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Effects of Cationic Diamidines on Polyamine Content and Uptake on *Leishmania infantum* in *In Vitro* Cultures

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ABSTRACT. The effect of a series of cationic diamidines recently synthesized by Ciba Geigy, bearing diarylic (CGP040215A and CGP039937A) or monoarylic moieties (CGP033829A, CGP035537A and CGP036958A), was analyzed on some metabolic targets and cell proliferation of *in vitro* cultures of *Leishmania infantum* promastigotes (insect form). The action of these compounds on intracellular polyamine pools and putrescine transport suggests that diarylic structures were more effective than their monoarylic counterparts in depleting polyamine levels and inhibiting putrescine transport, although these processes correlate poorly with the antiproliferative rate of these compounds. Finally, the displacement of cationic diamidines to kDNA observed in the presence of several concentrations of spermidine suggests a possible combined mode of action of these molecules, first depleting intracellular polyamine pools and, then, displacing spermidine from its site of interaction to kDNA. BIOCHEM PHARMACOL 52;6:835–841, 1996.

KEY WORDS. cationic diamidines; *Leishmania*; polyamines; DNA binding

Leishmania infantum is a unicellular parasite causative of both visceral and cutaneous leishmaniasis in human and dogs in Mediterranean countries. Drugs of choice against this group of diseases are pentavalent antimonials: pentostam (sodium stibogluconate) and glucantime (meglumine antimoniate), whose therapeutic indices are very favorable, although clinical resistance has been reported [1, 2]. Some unresponsive strains of *Leishmania* spp can be treated with second-line drugs, such as cationic diamidines (e.g. pentamidine [3, 4]), or poliene antibiotics such as amphotericin B [1], although close control of these treated with these drugs is always required due to the appearance of undesirable side effects.

The use of aromatic diamidinic compounds on diseases produced by parasitic protozoa, including opportunistic infections associated with acquired immune deficiency syndrome (AIDS), has been greatly increased in the last 10 years since aerosolized pentamidine was recommended in 1984 by the FDA as a drug of choice against *Pneumocystis carinii* pneumonia [5, 6]. Since the late 1980s, several laboratories looking for more active and safer drugs have synthesized a number of cationic analogues resembling pentamidine (i.e. either diguanidine, diamidine or dimidazoline structures) that have been tested on different etiologic agents with different results [7, 8]. In 1991, Tidwell *et al.* [9] showed that 1,4-di(4'amidino-phenoxy)butane was more effective than pentamidine against *P. carinii* pneumonia in experimentally immunosuppresed rats, showing the relevance of these structures to parasitic diseases.

Nevertheless, the mechanisms of action of these compounds are poorly understood. All these structures seem to be multitarget drugs and a major mode of action has yet to be discovered. One of the targets of aromatic diamidines is DNA. The cationic nature of these molecules at physiological pH allows them to bind in a nonintercalative way to the AT minor grove regions of DNA in both chromosomic and kinetoplastic DNA (kDNA), producing inhibitions in replication and transcription. In addition, other minor biochemical targets of aromatic diamidines have been described previously (i.e. their inhibitory effect on some proteases) (i.e. trypsin), topoisomerases, and thymylidate synthetase [4, 10].

It is well established that the biosynthetic pathway of naturally occurring polyamines (putrescine, spermidine, and spermine) is a key step in cell growth and differentiation. The inhibition of polyamine biosynthetic enzymes and uptake is considered a main target for the chemotherapy of several parasitic diseases, including trypanosomiasis and leishmaniasis [11, 12]. Several authors have shown a strong inhibitory effect of cationic diamidines (diminazene and pentamidine) on both SAMDC† [13–15], and putrescine transport [16, 17] in parasitic protozoa and host cells, depleting intracellular polyamine pools and reducing cell proliferation.

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† *Abbreviations:* SAMDC, S-adenosyl-L-methionine decarboxylase.

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Therefore, we have tested the effect of a series of cationic diamidines recently synthesized and successfully tested against tumor cell lines by Ciba Geigy [18], on growth, polyamine content, and putrescine transport in *L. infantum* promastigotes, to establish the relevance of these mechanisms to the mode of action of these compounds.

MATERIALS AND METHODS

Chemicals

The diamidine derivatives (CGP040215A, CGP039937A, CGP033829A, CGP035753A, and CGP036958A) shown in Fig. 1 were a generous gift from Dr. Regenass (Ciba Geigy, Basel, Switzerland) [18]. [1,4^{14}C]-putrescine and the scintillation cocktail NEF 989 were obtained from New England Nuclear (NEN, Dupont de Nemours, Germany). Culture medium 199, gentamicin sulfate (10 mg/mL), ethidium bromide, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and 2-hydroxyaminopropane were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Fetal calf serum, pronase, and proteinase K were purchased from Boehringer Mannheim (GmbH, Germany). All other reagents were of standard laboratory grade.

Organisms and Media

L. infantum (PEP 1G11 clone) promastigotes were originally isolated from popliteal lymph nodes of a naturally

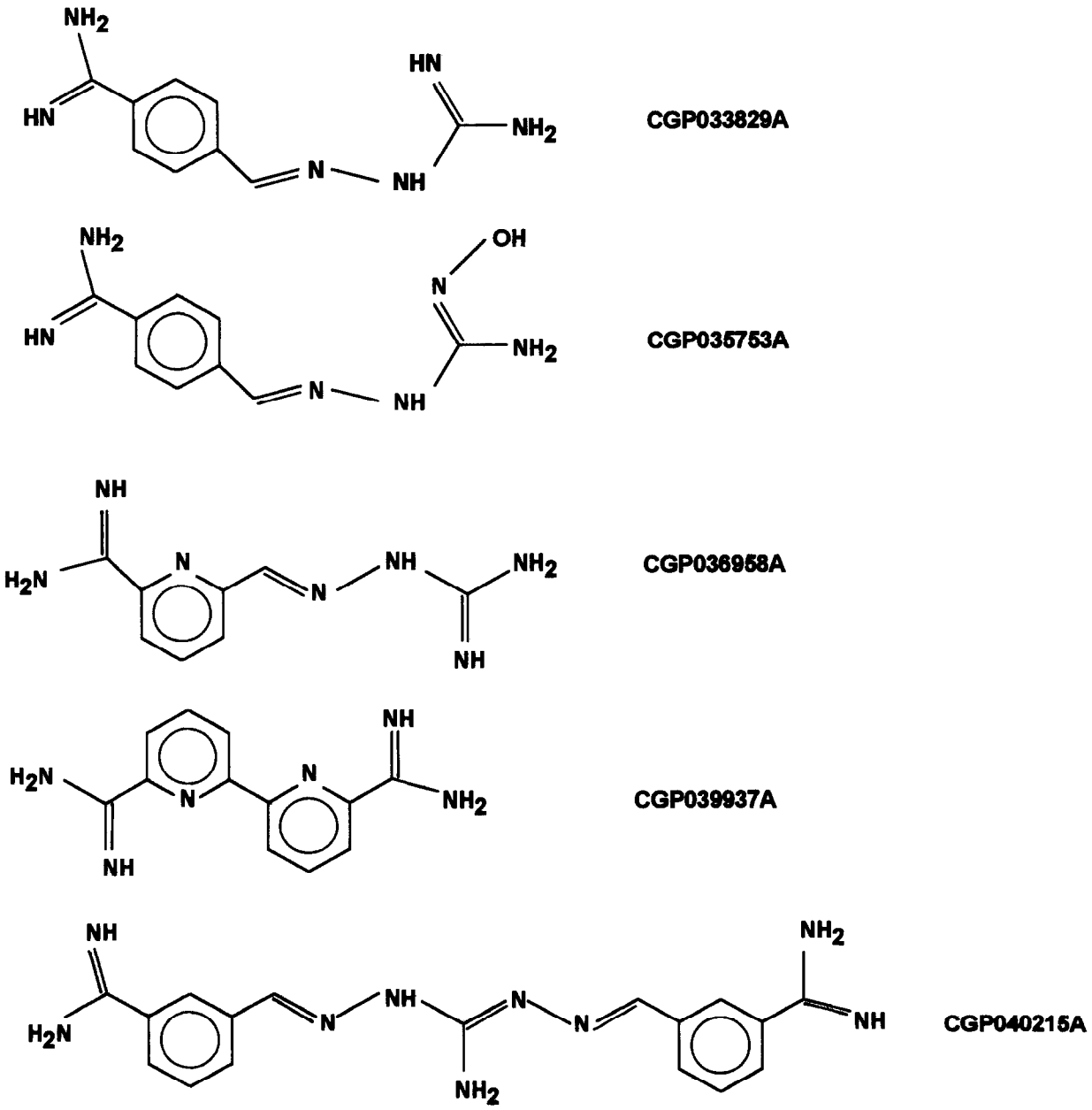

FIG. 1. Structural formulas of cationic diamidines used in this study.

infected dog and cloned by the agar plating method [19]. Promastigotes (insect form) were routinely cultured at 26.5°C in Medium 199 supplemented with 10% heat-inactivated feral calf serum and 1.5 μg/mL gentamicin sulfate. Cultures were subpassaged once a week with only a small number of subpassages to maintain genetic homogeneity. Cell growth was optically monitored by counting in improved Neubauer chambers. The doubling time calculated for these parasites was 12 hr.

Putrescine Uptake

Promastigotes cultured as above were harvested in midlogarithmic phase (48 hr subpassage) by centrifuging at 6000 × *g* for 10 min at 4°C. Pellets were washed 3 times in phosphate saline, 1% glucose (PSG buffer), resuspended in the same buffer at a cellular density of 5×10^6 promastigotes/mL and preincubated for 10 min at 30°C in the presence of several concentrations of each diamidine. The assays were carried out by incubating the cell samples with different concentrations of putrescine (2.8 to 32 μM) that included 0.16 μCi of 1,4[^{14}C]-putrescine (90.4 mCi/mmol) for 20 min, at the same temperature [16]. The reactions were stopped on ice and vacuum-filtered through Millipore nitrocellulose filters (0.45 μm 25 mm diameter), previously soaked in 0.1 M unlabelled putrescine to avoid unspecific adsorption. Filters were washed with 2 × 5 mL of PSG containing 2 mM unlabelled putrescine, air dried, and counted in a liquid scintillation spectrometer using NEF 989 aqueous scintillation cocktail. Uptake rate was expressed as pmol of putrescine internalized per 30 min and per 10^7 promastigotes. The unspecific binding to filters and the passive diffusion component were subtracted from total uptake rates by using blanks incubated at 0°C.

kDNA Binding and Displacement Assays

The binding of the cationic diamidines to kDNA isolated from *L. infantum* promastigotes was determined spectrophotometrically [20]. To obtain *L. infantum* kDNA, promastigotes were grown until stationary growth phase, harvested, and washed as described above. The last pellet was then treated with 0.1% Sarcosyl in MET buffer (0.1 M NaCl, 0.1 M EDTA, 0.01 M TrisHCl, pH 80) overnight at 60°C. After centrifugation at 15000 × *g*, the resuspended material was digested with pronase (1 mg/mL in MET buffer) and extracted twice in phenol/chloroform. The phenolic phase was treated with the same volume of ether and a solution of proteinase K (2 mg/mL in MET buffer containing 1% SDS) overnight. The purity and concentration of kDNA was monitored by the ratio of absorbances at 260 and 280 nm.

For binding assays, the cationic diamidines were dissolved at a concentration of 2×10^{-6} M in 3 mM Tris HCl buffer pH 7.0 containing 0.018 M NaCl, previously sterilized by filtration through 0.22 μm membranes. The displacement of each diamidine by spermidine was evaluated as the shift of absorbance at the maximum specific wavelength peak displayed by each compound at 0, 100, 125, 150, and 200 nmol/mL spermidine, using 20 μM of each cationic diamidine, and 7 μg of kDNA (lacking spermidine) after incubation of the reaction mix at 25°C for 5 hr.

Measurements of Polyamines

To determine the intracellular levels of polyamines in *L. infantum* parasites, cultures were grown until day 3, pulsed with different concentrations of each diamidine, and harvested 24 hr later. Cells were washed in ice-cold saline, and the last pellet was extracted with 0.5 mL of 10% trichloroacetic acid. After centrifugation, the supernatant was analyzed for polyamines by HPLC using the precolumn derivatization method with dansyl chloride previously described [21]. The amount of polyamine in each sample—expressed as nmol/mg of total protein—was determined using 2-hydroxyaminopropane as internal standard. Protein concentrations were determined by the Bradford procedure using bovine serum albumin as standard [22].

RESULTS

Drug Susceptibilities

The growth of *L. infantum* promastigotes was monitored microscopically in the presence of several concentrations of the cationic diamidines represented in Fig. 1. The parasite cultures were grown until the day 4 subpassage and counted using improved Neubauer chambers. Table 1 shows the inhibitory effect of such compounds at 3 different concentrations (i.e. 10 μM, 100 μM and 1 mM) and the EC_{50} values calculated by means of dose-response curves at a wider range of concentrations, fitted by semilogarithmic plots (regression coefficients were always higher than 0.98). Table 1 also shows that the inhibitory rates of these compounds ranged from 77 μM for CGP036958A to 158 μM for CGP040215A, being the EC_{50} of the other compounds comprised in a narrow margin around 100 μM each.

This suggests that the monoaryls (CGP035753A and CGP036958A) and monoheteroaryl (CGP033829A) were slightly more powerful antiproliferative compounds than their diaryl (CGP040215A) and diheteroaryl (CGP039937A) counterparts on *L. infantum* parasites. It is

TABLE 1. Inhibitory effect of the cationic diamidine compounds on the proliferative growth rate of *L. infantum* cultures

	Inhibition of growth (%)			
Compound	**10 μM**	**100 μM**	**1 mM**	**EC_{50} (μM)**
CGP040215 A	10	45	85	158
CGP039937 A	14	47	93	126
CGP033829 A	17	49	92	108
CGP035753 A	14	43	87	114
CGP036958 A	20	62	91	77

EC_{50} values were calculated by means of semilogarithmic plots and were the result of duplicate experiments.

also apparent that the heteroarylic moieties (CPG039937A and CGP035753A) confer higher antiproliferative capacity than the arylic residues displayed by CGP040215, CGP033829A, and CGP036958A.

Effect on Polyamine Content

To analyze the mode of action of this series of compounds, *L. infantum* promastigotes were grown for 48 hr in the absence of any drug and then pulsed with 10 μM, 100 μM, and 1 mM of each diamidine for 24 hr. Afterward, the parasites were harvested and analyzed for polyamines as described above. The polyamine pattern found on *L. infantum in vitro* cultures resembles the previously described profile: high concentrations of putrescine and spermidine and the complete absence of spermine, regarding the polyamine content of other Trypanosomatids. Table 2 shows the effect of cationic diamidines on putrescine and spermidine intracellular concentrations after exposure to each compound, as well as the putrescine/spermidine ratio. A dose-dependent depletion of intracellular putrescine and spermidine pools was found in *L. infantum* promastigotes using the cationic products selected in the study. Diarylic and diheteroarylic diamidines showed good results, reducing spermidine content (more than 50% with CGP040215A and almost 80% with CGP039937A) at only 10 μM, thus producing putrescine/spermidine ratios much higher than the untreated controls. However, the most effective antiproliferative compound used in this study, the monoheteroarylic diamidine (CGP033829A), showed high activity, depleting both putrescine and spermidine intracellular pools and, thereby, producing putrescine/spermidine ratios below the control cells at the 3 concentrations studied. Finally, the effect of monoarylic diamidines CGP035753A and CGP036958A were only significant at the highest concentrations assayed.

Effect on Putrescine Transport

The presence of an active, specific, and growth-regulated putrescine transport system in *L. infantum* has been previously described, as has the inhibitory effect of chemicals with diamidinic structure. *L. infantum* cultures were grown until 2 days subpassage (maximum peak of putrescine uptake throughout the growth cycle of this parasite), harvested, and washed for uptake assay (Table 3). The EC_{50} calculated by means of dose-response curves at different concentrations of each inhibitor were estimated to be 14.6 μM for CGP040215A, 32 μM for CGP03993A, respectively, and far higher for the monoarylic and monoheteroarylic structures, (i.e. between 200 and 300 μM).

Kinetic analysis of putrescine inhibition by the aromatic diamidines used in the present study was carried out by means of the Michaelis Menten approach at different concentrations of the diarylic diamidines, to calculate the K_i value and the inhibitory pattern. The Lineweaver-Burk plots represented in Fig. 2 show that the K_i calculated for CGP039937A was estimated to be 43 μM, showing a noncompetitive inhibition (Fig. 2A), being the K_i value estimated for CGP040215A of 13 μM with a mixed-competitive inhibitory pattern (Fig. 2B).

Displacement of Cationic Compounds by Spermidine

To know the amount of spermidine bound to kDNA, recently isolated kDNA from stationary-phase promastigotes

TABLE 2. Effect of cationic diamidines on polyamine (putrescine and spermidine) content after a 24-hr administration period

Compound		Polyamine concentration (nmol/mg protein)				
		Putrescine		Spermidine		Put/Spd
Control		347.6	(100)	239.9	(100)	1.45
CGP040215A	10 μM	209.2	(39.8)	104.7	(56.3)	2.00
	100 μM	186.6	(46.3)	103.3	(56.9)	1.81
	1 mM	97.6	(72.1)	66.6	(72.2)	1.45
CGP039937A	10 μM	213.4	(38.6)	56.1	(76.6)	3.80
	100 μM	163.5	(52.9)	84.1	(64.9)	1.94
	1 mM	104.9	(69.8)	80.0	(66.6)	1.31
CGP033829A	10 μM	263.5	(24.2)	191.0	(20.3)	1.38
	100 μM	96.4	(72.2)	178.5	(25.6)	0.54
	1 mM	48.4	(85.9)	93.7	(60.9)	0.52
CGP035753A	10 μM	398.5	(n.i.)	233.3	(0.5)	1.71
	100 μM	282.6	(18.7)	195.2	(18.6)	1.45
	1 mM	193.9	(44.2)	109.6	(54.3)	1.77
CGP036958A	10 μM	418.9	(n.i.)	190.4	(20.6)	2.20
	100 μM	259.7	(25.3)	137.6	(42.6)	1.89
	1 mM	187.7	(46.0)	152.3	(36.5)	1.23

Values in parentheses represent the percentage of inhibition with respect to control cells.

L. infantum parasites were grown until 48 hr subpassage, were pulsed with 10 μM, 100 μM, and 1 mM of each diamidine, and maintained for an extended period of 24 hr. Cells were harvested, washed, and analyzed for polyamines according to [21].

TABLE 3. Effect of cationic diamidines on putrescine transport of *L. infantum* promastigotes

Compound	Putrescine uptake (%)				
	10 μM	100 μM	1 mM	EC_{50} (μM)	K_i (μM)
CGP040215 A	55.4	8.0	0.2	14.6	13.0
CGP039937 A	61.5	33.1	4.1	32.0	43.2
CGP033829 A	100	97.8	10.0	228.0	n.d.
CGP035753 A	100	86.7	30.0	327.0	n.d.
CGP036958 A	100	81.02	14.0	205.0	n.d.

Putrescine uptake on control untreated parasites was estimated on pmol/30 min/10^7cells. n.d., not determined.

Cells were grown for 36 hr and then harvested and washed for putrescine uptake analysis. Results are the average of 3 determinations in duplicate.

were treated with isopropanol and stirred gently. After 2 extractions, the isopropilic phase was evaporated to dryness, resuspended in 200 μL distilled water, and analyzed for polyamines. Polyamine analysis of either kDNA pellet and alcoholic phase showed that only spermidine was bound to kDNA at a concentration of 0.571 nmol/μg kDNA. kDNA (7 μg) were incubated in the presence of 20 μM of each diamidine at different concentrations of spermidine. Figure 3A shows the displacement patterns when the monoarylic (CGP033829A and CGP035753A) and diarylic (CGP040215A) were used at the above-indicated spermidine concentrations. This panel shows that the diarylic structure was readily displaced by lower concentrations of spermidine than were the monoarylic compounds, the maximum binding strength to kDNA being displayed by CGP033829A. Panel 3B shows that the monoheteroarylic compound (CGP036958A) was more easily displaced by spermidine than was the diheteroarylic diamidine (CGP039937A), although the maximum binding strength was displayed by the well-known intercalative compound ethidium bromide.

DISCUSSION

The official use of pentamidine against *Pneumocystis carinii* pneumocystosis began 10 years ago [5, 6, 23]. Since then, several series of cationic diamidines resembling pentamidine have been synthesized [8, 9] in an attempt to improve the chemotherapeutic index of the parent compound and minimize its toxic side effects, which appear after chronic treatment [4]. Moreover, the interest in these drugs has been extended to other infectious diseases produced by parasites, particularly those produced by Trypanosomatids (i.e. *Trypanosoma* and *Leishmania* spp.), where current treatments are always very toxic for the host [1, 7, 8, 24]. However, the mode of action of aromatic diamidines has yet to be clarified. Previous reports show that the multitarget action of these chemicals is related to their cationic nature at physiological pH, which allows them to interact with macromolecules [4, 24].

The inhibitory effect of cationic diamidines on polyamine metabolism is one of the latest targets investigated, and deserves our interest due to its considerable relevance to proliferative processes. The irreversible inhibitory effect of berenil and pentamidine on host [13, 15] and *Trypanosoma brucei brucei* SAMDC [14] has been established and has also been confirmed on mammalian diamine oxidase [13, 25]—the first enzyme of putrescine catabolism and other normobiotic diamines—and *L. infantum* putrescine uptake [16, 17]. The results found on both putrescine uptake and polyamine levels in the presence of cationic diamidines (Tables 2 and 3, respectively) suggest that the antiproliferative effect of these molecules may involve both processes. The polyamine profile produced by cationic diamidines on *L. infantum* parasites after 24-hr exposure, suggests an inhibitory action on SAMDC, because intracellular polyamine levels were affected at the lowest concentration of each diamidine added. However, the polyamine pattern found in the cells after diamidine treatment is

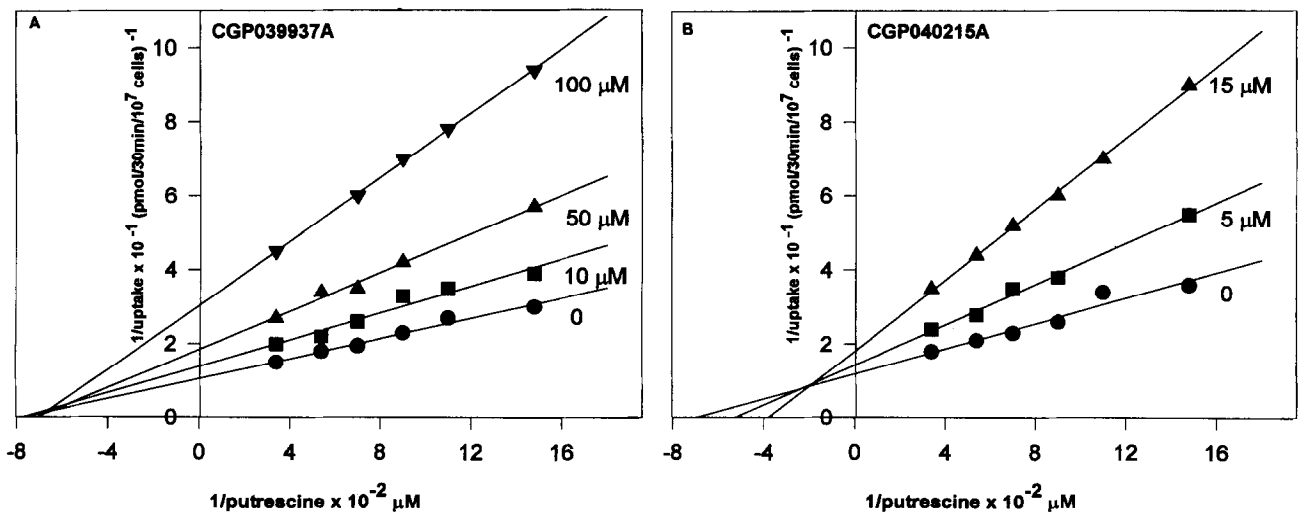

FIG. 2. Lineweaver-Burk plots of the inhibition produced by the two most active diamidinic compounds on *L. infantum* putrescine uptake. (A) CGP039937A, (B) CGP040215A. Each point represents the average of two separate experiments.

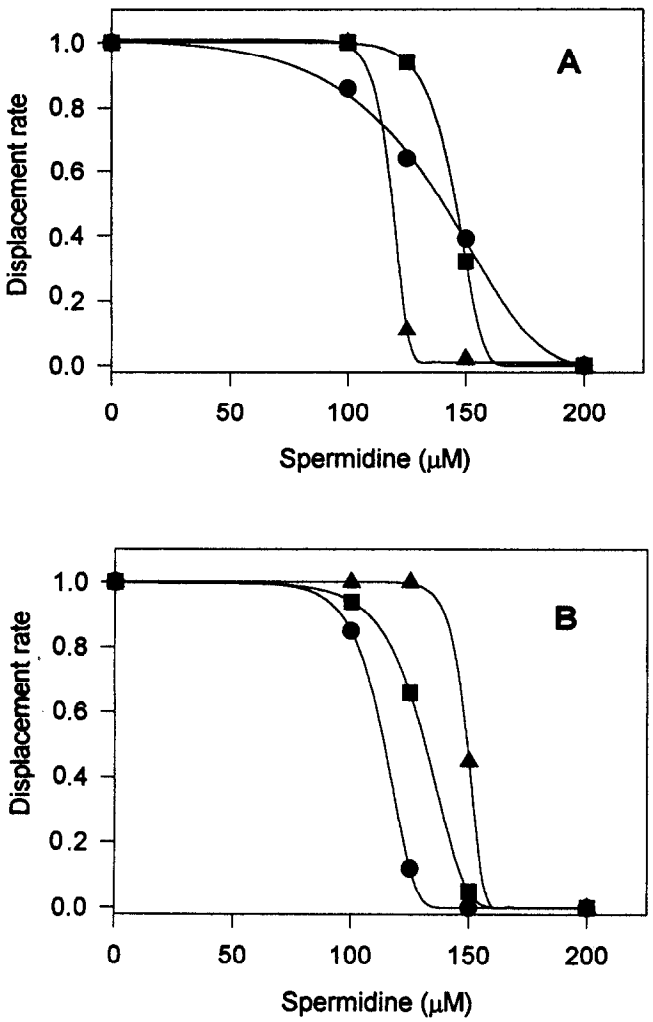

FIG. 3. Displacement of cationic diamidines from *Leishmania infantum* kDNA by different concentrations of spermidine. 7 μg of kDNA and 20 μM of each compound were incubated for 5 hr in the presence of 0, 100, 150, and 200 μM spermidine. Then, the shift of absorbance at the specific maximum wavelength peak was measured. 3A: (●) CGP035753A, (■) CGP039937A, (▲) ethidium bromide. B: (●) CGP033829A, (■) CGP036958A, (▲) CGP040215A. Each point is the average of duplicate experiments.

hardly conceivable based only on SAMDC inhibition. The standard polyamine profile produced by specific SAMDC inhibitors includes a deep depletion of spermidine levels and a characteristic increase of intracellular putrescine pools [26] that could not be obtained using the experimental protocol described in the present study. Unfortunately, *L. infantum* SAMDC could not be detected using the classic enzymatic assays previously described for both putrescine-activated [27] and independent enzymes [28]. Because the effect of cationic diamidines on intracellular polyamines was measured 24 hr after the addition of the compounds, corresponding to day 4 subpassage, the ornithine decarboxylase activity in the parasite was nil. The depletion of putrescine levels may be due to an exchange with the culture medium through an energy-independent mechanism, previously described in this species, or to a cellular leakage in response to the toxic effects of these compounds [29]. On the other hand, the inhibition of putrescine intracellular levels correlates well with the inhibitory effect on putrescine uptake. Although polyamine uptake can be considered a secondary antiproliferative target, its relevance in cell cultures or animals exposed to polyamine synthesis inhibitors such as tumor-bearing DFMO-treated rats [30], DFMO-treated *T. brucei*-infected mice [31] or DFMO-treated *L. infantum* promastigotes [16, 17] should be crucial. Only the diarylic forms with EC_{50} on putrescine uptake lower than 50 μM were able to reduce significantly the putrescine content at the lowest concentrations analyzed, where the monoarylic structures were almost ineffective.

An important question is whether or not the depletion of intracellular polyamines by cationic diamidines is related to their binding to kDNA and, thus, to their antiproliferative effect on *L. infantum* parasites. In 1981, Bacchi [11] suggested that the trypanocydal effect of certain drugs—including polyamine analogs—may take place by displacing intracellular polyamines. In agreement with this theory, cationic diamidines can, indeed, displace polyamines, especially spermidine, from their sites of interaction with DNA, thus blocking or inhibiting replication and/or transcription [32]. We evaluated how this polyamine can compete with aromatic diamidines on their site of interaction to *L. infantum* kDNA, at the intracellular homeostatic concentrations of this polyamine. Results obtained in Fig. 3, show how spermidine can displace all diamidines used in an inverse mode to their affinity constants. It is worth keeping in mind that the concentrations of spermidine able to displace the binding of these compounds to kDNA were in the physiological range of the parasites, and that the concentrations of the compounds used in these experiments were those able to deplete the spermidine pools and putrescine uptake in 20–50%. According to these results, a depletion primarily of spermidine pools and one of the sources of polyamines (putrescine uptake) can help the interaction of cationic diamidines to kDNA, producing a drop in the proliferative rate [33, 34].

Despite these results, which correlate with the depletion of polyamine levels and metabolism and the interaction of cationic diamidines with kDNA by displacement of spermidine in *L. infantum* parasites, more information is required to clarify the actual site of binding of these structures to other intracellular targets and their possible effect on replication and transcription processes, which would explain their antiproliferative effects on Trypanosomatids.

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