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ORIGINAL ARTICLE

Antibacterial, antifungal and antileishmanial activities of indolone-*N*-oxide derivatives

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An alarming increase in microbial resistance to traditional drugs and classical pharmacophores has spurred the search for new antimicrobial compounds. Indolone-*N*-oxides (INODs) possess a redox pharmacophore with promising, recently established, antimalarial activities. In this study, the anti-infectious properties of a series of INODs were investigated. The antibacterial activity was evaluated against five bacterial strains Gram-positive (*Staphylococcus aureus*, *Enterococcus hirae*), Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) and acid-fast (*Mycobacterium tuberculosis*). The antifungal activity was assessed using two fungal strains (*Aspergillus niger*, *Candida albicans*). The antileishmanial activity was tested against two leishmanial strains, axenically-cultured amastigote (*Leishmania infantum, Leishmania amazonensis*). The pharmacological activities are discussed as a function of structural and lipophilic characteristics. The Gram-positive bacterial strain *E. hirae* was found to be the most sensitive strain, whereas the Gram-negative *E. coli* was resistant to this family of compounds. One compound (64) was more potent than nalidixic acid against *E. hirae,* whereas another one (52) was equipotent as clotrimazole against *C. albicans*. INODs were microbe -cidal rather than -static. INODs showed good antitubercular activity in the low micromolar range (similar to ciprofloxacin). In addition, INOD-antiprotozoal potencies were confirmed against the leishmania parasite. INODs showed a broad spectrum of antimicrobial activity and offer a promising anti-infectious prototype worthy of being developed.
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INTRODUCTION

The emergence of microbial resistance to many drugs (multidrug resistance) increases owing to the use and misuse of antimicrobials in human medicine and animal husbandry in addition to the natural microbial mutations. The emergence of multidrug resistance strains and more recently extremely drug-resistant (XDR) mutants of certain bacterial strains, such as *Mycobacterium tuberculosis*, pose major difficulties in treatment.[1–3] However, despite a call for new antibiotic therapies, there has been a continued decline in the number of newly approved drugs.[2] There is no doubt that society needs new antibiotics and to overcome resistant strains, it seems crucial to search for new compounds with new pharmacophores and mechanisms of action.[4] Within this perspective, we recently designed and synthesized a series of indolone-*N*-oxides (INODs). This family of compounds has shown very promising antimalarial properties with low cytotoxicity.[5,6] The redox pharmacophore (nitrone function conjugated with a ketone group) is different from those of already commercialized antimicrobial agents. This pharmacophore possesses redox properties that create oxidative stress within the infected red blood cell leading to plasmodicidal activities.[7,8] Moreover, INODs show moderate binding affinity toward human serum albumin,[9] which may provide a basis for its rational use in clinical practice and for the development of new antimicrobial archetypes. INODs have the ability to generate radical adducts,[10] which may participate in the observed biological activities. In addition, the nitrone function may react with the glutathiyl radicals formed within the cells upon oxidation of glutathione[11] and these oxido-reducible molecules may disrupt the cellular redox homeostasis.

The objective of the present study was to explore the spectrum of activity of INOD compounds against several microorganisms, including five bacterial strains, two fungal strains and two leishmanial strains, to afford new hits for further pharmacomodulation.

MATERIALS AND METHODS

Chemicals

All the compounds tested were synthesized in our laboratory,[5] except compounds **69**, **70** and **71** that were kindly supplied by Idealp-Pharma (Lyon, France). Compound numbering was the same as in Nepveu *et al.*[5]

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Reagents for Leishmania and cell culture were purchased from Lonza (Basel, Switzerland) and reference drugs and G418 from Sigma-Aldrich (Saint Quentin Fallavier, France). Commercial reagents (Sigma-Aldrich) were used as received without additional purification.

Biological activities

Antibacterial (Gram-positive and Gram-negative)/antifungal assays. The tested compounds were dissolved in dimethylsulfoxide to obtain an initial concentration of 1000 μg ml^{-1}. Owing to compound availability or solubility problems, the initial concentrations were 100 and 500 μg ml^{-1} for compounds **64** and **21**, respectively. The resulting solutions were then diluted in microtiter plates in trypcase soy broth (Biomérieux, Craponne, France) for bacteria and Sabouraud broth (Biomérieux) for fungi. Gram-positive strains *Staphylococcus aureus* CIP 4.83 and *Enterococcus hirae* CIP 58.55, and Gram-negative strains *Pseudomonas aeruginosa* CIP 82118 and *Escherichia coli* CIP 53126 were used for the antimicrobial assays. For the antifungal assays, cultures of the yeast *Candida albicans* IP 48.72 and the mould, *Aspergillus niger* IP 1431.83 were used. The strains were obtained from the collection of the Pasteur Institute (Paris, France). Microbial suspensions were prepared in sterile distilled water to obtain final inocula of 10^6 cells ml^{-1} and 10^5 spores ml^{-1} for bacteria and fungi, respectively. The MICs and minimal germicidal concentrations (MBCs or MFCs) were determined after incubation of the bacterial strains at 37 °C and of the fungal strains at 30 or 22.5 °C for 24–48 h in the presence of serial dilutions of the test compounds (Table 1). The MIC was defined as the concentration at which no macroscopic sign of cellular growth was detected in comparison with the control without antimicrobial compound. The MBCs/MFCs were determined by subcultivating on corresponding agar plates after incubating the bacterial strains at 37 °C and the fungal strains at 30 or 22.5 °C for 24–48 h. The MBC/MFC was defined as the compound concentration at which no macroscopic sign of cellular growth was detected in comparison with the control without antimicrobial compound. All the experiments were carried out in duplicate at each concentration. In order to ensure that dimethylsulfoxide *per se* did not interfere with the antimicrobial activity of the products under assay, a control test was also carried out containing inoculated broth supplemented with only dimethylsulfoxide at the same concentration used in the assays. Bifonazole and clotrimazole were used as reference drugs in the antifungal assays and ciprofloxacin and nalidixic acid in the antibacterial assays. The MICs and minimal germicidal concentrations (MBC or MFC) determined are reported in Table 1.

Protocol of antitubercular assay. Inhibition of mycobacterial growth: The susceptibility of *M. tuberculosis* strain $H_{37}Rv$ to all the synthesized and commercial compounds was evaluated by determining the MIC. A colorimetric microassay based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) to formazan by metabolically active cells was used.[12,13] Briefly, serial twofold dilutions of each drug dissolved in dimethylsulfoxide were prepared in 7H9 broth (Middlebrook 7H9 broth base (Difco, Becton Dickinson, Le Pont de Claix, France)) using 96-well microtiter plates, and 100 μl of *M. tuberculosis* $H_{37}Rv$ suspension in 7H9 broth was added to each well. After 6 days of incubation, MTT was added (50 μl, 1 mg ml^{-1}). After 1 day of incubation, solubilization buffer was added to each well. The optical densities were measured at 570 nm. The MIC was determined as the lowest concentration of drug that inhibited bacterial growth (absorbance from untreated bacilli was taken as a control for growth). Reported MICs are an average of three individual measurements. When possible the IC_{50} (corresponding to concentration of product that inhibits 50% of the bacterial growth) was determined by using the GraphPad Prism 5.0 software. Ciprofloxacin was used as reference drug in the test.

Antileishmanial activity. Experiments were conducted on axenic amastigotes of *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012) and on luciferase-expressing axenic amastigotes of *L. infantum* kindly given by JL Lemesre (MHOM/MA/67/ITMAP-263).[14,15] Axenically grown amastigotes were maintained by weekly sub-passages in MAA/20 medium[16] at 37 °C with 5% CO_2 in 25 cm^2 tissue culture flasks. Cultures were initiated with 5×10^5 amastigotes (promastigotes) ml^{-1} in 25 cm^2 tissue culture flasks with 5 ml of medium. Transfectant axenic amastigotes of *L. infantum* were routinely maintained in the presence of 20 μg ml^{-1} of G418.

Drug susceptibility assays on L. amazonensis: The activity of the compounds was determined on axenic amastigotes of *L. amazonensis* by the MTT micromethod as previously described.[17] To assess intracellular antileismanial activity, medium in the wells containing the macrophages was replaced by the suspension of amastigotes using an infection ratio of 3/1 amastigotes/macrophages according to Sauvain *et al.*[18] Twenty-four hours after infection, a solution of the compounds to be tested was added to the cultures at various concentrations and maintained at 37 °C in 5% CO_2 for a further 48 h. The plates were fixed with methanol and stained with 10% Giemsa stain. The percentage of infected macrophages was determined microscopically at 1000 times magnification. The number of intracellular amastigotes was determined in 300 cells. IC_{50} was calculated as the dose giving a 50% reduction in the number of infected cells. All experiments were performed in triplicate. The cytotoxicity of the compounds was tested on murine peritoneal macrophages prepared as shown in Sauvain *et al.*[18] Briefly, non-inflammatory macrophages (10^5) were collected from 6-week-old male BALB/c mice. The adherent cells were incubated at 37 °C under 5% CO_2 and appropriate dilutions of compounds were added. The contact between drugs and macrophages alone lasted 48 h at 37 °C under 5% CO_2. The percentage of live macrophages was determined microscopically by the trypan blue dye exclusion test. CC_{50} was the drug concentration needed to cause a 50% decrease in the cellular viability.

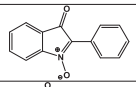
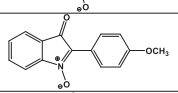
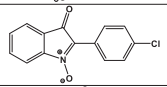
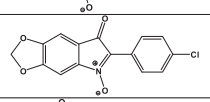
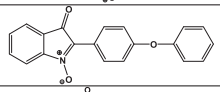
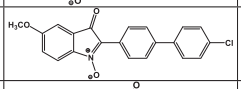
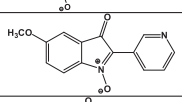
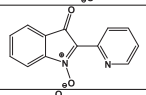
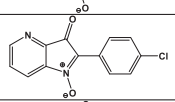
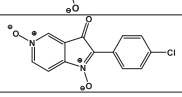
Drug susceptibility assays on L. infantum: Drug activity was evaluated on axenic amastigotes of *L. infantum* by the luciferase assay described elsewhere[15] with modifications. Briefly, exponentially growing luciferase-expressing axenic amastigotes of *L. infantum* were seeded in 96-well flat-bottom tissue culture plates to a final concentration of 10^6 ml^{-1} in medium alone or in the presence of various concentrations of tested drugs. The reference compound was amphotericin B. Parasites were grown at 37 °C with 5% CO_2 over 72 h in triplicate for each concentration of drug tested. After incubation, 50 μl of Steady Glo reagent (Promega, Charbonnières, France) were added to each well, incubated for 5 min, and read in a 1450-Microbeta Trilux plate reader (Wallac-PerkinElmer, Courtaboeuf, France). Growth-inhibition percentages were plotted as a semi-logarithmic function of drug concentration. The IC_{50} values were determined by linear regression analysis on the linear segments of the curves. Controls were carried out to assess the background (negative control) and parasite growth (positive control). Second, drug activity was measured on intra-macrophage parasites. Mouse leukemic monocyte macrophage cells (RAW 264.7) were infected with axenic amastigotes at a 16 : 1 parasite/macrophage ratio and the luciferase activity was measured as before. The cytotoxicity of compounds for RAW 264.7 cell growth was measured by [^{3}H]hypoxanthine (Amersham, Courtaboeuf, France) incorporation after a 48-h incubation with serial drug dilutions. Control cells were incubated in the presence of SDS. The amount of [^{3}H]hypoxanthine incorporated in the presence of drugs was compared with that of control cultures without the test compounds.

RESULTS AND DISCUSSION

Antibacterial (Gram-positive and Gram-negative)/antifungal activities

The results are summarized in Table 1. Based on the MIC, seven compounds out of ten showed moderate to good activity (MIC ≈ 3–31 μg ml^{-1}) against the Gram-positive strain, *E. hirae*. Six compounds also had moderate activity (MIC ≈ 6–62 μg ml^{-1}) against another tested Gram-positive strain, *S. aureus*. None of the compounds tested had activity against Gram-negative *E. coli*. Three compounds were found to exhibit moderate to low activity against Gram-negative strain, *P. aeruginosa*. Compound **64** was the most active (MIC ≈ 3–6 μg ml^{-1}) on the two tested Gram-positive bacterial strains. Seven compounds out of ten showed moderate to good activity (MIC ≈ 3–31 μg ml^{-1}) against the fungal strain *C. albicans*. Four compounds showed moderate activity (MIC ≈ 15–62 μg ml^{-1}) against the fungal strain *A. niger*. Compound **52** was the most active (MIC ≈ 3 μg ml^{-1}) against *C. albicans*, whereas compound **69** was the

Table 1 Antibacterial and antifungal activity of INOD derivatives (all the concentrations expressed in μg ml^{-1})

Compound N°	*Chemical Structure*	*Log* P_{calc} [a]	*S. aureus*		*P. aeruginosa*		*E. hirae*		*E. coli*		*A. niger*		*C. albicans*	
			MIC [b]	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MFC*	*MIC*	*MFC*
52		2.06	15.63	> 500	> 125	125	7.81	> 125	> 125	> 500	62.5	/	3.9	7.81
4		2.03	> 125	> 500	> 125	> 250	31.25	> 125	> 125	> 500	> 125	> 500	> 125	> 125
64		2.68	6.25	25	> 12.5	> 25	3.13	6.25	> 12.5	> 50	› 12.5	> 50	> 12.5	> 12.5
1		2.09	62.5	> 500	62.5	125	31.25	> 125	> 125	> 500	> 125	/	31.25	> 125
2		3.49	62.5	> 500	> 125	> 250	15.63	> 125	> 125	> 500	> 125	> 500	> 125	> 125
21		4.14	31.25	> 250	31.25	> 125	31.25	> 62.5	> 62.5	> 250	› 62.5	> 250	31.25	> 62.5
70		0.95	7.81	125	62.5	125	7.81	15.63	> 125	> 500	> 125	250	7.81	15.63
69		1.24	250	250	500	> 500	250	> 500	500	500	15.63	62.5	7.81	15.63
65		1.86	250	500	500	> 500	250	> 500	500	> 500	31.25	> 500	15.63	15.63
66		1.02	250	500	500	> 500	500	> 500	500	> 500	31.25	> 500	15.63	15.63
CP			≤ 3.125	≤ 3.125	≤ 3.125	≤ 3.125	≤ 3.125	6.25	≤ 3.125	≤ 3.125	/	/	/	/
N.A.			> 125	> 125	> 125	> 125	6.25	6.25	3.125	3.125	/	/	/	/
BL			> 125	> 125	> 125	> 125	> 125	> 125	> 125	> 125	3.125	/	> 125	> 125
CL			> 125	> 125	> 125	> 125	> 125	> 125	> 125	> 125	1.562	/	3.125	6.25

Abbreviations: BL, bifonazole; CL, clotrimazole; CP, ciprofloxacin; MFC, minimum fungicidal concentration (μg ml^{-1}); N.A., nalidixic acid.
The shaded cells rapidly distinguish the most active compound for each microbial strain.
[a]Log P_{calc} calculated with VCCLAB (http://www.virtuallaboratory.org/lab/alogps/start.html).
[b]The highest tested drug concentration was 50 μg ml^{-1}.

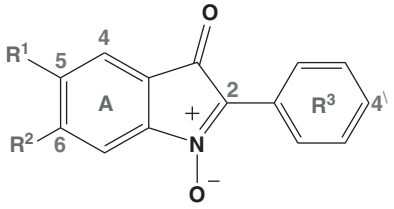


Figure 1 Structure of INOD analogs. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

most active (MIC ≈ 15 μg ml^{-1}) against *A. niger*. Based on the MBC/MIC and MFC/MIC ratios,[19,20] INOD most active compounds showed bactericidal and fungicidal activity rather than bacteriostatic or fungiostatic activity. No correlation was found between lipophilicity and the antimicrobial activity of the compounds (Table 1).

Figure 1 shows the structure of 2-phenylindolone-*N*-oxide derivatives. The phenyl group (A) of the indolone moiety was found to be essential for antibacterial activity. Replacement of the phenyl group (A) either by 3-pyridyl (*N* at position 4 of the indolone moiety, compound **65**) or by pyridinium-*N*-oxide (*N* at position 5 of the indolone moiety, compound **66**) led to significant loss of antibacterial activity but the antifungal activity was almost retained or even improved compared with the counterpart compound **64**.

2-phenylindolone-*N*-oxide (compound **52**) was the most active anti-Candida derivative, while replacement of the phenyl group (R^3) by 3-pyridyl (compound **69**) slightly decreased the activity against *C. albicans* but improved the activity against *A. niger*. Functionalization of the phenyl moiety (R^3) by groups having a positive mesomeric effect (OCH_3), compound **4**, leads to a significant loss of the activity, while groups with negative inductive effect (Cl), compound **64**, improve the antibacterial activity against Gram-positive strains only.

Antitubercular activity

The discussion of the results on antitubercular activity is separated from the above on Gram-stain bacteria, as mycobacteria have a unique acid-fast cell wall composed in part of a thick waxy layer of hydrophobic (C70-90) mycolic acids (that is, high lipid content in its cell wall). This major difference explains the subsequent selective activity depending mostly on compound lipophilicity (Log *P*) and on the different substituents of the heterocyclic core (structure–activity relationship). Log *P*-values were calculated using the VVCLAB software.[21]

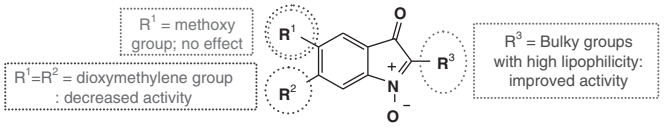


Figure 2 Effect of substituents on antitubercular activity of INOD derivatives. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

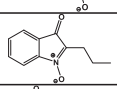
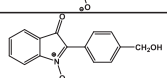
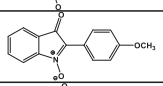
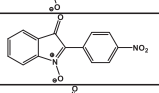
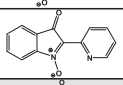
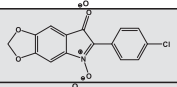
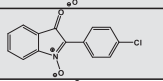
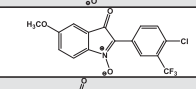
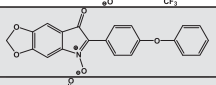
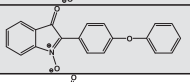
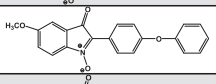
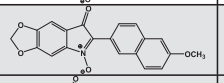
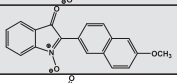
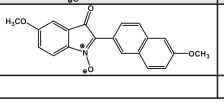
Structure-activity relationship: effect of substituents on the biological activity. Figure 2 shows the basic structure of the INOD derivatives. The non-substituted compound **71** where $R^1 = R^2 = R^3 = H$ exerts moderate activity (MIC = 12.5 μM). From Table 2, replacement of R^3 (H) by an aliphatic chain (*n*-propyl) leads to a significant loss of activity (MIC = 100 μM, compound **50**) and the replacement of R^3 (H) by one aryl group (2-pyridyl/4-nitrophenyl/4-methoxyphenyl/4-hydroxymethylphenyl/4-chlorophenyl, compounds **69**, **56**, **4**, **3** and **64**, respectively) leads to modest activity (MIC ≈ 50-25 μM), whereas replacement of R^3 (H) by bulky groups with high lipophilicity (log P > 3) (4-phenoxyphenyl/6-methoxynaphtyl, compounds **2** and **5**, repectively) leads to good activity (MIC ≈ 6.25-3.2 μM). Replacement of R^1 (H) by a methoxy group has almost no effect on the activity (compounds **2** and **5** versus **24** and **20**, respectively).

From Table 2, it can be seen that replacement of $R^1 = R^2 = H$ by a dioxymethylene group (O-CH_2-O) leads to significant decrease or loss of the activity (compounds **1**, **7** and **6** versus **64**, **2** and **5**, respectively, shaded zones in Table 2). This may be explained by the decrease in the lipophilicity (that is, compounds containing the dioxymethylene group are less lipophilic compared with their analogs without the dioxymethylene group). However, compound **7**, which is relatively lipophilic (Log *P* = 3), has low activity (MIC = 50 μM). Therefore, other factors may contribute to their potency (MIC): steric hindrance, relative rigid structure of the dioxymethylene group compared with the 'freely rotatable' methoxy group. Figure 3 shows the relationship between the lipophilicity (Log *P*) and the antitubercular activity (MIC). Compounds that are more lipophilic are more active. At this point of the study, we could suggest that these compounds are active because of their lipophilicity. As mycobacteria themselves are greasy, and have a thick external lipid layer, the lipophilic INOD derivatives may have strong affinity for the mycobacteria and be able to cross the usually impermeable cell wall. Cell uptake studies will answer this question in the future.

Compound **5**, with an unsubstituted phenyl group (of the indolone moiety) and with a bulky naphtyl group on R^3, was the most active INOD (MIC = 3.2 μM) with low cytotoxicity against two mammalian cell lines (CC_{50}/MCF7 = 15.3 μM and CC_{50}/KB = 233 μM)[5] and against mouse peritoneal macrophages (CC_{50} = 12.2 μM, Table 3). Therefore, compound **5** was found to be a promising antitubercular candidate and needs further investigation against multi-drug resistant strains and other Mycobacterium strains.

The main key structural features for optimal anti-infectious activity reside in the INOD moiety (Figure 1). Slight modifications (or substitutions at positions 4, 5, 6 or R^3) increase the activity against certain strains or species at the expense of decreasing activity against others. For example, replacement of the phenyl group (R^3) by 2-pyridyl group leads to the most active compound **69** against *A. niger*, whereas it was almost inactive against other bacterial strains tested, even against *M. tuberculosis*. The basic structure (2-phenylindolone-*N*-oxide, compound **52**) was the most active against *C. albicans*. Substitution of hydrogen at position 4\ of the phenyl

Table 2 Effect of substituents on the biological activity of INOD derivatives against *M. tuberculosis*

Compound N°	*Chemical Structure*	*IC_{50} (μM)*	*MIC (μM)*	*Log P_{cale}*
71		5.7	12.5	-0.10
50		39.1	100	1.41
3		15.4	50	1.29
4		10.2	50	2.01
56		12.4	50	1.96
69		17	50	1.24
1		14.6	50	2.07
64		7.9	25	2.54
11		6.6	12.5	3.32
7		13.4	50	3.00
2		2.7	6.25	3.51
24		2.7	6.25	3.51
6		ND	> 100	2.51
5		1.8	3.2	3.03
20		2.8	6.25	3.03
Ciprofloxacin		1.6	2.5	

The shaded cells show the effect of compounds containing dioxymethylene group against their counterpart (without dioxymethylene group).

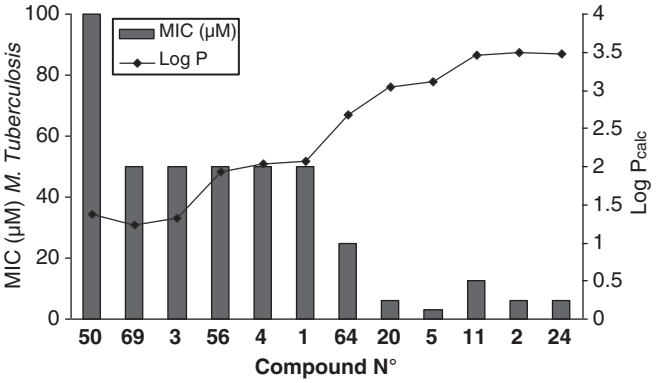
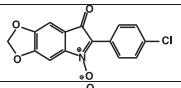
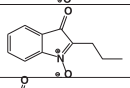
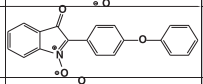
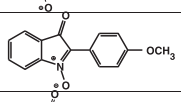
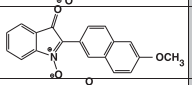
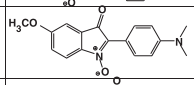
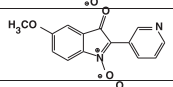
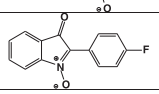


Figure 3 Relationship between MIC against *M. tuberculosis* and the lipophilicity of the tested compounds. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 3 Antileishmania and cytotoxicity activities of INODs

Compound N°	*Chemical Structure*	*L. amazonensis (axenic amastigote) IC_{50} ($\mu g\ ml^{-1}$)*	*Cytotoxicity Mouse peritoneal macrophage CC_{50} ($\mu g\ ml^{-1}$)*	*Selectivity Index*[a]	*L. infantum (axenic amastigote) IC_{50} ($\mu g\ ml^{-1} \pm$ s.e.)*	*Cytotoxicity macrophages (RAW) CC_{50} ($\mu g\ ml^{-1} \pm$ s.e.)*	*Selectivity Index*	*L. infantum intra-macrophagic stage (RAW) IC_{50} ($\mu g\ ml^{-1} \pm$ s.e.)*
1		2.3	1.2	0.5	0.43 ± 0.01	1.2 ± 0.1	2.8	0.19 ± 0.003
50		Inactive	2.5	-	Non-tested	Non-tested	-	Non-tested
2		0.9	4.3	4.8	0.16 ± 0.01	0.5 ± 0.0	3.1	0.20 ± 0.01
4		0.7	9.0	12.9	0.49 ± 0.02	2.6 ± 0.7	5.3	1.73 ± 0.11
5		2.3	3.7	1.6	0.46 ± 0.05	0.7 ± 0.0	1.5	5.01 ± 0.08
26		0.9	18.7	20.8	0.21 ± 0.02	2.1 ± 0.3	10.0	3.09 ± 0.07
70		0.4	2.7	6.7	0.56 ± 0.05	2.5 ± 0.0	4.5	2.20 ± 0.07
71		Inactive	40.1	-	Non-tested	Non-tested	-	Non-tested
53		2.2	2.7	1.2	Non-tested	Non-tested	-	Non-tested
AmphB	924.1	0.1	3.9	39	0.08	1.6 ± 0.2	20.0	0.01

The shaded cells easily distinguish between the two leishmanial strains.
[a]Selectivity index was defined as the ratio of CC_{50} value in macrophages to IC_{50} value in *Leishmania* axenic amastigotes. A full color version of this table is available at *The Journal of Antibiotics* journal online.

group (R^3) by chlorine gives the most active compound (**64**) against Gram-positive strains. The presence of a bulky aryl group at R^3 (along with high lipophilicity) gives the most active compound **5** against *M. tuberculosis*.

Antileishmanial activity

The antiparasitic activity of INOD derivatives was first evaluated *in vitro* against different *Plasmodium falciparum* strains.[5,6] In this current study, the antiprotozoal activity was extended and tested against *Leishmania* using two strains (*L. infantum and L. amazonensis* are the causative agents of visceral leishmaniasis and diffuse cutaneous leishmaniasis, respectively) (Table 3). The antileishmanial activity was evaluated in two steps: initially, at the axenic amastigote stage and finally at the intramacrophagic amastigote stage. The *L. infantum* strain was found to be more susceptible to the tested compounds than the *L. amazonensis* strain. Compound **2** was the most active against axenic forms of *L. infantum* with an $IC_{50} = 0.16\ \mu g\ ml^{-1}$ (amphotericin B: $IC_{50} = 0.08\ \mu g\ ml^{-1}$). Compounds **1** and **2** showed interesting results. First, the IC_{50} values for the inhibition of both axenic and intra-macrophage parasite growth were relatively low ($< 0.5\ \mu g\ ml^{-1}$). Second, the cytotoxicity values (compounds **1**, **2** and **4**) against the same macrophage cell line (RAW) were higher than the corresponding IC_{50} for the inhibition of intra-macrophage parasite growth (Table 3).

Most of the tested compounds showed satisfactory activity ($IC_{50} \approx 0.4$–$2.2\ \mu g\ ml^{-1}$) against *L. amazonensis* at the axenic amastigote stage combined with low cytotoxicity against macrophages, particularly compounds **2**, **4** and **26**. Compounds **50** and **71** were inactive. Two compounds (**4** and **26**) were selected for further investigation at the intra-macrophagic amastigote stage in mouse peritoneal macrophages (data not shown). These two compounds were found to be inactive; this could be attributed to a differential metabolic rate of the immortalized macrophage cell line used (RAW 264.7) compared with murine peritoneal macrophages. Interestingly, INOD derivatives were previously reported to interact with thiol-containing compounds.[7] Therefore, depending on the redox status of the host cell and its metabolic activity, these compounds may be reduced and/or degraded intracellularly even before reaching the parasitic target. This could explain the differential activities on the two stages (axenic and intramacrophagic) and the differential activity between the two macrophage cell lines (peritoneal and RAW). A second hypothesis relies on the differential capacity of these compounds to induce macrophage activation according to the type of macrophage used (peritoneal or immortalized). The production of interferon-γ and the subsequent generation of NO and reactive oxygen species is known to be critical for the elimination of *Leishmania*.[22] It has already been shown that RAW cells stimulated by a thiol-activated toxin produced NO and expressed iNOS, via sufficient endogenous interferon-γ production.[23] To investigate further, compound **4** could be tested not on normal macrophages but rather on inflammatory peritoneal macrophages.

Structure–activity relationship. No relation between antileishmanial activity and lipophilicity could be established. However, some structural features were mandatory for antiparasitic activity. The

2-phenylindolone-*N*-oxide (Figure 1) was essential for the minimal antileishmanial activity. Replacement of the 2-phenyl group by hydrogen (compound **71**) or an aliphatic chain (*n*-propyl, compound **50**) leads to loss of activity against *L. amazonensis* (Table 3). Modifications of the 2-phenyl group or the phenyl group of the indolone moiety did not greatly affect the antileishmanial activity against the two leishmanial strains.

CONCLUSION

INOD derivatives exhibiting various pharmacological activities with antiplasmodial activities have been recently established. Studies on the anti-infectious properties of INODs were extended against different microorganisms. The most susceptible bacterial strain was the Gram-positive *E. hirae*, with compound **64** being more potent than nalidixic acid against *E. hirae*, whereas the most resistant strain was the Gram-negative *E.coli*. The antifungal properties of this family were found promising with good activity against either *A. niger* or *C. albicans*. Compound **52** had comparable activity as clotrimazole against *C. albicans*. The antitubercular activities of this family were in the low micromolar range and largely correlated with the lipophilicity of the tested compounds. The antiparasitic activities were studied against two *Leishmanial* strains. The redox pharmacophore (INOD) of this antimicrobial archetype may disrupt the redox systems of the pathogen, which may represent new mechanisms of action. These results point to a valid prototype for which pharmacomodulation may lead to the discovery of new compounds active against the desired range of pathogens.

ACKNOWLEDGEMENTS

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