Chapter 3

Symmetrical Pyridinium-Phanes and –Diazacyclophanes — Promising Heterocyclic Scaffolds for the Development of Anti-Leishmanial Agents

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Additional information is available at the end of the chapter

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Abstract

There is an urgent need for better drugs for a more successful fight against leishmaniasis, one of the most important neglected diseases caused by the parasite *Leishmania*. We have recently synthesized several symmetrical pyridinium compounds belonging to two different series: bis-pyridinium and bis-quinolinium acyclic structures and bis-pyridinium diazacyclophanes derivatives. The first series of bis-pyridinium derivatives have been found to display activity against promastigotes and intracellular amastigotes of *Leishmania donovani* and *Leishmania major*, with EC_{50} values lower than 1 μM. The majority of compounds show a similar behavior in both *Leishmania* species, being slightly more active against intracellular amastigotes of L. *major*. The series of bis-pyridinium diazacyclophanes can be considered as rigid analogues of the previous bis-cationic ones. The activity of these compounds has also been evaluated against promastigotes and intracellular amastigotes of *L. donovani* and *L. major*. All the diazacyclophanes are more active against *L. major*, with EC_{50} values of between 1 and 17 μM in intracellular amastigotes, and in some cases they present a higher selectivity index than the reference anti-leishmanial drugs such as amphotericin B and miltefosine. In conclusion, these bis-quaternary compounds represent promising candidates as potential therapeutic agents against leishmaniasis.

Keywords: Pyridinium phanes, diazacyclophanes, leishmaniasis, *Leishmania* chemotherapy

1. Introduction

Leishmaniasis is a major group of neglected tropical diseases caused by the protozoan parasite *Leishmania*. Currently it affects 12 million people in 98 countries, and around 350 million people

worldwide are at risk of infection [1]. Leishmaniasis is responsible for a variety of pathologies that have been classified in three main clinical manifestations including cutaneous (CL), mucocutaneous (MCL), and visceral (VL) leishmaniasis, ranging from self-healing cutaneous lesions to fatal visceral infection [2].

All *Leishmania* species are digenetic parasites that exist as both insect vector (promastigotes) and mammalian forms (intracellular amastigotes). The digenetic life cycle of *Leishmania* consists of flagellated, motile, extracellular promastigote form that proliferates in the midgut of phlebotomine sand fly family vectors, which infect mammalian host and transform into the non-motile, intracellular amastigote form that resides in phagolysosomes of macrophages and other reticuloendothelial cells.

Since an effective vaccine against leishmaniasis is not available, chemotherapy is at present the only effective way to treat all forms of the disease. The recommended first-line therapies for leishmaniasis include pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate, amphotericin B (AmB), paromomycin, and miltefosine (Figure 1), all of which have different types of limitations including toxicity, price, efficacy, and emerging resistance [3], which emphasizes the importance of developing new drugs against leishmaniasis. Pentamidine [1,5-bis(4-amidinophenoxy)pentane] is an aromatic diamidine (Figure 1) widely used for the treatment of sleeping sickness caused by *Trypanosoma brucei* [4]. It was used as a second-line drug against VL in cases of antimony failure, but its use against leishmaniasis is now limited to the treatment of some forms of CL in South America [5]. Pentamidine acts at the mitochondrial level of the parasite by accumulating within the mitochondria and binding to DNA, thus interfering with the replication and transcription [6]. Novel diamidine derivatives with improved pharmacokinetic properties have been under development in recent years [7, 8].

New diamidine and choline-derivative dications have been developed recently in order to find new drugs with improved activity against leishmaniasis and lower toxicity [9–12] (Figure 2).

Chemistry is a science on which all the other sciences are based. An understanding of biology requires knowledge of chemistry. The majority of the leishmaniasis reviews are concentrating on the biology of the processes and very little on the chemistry. We would like to fill this gap and we will focus on the chemical structures that could be useful to the medicinal chemists working in this important area of research.

Here we present the anti-leishmanial activity of a set of symmetrical bis-pyridinium compounds with cyclic or acyclic structures. Both types of compounds can be named according to the IUPAC nomenclature for phanes, a method based on assembling names that describe component parts of a complex structure.

2. Symmetrical bis-pyridinium compounds

We have previously designed and synthesized a set of symmetrical bis-pyridinium compounds, which consist of a linker and two cationic heads which are 4-substituted pyridinium

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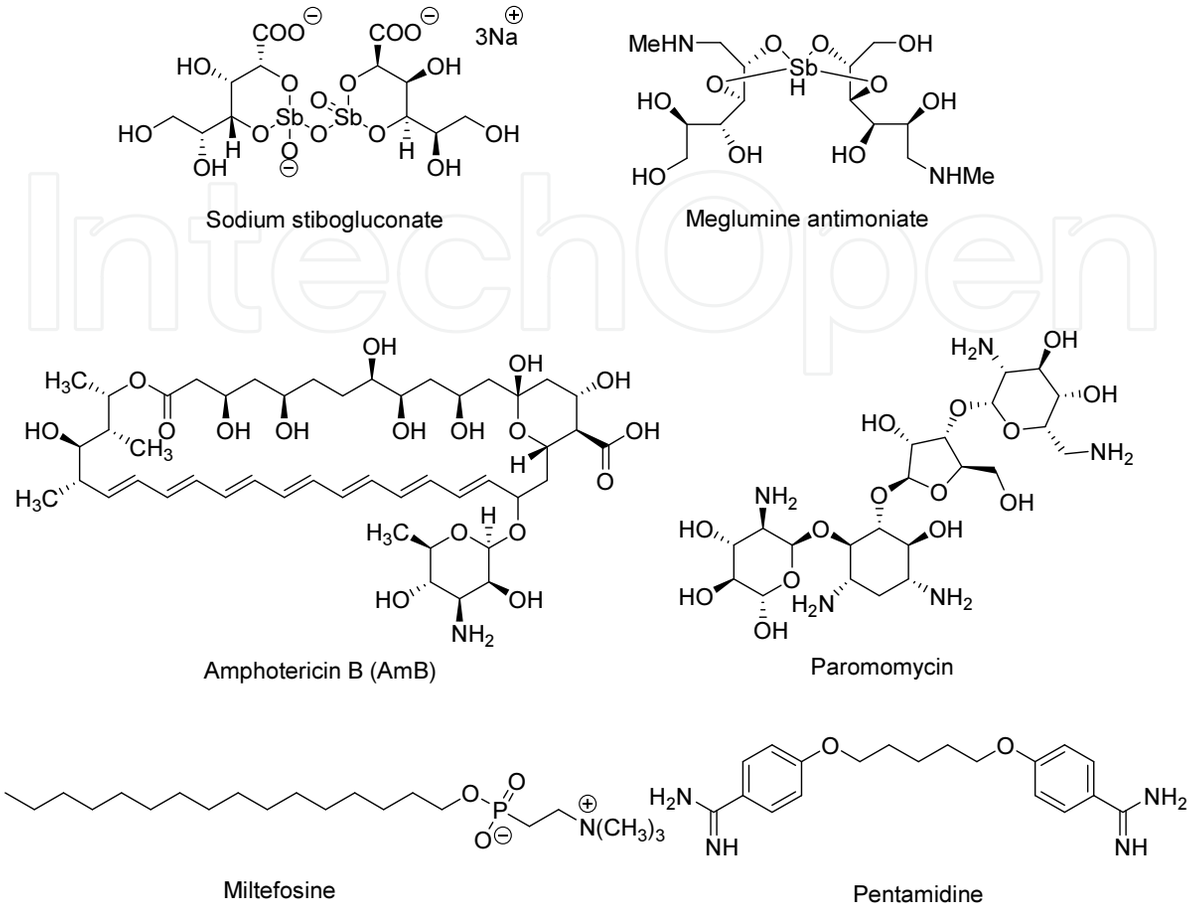

Figure 1. Anti-leishmanial drugs.

or quinolinium rings with cyclic or acyclic amino groups, as inhibitors of the human choline kinase (ChoK) (Table 1), the first enzyme in the CDP–choline pathway that synthesizes phosphatidylcholine, the major phospholipid in eukaryotic cell membranes. In humans, choline kinase exists as three isoforms (ChoKα1, α2, and β). Specific inhibition of ChoKα has been reported to selectively kill the tumor cells. Ten symmetrical bis-pyridinium and bis-quinolinium derivatives were tested for their ability to inhibit human ChoKα2, and **VGP-118** and **VGP-150** were identified as highly potent choline kinase inhibitors with EC_{50} values of 80 nM. Kinetic enzymatic assays indicated a mixed, predominantly competitive, inhibition mechanism for these compounds. These novel compounds showed strong anti-proliferative activity (EC_{50} of 1 μM) on the human breast cancer SKBR3 cell line [13].

In addition, these compounds can be considered as structural analogues of pentamidine in which the amidino moiety, which is protonated at physiological pH, has been replaced by a positively charged nitrogen atom as a pyridinium ring. In view of this structural resemblance and with the intention of identifying potential drugs against leishmaniasis, we analyzed the anti-leishmanial activity of these bis-pyridinium derivatives.

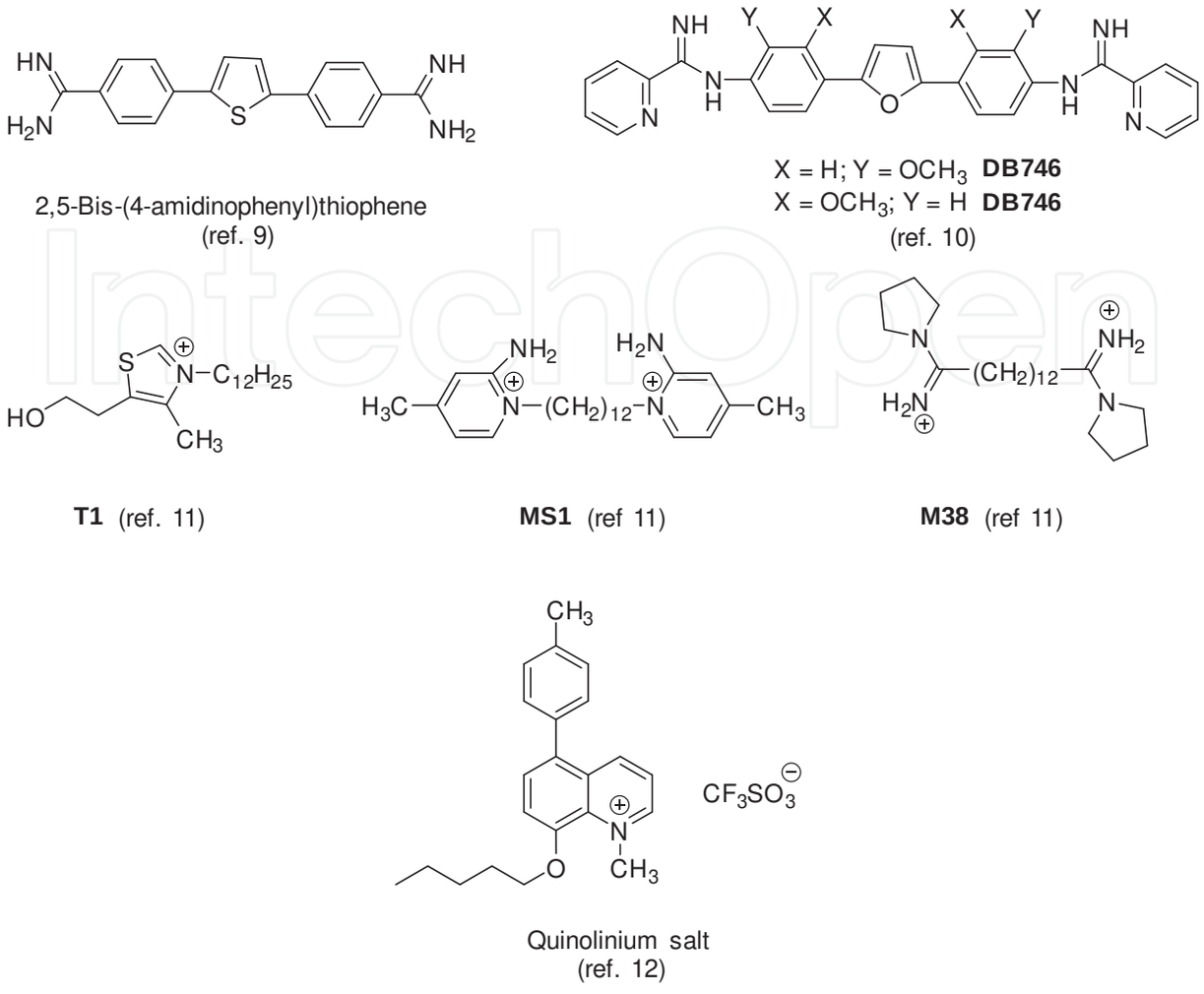

Figure 2. Potent diamidine and charged derivatives with improved activity against leismaniasis.

2.1. Susceptibility analysis in *Leishmania* lines

The anti-leishmanial activity of the ten choline kinase inhibitors was evaluated against promastigotes and intracellular amastigotes of *Leishmania donovani* and *Leishmania major* in order to identify the potential hits for further optimization. The cytotoxic effect of these compounds was also investigated on the human monocytic cell line THP-1, the host cell used in the assay with intracellular amastigotes. Selectivity indexes (SI) were calculated as the ratio of the EC_{50} (the concentration of compound required to inhibit growth by 50%) for THP-1 to the EC_{50} for intracellular amastigotes. Table 2 shows the results, where miltefosine and AmB were used as the reference anti-leishmanial drugs. Most assayed compounds exhibit a specific high activity against promastigotes and intracellular amastigotes of *L. major*, with EC_{50} values between 0.09 and 0.42 μM in amastigotes, except for compounds **VGP-106** and **VGP-118** (EC_{50} 13.07 and 6.21 μM, respectively). With regard to *L. donovani*, all assayed compounds display EC_{50} values below 1 μM in promastigotes, except compound **VGP-138** (EC_{50} 2.11 μM). Although these values are slightly higher in intracellular amastigotes, they are similar to those for the anti-leishmanial drug miltefosine [14].

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Our analysis of the effect on THP-1 cells showed that bis-pyridinium derivatives (**VGP-106**, **VGP-114**, **VGP-118**, **VGP-130**, **VGP-138**) are less cytotoxic than the bis-quinolinium counterparts (**VGP-146**, **VGP-150**, **VGP-162**, **VGP-174**, **VGP-182**), with a higher SI than miltefosine (Table 2).

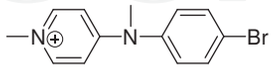
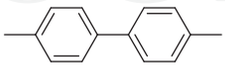
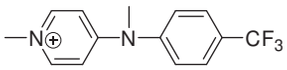
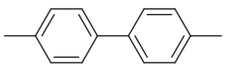
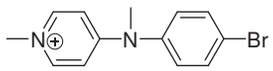
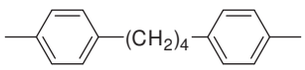
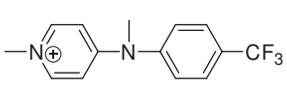
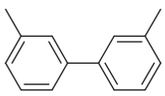
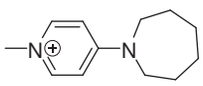
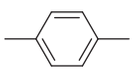
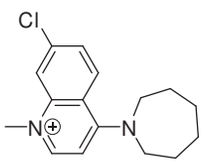
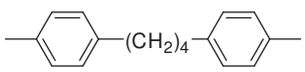
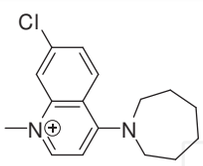
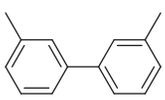
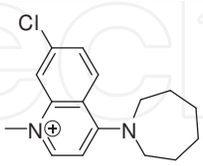
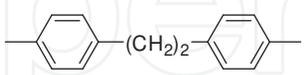
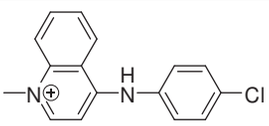
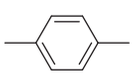
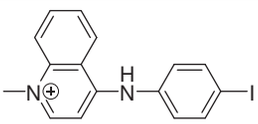
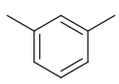
	Het–Linker–Het	
Compound	**Het**	**Linker**
VGP-106		
VGP-114		
VGP-118		
VGP-130		
VGP-138		
VGP-146		
VGP-150		
VGP-162		
VGP-174		
VGP-182		

Table 1. Structure of the bis-cationic compounds

	EC_{50} promastigotes (μM)		EC_{50} amastigotes (μM) [SI][b]		THP-1 toxicity EC_{50} (μM)
Compound	***L. major***	***L. donovani***	***L. major***	***L. major***	
VGP-106	21.55 ± 3.72	0.36 ± 0.09	13.07 ± 6.30 [15.8]	0.86 ± 0.46 [240.2]	206.54 ± 9.89
VGP-114	0.47 ± 0.04	0.61 ± 0.09	0.10 ± 0.03 [1000.6]	0.85 ± 0.04 [117.7]	100.06 ± 8.57
VGP-118	29.15 ± 5.73	0.65 ± 0.19	6.21 ± 1.02 [2.4]	0.18 ± 0.03 [85.3]	15.35 ± 3.99
VGP-130	0.50 ± 0.07	0.73 ± 0.11	0.09 ± 0.02 [903.7]	2.02 ± 0.05 [40.3]	81.34 ± 10.65
VGP-138	0.74 ± 0.19	2.11 ± 0.48	0.30 ± 0.16 [586.8]	4.01 ± 0.43 [43.9]	176.05 ± 20.75
VGP-146	0.21 ± 0.06	0.33 ± 0.07	0.10 ± 0.04 [156.1]	0.42 ± 0.01 [37.2]	15.61 ± 3.26
VGP-150	0.36 ± 0.11	0.77 ± 0.04	0.09 ± 0.03 [267]	0.55 ± 0.16 [43.7]	24.03 ± 5.42
VGP-162	0.40 ± 0.08	0.35 ± 0.02	0.37 ± 0.03 [29.6]	1.00 ± 0.08 [11.0]	10.97 ± 2.41
VGP-174	1.70 ± 0.01	0.34 ± 0.03	0.41 ± 0.05 [6.1]	0.86 ± 0.03 [2.8]	2.47 ± 0.05
VGP-182	2.51 ± 0.01	0.92 ± 0.2	0.42 ± 0.12 [11.2]	0.52 ± 0.12 [9.1]	4.71 ± 0.23
AmB	0.32 ± 0.02	0.21 ± 0.01	0.24 ± 0.01 [59.7]	0.28 ± 0.13 [51.1]	14.32 ± 4.10
Miltefosine	16.65 ± 1.23	6.60 ± 1.57	10.61 ± 0.89 [2.5]	0.88 ± 0.14 [30.5]	26.86 ± 3.08

[a]Parasites were grown for 72 h at 28 °C (promastigotes) or 37 ºC (intracellular amastigotes) in the presence of increasing concentrations of compounds. THP-1 cells were grown for 72 h at 37 ºC, in the presence of increasing concentrations of compounds. Promastigotes and THP-1 viability was determined using an MTT-based assay. Number of intracellular amastigotes was determined by nuclear staining. AmB and miltefosine were used as standard anti-leishmanial agents. Data are means ± SD of three independent determinations.

[b]Selectivity indexes [SI] were calculated by dividing the EC_{50} THP-1 by that for intracellular amastigotes. Compound **VGP-106** (grey color) was selected for further studies of the mechanism of action.

Table 2. Anti-leishmanial activity and toxicity in THP-1 cells of symmetrical bis-pyridinium compounds.[a]

Compound **VGP-106** was identified as a representative compound that displayed a potent activity against *L. donovani* intracellular amastigotes. As the least cytotoxic of the set of compounds assayed for THP-1 cells, it was selected to further elucidate their mechanism of action in this protozoan parasite [14].

2.2. Drug susceptibility assay of *L. donovani* lines overexpressing CEK or EK

Considering that the *Leishmania* genome includes two homologous enzymes of human ChoK, namely, choline/ethanolamine kinase (CEK) and ethanolamine kinase (EK), we decided to study whether there is a correlation between their ChoK inhibitory activity and anti-leishmanial activity. These proteins can be overexpressed in *L. Donovani* promastigotes by transfecting the parasites with a plasmid encoding the *Leishmania* CEK (pXG-CEK) or EK (pXG-EK) genes [14]. The susceptibility of transfected parasites to compound **VGP-106** was determined in both promastigotes and intracellular amastigotes. As can be seen from Table 3, there are no significant differences between the EC_{50} values of parasites overexpressing CEK or EK enzymes compared to control parasites. These results suggest that the mechanism of action of this compound in *Leishmania* is independent of the aforementioned enzymes [14]. If this were

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not the case, overexpression of these enzymes would have resulted in an increase in the EC_{50} value.

	EC_{50} (μM)	
Plasmid	**Promastigotes**	**Amastigotes**
pXG	0.36 ± 0.09	0.45 ± 0.03
pXG-CEK	0.36 ± 0.09	0.42 ± 0.05
pXG-EK	0.43 ± 0.05	0.35 ± 0.03

[a]Control (pXG) and transfected (pXG-CEK and pXG-EK) parasites were grown for 72 h at 28 °C (promastigotes) or 37 ºC (intracellular amastigotes) in the presence of increasing concentrations of compounds. Data are means ± SD of three independent determinations.

Table 3. Susceptibility to VGP-106 of *L. donovani* lines overexpressing CEK or EK.[a]

3. Symmetrical bis-pyridinium diazacyclophanes

Rigidification is a commonly used strategy to increase the activity of a drug or to reduce its side effects. A cyclophane is a hydrocarbon consisting of an aromatic unit (typically a benzene ring) and an aliphatic chain that forms a bridge between two non-adjacent positions of the aromatic ring.

We have synthesized a new family of symmetrical bis-pyridinium diazacyclophanes designed as cyclic analogues of previously reported acyclic bis-pyridinium derivatives, by cyclization through the exocyclic nitrogen atoms at position 4 of the pyridinium moiety via linker 2, which leads to the diazacyclophane targets (Figure 3) [15]. These compounds have been evaluated against *L. major* and *L. donavani*.

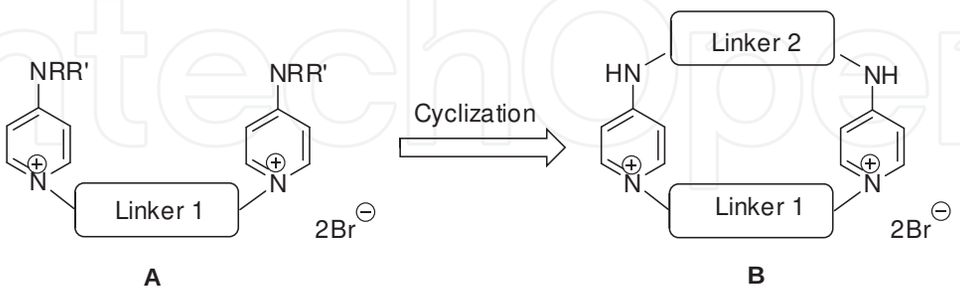
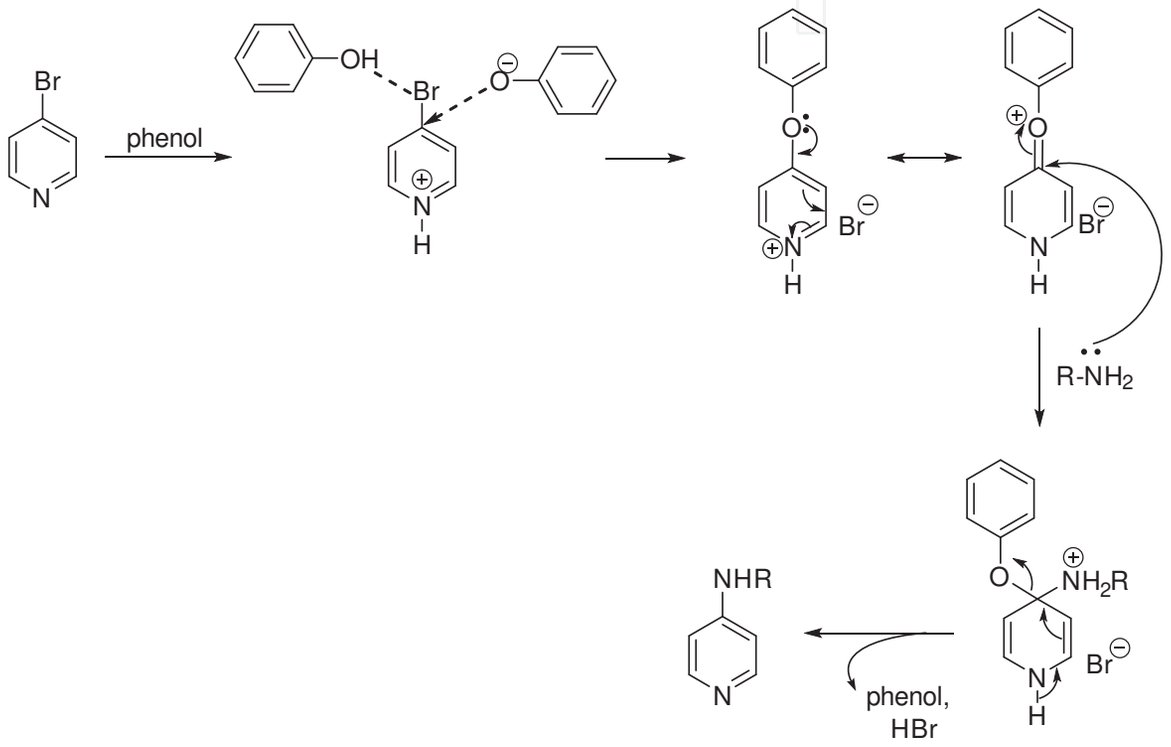


Figure 3. Structural variation that leads to symmetrical bis-pyridinium diazacyclophanes (**B**) from symmetrical acyclic bis-pyridinium derivatives (**A**).

This new compounds are symmetrical bis-pyridinium derivatives which differ from each other in the upper and lower spacers. Four different spacers were used: two are phenyl-*p*-diyl-methylene and phenyl-*m*-diylmethylene linkers, and the other two are aliphatic, such as the

1,5-pentanediyl and 3-oxa-1,5-pentanediyl moieties. At least one of the two spacers in every cyclophane is an aliphatic linker (Table 4).

The final compounds were synthesized according to Scheme 1. Dipyridines **1** and **2** were prepared from commercially available diamines and 4-bromopyridine in the presence of phenol under argon atmosphere, as previously described [11]. A reaction involving phenol as proton donor, solvating agent, and source of phenoxide ion is envisaged, as outlined in Scheme 1 [16]. As a reaction medium, phenol reduces both the reaction time and temperature of the halogen-replacement reactions.



Sheme 1. As a reaction medium, phenol reduces reaction time and temperature of halogen-replacement reactions, by acting as proton donor, solvating agent, and source of phenoxide ion.

The novel dipyridines (**3** and **4**) were prepared from commercially available pentane-1,5-diamine and bis-2-(aminoethyl)ether, and following the same synthetic protocol previously reported [11].

Cyclophanes were obtained by cyclization of dipyridines **1-4** and the dibromide derivatives in acetonitrile, according to our reported procedures [11]. The reaction was carried out by adding 4 mM solution of the dibromide drop by drop to the dipyridine in acetonitrile at the reflux temperature of the mixture for a period of 10–12 days, which favors the cyclization step and avoids the intermolecular reaction [17]. In order to shorten the reaction time, microwave was used. Thus, dipyridine and dibromide derivatives in acetonitrile were microwave-irradiated at 140 ºC for 20 min. Under these conditions, similar yields were obtained as compared to standard heating at the boiling point of the solvent (acetonitrile). Similarly bis-

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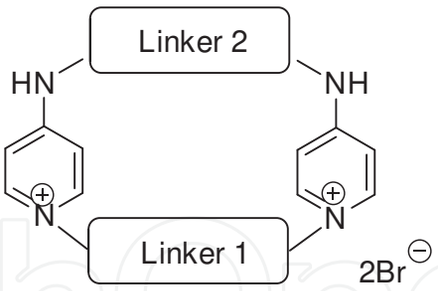


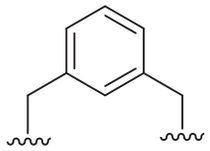
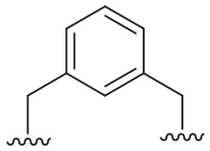
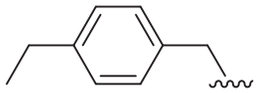
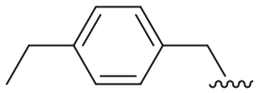
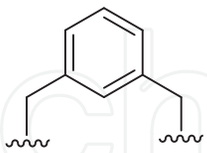
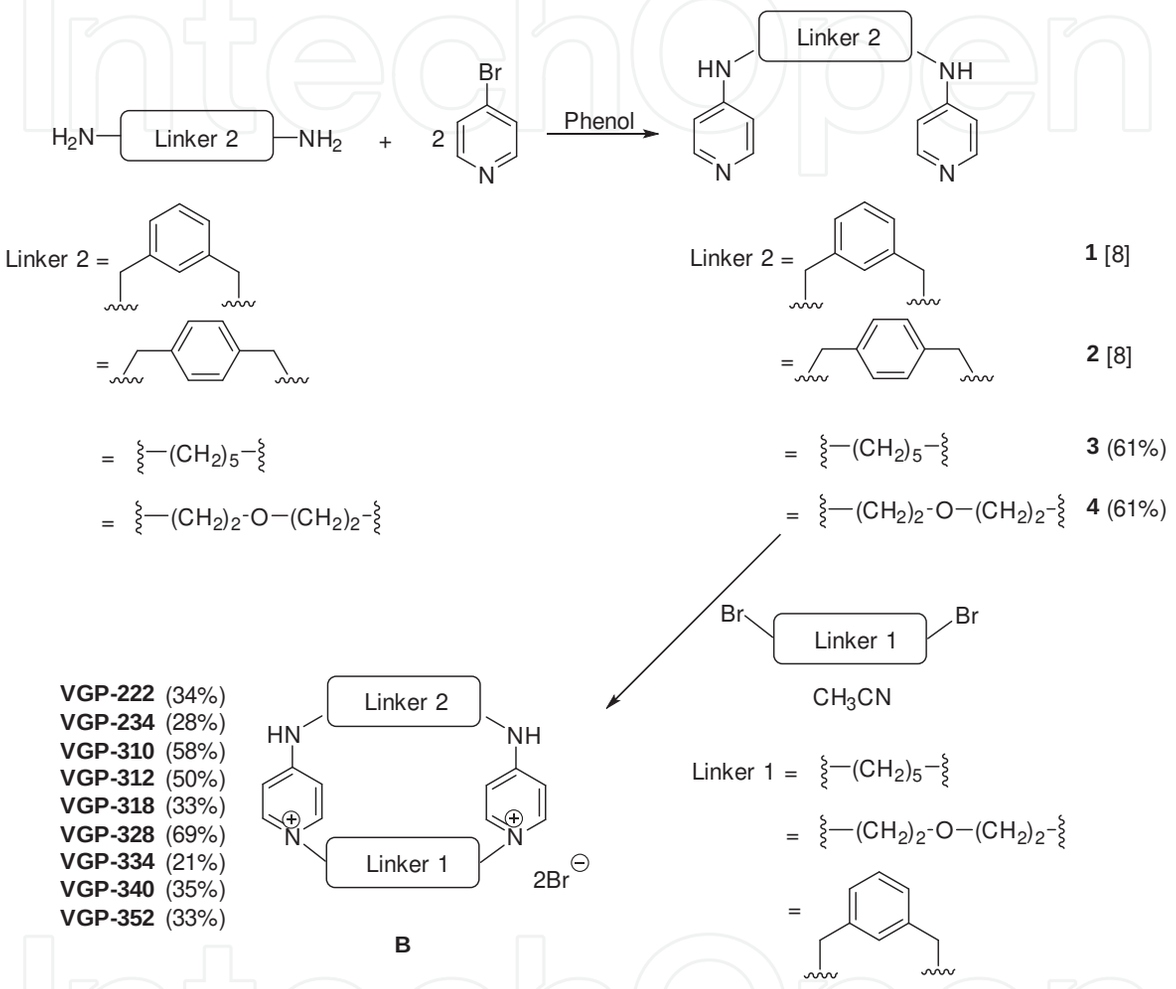
Compound	Linker 1	Linker 2
VGP-222	$-(CH_2)_5-$	
VGP-234	$-(CH_2)_2\text{-}O-(CH_2)_2-$	
VGP-310	$-(CH_2)_5-$	
VGP-312	$-(CH_2)_2\text{-}O-(CH_2)_2-$	
VGP-318	$-(CH_2)_5-$	$-(CH_2)_5-$
VGP-328		$-(CH_2)_2\text{-}O-(CH_2)_2-$
VGP-334	$-(CH_2)_5-$	$-(CH_2)_2\text{-}O-(CH_2)_2-$
VGP-340	$-(CH_2)_2\text{-}O-(CH_2)_2-$	$-(CH_2)_2\text{-}O-(CH_2)_2-$
VGP-352	$-(CH_2)_2\text{-}O-(CH_2)_2-$	$-(CH_2)_5-$

Table 4. Structures of the symmetrical bis-pyridinium diazacyclophanes.

quinolinium cyclophanes [18–20] needed to be purified by tedious reverse-phase preparative HPLC because conventional purification methods failed to give analytically pure samples for biological testing, despite having been obtained under high-dilution conditions (1–2 mM). In our case, this represents a great advantage for the accessibility of such an interesting class of compounds (Scheme 2).



Sheme 2. General synthesis of the symmetrical bis-pyridinium diazacyclophanes.

3.1. Anti-leishmanial activity

The final nine cyclophanes were tested as anti-leishmanial agents against promastigotes and intracellular amastigotes of *L. donovani* and *L. major* [15]. The results are shown in Table 5, where miltefosine and AmB were used as reference drugs.

All assayed compounds exhibit activity against promastigotes and intracellular amastigotes of *L. major* and *L. donovani*, being more active in *L. major*, with EC_{50} values lying in the range 1 and 17 μM in amastigotes. Compounds **VGP-310**, **VGP-318**, **VGP-334**, **VGP-340,** and **VGP-352** display EC_{50} values below 1 μM against promastigotes of *L. major*, an activity 100-fold higher

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than that obtained in promastigotes of *L. donovani*. However, the differences in activity decrease in the amastigote forms, because some of these compounds are less active in amastigotes than in promastigotes of *L. major* and more active in amastigotes than in promastigotes of *L. donovani*.

	EC_{50} promastigotes (μM)		EC_{50} amastigotes (μM) [SI][b]		THP-1 toxicity EC_{50} (μM)
Compound	***L. major***	***L. donovani***	***L. major***	***L. donovani***	
VGP-222	16.84 ± 1.20	51.97 ± 1.97	5.94 ± 0.93 [32.3]	13.53 ± 1.40 [14.2]	191.90 ± 8.12
VGP-234	5.97 ± 0.35	33.77 ± 4.68	8.67 ± 1.04 [22.5]	8.92 ± 1.96 [10.3]	195.17 ± 6.41
VGP-310	0.17 ± 0.01	26.41± 1.28	0.97 ± 0.27 [170.2]	38.33 ± 1.74 [4.3]	165.06 ± 21.29
VGP-312	26.48 ± 2.44	76.87 ± 9.59	17.15 ± 1.50 [12.9]	63.67 ± 5.21 [3.5]	221.89 ± 8.27
VGP-318	0.07 ± 0.01	10.64 ± 1.03	1.26 ± 0.30 [122.3]	7.62 ± 0.16 [20.2]	154.07 ± 5.95
VGP-328	2.87 ± 0.36	76.27 ± 4.96	1.61 ± 0.35 [120.8]	21.25 ± 2.03 [9.2]	194.41 ± 2.95
VGP-334	0.26 ± 0.02	31.47 ± 2.53	2.59 ± 0.23 [62.7]	33.19 ± 0.57 [4.9]	162.44 ± 6.07
VGP-340	0.19 ± 0.01	23.43 ± 0.57	2.24 ± 0.35 [57.2]	20.72 ± 1.07 [6.2]	128.22 ± 9.78
VGP-352	0.26 ± 0.01	31.41 ± 3.02	2.18 ± 0.05 [98.5]	12.95 ± 1.86 [16.6]	214.65 ± 13.80
AmB	0.32 ± 0.02	0.21 ± 0.01	0.24 ± 0.01 [59.7]	0.28 ± 0.13 [51.1]	14.32 ± 4.10
Miltefosine	16.65 ± 1.23	6.60 ± 1.57	10.61 ± 0.89 [2.5]	0.88 ± 0.14 [30.5]	26.86 ± 3.08

[a]Parasites were grown for 72 h at 28 °C (promastigotes) or 37 ºC (intracellular amastigotes) in the presence of increasing concentrations of compounds. THP-1 cells were grown for 72 h at 37 ºC, in the presence of increasing concentrations of compounds. Promastigote and THP-1 viability was determined using an MTT-based assay. Number of intracellular amastigotes was determined by nuclear staining. AmB, and miltefosine were used as standard anti-leishmanial agents. Data are means ± SD of three independent determinations.

[b]Selectivity indexes [SI] were calculated by dividing the EC_{50} THP-1 by that for intracellular amastigotes. Compound **VGP-318** (grey color) was selected for further studies of the mechanism of action.

Table 5. Anti-leishmanial activity and toxicity in THP-1 cells of symmetrical bis-pyridinium diazacyclophanes[a].

In general, from a structural point of view, compounds with two aliphatic linkers show better activity against promastigotes of *L. major* than compounds with an aromatic linker. However, the presence of an aromatic spacer increases the activity in intracellular amastigotes relative to the activity in promastigotes, except for **VGP-310**. This could be due to the higher lipophilicity of these structures that allowed a better penetration into THP-1 cells. Nevertheless, most compounds displayed higher activity in intracellular amastigotes than in promastigotes of *L. donovani.* Regarding the aliphatic linker, the presence of an oxygen atom in the linker did not involve significant differences in the activity. All diazacyclophanes exhibited very low toxicity against THP-1 cells (EC_{50} values between 128 and 220 μM) and some of them evince a higher selectivity index than the reference compounds.

Compound **VGP-318** was chosen as a representative compound to further investigate the mechanism of action of this new family of compounds [15]. This compound shows promising activity against intracellular amastigotes of *L. major* (EC_{50} 1.3 ± 0.3 μM), with a selectivity index (122) higher than those of AmB (51) and miltefosine (30). It is also the most active diazacyclophane derivative against intracellular amastigotes of *L. donovani* (EC_{50} 7.6 ± 0.2 μM).

3.2. Drug susceptibility assay of *L. donovani* lines overexpressing CEK or EK

As we have previously published that other bis-pyridinium diazacyclophanes were ChoK inhibitors and active anti-proliferative drugs [11], we performed a sensitivity test for **VGP-318** in promastigotes and intracellular amastigotes of *L. donovani* overexpressing the *Leishmania* enzymes CEK or EK. The sensitivity for **VGP-318** is similar in both promastigotes and intracellular amastigotes overexpressing CEK and EK versus control parasites (Table 6). This result suggests that the anti-leishmanial activity of these compounds is not related to the CEK and EK enzymes [15].

	EC_{50} (μM)	
Plasmid	**Promastigotes**	**Amastigotes**
pXG	13.50 ± 0.32	8.84 ± 0.18
pXG-CEK	11.51 ± 0.52	12.70 ± 2.03
pXG-EK	12.04 ± 0.42	10.54 ± 1.42

[a]Control (pXG) and transfected (pXG-CEK and pXG-EK) parasites were grown for 72 h at 28 °C (promastigotes) or 37 ºC (intracellular amastigotes) in the presence of increasing concentrations of compound. Data are means ± SD of three independent determinations.

Table 6. Susceptibility to VGP-318 of *L. donovani* lines overexpressing CEK or EK.[a]

3.3. Effect of VGP-318 on *Leishmania* metabolism

In order to investigate the anti-leishmanial mechanism of action of compound **VGP-318**, we focused the studies on the energetic metabolism of *Leishmania* promastigotes [15]. First, the effect of the compound on intracellular ATP levels was analyzed by the bioluminescence assay, which generates a luminescent signal proportional to the amount of ATP. In *L. major*, this assay showed a rapid decrease in the intracellular ATP levels which depends on the compound concentration (Figure 4A). However, no effect was observed on *L. donovani* after incubation with 30 μM for 3 h (Figure 4B). The decrease in the ATP levels may be caused mainly by an effect on the ATP synthesis or a release of the intracellular ATP due to the permeabilization of plasma membrane. However, under conditions that decrease 95% of the ATP relative to control (30 μM for 3 h), no sign of plasma membrane alteration was observed (Figure 5), showing that the drop of free intracellular ATP is not due to disruption of the plasma membrane and suggesting that this may be due to a defect in the ATP synthesis.

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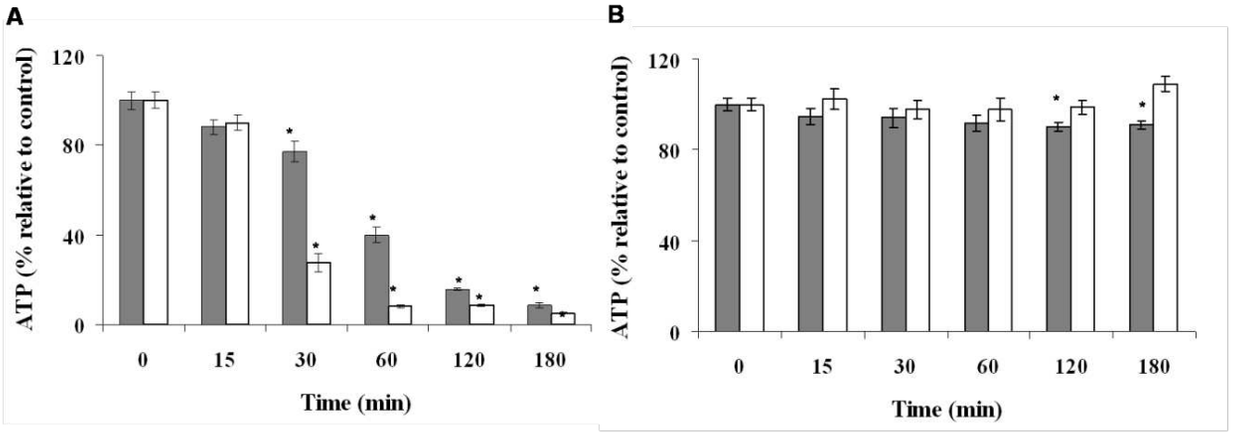

Figure 4. Effect of **VGP-318** on ATP levels in *Leishmania* promastigotes. Changes in intracellular ATP levels in *L. major* (A) or *L. Donovani* (B) promastigotes treated with 0.2 (black bar) or 30 μM (white bar) of compound **VGP-318** were determined using the bioluminescence assay. Data are means ± SD of three independent experiments. Significant differences were determined using Student's *t*-test (*p<0.01).

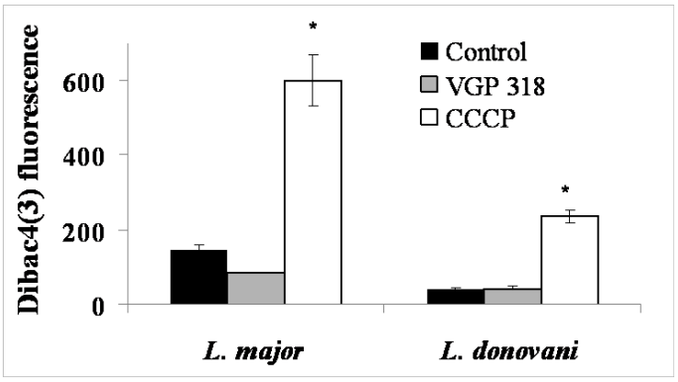

Figure 5. Compound **VGP-318** does not alter the plasma membrane potential in *Leishmania* lines. Promastigotes were incubated with 30 μM of compound **VGP-318** for 3 h and then treated with 2 μM of the specific plasma membrane potential probe DIBAC4(3) for 10 min at 28 ºC. Untreated parasites were used as control, and treatment with 10 μM CCCP was used as 100% depolarization of the plasma membrane potential. Data are means ± SD of three independent experiments. Significant differences were determined using Student's *t*-test (*p< 0.01).

In *Leishmania*, the ATP is mainly synthesized by mitochondrial oxidative phosphorylation [21]. The anti-parasitic activity of many drugs, such as pentamidine and miltefosine, is mediated by an alteration of the mitochondrial membrane potential ($\Delta\Psi_m$) [22, 23]. To determine whether the intracellular ATP decay was associated with an effect of **VGP-318** on the mitochondria, the variation of its electrochemical potential was monitored in parasites incubated with **VGP-318** using the JC-1 fluorescent marker. *L. major* parasites incubated for 1 h with 0.2 μM of **VGP-318** (conditions where there was 50% decay in the ATP levels) showed a significant decrease in JC-1 ratio compared with untreated parasites (Figure 6), evidencing a depolarization of the mitochondrial potential. However, in *L. donovani* promastigotes non-significant depolarization was observed after treatment with 30 μM of **VGP-318** for 3 h (Figure 6). The depolarization of

the $\Delta\Psi_m$ in *L. major* promastigotes suggests that this compound may cause damage in the mitochondria, leading to a fall in the intracellular ATP levels and the death of parasites.

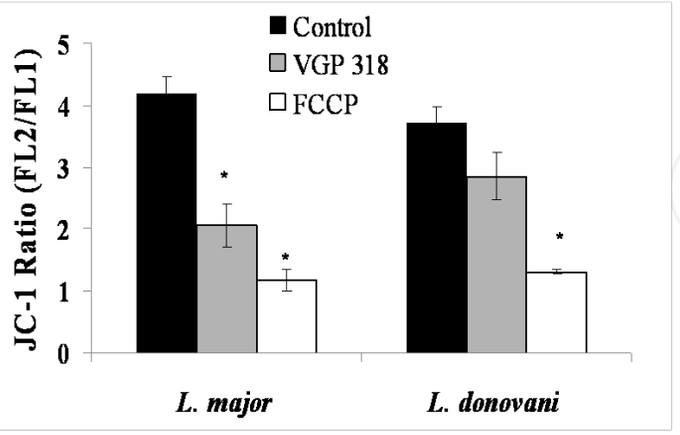

Figure 6. Effect of **VGP-318** on the $\Delta\Psi_m$ of *Leishmania* promastigotes. Promastigotes were treated with 0.2 μM (*L. major*) or 30 μM (*L. donovani*) of compound **VGP-318** for 3 h and then incubated with 5 μM JC-1 for 10 min for the ΔΨm determination. The FL2/FL1 fluorescence ratio was measured by flow cytometry analysis. Untreated parasites were used as control, and treatment with 10 μM FCCP for 10 min was used as full depolarization controls. Data are means ± SD of three independent experiments. Significant differences were determined using Student's *t*-test (**p*<0.01).

The lack of effect of compound **VGP-318** in *L. donovani* may be explained by the lower activity of this compound; **VGP-318** is 100-fold less active in promastigote forms of *L. donovani* than in *L. major*. Additionally, **VGP-318** induces a slight depolarization of the $\Delta\Psi_m$ in *L. Donovani* promastigotes, suggesting that a longer incubation time is necessary to produce significant mitochondrial damage leading to failure of ATP synthesis. Compound **VGP-318** has been highlighted very recently [24].

4. Conclusions

In the search of new drugs against leishmaniasis, we have synthesized and evaluated two set of symmetrical bis-pyridinium derivatives: (i) bis-pyridinium and bis-quinolinium acyclic structures which contain a linker and 4-substituted cyclic or acyclic amino groups in the two cationic heads and (ii) bis-pyridinium diazacyclophanes that are rigid derivatives with an upper spacer which joins the two exocyclic amino groups and a lower spacer joining the two positively charged nitrogen atoms. Restriction of conformational flexibility could be an important consideration for the design of anti-leishmanial agents. Global constraint was obtained by backbone cyclization in a tail-to-tail fashion. This popular tactic in medicinal chemistry remains in some extent empirical, but has met successes, mainly for the elaboration of working or preliminary pharmacophores.

All these bis-pyridinium salts show activity against promastigotes and intracellular amastigotes of the protozoan parasites *L. donovani* and *L. major* [14, 15]. Most acyclic compounds show

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a similar behavior in both species, being slightly more active against *L. major* amastigotes. All the cyclophanes are more active against promastigotes and amastigotes of *L. major* than *L. donovani*, although with a lower potency than the acyclic derivatives. However, in contrast to the variable toxicity of the acyclic compounds [14], all cyclophanes exhibit very low toxicity against mammalian cells THP-1 and some of them evince a higher safety margin than well-known anti-leishmanial drugs such as AmB and miltefosine [15].

Although we have studied certain aspects of the mechanism of action of these compounds [14, 15], it has not been determined any key target on which they are operating, which would be decisive for the rational design of new structures. Future work should be directed to carry out studies to elucidate the metabolism, pharmacokinetics, and mechanism of action of these compounds. On the other hand, it would be interesting to conduct a screening of a large number of symmetrical bis-pyridinium compounds that allows us to study structure–activity relationships. In any case, additional experiments are necessary for evaluating the toxicity and potency of these compounds by *in vivo* assays.

Note

Some parts of this chapter have been previously published in references [14, 15].

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References

[1] Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M. WHO Leishmaniasis Control Team, Leishmaniasis worldwide and global estimates of its incidence. Plos One 2012;7e35671. Doi: 10.1371/journal.pone.0035671.

[2] Leishmaniais. http://www.who.int/leishmaniasis/en/ (accessed 24 August 2015).

[3] Seifert K. Structures, targets and recent approaches in anti-leishmanial drug discovery and development. Open Medicinal Chemistry Journal 2011;5, 31–39. Doi: 10.2174/1874104501105010031.

[4] Fairlamb AH, Chemotherapy of human African trypanosomiasis: current and future prospects. Trends in Parasitology 2003;19, 488–494. Doi: 10.1016/j.pt.2003.09.002.

[5] van der Meide WF, Sabajo LOA, Jensema AJ, Peekel I, Faber WR, Schallig HD, Fat RF. Evaluation of treatment with pentamidine for cutaneous leishmaniasis in Suriname. International Journal of Dermatology 2009;48, 52–58. Doi: 10.1111/j.1365-4632.2009.03883.x.

[6] Sun T, Zhang Y. Pentamidine binds to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation. Nucleic Acids Research 2008;36 1654–1664. Doi: 10.1093/nar/gkm1180.

[7] Patric DA, Bakunov SA, Bakunova SM, Kumar EV, Chen H, Jones SK, Wenzler T, Barzcz T, Werbovetz KA, Brun R, Tidwell RR. Synthesis and antiprotozoal activities of dicationic bis(phenoxymethyl)benzenes, bis(phenoxymethyl)-naphthalenes, and bis(benzyloxy)-naphthalenes. European Journal of Medicinal Chemistry 2009;44, 3543–3551. Doi: 10.1016/j.ejmech.2009.03.014.

[8] Patric DA, Bakunov SA, Bakunova SM, Jones SK, Wenzler T, Barzcz T, Kumar A, Boykin DW, Werbovetz KA, Brun R, Tidwell RR. Synthesis and antiprotozoal activities of benzyl phenyl ether diamidine derivatives. European Journal of Medicinal Chemistry 2013;67, 310–324. Doi: 10.1016/j.ejmech.2013.06.033.

[9] Brendle JJ, Outlaw A, Kumar A, Boykin DW, Patrick DA, Tidwell RR, Werbovetz KA. Antileishmanial activities of several classes of aromatic dications. Antimicrobial Agents and Chemotherapy 2002;46797–46807. Doi:10.1128/AAC.46.3.797-807.2002.

[10] Rosypal AC, Werbovetz KA, Salem M, Stephens CE, Kumar A, Boykin DW, Hall JE, Tidwell RR. Inhibition by Dications of *in vitro* growth of Leishmania major and Leishmania tropica: causative agents of old world cutaneous leishmaniasis. Journal of Parasitology 2008;94, 743–749. Doi: 10.1645/GE-1387.1.

[11] Ibrahim HM, Al-Salabi MI, El Sabbagh N, Quashie NB, Alkhaldi AA, Escale R, Smith TK, Vial HJ, de Koning HP. Symmetrical choline-derived dications display strong anti-kinetoplastid activity. Journal of Antimicrobial Chemotherapy. 2011;66, 111–125. Doi: 10.1093/jac/dkq401.

[12] Bringmann G, Thomale K, Bischof S, Schneider C, Schultheis M, Schwarz T, Moll H, Schurigt U. A novel Leishmania major Amastigote Assay in 96-well format for rapid drug screening and its use for the discovery and evaluation of a new class of Leishmanicidal quinolinium salts. Antimicrobial Agents and Chemotherapy 2013;57, 3003–3011. Doi:10.1128/AAC.02201-12.

[13] Gómez-Pérez V, McSorley T, See Too WC, Konrad M, Campos JM. Novel 4-Amino Bis-pyridinium and bis-quinolinium derivatives as choline kinase inhibitors with antiproliferative activity against the human breast cancer SKBR-3 cell line. ChemMedChem 2012;7, 663–669. Doi: 10.1002/cmdc.201100505.

[14] Gómez-Pérez V, Manzano JI, García-Hernández R, Castanys S, Campos Rosa JM, Gamarro F. 4-Amino bis-pyridinium derivatives as novel anti-leishmanial agents. Antimicrobial Agents and Chemotherapy 2014;58, 4103–4112. Doi: 10.1128/AAC.02481-13.

[15] Gómez-Pérez V, Manzano JI, García-Hernández R, Castanys S, Gamarro F, Campos Rosa JM. Design, synthesis and anti-leishmanial activity of novel symmetrical bispyridinium cyclophanes. European Journal of Medicinal Chemistry 2015;89, 362–369. Doi: 10.1016/j.ejmech.2014.10.040.

[16] Surrey AR, Cutler RK. The role of phenol in the reaction of 4,7-dichloroquinoline with novol diamine. Journal of the American Chemical Society 1951;73, 2623–2626. Doi:10.1021/ja01150a060.

[17] Conejo-García A, Campos JM, Sánchez-Martín RM, Gallo MA, Espinosa A. Bis-pyridinium cyclophanes: novel templates for human choline kinase inhibitors. Journal of Medicinal Chemistry 2003;46 3754–3757. Doi:10.1021/jm030792i.

[18] Campos Rosa J, Galanakis D, Ganellin CR, Dunn PM, Jenkinson DH. Bis-Quinolinium Cyclophanes: 6,10-Diaza-3(1,3),8(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane (UCL 1684), the first nanomolar, non-peptidic blocker of the apamin-sensitive $Ca^{2\pm}$-activated $K^{\pm}$ Channel. Journal of Medicinal Chemistry 1998;41, 2–5. Doi: 10.1021/jm970571a.

[19] Campos Rosa J, Galanakis D, Piergentili A, Bhandari K, Ganellin CR, Dunn PM, Jenkinson DH. Synthesis, molecular modeling, and pharmacological testing of bis-quinolinium cyclophanes: Potent, non-peptidic blockers of the apamin-sensitive Ca^{2+}-activated K^{+}channel. Journal of Medicinal Chemistry 2000;43, 420–431. Doi:10.1021/jm9902537.

[20] Chen JQ, Galanakis D, Ganellin CR, Dunn PM, Jenkinson DH. Bis-quinolinium cyclophanes: 8,14-diaza-1,7(1,4)-diquinolinacyclotetradecaphane (UCL 1848), a highly potent and selective, non-peptidic blocker of the apamin-sensitive Ca^{2+}-activated K^{+} channel. Journal of Medicinal Chemistry 2000;43, 3478–3481. Doi: 10.1021/jm000904v

[21] Tielens AG, van Hellemond JJ. Surprising variety in energy metabolism within Trypanosomatidae. Trends Parasitology 2009;25, 482–490. Doi: 10.1016/j.pt.2009.07.007.

[22] Mukherjee A, Padmanabhan PK. Sahani MH. Barrett MP. Madhubala R. Roles for mitochondria in pentamidine susceptibility and resistance in Leishmania donovani, Molecular and Biochemical Parasitology 2006;145, 1–10. Doi:10.1016/j.molbiopara. 2005.08.016.

[23] Luque-Ortega JR, Rivas L. Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in Leishmania donovani promastigotes. Antimicrobial Agents and Chemotherapy 2007;51, 1327–1332. Doi: 10.1128/AAC.01415-06.

[24] Azevedo Cardoso E, Rodrigues da Silva A, de Carvalho GC, Guerra Manssour Fraga A, de Castro Barbosa ML, Souza dos Santos AL, Castro HC, Lione V. Leishmaniasis: history, evolution of treatment and the need for new drugs. Current Biotechnology 2015;4279–4288. Doi:10.2174/2211550104666150303231736.