

Synthesis and Structure–Activity Analysis of New Phosphonium Salts with Potent Activity against African Trypanosomes

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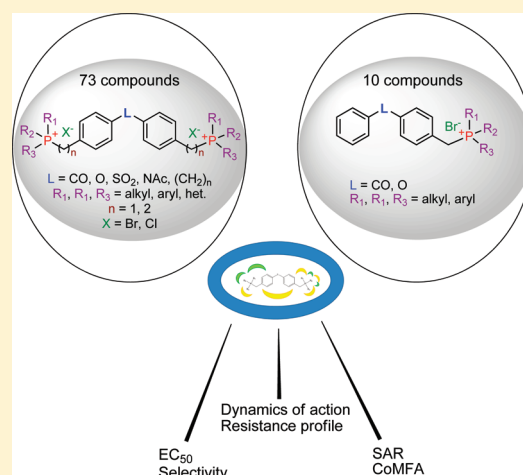
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Supporting Information

ABSTRACT: A series of 73 bisphosphonium salts and 10 monophosphonium salt derivatives were synthesized and tested in vitro against several wild type and resistant lines of *Trypanosoma brucei* (*T. b. rhodesiense* STIB900, *T. b. brucei* strain 427, *TbAT1-KO*, and *TbB48*). More than half of the compounds tested showed a submicromolar EC₅₀ against these parasites. The compounds did not display any cross-resistance to existing diamidine therapies, such as pentamidine. In most cases, the compounds displayed a good selectivity index versus human cell lines. None of the known *T. b. brucei* drug transporters were required for trypanocidal activity, although some of the bisphosphonium compounds inhibited the low affinity pentamidine transporter. It was found that phosphonium drugs act slowly to clear a trypanosome population but that only a short exposure time is needed for irreversible damage to the cells. A comparative molecular field analysis model (CoMFA) was generated to gain insights into the SAR of this class of compounds, identifying key features for trypanocidal activity.



INTRODUCTION

Human African trypanosomiasis (HAT or sleeping sickness) is a parasitic disease caused by infection with two subspecies of trypanosomes: *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. HAT is endemic in 36 countries of sub-Saharan Africa and, together with the corresponding condition in domestic animals, is a major cause of suffering and poverty for the affected population. If left untreated, the disease is usually fatal.¹ However, the treatment options are scarce, as few drugs are available (i.e., suramin, pentamidine, melarsoprol, eflornithine, and recently, the combination therapy nifurtimox–eflornithine). These are far from being ideal because of ineffectiveness to some trypanosome species or stages of the infection, as well as toxicity, the parenteral mode of administration, and the emergence of resistance.^{2,3}

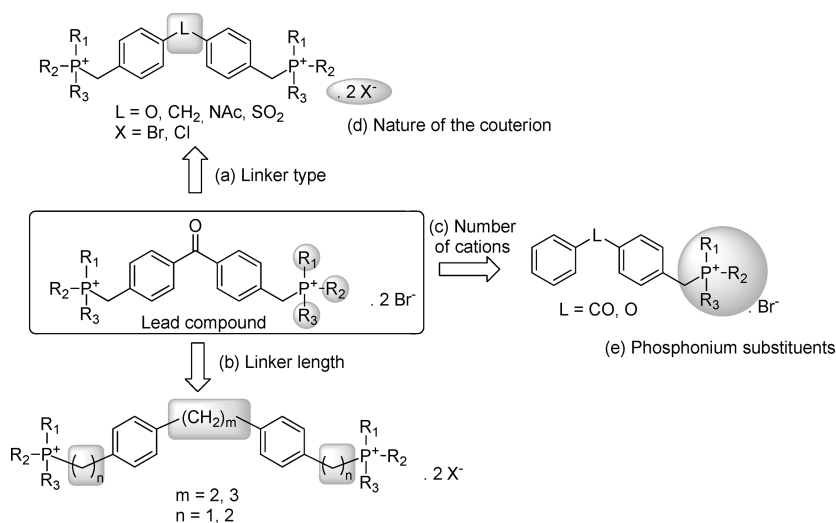
The antitrypanosomal activity of benzyltriphenylphosphonium salts against *T. brucei* was first reported in 1979 by Kinnamon et al.⁴ Some compounds were curative in a murine model of *T. b. rhodesiense* infection. However, the study of the potential of phosphonium salts as antiprotozoal agents was not followed up by any research group as shown by the lack of

literature on this subject in the past 30 years. We recently regained interest in the antiparasitic activity of phosphonium salts with the discovery of a series of benzophenone-derived bisphosphonium salt derivatives that showed a marked antileishmanial activity in vitro.⁵ In *Leishmania*, the best compound of the series (i.e., 4,4'-bis((tri-*n*-pentylphosphonium)methyl)benzophenone dibromide) was found to target the mitochondria of the parasite, inhibiting complex II of the respiratory chain.⁵ Since some of these benzophenone compounds also showed interesting activity against *T. b. rhodesiense*,⁶ we decided to prepare new derivatives to systematically examine the structure–activity relationship of antitrypanosomal phosphonium compounds. Hence, we synthesized 60 new phosphonium salt derivatives with variations in the following parts of the lead structure: (a) linker type, (b) linker length, (c) number of cations, (d) nature of the counterion, and (e) nature of the phosphonium groups substituents R₁, R₂, R₃ (Chart 1).

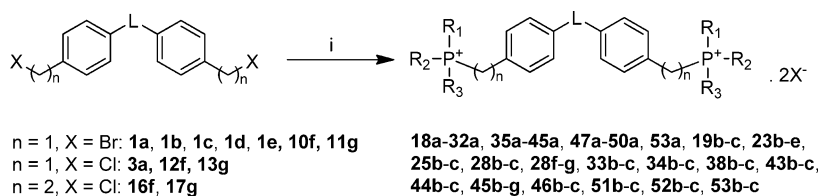
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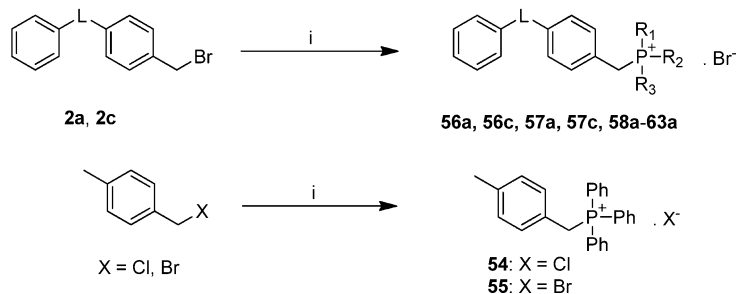
Chart 1. General Structure of Benzophenone-Derived Bisphosphonium Salt Derivatives with Antileishmanial and Antitrypanosomal Activity⁵ and New Series Being Studied



Scheme 1. Synthesis of the Phosphonium Salt Derivatives^a



$L = CO$ (a), CH_2 (b), O (c), SO_2 (d), NAC (e), $(CH_2)_2$ (f), $(CH_2)_3$ (g)



^aReagents and conditions: (i) $R_1R_2R_3P$ (excess), DMF or toluene, Δ . See Tables 1–4 for substituents pattern.

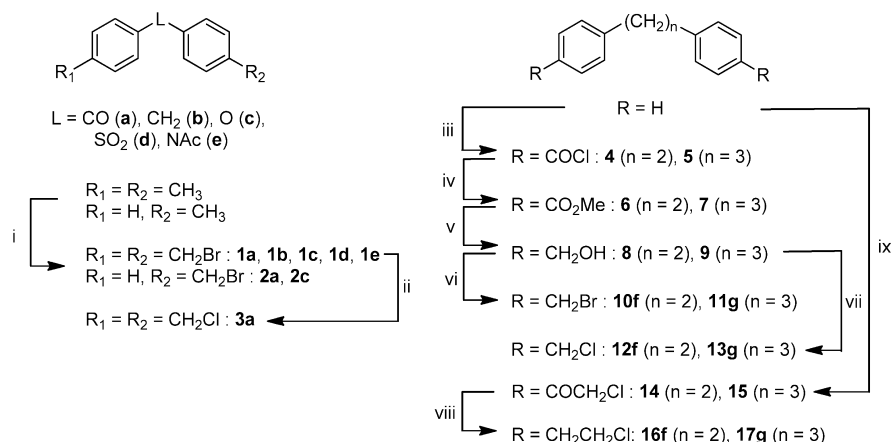
The compounds were tested for in vitro activity against *T. b. rhodesiense* (strain STIB900) and on a standard panel of *T. b. brucei* lines (s427, *TbAT1-KO*, and *TbB48*) with decreasing sensitivity to most diamidine and arsenic-based drugs due to loss of specific drug transporters (see Experimental Section for details).^{7,8} This is important because, like diamidines, most of the compounds described here are dications. In fact, the drug transporters may contribute positively to the selectivity of the compounds under development or, conversely, be a cause of drug resistance.⁹ Resistance to first line diamidines such as diminazene aceturate is linked to loss of these transporters¹⁰ and represents a genuine threat to the treatability of trypanosomiasis.² Thus, it is essential to ascertain that no cross-resistance between new compounds and existing therapy will arise. In addition, we established the dynamics of the action of phosphonium salts on trypanosomes: trypanocidal or trypanostatic, fast action or slow, minimum time of exposure,

etc. Finally, we sought to understand the SAR behind this class of phosphonium compounds by performing CoMFA.

RESULTS

Chemistry. The synthesis of the target compounds is based on the nucleophilic substitution of a (bis)halogenated precursor (i.e., linker) with a trisubstituted phosphine giving rise to the corresponding phosphonium salts (Scheme 1). Hence, the (bis)halogenated precursors must hold the different structural characteristics (i.e., linker type, linker length, number of cations, type of counterion) one wants to introduce in the lead structure.

The different halogenated linkers were synthesized as shown in Scheme 2. The 4,4'-bisbromomethyl linkers **1a**,⁶ **1b**,¹¹ **1d**,¹² and **1e**¹³ were synthesized by *N*-bromosuccinimide (NBS) bromination of the 4,4'-dimethylphenyl precursors as previously reported. The linkers **1c** and **2c** were commercially available. The 4,4'-bischloromethyl linker **3a** was obtained by reaction of

Scheme 2. Synthesis of the Linkers^a

^aReagents and conditions. (i) NBS, ^tBuOOH, CCl₄, reflux, 20–52%; (ii) BiCl₃, 1,2-dichloroethane, Δ, 60–72%; (iii) (1) oxalyl chloride, AlCl₃, CH₂Cl₂, –15 °C, 5 h; (2) chlorobenzene, reflux, 5 h, 80–84%; (iv) MeOH, rt, quantitative; (v) LiAlH₄, THF, reflux, 18 h, 90–94%; (vi) thionyl bromide, CH₂Cl₂, rt, 2 h, 82–95%; (vii) thionyl chloride, CH₂Cl₂, rt, 2 h, 68–97%; (viii) Et₃SiH, CF₃CO₂H, 0 °C → 45 °C → rt, 61–90%; (ix) ClCOCH₂Cl, AlCl₃, CH₂Cl₂, reflux, 3 h, 94%.

the corresponding 4,4'-bisbromomethyl linker **1a** with BiCl₃ in anhydrous 1,2-dichloroethane.¹⁴ The diphenylethane (**10f**, **12f**) and diphenylpropane (**11g**, **13g**) linkers were synthesized as shown in Scheme 2. Friedel–Crafts acylation of diphenylethane and diphenylpropane (oxalyl chloride/AlCl₃) followed by decarbonylation in refluxing chlorobenzene gave **4** and **5**,¹⁵ respectively. The acyl chlorides were stirred in methanol at room temperature to yield the methyl esters **6** and **7** quantitatively. Lithium aluminum hydride reduction of **6** and **7** gave excellent yields of the diols **8** and **9**. These were converted to the dibromide (**10f**, **11g**) and dichloride (**12f**, **13g**) using thionyl bromide and thionyl chloride, respectively. The synthesis of the 4,4'-bis(2-chloroethylphenyl) linkers **16f** and **17g** started with Friedel–Crafts acylation of diphenylethane and diphenylpropane with chloroacetylchloride/AlCl₃. Reduction of **14** and **15** with triethylsilane in trifluoroacetic acid yielded the expected products **16f** and **17g**, respectively.

The bisphosphonium salts **18–53(a–e)** were synthesized in good yields by reaction of the corresponding 4,4'-bischloromethyl (**3a**, **12f**, **13g**), 4,4'-bisbromomethyl (**1a–e**, **10f**, **11g**), or 4,4'-bischloroethyl linker (**16f**, **17g**) with an excess of commercially available trisubstituted phosphine in anhydrous DMF at 100 °C (150 °C for **16f** and **17g**) (Scheme 1). The monophosphonium salts **54**, **55**, **56a–63a**, and **56c–57c** were prepared in the same way from 4-chloromethyltoluene, 4-bromomethyltoluene, 4-bromomethylbenzophenone (**2a**), and 1-bromomethyl-4-phenoxybenzene (**2c**), respectively. A lower reaction temperature (50 °C) was used with bis- and tris-2-methoxyphenylphosphine (**42a**, **43a–c**, and **61a**) to avoid the formation of several byproducts. The salts were isolated by crystallization from the reaction mixture or by semipreparative HPLC–MS for **64** and **65**. Compounds **18a–24a**, **26a**, **28a–32a**, **36a–38a**, **48a**, and **49a** were synthesized as reported earlier.⁵

In Vitro Antitrypanosomal Activity. The new compounds were tested for in vitro activity against *T. b. rhodesiense* (strain STIB900) and a standard panel of *Trypanosoma brucei* lines (s427, TbAT1-KO, and *T. b.* B48) with decreasing sensitivity to diamidine drugs. The small differences in phosphonium compounds EC₅₀ values observed between *T. b. rhodesiense* STIB900 and *T. b. brucei* s427 are most probably

due to the slightly different assay procedures used in the two laboratories involved in this work.¹⁶ Alternatively, they could reflect the minor biochemical differences between the two strains used.

In general, compounds with three short alkyl substituents (Me, Et, or Pr) on the phosphonium cations were poorly active against both trypanosome species (EC₅₀ ≥ 10 μM), whatever the linker was (Table 1, entries 1–6; Table 4, entries 7, 9). The activity was enhanced with the increase in chain length (*n*-octyl ~ *n*-hexyl > *c*-hexyl ~ *n*-pentyl > *n*-Bu > *i*-Bu), reaching a maximum for six- to eight-carbon substituents (Table 1, entries 7–18). Replacement of the alkyl substituents with phenyl rings increased the activity in the order (R₁, R₂, R₃ = alkyl) < (R₁, R₂ = alkyl; R₃ = Ph) < (R₁ = alkyl; R₂, R₃ = Ph) (Table 1, entries 19–32).

Since the best activities and selectivities were obtained with phenyl substituents, we decided to study whether the presence of substituents on the aromatic ring would influence the antitrypanosomal activity. As shown in Table 2, the best results were obtained with the 4-Me substituted phenyl rings (entries 16–25). Substitution pattern for the phenyl ring, in order of decreasing activity, was 4-Me > 3-Me > H and 2-OMe > 4-OMe > 4-Cl ~ 4-F > 4-CF₃. Within this series, the order of decreasing activity as a function of the linker was O ~ (CH₂)₃ > (CH₂)₂ ~ CH₂ > CO > SO₂ ~ NAc.

Bioisosteric replacement of the phenyl substituents of the phosphonium cation by 2-thienyl groups (Table 3, entries 1 and 2) or 1-naphthyl substituents (Table 3, entries 10–12) maintained the activity. On the contrary, the introduction of an oxygenated (2-furanyl) or amino (2-pyridyl) heterocycle (entries 3 and 4) was detrimental to the activity, presumably because these are more polar and the substituents can engage in hydrogen bonds. Likewise, the replacement of one phenyl substituent by a more polar phenylsulphonate or pentafluorophenyl ring abolished the trypanocidal activity (Table 3, entries 6–9).

Interestingly, removal of one of the phosphonium cations did not reduce the antitrypanosomal activity (Table 4). On the contrary, lower EC₅₀ values were observed for **56a**, **56c**, **57c**, **59a**, and **62a** compared with their bisphosphonium salt analogues (**45a**, **45c**, **46c**, **38a**, and **25a**, respectively).

Table 1. Antitrypanosomal Activity of Bisphosphonium Salts Having Aliphatic and Phenyl Substituents

entry	compd	L	R ₁	R ₂	R ₃	T. b. rhod. ^a 0.0075	cytox L6 ^b 0.012	EC ₅₀ (μM) (selectivity index) ⁱ				SI ^h				
								T. b. brucei WT ^c	TbATI-KO ^d	RF ^e	T. b. B48 ^f		RF ^e	cytox HEK ^g		
1	18a	CO	Me	Me	Me	127	>173	>100	0.008 ± 0.002	0.009 ± 0.003	1.1	0.567 ± 0.086	70.9	nd ^f	>300	>14
2	19a	CO	Et	Et	Et	77.8	>149	>100	0.517 ± 0.132	2.39 ± 0.84	4.6	2.617 ± 0.714	5.1	>300	>300	>8
3	19b	CH ₂	Et	Et	Et	18.7	48.3	21.5 ± 0.3	18.5 ± 0.4	19.3 ± 0.2	0.9	19.3 ± 0.2	0.9	>300	>300	>8
4	19c	O	Et	Et	Et	31.9	101.6	36.5 ± 3.5	34.9 ± 0.2	36.6 ± 0.6	1.0	36.6 ± 0.6	1.0	>300	>300	>8
5	20a	CO	n-Pr	n-Pr	n-Pr	8.5	>130	19.73 ± 4.11	27.15 ± 3.82	31.32 ± 4.15	1.4	31.32 ± 4.15	1.6	nd	nd	>620
6	21a	CO	Pr	Pr	Pr	9.6	>130	20.55 ± 5.19	33.41 ± 6.20	38.82 ± 6.41	1.6	38.82 ± 6.41	1.9	nd	nd	>110
7	22a	CO	n-Bu	n-Bu	n-Bu	0.355 (315)	111.7	0.48 ± 0.11	1.32 ± 0.46	1.63 ± 0.46	3.4	1.63 ± 0.46	3.4	>300	>300	>429
8	23a	CO	Bu	Bu	Bu	1.46	>116	2.62 ± 0.69	8.34 ± 3.08	6.30 ± 1.39	2.4	6.30 ± 1.39	2.4	>300	>300	>507
9	23b	CH ₂	Bu	Bu	Bu	0.326 (141)	45.9	0.70 ± 0.03	0.88 ± 0.10	0.88 ± 0.06	1.3	0.88 ± 0.06	1.3	>300	>300	>507
10	23c	O	Bu	Bu	Bu	1.62	51.1	0.59 ± 0.06	0.80 ± 0.10	1.06 ± 0.34	1.8	1.06 ± 0.34	1.8	>300	>300	>507
11	23d	SO ₂	Bu	Bu	Bu	6.31	>111	16.7 ± 0.5	13.6 ± 2.2	13.3 ± 1.6	0.8	13.3 ± 1.6	0.8	nd	nd	>507
12	23e	NAC	Bu	Bu	Bu	11.44	>112	nd	nd	nd	nd	nd	nd	nd	nd	>507
13	24a	CO	n-pentyl	n-pentyl	n-pentyl	0.289 (30)	8.7	0.20 ± 0.03	0.17 ± 0.01	0.18 ± 0.01	0.8	0.18 ± 0.01	0.9	66.8 ± 4.9	338	>507
14	25a	CO	n-hex	n-hex	n-hex	0.112 (10)	1.11	0.038 ± 0.001	0.047 ± 0.007	0.035 ± 0.003	1.2	0.035 ± 0.003	0.9	16.6 ± 0.7	438	>507
15	25b	CH ₂	n-hex	n-hex	n-hex	0.180 (5)	0.964	0.033 ± 0.006	0.033 ± 0.005	0.039 ± 0.002	1.2	0.039 ± 0.002	1.2	18.1 ± 2.6	543	>507
16	25c	O	n-hex	n-hex	n-hex	0.109 (9)	1.03	0.037 ± 0.004	0.045 ± 0.005	0.041 ± 0.002	1.1	0.041 ± 0.002	1.1	22.1 ± 1.1	593	>507
17	26a	CO	c-hex	c-hex	c-hex	0.197 (166)	32.7	0.14 ± 0.03	0.23 ± 0.05	0.25 ± 0.04	1.6	0.25 ± 0.04	1.8	>300	>2100	>507
18	27a	CO	n-octyl	n-octyl	n-octyl	0.22 (5)	1.0	0.042 ± 0.002	0.061 ± 0.001	0.045 ± 0.001	1.4	0.045 ± 0.001	1.1	27.0 ± 1.4	639	>507
19	28a	CO	Me	Me	Ph	8.8	>139	19.17 ± 7.17	28.77 ± 5.62	24.55 ± 4.75	1.5	24.55 ± 4.75	1.3	nd	nd	>507
20	28b	CH ₂	Me	Me	Ph	2.586	>142	5.45 ± 1.09	4.60 ± 0.80	3.84 ± 1.19	0.8	3.84 ± 1.19	0.7	>300	>300	>507
21	28c	O	Me	Me	Ph	3.827	84.5	7.22 ± 0.16	6.52 ± 0.39	6.15 ± 0.89	0.9	6.15 ± 0.89	0.9	>300	>300	>507
22	28f	(CH ₂) ₂	Me	Me	Ph	1.20 (40)	48.6	2.59 ± 0.64	2.62 ± 0.16	1.8 ± 0.58	1.0	1.8 ± 0.58	0.5	>300	>300	>507
23	28g	(CH ₂) ₃	Me	Me	Ph	1.22 (49)	59.5	1.27 ± 0.33	1.37 ± 0.11	1.09 ± 0.38	1.1	1.09 ± 0.38	0.9	>300	>300	>507
24	29a	CO	Et	Et	Ph	2.9	>128	8.59 ± 2.16	17.75 ± 2.03	15.17 ± 3.72	2.1	15.17 ± 3.72	1.8	>300	>300	>507
25	30a	CO	c-hex	c-hex	Ph	0.32 (72)	22.9	0.24 ± 0.03	0.26 ± 0.04	0.29 ± 0.03	1.1	0.29 ± 0.03	1.2	>300	>300	>507
26	31a	CO	Me	Ph	Ph	0.656 (177)	115.9	1.53 ± 0.38	2.08 ± 0.63	2.05 ± 0.38	1.4	2.05 ± 0.38	1.3	>300	>300	>507
27	32a	CO	Et	Ph	Ph	0.554 (>204)	>113	0.97 ± 0.27	2.10 ± 0.54	1.76 ± 0.30	2.2	1.76 ± 0.30	1.8	>300	>300	>507
28	33b	CH ₂	n-Pr	Ph	Ph	0.799 (4.1)	3.27	0.373 ± 0.003	0.40 ± 0.01	0.43 ± 0.08	1.1	0.43 ± 0.08	1.1	>300	>300	>507
29	33c	O	n-Pr	Ph	Ph	0.567 (5)	2.9	0.25 ± 0.03	0.28 ± 0.01	0.3 ± 0.01	1.1	0.3 ± 0.01	1.2	233 ± 31	914	>507
30	34b	CH ₂	Pr	Ph	Ph	0.534 (6.4)	3.44	0.44 ± 0.04	0.40 ± 0.03	0.43 ± 0.09	0.9	0.43 ± 0.09	1.0	>300	>300	>507
31	34c	O	Pr	Ph	Ph	0.585 (8.6)	5.0	0.33 ± 0.02	0.33 ± 0.01	0.33 ± 0.01	1.0	0.33 ± 0.01	1.0	>300	>300	>507
32	35a	CO	c-hex	Ph	Ph	0.43 (155)	65.8	0.30 ± 0.03	0.41 ± 0.01	0.33 ± 0.03	1.4	0.33 ± 0.03	1.1	>300	>300	>507

Table 1. continued

^a*T. b. rhodesiense* STIB900 trypanomastigotes. ^bRat skeletal myoblast L6 cells. ^c*T. b. brucei* s427 trypanomastigotes. ^d*T. b. brucei* knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate. ^eResistance factor compared to WT. ^fThe B48 strain is a mutant derived from the TbAT1-KO strain with a nonfunctional high affinity pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine, and melaminophenyl arsenicals. ^gHuman embryonic kidney (HEK) cells. ^hSelectivity index = $[\text{EC}_{50}(\text{HEK cells})/\text{EC}_{50}(T. b. brucei \text{ WT})]$. ⁱSelectivity index = $[\text{EC}_{50}(\text{L6-cells})/\text{EC}_{50}(T. b. rhodesiense)]$. ^jNot determined.

Regarding the substituents pattern, the same SAR as for the bisphosphonium analogues was observed (i.e., *p*-tolyl > *m*-tolyl and *n*-hex > *i*-Bu \gg Et). In this series, replacement of the carbonyl group by an oxygen linker enhanced the in vitro activity 20- to 40-fold against *T. b. rhodesiense* (compare **56a** vs **56c**, **57a** vs **57c**). The diphenyl ether analogues **56c** and **57c** were the most active compounds of all the series with EC₅₀ values in the low nanomolar range against wild type and resistant *T. b. brucei* lines (Table 4, entries 4 and 6), respectively. Despite their submicromolar and low micromolar cytotoxicity toward L6 cells and HEK cells, respectively, both compounds still have a reasonable therapeutic window with a selectivity index (SI) vs L6-cells of 131 and 62 and vs HEK-cells of 1145 and 876, respectively.

As far as the counterion is concerned, no significant difference in in vitro activity was observed between the bromide and the chloride salt of the compounds (Table 2, entries 16–22). In addition, a longer linker (i.e., ethylene instead of methylene linker) between the phosphonium cation and the central diphenyl core hardly affected the activity as shown by the nanomolar EC₅₀ values of compounds **65** (73 nM vs 63 nM for **45g**) and **64** (27 nM vs 50 nM for **45f**) (Table 5).

In most cases, the compounds displayed an acceptable selectivity index toward HEK cells (>100). However, higher cytotoxicity was found with L6 cells, indicating a notable difference in susceptibility to phosphonium compounds between the two cell lines.

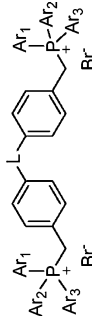
Cross-Resistance and Drug Transport. Importantly, EC₅₀ values were found to be statistically identical for the s427, TbAT1-KO, and B48 strains in all cases (Student *t*-test, *P* > 0.05). This indicates that there is no cross-resistance between the phosphonium cations and the crucial diamidine and melaminophenyl arsenical classes of trypanocides. However, the resazurin assay employed to generate EC₅₀ values provides a single reading after an initial 48 h incubation period with test compound followed by a further 24 h in the additional presence of the dye. While this allows for high throughput and reproducible EC₅₀ values, it gives no information on how speedily the compounds act on the cells. The relationship between drug concentration and minimal exposure time is important, as it has major implications for potential drug development: should the drug be required to be present in circulation for 48 h at a concentration greater than EC₉₀, this would inevitably require a far higher dosage, and more frequent administration, than achieving such a dose for 2 h. We thus conducted a series of experiments to assess differential action on the cell lines by a small selection of phosphonium salts. We used incubations with the viability reporter dye propidium iodide (PI), which gives a fluorescent signal upon cell entry and binding to nucleic acid.¹⁷ Figure 1 shows the effects on cellular integrity by **24a**, recorded in real time for a period of >8 h. This revealed that for all three strains only concentrations of $\geq 3.3 \mu\text{M}$ affected viability over the course of the experiment, despite EC₅₀ values of $\leq 0.20 \mu\text{M}$. Yet the speed at which the cell membrane integrity was compromised appeared to be slightly less in the diamidine resistant strains. Similar observations were made for **25b**, **25c**, **26a**, **35a**, **36a**, **38a**, **43a**, **43c**, **45d**, **45e**, **47a**, and **55** (see Supporting Information).

Given the very similar sensitivities, it must be deemed highly unlikely that the cellular entry of these cations depends critically on either of the two diamidine transporters (i.e., TbAT1/P2 aminopurine transporter and the high affinity pentamidine transporter, HAPT1) that are known to be, by

Table 2. Antitrypanosomal Activity of Bisphosphonium Salts having Substituted Phenyl Substituents^a

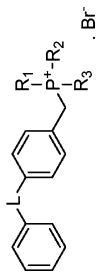
entry	compd	L	R ₁	R ₂	R ₃	EC ₅₀ (μM) (selectivity index) ^f							cytox HEK ^g	SI ^h
						<i>T. b. rhodesiense</i> ^a	cytox L6 ^b	<i>T. b. brucei</i> WT ^c	TbAT1-KO ^d	RF ^e	<i>T. b. B48</i> ^f	RF ^e		
1	36a	CO	H	H	H	0.260 (282)	73.3	0.24 ± 0.07	0.36 ± 0.07	1.5	0.38 ± 0.06	1.6	>300	>1250
2	37a	CO	H	H	2-Me	0.16 (88)	14.0	0.20 ± 0.01	0.24 ± 0.03	1.2	0.22 ± 0.02	1.1	214.3 ± 9.5	1098
3	38a	CO	H	H	4-Me	0.26 (62)	16.3	0.180 ± 0.002	0.182 ± 0.006	1.0	0.183 ± 0.005	1.0	261.3 ± 24.3	1449
4	38b	CH ₂	H	H	4-Me	0.200 (22)	4.47	0.24 ± 0.04	0.19 ± 0.03	0.8	0.18 ± 0.02	0.7	>300	>1250
5	38c	O	H	H	4-Me	0.142 (23)	3.34	0.082 ± 0.014	0.077 ± 0.002	0.9	0.064 ± 0.009	0.8	240.3 ± 6.7	2930
6	39a	CO	4-Cl	4-Cl	4-Cl	0.41 (18)	7.2	0.94 ± 0.14	1.37 ± 0.02	1.5	0.95 ± 0.13	1.0	63 ± 8	67
7	40a	CO	4-F	4-F	4-F	0.45 (172)	77.4	0.85 ± 0.17	2.10 ± 0.03	2.5	1.24 ± 0.14	1.5	>300	>350
8	41a	CO	4-OMe	4-OMe	4-OMe	0.29 (40)	11.8	0.32 ± 0.07	0.52 ± 0.02	1.6	0.23 ± 0.04	0.7	172 ± 7	529
9	42a	CO	2-OMe	2-OMe	H	0.23 (28)	6.4	0.24 ± 0.04	0.32 ± 0.02	1.3	0.19 ± 0.04	0.8	107 ± 15	438
10	43a	CO	2-OMe	2-OMe	2-OMe	0.20 (45)	9.0	0.11 ± 0.02	0.16 ± 0.01	1.5	0.08 ± 0.02	0.7	131 ± 17	1175
11	43b	CH ₂	2-OMe	2-OMe	2-OMe	0.209 (5)	1.08	0.080 ± 0.004	0.074 ± 0.003	0.9	0.066 ± 0.002	0.8	204 ± 13	2561
12	43c	O	2-OMe	2-OMe	2-OMe	0.153 (8)	1.27	0.056 ± 0.011	0.057 ± 0.008	1.0	0.045 ± 0.004	0.8	141 ± 5	2518
13	44a	CO	4-CF ₃	4-CF ₃	4-CF ₃	16.4	25.0	5.05 ± 1.84	8.51 ± 1.98	1.7	2.73 ± 0.76	0.5	>300	>60
14	44b	CH ₂	4-CF ₃	4-CF ₃	4-CF ₃	1.72	7.52	0.23 ± 0.06	0.37 ± 0.09	1.6	0.28 ± 0.04	1.2	77.1 ± 8.6	338
15	44c	O	4-CF ₃	4-CF ₃	4-CF ₃	5.26	9.93	0.24 ± 0.06	0.32 ± 0.05	1.3	0.28 ± 0.05	1.2	174 ± 23	723
16	45a	CO	4-Me	4-Me	4-Me	0.22 (49)	10.8	0.11 ± 0.02	0.018 ± 0.001	1.6	0.09 ± 0.02	0.8	141 ± 10	1266
17	45a (Cl ⁻ salt)	CO	4-Me	4-Me	4-Me	0.084 (14.5)	1.22	0.094 ± 0.023	0.065 ± 0.002	0.7	0.073 ± 0.025	0.8	24 ± 1.2	254
18	45b	CH ₂	4-Me	4-Me	4-Me	0.099 (24)	2.33	0.080 ± 0.002	0.080 ± 0.003	1.0	0.074 ± 0.001	0.9	233 ± 14	2905
19	45b	(CH ₂) ₂	4-Me	4-Me	4-Me	0.084 (7.8)	0.656	0.15 ± 0.06	0.15 ± 0.02	1.0	0.15 ± 0.06	1.0	32.4 ± 2.4	213
20	45f (Cl ⁻ salt)	(CH ₂) ₂	4-Me	4-Me	4-Me	0.050 (33.8)	1.69	0.058 ± 0.026	0.030 ± 0.008	0.5	0.045 ± 0.018	0.8	23.3 ± 0.4	404
21	45g	(CH ₂) ₃	4-Me	4-Me	4-Me	0.081 (22)	1.80	0.029 ± 0.005	0.049 ± 0.007	1.7	0.053 ± 0.003	1.8	25.8 ± 1.7	897
22	45g (Cl ⁻ salt)	(CH ₂) ₃	4-Me	4-Me	4-Me	0.063 (27.3)	1.72	0.100 ± 0.034	0.066 ± 0.005	0.7	0.062 ± 0.004	0.6	12.7 ± 0.5	127
23	45c	O	4-Me	4-Me	4-Me	0.170 (14)	2.34	0.024 ± 0.005	0.025 ± 0.004	1.0	0.021 ± 0.001	0.8	85.7 ± 6.4	3567
24	45d	SO ₂	4-Me	4-Me	4-Me	0.173 (127)	22.0	0.23 ± 0.01	0.253 ± 0.002	1.1	0.27 ± 0.01	1.2	>300	>1300
25	45e	NAC	4-Me	4-Me	4-Me	0.159 (137)	21.8	0.32 ± 0.01	0.30 ± 0.01	0.9	0.29 ± 0.01	0.9	>300	>930
26	46b	CH ₂	3-Me	3-Me	3-Me	0.231 (4)	0.939	0.14 ± 0.02	0.14 ± 0.01	1.0	0.13 ± 0.02	0.9	72.6 ± 7.1	524
27	46c	O	3-Me	3-Me	3-Me	0.488 (1.9)	0.942	0.057 ± 0.013	0.043 ± 0.004	0.7	0.088 ± 0.024	1.5	45.4 ± 12.4	792

^a*T. b. rhodesiense* STIB900 trypanomastigotes. ^bRat skeletal myoblast L6 cells. ^c*T. b. brucei* s427 trypanomastigotes. ^d*T. b. brucei* knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate. ^eResistance factor compared to WT. ^fThe B48 strain is a mutant derived from the TbAT1-KO strain with a nonfunctional high affinity pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine, and melaminophenyl arsenicals. ^gHuman embryonic kidney (HEK) cells. ^hSelectivity index = [EC₅₀(HEK cells)/EC₅₀(*T. b. brucei* WT)]. ⁱSelectivity index = [EC₅₀(L6-cells)/EC₅₀(*T. b. rhodesiense*)].

Table 3. Antitrypanosomal Activity of Bisphosphonium Salts having Aryl Groups or Heterocycle Substituents^a


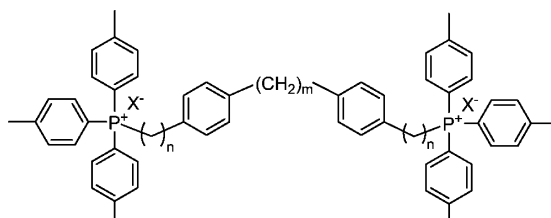
entry	compd	L	Ar ₁	Ar ₂	Ar ₃	T. b. rhod. ^a		cytox L6 ^b	EC ₅₀ (μM) (selectivity index) ^f				cytox HEK ^g	SI ^h
						T. b. rhod. ^a	T. b. brucei WT ^c		TbATI-KO ^d	RF ^e	T. b. B48 ^f	RF ^e		
1	36a	CO	Ph	Ph	Ph	0.260 (282)	0.24 ± 0.07	73.3	0.36 ± 0.07	1.5	0.38 ± 0.06	1.6	>300	>1250
2	47a	CO	2-thienyl	2-thienyl	2-thienyl	0.29 (58)	0.33 ± 0.06	16.6	0.52 ± 0.03	1.6	0.33 ± 0.05	1.0	260 ± 21	785
3	48a	CO	2-furanyl	2-furanyl	2-furanyl	13.81	36.9 ± 1.2	86.0	33.8 ± 1.2	0.9	21.7 ± 1.2	0.6	nd ^k	>400
4	49a	CO	Ph	Ph	2-pyridyl	0.607 (24)	0.70 ± 0.24	14.6	1.43 ± 0.41	2.0	1.33 ± 0.26	1.9	>300	>370
5	50a	CO	Bn	Ph	Ph	0.48 (67)	0.81 ± 0.12	32.0	1.02 ± 0.02	1.3	1.03 ± 0.22	1.3	>300	>110
6	51b	CH ₂	Ph	Ph	C ₆ F ₅	16.0	2.68 ± 0.05	p ^j	3.79 ± 0.09	1.4	2.74 ± 0.02	1.0	>300	>10
7	51c	O	Ph	Ph	C ₆ F ₅	12.8	23.2 ± 3.1	5.35	9.9 ± 1.9	0.4	6.68 ± 1.65	0.3	>300	>10
8	52b	CH ₂	Ph	Ph	3-(SO ₃ Na)Ph	>92	>100	>92	>100	>100	>100	>300	>300	>100
9	52c	O	Ph	Ph	3-(SO ₃ Na)Ph	>92	>100	90.5	>100	>100	>100	>300	>300	>100
10	53a	CO	1-naphthyl	1-naphthyl	1-naphthyl	0.40 (12)	0.18 ± 0.04	4.9	0.339 ± 0.005	1.8	0.18 ± 0.04	1.0	92 ± 4.0	502
11	53b	CH ₂	1-naphthyl	1-naphthyl	1-naphthyl	0.299 (12)	0.14 ± 0.02	3.43	0.17 ± 0.04	1.3	0.14 ± 0.01	1.0	163 ± 19	1207
12	53c	O	1-naphthyl	1-naphthyl	1-naphthyl	0.384 (9)	0.16 ± 0.01	3.45	0.14 ± 0.01	0.9	0.128 ± 0.002	0.8	114 ± 16	722

^aT. b. rhodensis STIB900 trypanostigotes. ^bRat skeletal myoblast L6 cells. ^cT. b. brucei s427 trypanostigotes. ^dT. b. brucei knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate. ^eResistance factor compared to WT. ^fThe B48 strain is a mutant derived from the TbATI-KO strain with a nonfunctional high affinity pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine, and melaminophenyl arsenicals. ^gHuman embryonic kidney (HEK) cells. ^hEC₅₀(HEK cells)/EC₅₀(T. b. brucei WT). ⁱSelectivity index = [EC₅₀(L6-cells)/EC₅₀(T. b. rhodensis)]. ^jPrecipitate in solution. ^kNot determined.

Table 4. Antitrypanosomal Activity of Monophosphonium Salts^a


entry	compd	L	R ₁	R ₂	R ₃	<i>T. b. rhod.</i> ^a	cytox L6 ^b	<i>T. b. brucei</i> WT ^c	TbATI-KO ^d	RF ^e	<i>T. b. B48</i> ^f	RF ^e	cytox HEK ^g	SI ^h
1	54					0.216 (48)	10.4	0.023 ± 0.001	0.024 ± 0.002	1.0	0.023 ± 0.001	1.0	>300	>12500
2	55					0.364 (35.5)	12.9	0.043 ± 0.001	0.033 ± 0.006	0.8	0.022 ± 0.000	0.5	>300	>7000
3	56a	CO	4-Me-Ph	4-Me-Ph	4-Me-Ph	0.081 (16.7)	1.35	0.064 ± 0.004	0.012 ± 0.002	0.2	0.011 ± 0.003	0.2	32.5 ± 3.6	505
4	56c	O	4-Me-Ph	4-Me-Ph	4-Me-Ph	0.002 (131)	0.263	0.015 ± 0.002	0.013 ± 0.0006	0.8	0.010 ± 0.001	0.7	17.6 ± 1.2	1145
5	57a	CO	3-Me-Ph	3-Me-Ph	3-Me-Ph	0.274 (9.5)	2.61	0.17 ± 0.05	0.15 ± 0.03	0.8	0.11 ± 0.03	0.7	48.2 ± 2.4	281
6	57c	O	3-Me-Ph	3-Me-Ph	3-Me-Ph	0.014 (62)	0.870	0.028 ± 0.002	0.021 ± 0.002	0.8	0.017 ± 0.0003	0.6	24.5 ± 2.3	876
7	58a	CO	^t Bu	^t Bu	^t Bu	4.48	>209	5.9 ± 0.7	6.1 ± 0.9	1.0	6.28 ± 0.85	1.1	>300	>50
8	59a	CO	4-Me-Ph	Ph	Ph	0.267 (29)	7.72	0.15 ± 0.03	0.15 ± 0.02	1.0	0.10 ± 0.01	0.7	76.8 ± 2.0	504
9	60a	CO	Et	Et	Et	>254	>254	>100	>100	1.0	>100	0.7	>300	>7000
10	61a	CO	2-MeO-Ph	2-MeO-Ph	2-MeO-Ph	0.376 (10)	3.97	0.21 ± 0.05	0.22 ± 0.06	1.1	0.15 ± 0.03	0.7	63.2 ± 4.4	306
11	62a	CO	<i>n</i> -hex	<i>n</i> -hex	<i>n</i> -hex	0.103 (15)	1.61	0.011 ± 0.004	0.008 ± 0.003	0.7	0.022 ± 0.011	1.9	13.4 ± 0.3	1198
12	63a	CO	Me	Me	Ph	16.94	>241	42.7 ± 6.2	46.6 ± 2.3	1.1	45.8 ± 12.8	1.1	>300	>7

^a*T. b. rhodensis* STIB900 trypomastigotes. ^bRat skeletal myoblast L6 cells. ^c*T. b. brucei* s427 trypomastigotes. ^d*T. b. brucei* knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate. ^eResistance factor compared to WT. ^fThe B48 strain is a mutant derived from the TbATI-KO strain with a nonfunctional high affinity pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine, and melaminophenyl arsenicals. ^gHuman embryonic kidney (HEK) cells. ^hSelectivity index = [EC₅₀(HEK cells)/EC₅₀(*T. b. brucei* WT)]. ⁱSelectivity index = [EC₅₀(L6-cells)/EC₅₀(*T. b. rhodensis*)].

Table 5. Antitrypanosomal Activity of Phosphonium Salts with Ethylene Linker^a


compd	salt form	m	n	EC ₅₀ (μM) (selectivity index) ⁱ								
				<i>T. b. rhod.</i> ^a	cytox L6 ^b	<i>T. b. brucei</i> WT ^c	TbAT1-KO ^d	RF ^e	<i>T. b.</i> B48 ^f	RF ^e	cytox HEK ^g	SI ^h
45f	Br ⁻	2	1	0.084 (7.8)	0.656	0.15 ± 0.06	0.15 ± 0.02	1.0	0.15 ± 0.06	1.0	32.4 ± 2.4	213
45g	Br ⁻	3	1	0.081 (22)	1.80	0.029 ± 0.005	0.049 ± 0.007	1.7	0.053 ± 0.003	1.8	25.8 ± 1.7	897
64	Cl ⁻	2	2	0.027 (40.3)	1.09	0.125 ± 0.027	0.094 ± 0.023	0.8	0.089 ± 0.017	0.7	12.8 ± 0.9	103
65	Cl ⁻	3	2	0.073 (8.8)	0.645	0.077 ± 0.022	0.043 ± 0.009	0.6	0.048 ± 0.007	0.6	5.6 ± 0.2	73

^a*T. b. rhodesiense* STIB900 trypomastigotes. ^bRat skeletal myoblast L6 cells. ^c*T. b. brucei* s427 trypomastigotes. ^d*T. b. brucei* knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate. ^eResistance factor compared to WT. ^fThe B48 strain is a mutant derived from the TbAT1-KO strain with a nonfunctional high affinity pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine, and melaminophenyl arsenicals. ^gHuman embryonic kidney (HEK) cells. ^hSelectivity index = [EC₅₀(HEK cells)/EC₅₀(*T. b. brucei* WT)]. ⁱSelectivity index = [EC₅₀(L6-cells)/EC₅₀(*T. b. rhodesiense*)].

their absence, the cause of resistance in the strain B48.^{8,18} We can speculate that, unlike diamidines, the positive charge(s) of the (bis)phosphonium compounds are highly dispersed and shielded by hydrophobic substituents, allowing transmembrane diffusion at an appreciable rate.¹⁹ It is noted, in this context,

that the antiparasitic activity seems to be proportionate with the level of shielding, with methyl and ethyl substituents having virtually no effect (18a, 19a). Yet some of the phosphonium compounds, such as 24a and 45e, were good inhibitors of the diamidines transporters, especially of the low affinity pentamidine transporter (LAPT1),²⁰ for which most of the compounds displayed higher affinity than the original substrate pentamidine (Table 6). As there was, however, no correlation

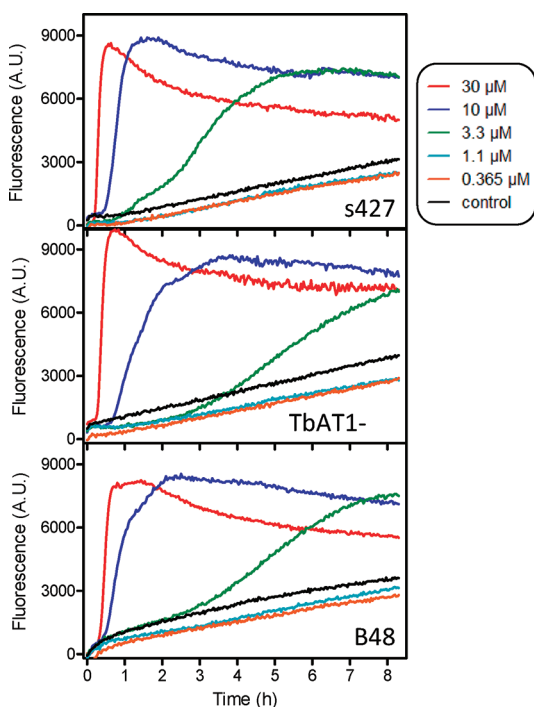


Figure 1. Real time monitoring of the effects of 24a on various strains of *T. b. brucei*. The experiment was conducted with 5×10^5 cells/well in a 96-well format, with 200 μL/well of HMI-9 medium containing 10% FBS and 9 μM PI. Fluorescence was determined every 2 min for a total of 250 cycles. Fluorescence was read using 544 and 620 nm filters for excitation and emission, respectively. Fluorescence values were determined in parallel for all three strains, using a single 96-well plate incubated in a Fluostar Optima fluorimeter at 37 °C and 5% CO₂. The slow increase in fluorescence in drug-free controls is attributable to the slow entry of PI into live trypanosomes.¹⁷

Table 6. Affinity of Selected Phosphonium Compounds on the HAPT1 and LAPT1 Diamidine Transporters of *T. b. brucei*^a

compd	K _d , HAPT1 (±SE) (μM)	K _d , LAPT1 (±SE) (μM)
pentamidine ^b	0.036 ± 0.006	56.2 ± 8.3
18a	>250	>250
24a	5.2 ± 0.9	2.2 ± 0.4
25a	ND ^c	25 ± 6
25b	ND ^c	9.5 ± 0.5
25c	53 ± 13	7.2 ± 4.0
26a	ND ^c	39 ± 7
35a	ND ^c	19 ± 6
36a	ND ^c	49 ± 6
38b	ND ^c	20 ± 4
45e	9.2 ± 1.3	3.6 ± 0.4
55	ND ^c	25 ± 11

^aExperiments were performed in triplicate and on at least three separate occasions, as described in refs 8 and 20. ^bTaken from ref 20. ^cND, not determined.

between inhibition of the transporters and the antiparasitic activity, we conclude that the activity is not dependent on entry through any of the known drug transporters, with diffusion being the likely route of entry.

Characterization of the Action of Phosphonium Salts on *T. b. brucei*. Figure 1 indicated that 24a only affected cellular viability during an 8 h experiment at concentrations >15-fold its EC₅₀. In fact, numerous additional bisphosphonium salts were tested over 8 h on *T. b. brucei* s427 and typically affected parasite viability only at concentrations 70–120 ×

EC₅₀ (see Supporting Information). The only exception was **55**, with only concentrations >700 EC₅₀ affecting parasite survival within this time frame. This indicates that monophosphonium salts may affect trypanosomes more slowly than the corresponding dications, probably because the single positive charge provides less driving force to cross inside-negative membranes. In fact, previous studies by Ross et al.¹⁹ have shown that lipophilic triphenylphosphonium dications are accumulated into mitochondria to a greater extent than their monocationic counterpart.

It is thus clear that while monophosphonium and bisphosphonium compounds act potently on trypanosomes, their effect on cellular integrity is relatively slow. In order to assess the timing of the antitrypanosomal effect of phosphonium compounds, we conducted manual cell counts on cultures incubated with various concentrations of test compound. We found that growth rates of *T. b. brucei* bloodstream forms were initially reduced upon incubation with phosphonium salts at concentrations of EC₅₀ or slightly over but typically recovered after 36 h; only at concentrations ≥2 EC₅₀ did the cells not recover from the initial growth inhibition and started to die after 12 h (Figure 2A).

Although trypanosomes died only slowly on incubation with phosphonium compounds, only a relatively short exposure time was required. Figure 2B shows that removal of **25c** after a mere

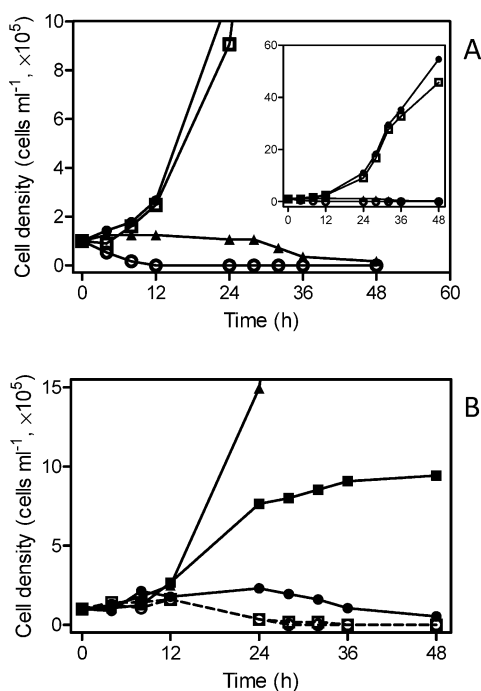


Figure 2. Effect of **25c** on cultures of *T. b. brucei* bloodstream forms. Cultures in the presence or absence of various concentrations of **25c** were set up at 10^5 cells/mL. Microscopic cell counts were performed in triplicate using a hemocytometer, and average values are shown. (A) Cells were incubated in the continuous presence of $0.05 \mu\text{M}$ (\square), $0.25 \mu\text{M}$ (\blacktriangle), or $1 \mu\text{M}$ **25c** (\circ), or no compound (control, \bullet). Inset shows the same data but with the full growth over 48 h on a different scale. (B) Reversibility of growth inhibition by $0.25 \mu\text{M}$ **25c** was determined by centrifugation 1100g after either 30 min (\blacksquare , \square) or 4 h (\bullet , \circ) and replacement with drug-free fresh medium (closed symbols, solid lines) or fresh medium with $0.25 \mu\text{M}$ **25c** (open symbols, dashed lines). A control culture (\blacktriangle , no drug) reached a density of 6×10^6 cells/mL after 48 h, as in panel A.

30 min of incubation allowed for only very limited resumption of growth, reaching a maximum density of 9.4×10^5 cells/mL at 48 h (i.e., 15% of the drug-free control). Removal of drug after 4 h did not lead to any resumption of growth at all, and the trypanosome population slowly dwindled over the next 44 h. Continuous exposure to the drug resulted in sterilization of the culture after approximately 30 h. Highly similar results were obtained with a number of other compounds including **25b** and **45c**.

CoMFA Models. In order to gain insights into the SAR of this class of phosphonium salts, we have derived two main CoMFA models based on the EC₅₀ values for *T. b. rhodesiense* and *T. b. brucei*, respectively (Tables 1–5). Both models are, at the least, able to discriminate activities, and they possess a moderate to high predictive power. Nevertheless our hypothesis that both end points have a similar behavior is confirmed by the modeling step. The two models are so alike that explaining both in detail becomes redundant, consistent with identical modes of action for these closely related species. Therefore, we are presenting here only the *T. b. brucei* model. For further reading about the *T. b. rhodesiense* model see the Supporting Information.

The training set (54 compounds) model statistics are fairly good: slope of 0.8905, y intercept of 0.0466, $r^2 = 0.891$, $F = 99.7$. Standard validations on the training set were conducted in several ways: “leave one out” using SAMPLS algorithm²¹ (SAMple-distance partial least squares (PLS) fitting; 10 groups “leave several out” (LSO), $q^2 = 0.43$, three components partial least squares (PLS) fitting and bootstrapping, averaged $r^2 = 0.856$, and averaged standard error (SE) of 0.343. Cross-validation values were not actually promising, so some suspicions of overfitting arose. However, the test set statistics (Figure 3), a set never used in

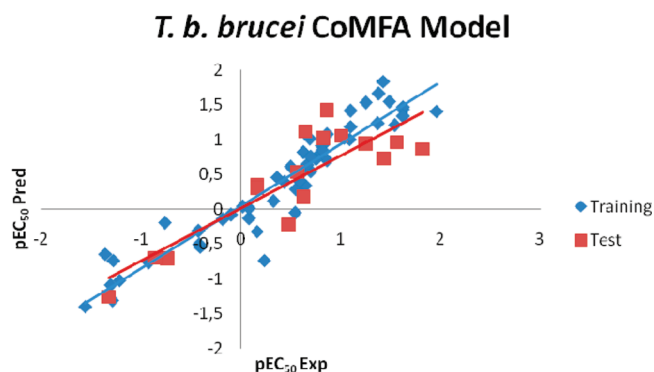


Figure 3. CoMFA model for *T. b. brucei*. Plot of $-\log_{10}[\text{EC}_{50} (\mu\text{M})]_{\text{experimental}}$ vs $-\log_{10}[\text{EC}_{50} (\mu\text{M})]_{\text{predicted}}$ for *T. b. brucei* WT. Outliers are not shown. Model statistics are commented in the text.

the modeling step, refuted this point. For *T. b. brucei* (pEC_{50} experimental vs pEC_{50} theoretical), the model statistics are as follows: slope of 0.7531, intercept of 0.0117, and $r^2 = 0.744$ (excluding outliers) or $r^2 = 0.67$ (including outliers). Such values fulfill the rules for predictive models proposed by Tropsha et al.²² (i.e., $r_0^2 = 0.743$) and can be considered as clearly predictive.

Aside from the good behavior of this model, a number of outliers were found within the test set: **48a**, **52b**, **52c**, and **63a**. Compound **48a** can be considered as an outlier because of its similar structure to **47a**, a rather active compound, while no other related structures were included. This fact clearly biased

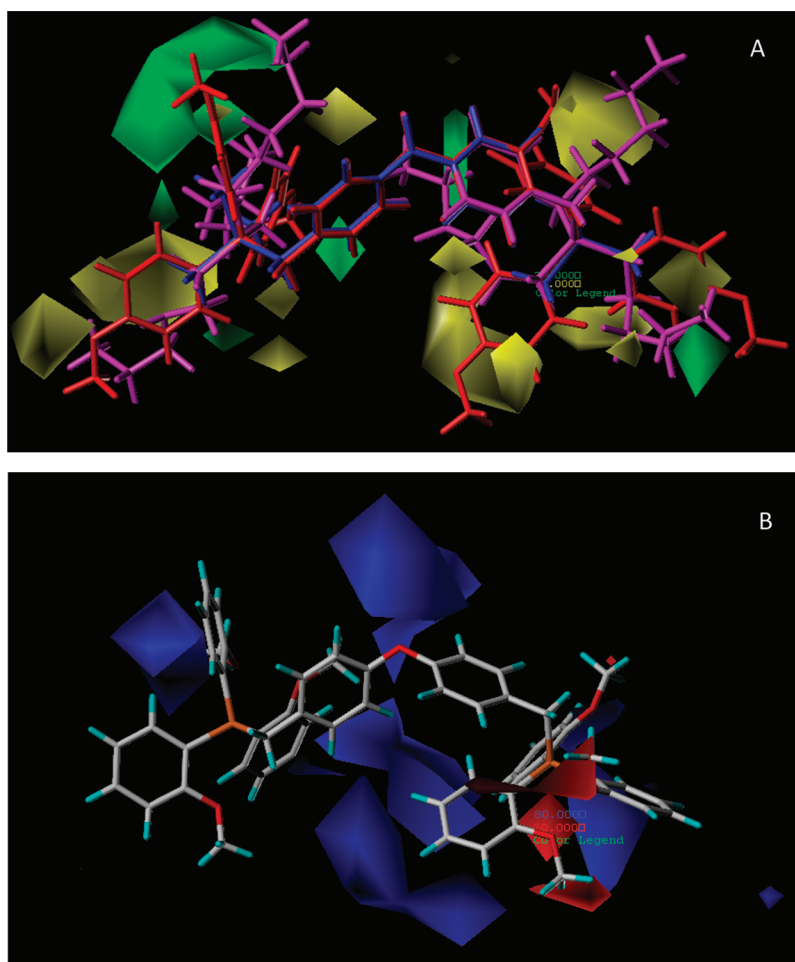


Figure 4. Steric and electrostatic CoMFA fields for *T. b. brucei*. (A) For steric fields, regions where increasing the volume of the molecule favors antitrypanosomal activity are green. Regions where increasing the volume decreases antitrypanosomal activity are yellow. Compounds **18a** (blue), **25a** (magenta), and **41a** (red) are shown. (B) Electrostatics field contours indicate an increase of anti-*T. brucei* activity with increasing positive (blue) and negative (red) charge, respectively. Compound **43c** is shown.

the model to predict a higher activity than it actually has. However, **48a** was still predicted as inactive.

52b and **52c** were not included in the modeling step because their activities were not determined at the time of generating this model. These compounds are outliers because they are the only ones with neutral charge (i.e., presence of the 3-phenylsulfonate substituent) and no compound alike was included in the model. The counterions are not taken into account to generate the CoMFA models (see Experimental Section). Finally, **63a** has clearly a lower activity than would be expected based on our model. This is probably due to the lack of lipophilic shielding around the phosphonium cation, reducing membrane permeation. For quantitative information see Table S2 in Supporting Information.

CoMFA fields derived from this model denote that the steric component is rather selective (Figure 4). Indeed the steric contribution to the CoMFA is 0.641. Bulk is strictly forbidden around the whole molecule except upon special positions on the phosphorus substituent. Such positions coincide with para and meta substitutions on six-membered aromatic rings which can also be easily occupied by long enough aliphatic chains. This fact explains why aliphatic chains are not active up to a certain number of carbons (≤ 4); molecules with such small substituents do not fill the preferred volume (see Figure 4A,

compound **18a** shown in blue), while they do not offer the essential lipophilic shielding to the phosphonium cation.

One issue derived from the good activities obtained for monocations is a remarkable loss of symmetry on the steric fields. While one end of the molecule is nearly surrounded by sterically unfavorable fields, the distal end is virtually the opposite. Such assumption is coherent with the submicromolar activities found for dications with symmetric aromatic substitution (e.g., see Figure 4A, compound **41a** shown in red). Here the favorable effect of bulk in one molecular end is compensated somehow by the same unfavorable bulk in the distal end. It is indeed the flexibility of aliphatic chains, the ability to avoid the restricted areas, and the predominant effect on the nanomolar activities of compounds such as **25a–c** (see Figure 4A, compound **25a** showed in magenta) compared with **26a**.

Electrostatic fields show that positive charge dispersion over the whole molecule favors the activity as we previously supposed. Here we see again a loss on the symmetry on the fields where a bit of negatively charged substituent, near the phosphonium (e.g., OMe, F, ...) and close to the more sterically impeded area, would favor the activity of these compounds. This is the case of compounds **43b** and **43c** bearing 2-methoxyphenyl substituents (see Figure 4B). In such cases this is the predominant effect confronted with the mere bulk offered

by only aromatic substitution, such as in **50a**. Surprisingly enough, there is no other negative favorable area in the model. This is combined with a huge dispersion of the positive charge upon the molecule even surrounding the negative surfaces. We can deduce then a minor influence of localized electrostatic interactions, such as hydrogen bonds, with the phosphonium salts activities.

DISCUSSION

Phosphonium salts bearing hydrophobic substituents are lipophilic cations with a delocalized charge. This affords specific properties to these molecules such as the capacity to cross biological membranes driven by electrical potential. Thus, they tend to accumulate in organelles with high membrane potential such as mitochondria.²³ In *Leishmania*, several of the benzophenone-derived bisphosphonium salts reported here were found to target mitochondria of the parasite, a key to their leishmanicidal action.⁵ Ongoing studies on its mechanism of action in *T. brucei* also point to a mitochondrial target; for instance, phosphonium compounds strongly affected the mitochondrial membrane potential (unpublished results).

Interestingly, the SAR of the series of compounds presented here shows some similarities with the SAR against *Leishmania*. For instance, for compounds with homoalkyl groups, optimum activity was observed with five to eight carbons, independent of the linker (Tables 1 and 4). In the same way, the combination of two phenyl groups and one alkyl substituent was enough to get submicromolar activity whereas the combination of one phenyl group and two short alkyl substituents (Me, Et) led to micromolar EC₅₀. These observations are clearly supported by the CoMFA models that show that a minimum bulk around the phosphonium cations is favorable to the activity, but this volume is also limited in size.

From these data, it is clear that a substantial level of lipophilic shielding around the phosphonium cation(s) is necessary to get high levels of activity against *Trypanosoma* and *Leishmania* species, presumably by increasing membrane permeation. No correlation was found between the antiparasitic activity and inhibition of the known *T. brucei* drug transporters (P2, HAPT1, and LAPT1). Although some bis-phosphonium compounds displayed surprisingly high affinity for the HAPT1 and LAPT1 transporters, most of the bis-phosphonium compounds would be considered too large to be a substrate (as opposed to be an inhibitor) of such transporters (e.g., molecular weights range between 800 and 1300 compared with 340 for pentamidine). HAPT1 and LAPT1 were first described to transport a range of diamidines,^{20,24} which, like the bis-phosphonium compounds described here, are dications. Our CoMFA models show the importance of considerable positive charge dispersion over a large surface for increased activity. On balance, we conclude that, similar to a series of dicationic choline-derived compounds on which we recently reported,²⁵ the antitrypanosomal activity is not dependent on entry through any of these transporters. In fact, it is highly probable that diffusion is the route of entry. This interpretation is much strengthened by the observation that substituents such as furan, pyridine, and phenylsulfonate, which can engage in H-bonding, display significantly reduced antitrypanosomal activities, presumably because of impeded cellular penetration. The data presented in Table 4 clearly show that monophosphonium compounds have as good and often slightly better activity against trypanosomes than the corresponding symmetrical bis-phosphonium analogues. However, this

improved activity is in many cases also coupled to somewhat increased toxicity against the mammalian cell lines employed. The redundancy of the second phosphonium group is consistent with the observation that longer, more flexible linkers (i.e., ethylene instead of methylene linker) between the phosphonium cation and the central diphenyl core hardly affected the antitrypanosomal activity. This observation is important because monophosphonium salts are smaller molecules with physicochemical properties (i.e., lower molecular weight, lower clogP) more likely to provide good pharmacokinetic behavior in vivo compared with the bis-phosphonium counterparts.

In their 1979 paper,⁴ Kinnamon and Steck reported that the monophosphonium compound **54** (chloride salt) displayed 100% curative activity (53 mg/kg sc) in a murine model of *T. b. rhodesiense* infection. The bromide analogue **55** was found to be less active in vivo than its chloride counterpart. However, no in vitro data were reported. The results of our in vitro screening are in agreement with these data, as **54** displayed between submicromolar and nanomolar activities against *T. b. brucei* and *T. b. rhodesiense*, respectively.

The excellent in vitro antitrypanosomal activity observed with this set of phosphonium compounds confirms the potential of this class of molecules. We show that phosphonium compounds irreversibly inhibit trypanosome growth, leading to certain clearance of the population after only a minimum exposure time. This situation is similar to that of the highly successful class of diamidine trypanocides, which also require only a brief exposure time to ultimately kill the trypanosomes after 1–2 days.²⁶ This result is important for potential therapeutic applications, as dosage can be kept relatively low and a single administration may be sufficient, similar to the single administration of the diamidine diminazene aceturate that is the routine treatment for animal trypanosomiasis.² In addition, the positive correlation with lipophilicity and thus membrane penetration should increase penetration into the central nervous system, essential for treatment of late stage trypanosomiasis.³

CONCLUSIONS

The in vitro screening of a set of 83 phosphonium salt derivatives against African trypanosomes allowed the discovery of several trypanocidal compounds with nanomolar activities and adequate selectivities vs HEK and L6 cells. SAR studies showed that bulky substituents around the phosphonium cations (i.e., either C5–C8 homoalkyl chains or phenyl rings) are necessary to get submicromolar activities, whereas the linkers have less influence on the antitrypanosomal activity. However, this statement is true as far as the bisphosphonium salts are concerned. On the contrary, the diphenyl ether linker seemed to be preferred in the case of monophosphonium compounds. In fact, **56c** and **57c** were the most active compounds of all the series with EC₅₀ values in the low nanomolar range against wild type and resistant *T. brucei* lines.

Phosphonium compounds displayed a very similar structure–activity relationship against *T. brucei* and *Leishmania*.⁵ This observation strongly suggests a similar mechanism of action against the two parasite species and indicates that this class may have broad antiprotozoal activity. This is the subject of ongoing investigations in our laboratories.

Importantly, the screening reported here confirmed that there is no cross-resistance between the new compounds and

existing diamidines and arsenical trypanocides, an essential condition for any new preclinical candidate.

Finally, the CoMFA models developed in this work should help in the design of new custom-made phosphonium derivatives with improved activity against African trypanosomes.

EXPERIMENTAL SECTION

Chemistry. All dry solvents were purchased from Aldrich or Fluka in Sure/Seal bottles. All reactions requiring anhydrous conditions or an inert atmosphere were performed under argon atmosphere. All reactions were monitored by thin layer chromatography (TLC) using silica gel 60 F₂₅₄ plates (Merck) or HPLC–MS. Chromatography was performed with Isolute SI prepacked columns. ¹H and ¹³C NMR spectra were recorded on a Bruker Advance 300 or Varian Inova 400 spectrometer. Chemical shifts of the ¹H NMR spectra were internally referenced to the residual proton resonance of the deuterated solvents: CDCl₃ (δ 7.26 ppm), D₂O (δ 4.6 ppm), CD₃OD (δ 3.49 ppm), and DMSO (δ 2.49 ppm). *J* values are given in Hz. Melting points were determined in open capillary tubes with a Stuart Scientific SMP3 apparatus or Mettler Toledo MP70 melting point system and are uncorrected. All compounds are >95% pure by HPLC or combustion analysis unless otherwise noted. Elemental analysis was performed on a Heraeus CHN-O rapid analyzer. Analytical results were within \pm 0.4% of the theoretical values unless otherwise noted. Analytical HPLC–MS was run with an Xbridge C18–3.5 μ m (2.1 mm \times 100 mm) column on a Waters 2695 separation module coupled with a Waters Micromass ZQ spectrometer using electrospray ionization (ESI⁺). The following HPLC conditions were used: column temperature of 30 °C, gradient time of 5 min, H₂O/CH₃CN (10:90 \rightarrow 90:10) (HCO₂H, 0.1%), flow rate of 0.25 mL/min, UV detection using diode array (λ = 190–400 nm). Semipreparative HPLC–MS was run with a SunFire Prep C18, 5 μ m (19 mm \times 150 mm) column on a Waters separation module (Waters 2545/SFO/2767) coupled to a Waters 3100 mass detector using ESI⁺. The fractions were collected with a Waters 2767 sampler manager.

1. General Procedure for the Synthesis of the Bisphosphonium Salts. A Kimax tube was charged with the appropriate bis-halogenated precursor (100 mg, \sim 0.28 mmol) and flushed with argon. Anhydrous DMF (3 mL) was added followed by the phosphine (1.12 mmol, 4 equiv). The tube was flushed with argon, stopped, and the reaction mixture was stirred at 100 °C for 20 h. A higher temperature (150 °C) and longer reaction time were necessary with the 4,4'-bischloroethyl linkers **16f** and **17g**. Different workup procedures were used depending on whether the product precipitated from the reaction mixture or not. For workup I, the mixture was allowed to cool to room temperature and the precipitated product was collected by filtration, rinsed successively with toluene and Et₂O, and dried under vacuum. For workup II, the reaction mixture was transferred to a flask. Then toluene (10–20 mL) was added to precipitate the product. The flask was stored in the refrigerator overnight. The supernatant was removed, and the precipitate was rinsed with toluene. Et₂O (10 mL) was added, and the precipitate was triturated with a spatula. The solid was collected, rinsed with Et₂O, and dried under vacuum.

4,4'-Bis((triethylphosphonio)methyl)diphenylmethane Dibromide (19b). The reaction was carried out in toluene following the general procedure with triethylphosphine and **1b**. The product was obtained as a white hygroscopic solid (107 mg, 65%) following workup I procedure and recrystallization in DMF/toluene. HPLC = 91% pure; mp 246–248 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.42 (dd, *J* = 7.7, 2.1, 4H, ArH), 7.07 (d, *J* = 7.7, 4H, ArH), 4.23 (d, *J* = 15.2, 4H, PCH₂), 3.88 (s, 2H, PhCH₂Ph), 2.46 (dq, *J* = 20.5, 7.7, 12H, CH₂CH₃), 1.23 (dt, *J* = 17.9, 7.7, 18H, CH₂CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 156.9, 131.9, 123.2, 119.9, 25.6, 25.0, 12.2 (*J* = 48.2), 6.2 (*J* = 5.4). LRMS (ESI⁺) *m/z* = 429.39 [(M – H)⁺], 214.93 [M²⁺, 100%]. Anal. Calcd for C₂₇H₄₄Br₂P₂: C, 54.93; H, 7.51; Br, 26.91. Found: C, 54.75; H, 7.72; Br, 26.20.

4,4'-Bis((trihexylphosphonio)methyl)benzophenone Dibromide (25a). The reaction was carried out following the general

procedure with trihexylphosphine and **1a** for 48 h at 80 °C. The product was obtained as a hygroscopic oily solid after workup II. Recrystallization from MeOH/Et₂O yielded a yellowish hygroscopic solid (27%). HPLC = 89% pure. ¹H NMR (300 MHz, CDCl₃) δ 7.63 (d, *J* = 7.7, 4H, ArH), 7.50 (d, *J* = 7.7, 4H, ArH), 4.67 (d, *J* = 16.1, 4H, PCH₂Ph), 2.29 (m, 12H, PCH₂CH₂), 1.6–1.1 (m, 48H, (CH₂)₄), 0.84 (m, 18H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 194.9, 136.5 (d, *J* = 3.4), 134.3 (d, *J* = 8.7), 130.6 (d, *J* = 4.8), 129.8 (d, *J* = 79.7), 31.1, 30.6 (d, *J* = 14.7), 27.0 (d, *J* = 44.4), 22.4, 21.9 (d, *J* = 4.6), 19.2 (d, *J* = 46.1), 14.0. LRMS (ESI⁺) *m/z* 779.86 [(M – H)⁺], 390.11 [M²⁺, 100%]. ESI-HRMS *m/z* 390.3233 [M²⁺] (C₅₁H₉₀OP₂ requires 390.3228).

4,4'-Bis((trihexylphosphonio)methyl)diphenylmethane Dibromide (25b). The reaction was carried out following the general procedure with trihexylphosphine and **1b**. The mixture was concentrated to \sim 1 mL to give a yellowish oil that was diluted with toluene (10 mL). Addition of Et₂O (10 mL) caused precipitation of an oily residue. The flask was stored in the refrigerator overnight. The supernatant was removed, and the oily precipitate was rinsed with toluene. Et₂O (10 mL) was added to the oily precipitate, which was triturated with a spatula to yield **25b** as a white solid (158.7 mg, 64%): mp 111–112 °C with previous softening (DMF/toluene); HPLC > 94% pure. ¹H NMR (300 MHz, CDCl₃) δ 7.38 (dd, *J* = 8.3, 2.0, 4H, ArH), 7.08 (d, *J* = 8.3, 4H, ArH), 4.23 (d, *J* = 15.1, 4H, PCH₂), 3.89 (s, 2H, PhCH₂Ph), 2.33 (br s, 12H, CH₂), 1.42 (br s, 24H, CH₂), 1.23 (br s, 24H, CH₂), 0.85 (t, *J* = 6.6, 18H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 140.8, 130.5 (d, *J* = 4.9), 130.0 (d, *J* = 2.8), 126.5 (d, *J* = 8.8), 41.1, 31.1, 30.6 (d, *J* = 14.6), 26.7 (d, *J* = 45.2), 22.4, 21.9 (d, *J* = 4.8), 19.1 (d, *J* = 46.3), 14.0. LRMS (ESI⁺) *m/z* 765.87 [(M – H)⁺], 383.04 [M²⁺, 100%]. ESI-HRMS *m/z* 383.3357 [M²⁺] (C₅₁H₉₂P₂ requires 383.3332).

4,4'-Bis((phenyldimethylphosphonio)methyl)diphenylethane Dibromide (28f). The reaction was carried out following the general procedure with phenyldimethylphosphine (68.94 mg, 0.497 mmol) and **10f** (73.2 mg, 0.199 mmol). The mixture was concentrated under vacuum until the formation of an oily solid which was diluted with DMF. Et₂O was added to precipitate the product as a white hygroscopic solid. The flask was allowed to stand in the freezer overnight. The solid was collected, rinsed with Et₂O, and dried under vacuum (106.5 mg, 98%). HPLC > 95% pure; mp >300 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.80 (dd, *J* = 12.4, 7.5, 4H, ArH), 7.66 (m, 2H, ArH), 7.57 (m, 4H, ArH), 6.98 (dd, *J* = 3.0, 7.2, 4H, ArH), 6.79 (d, *J* = 7.5, 4H, ArH), 4.26 (d, *J* = 15.4, 4H, PCH₂), 2.72 (s, 4H; CH₂CH₂), 2.30 (d, *J* = 13.8, 12H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 141.4 (d, *J* = 4.1), 134.6 (d, *J* = 1.8), 131.3 (d, *J* = 9.7), 130.2 (d, *J* = 5.2), 130.0 (d, *J* = 12.4), 129.4 (d, *J* = 2.9), 125.4 (d, *J* = 9.1), 119.8 (d, *J* = 83.6), 36.9, 31.2 (d, *J* = 48.0), 7.5 (d, *J* = 55.6). LRMS (ESI⁺) *m/z* = 483.00 [(M – H)⁺]. ESI-HRMS *m/z* 242.1241 [M²⁺] (C₃₂H₃₈P₂ requires 242.1219).

4,4'-Bis((phenyldimethylphosphonio)methyl)diphenylpropane Dibromide (28g). The reaction was carried out following the general procedure with phenyldimethylphosphine (66 mg, 0.478 mmol) and **11g** (73.1 mg, 0.191 mmol). The mixture was concentrated under vacuum until the formation of an oily solid which was diluted with acetone. Et₂O was added to produce precipitation of the product as a dark brown hygroscopic solid (67.6 mg, 54%). HPLC = 90% pure. ¹H NMR (300 MHz, CDCl₃) δ 7.86–7.66 (m, 7H, ArH), 7.66–7.57 (m, 3H, ArH), 6.98 (t, *J* = 6.9, 8H, ArH), 4.29 (d, *J* = 15.3, 4H, PCH₂), 2.58–2.45 (m, 4H, PhCH₂CH₂), 2.37 (d, *J* = 13.7, 12H, CH₃), 1.80 (dd, *J* = 19.2, 11.6, 2H, PhCH₂CH₂). ¹³C NMR (75 MHz, CDCl₃) δ 142.4 (d, *J* = 4.3), 134.6 (d, *J* = 3.2), 131.9 (d, *J* = 9.6), 130.5 (d, *J* = 5.5), 130.1 (d, *J* = 12.2), 129.2 (d, *J* = 3.4), 125.3 (d, *J* = 9.4), 120.2 (d, *J* = 83.0), 35.1, 32.3, 31.3 (d, *J* = 48.1), 7.8 (d, *J* = 55.5). LRMS (ESI⁺) *m/z* = 497.48 [(M – H)⁺], 248.95 [M²⁺, 100%]. ESI-HRMS *m/z* 249.1316 [M²⁺] (C₃₃H₄₀P₂ requires 249.1297).

4,4'-Bis((diphenyl-*p*-tolylphosphonio)methyl)diphenyl Ether Dibromide (38c). The reaction was carried out following the general procedure with diphenyl-*p*-tolylphosphine and **1c**. The product was obtained as a white solid (187 mg, 66%) following workup II procedure: mp 230 °C (dec); HPLC > 97% pure. ¹H NMR

4,4'-Bis((trip-tolylphosphonio)ethyl)diphenylpropane Dichloride (65). The reaction was carried out following the general procedure with tri-*p*-tolylphosphine and 17g for 19 days. The mixture was evaporated to dryness forming a yellowish oil which was purified by semipreparative HPLC–MS, eluting with H₂O/CH₃CN. The following HPLC conditions were used: column temperature of 25 °C, gradient time of 15 min, CH₃CN/H₂O (30:70 → 70:30) (HCO₂H, 0.1%), flow rate of 0.24 mL/min, UV detection using diode array (λ = 240 nm). t_R = 10.32 min. The fractions containing the pure product were combined and lyophilized to give a white hygroscopic solid (17.3 mg, 17%). HPLC > 99% pure. ¹H NMR (300 MHz, CDCl₃) δ 7.72–7.55 (m, 12H, ArH), 7.53–7.43 (m, 12H, ArH), 7.14 (d, J = 7.4, 4H, ArH), 7.04 (d, J = 7.4, 4H, ArH), 3.78 (br m, 4H, PCH₂), 2.95 (br m, 4H, CH₂), 2.60–2.41 (m, 22H, CH₂ and CH₃), 1.86 (dt, J = 14.3, 7.4, 2H, CH₂CH₂CH₂). ¹³C NMR (75 MHz, CDCl₃) δ 146.4 (dd, J = 5.1, 3.0), 142.3 (d, J = 2.8), 135.5 (d, J = 24.9), 133.6 (dd, J = 10.4, 2.6), 132.2 (d, J = 10.3), 129.3 (d, J = 12.5), 128.6 (d, J = 7.2), 115.1 (dd, J = 88.4, 7.2), 34.6, 33.0, 28.1 (d, J = 3.1), 24.8 (d, J = 50.1), 22.0. LRMS (ES⁺) m/z = 857.88 [(M – H)]⁺, 429.21 [M²⁺, 100%]. ESI-HRMS m/z 429.2277 [M²⁺] (C₆₁H₆₄P₂ requires 429.2236).

2. General Procedure for the Synthesis of the Mono-phosphonium Salts. The appropriate halogenated precursor (100 mg, ~0.36 mmol) was added to a Kimax tube and dissolved in anhydrous DMF (3 mL) under argon atmosphere. The phosphine was then added (0.72 mmol, 2 equiv), the tube was stopped, and the reaction mixture was stirred at 100 °C for 20 h. Next, the reaction mixture was transferred to a flask, and DMF was evaporated under vacuum. Subsequently, toluene (10–20 mL) was added to precipitate the product and the flask was stored in the refrigerator overnight. The supernatant was removed, and the precipitate was rinsed with toluene. Et₂O (10 mL) was added, and the precipitate was triturated with a spatula. The solid was collected, rinsed with Et₂O, and dried under vacuum.

(4-Benzoylbenzyl)tri-*p*-tolylphosphonium Bromide (56a). The reaction was carried out following the general procedure with 2a and tri-*p*-tolylphosphine. The product was obtained as a white solid following workup II procedure (117.6 mg, 56%). HPLC > 95% pure; mp 233 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.68 (dd, J = 3.0, 6.5, 2H, ArH), 7.62–7.50 (m, 9H, ArH), 7.47–7.32 (m, 8H, ArH), 7.28 (dd, J = 2.3, 8.0, 2H, ArH), 5.44 (d, J = 15.0, 2H, PCH₂), 2.42 (s, 9H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 196.3, 146.4 (d, J = 3.0), 137.2 (d, J = 3.8), 137.1, 134.3 (d, J = 10.3), 132.8, 132.7, 131.7 (d, J = 5.4), 130.9 (d, J = 13.0), 130.2 (d, J = 3.1), 130.1, 128.4, 114.4 (d, J = 88.7), 31.1 (d, J = 48.3), 21.9. LRMS (ES⁺) m/z = 499.44 [M⁺]. ESI-HRMS m/z 499.2203 [M⁺] (C₃₅H₃₂OP requires 499.2185).

(4-Phenoxybenzyl)tri-*p*-tolylphosphonium Bromide (56c). The reaction was carried out following the general procedure with 2c and tri-*p*-tolylphosphine. The product was obtained as a white solid following workup II procedure (94.7 mg, 87%). HPLC > 95% pure; mp 192 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.56 (dd, J = 7.8, 12.2, 6H, ArH), 7.47–7.00 (m, 11H, ArH), 6.93 (m, 2H, ArH), 6.76 (d, J = 8.1, 2H, ArH), 5.20 (d, J = 13.9, 2H, PCH₂), 2.45 (s, 9H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 157.5 (d, J = 3.8), 156.7, 146.3 (d, J = 2.8), 134.4 (d, J = 10.1), 133.1 (d, J = 5.3), 131.0 (d, J = 12.9), 129.1, 123.8, 122.0 (d, J = 8.5), 119.1, 119.0 (d, J = 2.9), 114.8 (d, J = 88.3), 30.6 (d, J = 48.4), 22.0. LRMS (ES⁺) m/z = 487.40 [M⁺]. ESI-HRMS m/z 487.2206 [M⁺] (C₃₄H₃₂OP requires 487.2185).

(4-Phenoxybenzyl)tri-*m*-tolylphosphonium Bromide (57c). The reaction was carried out following the general procedure with 2c and tri-*m*-tolylphosphine. The product was obtained as a white solid following workup II procedure (65.2 mg, 60%): mp 228.9 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.58–7.46 (m, 11H, ArH), 7.29 (dd, J = 14.5, 6.2, 3H, ArH), 7.04 (dd, J = 8.7, 2.4, 3H, ArH), 6.93 (d, J = 7.6, 2H, ArH), 6.75 (d, J = 8.2, 2H, ArH), 5.29 (d, J = 13.8, 2H, PCH₂), 2.41 (s, 9H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 157.7, 156.6, 140.6 (d, J = 12.4), 135.9 (d, J = 2.9), 134.6 (d, J = 9.7), 133.1 (d, J = 5.4), 131.6 (d, J = 9.7), 130.4 (d, J = 13.2), 128.9, 123.9, 121.9 (d, J = 8.6), 119.3, 118.8 (d, J = 3.1), 117.9 (d, J = 84.8), 30.4 (d, J = 47.1), 21.6. LRMS (ES⁺) m/z = 487.40 [M⁺]. Anal. Calcd for C₃₂H₅₀BrOP: C, 71.96; H, 5.68; Br, 14.08. Found: C, 72.18; H, 5.71; Br, 13.93.

(4-Benzoylbenzyl)tri-*n*-hexylphosphonium Bromide (62a).

The reaction was carried out following the general procedure with 2a and tri-*n*-hexylphosphine. The product was obtained as a white solid following workup II procedure (84.3 mg, 52%). HPLC > 95% pure; mp 109.1 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.81–7.64 (m, 6H, ArH), 7.63–7.55 (m, 1H, ArH), 7.47 (t, J = 7.5, 2H, ArH), 4.58 (d, J = 15.9, 2H, PhCH₂), 2.51–2.29 (m, 6H, PCH₂CH₂), 1.42 (s, 12H, CH₂), 1.33–1.12 (m, 12H, CH₂), 0.84 (t, J = 6.7, 9H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 195.9, 137.6 (d, J = 3.6), 137.0, 133.7 (d, J = 8.9), 133.0, 131.0 (d, J = 2.9), 130.4 (d, J = 4.9), 129.3 (d, J = 115.5), 31.1, 30.6 (d, J = 14.8), 22.4, 22.0 (d, J = 4.8), 19.2 (d, J = 46.1), 14.0. LRMS (ES⁺) m/z = 482.36 [(M + H)]⁺. Anal. Calcd for C₃₂H₅₀BrOP: C, 68.56; H, 8.81; Br, 14.25. Found: C, 68.59; H, 8.90; Br, 14.41.

Biology. In Vitro Antitrypanosomal Activity. The in vitro trypanocidal and cytotoxic activities were determined using the Alamar blue assay.²⁷ Detailed experimental protocols for these assays with *T. b. rhodesiense* STIB900 and L6 cells have been previously reported.²⁸ A slight modification of this protocol was used for the assays with wild type and resistant *T. b. brucei* strains, as described.²⁹ The TbAT1-KO strain is derived from the wild type strain *T. b. brucei* Lister 427 (s427) by deletion of the *TbAT1* gene.¹⁸ The B48 strain is a mutant derived from TbAT1-KO by in vitro selection to high levels of pentamidine and does not express a functional high affinity pentamidine transporter (HAPT1).⁸ Monitoring of trypanosome cellular integrity with propidium iodide was performed exactly as described previously.²⁵

Pentamidine Transport Assays. Uptake assays used [³H]-pentamidine (Amersham) at either 30 nM final concentration for the assessment of HAPT1-mediated transport or at 1 μ M for the assessment of LAPT1-mediated transport, exactly as described.^{20,30} Briefly, trypanosomes were grown in HMI-9 with 10% fetal bovine serum (FBS) until late log phase, harvested, and washed into assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl₂, 0.07 mM MgSO₄, 5.8 mM NaH₂PO₄, 0.3 mM MgCl₂, 23 mM NaHCO₃, 14 mM glucose, pH 7.3) prior to use in the assay. Cells were incubated with radiolabel for 60 and 120 s for HAPT1 and LAPT1 assays, respectively. Incubations were stopped by the addition of 2 mM ice-cold unlabeled pentamidine and immediate centrifugation through oil. Quantification was through scintillation counting, and curves were fitted to sigmoid curves using the Prism 5.0 software package. All experiments were performed in triplicate and on at least three fully independent occasions.

CoMFA Models. The set of compounds (78 in total) were converted to 3D structures using LigPrep,³¹ considering pH 7.0 for the protonation state. Nevertheless, only one compound within the set, 49a, showed an additional protonation at the fixed pH for the pyridine moiety. Both states were modeled, and it turned out that the protonated state was more consistent with the modeled data. We removed all counterions, which is why compounds 45a, 45f, 45g, 64, and 65 were not included in the modeling step. Atomic charges used for the CoMFA models were calculated at the AM1³² semiempirical level using Gaussian 03.³³

The whole set was divided into two subsets, training compounds and test compounds, in order to properly validate the models. It was considered as important to have a good balance between active and inactive compounds within the test set for the sake of keeping a predictive model. With this goal in mind the whole set was divided into active, less active, and inactive compounds, doing the random selection upon these three subsets and trying to keep a similar number of compounds among the three subsets. However, during the modeling step the activities (EC₅₀) of some compounds were still to be determined, so these compounds were included in the test set.

The molecular alignment was built with the flexible molecular overlay method (50% steric, 50% electrostatic) as included in Discovery Studio, Accelerlys,³⁴ using the selected conformations of the more active compound in the training set as reference. Erroneously fitted compounds were realigned manually defining pairs of atoms ensuring a maximum molecular surface overlay. The resulting conformations on the whole set were checked to verify that no unusual energy penalty is paid for the resulting conformations.

Four different alignments were used within this work based on the four more stable conformations, while keeping significantly different root mean square deviations, found for the more active compound. Here we only present the results on one alignment, the one corresponding to the lowest energy conformations, given that it yields the better results. In any case no remarkable differences were found for the four calculated molecular overlays. CoMFA descriptors calculation (standard CoMFA, HB, and index), PLS model fitting, region focusing, and models validations were conducted in the standard manner described within the SYBYL suite,³⁵ and we found that standard CoMFA descriptors were perfectly able to model the proposed end point. Energetic cut-offs for CoMFA descriptors were set to 30 kcal mol⁻¹, which was more suitable for *T. b. brucei* than other calculated values.

■ ASSOCIATED CONTENT

■ Supporting Information

Synthesis and characterization of the new phosphonium salt derivatives **19c**, **23b–e**, **25c**, **28b,c**, **33b,c**, **34b,c**, **35a**, **38b**, **39a**, **40a**, **42a**, **43a**, **44a–c**, **45a,b,d,f,g**, **46b,c**, **50a**, **51b,c**, **52b,c**, **53a,c**, **57a–61a**, **63a** and the linkers **2a**, **3a**, **10f**, **11g**, **12f**, **13g**, **16f**, and **17g**; QSAR results and molecular overlay; effect of compounds **25b,c**, **26a**, **35a**, **36a**, **38a**, **43a,c**, **45d,e**, **47a**, and **55** on parasite viability as determined with the propidium iodide assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS USED

CoMFA, comparative molecular field analysis; EC₅₀, 50% effective concentration; HAPT, high affinity pentamidine transporter; HAT, human African trypanosomiasis; HEK, human embryonic kidney; LAPT, low affinity pentamidine transporter; NBS, *N*-bromosuccinimide; PI, propidium iodide; PLS, partial least squares; rmsd, root-mean-square deviation; SAR, structure–activity relationship; SE, standard error; SI, selectivity index

■ REFERENCES

- (1) *Working To Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases*; World Health Organization: Geneva, 2010.
- (2) Delespau, V.; de Koning, H. P. Drugs and drug resistance in African trypanosomiasis. *Drug Resist. Updates* **2007**, *10*, 30–50.
- (3) Rodgers, J. Human African trypanosomiasis, chemotherapy and CNS disease. *J. Neuroimmunol.* **2009**, *211*, 16–22.
- (4) Kinnamon, K. E.; Steck, E. A.; Rane, D. S. A new chemical series active against African trypanosomes: benzyltriphenylphosphonium salts. *J. Med. Chem.* **1979**, *22*, 452–455.
- (5) Luque-Ortega, J. R.; Reuther, P.; Rivas, L.; Dardonville, C. New benzophenone-derived bisphosphonium salts as leishmanicidal leads targeting mitochondria through inhibition of respiratory complex II. *J. Med. Chem.* **2010**, *53*, 1788–1798.
- (6) Dardonville, C.; Brun, R. Bisguanidine, bis(2-aminoimidazoline), and polyamine derivatives as potent and selective chemotherapeutic agents against *Trypanosoma brucei rhodesiense*. Synthesis and in vitro evaluation. *J. Med. Chem.* **2004**, *47*, 2296–2307.
- (7) de Koning, H. P. Ever-increasing complexities of diamidine and arsenical crossresistance in African trypanosomes. *Trends Parasitol.* **2008**, *24*, 345–349.
- (8) Bridges, D. J.; Gould, M. K.; Nerima, B.; Mäser, P.; Burchmore, R. J. S.; De Koning, H. P. Loss of the high-affinity pentamidine transporter is responsible for high levels of cross-resistance between arsenical and diamidine drugs in african trypanosomes. *Mol. Pharmacol.* **2007**, *71*, 1098–1108.
- (9) Lüscher, A.; de Koning, H. P.; Mäser, P. Chemotherapeutic strategies against *Trypanosoma brucei*: Drug targets vs. drug targeting. *Curr. Pharm. Des.* **2007**, *13*, 555–567.
- (10) de Koning, H. P.; Anderson, L. F.; Stewart, M.; Burchmore, R. J.; Wallace, L. J.; Barrett, M. P. The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in african trypanosomes. *Antimicrob. Agents Chemother.* **2004**, *48*, 1515–1519.
- (11) Golden, J. H. Poly-*p*-xylylene and related compounds. *J. Chem. Soc.* **1961**, 1604–1610.
- (12) Jung, H. K.; Lee, J. K.; Kang, M. S.; Kim, S. W.; Kim, J. J.; Park, S. Y. Synthesis and properties of poly(*p*-phenylenevinylene-*co*-sulfonylene) for a blue light-emitting diode. *Polym. Bull.* **1999**, *43*, 13–20.
- (13) Wakselman, M.; Domé, M. Alkylations en milieu aqueux par les halogénures benzyliques aminés ou amidés. 1. Synthèse de réactifs mono- et bialkylants. *Bull. Chem. Soc. Fr.* **1975**, 571–575.
- (14) Boyer, B.; Keramane, E. M.; Arpin, S.; Montéro, J.-L.; Roque, J.-P. BiX3 as an efficient and selective reagent for the halogen exchange reaction. *Tetrahedron* **1999**, *55*, 1971–1976.
- (15) Grützmacher, H. F.; Neumann, E.; Ebmeyer, F.; Albrecht, K.; Schelenz, P. [3.2]Paracyclophane-10-enes and [3.2.3.2]-paracyclophane-10,27-dienes: a convenient synthesis by the McMurry reaction and dynamic stereochemistry. *Chem. Ber.* **1989**, *122*, 2291–2297.
- (16) Berg, M.; Kohl, L.; Van der Veken, P.; Joossens, J.; Al-Salabi, M. I.; Castagna, V.; Giannese, F.; Cos, P.; Versee, W.; Steyaert, J.; Grellier, P.; Haemers, A.; Degano, M.; Maes, L.; de Koning, H. P.; Augustyns, K. Evaluation of nucleoside hydrolase inhibitors for treatment of African trypanosomiasis. *Antimicrob. Agents Chemother.* **2010**, *54*, 1900–1908.
- (17) Gould, M. K.; Vu, X. L.; Seebeck, T.; de Koning, H. P. Propidium iodide-based methods for monitoring drug action in the kinetoplastidae: comparison with the Alamar Blue assay. *Anal. Biochem.* **2008**, *382*, 87–93.
- (18) Matovu, E.; Stewart, M. L.; Geiser, F.; Brun, R.; Mäser, P.; Wallace, L. J. M.; Burchmore, R. J.; Enyaru, J. C. K.; Barrett, M. P.; Kaminsky, R.; Seebeck, T.; De Koning, H. P. Mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. *Eukaryotic Cell* **2003**, *2*, 1003–1008.
- (19) Ross, M. F.; Da Ros, T.; Blaikie, F. H.; Prime, T. A.; Porteous, C. M.; Severina, I. I.; Skulachev, V. P.; Kjaergaard, H. G.; Smith, R. A.

J.; Murphy, M. P. Accumulation of lipophilic dications by mitochondria and cells. *Biochem. J.* **2006**, *400*, 199–208.

(20) de Koning, H. P. Uptake of pentamidine in *Trypanosoma brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. *Mol. Pharmacol.* **2001**, *59*, 586–592.

(21) Bush, B. L.; Nachbar, R. B. Sample-distance partial least squares: PLS optimized for many variables, with application to CoMFA. *J. Comput.-Aided Mol. Des.* **1993**, 587–619.

(22) Golbraikh, A.; Tropsha, A. Beware of q²! *J. Mol. Graphics. Modell.* **2002**, *20*, 269–276.

(23) Ross, M.; Kelso, G.; Blaikie, F.; James, A.; Cochemé, H.; Filipovska, A.; Da Ros, T.; Hurd, T.; Smith, R.; Murphy, M. Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology. *Biochemistry (Moscow)* **2005**, *70*, 222–230.

(24) Teká, I. A.; Kazibwe, A. J. N.; El-Sabbagh, N.; Al-Salabi, M. I.; Ward, C. P.; Eze, A. A.; Munday, J. C.; Mäser, P.; Matovu, E.; Barrett, M. P.; De Koning, H. P. The diamidine diminazene aceturate is a substrate for the high-affinity pentamidine transporter: implications for the development of high resistance levels in trypanosomes. *Mol. Pharmacol.* **2011**, *80*, 110–116.

(25) Ibrahim, H. M. S.; Al-Salabi, M. I.; Sabbagh, N. E.; Quashie, N. B.; Alkhalidi, A. A. M.; Escala, R.; Smith, T. K.; Vial, H. J.; de Koning, H. P. Symmetrical choline-derived dications display strong anti-kinetoplastid activity. *J. Antimicrob. Chemother.* **2011**, *66*, 111–125.

(26) Ward, C. P.; Wong, P. E.; Burchmore, R. J.; De Koning, H. P.; Barrett, M. P. Trypanocidal furamide analogues: influence of pyridine nitrogens on trypanocidal activity, transport kinetics, and resistance patterns. *Antimicrob. Agents Chemother.* **2011**, *55*, 2352–2361.

(27) Ráz, B.; Iten, M.; Grether-Bühler, Y.; Kaminsky, R.; Brun, R. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. *Acta Trop.* **1997**, *68*, 139–147.

(28) Dardonville, C.; Fernandez-Fernandez, C.; Gibbons, S. L.; Jagerovic, N.; Nieto, L.; Ryan, G.; Kaiser, M.; Brun, R. Antiprotozoal activity of 1-phenethyl-4-aminopiperidine derivatives. *Antimicrob. Agents Chemother.* **2009**, *53*, 3815–3821.

(29) Rodenko, B.; Van Der Burg, A. M.; Wanner, M. J.; Kaiser, M.; Brun, R.; Gould, M.; De Koning, H. P.; Koomen, G. J. 2,N6-disubstituted adenosine analogs with antitrypanosomal and antimalarial activities. *Antimicrob. Agents Chemother.* **2007**, *51*, 3796–3802.

(30) Wallace, L. J. M.; Candlish, D.; De Koning, H. P. Different substrate recognition motifs of human and trypanosome nucleobase transporters. Selective uptake of purine antimetabolites. *J. Biol. Chem.* **2002**, *277*, 26149–26156.

(31) *LigPrep*, version 2.5; Schrödinger, LLC: New York, NY, 2011.

(32) Mittal, R. R.; Harris, L.; McKinnon, R. A.; Soric, M. Partial charge calculation method affects CoMFA QSAR prediction accuracy. *J. Chem. Inf. Model.* **2009**, *49*, 704–709.

(33) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, revision C; Gaussian, Inc.: Wallingford, CT, 2004.

(34) *Discovery Studio*; Accelrys Software Inc.: San Diego, CA, 2010.

(35) SYBYL-X, version 1.2; Tripos Inc. (1699 South Hanley Road, St. Louis, MO, 63144, U.S.).