ANTILEISHMANIAL ACTIVITY OF POLYCYCLIC DERIVATIVES

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Summary:

33 polycyclic derivatives have been studied and tested on *Leishmania donovani* and *L. major* promastigotes. Their antileishmanial activity was assessed *in vitro* and an assay of their cytotoxicity was realized on human myelomonocytic cell line. The reference molecules used in the assays were amphotericin B and pentamidine. Among the compounds tested, 29 possess an antileishmanial activity; 25 of those were more active against *L. donovani* than amphotericin B, and nine were as effective as amphotericin B against *L. major*. Many synthesized derivatives were more active against *L.donovani* than against *L. major*. The cytotoxicity studies have shown that among the thirty-three derivatives tested, 12 molecules have an IC_{50} towards THP-1 cells about equal than that reference drugs, the 21 other derivatives are much less toxic. A 3D QSAR study was undertaken and has permitted to predict activity against *L. donovani* and *L. major* and to highlight critical area to optimize activity against the two species.

KEY WORDS : *Leishmania donovani*, *Leishmania major*, promastigote, polycyclic derivative.

Résumé : ACTIVITÉ ANTILEISHMANIOSE DE DÉRIVÉS POLYCYCLIQUES

33 dérivés polycycliques ont été testés contre les promastigotes de Leishmania donovani *et* Leishmania major. *Leur activité antileishmaniose a été faite* in vitro *et leur toxicité a été réalisée sur des cellules monocytaires humaines. Les molécules de référence utilisées dans ces tests sont l'amphotéricine B et la pentamidine. Parmi les molécules testées, 29 ont montré une activité antileishmaniose ; 25 d'entre elles étaient plus actives contre* L. donovani *que l'amphotéricine B, et neuf étaient plus actives contre* L. major *que l'amphotéricine B. Beaucoup de dérivés synthétisés étaient plus actifs contre* L. donovani *que contre* L. major. *Les études de cytotoxicité ont montré que parmi les 33 molécules testées, 12 ont une IC50 pratiquement identique aux molécules de référence, les 21 autres dérivés sont beaucoup moins toxiques. Une étude QSAR 3D a permis de prévoir leur effet contre* L. donovani *et* L. major *et de définir des régions hautement actives pour optimiser l'activité contre ces deux espèces.*

MOTS CLÉS : Leishmania donovani, Leishmania major, *promastigote, dérivé polycyclique*.

INTRODUCTION

Leishmaniasis is a protozoal parasitic disease which leads to considerable mortality. It is a major public health problem particularly in Latin America; Africa and Asia (Jeronimo *et al.*, 2004; Marlet *et al.*, 2003; Querido, 2004), affecting the life of billions of people worldwide (Herwalt, 1999; World Health Organization, 2002).

Several *Leishmania* species can be implicated, with variable sensitivity to drugs. Among them, *Leishmania donovani* is responsible for visceral diseases which are amongst the most severe clinical lesions, and *Leishmania major* is responsible of cutaneous infections (Alrajhi *et al.*, 2002). Pentavalent antimony, pentamidine, amphotericin B or miltefosine have been used for the treatment of the infection (Murray, 2000; Croft & Yardley, 2002; Pearson, 2003; Soto *et al.*, 2004; Prasad *et al.*, 2004). However, pentamidine and amphotericin B have shown a high toxicity at the effective therapeutic doses (Maddux & Barriere, 1980; Soto-Mancipe *et al.*, 1994). Resistance to treatment with antimony-based agents occurs frequently, and resistance to other drugs like pentamidine or amphotericin B was mentioned (Sereno *et al.*, 1997; Durand *et al.*, 1998; Lira *et al.*, 1999; Thakur *et al.*, 2001; Basselin *et al.*, 2002; Khan *et al.*, 2002; Coelho *et al.*, 2004). So, no drug is totally active and safe. Therefore, the search for new antileishmanial compounds remains a priority.

Among the compounds which have been studied as antileishmanial drugs, heterocyclic compounds (Fournet *et al.*, 1993; Chibale *et al.*, 2001), and quinonic derivatives have been found to possess some *in vitro* activity against strains of *Leishmania* sp. (Jernigan *et al.*, 1996; Tournaire *et al.*, 1996; Valderrama *et al.*, 1999). We have studied the antileishmanial potency of a

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series of 33 polycyclic compounds (Figs 1 and 2), and we wish to describe here our work on the *in vitro* activity of these compounds against the promastigote forms of two strains of *Leishmania*, *L. donovani* and *L. major*, with pentamidine and amphotericin B as the references. In the first time, we have chosen to test these molecules against promastigote forms, in the aim to select the drugs having a antileishmanial potentiality. We also present a quantitative structure activity relationship (QSAR) study on these results.

MATERIALS AND METHODS

PARASITE

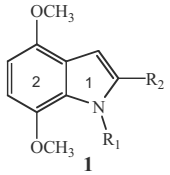
L. donovani (MHOM/ET67/L82:LV9) and *L. major* (MHOM/PT/92/CRE26) promastigotes were provi ded to us by Dr C. Bories (UMR 8076 CNRS, Châtenay-Malabry, France). The parasites were maintained in culture in RPMI 1640 medium (Sigma, L'Isle d'Abeau, France) enriched with 20 % foetal calf serum (Perbio Science, Brebières, France), 100 units of penicillin/mL and 100 µM streptomycin/mL (Sigma, L'Isle d'Abeau, France) at 27° C in an atmosphere of 95 % air/5 % CO_2.

ANTILEISHMANIAL ACTIVITY

The activity against promastigotes of the two strains was assessed in 96-well plates (Falcon) at 27° C using CellTiter 96^R Aqueous Non-Radioactive Cell Proliferation Assay (Promega Charbonnières les Bains, France), a colorimetric method. 10^5 leishmanies were placed in fresh medium and 100 µL of this suspension was distributed per well. Compounds were dissolved in DMSO and then diluted at the appropriate concentration in the standard culture medium. Median inhibitory concentration (IC_{50}), defined as the concentration of drug necessary to inhibit 50 % of parasite growth, was evaluated after 48 h, the drug being tested as a serial four-fold dilution from 0.01 to 1 µM and six replicate cultures being set up at each concentration.

ASSAYS OF CYTOTOXICITY

These assays were conducted on a human myelomonocytic cell line THP-1 (European collection of animal cell culture number 88081201: Sophia-Antipolis, France). These non-adherent cells were suspended in RPMI 1640 medium (DAP, Vogelgrun, France) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10 % fetal calf serum (DAP). The growth of THP-1 cells was assessed in 96-well plates at 37° C using the method described above for parasites.



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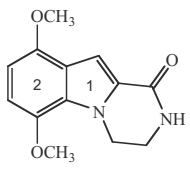
Compound	R_1	R_2
1a	$(CH_2)_2NHCOOC(CH_3)_3$	H
1b	$(CH_2)_2Cl$	$COOC_2H_5$
1c	$(CH_2)_2Br$	$COOC_2H_5$
1d	$(CH_2)_2N_3$	H
1e	H	H
1f	H	COOH
1g	H	$COOC_2H_5$
1h	$(CH_2)_2Br$	H



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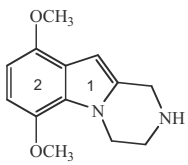

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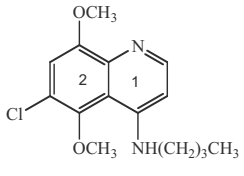

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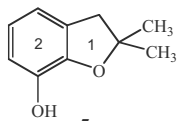

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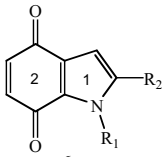
Fig. 1. – Structure of non-quinonic compounds 1-5.



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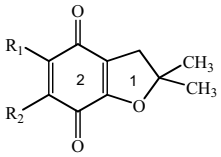
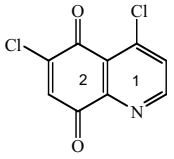
Compound	R_1	R_2
6a	$(CH_2)_2NHCOOC(CH_3)_3$	H
6b	$(CH_2)_2Cl$	$COOC_2H_5$
6c	$(CH_2)_2Br$	$COOC_2H_5$
6d	$(CH_2)_2N_3$	H



7 : $R_1 = R_2 = H$
8 : $R_1 = Br$, $R_2 = H$
9 : $R_1 = H$, $R_2 = Br$



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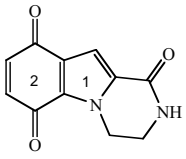

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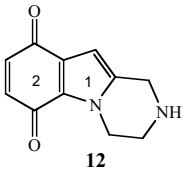

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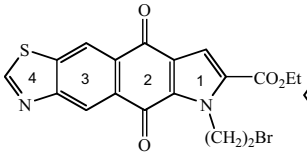

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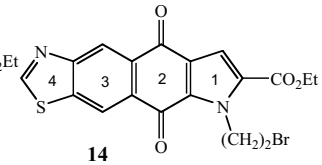

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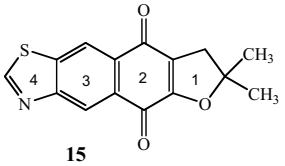

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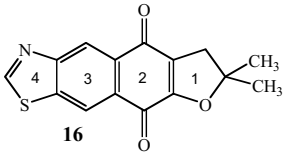

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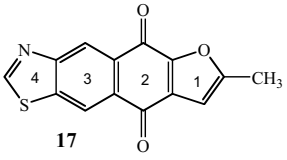

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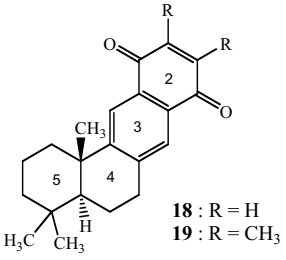
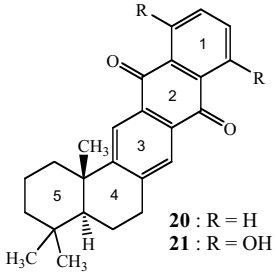
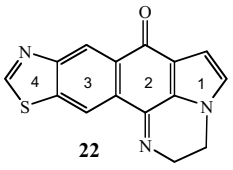


18 : R = H
19 : R = CH_3

Quinonic pentacyclic derivatives



20 : R = H
21 : R = OH



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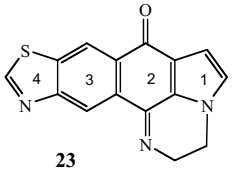

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Fig. 2. – Structure of quinonic bi- or polycyclic derivatives 6-23.

COMPOUNDS

The tested compounds were synthesized according to the method described previously: 1a, 1d, 1e, 1f, 1g, 1h, 6d, 6a, 22, 23 (Tapia *et al.*, 2003b) 1b, 1c, 2, 3, 6b, 6c, 11, 12, 13, 14, (Tapia *et al.*, 2002b) 4 (Tapia *et al.*, 2002c) 5, 7, 8, 9, 17, 15, 16 (Tapia *et al.*, 2003a) 10 (Tapia *et al.*, 2002a) 18, 19, 20, 21 (Cuellar *et al.*, 2003). Amphotericin B and Pentamidine were provided from Sigma (L'Isle d'Abeau, France).

MOLECULAR MODELING

Geometric optimization of each molecule was performed with the Sybyl molecular modeling package with the Merck Molecular Force Field (MMFF94). During the optimization dielectric constant was set to 80.0 and electrostatic cutoff set to 20 Å.

RESULTS

All the products, except 10, 11, 13, 14, possess an antiprotozooal activity against both *Leishmania* with an IC_{50} < 0.080 µM (Tables I and II). Among these twenty-nine compounds, twelve are non quinonic (1-5) and seventeen are quinonic derivatives (6-9, 12, 15-23). Solely four of them are less active against *Leishmania donovani* than amphotericin B: 1b, 1c, 6b, 6c, which are chlorinated or brominated molecules. Against *Leishmania major*, only nine compounds were as effective as amphotericin B: 1a, 3-5, 6a, 19, 21-23. Four compounds (1e, 8, 10, 17) exhibited an IC_{50} against *L. donovani* much lower than that found against *L. major*, and only one (22) showed an opposite behavior.

Compound	*L. donovani*	*L. major*	THP-1 cells
Amphotericin B	0.020[a] ± 0.002	0.005 ± 0.002	0.015 ± 0.003
Pentamidine	0.001 ± 0.000	0.002 ± 0.001	0.004 ± 0.001
1a	0.007 ± 0.001	0.008 ± 0.002	0.110 ± 0.011
1b	0.029 ± 0.004	0.027 ± 0.005	0.121 ± 0.015
1c	0.030 ± 0.004	0.030 ± 0.003	0.110 ± 0.06
1d	0.005 ± 0.001	0.064 ± 0.005	0.099 ± 0.003
1e	0.003 ± 0.001	0.250 ± 0.012	0.392 ± 0.021
1f	0.009 ± 0.001	0.076 ± 0.005	0.011 ± 0.002
1g	0.004 ± 0.001	0.062 ± 0.002	0.096 ± 0.005
1h	0.004 ± 0.001	0.045 ± 0.005	0.011 ± 0.003
2	0.004 ± 0.001	0.053 ± 0.006	0.001 ± 0.00
3	0.005 ± 0.002	0.006 ± 0.002	0.152 ± 0.015
4	0.004 ± 0.001	0.007 ± 0.001	0.011 ± 0.005
5	0.006 ± 0.002	0.005 ± 0.001	0.010 ± 0.002

[a]IC50 represents the means ± standard deviations determined at least three time in three different experiments.

Table I. – IC50 (µM) of reference molecules and of non-quinonic compounds **1-5** against *Leishmania donovani* (MHOM/ET67/L82:LV9), *L. major* (MHOM/PT/92/CRE26) and human myelomonocytic cell line, THP-1 cells.

Compound	*L. donovani*	*L. major*	THP-1 cells
6a	0.007[a] ± 0.002	0.006 ± 0.001	0.081 ± 0.010
6b	0.030 ± 0.002	0.060 ± 0.003	0.015 ± 0.003
6c	0.050 ± 0.003	0.090 ± 0.005	0.015 ± 0.004
6d	0.005 ± 0.001	0.043 ± 0.003	0.817 ± 0.015
7	0.010 ± 0.002	0.012 ± 0.002	0.123 ± 0.015
8	0.006 ± 0.001	0.198 ± 0.009	0.085 ± 0.003
9	0.010 ± 0.001	0.013 ± 0.003	0.053 ± 0.003
10	0.080 ± 0.002	0.150 ± 0.021	0.020 ± 0.002
11	0.250 ± 0.009	0.240 ± 0.032	0.110 ± 0.010
12	0.040 ± 0.003	0.035 ± 0.002	0.085 ± 0.003
13	NI[b]	0.300 ± 0.017	0.125 ± 0.005
14	NI[b]	> 3	0.153 ± 0.006
15	0.014 ± 0.002	0.011 ± 0.001	0.007 ± 0.001
16	0.008 ± 0.001	0.010 ± 0.001	0.007 ± 0.001
17	0.009 ± 0.002	2.384 ± 0.52	1.466 ± 0.009
18	0.004 ± 0.001	0.010 ± 0.002	0.002 ± 0.001
19	0.006 ± 0.001	0.009 ± 0.001	0.608 ± 0.009
20	0.005 ± 0.001	0.005 ± 0.001	0.048 ± 0.002
21	0.005 ± 0.001	0.010 ± 0.002	0.012 ± 0.003
22	0.210 ± 0.009	0.008 ± 0.001	0.615 ± 0.009
23	0.006 ± 0.002	0.007 ± 0.001	0.083 ± 0.003

[a]IC50 represents the means ± standard deviations determined at least three time in three different experiments.
[b] NI = non-inhibitor.

Table II. – IC50 (µM) of quinonic compounds 6-23 against *Leishmania donovani* (MHOM/ET67/L82:LV9), *L. major* (MHOM/PT/92/CRE26) and human myelomonocytic cell line, THP-1 cells.

The cytotoxicity studies have shown that if 1f, 1h, 2, 4, 5, 6b, 6c, 10, 15, 16, 18, 21 have an IC_{50} towards THP-1 cells about equal than that of amphotericin B and pentamidine, the 21 other derivatives were much less toxic.

MOLECULAR MODELING STUDY

Despite a certain molecular diversity, all the tested compounds have common cycles in their structure which can be easily superposed. The geometry optimization of the 33 molecules was performed with the Merck Molecular Force Field (MMFF94). After energy minimization of all structures were carefully checked and added to a chemical structural database. Each structure was carefully checked to verify that the conformation is the lowest energy conformer. The database was aligned by rigid alignment using atoms of the central ring. The position of central ring allows having good quality alignment for the skeleton of molecule. In a second step, laterals chains of molecule were also align by rotation of free torsion angle bond to the common conformation. For each molecule which to conformation was modified, an energy minimization was performed and the internal energy was check in order to have a difference less than 20 $kJ.mol^{-1}$ to have reliable conformer. This alignment was saved in the structural database. The COmFA method is a 3D QSAR technique which determines activity relationship using molecular field computed on the 3D model stored in the database. This method is particularly well adapted to treat

a set of molecules with a common core. We divided and rank molecules in five classes in function of their IC_{50} on *Leishmania major*, starting from active (class 1) to inactive (class 5). The inhibitors with a IC_{50} comprised between 0 and 8 nM were in class 1, between 9 and 30 nM are in class 2, between 31 and 60 nM are in class 3, between 61 and 300 nM are in class 4, and with an IC_{50} greater than 301 nM are in class 5. Starting form this database all aligned structures were put in a parallelepipedic grid, and using as probe a C3 atoms with a +2 as partial charge the energy of electrostatic and the steric interaction energy is used as descriptor. A partial charge of +2 is used than a +1 to give a predominant weight at electrostatic description, this allow having a better representation of polarity and potent hydrogen bonding in the final structure activity relationship equation. The weight of steric and electrostatic descriptor is checked after statistical analysis and does not exceed 65 % of electrostatic to have an equilibrated system. A Simca statistical method determines relationship between descriptor and class number. The Partial Leap Square method was chosen with an optimal number of components set to 6. The value of the class for one molecule was predicted by cross validation using the leave one out method. Results were given in Table III.

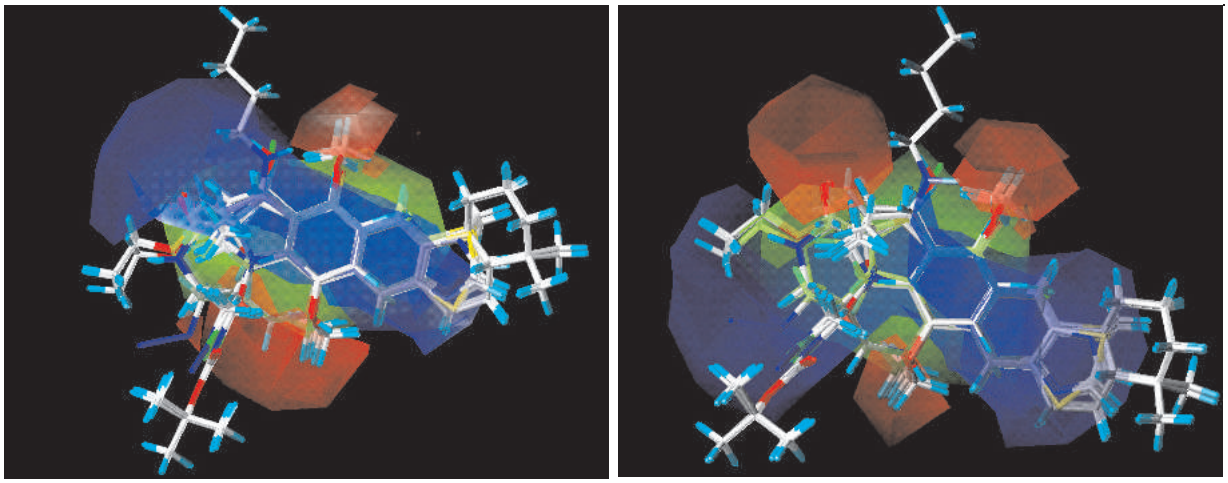
Major contributions were plot in the space around the molecules. There were four types of area, which are green, favorable contribution and yellow unfavorable contribution for activity from the steric contribution of the probe, and for the electrostatic contribution, blue are favorable and red unfavorable for activity. The result on *Leishmania donovani* and *Leishmania major* are drawing on the Figure 3.

DISCUSSION

The control of leishmaniasis remains a problem. Among drugs employed, amphotericin B, one of our two references, is a polyene antibiotic currently recommended as a second-line treatment for visceral and mucocutaneous leishmaniasis, but its potency was dependent of the immune status of the patient

	Based on IC_{50} from *L. major*			Based on IC_{50} from *L. donovani*		
Compounds	**Experimental**	**Predicted**	**Error**	**Experimental**	**Predicted**	**Error**
1a	2	2	0	1	1	0
1b	2	2	0	2	2	0
1c	3	2	1	2	2	0
1d	4	4	0	1	1	0
1e	4	4	0	1	1	0
1f	4	4	0	2	2	0
1g	4	4	0	1	1	0
1h	3	4	1	1	1	0
2	3	4	1	1	1	0
3	1	3	2	1	1	0
4	1	1	0	1	1	0
5	1	2	1	1	1	0
6a	1	2	1	1	1	0
6b	1	2	1	2	2	0
6c	2	2	0	3	2	1
6d	3	3	0	1	1	0
7	2	2	0	2	2	0
8	4	2	2	1	2	1
9	2	2	0	2	2	0
10	4	4	0	4	3	1
11	4	4	0	4	3	1
12	3	4	1	3	2	1
13	5	5	0	5	5	0
14	5	5	0	5	5	0
15	2	2	0	2	2	0
16	2	2	0	1	1	0
17	5	5	0	2	2	0
18	2	2	0	1	1	0
19	2	2	0	1	1	0
20	1	1	0	1	1	0
21	2	2	0	1	1	0
22	2	2	0	4	4	0
23	1	2	1	1	1	0

Table III. – The first column is the family number based on the IC_{50}. In the second column there are the cross-validated prediction for molecule and the third column is the error between experimental and predicted. This calculation is done for the two species.



L. donovani ***L. major***

Fig.3. – Favorable and unfavorable bulk for antileishmanial activity found by SIMCA analysis. Green volumes are favorable contribution to steric term. Blue volumes are favorable contribution to activity for electrostatic terms and Red is unfavorable. The analysis was performed for the two species.

(Maddux & Barriere, 1980). Amphotericin B-arabinogalactan water-soluble derivatives have been developed for treated leishmanial infections in mice (Golenser *et al.*, 1999). Since several years, the drug research against *Leishmania* sp. was intensified. Among these molecules, azithromycin, an antibiotic from the family of macrolide substances, had showed a potential activity against *L. major* (MHOM/IL/80Friedlin) (Krolewiecki *et al.*, 2002). An important problem in the *Leismania* treatment is the difference susceptibility of the strains. By example, they appear to vary in their susceptibility to sterol-biosynthesis inhibitors (Urbina, 1997). Various compounds of the acridine family (9-amino-6-chloro-2-methoxyacridine series and their unsubstituted bisacridine derivatives) have shown a potent toxicity towards *Leishmania* parasites (Di Giorgio *et al.*, 2003; Grisard *et al.*, 2000; Murray, 2000; Tapia *et al.*, 2003a). Chalcones, or 1, 3-diaryl-2-propen-1-ones, are natural or synthetic compounds belonging to the flavonoid family. They possess a broad spectrum of biological effects. Lunardi *et al.* (2003) have studied substituted derivatives and have noted an activity against *Leishmania sp* and the importance of the position of the substituents in this activity. The chalcones would inhibited fumarate reductase in *L. major* and *L. donovani* (Chen *et al.*, 2001).

Some heterocyclic quinones have been reported to be active in vitro against virulent strains of *Leishmania* sp. (Tournaire *et al.*, 1996; Valderrama *et al.*, 1999; Tapia *et al.*, 2003a; Tapia *et al.*, 2002b; Tapia *et al.*, 2003b), it is why we have explored the antileishmanial activity of various quinonic compounds. We have performed *in vitro* tests in order to identify three-dimensional structural similarities between these compounds that may explain their potency against *L. donovani*, or *L. major* or both strains. Indeed, we noted that our compounds were more often active against *L. donovani* than against *L. major*. This showed the importance of the strain concerned to test the efficacy of products. We have noted for some molecules a large difference of their IC_{50} towards *L. donovani* and THP-1 cells.

A 3D QSAR approach was made and will allow a better knowledge of the area and the fragments which can increase the affinity to the target.

An analysis of the Table III revealed that 23 molecules were predicted in the right class, eight molecules predicted with an error of one class, and two with an error of two classes. For all well unpredicted molecules except 8 and 1c the system overvalues the IC_{50}. Five molecules of class 1 were predicted in class 2, but the class 1 and 2 were very narrow. Class 1 and 2 were constituted by very active molecules, but the system never undervalues the IC_{50} which was a good point to select promising new potential compounds.

The same calculations were made with the IC_{50} measured on *Leishmania donovani* with the same five classes. On the 33 derivatives, 28 are well predicted and only five are predicted with a maximum error of one class. For this prediction, the values are underestimated but no molecules were predicted by mistake in the class 1.

By considering the structure, rings are numbered as display on Figures 1 and 2. An analysis of the Figure 3 shows few common points and one major difference. The blue and the green zone are roughly the same; the only big difference is on the upper part of the cycle 1. This region was favorable for activity on *L. donovani* and unfavorable for *L. major*. In this region a few compound had chemical function which accepted hydrogen bond or a hydrogen bond donor. This area

was particularly important about the electrostatic term. The steric favorable (green) volume was the same on the two cases; it explained that the active site has the same volume in the biologic receptor of the two species, but the nature of amino acids which were in the interface of the site was different. A hydrogen bond donor or acceptor amino acid for *L. donovani* was located in upper front of the first cycles, and hydrophobic area (red) was bigger on the upper of this figure. For *L. major* the hydrophobic area was located on the upper front of the first cycles and less on the lower part. For *L. major* the right part of the site had potential hydrogen bond capacity, and not for *L. donovani*. These differences could explain the difference of activity between the two species of *Leishmania*.

The analysis of the different area confirms measured IC_{50}. There are active compounds with two, three, four or five cycles. The number of cycles does not affect the IC_{50}, but the system predicted a critical size around the bigger series of inhibitors. The analysis of compounds 17, 22, 23, indicates that to have an activity against *L. donovani* the nitrogen atom on the cycle number 4 (which has a partial negative charge) must be the same side as the heteroatom on the cycle number 1. The analysis confirms that the favorable electrostatic volume is less bulky for *L. donovani* than for *L. major* on this cycle 1, this volume does not include the atom in opposite side of the heteroatom of the cycle 1. Compounds are very active on the *L. major* if there is a group bonded on the hetero atom of the ring 1 (*i.e.* compound 3 *vs* compound 1g). This group must be uncharged in the front of the ring 1, the red volume (Fig. 3) being more important for *L. major* than for *L. donovani* at this location. For *L. major* a blue volume is more important above the ring number 1 and explains the high activity for compounds with group in this area. These results may facilitate the rational design of new polycyclic derivatives active against *Leishmania*. Our *in vitro* results have shown that many of the 33 tested molecules possess an antileishmanial potency on promastigote forms of *L. donovani* and *L. major*. This study we permitted us to note among the synthesized molecules, the most interesting. In a second step, but it will be interesting, to test them *in vivo* to confirm our preliminary results which can be very important in the treatment of leishmaniasis especially against *L. donovani* which causes 91 % human mortality when untreated (Werneck *et al*., 2003).

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