*Journal of Enzyme Inhibition and Medicinal Chemistry*, June 2006; 21(3): 305–312

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# Microtubule target for new antileishmanial drugs based on ethyl 3-haloacetamidobenzoates

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*(Received 24 January 2006; in final form 13 February 2006)*

**Abstract**
A new family of antimicrotubule drugs named (3-haloacetamidobenzoyl) ureas and ethyl 3-haloacetamidobenzoates were found to be cytotoxic to the *Leishmania* parasite protozoa. While the benzoylureas were shown to strongly inhibit *in vitro* mammalian brain microtubule assembly, the ethyl ester derivatives were characterized as very poor inhibitors of this process. Ethyl 3-chloroacetamidobenzoate, MF29, was found to be the most efficient drug on the promastigote stage of three *Leishmania* species ($IC_{50}$: 0.3–1.8 μM). MF29 maintained its activity against the clinical relevant intracellular stage of *L. mexicana* with $IC_{50}$ value of 0.33 μM. It was the only compound that exhibits a high activity on all the *Leishmania* species tested. This compound appeared to alter parasite microtubule organisation as demonstrated by using antibodies directed against microtubule components and more precisely the class of microtubule decorated by the MAP2-like protein. It is interesting to notice that this MAP2-like protein was identified for the first time in a *Leishmania* parasite

**Keywords:** *Leishmania, antileishmanials, benzoylureas, ethyl 3-haloacetamidobenzoates, cytotoxicity, microtubule*

## Introduction

Leishmaniasis is a vector borne disease constituting a major world public health problem. It is estimated that approximately up to 20–30 millions people in 88 countries are infected by one of the various forms of leishmaniasis [1,2]. Unfortunately, the therapeutic armentarium remains very limited; up to now it mainly uses pentavalent antimonials (Pentostam®, Glucantime®), azole derivatives (Sporanox®) and antibiotic drugs (fungizone)® [1]. Antimonials require the use of high doses, long courses of treatment and parenteral administration; moreover most of these agents exhibit toxic effects or induce appearance of resistant strains of *Leishmania* sp. Consequently there is still a real need for new active compounds that would provide therapeutic benefits for this parasitosis, with less side effects [3].

Abundant and functionally distinct microtubules characterize this protozoan. Flagellar microtubules are involved in locomotion; subpellicular microtubules participate in the maintenance of cell shape and nuclear spindle microtubules are required in cell division process [4]. Tubulin is the proposed cellular target of several clinically useful agents ranging from anticancer to antihelmintic drugs. The antitubulin herbicid trifluralin has previously been successfully tested against *Leishmania*, *Trypanosoma* and other protozoan parasites. It interferes with *Leishmania* growth and infection *in vitro* and binds selectively to *Leishmania* but not to host macrophage tubulin [5]. Moreover, taxol was shown to cause *Leishmania donovani* promastigotes to accumulate in the G2/M phase of the cell cycle [6]. Despite the fact that differences in drug susceptibility exist between

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2006 Taylor & Francis
DOI: 10.1080/14756360600700699

mammalian and kinetoplastid tubulin, as suggested by several reports, the identification and characterization of prospective drug targets is of vital importance [7,8]. Differentiation of *Leishmania* promatigotes to infective amastigotes has been reported to be accompagnied by different tubulin gene expression in particular for the α tubulin expression in the *in vitro*-generated arsenite-resistant strain. This resistant strain was two-fold more sensitive to some antimicrotubular agents than the wild type strain.

This communication reports different data which suggest that a new family of antimicrotubule drugs derived from 3-haloacetamidobenzoïc acids, already shown very active against cancerous cells [9], may represent potential agents for leishmaniasis chemotherapy.

## Materials

*Parasites*

*Leishmania mexicana (MHOM/MX/95/NAN1), L. major* (MHOM/SE/96/NAN2), and *L. infantum* (MHOM/FR/91/LEM2259) promastigotes were maintained *in vivo* by passage in hamster and grown *in vitro* in Schneider's drosophila medium (Sigma, St Quentin Fallavier, France) supplemented with 15% foetal bovine serum (FBS), 100 UI/ml penicillin and 50 μg/ml streptomycin (Medium A) at 26°C by passage every 7 days.

*Animals*

Seven week old Male BALB/c mice (Centre d'élevage R. Janvier, Le Genest-St, France) were housed in microisolator cages under a specific pathogen-free environment in the Animal Care Facility of the Pharmacy and Medicine Faculty, Nantes University.

*Drugs*

The following (3-haloacetamidobenzoyl) ureas were used: (**MF191**, halogen = Br) and (**MF569**, I) and ethyl 3-haloacetamidobenzoates (**MF708**, I), (**MF56**, Br) and (**MF29**, Cl) (see Figure 1) [9]. They were dissolved in a mixture of *N*,*N* dimethylacetamido, propylene glycol and Tween 80 (v,v,v, 1:2:1). Taxol and the reference antileishmanial drug antimoniate meglumine (Glucantime®) were purchased from Specia Rhone Poulenc, Paris, France.

## Methods

*Assay of microtubule assembly*

Rat brain microtubules were prepared by a temperature-dependent *in vitro* procedure described by Shelanski *et al*. [10] and slightly modified by Fellous *et al*. [11]. Tubulin was purified by the method of Weingarten *et al*. [12] except that the cation exchanger Fractogel® EMD SO3-650 (M) from Merck was used instead of phosphocellulose P11 from Whatman. $MAP_2$ was purified as described by Fellous *et al*. [11]. Microtubule assembly was measured by monitoring the changes in turbidity. Experiments to measure the rate and extent of microtubule assembly were performed in an UVICON spectrophotometer equipped with an automatic thermostated six sample changer connected to a circulating water bath set at 37°C. The optical density was determined at 345 nm every 30 s of the incubation period.

*Leishmania growth inhibition assay*

*Leishmania* promastigotes in exponential phase were seeded into 96-well plates and exposed for 96 h at 26°C to different drug concentrations in triplicate. The antiproliferative effect was determined by a colorimetric assay based on the conversion of a tetrazolium dye (MTT) (Sigma) into a blue formazan product by mitochondrial dehydrogenase [13]. The activity on the clinical relevant intracellular amastigote stage was determined in a BALB/c mice peritoneal infected macrophage model [14]. Parasite burden reduction was evaluated by microscopical observation after 96 h contact with the drugs. The inhibitory concentration ($IC_{50}$) which reduced cellular division by 50% compared to controls was determined by linear regression analysis and expressed as mean +/− standard deviation.

*Cytotoxicity in MRC-5 and MDA-MB231 cell lines*

The MRC-5 cell line derived from normal lung tissue of a 4 week-old male foetus by J.P. Jacobs was obtained

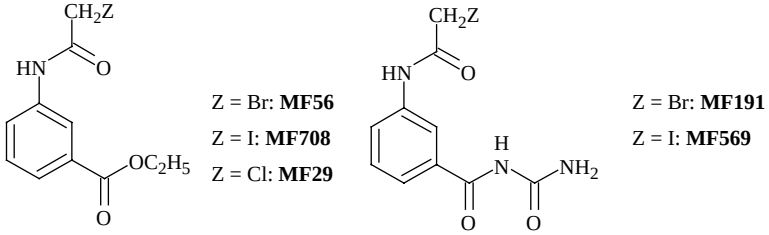


Figure 1. Structures of **MF56**, **MF708**, **MF29**, **MF191** and **MF569**.

from Biomerieux, Paris. For the toxicity evaluation, the cells were cultivated at 37°C in RPMI 1640, supplemented with 5% FBS. Cytotoxicity was estimated by a modified fluorometric assay using alamar Blue (Interchim, Montluçon, France) after 72 h contact with the drugs [15]. The MDA-MB231 cell line is a human mammary cell line established by Chandrasekaran [16]. The cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For toxicity evaluation, the cells were plated at a density of 1000 cells per well in 96-well microtiter culture plates for 96 h at 37°C. The quantification of surviving cells in the presence of increasing concentration of drug was determined by measurement of the optical density at 410 nm after incubation for 1 h at 37°C in a solution of *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminidine (0.75 mM) triton (0.05%) followed by an addition of glycine buffer (glycine 50 mM, EDTA 5 mM, pH 10.4).

The inhibitory concentration ($IC_{50}$) which reduced cellular division by 50% compared to the control was determined by linear regression analysis and expressed as mean +/− standard deviation.

### *Scanning Electron Microscopy (SEM)*

Promastigotes were prepared for SEM by fixation in 0.25% glyceraldehyde and 1% osmic acid, and dehydrated through a series of increasing concentrations of ethyl alcohol. After substitution by ethyl alcohol and acetone, they were critical-point dried using carbon dioxide substitution. The parasites were coated with gold palladium and studied using a JEOL field emission scanning electron microscope (ISM 6400F) at 12–15 kV.

### *Immunocytochemistry*

*Leishmania* promastigotes were treated with **MF29** at 10 μg/mL during 1 h and fixed with methanol on poly-D-lysine coated slides (10 min at −20°C). PBS with 3% BSA (Bovine Serum Albumin) and 0.1% saponin was used as a blocking, permeabilizing and antibody dilution buffer. Hydrogen peroxide 4% treatment during 10 min was used to inhibit the endogenous peroxidases. The monoclonal antibody N 356 (Amersham, Pharmacia) directed against α tubulin diluted 1/1000 and a monoclonal antibody directed against rat brain $MAP_2$ diluted 1/500 [17] were left on slides overnight at 4°C in a moist room. Biotinylated secondary antibodies were incubated 45 min at room temperature. A signal amplification was provided by a streptavidine peroxidase complex (Strept ABC kit, DAKO). The signal was detected by a colorimetric method using 3-amino-9-ethylcarbazole Photographs were taken with a tri CCD camera (LH 750 RC3, Lheisa Electronic System).

### In vivo *antileishmanial activity*

BALB/c mice were challenged intradermally in the left hint foodpad with $2 \times 10^6$ *L. major* stationary promastigotes for the cutaneous model or intravenously in the caudal vein with $10^7$ *L. infantum* stationary promastigotes for the visceral model. One month after infection, treated mice were dosed intraperitonealy with 10 mg/kg of body weight with **MF29** in its solvent, subcutaneously with antimoniate meglumine in 0.9% NaCl for reference treatment, both once a day for ten consecutive days. The control groups received the respective solvents.

For the cutaneous model, the resolution of infection was monitored by measuring the thickness of the infected foodpad with a dialcaliper. Twenty days after completion of drug administration mice were sacrificed. An *in vitro* limiting-dilution culture assay was used to quantitate the number of viable *L. major* parasites in the lesion footpad tissue as described previously [18]. The parasite burden was expressed as the $\log_{10}$ of the number of parasite per gram of tissue. Amastigote burden was also calculated from MGG-stained smears prepared from liver (1000 cells), spleen (500 fields) and poplitea lymph node (500 fields) tissues. The efficacy of drugs was expressed as inhibition percentage of parasite load in treated mice in comparison to the control.

For the visceral model, infected mice were weighed twice a week and sacrificed 7 days after completion of drug administration. Parasite burdens in liver and spleen homogenates were determined by using the culture microtitration method as summarized above for the footpad.

### *Statistical analysis*

The results are expressed as means ± standard errors and were analysed with the unpaired Student's test. Results were considered significant at $p < 0.05$.

## Results

### *Effects of MF 708, MF 56 and MF 29 on microtubules reconstituted* in vitro

Among the different 3-haloacetamidobenzoic acid derivatives studied in this report only the benzoylurea derivatives (**MF191**, **MF569**) were previously found to significantly inhibit microtubule assembly [19]. It was therefore necessary to check whether the ethyl benzoate derivatives (**MF708**, **MF56**, **MF29**) had any effect on microtubule assembly. When microtubules, purified from rat brains, were reconstituted *in vitro* at 37°C in the presence of 3.33 μmoles of drug (Figure 2A-B-C-D), **MF191** markedly inhibits tubulin assembly process while **MF708** inhibits slightly this process. At this concentration, no inhibition was observed with MF56 and **MF29**. **MF56** begins to have

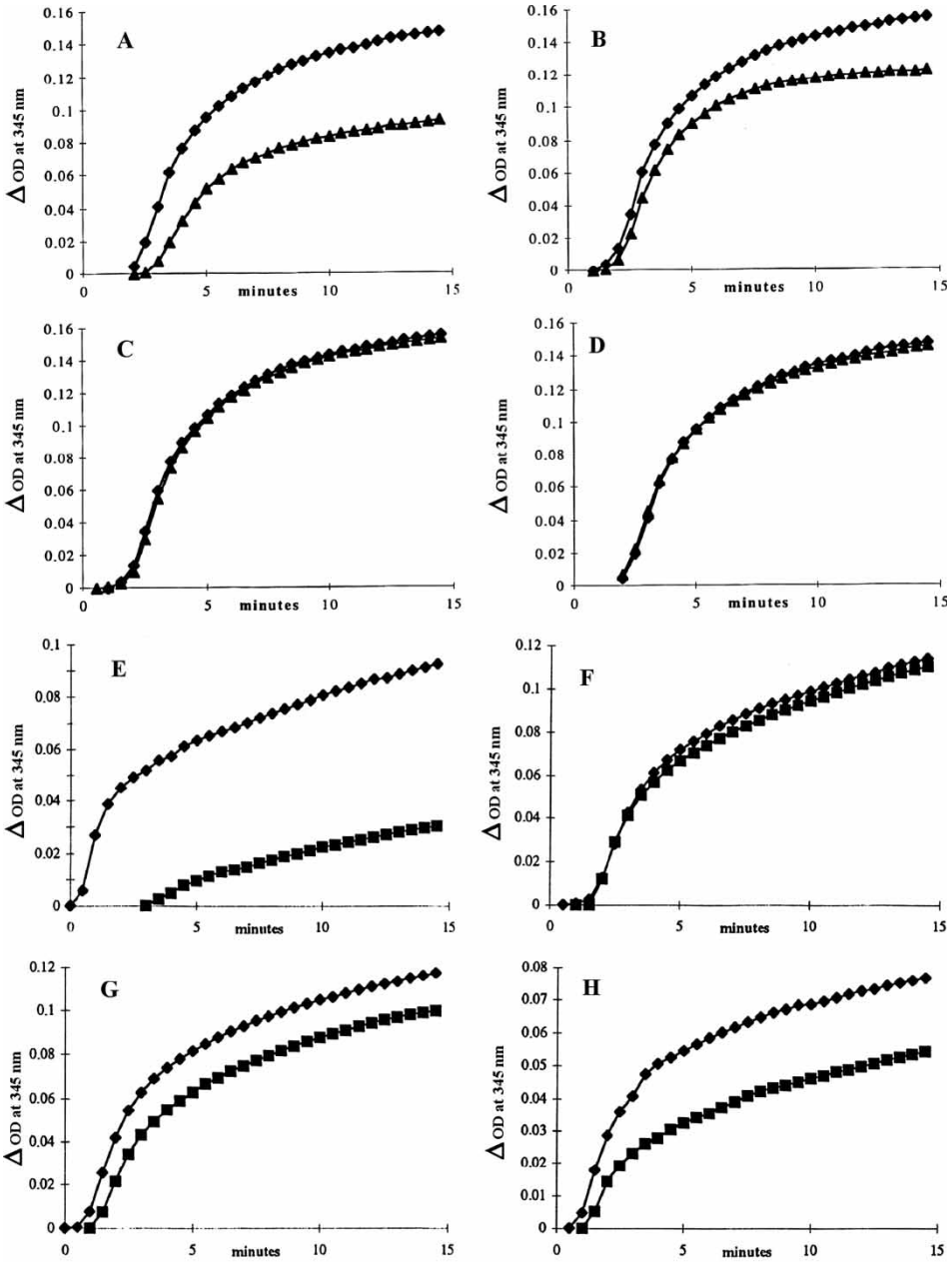


Figure 2. Effects of **MF191**, **MF708**, **MF56** and **MF29** on microtubule polymerization. Microtubules prepared from rat brain as described in Material and Methods at the concentration of 1.2 mg/ml at 37°C in the absence (◆–◆) or in the presence (■–■) of **MF191** (panel A), **MF708** (panel B), **MF56** (panel C) and **MF29** (panel D) at the concentration of 3,33 μM of drug. Microtubules were reconstituted *in vitro* with purified tubulin (1 mg / ml) and $MAP_2$ (0,1 mg /ml) in the absence (◆–◆) or in the presence (■–■) of 3,33 μM MF191 (panel E), 88 μM of **MF29** (panel F), 888 μM of **MF29** (panel G and H). In panel H, purified tubulin was preincubated 30 min at 4°C with 888 μM of **MF29** before the addition of the drug.

an inhibitory effect on tubulin assembly when the drug concentration reaches 90 μM and a significant inhibition was observed at 450 μM. No inhibition of tubulin assembly was observed with **MF29** used at this high concentration of drug (data not shown).

However different results were obtained when *in vitro*-reconstituted microtubules were not a heterogenous population of different classes of microtubules but a specific class of microtubules. When purified rat brain tubulin and $MAP_2$, used as a promotor of tubulin polymerization into microtubules were incubated in the presence of increasing concentrations of **MF29**, some inhibitory effect was observed (Figure 2E-F-G-H). Strong inhibitory effect was observed with 3.33 μM of MF191 (Figure 2E) while no inhibition was obtained at a 26-fold higher concentration of MF29 (Figure 2F); **MF29** begins inhibiting $MAP_2$-induced tubulin assembly at a very high concentration (888 μM) (Figure 2G). A significant inhibition was only obtained when the

Table I. *Leishmania* susceptibility ($IC_{50}$ = μM) to 3-haloacetamidobenzoic acid derivatives.

| Compounds | Promastigotes | | | Intracellular Amastigotes |
|---|---|---|---|---|
| | *L. mexicana* | *L. major* | *L. infantum* | *L. mexicana* |
| MF 191 | 1.8 ± 0.2 | 9.1 ± 0.7 | 148.5 ± 9.6 | 0.3 ± 0.0 |
| MF 569 | 1.6 ± 0.4 | ND | 21.1 ± 4 | 1.2 ± 0.3 |
| MF 708 | 1.2 ± 0.3 | ND | 17.0 ± 4 | 0.6 ± 0.2 |
| MF 56 | 1.6 ± 0.1 | ND | 8.6 ± 2 | 0.1 ± 0.0 |
| MF 29 | 0.3 ± 0.1 | 1.8 ± 0.3 | 0.9 ± 0.4 | 0.33 ± 0.0 |
| GLUC | 11700 ± 1.2 | ND | ND | 133 ± 23 |

ND = not determined

purified tubulin was preincubated at 4°C for 30 min, in the presence of a high concentration of **MF29** (888 μM), before addition of $MAP_2$ and incubation at 37°C to produce microtubules (Figure 2H).

### In vitro *antileishmanial activity and cytotoxicity*

The antileishmanial activity of 3-haloacetamidobenzoic acid derivatives was evaluated against *Leishmania mexicana*, *L. major* and *L. infantum* (Table I). **MF29** displayed a high activity on the three *Leishmania* species with $IC_{50}$ values lower than 1.8 μM. **MF29** remained the most effective drug against *L. infantum* (160-fold more active than **MF191**) and the increase in level of activity was less pronounced against *L. mexicana* and *L. major* (5–6 fold). **MF29** was 140-fold more active than taxol ($IC_{50}$ = 43.4 ± 7 μM) against *L. mexicana* promastigotes. In respect of the intracellular amastigote stage, all the compounds exerted high activity (0.1 – 0.6 μM) and **MF29** was 3- and 400-fold as active as taxol (0.90 ± 0.2 μM) and meglumine antomoniate, respectively.

The ratios of **MF29** $IC_{50}$ values in MRC-5 ($IC_{50}$ = 90 μM) or MDA-MB231 cell lines (Table II) and *L. mexicana* amastigotes indicates that **MF29** is highly selective against the parasite. In contrast, taxol exhibited a too high cytotoxicity spectrum.

### *Scanning Electron Microscopy*

The effects of **MF29** on *L. mexicana* were analysed by scanning electron microscopy. As shown in Figure 3 (A,B), **MF29**-treated parasites with 5 μg/mL for 1 hour showed a porous body surface without any alteration of the flagella structure contrary to results obtained with other antitubulin compounds such taxol or sinefungine.

Table II. Cytotoxicity of taxol and 3-haloacetamidobenzoic acid derivatives against MDA-MB231 cells.

| Drug | $IC_{50}$ (μM) |
|---|---|
| taxol | 0.0015 |
| MF 191 | 0.027 |
| MF 569 | 0.025 |
| MF 708 | 0.16 |
| MF 56 | 0.35 |
| MF 29 | 1 |

### *Immunocytochemistry analysis*

Immunostaining of *L. mexicana* promastigotes, either treated or untreated by **MF29**, revealed an alteration of microtubule organization induced by drug treatment. In untreated promastigotes subpellicular microtubules, stained by a monoclonal antibody directed against mammal α tubulin, appeared as a continuous structure surrounding the parasite cell body (Figure 4 insert), while, in promastigotes treated by **MF29** (10 μg/mL), the same subpellicular microtubules

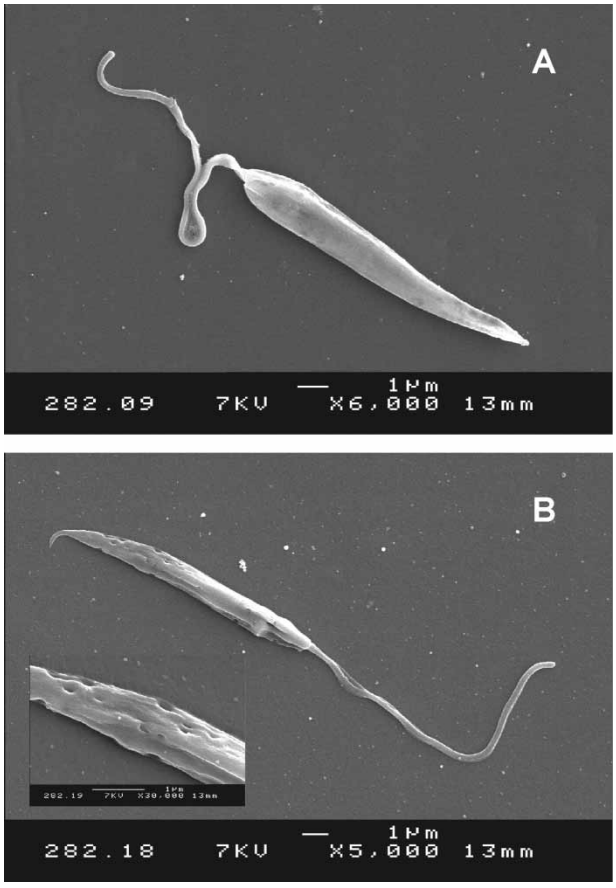


Figure 3. Effects of **MF29** on *L. mexicana* by scanning electron microscopy (control: A, treated: B).

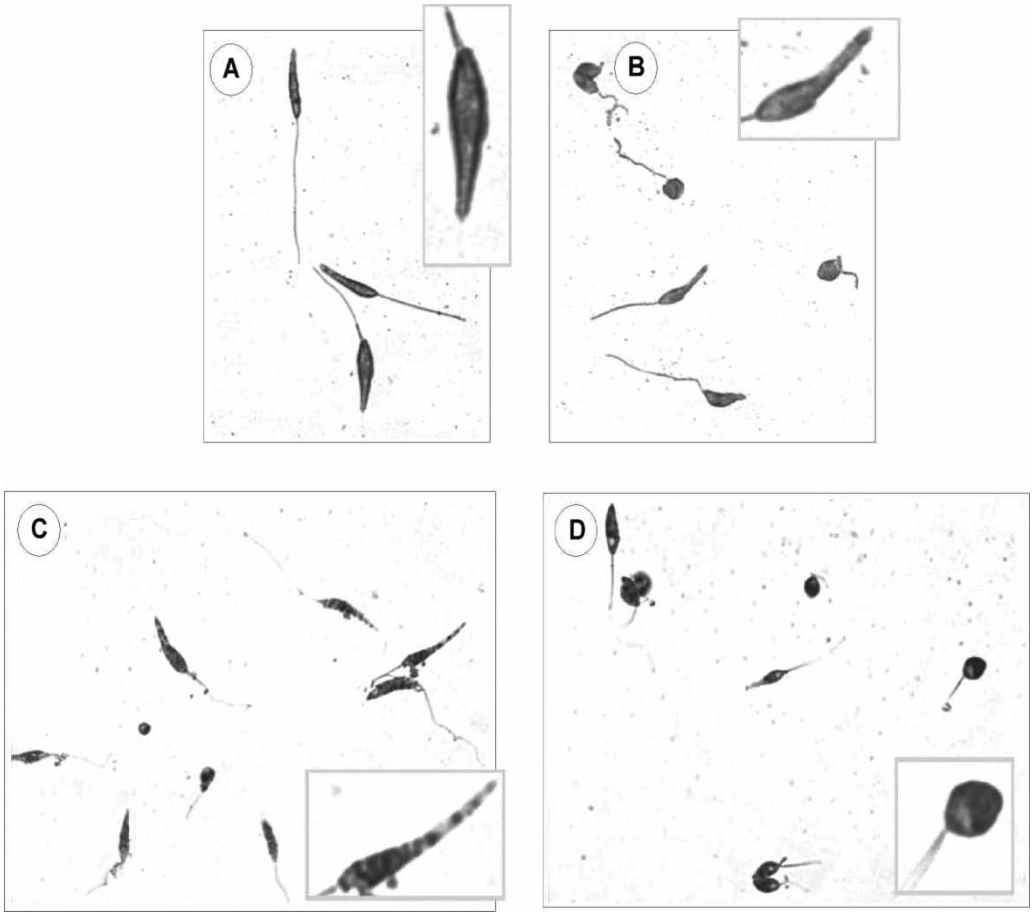


Figure 4. Effects of **MF29** on *L. mexicana* by immunocytochemistry. Immunocytochemistry of *Leishmania mexicana* promastigotes treated or not treated by **MF29** (panels A, B, C, D) was performed as described in Material and Methods. Promastigotes not treated (panels A, C) or treated by **MF29** (panels B, D) were labelled by a monoclonal antibody against α tubulin (panels A, B) or a monoclonal antibody against $MAP_2$ (panels C, D).

appeared discontinuous, suggesting that **MF29** might disorganize, at least partially, this class of microtubules (Figure 4 B); drug treatment appeared also to alter intracellular microtubules as indicated by the round shape of several cell bodies and the decrease of immunostaining intensity with anti-α tubulin antibody (Figure 4 B insert). By contrast with other antileishmanial drugs, the flagella were not significantly altered by **MF29** treatment. Immunostaining analysis also demonstrated that microtubule decorated by associated protein immunologically related to rat brain $MAP_2$ is disorganized by **MF29**. By using a monoclonal antibody directed against rat brain $MAP_2$, it has been shown that the $MAP_2$-like protein is distributed in the whole parasite cell body (Figure 4 C). In treated promastigotes, an heterogeneous immunostaining was observed with a $MAP_2$ labelling intensity very low in one pole and very high at the opposite pole (Figure 4 D insert).

### In vivo *antileishmanial activity*

To investigate *in vivo* antileishmanial activity, the well recognized *L. major*-infected BALB/c mice model was used with **MF29** at the dose of 10 mg/kg during 10 days. The parasite load in the liver, the spleen and the poplitea ganglion was reduced by 96%, 97% and 59%, respectively. In this model, the reference drug antimoniate meglumine (Glucantime®) showed a less marked reduction in parasite organ load: 74%, 82%, and 44% respectively (Figure 5). In the *L. infantum* visceral model, the reference drug Glucantime® and **MF29** treatment showed similar results in parasite organ load reductions. The inhibitory effects of both compounds were less in the liver, where the parasite load was slightly decreased by 25% and 30.6% respectively. On the other hand, both compounds exerted a moderate reduction of the spleen parasite burdens by 56% and 66%.

## Discussion

Some of the 3-haloacetoamidobenzoylureas were previously found to have an anticancer activity in several tumor cell lines [9,19]. The antimitotic effect of two compounds of the family, **MF191**, a (3-bromoacetamidobenzoyl) urea and **MF569**, a (3-iodoacetamidobenzoyl) urea seem to be

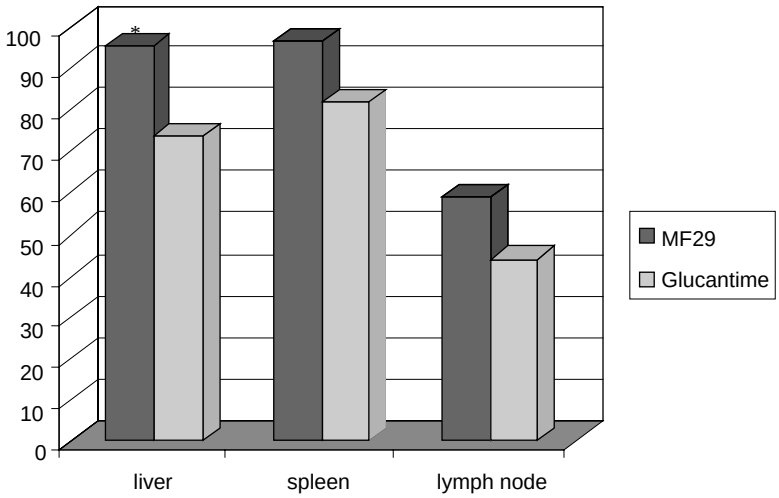


Figure 5. In vivo activity of **MF29** on cutaneous murine leishmaniasis in BALB/c mice. Inhibitory effect (%) of **MF29** and meglumine antimoniate in tissue parasite burden in liver (1000 cells), spleen (500 fields) and poplitea lymph node (500 fields) smear tissues after intraperitoneal (**MF29**) or subcutaneously (meglumine antimoniate) administration of 10 mg/kg during 10 consecutive days to BALB/c mice. * $p < 0.05$.

essentially related to their antimicrotubule properties. The two drugs inhibit *in vitro* tubulin assembly without interfering with microtubule disassembly. On the contrary, three other compounds have been described in this report to have either the capacity to inhibit very slightly *in vitro* rodent microtubule assembly (**MF708, MF56**) or not to have any inhibiting activity (**MF29**) except when used at very a high concentration of drug. However we demonstrated that these drugs are very active on *Leishmania* parasites. They have an anti-proliferative and anti-microtubule actions as shown by both growth inhibition assays and immunocytochemistry data. To explain this discrepancy it is tempting to hypothesize that parasite tubulins are better targets for the drugs than rodent brain tubulin. Tubulins belong to a multigenic family characterized by a high level of heterogeneity produced not only at a transcriptional but also at a post-transcriptional level [20–22]. This heterogeneity is partially responsible for differences in the affinity of several antimicrotubule drugs for tubulin populations which differ by their content in tubulin isotypes [23–25]. Some heterogeneity of *Leishmania* tubulin has also been described [26,27]. *Leishmania* parasites could contain a pool of tubulin isotypes which differ markedly from rat brain tubulin as proposed in dinitroaniline microtubule inhibitor experiments [28]. As for dinitroaniline, our compounds are ineffective against fungal proliferation (data not shown).

In this report, we have noticed that the level of the promastigote antimitotic activity of (3-bromoacetamidobenzoyl) urea differs from one parasite species to another. These data strongly suggest that parasite tubulin target of this compound could present an heterogeneity which characterizes each species of *Leishmania* parasites. The correlation between tubulin isotype content in the different parasite species and the activity of the different 3-haloacetamidobenzoïc acid derivatives has to be established. **MF29** appears to be the most efficient drug against *Leishmania* growth. This could be explained by a tubulin isotype abundant in the three *Leishmania* species analysed in this report (*L. mexicana*, *L. major* and *L. infantum*) and very reactive to **MF29**.

Microtubule components other than tubulin may influence the effect of a drug which binds to tubulin. Microtubule associated proteins (MAPs) have this property and their effect has been very well documented for several antimicrotubule drugs [29–31]. It is therefore probable that in the case of *Leishmania*, the different classes of microtubules may react differently to **MF29**. The immunochemistry analysis has shown that some intracellular microtubules decorated by microtubule-associated proteins immunologically related to rat brain $MAP_2$, discovered for the first time in *Leishmania,* appear to be sensitive to **MF29**. Interestingly this observation may be correlated with our observation that when **MF29** had some effect on microtubule assembly, this effect was not observed with a heterogeneous population of microtubules but only when tubulin was promoted by $MAP_2$. This means that even when some tubulin isotypes are not good targets for **MF29**, the presence of a certain amount of a microtubule-associated protein close to $MAP_2$ may increase the sensitivity of these tubulins to the drug. However this increase is very limited. Another interesting point concerns our observation that in *Leishmania*, microtubules decorated by $MAP_2$ are more sensitive to **MF29** in *L. mexicana* than in *L. major*. This may be explained by the fact that in addition to possible differences in tubulin isotype content, the $MAP_2$-like

protein might be different in the two *Leishmania* species and modulate differently the affinity of tubulin for the drug. Another possibility is that other MAPs present in *L. mexicana* have a higher capacity than $MAP_2$ to modulate positively the affinity of tubulin for **MF29**.

During *in vivo* experimentation, we also observed some heterogeneity of **MF29** action on infected mice. Mice infected by *L. infantum* are less sensitive to **MF29** than mice infected by *L. major*. Microtubule component heterogeneity may represent one of the factors responsible for this variation in MF29 efficiency.

In conclusion, 3-haloacetamidobenzoic acid ethyl esters are good lead compounds for treatment of leishmaniasis because they exert high activity without any toxic effect against mammalian cells. Our results provide valuable data for further development of a therapeutic project focused on specific microtubule polymerization inhibitors against *Leishmania*.

## Acknowledgements

We would like to thank Paule Berda and Marlene Biron for their helpful technical assistance.

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