IN VITRO AND IN VIVO ANTILEISHMANIAL ACTIVITY OF 2-AMINO-4,6-DIMETHYLPYRIDINE DERIVATIVES AGAINST *LEISHMANIA MEXICANA*

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Summary:

Leishmania mexicana promastigote and intracellular amastigote growths were inhibited by the water-soluble furan-2-carboxamide issued from the pharmacophore 2-amino-4,6-dimethylpyridine with IC_{50} values of 69 ± 2 and $89 \pm 9 \mu M$, respectively. This compound was also tested against established *L. mexicana* infection in susceptible BALB/c mice; an intraperitoneal administration of 10 mg/Kg/day during five consecutive days induced a high reduction in the amastigote burden of the poplitea lymph node ($81 \pm 6.4 \%$), the spleen ($80 \pm 1.6 \%$) and the liver ($73 \pm 9 \%$). Approach of the mechanism of antileishmanial activity of this compound, assessed by the flow cytometry, showed a reduction in the protein and DNA synthesis. Finally, an actual increase of the *in vitro* antileishmanial activity was obtained by replacement of the amidic function by an imidazolidin-2-one moiety. In this new series, two of the N-substituted derivatives showed IC_{50} values of 13 ± 0.5 and $7 \pm 3 \mu M$ in intracellular amastigotes constituting new promising compounds for further studies.

KEY WORDS : *Leishmania mexicana*, antileishmanial activity, 2-amino-4,6-dimethylpyridine, flow cytometry.

Résumé : ACTIVITÉ ANTILEISHMANIENNE DE DÉRIVÉS DE LA 2-AMINO-4,6-DIMÉTHYLPYRIDINE *IN VITRO* ET SUR UN MODÈLE MURIN DE *LEISHMANIA MEXICANA*

Un furane-2-carboxamide hydrosoluble issu de la 2-amino-4,6-diméthylpyridine présente des valeurs respectives de CI_{50} de 69 ± 2 et $89 \pm 9 \mu M$ sur la forme promastigote et sur les amastigotes intracellulaires de *Leishmania mexicana*. Sur le modèle *L. mexicana* chez la souris BALB/c, le composé provoque après un traitement de cinq jours par voie intrapéritonéale à la dose de 10 mg/kg/jour, une suppression de la charge parasitaire dans le ganglion poplité ($81 \pm 6.4 \%$) ainsi que la charge splénique ($80 \pm 1.6 \%$) et hépatique ($73 \pm 9 \%$). Le mécanisme d'action de ce composé semblerait mettre en jeu une réduction de la synthèse des protéines et de l'ADN. Enfin, l'action anti-leishmanienne *in vitro* a pu être augmentée par l'introduction d'un motif imidazolidine-2-one dans la structure de référence. Dans cette nouvelle série, deux composés, présentant des valeurs de CI_{50} de 13 ± 0.5 et $7 \pm 3 \mu M$ sur la forme intracellulaire, constituent de nouveaux antileishmaniens prometteurs justifiant la poursuite des recherches.

MOTS CLÉS : *Leishmania mexicana*, activité antileishmanienne, 2-amino-4,6-diméthylpyridine, cytométrie de flux.

The therapeutic armamentarium against leishmaniasis is very restricted, using mainly up to now antimonials, azole derivatives which show high toxicity. Moreover, failure and relapses under pentavalent salts and some resistances have been reported (Berman, 1997). Consequently there is a real need for new compounds that could provide better alternatives for the treatment of leishmaniasis.

In this work we have evaluated the *in vitro* proliferative inhibition and the *in vivo* effectiveness against *Leishmania mexicana* of the water-soluble N-(4,6-dimethylpyridin-2-yl) (furan-2-carboxamide) issued from the pharmacophore 2-amino-4,6-dimethylpyridine. The effect of this compound on parasite cellular

cycle and protein synthesis was also investigated by flow cytometry. On going pharmacomodulation in 2-amino-4,6-dimethylpyridine derivatives consisting in the replacement of the amidic function by an imidazolidinone moiety led to the discovery of new highly efficient compounds.

EXPERIMENTAL METHODS AND RESULTS

IN VITRO ANTILEISHMANIAL ACTIVITY

Leishmania mexicana (MHOM/MX/95/NAN1) and *L. infantum* (MHOM/FR/91/LEM2259) are maintained in Schneider's drosophila medium plus 10 % heat-inactivated foetal calf serum, penicillin (100 UI/ml) and streptomycin (100 μg /ml) at 26°C by passage every seven days. Drugs were synthesized in the Laboratory of Medicinal Chemistry of the Faculty of Pharmacy, University of Nantes (Abdala *et al.*, 2000). Promastigotes of *Leishmania* spp (2×10^6 /ml) were seeded into a 96 well plate and exposed for 96 h to the 2-amino-4,6-dimethylpyridine derivatives. The anti-

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2-amino-4,6-dimethylpyridine derivatives	IC ₅₀ (µM)		
	Promastigotes		Intracellular amastigotes
	<i>L. mexicana</i>	<i>L. infantum</i>	<i>L. mexicana</i>
Furan-2-carboxamide (I)	69 ± 2	60 ± 1	89 ± 9
Imidazolidinones (II)			
1	42 ± 7	> 100	88 ± 0.2
2	32 ± 3	> 100	13 ± 0.5
3	53 ± 4	35.5 ± 0.4	> 100
4	36 ± 2	29.3 ± 0.7	7 ± 3
Meglumine antimoniate	11750 ± 1.3	ND	133 ± 23

Table I. – *In vitro* activities of 2-amino-4,6-dimethylpyridine derivatives (I and II) against *Leishmania* promastigotes and intracellular amastigotes (IC₅₀ µM).

proliferative effect was determined by a modified colorimetric method based on the conversion of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (Le Pape *et al.*, 1999). The activity against the intracellular stage of the parasite was determined in BALB/c mice peritoneal macrophages. Cultures of 10⁶ macrophages were challenged for 24 h with stationary promastigotes at 37° C and 5 % CO₂. Cytotoxicity was determined by exposing infected macrophages to the studied molecules for 96 hours (Katiyar *et al.*, 1992). In this study, the furan-2-carboxamide (I) issued from the 2-amino-4,6-dimethylpyridine afforded the following IC₅₀ values: 69 ± 2 and 89 ± 9 µM, respectively, for *L. mexicana* promastigotes and intracellular amastigotes (Table I). Its activity against intracellular stage was comparable to that of the meglumine antimoniate (Specia Rhone-Poulenc) (IC₅₀: 133 ± 23 µM). In the imidazolidin-2-one series, the two most potent derivatives against *L. mexicana* promastigotes, were the N³-(2-bromobenzyl) (2) and tolylsulfonyl (4) derivatives with IC₅₀ values of 32 ± 3 and 36 ± 2 µM, respectively. The high activity of compound 4 was maintained against *L. infantum* promastigotes. Contrary to most studied compounds, the activity spectrum of these two imidazolidin-2-one derivatives elicited higher activity against the intracellular stage, the only one found in the mammalian host, with IC₅₀ values of 13 ± 0.5 and 7 ± 3 µM, respectively. Both derivatives 2 and 4 were 10- and 20-fold more active than the standard drug against intracellular amastigotes. Moreover, toxicity assessment towards a human lung cell line (MRC-5) showed an IC₅₀ value superior to 300 µM for the furan-2-carboxamide. All the imidazolidin-2-one derivatives exhibited MRC-5 IC₅₀ values superior to 100 µM, except for compound 1 (IC₅₀: 31 µM).

FLOW CYTOMETRY

The effects of furan-2-carboxamide derivative on the intracellular metabolism of *L. mexicana* promasti-

Fig. 1. – Example of histograms of DNA fluorescence corresponding to the distribution of the cells in different phases of the cell cycle in the control culture (1a) and in culture treated with pentamidine (1b) or furan-2-carboxamide (I) (1c), after a 28 hours incubation period. Ten thousands cells were acquired for each analysis.

gotes were estimated by determining cell cycle and total protein contents and compared to those of pentamidine and allopurinol (Crissman *et al.*, 1985). Promastigotes exposed to this compound were fixed with 70 % methanol, centrifuged at 600 g and suspended in 1 ml RNase solution (50 µg/ml in PBS) at 37° C for 30 min. DNA and total protein content was stained by propidium iodide (200 µg/ml) and fluorescein isothiocyanate (FITC: 1 µg/ml), respectively. The functional parameters reflecting parasite metabolism were quantified by the average intensity of red (DNA content) or green fluorescence (protein content) observed for each distribution (Azas *et al.*, 1997). Concerning the cellular metabolism, furan-2-carboxamide was shown to cause a weak decrease of protein synthesis in comparison with that of allopurinol, a purine analog inhibitor of RNA. Likewise, the kinetic study of the DNA synthesis and the cellular cycle indicated a small reduction in the S cell stage percentage, by contrast to pentamidine, a powerful inhibitor of polyamine synthesis that blocks the parasite at the G0-G1 phase (Fig. 1).

IN VIVO ANTILEISHMANIAL ACTIVITY

BALB/c mice were challenged intradermally with 5x10⁶ stationary promastigotes. One month after infec-

Fig. 2. – Inhibitory effect of furan-2-carboxamide (I) in tissue parasite burden in spleen smears (amast./500 fields), liver smears (amast. $\times 10^{-1}$ /1000 cells), and poplitea lymph node (amast./50 fields) after intraperitoneal administration of 10 mg/kg daily, during five consecutive days to BALB/c mice randomly sorted into two groups of five animals. Values as means $\pm$ standard error.

tion, treated mice were dosed intraperitoneally once a day with 10 mg/kg of body weight with furan-2-carboxamide and the controls received 0.9 % saline solution, both for five consecutive days. Twenty days after completion of drug administration, mice were sacrificed. The assessment of the amastigote burden was carried out with MGG-stained smears prepared from liver, spleen and poplitea lymph node tissues. The percent efficacy was calculated as mean amastigote load in treated mice/mean amastigote load in control (Croft *et al.*, 1987). The resolution of the infection was also followed by measuring the thickness of the infected footpad with a dial caliper (Hill *et al.*, 1983). During the development of the primary infection in BALB/c mice, the increase in lesion size was progressive, inducing extensive swelling before treatment which started on the 4th week after infection. Ten days after treatment completion, stabilization and transitory decrease in the footpad swelling was observed. Thereafter, their primary swelling progression was parallel to that observed in controls, although control mice lesions progressed to local tissue necrosis and in one case limb mutilation. However, an important decrease in parasitic charge with inhibitory percentage values of 72.7 ± 9.9 in liver, 80.2 ± 1.6 in spleen, and 81.1 ± 6.4 % in poplitea lymph node was shown (Fig. 2). No wasting due to infection was detected during the course of the disease in *L. mexicana*-infected BALB/c mice.

DISCUSSION

The level of antileishmanial activity of the 2-amino-4,6-dimethylpyridine derivatives was remarkably enhanced by the replacement of the amidic function by the imidazolidin-2-one ring II (derivatives 1–4). The non toxicity and hydrosolubility of compound I, have prompted us to carry out *in vivo* studies. As in the *L. donovani* visceral murin model (Le Pape *et al.*, 1999), this derivative also exhibited effectiveness against *L. mexicana*, in spite of the short-termed treatment and the infection which evolved for four weeks prior to drug administration. In a cautious effort to optimize *in vivo* antileishmanial activity, a longer time treatment could be of interest (20 days). It was previously reported by us that the furan-2-aryl-carboxamide derivatives inhibit the inflammatory response in rat experimental brain oedema through a phospholipase A₂ (PLA₂) inhibition (Robert *et al.*, 1992). In this regard, it had been reported that an inflammatory response induced by traumatism, promotes cutaneous metastasis in animals infected with *L. panamensis* (Travi *et al.*, 1996) and that indomethacin, a powerful anti-inflammatory agent, inhibits visceral dissemination of *L. major* (De-Freitas *et al.*, 1999). Thus, it is thought that a similar phenomenon could have occurred in our experimental model through a host PLA₂ inhibition, in addition to a direct antiparasitic activity. The hypothesis concerning the PLA₂ involvement could be supported by the two following observations. Firstly, arachidonic acid liberated by the PLA₂ action seems to be essential for the antibody-mediated phagocytosis in monocytes (Lenartz *et al.*, 1997); therefore, remaining out of the host cell, this obligatory intracellular parasite could openly be exposed to cytotoxic factors. Secondly, the production of host PGE₂ elicited by the parasite from free arachidonic acid, may drive a Th₂-cell response which could increase the severity of the infection by a down regulation of T-cell function (Farell & Kirkpatrick, 1987).

In conclusion, we showed that the furan-2-carboxamide reduced the parasite burden in *L. mexicana* BALB/c model. In the new serie of imidazolidinones, the N³-(2-bromobenzyl) and tolylsulfonyl derivatives constitute new promising compounds for further studies.

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