC_{50} values for *L. major* axenic amastigotes.

4.2.2. Cytotoxicity evaluation

The toxicity of compounds to mammalian cells was determined with the MTT assay using cultured human fibroblast cell line. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in 5% CO_2. The cells (10^5 cells /well) were incubated into 96-well plates overnight in a humidified 5% CO_2 atmosphere at 37 °C to ensure cell adherence. After 24 h, cells were treated with increasing concentrations of each compound. Non-treated cells were included as a negative control. After 72 h incubation with each compound, the MTT solution (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. Then the medium and MTT were removed, cells were washed by PBS and 200 µL of DMSO was added to dissolve the formazan crystals. The absorbance was measured using a PolarStar plate reader at 570

nm. The toxic dose of compound that results in 50% inhibition of control cell growth (TD_{50} value) was determined using the program Quest™ IC_{50} Calculator. The TD_{50} values were used to generate the cytotoxic index, the ratio of human cell cytotoxicity to antileishmanial activity (TD_{50}/IC_{50}). The inhibitory concentration 50% (IC_{50}) is the drug concentration that results in 50% growth reduction of parasites compared to untreated controls.

Author Contribution Statement

This article was extracted from the MSc research project of Foroogh Rezaei. She performed all the experiments by herself and wrote the article along with Lotfollah Saghaei and Razieh Sabet. Afshin Fassihi was the supervisor of the chemistry part of the research and Gholamreza Hatam was the supervisor of the biological part of the project.

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