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In vitro activity of the β-carboline alkaloids harmane, harmine, and harmaline toward parasites of the species *Leishmania infantum*

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Abstract

Harmane, harmine, and harmaline were investigated for their in vitro antileishmanial activity toward parasites of the species *Leishmania infantum*. Harmane and Harmine displayed a moderate antiproliferative activity toward human monocytes and exerted a weak antileishmanial activity toward both the promastigote and the amastigote forms of the parasite. Their mechanism of action on the promastigote form of the parasite involved interactions with DNA metabolism leading to an accumulation of parasites in the $S–G_2M$ phases of the cell-cycle. Harmaline, at the contrary, was deprived from toxicity toward human cells and *Leishmania* promastigotes, however it exerted a strong antileishmanial activity toward the intracellular amastigote form of the parasite. This property was shown to partly result from the capacity of the molecule to prevent parasite internalization within macrophages by inhibiting *Leishmania* PKC activity.

1. Introduction

With an estimated amount of more than 500,000 new cases occurring annually (Grimaldi et al., 1989) , visceral leishmaniasis, the vector-borne parasitic disease resulting from infection of macrophages by obligate intracellular parasites of the genus *Leishmania*, is still considered as one of the most severe reemerging affections by the World Health Organization. Clinical manifestations and evolution of the illness have been shown to depend greatly on the etiological agent, nevertheless most of infected individuals develop long-lasting disease characterized by weight loss, cough, fever, hepatosplenomegaly, and lethargy, with a mortality that approaches 90% for untreated patients (Herwalt, 1999). Since the 1940s, pentavalent antimonial compounds constitute the first-line treatment for all forms of leishmaniasis and, in case of therapeutic resistance to these compounds, amphotericin B desoxycholate and liposomal amphotericin B may also be used (Croft and Yardley, 2002). However, both of these treatments, that are expensive and complicated by the fact that they are given IV or IM, are not entirely satisfactory (Murray, 2000). On this basis, it is of paramount importance to study new antileishmanial drugs.

In developing countries, a large proportion of the population still relies on traditional medicine to satisfy their primary health care needs. Medicinal plants, the oldest known health-care products, may also constitute an important basic material for pharmacological research and drug development. Initially known for their potent psychoactive and hallucinogen abilities, harmane, harmine, and harmaline the β-carboline alkaloids isolated from various medicinal plants such as *Grewia bicolor* (Jaspers et al., 1986), *Tribulus terrestris* (Bourke et al., 1992) or *Passiflora incarnata* (Bennati, 1971), have been shown to exert a wide range of pharmacological properties including antimicrobial (Aassila et al., 2003) and anti-HIV activities (Ishida et al., 2001). Recently, Rivas et al. (1999) and Freiburghaus et al. (1996) demonstrated that harmane could exert antiproliferative effects toward parasites of the genus *Trypanosoma*, suggesting that β-carboline derivatives could also present interesting antiparasitic properties. This statement

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was confirmed by the study of Evans and Croft (1987), demonstrating that harmaline exerted interesting in vitro and in vivo antileishmanial activity. In the present study, we compared the antileishmanial activity of harmaline to those of other β-carboline alkaloids such as harmane and harmine on the protozoan parasite *Leishmania infantum*. Then, we investigated their possible mechanisms of action on both the promastigote and the amastigote-macrophage models.

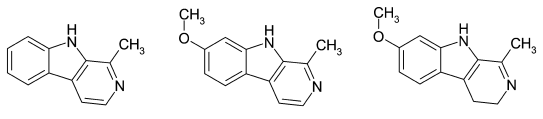
2. Materials and methods

2.1. Strains and reagents

Harmane, harmine, and harmaline were purchased by Sigma St. Louis, MO, USA. Their chemical structures are described in Fig. 1. Amphotericin B (Sigma), pentamidine (Sigma), allopurinol (Sigma), ketoconazole (Calbiochem, San Diego, USA), carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone FCCP (Sigma), and RO-32-0432 (Calbiochem, Paris) were used as standard drugs for positive controls. All compounds were dissolved in sterile dimethyl sulfoxide (analytical grade, Sigma) and stored frozen at −70 °C until used. Antileishmanial activity was assessed on the referenced strain *L. infantum* (MHOM/FR/78/LEM75).

2.2. Mathematical models for prediction of physicochemical and biological properties

Predictive values calculated for lipophilicity (LogP representing *n*-octanol/water partition coefficient) and solubility in water (LogS), were estimated by mathematical methods using ALOGPS v.2.0 software according to the methodology described by Tekto et al. (2001). Prediction of acid–base ionization constant pK_a was performed by Pallas 2.1 software (Compudrug International, San Francisco USA). Predictive values of antileishmanial activity were investigated using the chemistry software server PASS for prediction of biological activities spectrum (http://www.ibmh.msk.su/PASS/), according to the mathematical model and the database developed by Poroikov et al. (2000) and Lagunin et al. (2000).



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	Compounds	n-octanol/water partition coefficient LogP	Solubility in water LogS	pKa	
				Pyrrole group	Pyridinium group
1	Harman	2.71	−2.71	14.92	8.08
2	Harmine	3.22	−3.54	15.0	8.55
3	Harmaline	3.39	−3.33	16.29	–

Fig. 1. Structure and physicochemical properties of β-carboline alkaloids.

2.3. Antileishmanial activity on promastigotes

Leishmania infantum promastigotes in late log-phase were incubated in RPMI medium supplemented with 12% foetal calf serum, at an average of 10^5 cells/ml and a range of alkaloid concentrations was aseptically incorporated into duplicate cultures (final DMSO concentration less than 5%). Following a 48 h incubation period at 25 °C, promastigote growth was estimated by counting parasites with an hemacytometer. IC_{50} was determined as the concentration of drug necessary to inhibit 50% of parasite growth.

2.4. Antileishmanial activity on intracellular amastigotes

Intracellular amastigote cultures were performed in human monocyte-derived macrophages according to the methodology previously described by Ogunkolade et al. (1990). Maturation of monocytes into adherent macrophages was performed by treating exponentially growing monocytes (10^5 cells/ml) with 1 μM phorbol myristate acetate (Sigma). After a 48 h incubation period at 37 °C (5% CO_2) in chamber-slides (Fisher, Paris, France), cells were rinsed with fresh medium and suspended in RPMI medium containing stationary-phase promastigotes (cells/promastigotes ratio = 1/10). After a 24 h incubation period at 37 °C (5% CO_2), promastigotes were removed by 4 successive washes with fresh medium. Adapted dilutions of chemical compounds were added in duplicate chambers and cultures were incubated for 96 h at 37 °C (5% CO_2). Negative controls treated by solvent (DMSO) and positive controls containing a range of amphotericin B concentrations were added to each set of experiments. At the end of the incubation period, cells were harvested with analytical grade methanol (Sigma) and stained with 10% Giemsa stain (Eurobio, Paris, France). The percentage of infected macrophages in each assay was determined microscopically at 1000 times magnification by counting at least 300 cells in each sample. IC_{50} was defined as the concentration of drug necessary to produce a 50% decrease of infected macrophages.

2.5. Toxicity toward human monocytes

The toxicity of β-carboline alkaloids was assessed on human monocytes maintained in RPMI medium (Eurobio, Paris, France) supplemented with 10% foetal calf serum (Eurobio, Paris, France) at 37 °C in 5% CO_2 and replicated every 7 days. A range of concentrations was incorporated in late log-phase monocytes (10^5 cells/ml) and cultures were incubated at 37 °C with 5% CO_2. After a 72 h incubation period, cell growth was measured by

counting monocytes in an hemacytometer. IC_{50} was defined as the concentration of drug required to induce a 50% decrease of cell growth. An in vitro Selective Index (*SI*), corresponding to the ratio between antiparasitic and cytotoxic activities, was calculated according to the following formula $SI = IC_{50}$ versus human monocytes/ IC_{50} versus intracellular amastigotes.

2.6. Effects of β-carboline alkaloids on promastigote cell cycle and protein synthesis

Cell cycle and protein synthesis were assessed according to the protocol described by Crissman et al. (1990). Duplicate cultures of *L. infantum* promastigotes (10^5 cells/ml) in late log-phase were incubated at 25 °C in RPMI medium supplemented with 12% foetal calf serum and various concentrations of alkaloids were added. After a 28 h incubation period, corresponding to the length of time necessary to a maximal DNA and protein synthesis in promastigotes, cells were fixed with 70% methanol, centrifuged at 1500 rpm for 5 min, and suspended in 1 ml RNase solution (50 μg/ml in PBS buffer) at 37 °C for 30 min. DNA was stained with 5 μl propidium iodide (1 mg/ml) and total protein content was stained by 5 μl fluorescein isothiocyanate (FITC, 1 mg/ml).

2.7. Effects of β-carboline alkaloids on intracellular lipid droplets

Neutral lipid and polar lipid droplets were assessed according to the methodology described by Greenspan et al. (1985). *L. infantum* promastigotes (10^5 cells/ml) in late log-phase were incubated at 25 °C in RPMI medium supplemented with 12% foetal calf serum and various concentrations of alkaloids were added in duplicate. After a 48 h incubation period, cells were centrifuged at 1500 rpm during 5 min and suspended in PBS buffer. Nile Red (Sigma) 1 μg/ml final concentration was incorporated into each assay, cells were incubated at room temperature during 7 min and submitted to three successive washings with PBS buffer. Lipid droplets were analysed by flow cytometry. Fluorescence measured in parasites incubated with 5 μM ketoconazole was taken as positive control.

2.8. Effects of β-carbolines on plasma membrane potential

Membrane potential was analysed according to a methodology described by Shapiro (2000) and adapted by Azas et al. (1997), using a carbocyanine dye, 3,3′-dipentyloxacarbocyanine iodide $DIOC_{5(3)}$.Duplicate cultures of promastigotes in exponential growing phase containing a range of alkaloid concentrations were incubated for 3 h at 25 °C. At the end of the incubation period, $DIOC_{5(3)}$ was incorporated into each assay at the final concentration of 0.5 μM and promastigotes were analysed by flow cytometry. Fluorescence measured in parasites incubated with 7.5 μM amphotericin B was taken as positive control.

2.9. Effects of β-carbolines alkaloids on non-protein thiol contents

Promastigotes in late log-phase were incubated at 25 °C in RPMI medium supplemented with 10% foetal calf serum at an average density of 10^5 cells/ml and a range of alkaloid concentrations was incorporated into duplicate cell cultures. After a 24 h incubation period, non-protein thiols were assessed according to the technique developed by O'Connor et al. (1988): parasites were rinsed in ice-cold PBS buffer and centrifuged 5 min at 1500 rpm. Then the cell pellet was suspended in 1 ml mercury orange/acetone (100 μM) and incubated on ice for 5 min. Following incubation, the suspensions were centrifuged twice and suspended in ice-cold PBS until analysis.

2.10. Effects of β-carboline alkaloids on promastigote mitochondrial potential

Variations in the mitochondrial potential induced by β-carboline derivatives were measured by tetramethylrosamine (Molecular Probes) incorporation. Duplicate promastigote cultures (5×10^5 cells/ml) in exponential log-phase were treated with various concentrations of alkaloid derivatives and incubated at 25 °C during 30 min. At the end of the incubation period, parasites were harvested by 10 min centrifugation (1500 rpm) and suspended in fresh medium containing tetramethylrosamine 200 nM final concentration (Sigma). Following 30 min of incubation at 25 °C, cells were centrifuged, rinsed with fresh medium and analysed by flow cytometry. Fluorescence measured in parasites incubated with 7.5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone) was taken as positive control.

2.11. Flow cytometric analysis

Cells were run on a FacScan analytical flow cytometer (Becton–Dickinson, Paris, France) equipped with a 15-mV, 488 nm air-cooled argon ion laser. Ten thousand cells were acquired for each analysis, on the basis of their green or red fluorescence. The percentages of parasites in the different phases of the cell cycle were analysed by using the modfit software (Becton–Dickinson, Paris, France).

2.12. Effects of harmaline on promastigote and extracellular amastigote viability

Promastigotes were incubated in RPMI medium supplemented with 12% foetal calf serum and incubated

at 25 °C. Amastigotes were obtained from human macrophages previously infected with promastigotes according to the protocol described by Ogunkolade et al. (1990). They were transferred into RPMI medium supplemented with 20% foetal calf serum titrated to pH 5.5 and incubated at 37 °C (5% CO2). Under these conditions, extracellular amastigotes could be maintained during more than one week. Various concentrations of harmaline were aseptically incorporated into duplicate promastigote and extracellular amastigote cultures and incubated at 25 and 37 °C, respectively. Following a 48 h incubation period, parasite viability was estimated by flow cytometry after staining with 1 μM propidium iodide.

2.13. Effects of harmaline on nitric oxide production by macrophages

Maturation of human monocytes into adherent macrophages was performed by treating exponentially growing monocytes (10^5 cells/ml) with 1 μM phorbol myristate acetate (Sigma). After a 48 h incubation period at 37 °C (5% CO_2) in chamber-slides (Fisher, Paris, France), cells were rinsed with fresh medium and suspended in RPMI medium containing various concentrations of alkaloids, in presence or absence of 10 U/ml human recombinant interferon-γ. After 48 h at 37 °C, NO production was measured by assessing the nitrite content of culture supernatants by the method described by Ding et al. (1988): 100 μl of fresh Griess reagent were added to equal volumes of culture supernatants and the optical density at 540 nm was measured after 15 min of incubation at room temperature. Nitrite concentrations were determined by using $NaNO_2$ diluted in RPMI as the standard.

2.14. Effects of harmaline on Leishmania internalization by macrophages

Maturation of monocytes into adherent macrophages was performed by treating exponentially growing monocytes (10^5 cells/ml) with 1 μM phorbol myristate acetate (Sigma). After a 48 h incubation period at 37 °C (5% CO_2) in chamber-slides (Fisher, Paris, France), cells were rinsed with fresh medium. Various concentrations of harmaline were incorporated into duplicate cultures of *Leishmania* promastigotes in late log-phase and incubated at 25 °C. After one hour, harmaline was removed by 3 successive washes and promastigotes were added in macrophage cultures (cells/promastigotes ratio = 1/10). Following 18 h at 37 °C (5% CO_2), *Leishmania*-macrophage co-cultures were fixed with methanol and stained with 10% Giemsa stain. The percentage of macrophages containing intracellular parasites was analysed microscopically at 1000 times magnification by examining 300 cells at least.

2.15. Effects of harmaline on Leishmania protein kinase C activity

The effects of harmaline on protein kinase C (PKC) activity were assessed by an enzyme-linked immunosorbent assay kit (calbiochem, Paris, France) that used a synthetic PKC pseudosubstrate (RFAARKGSLRQK NV) and a monoclonal antibody that recognized the phosphorylated form of the peptide. Various concentrations of harmaline were incorporated into duplicate cultures of *Leishmania* promastigotes in exponential growing phase and incubated during one hour at 25 °C. PKC activity measured in promastigotes treated with 50 nM of RO-32-0432, a specific PKC inhibitor, was taken as positive control. At the end of the incubation period, promastigotes were centrifuged at 1500 rpm for 5 min and suspended in 100 μl buffer containing 25 mM Tris–HCl, pH 7, 3 mM $MgCl_2$, 0.1 mM ATP, 2 mM $CaCl_2$, 50 μg/ml phosphatidylserine, 5 mM β-mercaptoethanol, 1 mM EGTA, 0.5 mM EDTA, 1 mM PMSF, and 10 mM benzamidine. The mixtures were incubated during 30 min at 25 °C and the biotinylated antibody was added into each well. PKC activity was revealed by a peroxidase-conjugated streptavidin antibody coupled to the *ortho*-phenylenediamine substrate and measured at 492 nm.

3. Results

Complete data concerning the physicochemical properties of β-carboline alkaloids are reported in Fig. 1. Lipophilicity was estimated by prediction of *n*-octanol/water partition coefficient LogP, defined as the ratio of concentration in an immiscible solvent such as *n*-octanol to the concentration in the aqueous phase. Both alkaloids exhibited *n*-octanol/water partition coefficients greater than 1, indicating that they possessed high lipophilic properties, consequently they were poorly soluble in water. Prediction of the acid–base ionization constants pK_a demonstrated that harmane displayed the lowest pK_a value, indicating that its absorption in biological organisms could be easier than those of harmine and harmaline.

Predictive values for biological activities as well as antileishmanial and cytotoxic properties of β-carboline alkaloids are reported in Table 1. Predictive probabilities *Pa* were obtained by comparing the chemical structure of each compound with structures or substructures of more than 30,000 well-known biologically active drugs. They represented the probability *Pa* of each compound to be active and illustrated its degree of similarity with well-known antileishmanial molecules. On this basis, $Pa > 0.7$ signified that the corresponding compound was very likely to reveal activity in experiments, $0.5 < Pa < 0.7$ indicated that the compound was

Table 1
Antileishmanial activity and toxicity of β-carboline alkaloids

	Predictive antileishmanial activity *Pa*	Toxicity versus monocytes (μM)		Antileishmanial activity IC_{50} (μM)		*SI*
		LC_{50}	IC_{50}	Promastigotes	Amastigotes	
Harmane	0.471	120.8	22.1	19.2	0.27	81
Harmine	0.502	82.5	17.5	3.7	0.23	73
Harmaline	0.517	>200	>200	116.8	1.16	>170
Amphotericin B		21.2	13.5	0.036	0.023	585

Pa, predictive values of antileishmanial activity calculated according to the mathematical model and the database developed by Poroikov et al. (2000) and Lagunin et al. (2000) by comparison with the molecular structures of well-known antileishmanial agents.

LC_{50}, concentration that reduced by 50% the percentage of viable monocytes.

IC_{50}, concentration that reduced by 50% the number of monocytes, promastigotes or infected macrophages.

SI, *selectivity index* corresponding to the ratio between antiparasitic and cytotoxic activities, according to the following formula $SI = IC_{50}$ versus human monocytes/IC_{50} versus intracellular amastigotes.

likely to reveal activity in experiments, while $Pa < 0.5$ implied that the compound was unlikely to reveal activity in experiments. Predictive probabilities *Pa* presented in Table 1 were between 0.4 and 0.6: they showed that all alkaloids were likely to reveal interesting antileishmanial activity. Harmaline displayed the higher value of *Pa* (0.517), indicating that its chemical structure was closely similar to those of active compounds.

Cytotoxicity was assessed on human monocytes. Harmane and harmine exhibited a moderate cytotoxicity ($LC_{50} = 120.8$ and 82.5 μM, respectively) associated with a strong antiproliferative activity ($IC_{50} = 22.1$ and 17.5 μM, respectively), while harmaline appeared far less active (LC_{50} and $IC_{50} > 200$ μM).

The antileishmanial activity of alkaloids was evaluated on both extracellular flagellated promastigote and intracellular amastigote forms. Harmine appeared the most efficient compound toward the promastigote stage of the parasite ($IC_{50} = 3.7$ μM), however both harmane and harmine strongly inhibited the growth of intracellular amastigotes ($IC_{50} = 0.27$ and 0.23 μM, respectively) with a moderate selectivity ($SI = 81$ and 73, respectively). Harmaline weakly inhibited the growth of *Leishmania* promastigotes ($IC_{50} = 116.8$ μM), but it showed an interesting amastigote-specific activity with a $IC_{50} = 1.16$ μM and a Selective Index greater than 170.

The effects of β-carboline alkaloids on *Leishmania* biochemical pathways are presented in Table 2. Cell-cycle was investigated after a 24 h treatment as compared to the well-known antileishmanial agent pentamidine. Results showed that, in contrast to pentamidine which induced more than 40% of S–G_2M phase drop, the β-carbolines harmane, harmine, and harmaline increased of the percentages of promastigotes in the S–G_2M phase of the cell-cycle. Harmane was the most efficient compound, since it induced a 33% increase of S–G_2M phases as compared to the control culture while the effects of harmine and harmaline on the cell-cycle were less evident. Total protein, non-protein thiol, and lipid droplet contents were assessed as compared to the effects of pentamidine, allopurinol, and ketoconazole. Results demonstrated that β-carboline alkaloids significantly affected neither protein synthesis nor non-protein thiol metabolism. They also indicated that lipid metabolism was not strongly modified after a 24 h incubation period. The effects of harmane, harmine, and harmaline on plasma or mitochondria membrane potentials were studied as compared to those of amphotericin B and FCCP. No direct activity could be observed on the external and mitochondrial membranes of *Leishmania* parasites.

The effects of harmaline on the viability of promastigotes and axenically growing amastigotes are reported in Fig. 2. The β-carboline alkaloid appeared weakly toxic toward both the extracellular amastigote and promastigote forms of the parasite with IC_{50} upper

Table 2
Effects of β-carboline alkaloids on *Leishmania* biochemical pathways

	Leishmania intracellular entities observed at IC_{50} (Percentages compared to the control culture)						
	S + G_2M phases	Non-protein thiols	Protein contents	Membrane potential	Polar lipids	Neutral lipids	Mitochondrial potential
Harmane	133.5	94.2	91.6	99.4	93.1	99.9	99.8
Harmine	128.2	93.1	95.4	98.9	100.4	100.1	98.9
Harmaline	105.4	95.6	98.3	99.2	97.2	98.6	100.1
Pentamidine	58.4	70.4	—	—	—	—	—
Allopurinol	—	—	48.2	—	—	—	—
Amphotericin B	—	—	—	60.1	—	—	—
Ketoconazole	—	—	—	—	78.2	90.1	
FCCP	—	—	—	—	—	—	12.4

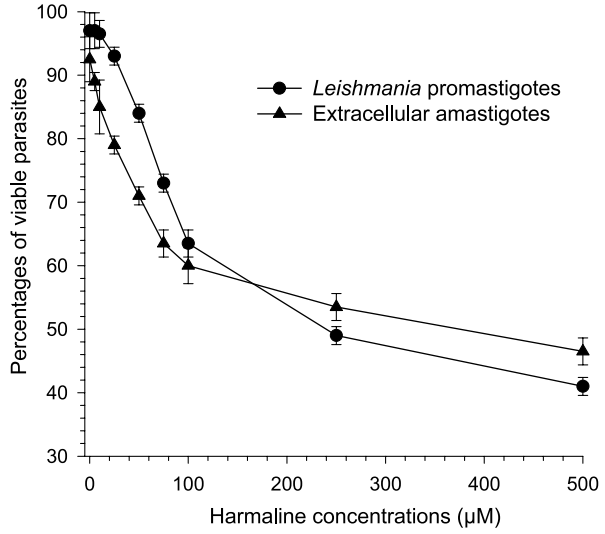

Fig. 2. Effects of harmaline on the viability of *Leishmania* promastigotes and extracellular amastigotes.

than 100 μM, suggesting that the compound could exert its antileishmanial activity by interfering with parasite–macrophage interactions. As a consequence, the effects of harmaline on NO production by macrophages and *Leishmania* internalization were investigated. Results observed in macrophage culture medium demonstrated that no increase in NO concentrations could be observed in both inactivated and LPS activated macrophages (data not shown) after pre-treatment by harmaline and established that harmaline did not prevent *Leishmania* infection by inducing NO production in macrophages. Results concerning internalization of *Leishmania* promastigotes into macrophages are displayed in Fig. 3. After one hour pre-treatment of promastigotes by harmaline, a strong reduction of parasite internalization could be observed, suggesting that the β-carboline alkaloid could inhibit parasite recognition and/or penetration within macrophages. Assessment of PKC activity confirmed this statement since a dose-related inhibition of PKC activity could be observed in *Leishmania* promastigotes after one hour pre-treatment with harmaline.

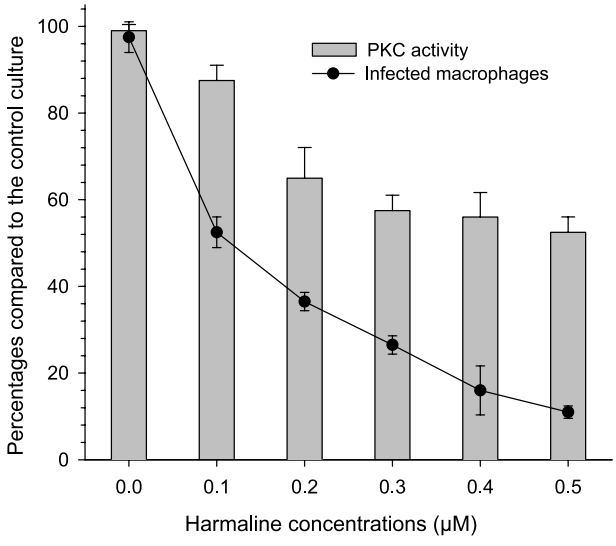

Fig. 3. Effects of harmaline on *Leishmania* internalization by macrophages and PKC activity in promastigotes.

4. Discussion

Due to their presence in a wide range of foodstuff, beverages, and smokes, harmane, harmine, and harmaline, the β-carboline alkaloids occurring in medicinal plants are now considered as common dietary contaminants (Herraiz, 2000). However, they display a wide range of biological properties including genotoxic (Boeira et al., 2001), spasmolytic (Shi et al., 2001), antiplatelet (Tsuchiya et al., 1999), vasorelaxant (Shi et al., 2000), and tremorogenic (Totsuka et al., 1999) activities.

Results observed in the present study confirmed that some elements of the β-carboline alkaloid series could reveal interesting in vitro antileishmanial activities. Harmane and harmine exhibited a moderate antiproliferative activity toward human cells in relation with a possible anticancer activity. They also inhibited the growth of both the promastigote and the amastigote stages of *Leishmania* parasites with a moderate selectivity. In promastigotes, their antiproliferative activity was principally due to interactions with DNA metabolism and resulted in a significant accumulation of parasites in the S–G_2M phases of the cell cycle. This mechanism of action, probably due to the planar structure of the molecules, was not specific of the protozoan parasite since it has been demonstrated that harmane and harmine could interact directly or indirectly with DNA of bacteria and mammalian cells by intercalating with the base-pairs or inhibiting DNA enzymes such as topoisomerases (Funayama et al., 1996). The principal consequences of this interaction consisted in genotoxic and mutagenic events including induction of SOS response and frameshift mutations in bacteria (Oda et al., 1988) and production of single and/or double strand breaks and chromosome aberrations in mammalian cells (Boeira et al., 2001). The chemical structures and the toxicokinetic properties of the two molecules have been shown very close since harmine constituted the main metabolite of harmane after oral administration in rats (Gyan et al., 2001). Nevertheless, harmane induced a stronger S–G_2M phase arrest in *Leishmania* promastigotes than harmine, suggesting that the presence of a methoxy side chain on the polyheterocyclic molecule decreased the capacity of the compound to interact with macromolecules. This difference could explain the fact that harmane presented higher in vitro mutagenicity than harmine (Picada et al., 1997).

Additional effects on the cytoplasmic targets of *Leishmania* could not be detected in the present study, however inhibition of several non-investigated enzymatic activities should not be excluded since β-carboline alkaloids have been shown to inhibit monoamine oxidases in mammalian cells (Glover et al., 1982). Harmaline exhibited a weak antiproliferative activity toward human cells and promastigotes, however it revealed a strong antiproliferative activity toward the intracellular amastigote stage of the parasite, suggesting that it could exert another interaction mechanism with *Leishmania* parasite that does not imply the classical DNA intercalation binding. This mechanism, probably due the steric hindrance resulting from the absence of one double bound in harmaline, was associated with a low toxicity toward human cells and could result in modulation of amastigote-specific biochemical pathways or host–parasite relationships. Additional experiments performed on promastigotes and axenically growing amastigotes did not confirmed the first hypothesis since harmaline did not exert a strong activity toward extracellular amastigotes. However, they demonstrated that the compound protected human macrophages from *Leishmania* parasitism by reduction of parasite internalization within macrophages. Assessment of PKC activity indicated moreover that harmaline could inhibit the action of PKC in *Leishmania* promastigotes.

Protozoa of the genus *Leishmania* are obligate intracellular parasites that multiply almost exclusively inside cells of the mononuclear phagocytic system of mammalian hosts. After the inoculation of infective promastigotes by the bite of parasitized sand fly through the dermis, the survival of the parasites has been shown to depend greatly on their capacity to overcome the immune system of the host (Bogdan et al., 1996) and to adhere and internalize into resident or recruited macrophages (Sibley and Norma, 2000). For this purpose, parasites have developed various phosphorylation reactions that contribute to escape mechanisms (Hammarton et al., 2001) and participate to *Leishmania* growth, metabolism, differentiation, and interaction with host cells (Bente et al., 2003; Hermoso and Jaffe, 1993). In particular, protein phosphorylation by enzymes of the protein kinase C family has been established as an important step in the processes of adhesion to macrophage membrane and internalization (Vannier-Santos et al., 1995) and various studies have demonstrated that variations in *Leishmania* PKC activity could greatly modulate parasite burden in macrophages. Results observed in the present study suggested that inhibition of promastigote internalization within macrophages could result from inhibition by harmaline of PKC activity. However, additional effects on other *Leishmania* protein kinases involved in host cell invasion and intracellular growth could be envisaged since only a slight drop in PKC activity could be observed. In particular, inhibition of MAP kinases involved in parasite growth in the parasitophorus vacuole of macrophages (Wiese, 1998) could explain the antileishmanial activity of the compound on established macrophage infection.

In conclusion, results observed in the present study confirmed the antileishmanial activity of β-carboline alkaloids. Due to their strong toxicity and their possible genotoxicity in mammalian cells, harmane and harmine could not represent interesting therapeutic antileishmanial agents, however their presence in several medicinal plants might contribute to prevent antileishmanial infections in populations using traditional medicine. Harmaline, the most active compound, was deprived from toxicity toward human cells and exhibited a non-conventional mechanism of action based on inhibition of PKC activity in *Leishmania* promastigotes leading to a reduction of parasite internalization within macrophages. Chemically induced modulation of parasite recognition by macrophages or reduction of macrophage phagocytic properties could influence parasite infection, and various compounds, including oxidant molecules and neuropeptides, demonstrated in vivo antileishmanial activities in relation with inhibition of parasite internalization (Sibley and Norma, 2000). On this basis, inhibition of *Leishmania* internalization within macrophages could represent a promising mechanism of action for β-carboline alkaloids and other antileishmanial candidates.

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