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Inhibition of Parasite Protein Kinase C by New Antileishmanial Imidazolidin-2-one Compounds

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The protein kinase C (PKC) family of isoenzymes mediate a wide range of signal transduction pathways in many different cells lines. Little is known regarding the presence and functional roles of PKC in *Leishmania* spp. Here we report the inhibition of parasite PKC by new imidazolidinone compounds. The most active derivative 7 showed an important activity (IC_{50} = 9.9 μM) **against the clinical relevant stage of parasites in comparison with Glucantime ®** (IC_{50} = 464.5 μM), **without inducing toxicity on human fibroblast cells** (IC_{50} = 102 μM). **Pretreatment of intact parasites with 10 μM of compound 7 inhibited 80% of PKC activity. At the same concentration, this compound inhibited 70% of the parasite-host cell invasion process. An *in vivo* model showed that compound 7 reduced the liver parasite burden by 25% and spleen parasite burden by 44%. These results provide the first evidence that PKC plays a critical role in the invasion process. Thus *Leishmania* PKC activity could be a relevant therapeutic target and the imidazolidinones novel antileishmanial candidates.**

Keywords: Leishmania; Imidazolidin-2-one compounds; Invasion process; Protein kinase C

INTRODUCTION

Protein phosphorylation by PKC is among the most important control mechanisms of cellular processes.[1,2] Usually, acting on lateral chains of serine or threonine, PKC uses ATP as a phosphate donor. PKC consists of a family of ten isoenzymes that have been currently identified as PKCs α, β_I, β_{II}, γ, δ, ϵ, ξ, η, θ, λ.[3,4] Isotypes α, β_I, β_{II} and γ contain a catalytic domain including an ATP binding site, and a regulatory domain accommodating a Ca^{++}, diacylglycerol (DAG) and phospholipid binding sites. In protozoan parasites, PKC has been implicated in some cellular processes.[5,6] A PKC participates in *Toxoplasma gondii* and *Entamoeba histolytica*-host cells invasion process.[7,8] *Trypanosoma brucei* possesses a PKC that may be involved in parasite growth and differentiation.[9] In some reports, inhibition of cellular responses by potential PKC inhibitors such as H7, staurosporine and K252a has been currently questioned because of their poor selectivity.[10,11] Nevertheless, H7 inhibits PKC in the intact cell only at very high concentrations, whereas staurosporine and K252a are known to inhibit a wide range of serine-threonine kinases and tyrosine kinases.

Little is known regarding the presence and functional roles of PKC in *Leishmania*. We have recently identified the presence of a PKC in *Leishmania* promastigotes and its participation in the early events of the parasite-macrophage interaction process.[12] The involvement of PKC in regulation of cell interactions, ligand binding and signal transduction makes it an attractive previously unknown target for drug development against intracellular parasites.

New imidazolidin-2-one compounds have previously demonstrated *in vitro* and *in vivo* activity against *L.(L). mexicana*.[13] As previously stated in mammalian cells,[14] its mechanism of action could

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Abbreviations: PKC, protein kinase C; MTT, 3-(4-5-Dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether); N,N,N′, N′-tetraacetic acid; PMSF, phenylmethane sulphonyl fluoride.

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originate from direct interference with the parasite phospholipase A_2 (PLA_2) activity or more likely from blockade of an activator located upstream from the PLA_2 activation pathway, such as PKC.

In this report, we have evaluated the *in vitro* and *in vivo* antileishmanial activity of N^1-(2-thiazolyl) and (5-isoxazolyl)-imidazolidin-2-ones and their effect on parasite PKC activity.

MATERIALS AND METHODS

Chemicals

Imidazolidin-2-one derivatives were synthesized at the Laboratory of Medical Chemistry of the Faculty of Pharmacy, University of Nantes, France. PKC specific inhibitor (RO-32-0432) and PKC enzyme-linked immunosorbent assay were purchased from Calbiochem, France. Schneider's insect medium, fetal calf serum, penicillin, streptomycin, aprotinin, leupeptine, MTT and PMSF were obtained from Sigma, St. Quentin Fallavier, France. Meglumine antimoniate (Glucantime®) was obtained from Specia Rhône-Poulenc, France.

Biological Materials

Animals

Male Balb/c mice (R. Janvier, Le Genest, France) were handled in accordance with existing protocol for animal treatment as embodied in the Guiding Principles of Biochemical Research.

Parasites

L.(L). infantum (MHOM/FR/91/LEM2259) strain was cultured at 26°C in Schneider's insect medium supplemented with 15% of fetal calf serum, penicillin (100 UI/ml) and streptomycin (100 μg/ml). Promastigotes were harvested in the Log stage (3–4 days) or stationary stage (5–7 days).

In Vitro Antileishmanial Activity

Logarithmic *L.(L). infantum* promastigotes (2×10^6/ml) were inoculated into 96-well plates and exposed to different concentrations of imidazolidinone derivatives. Cultures were incubated for 96 h at 26°C. The anti-proliferative effect was determined by the MTT method based on the tetrazolium salt reduction by mitochondrial dehydrogenases. Absorbance was determined at 570 nm[15] and IC_{50} values were determined by linear regression.

Antileishmanial activity in intracellular amastigotes was studied in a *Leishmania*-Balb/c infected macrophages model, incubating at 37°C in 5% CO_2. After treatment with imidazolidinone derivatives for 96 h, cells were fixed and stained with May-Grünwald-Giemsa.[16] Results were expressed as the percentage reduction of infected cells and parasite loads in comparison with controls.

In Vivo Antileishmanial Activity

Mice were injected intravenously with 2×10^6 stationary promastigotes in a volume of 100 μl. One week after infection, mice (8 animals/group) were treated intraperitoneally with a single injection (200 μl) of 10 mg of drug per Kg of body weight during ten consecutive days. Meglumine antimoniate (10 mg/Kg) was also diluted into 0.9% NaCl solution (1:15). Glucantime® injections were administred subcutaneously (200 μl/mouse). Infected mice were sacrified at the fourth week post infection and the liver and spleen were removed and weighed. A limiting-dilution culture assay was performed in order to quantitate the number of viable *L.(L). infantum* parasites in the tissue. Results were expressed as (-)log parasite titer.

Cytotoxicity

Cytotoxicity of compounds was studied with human fibroblasts (MRC5). After incubation for 96 h with the compounds, the cytotoxic effect was measured with an Alamar blue® fluorochrome reagent (Interchim, Montluçon, France).[17]

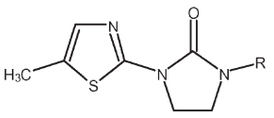
Protein Kinase C Assay

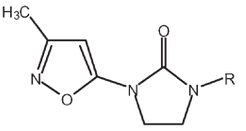
Inhibition of protein kinase C activity was measured by an enzyme-linked immunosorbent assay that uses a synthetic PKC pseudosubstrate (RFAARKGSL-RQKNV) and a monoclonal antibody (2B9) that recognizes the phosphorylated form of the peptide. After treatment with imidazolidin-2-one or a PKC-specific inhibitor (RO-32-0432), intact promastigotes were suspended in 100 μl of mixture buffer containing 25 mM Tris-HCl, pH 7.0, 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. After incubation for 30 min at 30°C, the biotinylated antibody 2B9 was added to each well. PKC activity was revealed by a peroxidase-conjugated streptavidin antibody coupled to ortho-phenylenediamine substrate. Plates were read at 492 nm. PKC activity was expressed in U/min/mg of protein.

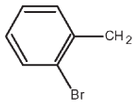
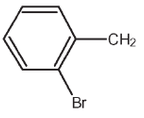
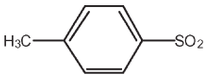
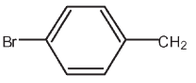
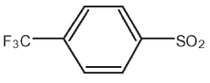
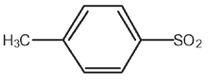
Invasion Inhibition Assay

Inhibition of parasite invasion by imidazolidinone derivatives was studied in a *L.(L). infantum*-Balb/c infected macrophages model. Adherent Balb/c macrophages were cultured in RPMI 1600 medium

TABLE I *In vitro* antileishmanial activity on *L.(L). infantum promastigotes* and cytotoxicity (MRC5 cells) of imidazolidin-2-one compounds.





Compound No.	R	*L.(L). infantum**	MRC5 cells*	Compound No.	R	*L.(L). infantum**	MRC5 cells*
1	H	163.8	131.6	**5**	H	179.4	108.3
2		20.4	96.8	**6**		33.9	55.3
3		17.0	91.0	**7**		9.5	102.0
4		76.6	84.6	**8**		93.2	52.3

Results were expressed in μM. Data are means of triplicates. *IC_{50}.

supplemented with 15% fetal calf serum and antibiotics. Stationary promastigotes (2×10^6 cells/ml) were pretreated for 1 h with RO-32-0432, a PKC inhibitor, or imidazolidin-2-one compounds. Tested concentrations were not toxic for the parasites. After 3 washes, promastigotes were added to 24-wells microplates (2 wells/concentration) containing adherent macrophages (parasite:macrophage ratio, 2:1). After a 18-h contact period and three washes, infected macrophages were stained with May–Grünwald–Giemsa. Results were expressed as the percentage of infected cells in comparison with controls. At least 100 cells from each well were counted.

RESULTS AND DISCUSSION

Leishmaniasis is a vector born disease constituting a major world public health problem. More than 300 million people live and travel to tropical and subtropical risk areas and nearly 12 million people are infected and three million suffer from the disease.[18,19] Unfortunately, the therapeutic armentarium remains very limited. The first line compounds are the two pentavalent antimonials, sodium stibogluconate and meglumine antimoniate. The second line drugs are composed of pentamidine and amphotericin B. Because of the frequent relapses, adverse effects, high toxicity and resistance emergence to standard therapy, several investigations are directed towards searching for new compounds and parasite targets for better and safer treatment.

The *in vitro* antiproliferative effect of imidazolidin-2-one compounds against *L.(L). infantum* promastigotes is summarized in Table I. IC_{50} values varied from 9.5 to 179.4 μM. The most active derivative **7** showed high activity (IC_{50} = 9.9 μM) against the clinically relevant stage of the parasite (intracellular amastigotes) in comparison with the reference treatment compound (IC_{50} = 464.5 μM). Activity–toxicity relations showed that **7** possesses a high therapeutical index with IC_{50} = 102 μM on human fibroblast cells (MRC5)

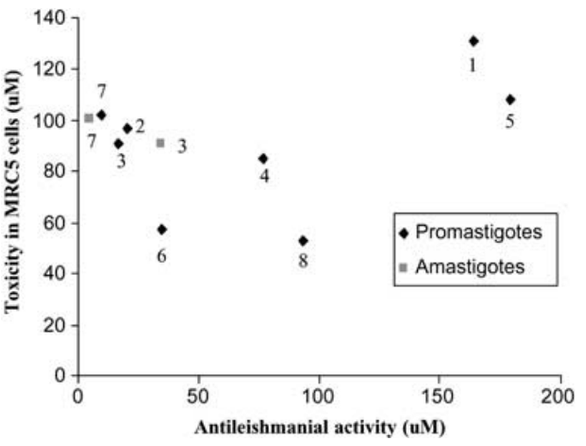


FIGURE 1 Relationship between antileishmanial activity and toxicity in MRC5 cells for imidazolidin-2-one compounds.

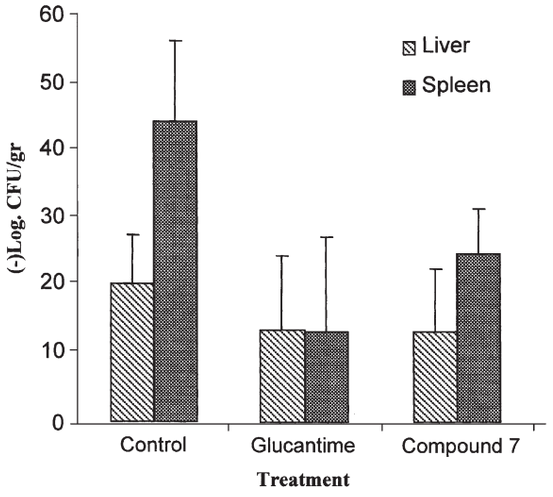


FIGURE 2 Inhibitory effect of imidazolidin-2-one compound **7** in liver and spleen cultures. (CFU = colony forming unit).

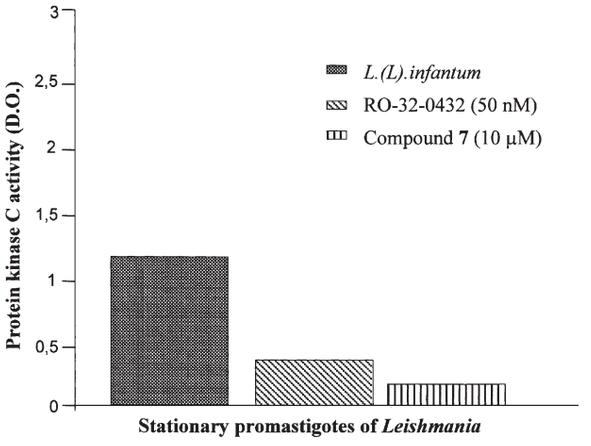


FIGURE 3 Inhibition of *L.(L). infantum* Ecto-PKC activity from stationary promastigotes by treatment with RO-32-0432 or compound **7**.

(Figure 1). In order to determine the *in vivo* activity of compound **7**, a *L.(L). infantum*-infected Balb/c mice model was used. The parasite means for growth in liver and spleen cultures in controls and treated mice are gathered in Figure 2. Compound **7** reduced the liver parasite burden by 25% and spleen parasite burden by 44%. In contrast, Glucantime® showed a reduction of 35% and 70%, respectively.

Previous studies have bought to the fore that these antileishmanial drugs have brought about under the same conditions an anti-inflamatory effect through PLA_2 inhibition.[13] These studies suggested that imidazolidin-2-one compounds have a direct antiparasitic effect through a perturbation of phospholipid membrane homeostasis. Because PLA_2 activity is increased after phosphorylation by PKC, new compounds of the imidazolidin-2-one series were tested against *L.(L). infantum* PKC. Figure 3 shows that pretreatment of intact parasites with 50 nM of the specific standard PKC inhibitor RO-32-0432 inhibed 72% of PKC activity. However when parasites were treated with the imidazolidin-2-one compound (**7**) at a concentration around its antileishmanial IC_{50} (10 μM), PKC activity was reduced by 80%. Percentage inhibition obtained on PLA_2 activity is less significant compared with that obtained on PKC activity. These results seem to confirm that PLA_2 inhibition could reflect an effect on parasite PKC. In the kinetoplastid parasites such as *Leishmania* spp. and *Trypanosoma* spp., the morphological and metabolical changes during their life cycle include cell surface-receptor-coupled signal transduction pathways. However, little is known about the second messengers such as PKC isoforms and their biological functions. We have previously demonstrated that an ecto-PKC α, β_I, β_{II} is present in stationary *Leishmania* promastigotes.[12] This enzyme activity is also detected in both membrane and cytosolic fractions of parasite. As occurs in *Trypanosoma cruzi* and *Trypanosoma brucei* PKC,[6,9] this *L.(L). infantum* PKC enzyme is Ca^{2+}/phophatidylserine-dependent for activity.

Leishmania protozoan are intracellular obligate parasites that possess many types of surface molecules involved in attachment and invasion. We have previously demonstrated that *Leishmania*-PKC is implicated in the invasion process by specific enzyme inhibition with RO-32-0432 inhibitor, before the early events of parasite-macrophage contact.[12]

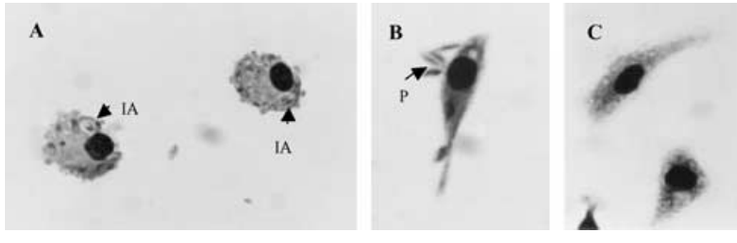


FIGURE 4 Invasion model showing non-treated promastigotes (A) which can invade macrophages and be transformed into intracellular amastigotes (IA). Imidazolidin-2-one treated promastigotes remain attached to cell membrane (B) and cannot invade macrophages (C).

Instead, RO-32-0432 leads to a 50% reduction of infected macrophage numbers at 100 nM. In this work we showed that the imidazolidin-2-one compound **7** inhibited the invasion process; at 10 μM, this compound was able to reduce the number of cells by 70% (Figure 4).

Taken together, these results provide the first evidence that PKC and protein phosphorylation play a critical role in attachment and internalization steps of parasitic invasion. In consequence, *Leishmania* PKC could be a relevant therapeutic target and N^1-(2-thiazolyl) and (5-isoxazolyl)-imidazolidin-2-ones novel antileishmanial candidates for further pharmacomodulation.

Acknowledgements

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