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Short communication

A comparative study of mesoionic compounds in *Leishmania* sp. and toxicity evaluation

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Abstract

In this first study, a series of mesoionic compounds like 1,3,4-thiadiazolium-2-phenylamine derivatives were synthesized and studied in *Leishmania amazonensis*. The cytotoxic effects of these compounds on the host cells were investigated and the antileishmanial *in vitro* activity was compared with other species of *Leishmania* (*Leishmania chagasi* and *Leishmania braziliensis*). The compounds presented lower toxicity in murine macrophages than the reference drug pentamidine. The halogen derivatives **5**, **6**, **8** and **13** (4-F, 4-Cl, 4-Br and 3-Cl) were the most active compounds among all the species tested.

Keywords: Mesoionic derivatives; Toxicity; Antileishmanial activity

1. Introduction

Leishmaniasis is a complex of disease syndromes, with a spectrum that has classically been divided into visceral, cutaneous, diffuse and mucocutaneous forms, caused by parasites of the genus *Leishmania*. The disease is endemic in many tropical and subtropical regions of the world. It is estimated that 12 million people are infected by over 20 species with about two million cases reported annually and about 350 million people live in endemic areas under the risk of infection [1]. The relevance of this parasitic disease is that the incidence of new cases is increasing daily and currently is emerging as a common and serious opportunist infection in human immunodeficiency virus (HIV)-infected patients [2]. The treatment of choice is pentavalent antimony (Sb^V) in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime). The long course of treatment of Sb^V often causes side effects such as myalgia, pancreatitis, cardiac arrhythmia and hepatitis leading to the reduction or cessation of treatment. Pentamidine isethionate, an aromatic diamidine has been used as a second line drug for antimony-resistant cases, but the toxicity has always been a limitation on its use with reports of hypoglycemia, diabetes, and nephrotoxicity [3,4]. Recently, miltefosine an alkylphosphocholine group has been found to have antineoplasic activity, and it is the first effective orally administered treatment for visceral leishmaniasis. However, unresponsive strains of *Leishmania* sp. have been reported against this drug too [5]. The development of new leishmanicidal agents is extremely important, considering the high toxicity of the clinical drugs and in some cases a incomplete efficiency.

In order to find new drugs against leishmaniasis, we have been engaged in a program of investigation of mesoionic compounds. In a previous work we synthesized some salts of mesoionic derivatives of 1,3,4-thiadiazolium-2-aminide class and assayed them against *Leishmania amazonensis* [6].

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In the present work, we continue evaluating the effectiveness of these mesoionic derivatives against other epidemiologically important New World species, as *Leishmania braziliensis* and *Leishmania chagasi*. In addition, we also studied the cytotoxic effect of these compounds against mouse peritoneal macrophage.

2. Chemistry

The chemistry of mesoionic rings, especially their use as masked dipoles, has been a fruitful area of research since the late 1950s [7]. Mesoionic compounds (subclass of betaines) are five (possibly six) membered heterocyclic that cannot be satisfactorily represented by Lewis structures. This implies a certain degree of aromatic character in the positively charged exocyclic atom [8]. Their structures having well separated regions of positive and negative charge, associated with a polyheteroatomic system, enable them to interact with biomolecules. Although the molecules are internally charged, they are neutral overall, and therefore can cross biological membranes *in vivo*. These characteristics have been revealed by interesting biological activities including anti-inflammatory, analgesic, antibacterial, antifungal and antitumoral activities. In addition, all the different classes of mesoionic compounds have received considerable attention and have been extensively studied because of their unique structures, reaction behavior, biological activities and possible medicinal use [6,9,10,11].

On the other side, potent antiplatelet, fibrinolytic, broncholytic and anticancer effects, and even those on the cardiovascular system [10,12], could be intimately related to the presence of specific substituent groups on the ring or to the ability to release nitric oxide [13] from the structure. In addition, nitric oxide of macrophages is identified as potent effector molecule against extra and intracellular *Leishmania* forms [14].

3. Pharmacology

In spite of the different types of biological activities that have been assigned to mesoionic compounds include all these sydnones, sydnonimines, isosydnones, oxatriazoles and thiadiazole classes, much of action mechanisms at molecular and cellular levels remain to be elucidated. However, a large number of mesoionic xanthine analogues inhibit platelet aggregation through their activity as phosphodiesterase inhibitors [15]. On the other side, effects of other mesoionic xanthine analogs on *Trypanosoma musculi* development in mice showed significantly lower parasitemia [16]. Mesoionic oxatriazole derivatives constitute a class of NO-donors, some of which stimulate selectively guanylate cyclase abiding either platelets or leukocytes or lung tissues [12]. A number of 3-alkylsydnones were found by Kier and Roche [7] to be potent central nervous stimulants. In earlier studies by Grynberg et al. [11] have shown that mesoionic compound, 4-phenyl-5-(4-nitrocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride (MI-D), could enhance survival of Ehrlich and S-180 tumor-bearing mice, preventing the growth of the tumor, with no significant concomitant alterations in the haematological parameters in test animals. In addition, other authors [9] reported the effect of MI-D on some energy-linked functions in isolated rat liver mitochondria. A number of compounds with anticancer effects have been found to be effective against tripanosomatids. The miltefosine initially developed as an anticancer drug, and its antileishmanial activity was discovered in the mid-1980s in experimental models [17,18], these findings led to oral treatment of visceral leishmaniasis [5]. The mesoionic compounds like 1,3,4-thiadiazolium derivatives were also active against melanoma cells [10] and in parallel in previous studies carried out in our laboratory showed the promising activity against *L. amazonensis* [6].

4. Results and discussion

In this report the effect of 4-phenyl-5-(4- or 3-*R*-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine mesoionic compounds where $R_4 = H$, 4′-OCH_3, 4′-NO_2, 4′-F, 4′-Cl, 4′-Br, 4′-CN, 4′-OCH_2CH_3, and 4′-OH, and $R_3 = 3'$-OCH_3, 3′-NO_2, 3′-Cl, and 3′-Br (Fig. 1) have been compared against three different species of *Leishmania* from the New World. In addition it should be taken into account that *L. amazonensis* has been associated to all clinical forms of leishmaniasis [19], *L. braziliensis* usually caused mucocutaneous disease and is endemic in the State of Rio de Janeiro and *L. chagasi* is the causal agent of visceral leishmaniasis [20]. Based on the above-mentioned statements a different sensitivity of these parasites to the assayed drugs was expected. Indeed, it can be observed that the activity of these mesoionic derivatives series differs against each of the tested species and were more effective against *L. amazonensis* promastigotes [6], than *L. braziliensis* and *L. chagasi*. Table 1 contains antileishmanial data from mesoionic compounds, 14 salt series, in which the nature and position of the group of the 5-cynnamoyl ring were varied (Fig. 1). Each of these derivatives exhibited activity against the three species of *Leishmania* with the reference drug pentamidine (from Filaxis Lab.) having an ED_{50}s of 23.64, 64.18 and 27.50 μM, respectively, for the *L. amazonensis*, *L. braziliensis* and *L. chagasi*. Four of these halogen derivatives such as fluoride, chloride and bromide (4-F, 3-Cl, 4-Cl and 4-Br) exhibited very potent antileishmanial activity with ED_{50}s around or less than 10 μM among the three species. Among the halogens, fluorine is the strongest electron-withdrawing element presenting low ED_{50}s within 0.92–3.42 μM. However, this was not confirmed for non-halogen groups with strong electron-withdrawing power such as nitro (3-NO_2 and 4-NO_2) and cyano (4-CN) presented moderate antileishmanial activity. The methoxy (3-OCH_3 and 4-OCH_3) group with lower electron-withdrawing power than the previous ones presented more potent activity against *L. amazonensis*. In addition, moving the methoxy group from *para* to *meta* positions resulted in an increase of activity for the three tested species. The presence of the electron-donating methyl group (4-CH_3) results in strong anti-*L. amazonensis* activity and moderate activity for *L. braziliensis* and *L. chagasi*. The overall activity profile

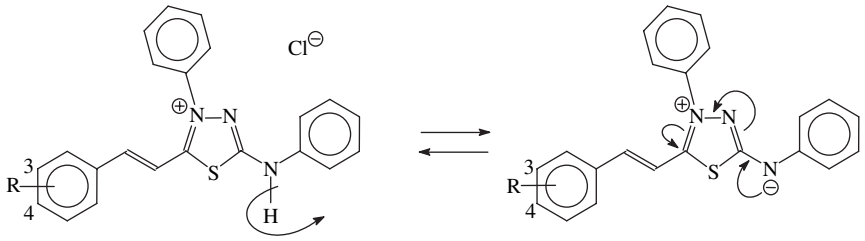

Fig. 1. Chemical structures of the mesoionic derivatives.

of compounds (**1**–**14**) demonstrated that there is a distinct difference in their ED_{50} values. Thus the biological activity was influenced by the type of the group attached to the 3- and 4-position of the 5-cinnamoyl-1,3,4-thiadiazole nucleus and a structure–activity relationship study could be crucial. For example, studies regarding the antimelanoma activity of 1,3,4-thiadiazolium mesoionics [10] have shown that the nitro group (4-NO_2) is important for the antitumor activity.

Nevertheless, the mechanisms of action of 1,3,4-thiadiazole mesoionic compounds are poorly understood. It was observed by Stewart and Kier [21] that compounds with long hydrocarbon chains or bulky group in 5-position had low activity on bacteria. However, in this study we observed that 5-cinnamoyl group unsubstituted exhibit potential activity (compound **1**) for all the assayed species. This could be explained by the planar structure due the resonance extended to unsaturated cinnamoyl group. In addition, the halogens (4-F, 4-Cl and 4-Br) of this aromatic group have a higher activity against *L. braziliensis* and *L. chagasi* when compared to an unsubstituded 5-cinnamoy-1,3,4-thiadiazole derivatives. In the case of *Leishmania*, the mechanisms of action of these compounds do not seem to be related to the biosynthesis of ergosterol [22] because in culture of promastigotes of *Leishmania* sp. treated with these compounds there was no change in the concentration of ergosterol while compared to the culture without treatment (unpublished data). However, mesoionic compounds are known as NO-donors [12] and their mechanism of action seems to be *via* activation of macrophages once some mesoionic derivatives were capable to induce NO production in macrophage cultures (unpublished data). On the other side, it is interesting to notice the considerable differences among different species of *Leishmania* (ED_{50} values ranging from 0.52 to 144.68 μM for 4-Br substituent) demonstrated in these studies. Variation in species sensitivity have also been demonstrated *in vitro* with *Leishmania donovani*, *Leishmania aethiopica*, *Leishmania tropica*, *Leishmania mexicana*, *Leishmania panamensis* and *Leishmania major* (EC_{50} values between 2.63 and 37.17 μM) to miltefosine test [23]. Findings of this nature could have important implications on clinical outcome. In addition, it was shown that the ambition to develop a single drug effective against all forms of leishmaniasis is unlikely to be fulfilled. Fortunately, the high *in vitro* leishmanicidal activity of compounds **5**, **6** and **13** (4-F, 4-Cl and 3-Cl) and lower toxicity evaluated on mouse peritoneal macrophages (Table 1), make these compounds a promising lead for development of an effective therapeutic agent. In conclusion, this study will be completed by additional tests in macrophage-amastigote *in vitro* models or *in vivo* mouse models. The structures of mesoionic derivatives are very sensitive to pH changes (Fig. 1). The promastigotes were grown in

Table 1
ED_{50} values of mesoionic compounds on promastigotes of *L. amazonensis*, *L. braziliensis*, *L. chagasi* and murine macrophage

Compound	4-R	3-R	[a]ED_{50} (μM)			TD_{50} (μM) toxicity
			L. amazonensis	*L. braziliensis*	*L. chagasi*	
1	H	H	0.46	49.61	22.76	3.06
2	CH_3	H	0.98	20.74	31.11	4.94
3	OCH_3	H	0.17	46.20	8.31	1.13
4	NO_2	H	1.0	70.77	22.83	5.49
5	F	H	0.92	5.1	3.42	5.82
6	Cl	H	1.51	6.23	13.17	11.98
7	CN	H	27.8	28.12	15.96	441.26
8	Br	H	0.87	2.93	9.97	6.59
9	OCH_2CH_3	H	1.49	12.96	33.33	9.73
10	OH	H	7.58	375.92	415.23	31.98
11	H	OCH_3	0.04	30.64	4.75	0.61
12	H	NO_2	1.58	221.46	23.6	5.54
13	H	Cl	0.48	1.72	5.17	6.23
14	H	Br	0.52	25.95	144.68	2.47
Pentamidine (Filaxis Lab.)	–	–	23.64	64.18	27.5	2.21
Pentamidine (May & Baker Lab.)	–	–	0.46	–	–	–

[a] Data for ED_{50} of *L. amazonensis* have been reported previously.

neutral pH while *in vitro* (intracellular models) the environment presents pH < 7.0. In this case differences in the results will be expected since we do not know which kind of change these compounds undergo inside the vertebrate host cell. Concerning the *in vivo* assay many factors such bioavailability, toxicity, and drug metabolism [24] may influence the animal model. Furthermore, it is also difficult to determine whether *in vitro* drug activity will correlate with *in vivo* activity, especially if compared with the three species of *Leishmania*.

5. Experimental

5.1. Chemistry

The preparation of 14 salt series, 4-phenyl-5-(4- or 3-*R*-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides was carried out by published procedures [6,25]. All these derivatives were fully characterized by IR, ^{1}H, and ^{13}C NMR spectroscopy and mass spectrometry. Pentamidine was obtained from Filaxis Lab., Argentina and all others chemicals were obtained from Sigma.

5.2. Parasites

Promastigotes of *L. amazonensis* (MHOM/BR/LTB0016 strain), *L. braziliensis* (MCAN/BR/98/R619 strain) and *L. chagasi* (MCAN/BR/97/P142 strain) were grown at 26 °C in Schneider's *Drosophila* medium pH 7.2 (Sigma, MO, USA) supplemented with 10–20% (v/v) heat-inactivated foetal calf serum (FCS). Parasites were harvested from the medium in the late log phase, counted in a Neubauer's chamber and adjusted to a concentration of 4×10^6 parasites/mL, for the drug assay.

5.3. Drug assay

The assay was carried out in 96-well flat-bottom microtrays with a volume of 200 μL/well. The 14 compounds solubilized in DMSO (the highest concentration used was 1.6% v/v, not hazardous to the parasite) were added to the culture, in a concentration range from 320 to 0.16 μg/mL. After 24 h of incubation at specific temperature, the number of surviving parasites was then counted in Neubauer's chamber and the percentage of growth inhibition was calculated comparing to the controls with DMSO without the compounds and the parasites alone. The LD_{50}/24 h values were determined by logarithmic regression from the percentages of inhibition. All tests were done in triplicate and pentamidine isethionate was used as reference drug.

5.4. Cytotoxicity assays

The cytotoxicity effect of the 14 mesoionic derivatives expressed as cell viability was assayed on mice's peritoneal macrophages. The cells were isolated from peritoneal cavity of BALB/c mice with cold RPMI 1640 medium, supplemented with 1 mmol L^{-1} L-glutamine, 1 mol L^{-1} HEPES, penicillin G (10^5 IUI^{-1}), streptomycin sulfate (0.10 g L^{-1}). The 2×10^5 cells/well were cultivated on microplate and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 2 h of incubation no adherent cells were then removed and the adhered macrophages were washed twice with RPMI. Compounds were added to the cell culture at the respective EC_{50}/24 h for *L. amazonensis* and to the cells incubated for 24 h. Then, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT was added and after 2–4 h the reaction was interrupted with DMSO. The results could be read in spectrophotometer with wavelength of 570 nm [26].

5.5. Statistical analysis

Significance was determined using a non-paired Student's *t*-test. Differences were considered to be significant when $p < 0.05$. Each experiment was triplicated.

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