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Conjugation of Quinones with Natural Polyamines: Toward an Expanded Antitrypanosomatid Profile

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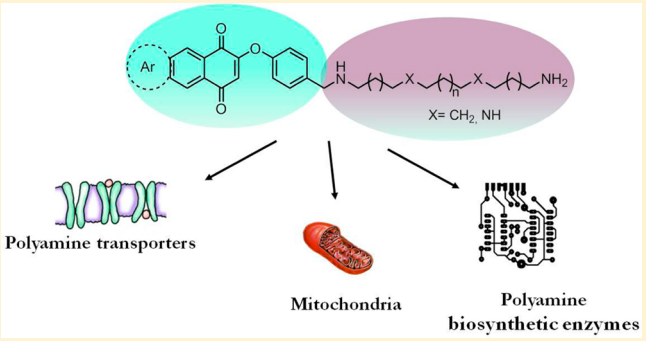
Supporting Information

ABSTRACT: A combinatorial library of quinone−polyamine conjugates designed to optimize the antitrypanosomatid profile of hit compounds **1** and **2** has been prepared by a solid-phase approach. The conjugates were evaluated against the three most important human trypanosomatid pathogens (*Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, and *Leishmania donovani*), and several showed promising activity. A subset also inhibited trypanothione reductase in vitro and induced oxidase activity of the enzyme. A highly potent analogue (**7**) was identified with activity against *T. brucei* as low as 70 nM and a selectivity index of 72. Interestingly, the presence of a cadaverine tail confers to **7** the ability to target mitochondrial function in Leishmania. In fact, in *L. donovani* promastigotes, we verified for **7** a decrease of cytoplasmic ATP and mitochondrial potential. Therefore, the current results support the suitability of the conjugation approach for the development of novel polyamine conjugates with enhanced therapeutic potential.



INTRODUCTION

Natural products still represent an essential source for drug discovery and over the years have provided design principles for chemical library development in several therapeutic areas,[1] including neglected tropical diseases (NTD).[2,3] The identification of natural products-based compound collections has been revealed as particularly successful if it can build on the link between a given chemical class and the desired biological activity.[4] The connection between quinones and antiparasitic activity[5,6] has been recently exploited by us for the design of a library of antitrypanosomatid compounds.[7] Trypanosomatids are parasitic protozoa that cause widespread and severe NTD, including human African trypanosomiasis (HAT or sleeping sickness) caused by *Trypanosoma brucei*, Chagas disease caused by *Trypanosoma cruzi*, and various forms of leishmaniasis caused by species of the *Leishmania* genus.[8,9] By directly testing the synthesized quinones in a phenotypic assay, we decided to go for a forward chemical genetics approach and to fish out the molecular targets responsible for the desired activity as a second step. Using whole-parasite-based assays for the screening of molecules has been steadily declining over the last 20 years but is now undergoing a resurgence, mainly due to improvements in assay technologies.[10] This approach, in principle, has the merit of circumventing some of the obstacles that have emerged for the exploitation of novel but chemically unvalidated targets identified from genetic or genomic methodologies.[11] On the other hand, it should be mentioned that the subsequent target identification step is time-consuming and not always straightforward.[10]

We designed two parallel series of quinones (naphtho- and anthraquinones) with different redox properties.[7] Despite the small compound collection synthesized, several were active against trypanosomatid parasites at concentration ranges similar to those of the corresponding reference drugs.[7] In particular, 2-phenoxy-anthraquinone and 2-phenoxy-naphthoquinone (**1** and **2** in Chart 1) showed remarkable nanomolar IC_{50} values against the subspecies *T. b. rhodesiense*,[7] although the selectivity

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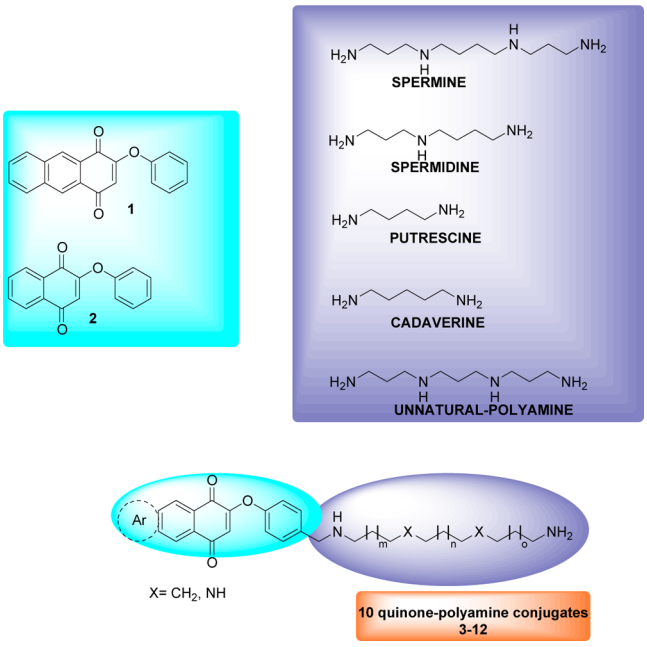
Chart 1. Design Strategy for Conjugates 3−12



index (SI) between parasite and mammalian cells was lower than that required by WHO/TDR (>100).[12] Thus, **1** and **2** could be considered as starting hits providing initial input into further medicinal chemistry. At this point, in a typical target-based approach, computational studies based on the binding modes can suggest strategies to improve selectivity, while in our case, the absence of target information made such a hit to lead optimization process impossible. For this reason, while trying to fish the target(s) of our compounds through a chemical proteomics approach, we were forced toward a ligand-based drug design strategy.

We have previously demonstrated that conjugation of several chemical scaffolds to a polyamine tail potentiates the initial pharmacological profile.[13] For instance, polyaminoquinones emerged as readily accessible and easily diversified scaffolds for anticancer lead discovery,[14] whereas tetraamine-based hybrids produced novel inhibitors of muscular nicotinic acetylcholine receptors[15] or acetylcholinesterase enzyme.[16] As a further example, by conjugating curcumin congeners with different polyamine motifs as vehicle tools, an improved mitochondrial neuroprotectant activity was recently achieved.[17] In the case of compounds against trypanosomatid diseases, the strategy of conjugating a polyamine tail to the structures of **1** and **2** emerged as particularly promising for the following reasons: (i) numerous naturally[18,19] and synthetic polyamines[20] and their conjugates have been reported to be active against trypanosomatid parasites; (ii) enzymes involved in polyamine synthesis and metabolism are validated targets for drug development;[21−24] (iii) the polyamine tail could exploit the parasite's polyamine transporters to improve the intracellular uptake of the conjugate;[25] and (iv) polyamine conjugation, as it increases the cationic character of the molecule, may promote its accumulation directly into the mitochondrion;[17] hence, by preferentially targeting an organelle essential to fulfill the energetic requirements of the parasite, an enhanced quinone-mediated damage might be envisioned.

On this basis, we designed and synthesized a combinatorial library of quinone−polyamine conjugates (Chart 1). We selected the para position of the phenoxy ring of **1** and **2** as the anchor point for the polyamine fragment, as we verified that this position can accommodate functional groups (unpublished data). Among the series, eight derivatives bear the natural polyamines spermine, spermidine, putrescine, and cadaverine (**3**, **5−8**, and **10−12**), which differs in the number of protonable nitrogen atoms and chain length. Another two compounds (**4** and **9**) present an unnatural tetramine, possibly serving as a negative control (see Table 1 for structures). Indeed, as natural polyamines display little diversity in their sequence and length as compared to other natural biopolymers (peptides, nucleic acids, and oligosaccharides), it is likely that the methylene units affect the recognition properties toward the selected biological targets.[26] Here, we report on the synthesis and the biological investigation of **3−12** aiming at an improved antitrypanosomatid profile.

RESULTS AND DISCUSSION

Chemistry. Being aware of putative synthetic problems that conjugation with the multifunctional polyamine system might raise, our initial investigations were directed toward a solid-phase organic synthesis (SPOS). With respect to classical solution synthesis, this approach makes isolation/purification steps much easier.[27] Moreover, SPOS is easily amenable to parallel library synthesis. On the other hand, it presents a major shortcoming; that is, the resins used are rather expensive, and therefore, at this preliminary stage, they may not exactly comply with the low-cost criteria required for their implementation in the field of NTD.

The synthesis of polyamine−quinone conjugates **3−12** has been achieved on a trityl-derivatized resin in good yield, using a mild borane reduction protocol to access the polyamine chains from polyamide precursors.[28,29] Successive steps of amino acid coupling and deprotection provided polyamide derivatives that were reduced, selectively protected at the amino groups, and finally linked to the quinone nuclei via phenolic intermediates (Scheme 1).

In detail, the intermediates **13−15** were obtained by reacting PS-trityl chloride resin with the appropriate diamines, 1,3-diaminopropane (x = 1), 1,4-diaminobutane (x = 2), and 1,5-diaminopentane (x = 3). To the resin **13** (x = 1), N-Fmoc-γ-aminobutyric acid (n = 3) or N-Fmoc-β-alanine (n = 2) were added in the presence of hydroxybenzotriazole (HOBt), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and *N*,*N*-diisopropylethylamine (DIPEA). Subsequent treatment with a 20% piperidine/DMF solution afforded the resin-bound amides **16** (x = 2), and **17** (x = 3). The coupling procedure was repeated with the addition of N-Fmoc-β-alanine, and then, removal of the N-Fmoc protecting group by a 20% piperidine/DMF solution gave the resin-bound diamides **18** (x = 2) and **19** (x = 3). The Fmoc-peptide coupling was easily monitored using the Kaiser test. To the resins **14**, **15**, and **17−19**, 4-hydroxybenzoic acid, HOBt, HBTU, and DIPEA were added affording the derivatives **20−24** that have been submitted to the amide bond reduction by using a 1 M diborane (BH_3) solution in THF.[30] A reaction time of 72 h at 65 °C under nitrogen atmosphere was necessary to achieve a complete transformation. Typical procedures for the reduction of amides by excess borane require a strongly acidic workup to cleave the resulting borane−amine intermediates and isolate the free amines.[31] These conditions are not compatible with most supports used in SPOS. In contrast, the mild piperidine workup allows the use of resins with acid-

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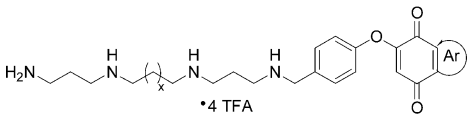
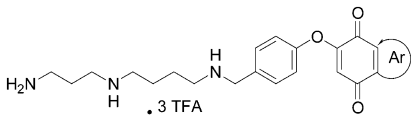
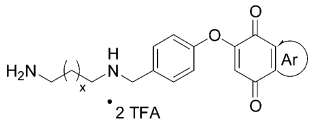
Table 1. Antitrypanosomatid Activity and Cytotoxicity of 3−12 and Reference Compounds, Expressed as IC_{50} (μM)[a]



3, 4, 8, 9



5, 10



6,7, 11, 12

	code	Ar	x	T. b. rhod	SI[b] T. b. rhod	T. cruzi	SI[b] T. cruzi	L. donovani				L6
								Axenic amastig	SI[b] Axenic amastig	Promastig	SI[b] Promastig	
1				0.05	19.80	4.66	0.21	0.34	2.91	2.81	0.35	0.99
2				0.08	74.00	1.26	4.7	1.26	4.70	0.74	8.00	5.92
3	Spermine-anthra		2	0.32	22.53	34.77	0.21	3.35	2.15	16.06	0.45	7.21
4	Unnatural-anthra		1	0.24	20.29	17.40	0.29	2.40	2.03	9.08	0.53	4.87
5	Spermidine-anthra			0.23	27.35	39.39	0.16	3.13	2.01	15.4	0.41	6.29
6	Putrescine-anthra		2	0.35	16.00	18.60	0.30	2.82	1.98	3.59	1.56	5.60
7	Cadaverine-anthra		3	0.07	72.00	17.43	0.29	2.44	2.06	3.62	1.39	5.04
8	Spermine-naphtho		2	0.45	15.67	24.10	0.29	5.79	1.22	>30	<0.23	7.05
9	Unnatural-naphtho		1	0.46	14.04	18.76	0.34	7.27	0.89	>30	<0.21	6.46
10	Spermidine-naphtho			0.72	12.75	52.16	0.18	7.17	1.28	>30	<0.31	9.18
11	Putrescine-naphtho		2	0.22	50.45	29.98	0.37	6.81	1.63	8.72	1.27	11.10
12	Cadaverine-naphtho		3	0.26	35.00	19.97	0.45	6.63	1.37	7.72	1.18	9.10
melarsoprol				0.01	1830.0							18.3
benznidazole						1.70	>225					>384
miltefosine								0.31	461.6			143.1
podophyllotoxin												0.0017

[a] IC_{50} values represent the concentration of a compound that causes 50% growth inhibition and are the mean of two independent determinations that varied by less than a factor of 2. The experimental error is within ±50%. [b] SI = (IC_{50} for L6)/(IC_{50} for the respective protozoan parasite).

sensitive trityl linkers.[32] Aliquots of the polyamines and their amide precursors were cleaved from the resins using a 5% trifluoroacetic acid (TFA) solution in CH_2Cl_2 and analyzed (NMR and MS) to ascertain the efficiency of this operation.

The amino groups of the resulting resin-bound polyamines **25**−**29** were protected using di-*tert*-butyl dicarbonate $(Boc)_2O$ and DIPEA to afford **30**−**34**. Using these conditions, besides the amino group, the hydroxyl functional group has been protected as well. To achieve the selective deprotection of the phenolic OH, the different reactivity between carbamate and carbonate group was exploited. The carbonate function was then selectively removed in a basic condition by using a 25% piperidine/CH_2Cl_2 solution to afford the derivatives **35**−**39**.[33] The characterization of the Boc-protected intermediates required a different cleavage method: a portion of the protected precursors was cleaved from the resins using a mild cleavage cocktail (CH_3COOH/2,2,2-trifluoroethanol/CH_2Cl_2 1:2:7),[34] to avoid the removal of the Boc-protecting group, and analyzed (NMR and MS) to validate the efficiency of the synthesis of derivatives **35**−**39** .

Finally, these phenol derivatives underwent a nucleophilic substitution with 2-bromo-1,4-anthraquinone and 2-bromo-1,4-naphthoquinone[7] in the presence of NaH and DMF, heating at 50 °C for 6 h, to give the PS-Trityl resin-bound polyamine-anthraquinones (**40**−**44**) and naphthoquinones derivatives (**45**−**49**). Final cleavage of **40**−**49** with a freshly prepared cocktail of TFA/water/triisopropylsilane (i-Pr_3SiH) (95:2.5:2.5) provided crude **3**−**12** as trifluoracetate ammonium salts, which were then purified by two rounds of precipitation

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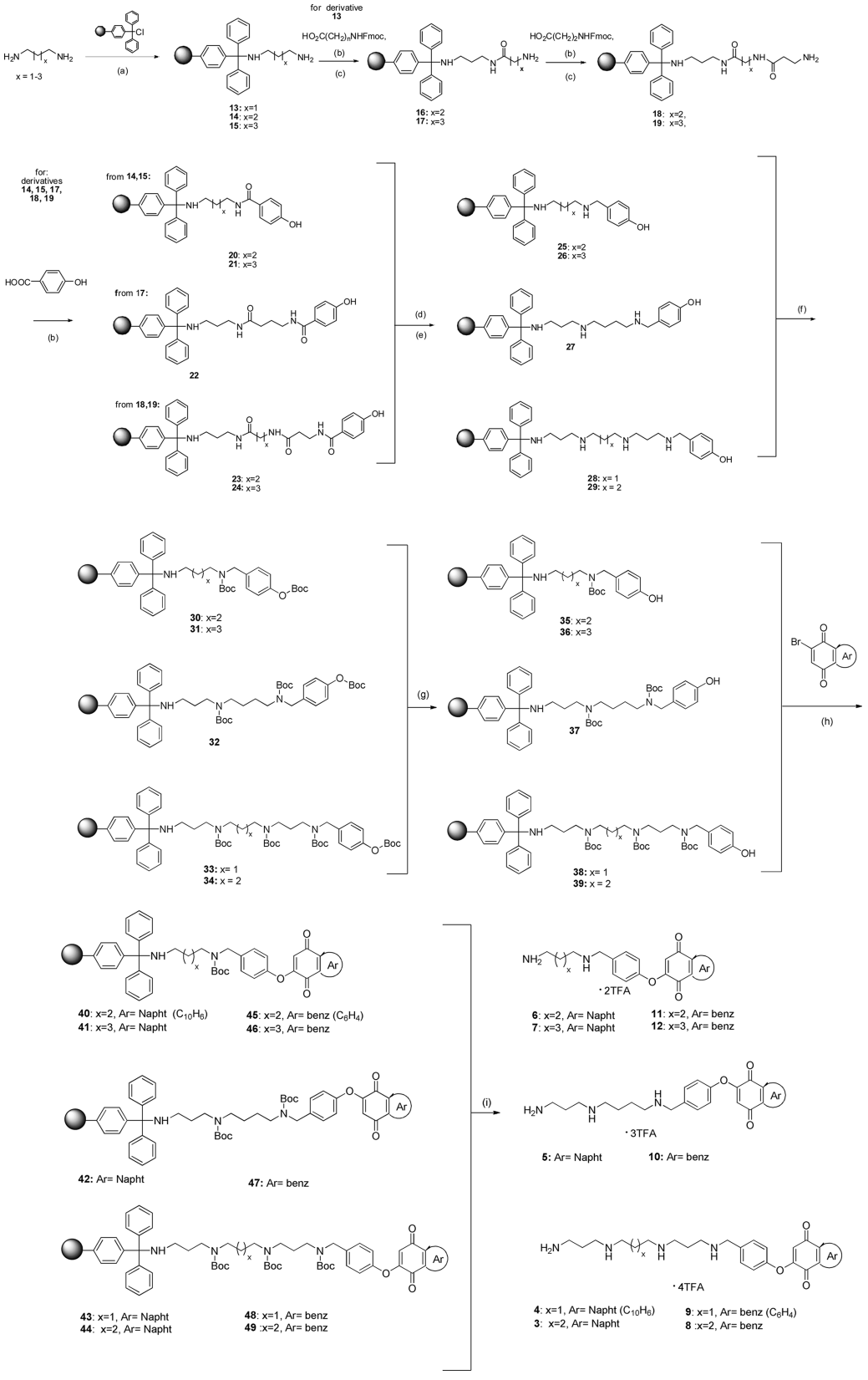
Scheme 1[a]



[a]Reagents and conditions: (a) DCM, room temperature, 3 h. (b) HOBt, HBTU, DIPEA, DMF, room temperature, 5 h. (c) 20% Piperidine/DMF, room temperature, 30 min. (d) 1 M BH_3/THF, 65 °C, N_2 atmosphere, 72 h. (e) Piperidine, 65 °C, 16 h. (f) $(Boc)_2O$, DIPEA, DCM, room temperature, 16 h. (g) 25% Piperidine/DCM, 50 °C, 6 h. (h) NaH, DMF, 50 °C, 2 h. (i) TFA/i-Pr_3SiH/H_2O (95:2.5:2.5), room temperature, 4 h.

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with methanol/ether, affording the final compounds with an overall yield ranging from 12 to 43%.

Biological Activity. As a first step, we evaluated the activity of **3**−**12** against three trypanosomatids: *T. b. rhodesiense* (trypomastigote stage, STIB 900 strain), *T. cruzi* (intracellular amastigote stage, Tulahuen C2C4 strain), and *L. donovani* (axenic amastigote stage, MHOM-ET-67/L82, and promastigote stage MHOM/SD/00/1S-2D) in a phenotypic screening. Furthermore, their toxicity against mammalian cells was assessed against a primary cell line derived from rat skeletal myoblasts (L6). An analysis of the data reported in Table 1 highlights for all of the newly synthesized compounds low, moderate, and high activity against *T. cruzi*, *L. donovani*, and *T. brucei*, respectively. Among them, **7** exhibited the best IC_{50} value against all of the three parasite strains. The values of **7** were only 7−10-fold higher than those of the corresponding reference drugs melarsoprol, benznidazole, and miltefosine.

However, as a general trend, most of the new conjugates were less potent than the starting hits **1** and **2**, indicating that the introduction of a polyamine chain did not lead to the expected improvement of the antiparasitic activity (Table 1). In addition, the presence of polyamine chains of different length and number of amino functions did not seem to have a marked impact on the activity profile. In fact, despite distinct charges (two, three, and four protonable nitrogen atoms) and structural properties, the activities of all conjugates varied only by ≤1 order of magnitude: from 0.07 to 0.7 μM for *T. brucei*, from 17 to 52 μM for *T. cruzi*, and from 2 to 7 μM for *L. donovani*. Regarding the cytoxicity profile, the determined IC_{50} values were within a concentration range of 5−11 μM. Consequently, the resulting SIs against *T. brucei* and *L. donovani* were low or negligible, respectively. In the case of *T. cruzi*, it turned out that **3**−**12** were even more toxic against the mammalian cells than against the parasite. The same was true for **9** against amastigote *L. donovani* and for **3**−**5** and **8**−**10** against the promastigote form. In no case did SI values exceed that of the reference drug.

When comparing the activities of **3**−**12** and **1** and **2** toward *T. cruzi* or *L. donovani*, no improvement was observed. This disproved our initial assumption that the conjugates could exploit the polyamine transporters for a facilitated entry and accumulation into the parasites.[35] On the other hand, **3**−**12** still maintain remarkable activity against *T. brucei*, demonstrating that the introduction of a polyamine chain is not so detrimental for the activity against this parasite. Notably, the anthraquinone−cadaverine conjugate **7**, showing an IC_{50} and SI value of 0.07 μM and 72, respectively, is the only compound in the series that is potent and at the same time reasonably selective for *T. b. rhodesiense*. More importantly, if we compare its profile with the corresponding unconjugated **1**, an increase of the SI (from 20 to 72) has been achieved, while the antiparasitic activity remained comparable (0.05 vs 0.07 μM). Unfortunately, *T. b. rhodesiense* is the only parasite for which an efficient polyamine transport mechanism has not yet been characterized, and even some evidence demonstrate the absence of a polyamine transport.[36] Actually, the lack of polyamine transporters contributes to the sensitivity of *T. brucei* to eflornithine, which acts by interfering with the polyamine metabolic pathway. Thus, further knowledge on the structural requirements for an effective polyamine transporters-mediated delivery of **3**−**12** is required.

Because of the presence of the cadaverine unit, the high activity observed for **7** is compatible with a mechanism of action typical of compounds mimicking parasite polyamines. Thus, as hypothesized, it is possible that the antitrypanosomal activity of **7** relates to its inhibition of one of the several enzymes involved in the polyamine pathway. Clearly, this could apply also to homologues of the two series but not to **1** and **2**, which lack the polyamine moiety. The bis(glutathionyl)-spermidine derivative trypanothione is a polyamine unique to trypanosomatids. It is involved in protecting the parasites against oxidative stress.[37] The enzymes of the trypanothione metabolism and in particular trypanothione reductase (TR) are attractive drug targets for the development of new antiparasitic drugs.[38] Quinones bearing protonable amino groups are effective inhibitors of *T. cruzi* TR.[39]

Thus, a small subset of diverse conjugates was selected to evaluate their effects on *T. brucei* TR. The most trypanocidal **7**, its lower homologue **6**, the triamine **5**, and two napthoquinones (**8** and **12**) were studied in comparison with **1** and **2** in the enzymatic assay as described previously.[40] All conjugates proved to be more effective inhibitors of TR than the unconjugated **1** and **2** (Figure 1). At fixed inhibitor

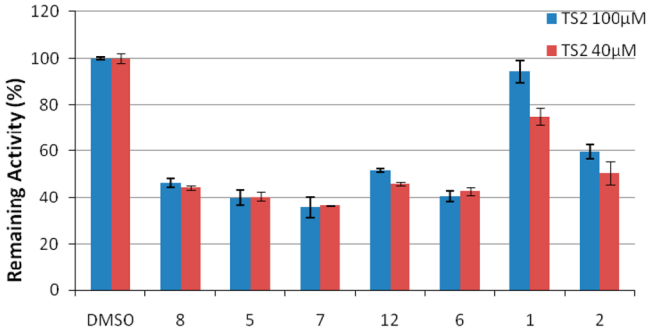

Figure 1. Inhibition of *Tb*TR by **5**−**8** and **12**. The activity was measured following the NADPH consumption at 340 nm as described in the Experimental Section. The assays contained a fixed concentration of 100 or 40 μM TS_2 and 5 μM inhibitor. The controls contained the same amount of DMSO used to dissolve the compounds. The remaining activity (%) refers to that of the enzyme in the presence of pure DMSO.

concentrations of 5 and 100 μM or 40 μM trypanothione disulfide (TS_2), the remaining activity varied between 36 and 52% for the polyamines and between 51 and 94% for **1** and **2**. Their higher activity with respect to **1** and **2** supports the view that the presence of a polyamine chain is critical for binding TR. Notably, no difference in the degree of inhibition at the two TS_2 concentrations was observed for conjugates **5**−**8** and **12**, suggesting an inhibition mechanism independent from the substrate concentration. Indeed, a Lineweaver−Burk plot analysis revealed that **7** behaves as a noncompetitive inhibitor, with a calculated[41] K_i value of 3.4 μM (Figure 2A). Because the lines did not precisely cut on the *x*-axis, we evaluated the data also assuming mixed type inhibition, which yielded nearly identical K_i and K_i' values of 3.4 and 3.9 μM, respectively. In addition, the calculated apparent K_m values for TS_2 in the presence of 5 and 10 μM **7** were 14.4 and 14.9 μM, respectively, and therefore corresponded to the K_m value of 14.4 μM obtained for the free enzyme. Thus, compound **7** is most probably a noncompetitive inhibitor of TR. Derivative **12**, which differs from **7** only by the naphthoquinone scaffold, showed a similar inhibitory potency, but in this case, the lines in the double reciprocal plot clearly cut above the *x*-axis (Figure 2B), suggesting a mixed type noncompetitive inhibition. K_i and

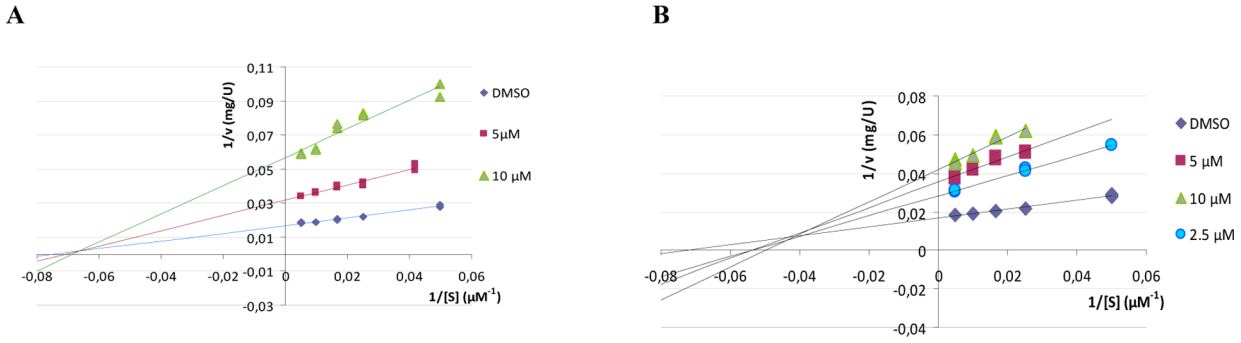

Figure 2. Lineweaver–Burk plots for the inhibition of *Tb*TR by the antraquinone conjugate **7** (A) and naphthoquinone conjugate **12** (B). The assays contained 100 μM NADPH and two or three different fixed concentrations of inhibitor as indicated in the graph, and the TS_2 concentration was varied.

K_i' of 2.8 and 4.6 μM, respectively, were calculated. Thus, the presence of two different redox-active moiety (naphtho- vs anthraquinone) may slightly affect the type of inhibition.

Quinones have been verified to act also as subversive substrates of TR.[39] Thus, it was highly conceivable that our compounds, in addition to inhibiting TS_2 reduction, undergo redox cycling in the presence of molecular oxygen, acting as a subversive substrate. In this reaction, the quinone is reduced by TR to a semiquinone (one-electron reduction) or to a hydroquinone (two-electron reduction), causing futile consumption of NADPH. The semiquinone formed can then react with molecular oxygen, producing superoxide anion radicals. The combination of TR inhibition and redox cycling should lead to stronger trypanocidal properties. Thus, our derivatives were studied for their ability to induce the intrinsic oxidase activity of TR by following the consumption of NADPH in the absence of TS_2 (Figure 3). Interestingly, naphthoquinones **8**

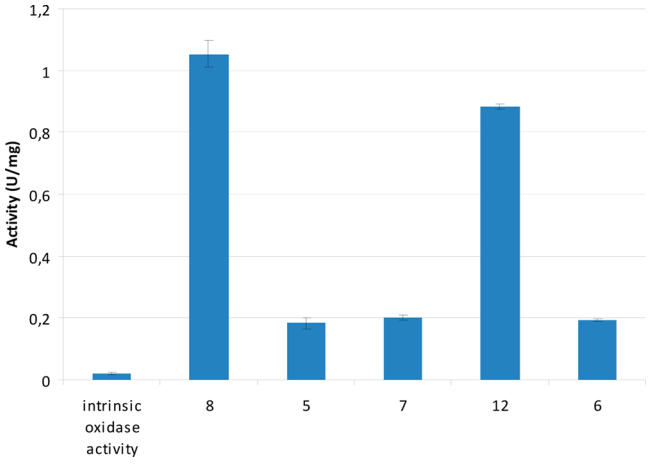

Figure 3. Compounds **5**–**7**, **8**, and **12** as subversive substrates of *Tb*TR. The assays contained 100 μM NADPH and 5 μM concentration of the compound but no TS_2. The intrinsic oxidase activity of TR (NADPH consumption in the absence of TS_2 or a subversive substrate) was 0.02 U/mg.

and **12** were much more effective in increasing TR oxidase activity than anthraquinones **5**–**7**. One may thus speculate that naphthoquinones are more easily reduced by TR than anthraquinones because of a more favorable redox potential.[42]

As a further possible action mediated by the polyamine chain, we anticipated for **2**–**12** a peculiar mitochondrial tropism. Because mitochondria maintain at rest a potential of −180 mV, the polycationic chain should drive the accumulation of the conjugates inside the matrix toward a diffusion equilibrium, as reported for membrane-permeable lipophilic cation.[43] Drugs acting at a mitochondrial level can be particularly effective against trypanosomatids infections (see ref 44 for a recent review), and mitochondrial damage was assessed for other polyamines, such as 1,4-diamino-2-butanone, in *L. amazonensis*.[45] As a special characteristic, these pathogenic protozoa contain a single mitochondrion, in comparison with mammalian cells, which possess hundreds to thousands of copies of this organelle. Therefore, the proper function of the single mitochondrion in trypanosomatid parasites is very vital as compared with mammalian cells, because the presence of numerous mitochondria ensures compensation for functionally impaired ones.[44] To test the hypothesis that the presence of a cadaverine moiety in **7** might address the cydal activities of **1** to the mitochondria, we used *Leishmania* promastigotes as a model system. In fact, in this parasite stage, the mitochondrial synthesis of ATP accounts for nearly 70% of the total production,[46] and thus, they represent a good model for the identification of drugs affecting mitochondrial ATP production. Furthermore, we have previously developed an engineered parasite strain (3-Luc promastigotes), which affords a real-time monitoring of changes in the cytoplasmic free ATP pool.[47] Accordingly, the effects on mitochondrial functionality of **1** and **7** in *Leishmania* promastigotes were studied by complementary bioenergetics and morphological approaches, already used for characterizing the mechanism of action for recently reported mitochondria-targeted bisphosphonium salts.[48]

First, the cytotoxic activity against *Leishmania* promastigotes was evaluated. Intriguingly, **1** and **7** showed almost identical IC_{50} values (2.81 vs 3.62 μM, respectively). The in vivo luminescence measured in 3-Luc promastigotes expressing a cytoplasmic form of luciferase showed that **7** induced a fast and concentration-dependent drop in the free cytoplasmic ATP content (Figure 4), reaching the end point 15–20 min after addition. This effect was much more pronounced than that shown by **1** at the same concentrations (Figure 4). To determine whether **1** and **7** differently affect the mitochondrial electrochemical potential ($\Delta\psi_m$) essential to drive ATP synthesis, we measured the $\Delta\psi_m$ by using the fluorescent probe Rhodamine 123, in conjunction with flow cytometry analysis. Figure 5 shows that in *L. donovani* promastigotes treated with a concentration causing about 80% of inhibition of MTT reduction (25 μM), again **7** decreased Rhodamine fluorescence more markedly than **1**, reaching values similar to those induced by KCN. This points toward a privileged

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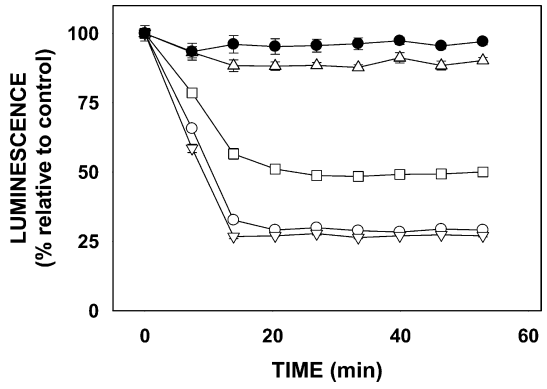

Figure 4. Decrease of luminescence caused by **1** and **7**. *L. donovani* 3-Luc promastigotes were prepared as described in the Experimental Section. DMNEP-luciferin was added, and when luminescence reached a steady state, drug was added (t = 0, 100% luminescence). Compound **1**: 25 μM (●). Compound **7**: 2.5 (△), 5 (□), 15 (○), and 25 μM (▽).

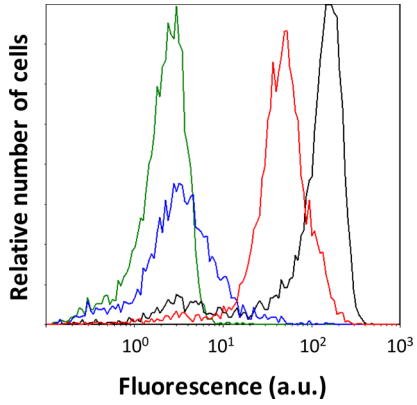

Figure 5. Variation of the mitochondrial ψ_m by equipotent concentrations of **1** and **7** on *L. donovani* promastigotes, as assessed by Rhodamine 123 accumulation. Parasites were treated for 4 h with the compounds at a concentrations causing 80% inhibition of MTT reduction, and Rhodamine 123 uptake was monitored by cytofluorometry (λ_{EXC} = 488 nm, and λ_{EM} = 525 nm). See the Experimental Section for further details. Black trace, control parasites; blue trace, 10 mM KCN as control for depolarized mitochondria; red trace, 25 μM **1**; and green trace, 25 μM **7**.

mitochondrial damage caused by **7**, probably because of the presence of the polyamine chain, with dissipation of the electrochemical gradient across the inner mitochondrial membrane (responsible for the lower Rhodamine 123 accumulation). Because $\Delta\psi_m$ is the driving force for ATP synthesis, its inhibition leads to a lower luminescence of the 3-Luc *L. donovani* promastigotes. In a further step, the morphological damage inflicted to the promastigote cells by **1** and **7** was visualized by transmission electron microscopy (Figure 6). Some mitochondria swelling was observed, especially for compound **1**, whereas for compound **7**, accumulation of vesicular material inside the flagellar pocket is evident (Figure 6B,C, respectively). Notably, the appearance of vesicular material was also observed for other compounds that inhibited the bioenergetic metabolism of Leishmania, such as edelfosine.[49] Nevertheless, an alternative explanation will be an enhanced interaction of **7** with membranes, as the combination of a polyamine tail and a quinone moiety, rather hydrophobic, will lead to a higher amphipatic structure and a consequent membrane interaction and perturbation of vesicle trafficking.

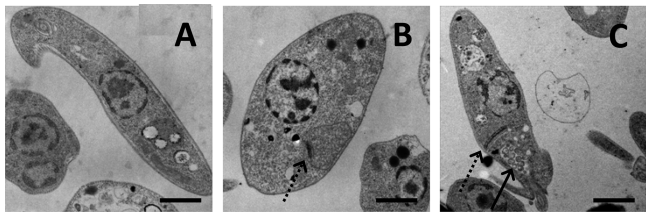

Figure 6. Electron microscopy of *L. donovani* promastigotes treated with **1** and **7**. Promastigotes were incubated with the respective compound for 4 h at a concentration causing 60−70% inhibition in MTT reduction. (A) Control parasites, (B) 5 μM **1**, and (C) 5 μM **7**. Solid arrow points toward the flagellar pocket, with abundant vesicles, whereas the kinetoplast is localized by the dotted arrow.

Taken together, the results shown by **7** are consistent with the idea that the insertion of a cadaverine tail might ameliorate the global antitrypanosomatid profile of **1**. The polyamine seems to confer to **7** intriguing biological features, such as a mitochondrial activity on the parasite that may contribute to the reduced toxicity against mammalian cells. However, the fact that the different polyamine derivatives studied here behave differently is not so clear-cut. In the case of **12**, the conjugation of a cadaverine tail to a naphthoquinone confers a rather contrary effect, **12** being 20 times less active than **2** against the *T. brucei* parasite. In general, there does not appear to be any simple and straightforward SAR that would explain the activities of all of the compounds examined. However, as reported for other polyamine conjugates,[50] the lack of unequivocal correlations within the series probably refers to the fact that for these molecules overall activity profile is multimodal, involving uptake at the plasma membrane (highly dependent on the parasite under study and on the number of charges), possible partitioning into different cellular compartments, and interaction with molecular targets.

In addition, the idea that the conjugates might exploit the polyamine transporters is not supported by the data obtained in the phenotypic screening. Although it has been verified that the specificity of the polyamine transport system is not stringent, it is likely that the characteristics essential for recognition have not been preserved in our conjugates.

■ CONCLUSIONS

A polyamine conjugate is a bifunctional molecule composed of a polyamine, which in addition to its peculiar activity profile might also act as a vector, appended to pharmacophoric elements with specific biological functions.[51] The covalent linkage of polyamines to several anticancer fragments has led to an improved profile, by up-regulating efficacy and down-regulating adverse effects.[52−54] However, it would be naive to assume that the biological profile of a conjugate exactly corresponds to the sum of those of the starting fragments. This would happen only when the chemical linkage preserved the initial fragment functions and did not affect the partitioning and trafficking of the polyamine.[55] Depending on the nature of the starting fragment and the polyamine itself, the combination gives rise to a completely new structure with novel chemical and physicochemical features and, thus, an individual biological profile.

The conjugation approach in Trypanosomatid parasites proved not to be as successful as we anticipated, although

our study has revealed cadaverine derivative **7** as an interesting hit compound toward *T. brucei*. Likely, the improved antiparasitic activity shown by **7** may be due to a fine-tuning between hydrophobicity and charge, as the first one is required for the passive translocation across the membranes, whereas the second is responsible for the mitochondria localization and the interactions with intracellular targets. This consideration might account for the generally lower activity shown by the tetraprotonated and triprotonated derivatives with respect to the diamine ones (**3**–**5** vs **7** and **8**–**10** vs **11** and **12**).

In conclusion, despite evidencing some critical issues, we have further highlighted the role of polyamine conjugation as a promising strategy in drug discovery: the versatile biological and chemical properties of polyamines foreshadow a myriad of conjugates yet to be tried.

EXPERIMENTAL SECTION

Chemistry. All reagents used were commercially available and were employed without further purification. Melting points were taken in glass capillary tubes on a Buchi SMP-20 apparatus and are uncorrected. ESI-MS spectra were recorded on Perkin-Elmer 297 and WatersZQ 4000. ^{1}H NMR and ^{13}C NMR were recorded at Varian VRX 200, 300, and 400 MHz. Chemical shifts are reported in parts per million (ppm) relative to peak of tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm, Merck) column chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F254) and then visualized in an iodine chamber or with a UV lamp. The term "dried" refers to the use of anhydrous sodium sulfate. Compounds were named following IUPAC rules as applied by Beilstein-Institute AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry. Reagents used in SPOS like Fmoc-protected amino acids and PS-Trityl chloride resin (200–400 mesh, 1% DVB, 1.42 mmol/g) were purchased from Novabiochem (San Diego, California) and RAPP Polymere GmbH (Tuebingen, Germany), respectively. All glassware used in solid-phase reactions had been silanized by treatment with 20% chlorotrimethylsilane/toluene for 12 h and then dried under vacuum. Polypropylene filter vessels were obtained from Bio-Rad. THF was dried by distillation over sodium/benzophenone ketyl, CH_2Cl_2 over sodium hydride. Anhydrous DMF was obtained commercially from Aldrich. NMR spectra were recorded at Varian Inova 300, 400, or 600 MHz. Low- and high-resolution ESI-MS were carried out on a Agilent 1100 MSD and ZabSpecETOF spectrometers, respectively. Satisfactory elemental analyses were obtained for all new compounds, confirming >95% purity.

PS-Trityl-NH$(CH_2)_5NH_2$ (15). 1,5-Diaminopentane (12.5 mL, 106.5 mmol) was dissolved in 10 mL of dry CH_2Cl_2 in a polypropylene (PP) filter vessel. PS-Trityl chloride resin (1.50 g, 2.13 mmol, 1.42 mmol/g) was then added to the solution in 4 portions over 1 h with vortexing in between additions. After it was vortexed for an additional hour, 4 mL of methanol was added followed by another 20 min of vortexing. The resin was then filtered and rinsed with MeOH, 1:4 Et_3N/DMF, MeOH, and CH_2Cl_2 (three times each) and dried under high-vacuum for over 12 h to give **15** (1.58 g, 1.35 mmol/g). A ninhydrin assay gave a positive result.

PS-Trityl-NH$(CH_2)_5NH_2$ Resin-Bound Polyamide 21. To the resin **15** (0.47 g, 0.64 mmol, 1.35 mmol/g) in a PP filter vessel, a solution of 4-hydroxybenzoic acid (0.35 g, 2.56 mmol), HOBt (0.35 g, 2.56 mmol), and HBTU (0.97 g, 2.56 mmol) in 10 mL of dry DMF was added. The vessel was shaken for 5 min, and then, a solution of DIPEA (0.89 mL, 5.12 mmol) was added. After it was vortexed for 5 h, the resin was filtered and rinsed with DMF, MeOH, and CH_2Cl_2 (three times each) and dried under vacuum for 12 h to give **21** (0.56 g, 1.14 mmol/g). A ninhydrin assay gave a negative result. A portion of the final product was cleaved from the resin, and its ES-MS analysis validated the synthesis efficiency.

PS-Trityl Resin-Bound Polyamine 26. The resin-bound amide **21** (0.56 g, 0.64 mmol, 1.14 mmol/g) was weighed into a 25 mL silanized round-bottom flask equipped with a condenser and a stir bar. The borane solution (1 M in THF, 24 mL, 24 mmol) was added dropwise at room temperature under nitrogen atmosphere. The suspension was gently refluxed at 65 °C for 72 h. After it was cooled to room temperature, the suspended resin was rapidly transferred into a PP filter vessel via a silanized pipet using dry THF to rinse out the flask and to wash the resin extensively. The exchange of amine–borane adduct was carried out following the piperidine workup: the resin was washed with THF (five times) and piperidine (three times) and then, using piperidine (12 mL), transferred into another silanized round-bottom flask heating at 65 °C for 16 h. The resin was transferred into a vessel, filtered, washed with THF, MeOH, and CH_2Cl_2 (five times each), and dried under high-vacuum overnight to give the resin-bound polyamine **26** (0.51 g, 1.256 mmol/g). A portion of the final compound was cleaved from the resins, and its ES-MS analysis validated the synthesis efficiency.

PS-Trityl Resin-Bound Boc-Protected Polyamines 31. To the resin-bound polyamine **26** (0.51 g, 0.64 mmol, 1.25 mmol/g) in a PP filter vessel, a solution of DIPEA (0.9 mL, 5.44 mmol) in 4 mL of dry CH_2Cl_2 was added. After it was shaken for 1 min, a 4 mL dry CH_2Cl_2 solution of di-*tert*-butyl dicarbonate [$(Boc)_2O$, 2.38 g, 10.9 mmol] was added. The suspension was vortexed overnight. Then, the resin was filtered, washed with DMF, MeOH, and CH_2Cl_2 (five times each), and dried under vacuum for over 12 h to afford the PS-Trityl resin-bound Boc-protected polyamine **31** (0.63 g, 1.02 mmol/g). A portion of the final product was cleaved from the resins using a mild cleavage cocktail (CH_3COOH/2,2,2-trifluoroethanol/CH_2Cl_2 1:2:7) to avoid the Boc-protecting groups removal, and its ES-MS analysis validated the synthesis efficiency.

PS-Trityl Resin-Bound Boc-Protected Polyamine 36. The PS-Trityl resin-bound Boc-protected polyamine **31** (0.63 g, 1.02 mmol/g) weighed into a silanized round-bottom flask was suspended into a 25% piperidine/CH_2Cl_2 solution and gently stirred for 6 h at 40 °C. Then, the resin was quickly transferred into a PP filter vessel via a silanized pipet, filtered, washed with DMF, MeOH, and CH_2Cl_2 (five times each), and dried under vacuum overnight to afford the PS-Trityl resin-bound Boc-protected polyamine **36** (0.49 g, 1.31 mmol/g). A portion of the final derivatives was cleaved from the resins using a mild cleavage cocktail (CH_3COOH/2,2,2-trifluoroethanol/CH_2Cl_2 1:2:7) to avoid the Boc-protecting group removal, and its ES-MS analysis validated the synthesis efficiency.

PS-Trityl Resin-Bound Polyamine–Anthraquinone 41 and PS-Trityl Resin-Bound Polyamine–Naphthoquinone 46. The PS-Trityl resin-bound Boc-protected polyamine **36** (0.49 g, 1.31 mmol/g), weighed into a silanized round-bottom flask, was suspended in dry DMF (25 mL). NaH (5 equiv) was carefully added, and the mixture was heated at 50 °C for 2 h. Then, 5 equiv of 2-bromo-1,4-anthraquinone (for **41**) or 2-bromo-1,4-naphthoquinone (for **46**) was added. After it was gently stirred for 48 h, the resin was quickly transferred into a PP filter vessel, washed with DMF, MeOH, and CH_2Cl_2 (five times each), and dried under vacuum overnight to give the PS-Trityl resin-bound polyamine–anthraquinones **41** (0.27 g, 1.18 mmol/g) and PS-Trityl resin-bound polyamine–naphthoquinones **46** (0.28 g, 1.14 mmol/g).

Anthraquinone–Polyamine Conjugate (3–7) and Naphthoquinone–Polyamine Conjugates (8–12). PS-Trityl resin-bound polyamine–anthra-/naphthoquinones derivatives **40**–**49** were weighed into a 25 mL silanized round-bottom flask and stirred in a freshly prepared TFA/H_2O/*i*-Pr_3SiH (95:2.5:2.5) cleavage cocktail (10 mL) for 2 h at room temperature. After the solution was removed by a pipet from the flask, the above-mentioned cleavage cocktail (10 mL) was added to the resins left. The suspensions were then stirred for an additional 2 h. The contents were filtered through a glasswool plug, and the resins were rinsed extensively with TFA/MeOH/CH_2Cl_2 (5:30:65). The combined filtrates from two rounds of cleavage were evaporated and dried over high-vacuum for >12 h to give crude **3**–**12**

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as trifluoroacetate ammonium salts. Two rounds of precipitation with methanol/ether finally afforded **3**−**12** as yellow/brown solids with an overall yield ranging from 12 to 43%.

2-(4-((5-Aminopentylamino)methyl)phenoxy)anthracene-1,4-dione Trifluoroacetate Salt (7). Yield, 37%. ^{1}H NMR (CD_3OD, 400 MHz): δ 1.50−1.52 (m, 2H), 1.70−1.73 (m, 4H), 2.94−3.26 (m, 4H), 4.28 (s, 2H), 6.01 (s, 1H), 7.37 (d, J = 8.0 Hz, 2H), 7.66 (d, J = 8.0 Hz, 2H), 7.75−7.77 (m, 2H), 8.14−8.21 (m, 2H), 8.60 (s, 1H), 8.76 (s, 1H). ^{13}C NMR (CD_3OD, 400 MHz): δ 24.1, 30.2, 42.0, 118.2, 120.0, 128.5, 129.1, 129.2, 131.3, 134.56, 178.2, 185.9. MS (ESI$^+$) m/z: 415 $[M + H]^+$.

2-(4-((5-Aminopentylamino)methyl)phenoxy)naphthalene-1,4-dione Trifluoroacetate Salt (12). Yield, 19%. ^{1}H NMR (CD_3OD, 400 MHz): δ 1.48−1.50 (m, 2H), δ 1.72−1.77 (m, 4H), 2.94−3.09 (m, 4H), 4.26 (s, 2H), 5.90 (s, 1H), 7.33 (d, J = 6.0 Hz, 2H), 7.64 (d, J = 6.0 Hz, 2H), 7.83−7.85 (m, 2H), 8.03−8.05 (m, 1H), 8.16−8.18 (m, 1H). ^{13}C NMR (CD_3OD, 400 MHz): δ 21.8, 23.0, 35.5, 47.1, 48.7, 114.1, 122.6, 123.2, 124.4, 128.7, 129.5, 130.4, 132.1, 133.1, 152.0, 158.5, 179.0, 182.5. MS (ESI$^+$) m/z: 365 $[M + H]^+$.

Biological Assays. In vitro activity against *T. b. rhodesiense* (STIB900), *T. cruzi* (Tulahuen strain C2C4), axenic amastigotes of *L. donovani* (strain MHOM/ET/67/L82), and cytotoxicity assessment against L6 cells were determined as previously reported.[56]

Kinetic Analysis of TR. The pET3a-TbTryR plasmid[57] for the expression of tag-free *T. brucei* TR was kindly provided by Dr. Alan Fairlamb, Dundee. TS_2 was generated enzymatically as described previously.[58] TR activity (66 mU) was measured in a total volume of 1 mL of 40 mM Hepes, 1 mM EDTA, pH 7.5, in the presence of 100 μM NADPH and varying concentrations of TS_2 and/or inhibitors. The absorption decrease due to NADPH oxidation was followed at 340 nm and 25 °C. Stock solutions of the inhibitors were prepared in DMSO. The K_i and K_i' values were determined using the following equations:

Noncompetitive Inhibition **Mixed Inhibition.**

$$K_i = \frac{[I]}{\frac{V_{max}}{V_{max(obs)}} - 1} \qquad K_i = \frac{[I]}{\frac{K_{m(obs)}\left(1 + \frac{[I]}{K_i'}\right)}{K_m} - 1}$$

$$K_i' = \frac{[I]}{\frac{V_{max}}{V_{max(obs)}} - 1}$$

The ability of the compounds to induce the oxidase activity of TR was measured in the presence of 100 μM NADPH, 5 μM compound, and 1.36 U of TR in a total volume of 1 mL. Under these conditions, the spontaneous NADPH oxidation resulted in an absorption decrease of ≤0.0026/min. This value was subtracted from the enzyme catalyzed rate.

Activity on *L. donovani* Promastigotes. *L. donovani* promastigotes (strain MHOM/ET/67/L82) and its surrogated strain 3-Luc were grown at 26 °C in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum, gentamycin, penicillin, and 2 mM L-glutamine. For 3-Luc promastigotes, the growth medium was supplemented with Geneticin (30 μg/mL). Promastigotes were harvested at late exponential phase, washed twice in Hanks balanced salt solution supplemented with 10 mM D-glucose (pH 7.2) (HBSS + Glc), and resuspended in the same medium. Unless otherwise stated, the experiments were carried out in 96-microwell plates in a final volume of 100 μL and a parasite density of 2×10^7 cells/mL. Samples were made by triplicate, and experiments were repeated at least twice. Parasites were incubated with the corresponding drug at different concentrations, and the effect of the drug was assessed by MTT reduction as described.[59]

Assessment of in Vivo Free Cytoplasmic ATP Levels. Promastigotes of the 3-Luc strain, expressing a cytoplasmic form of firefly luciferase, were prepared as outlined in the previous section. DMNPE-Luciferin [D-luciferin1-(4, 5-dimethoxy-2-nitrophenyl) ethyl ester] (Invitrogen), a membrane-permeable cage substrate of luciferase, was added at a final concentration of 25 μM. Under these conditions, the limiting substrate for luminescence is the free-cytoplasmic concentration of ATP and is expressed as percentage of luminescence with respect to control parasites.[59]

Variation of Mitochondrial $\Delta\psi_m$. *L. donovani* were incubated with the corresponding drug for 4 h, washed, resuspended in HBSS + Glc containing 0.3 μg/mL of Rhodamine 123 [2-(6-amino-3-imino-3*H*-xanthen-9-yl)benzoic acid methyl ester, chloride] (Invitrogen), and incubated for 10 min at 37 °C. Afterward, to remove the nonincorporated drug, the parasites were washed twice with HBSS + Glc, resuspended at a density of 1×10^6 cells/mL, and analyzed in a Beckman Coulter FC500 MPL (λ_{EXC}= 488 nm, λ_{EM} = 525 nm). Parasites incubated with 10 mM KCN were taken as fully depolarized parasites.[48]

Electron Microscopy. Promastigotes *L. donovani* were incubated for 4 h with the corresponding drugs according to the standard protocol, washed twice in HBSS, fixed with 5% (w/v) glutaraldehyde in HBSS, and included with 2.5% (w/v) OsO_4 for 1 h.[48] Afterward, samples were gradually dehydrated in ethanol [30, 50, 70, 90, and 100% (v/v); 30 min each] and propylene oxide (1 h). Finally, samples were embedded in Epon 812 resin and observed in a Jeol-1230 electron microscope.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of the synthesis of intermediates (**13**−**49**), characterization data for final compounds **3**−**6** and **8**−**11**, and elemental analysis data for **3**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DIPEA, *N*,*N*-diisopropylethylamine; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; HOBt, hydroxybenzotriazole; NTD, neglected tropical diseases; SI, selectivity index; SPOS, solid-phase organic synthesis; TR, trypanothione reductase; TS_2, trypanothione disulfide

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