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PUTRESCINE UPTAKE INHIBITION BY AROMATIC DIAMIDINES IN *LEISHMANIA INFANTUM* PROMASTIGOTES

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Abstract—The effect of a series of aromatic diamidines has been tested on *Leishmania infantum* promastigotes in both culture growth and putrescine uptake. The EC_{50} values calculated by means of dose–response curves were 45, 80, 165, 259 and 600 μM for 4',6-diamidino-2-phenylindole (DAPI), dibromo propamidine, pentamidine 2-hydroxy stilbamidine and stilbamidine, respectively, although no inhibitory effects on cell growth were found at 1 mM propamidine, phenamidine and amicarbalide. When these compounds were kinetically analysed for putrescine uptake using Lineweaver–Burk plots, the K_i values reached were: DAPI, 15 μM; pentamidine, 3 μM; dibromo propamidine, 7 μM; 2-hydroxy stilbamidine, 21 μM; stilbamidine, 20 μM; propamidine, 25 μM; and phenamidine, 95 μM. Amicarbalide, however, was not able to reduce putrescine uptake to a significant extent, even at the highest concentration studied of 1 mM.

Key words: aromatic diamidines; putrescine uptake; *Leishmania infantum*

The role of PA† (put, spermidine and spermine) transport in eukaryotic cells is poorly understood due to "*de novo*" synthesis of PAs by a highly regulated biosynthetic pathway, made up of two key inducible enzymes, ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, as well as two constitutive ones, spermidine and spermine syntheses [1]. The requirement for an active transport system for highly charged molecules, like PAs at physiological pH, however, has recently been investigated although its actual function and mechanism is as yet unclear [2]. PA transport in both mammalian (host) and parasitic protozoa cells appears to be a multiple, energy-dependent regulated mechanism [3], closely controlled either by developmental cell processes [4] or intracellular PA pools [5, 6]. However, PA transport in lower eukaryotes, unlike mammals, is very specific for put, being scarcely inhibited by large amounts of spermidine, spermine or related compounds like methylglyoxal bis (guanylhidrazone) (MGBG) [7].

The study of specific inhibitors of PA transport may constitute a useful approach to evaluate the importance of this process in cell metabolism, PA content or proliferative status. So far, three groups of PA uptake inhibitors have been reported; (a) PA analogues [8], including MGBG [7] and bisbenzylpolyamines [9], (b) methylviologen (paraquat) and related herbicides [10] and (c) diminacene aceturate (Berenil) [11], a common antiparasitic (*Babesia sp, Pyroplasma sp*) drug, of the aromatic diamidine family, widely used in veterinary and human medicine [12]. Recently Berenil has been related to PA metabolism, as a micromolar inhibitor of *S*-adenosyl-methionine decarboxylase, the key enzyme of spermidine and spermine biosynthesis in both mammalian (host) [13] and parasitic cells [14], and diamine oxidase [15], the enzyme controlling the terminal catabolic degradation of put in mammalian cells [16].

A structure–activity screening was carried out among a series of aromatic diamidines related to Berenil on the putrescine uptake system of *Leishmania infantum* promastigotes, the aetiological agent of human and canine leishmaniosis in the Mediterranean Basin, in order to establish the relevance of the inhibitory kinetic constants on the proliferation rate of these parasites.

MATERIALS AND METHODS

Chemicals. Culture Medium 199, and gentamicin sulphate (10 mg/mL) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and foetal calf serum from Boehringer Mannheim GmbH (Germany), 1,4-[^{14}C]Put dihydrochloride (90.40 mCi/mmol) and scintillation cocktail for aqueous solutions NEF-989 was from New England Nuclear (Du Pont de Nemours, Germany). Unlabelled put was from Sigma. Aromatic diamidines: propamidine, DB-propamidine, 2-OH-stilbamidine, phenamidine and amicarbalide as diisethionate salts were a kind gift from May & Baker (Rhône Poulenc Rorer, Dagenham, U.K.). DAPI

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† Abbreviations: PA, polyamine; put, putrescine; DB-propamidine, dibromopropamidine; 2-OH-stilbamidine, 2-hydroxystilbamidine; DAPI, 4',6-diamidino-2-phenylindole; PSG, phosphate saline glucose.

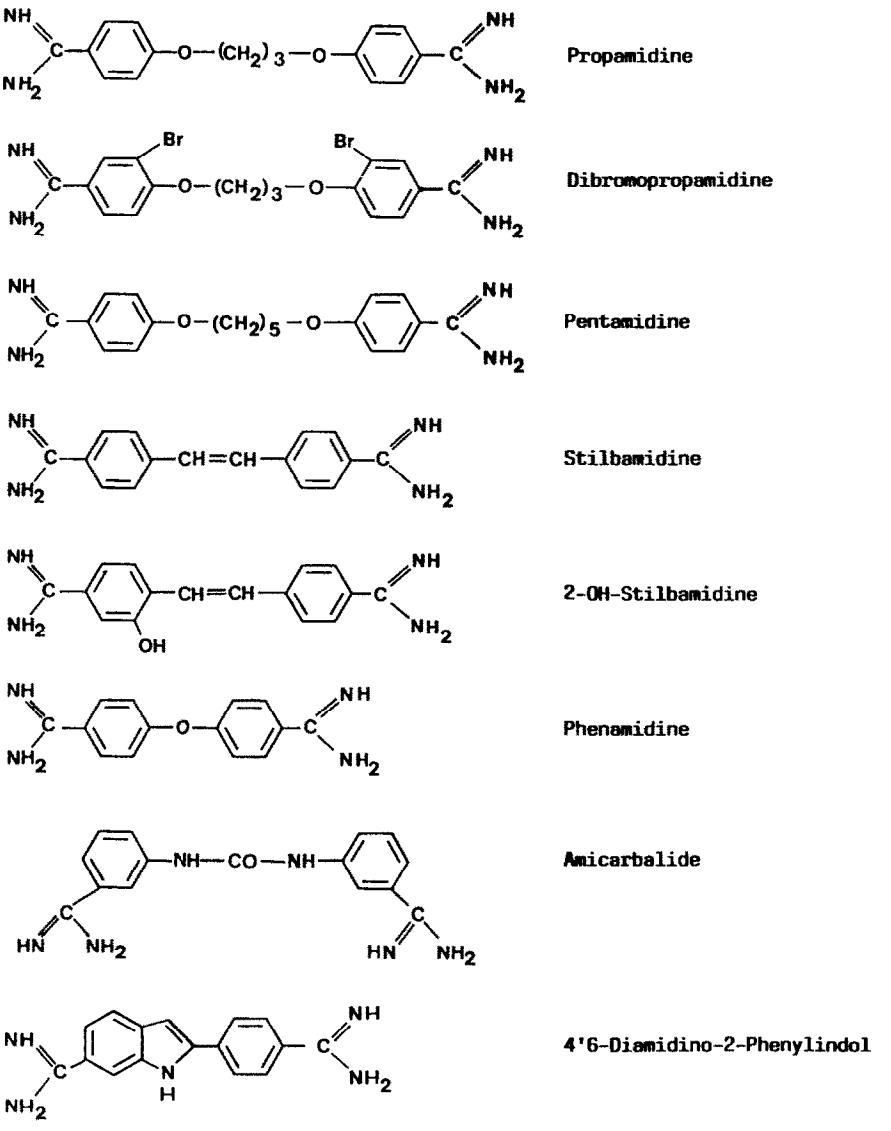


Fig. 1. Structure of the aromatic diamidines used in our study.

was obtained from Sigma and stilbamidine diisethionate was from Marion Merrel Dow Inc. (Cincinnati, OH, U.S.A.) (Fig. 1). All other reagents were of standard laboratory quality.

Organisms and media. Leishmania infantum (cell line PEP1G11) promastigotes, cloned according to the blood agar plating method [17], were originally isolated from the popliteal lymph nodes of a naturally infected dog and supplied by Dr Carrera Ferrer and Dr Alunda, Universidad Complutense de Madrid. Promastigotes were routinely cultured at 26.5° in Medium 199 supplemented with 10% of heat inactivated foetal calf serum and 1.5 μg/mL gentamicin sulphate. Cultures were subpassaged once a week with only a small number of subcultures. Cell growth was monitored by counting in an improved Neubauer chamber.

Put uptake. Promastigotes cultured as above were harvested in mid-logarithmic phase (48 hr subpassage) by centrifuging (6000 *g*, 10 min, cold) washing three times in PSG (1% w/v) buffer [18], resuspending in the same buffer at a cellular density of 5×10^6 promastigotes/mL and preincubating for 10 min at 30° with several concentrations of each diamidine and then pulsed with different concentrations of putrescine (from 2.8 to 32 μM) that included 0.16 μCi of 1,4-[^{14}C]put (90.40 mCi/mmol) for 20 min at the same temperature. The incubation samples were stopped on ice, then vacuum filtered through Millipore nitrocellulose filters (0.45 μm, 25 mm diameter), previously soaked in 0.1 M unlabelled put to avoid unspecific adsorption [11]. Filters were washed with 10 mL of PSG containing 2 mM put, air dried and counted in a

liquid scintillation spectrometer using NEF 989 scintillation cocktail. Uptake rate was expressed as pmol of put internalized per min and per 10^6 promastigotes. The unspecific binding to filters and the passive diffusion component were subtracted from total uptake rates using blanks incubated at 0°.

RESULTS

The clone IPG11 of *Leishmania infantum* promastigotes had a wide spectrum response to the selected aromatic diamidines (structures shown in Fig. 1) under the standard growth culture conditions. Table 1 displays the different antiproliferative sensitivities of *L. infantum* promastigotes expressed as EC_{50}, calculated by using dose–response curves determined on the 4th day subpassage. These values indicate the lack of effect of propamidine, phenamidine and amicarbalide with the highest concentration (1 mM) tested on the cultures. However, stilbamidine and its counterpart 2-OH-stilbamidine, the brominated analogue of propamidine (DB-propamidine), and mostly, pentamidine and DAPI displayed a stronger antiproliferative ability with EC_{50} values of 80 and 45 μM, respectively. When promastigotes were grown in the presence of appropriate amounts of each active diamidine able to reduce the cell growth by more than 90% plus 0.5 mM putrescine and/or spermidine, no growth rate difference was found from PA untreated cultures (data not shown), indicating either an unrelated PA mode of action or an inhibitory effect on PA uptake. In order to establish a possible relation of the effects of aromatic diiamidines with the inhibition of putrescine uptake, three concentrations: 10 μM, 100 μM and 1 mM of each diamidine were mixed with 5×10^6 cells/mL on day 2 subpassage in the appropriate assay buffer (Table 2) and the internalization rates were determined. A powerful dose-dependent inhibition pattern was evident using all the diamidines displayed in Fig. 1, except for amicarbalide, which at the highest concentration assayed (1 mM) did not reduce put uptake, this concentration being the limit of its solubility in the assay buffer. To determine the kinetic inhibitory constants of selected diamidines on put uptake a kinetic approach using Lineweaver–Burk plots was used. Figure 2A–F shows the double reciprocal plots of initial uptake rates vs putrescine concentrations (from 2.8 to 32 μM), calculated by subtracting the actual uptake values at 30° from the transport at 0°, at three concentrations of each inhibitor, estimated from the data displayed in Table 2. The inserts in Table 2 show the replots of slopes vs inhibitor concentrations made in order to calculate the K_i values.

In all cases studied a non-competitive inhibition pattern was achieved. From the data in Fig. 2A, the K_i value for propamidine (estimated as 25 μM) was calculated. The K_i for DB-propamidine was calculated as 7 μM (Fig. 2B). Figure 2C and D shows the K_i value for stilbamidine and 2-OH-stilbamidine calculated as 20 and 21 μM, respectively. The K_i for pentamidine was estimated to be 3 μM (Fig. 2E) and phenamidine was 95 μM (Fig. 2F). Finally, the K_i obtained for DAPI was 15 μM (Fig. 2G). Due to the absence of inhibitory capacity of amicarbalide at 1 mM (Table 2) as well as to the low solubility of this compound in the assay buffer at concentrations near 1 mM no kinetic determination was performed.

Table 1. Antiproliferative growth effect of aromatic diamidines on *Leishmania infantum* promastigotes

Compound	EC_{50} (μM)
Propamidine	NI
DB-propamidine	165
Pentamidine	80
Stilbamidine	600
2-OH-stilbamidine	250
Phenamidine	NI
Amicarbalide	NI
DAPI	45

NI, no inhibition at 1 mM.

Cells were incubated in the absence and presence of different concentrations of each diamidine on day 0, and EC_{50} value was determined by means of dose–response curves on the 4th day of subpassage. Results are the average of two separate experiments.

Table 2. Inhibition of put uptake in *Leishmania infantum* promastigotes by selected aromatic diamidines

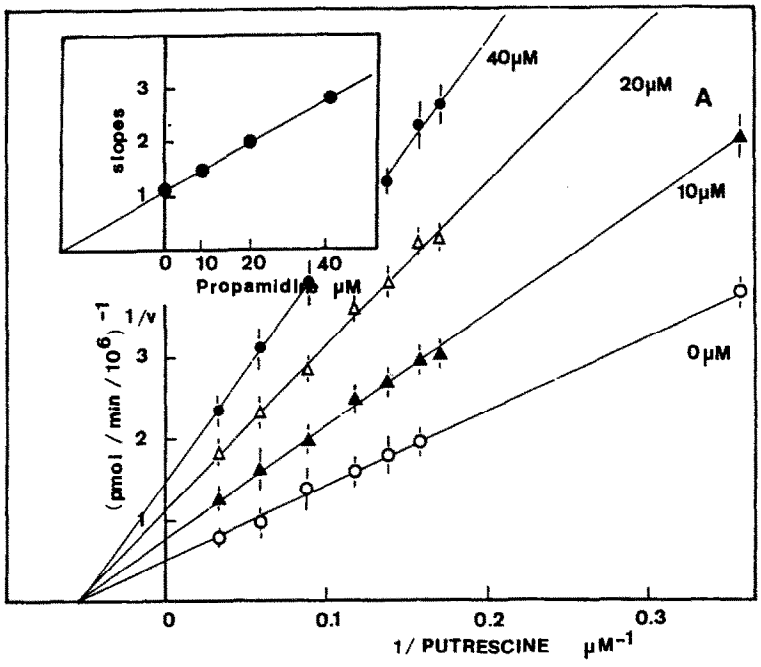
Compound	Inhibitor concentration 10 μM	100 μM	1 mM
Propamidine	101	16	1.1
DB-propamidine	50.7	31.8	0.2
Pentamidine	28.3	5.47	6.3
Stilbamidine	93.1	19.5	2.3
2-OH-stilbamidine	131	23.8	0.8
Phenamidine	131	76.6	10.4
Amicarbalide	140	114	96.2
DAPI	101.8	34.8	5.06

Cells were grown until 48 hr subpassage, harvested and washed as described in Material and Methods, and finally pulsed with the appropriate diamidine. Results are expressed as the percentage (%) of maximum transport in the absence of any inhibitor (100% of put transport was estimated in 1.09 pmol/min/10^6 promastigotes). Results are the average of two separate experiments.

DISCUSSION

Interest of the scientific community in aromatic diamidines or analogues recently synthetized to minimize toxic side-effects derives from the use of aerosolized pentamidine for the treatment of *Pneumocystis carinii* pneumonia in acquired immunodeficiency syndrome patients [19, 20]. Knowledge of the mode of action of molecules resembling pentamidine can contribute to their rational utilization in those pathologies in order to minimize their therapeutic risk.

The mode of action of aromatic diamidines has not been clarified. These structures appear to be multitarget drugs in both host and parasitic cells.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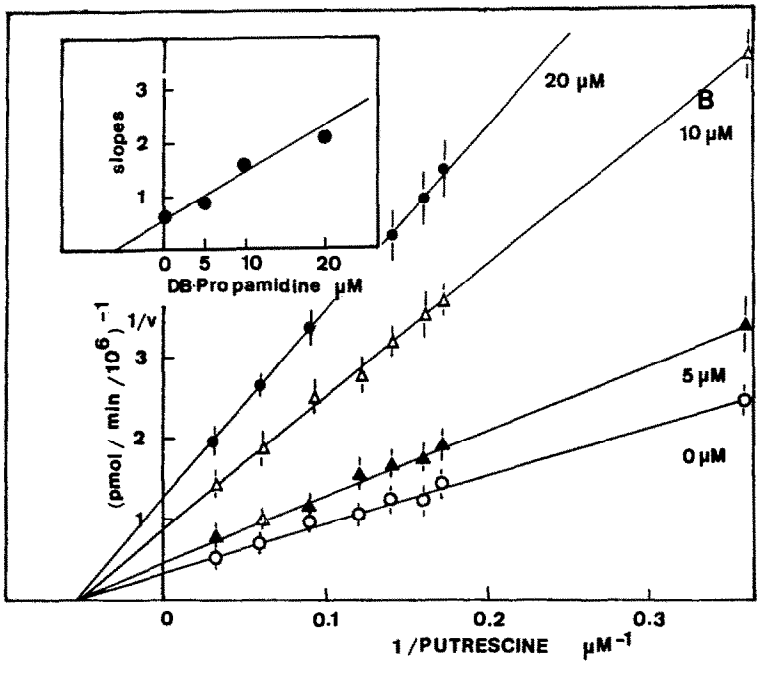
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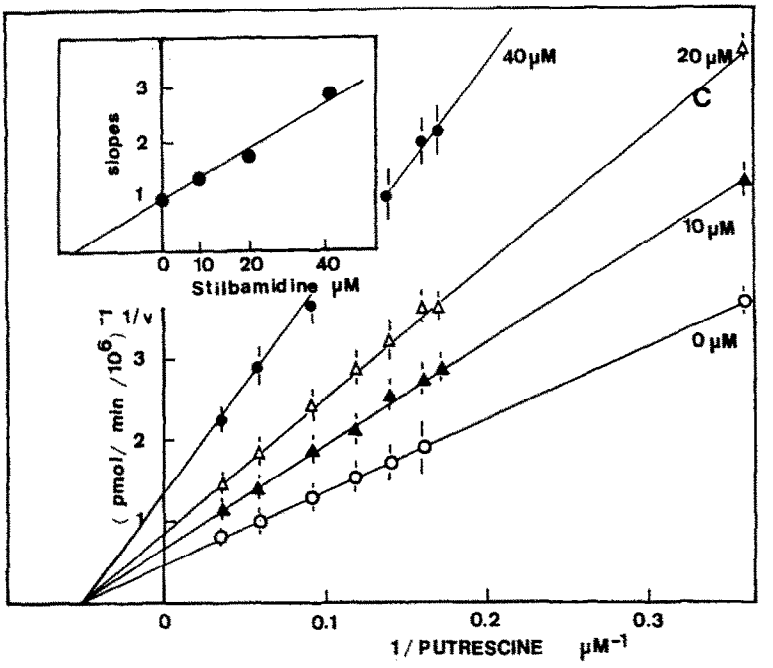
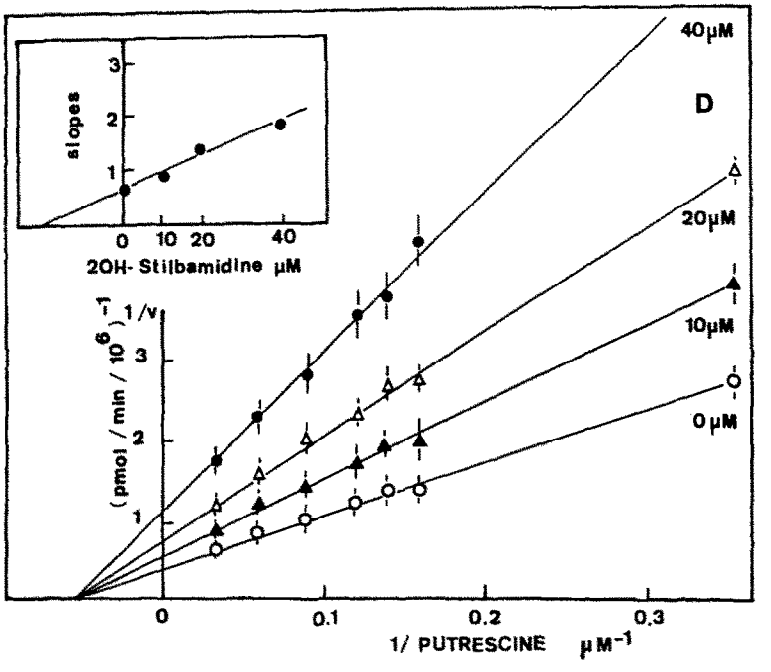
Fig. 2.





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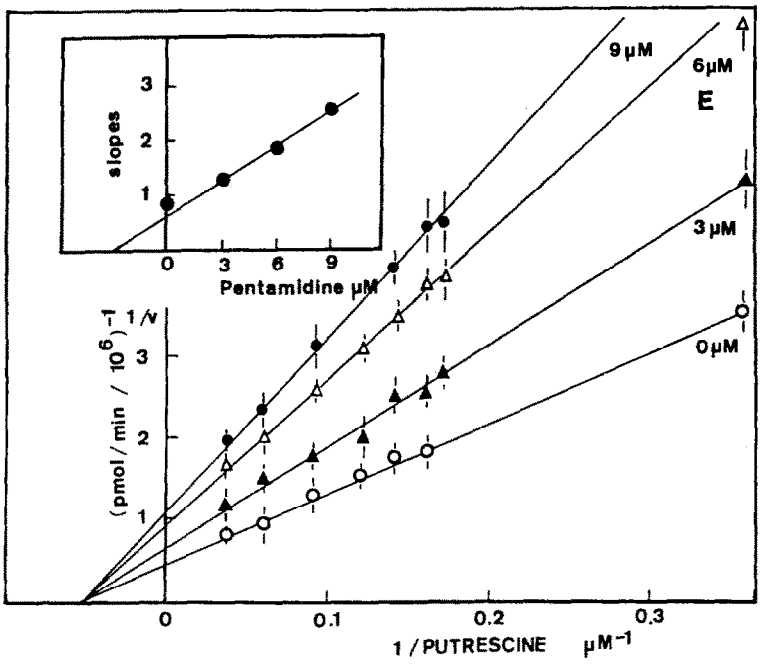

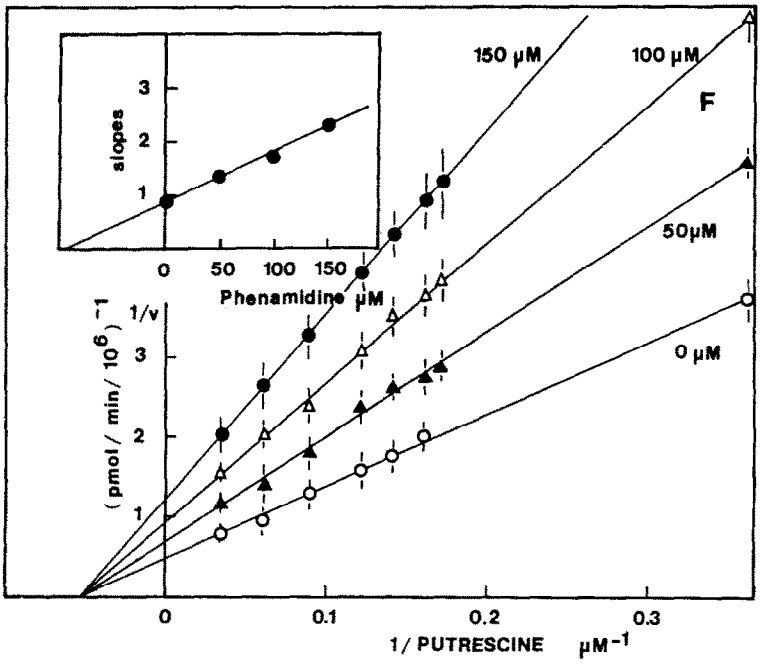

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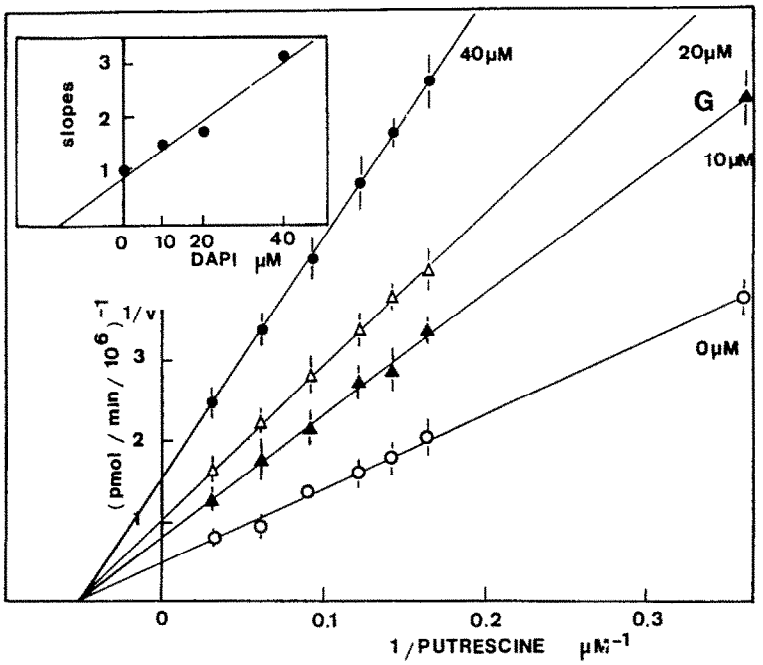

Fig. 2. Lineweaver–Burk plots of the inhibitory effect of different concentrations of aromatic diamidines on put transport of *Leishmania infantum* promastigotes. K_m value for put transport was estimated to be 17 mM. (A) Propamidine; (B) DB-propamidine; (C) stilbamidine; (D) 2-OH-stilbamidine; (E) pentamidine; (F) phenamidine and (G) DAPI. Inserts represent the slopes vs inhibitor concentration replots, carried out in order to determine the K_i value [27]. Each point is the average of three determinations from at least two different experiments.

These compounds have been described as planar intercalatory molecules to poly(dA)-poly(dT) rich regions of DNA [21]. Moreover, aromatic diamidines have shown a powerful inhibitory effect on PA metabolism, inhibiting the SAMDC biosynthesis competitively [13, 14], the enzyme in charge of spermidine and spermine biosynthesis or inhibiting diamine oxidase, the enzyme of terminal PA metabolic [15, 22] pathways.

Previous reports on Berenil utilization in veterinary medicine, have shown the antiproliferative effect of this compound could be due not only to PA metabolism depletion but also to inhibition of PA uptake since the addition of this diamine with spermidine was not able to reverse the inhibition of growth [23]. However, the results presented here reveal only a slight relationship between the antiproliferative rate of diamidines and put uptake inhibition that can be considered a side-effect of these compounds rather than a main target of action.

The structure–activity relationships between this family of compounds and the inhibition of *L. infantum* growth and/or putrescine influx. In agreement with these results a classification of these compounds in three different groups can be made. A first group including amicarbalide and phenamidine shows no antiproliferative effect, and lacks (amicarbalide) or shows only a little affinity for put uptake (phenamidine). Another group, made up of stilbamidine, 2-OH-stilbamidine and propamidine with a relatively high EC_{50} (from no effect at 1 mM for propamidine to 250 μM for 2-OH-stilbamidine) shows a medium affinity for the put uptake system (K_i *ca.* 20 μM). Finally, a third group of compounds with a high antiproliferative ability (EC_{50} ranging from 45 to 165 μM), and high affinity for the put influx mechanism, is made up of pentamidine, DAPI and DB-propamidine.

Previous studies have shown some structural requirements for put uptake inhibition for diamines, mainly related to a more favourable distance between the amine moieties, in eukaryotic cultures and mammalian tissues [24]. Consequently several related diamines, such as aromatic diamidines, may have different affinities for these receptors, thereby blocking put internalization to differing degrees. However this system could be used by these substrates as a specific transport system with different affinities for each structure, like the pentamidine transport system in *Trypanosoma brucei* [25], which is energy-dependent and competitively inhibited by stilbamidine. Nevertheless the non-competitive pattern found in all the diamidines used in the study could be better explained from their affinities for the receptor molecule, as well as by interference with some ATP synthetic metabolic reaction not yet reported.

Although the antiproliferative rate seems to be poorly related to the affinity for the putrescine carriers, some conclusions could be derived from the present results. (1) The diamidine substituting groups in the aromatic rings must be in *para* position to attain some inhibitory capacity on put uptake. (2) The bulk bromine *ortho* substituting group in the

propamidine molecule improved both affinity for the carrier and the antiproliferative rate, as has been obtained against *Giardia lamblia* cultures [20]. (3) Similar considerations could be made for the hydroxyl derivative of stilbamidine, which has a lower EC_{50} value, while maintaining a close affinity for the put carrier.

In view of the poor correlation between the inhibition of exogenous put uptake and the antileishmanial activity of these compounds, their effect on put uptake should be considered as a side-effect of these compounds, pointing to alternative mechanisms, like SAMDC inhibition or, as has been described in trypanosomes, inhibition of type II topoisomerases [26].

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