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ORIGINAL ARTICLE

Toxicity of betulin derivatives and *in vitro* effect on promastigotes and amastigotes of *Leishmania infantum* and *L. donovani*

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The toxicity and antileishmanial activity of 20 betulin derivatives were studied. The toxicity of betulin and synthesized compounds was determined using a bacterial test (Microtox) and two mammalian cell lines (CHO-K1 and J774). The antileishmanial activity of compounds (50 μM) was examined in both the promastigote and intracellular amastigote stages of *Leishmania infantum* and *L. donovani*. No correlation was found among the toxicity tests. All the compounds showed significant antipromastigote activity. The antiproliferative capacity of derivatives was dependent on the parasite stage studied, and no substantial differences were found between *Leishmania* species. Betulin, 3,28-di-*O*-acetylbetulin and L-aspartyl amide of betulonic acid showed moderate activity against amastigotes. The highest inhibition of intracellular amastigote multiplication was achieved with a low micromolar concentration (IC$_{50}$ ca 9 μM) of heterocyclic betulin derivative 3,28-di-*O*-acetyllup-13(18)-ene with N-ethyltriazolo moiety 16, without significant toxicity for mammalian cells. These results point to the interest of this lead compound for further *in vitro* and *in vivo* tests.

INTRODUCTION

Leishmaniases are a group of vector-borne parasitic diseases caused by different species of *Leishmania* and present in all inhabited continents. It is estimated that around 12 million people are currently infected; over 350 million live in risk areas and 2 million new cases occur every year.[1] Leishmaniasis has a range of clinical presentations, from self-healing dermal processes (*L. major* and *L. tropica*) to visceral infections that are fatal if left untreated (*L. donovani*). The infection was traditionally linked to tropical and subtropical regions but nowadays distribution is more widespread, as it is a common infection in immunocompromised patients.[2] Human migrations due to war and famine have expanded the area of sandfly vectors, and human-induced environmental modifications have either led to the infection being found in areas where it was not previously present[3] or have exacerbated its presence in other areas.[4] Apart from human cases, dog infections (*L. infantum* and *L. chagasi*) are of primary concern, both for public health considerations (zoonotic infections) and for strictly veterinary reasons. Conservative estimates suggest that in the Mediterranean region, at least 5–7% of the total dog population is infected[5] and that there are 'hot spots' with a canine prevalence of over 30%.

Different methods are employed to limit the spread of leishmaniasis (for example, medicated dog collars, insecticides where allowed) but, for the most part, the main system of control of infected individuals (humans and dogs) continues to be chemotherapy. A large number of drugs have been used, but the therapeutics of leishmaniasis is hampered by the toxicity of some of the compounds, difficulties in administration in remote areas, the intraspecific variability of *Leishmania* drug sensitivities and the development of resistances, particularly in the case of *L. donovani* in India.[6,7] Moreover, in many countries, the use of first-line drugs (antimonials and amphotericins) is precluded by their high price.[8] Despite impressive advances in the understanding of the biology and molecular biology of *Leishmania* spp, these have not yielded any substantial improvements on chemotherapy; generally speaking, the compounds either already in use or undergoing clinical trials are incidental findings rather than the result of a directed research effort.[9] In 2004, World Health Organization/Tropical Diseases Research (TDR) indicated that liposomal amphotericin, paromomycin and miltefosine were the most promising drugs against *Leishmania* on the market (http://apps.who.int/tdr/svc/diseases/leishmaniasis). These three drugs, together with antimonials in those areas where resistance has not developed, constitute the

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reference treatments for leishmaniasis. However, none of these compounds is new. Amphotericin B has been used for decades as an effective treatment against leishmaniasis (in addition to its fungicidal efficacy); its main drawback, related to its toxicity, is that it has to be administered slowly under hospital conditions. Miltefosine (hexadecylphosphocholine) was originally developed as an anticancer agent, whereas the aminoglycoside antibiotic paromomycin (aminosidine) has been used as an antimicrobial since the 1960s. The need to explore new treatments is therefore recognized as a leading priority for research on leishmaniasis.[10–13] Betulin (lup-20(29)-ene-3β,28-diol) is a pentacyclic triterpene widely distributed in nature. The outer bark of the birch (*Betula* sp) is particularly rich in this substance, which, depending on the species, may represent more than 25% of the bark by weight. This compound can be transformed into betulinic acid either by chemical transformation or biotransformation.[14,15] It has been suggested that betulinic acid has anticancer, anti-HIV and antimicrobial properties.[16,17] Some related compounds have shown antileishmanial activity against *L. donovani* (promastigotes and amastigotes)[18] and *L. amazonensis*.[19] More recently, studies have been carried out on the effect of a high number of betulinic derivatives on axenic amastigotes and amastigotes of *L. donovani* in the THP-1 cell line.[20] It was found that the compound with greater antileishmanial capacity (heterocycloadduct between 3.28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine) showed a 50% growth inhibition (IC$_{50}$) of 8.9 μM against amastigotes, although the compound showed a 30% toxicity for the THP-1 macrophage line. This toxicity possibly rules out its potential therapeutic use. In axenic amastigotes, the same group[21] observed that betulonic acid exhibited the highest antileishmanial activity. *L. infantum* and *L. donovani* are closely related species. However, there are epidemiological and pathological differences between the infections caused by each agent. Moreover, given the inconsistencies of many drug-screening reports (the differential effect on promastigotes and amastigotes; the relative value of axenic amastigotes), it has been considered to be of interest to test an available series of betulin derivatives against promastigotes and amastigotes of *L. infantum* cultured in J774 macrophages.

MATERIALS AND METHODS

Parasites
Autochthonous isolates of *L. infantum* (UCM 6 and 10), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health of the Faculty of Veterinary Medicine (UCM), and 'Khartoum 1246' isolate from *L. donovani*, provided by Dr Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid), were routinely maintained as promastigotes in RPMI 1640 medium (Lonza Group, Switzerland) at 26 °C supplemented with heat-inactivated (30 min at 56 °C) fetal bovine serum (Sera Laboratories International, Horsted Keynes, UK) and 100 U ml^{-1} penicillin+100 μg ml^{-1} streptomycin (BioWhittaker, Verviers, Belgium) in 25-ml culture flasks.

Macrophages
J774 cells (murine monocyte-like cell line) were grown in RPMI 1640 medium in 25-ml flasks supplemented with fetal bovine serum and antibiotics, as above, in a humidified 5% CO$_2$/air atmosphere at 37 °C.

Compounds tested
The 20 betulin derivatives tested in the present study were synthesized in the Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, Finland (Table 1). The chemical synthesis and detailed characterization data of the betulin derivatives are reported in our previous publications.[15,20,22] Lyophilized compounds were resuspended in dimethyl sulfoxide for a 10-mM stock solution. The concentration of dimethyl sulfoxide in the

working solution was no greater than 0.5% in any experiment. A standard concentration of 50 μM was used in the first screenings after Chowdhury *et al*.[18] Approximate IC$_{50}$ of compound 16 was estimated with a range of concentrations (0, 10, 25 and 50 μM).

Promastigote assay
Promastigotes (10^6 parasites ml^{-1}) were cultured in 10-ml sterile polystyrene tubes. Standard dilution of the compound (50 μM) was added up to 2 ml final volume. After 72 h at 26 °C, live promastigotes were counted in an improved Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Results of growth inhibition were expressed as percentage (%) of growth in untreated control cultures. Cultures were performed at least in triplicate, and amphotericin B was included as control.

Amastigote assay
In the amastigote assay, infection was carried out using a modification of the method developed by us.[23] Briefly, 5×10^3 J774 cells per well were cultured in 16-well Lab-Tek chambers (Nunc, Roskilde, Denmark) for 16 h. Stationary phase *Leishmania* promastigotes (days 8–11) were centrifuged without brake (660×*g*, 10 min) through a Ficoll gradient (0, 10 and 20%) in fresh RPMI medium. Parasites recovered from the top were washed with fresh medium and opsonized with 5% normal mouse serum in a solution of 1:1(v/v) RPMI medium and HBSS 0.15 mM CaCl$_2$ and 1 mM MgCl$_2$ for 30 min. The washed parasites were added to the macrophage cultures (parasite: macrophage ratio 10:1) and maintained at 33 °C in 5% CO$_2$ overnight. Non-internalized promastigotes were eliminated by washing three times with fresh medium. Compounds were added (50 μM) and maintained for 48 h for preliminary screenings or for 72 h in subsequent determinations. Unless otherwise stated, mitomycin C (Calbiochem, Merck, USA) at 1 μg ml^{-1} was added at each stage to avoid host cell proliferation. Slides were fixed and stained (Diff-Quick, Dade Behring, Switzerland), and the number of amastigotes per 100 cells and percentage of infected cells were determined. Cultures were performed at least in triplicate, and activity controls with Fungizone (deoxycholate-dispersed amphotericin B, Bristol-Myers Squibb, Rueil-Malmaison, France; 0.2 μg ml^{-1} amphotericin B base) were included.
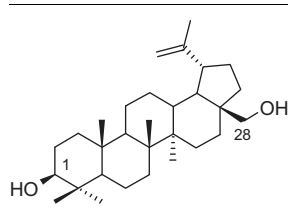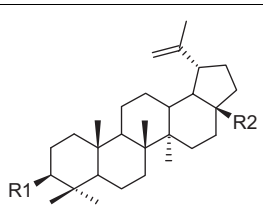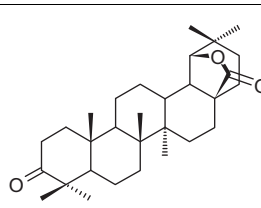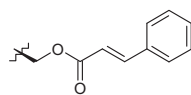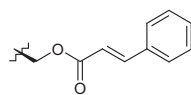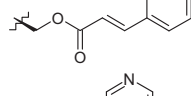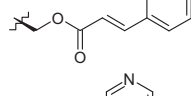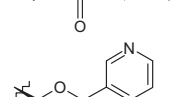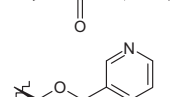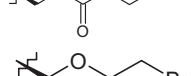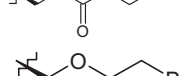
Cell cytotoxicity assays
Alamar blue reduction (J774 cells). J774 cells were counted in an improved Neubauer chamber (using trypan blue vital staining) and 1.25×10^4 cells per well in 200 μl were placed in a 96-well plate with the standard dilution of the compound (50 μM). After 48 h, the medium was eliminated and 200 μl of fresh supplemented medium plus 22 μl of Alamar Blue reagent (Serotec, Oxford, UK) were added. Absorbance (570 and 600 nm) was measured to calculate growth inhibition (%; IC$_{50}$).[24,25] Negative control cultures without compound were included. Cultures were performed in triplicate.

Neutral red assay (CHO-K1 cells). Cytotoxicity was estimated in CHO-K1 cells using the neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assay.[26] This assay measures cellular transport based on the dye uptake by living cells. The absorbance change is directly proportional to the number of viable cells. For this analysis, 2×10^3 cells per well were cultured in 96-well plates and grown at 37 °C in a 5% CO$_2$ humid atmosphere in a complete culture medium for 4 h. This medium was then replaced with betulin derivatives. After 24 h, the medium was removed and fresh medium containing 40 $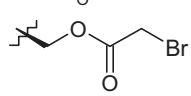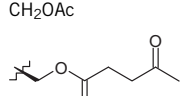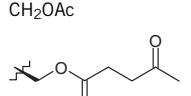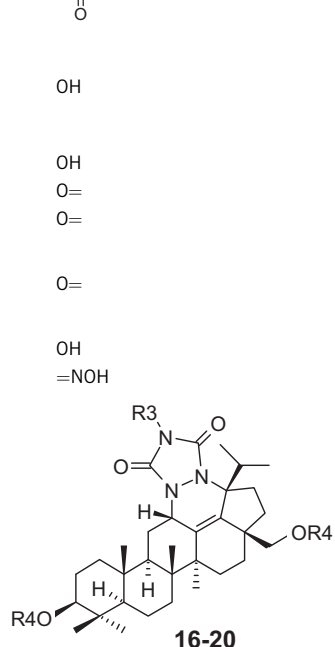\mu$g ml^{-1} RN dye (Sigma, St Louis, MO, USA) was added. After a 3 h incubation, cells were washed with phosphate-buffered saline (pH 7.2). Color was developed by the addition of 0.1 ml 1% acetic acid in 50% ethanol. The plate was shaken for 10 min, and absorbance was measured at 540 nm using an automatic FluoStar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany). Ethanol (5%) was used as a positive control. The cytotoxicity percentage was calculated as $((A-B)/A \times 100)$, where A and B are the absorbance of control and treated cells, respectively.

Microtox toxicity test. The Microtox basic test was carried out as preliminary screening using the manufacturer's (Microbics, Carlsbad, CA, USA) protocol. A Microtox Model 500 Analyzer (AZUR Environmental, Carlsbad, CA, USA) was used to measure the luminosity from *Vibrio fischeri* after 15-min exposure

Table 1 Structures of betulin 1, semisynthetic betulin derivatives 2–15 and heterocyclic betulin derivatives 16–20

Compound	R1	R2
1	OH	CH2OH
2	OH	(E)-cinnamate ester
3	OH	nicotinate ester
4	OH	bromoacetate ester
5	OAc	OH
6	OAc	CH2OAc
7	levulinate ester	levulinate ester
8	OH	tetrahydropyranyl ether
9	OH	CO2H
10	O=	CHO
11	O=	CO2H
12	O=	aspartate diester amide
13	OH	CH=NOH
14	=NOH	CH=NOH

Compound	R3	R4
16	Et	Ac
17	Ph	Ac
18	Indan-5-yl	Ac
19	Me	COEt
20	Me	COi-Pr

to the tested compounds. Inhibition of light emission was measured in relative units of luminescence, and these data were used to calculate the median effective concentration (IC_{50}).

Statistical analysis
Results obtained within each species were analyzed by one-way analysis of variance test, and multiple comparisons were made using P-values corrected by

the Bonferroni method. To compare the results obtained between *Leishmania* species, the Student's *t*-test was performed. In both cases, the level of significance was $P < 0.05$. Statistical analysis was carried out with Statgraphics Centurion, Timberlabe Cons. Ltd, London, UK. Unless otherwise stated, all experiments were performed in triplicate.

RESULTS
Effect of betulin derivatives on promastigote stage
All the compounds tested were able to significantly reduce the multiplication of both parasite species (Table 2). In the series of simple betulin derivatives (compounds **2–15**) where modifications were done to hydroxyl groups at C-3 and C-28, only few derivatives showed improved activity when compared with betulin **1**. In the subgroup of betulin esters (**2–7**), only 28-*O*-cinnamoylbetulin **2** showed improved activity at 50% against both parasite species. In addition, 28-*O*-nicotinoylbetulin **3** was more effective against *L. infantum* and 3-*O*-acetylbetulin **5** against *L. donovani*, respectively. The rest of the ester derivatives showed weak-to-moderate activity, with inhibition between 20 and 40%. Ether derivative 28-*O*-tetrahydropyranylbetulin **8** was slightly less active than betulin **1**. Oxidation of hydroxyl groups of betulin **1** had also only minor effects on antileishmanial activities (compounds **9–11**). Betulonic aldehyde **10** and betulonic acid **11** with a ketone carbonyl as a functional group at C-3 were less active, and only betulinic acid **9** showed improved activity against *L. donovani*, but activity was not improved against *L. infantum*. L-aspartyl amide of betulonic acid **12** showed decreased activity against *L. donovani*. Betulin-28-oxime **13** was only weakly active, whereas betulin-3,28-dioxime **14** with two oxime groups, at C-3 and C-28, showed improved activity against *L. donovani*. The 28-oxoallobetulone **15** showed decreased activity against *L. infantum*, whereas activity against *L. donovani* remained unchanged. In general, there can been seen a suggestive trend that betulin derivatives with free hydroxyl group at

C-3 showed same or slightly improved activities against *L. infantum* (except for betulin-28-oxime **13**), whereas modification at C-3 decreased the activity. In the case of *L. donovani*, no such trend can be seen, although compounds with ketone carbonyl at C-3 (**10–13**) were less active than betulin **1**.

In the subgroup of heterocyclic betulin derivatives (**16–20**), clearer structure–activity relationship trend could be observed. Compounds with sterically less bulky R3 and R4 groups showed better activity, when compared with more hindered derivatives. Heterocycloadduct **16** with ethyl as an R3 group and acyl moieties as R4 groups showed better activity than **17** or **18**, which have bulky aromatic R3 groups. When comparing cycloadducts **19** with **20**, both of which have methyl as an R3 group, derivative having less bulky R4 was more active. Overall, antileishmanial activity of betulin derivatives was depending on the studied parasite species. Some derivatives that showed good activity against *L. infantum* were only moderate or weakly active against *L. donovani* promastigotes and vice versa. Approximate IC$_{50}$ value of amphotericin, included as positive control compound, was 0.07 μM for both species after 48 h incubation.

Toxicity of betulin derivatives
Toxicity of the compounds for *V. fischeri* and two mammalian cell lines (CHO-K1 and J774) was examined. In addition, a qualitative microscopic evaluation was performed using J774 cells. Differences in toxicity were found among the betulin derivatives tested, and no correlation was observed between the three methods employed. The Microtox test displayed the highest variation of inhibition ranging from 0 to 100% (Table 3). When CHO-K1 cells were exposed to the betulin-derived compounds assayed (50 μM), all the compounds except **18** were lacking in toxicity, with inhibition values below 10%.

Table 2 Effect of betulin derivatives (50 μM) on the *in vitro* multiplication of *Leishmania infantum* and *L. donovani* promastigotes

Compound[a]	L. infantum *promastigotes*	L. donovani *promastigotes*
1	42.8 ± 12.7	39.2 ± 11.5
2	53.3 ± 6.4	50.1 ± 8.9
3	53.8 ± 12.0	34.3 ± 3.3
4	39.5 ± 6.9	26.4 ± 5.3
5	32.9 ± 8.9	52.8 ± 5.1
6	21.5 ± 12.1	25.4 ± 5.3
7	35.3 ± 14.9	37.5 ± 14.1
8	36.4 ± 4.0	25.4 ± 0.9
9	36.0 ± 11.7	63.7 ± 10.2
10	26.5 ± 6.8	32.7 ± 14.0
11	30.6 ± 9.2	21.2 ± 7.0
12	42.9 ± 4.3[b]	12.6 ± 13.0[b]
13	22.1 ± 2.1	17.6 ± 3.9
14	33.8 ± 12.8	50.1 ± 6.4
15	24.0 ± 3.2	42.0 ± 20.5
16	25.5 ± 9.9	48.3 ± 11.5
17	30.8 ± 6.0	24.7 ± 3.1
18	18.4 ± 4.4	31.0 ± 9.3
19	63.8 ± 4.3	33.8 ± 16.7
20	34.6 ± 7.0[b]	20.9 ± 1.6[b]

Results (average ± s.d.) represent the percentage of growth of untreated control cultures.
[a]Numbering of compounds as in Table 1.
[b]Statistically significant difference ($P < 0.05$) in the inhibitory effect on promastigotes from both species.

Table 3 Toxic effects induced by selected betulin derivatives on Microtox test and mammalian cell (CHO-K1 and J774 cell lines) toxicity assays

Compound[a]	Microtox test I$_{max}$ (% v/v ± s.d.)	Mammalian cells I$_{max}$ (% average ± s.d.)	
		CHO-K1	J774
1	0	0	18.4 ± 0.4
2	40.1 ± 7.5	1.27 ± 1.2	0
3	7.7 ± 1.3	3.27 ± 2.4	46.0 ± 0.4
4	100.0 ± 0.0	11.1 ± 0.9	50.4 ± 0.8
5	8.0 ± 2.9	0	21.6 ± 4
6	0	0	0
7	71.76 ± 16.2	0	0
8	0	0	49.4 ± 0.9
9	6.9 ± 6.9	0	44.3 ± 1.0
10	2.86 ± 2.86	1.59 ± 1.2	48.8 ± 1.5
11	0	0	43.9 ± 1.1
12	12.0 ± 8.3	9.03 ± 1.5	0
13	12.6 ± 1.4	0	49.8 ± 1.8
14	0	0	46.0 ± 3.9
15	93.3 ± 6.7	0	34.4 ± 0.7
16	20.1 ± 6.8	4.15 ± 1.1	8.1 ± 1.0
17	0	0	0
18	28.6 ± 3.0	14.4 ± 1.1	0
19	10.5 ± 1.73	0	46.7 ± 2.1
20	0	0	10.3 ± 7.6

Values are shown as maximal inhibition (I$_{max}$) percentages ± s.d. with respect to untreated controls.
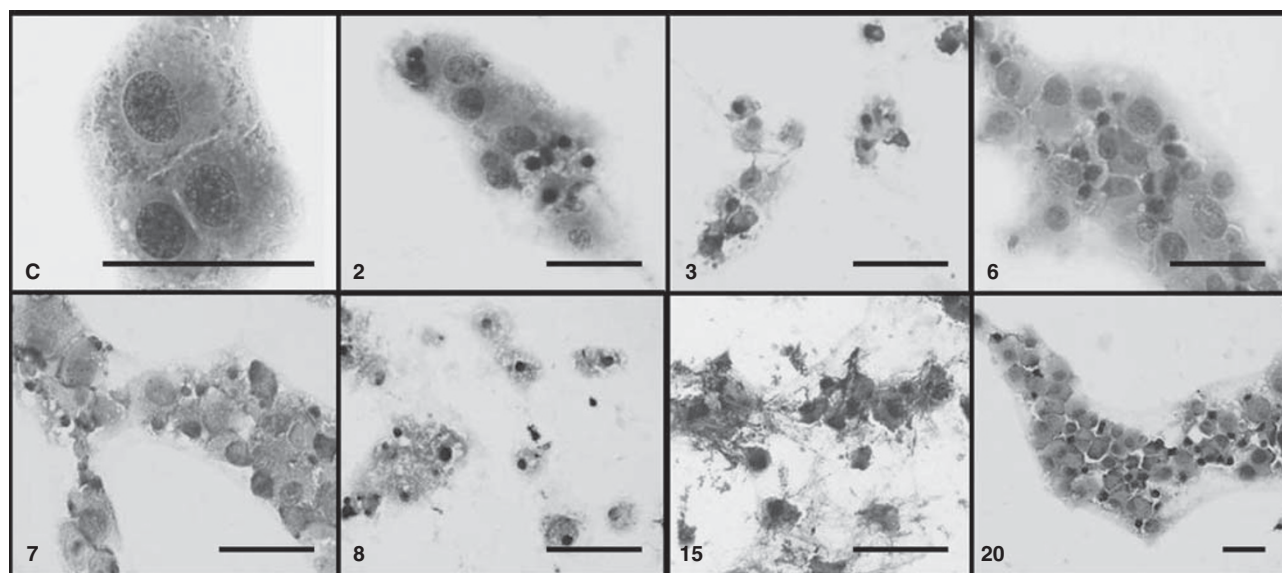[a]Numbering of compounds as in Table 1.

Figure 1 Representative microscopic fields of the *in vitro* effect of some betulin derivatives (50 μM) on the Diff-Quick-stained murine macrophage cell line J774 after 72-h exposition. C, untreated control culture. Numbers correspond to those in Table 1. Bar: 50 μm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

However, when the compounds were added to the macrophage murine line J774 and cell growth inhibition was determined by the Alamar Blue colorimetric method, the inhibitory effect was found to be much higher; after 48 h of coincubation with the betulin derivatives, nine compounds actually showed reductions of over 40% when compared with the untreated control cultures. Some compounds did not show any noticeable toxicity with this method (**2, 7, 16, 17** and **20**), and no apparent structure–activity relationship was observed between toxicity for the J774 cells and the compound subgroup (that is, derivatives having C-3 ketone carbonyl: compounds **10** and **11** were toxic, but L-aspartyl amide of betulinic acid **12** was non-toxic). However, all betulin derivatives having both hydroxyl groups acetylated (**6, 16, 17, 18**) were non-toxic to J774 cells. It is noticeable that even betulin **1** exhibited certain toxicity (18.4 ± 0.4% inhibition). Moreover, the effect was observed both with mitomycin and without the multiplication inhibitor. Toxicity for the macrophage was further assessed by microscopic examination. Unfortunately, only eight compounds could be used. However, in the cases examined, a good correlation was found between the Alamar Blue results and microscopy in J774 cells. Figure 1 shows the effect of some derivatives, with toxicity estimated by the colorimetric method ranging between 40–50% (**3, 4** and **15**), as compared with the scarce effect on cell integrity and staining pattern of non-toxic compounds (**2, 7, 8** and **20**).

Effect of betulin derivatives on *Leishmania* amastigotes in macrophages

Only compounds that showed an inhibitory effect on the mammalian cell line J774 ≤ 20% were extensively tested. All the compounds assayed were able to reduce the multiplication of intracellular amastigotes of *Leishmania* as compared with the uninfected control cultures (Table 4). In the subgroup of simple betulin derivatives (**1–15**), only four compounds (**1, 2, 6** and **12**) were tested against intracellular *L. infantum* and *L. donovani* amastigotes. Betulin **1** showed best activity (49.3 and 42.8%, respectively), whereas rest of the compounds showed similar or decreased activity.

When comparing intracellular amastigotes inhibition with promastigote inhibition activity, betulin **1** showed slightly improved activity

Table 4 Effect of some betulin derivatives (50 μM) on amastigotes of *Leishmania infantum* and *L. donovani* cultured in J774 macrophage cell line

	Amastigote % average ± s.d. reduction	
Compound[a]	L. infantum	L. donovani
1	49.3 ± 9.0	42.8 ± 22.1
2	44.0 ± 8.6	29.5 ± 6.3
3	ND	ND
4	ND	ND
5	ND	ND
6	25.5 ± 18.0	43.1 ± 11.7
7	ND	ND
8	ND	ND
9	ND	ND
10	ND	ND
11	ND	ND
12	44.9 ± 11.6	30.0 ± 3.8
13	ND	ND
14	ND	ND
15	ND	ND
16	70.4 ± 13.5	75.9 ± 5.8
17	51.2 ± 5.5	20.8 ± 20.6
18	40.7 ± 12.1	14.7 ± 9.7
19	ND	ND
20	ND	ND

Abbreviation: ND, not determined.
Values are means ± s.d. reduction (%) of untreated control cultures.
[a]Numbering of compounds as in Table 1.

against both *L. infantum* and *L. donovani* amastigotes, with 49.3 and 42.8% inhibition, respectively. Also, moderately active 3,28-di-*O*-acetylbetulin **6** and L-aspartyl amide of betulonic acid **12** were more active against amastigotes than promastigotes. On the other hand, 28-*O*-cinnamoylbetulin **2** was less active against amastigotes. From the subgroup of heterocyclic betulin derivatives (**16–20**), compounds **16**,

17 and **18** were screened against *Leishmania* amastigotes growing in macrophages. The activity of sterically less hindered cycloadduct **16** (over 70% inhibition against both *L. infantum* and *L. donovani* amastigotes, R3=Et) was clearly better than that of cycloadduct **17** (R3=Ph) or **18** (R3=indan-5-yl). The exploration of different concentrations of the most active compound (**16**) yielded an $\sim$50% IC_{50} of 9 μM (not shown). Under our conditions, IC_{90} was not achieved in the concentration range employed (0–50 μM). Amphotericin B showed an IC_{50} value of 0.09–0.1 μM for both *Leishmania* species.

DISCUSSION

Our results showed that some of the betulin derivatives tested had higher antileishmanial activity than the base compound of the series, betulin **1**, against amastigotes of *L. infantum* and *L. donovani* in macrophages. Of these, derivative **12** was previously tested with macrophages infected with *L. donovani* amastigotes,[21] and earlier results (53% inhibition at 50 μM) were in agreement with the present results (69% inhibition). Under our conditions, the highest inhibition activity against intracellular amastigotes of both *Leishmania* species was obtained with derivative **16**, IC_{50} value in the micromolar range (ca 9 μM). Better antileishmanial efficiency of betulin heterocycloadducts with less bulky *N*- and *O*-substituents is in accordance with our earlier results with *L. donovani* axenic amastigotes (strain MHOM/SD/1962/1S-Cl2d; IC_{50} 30 μM) and intracellular amastigotes in THP-1 macrophages, where this compound showed 53% inhibition at 25 μM.[20]

The activity found with **16** against intracellular *Leishmania*, without substantial toxicity for host cells, points to the interest of carrying out further tests of this compound on animal models. The mechanism of action of betulin and betulin derivatives has been only partially explored (it prevents DNA cleavage and the formation of topoisomerase–DNA complex),[18,27–29] and internalization by *Leishmania* is unknown. It is interesting to note that the most active derivative exhibited a higher inhibitory activity against amastigotes than against promastigotes. This finding may indicate the presence of a host cell-mediated mechanism and/or a mechanism involving different targets in hosts and parasite. This should be explored with the lead compound identified. The correlation found between the inhibition results against the amastigote from both *Leishmania spp.* was higher than that found between the amastigote and promastigote stages; results obtained in the intracellular stage in both species of *Leishmania* were comparable. This is consistent with the close intrahost life cycle and the similar sensitivities to well-established antileishmanial drugs (that is, amphotericin B).

Microscopic evaluation of toxicity against macrophages was as sensitive as the colorimetric method used (Alamar Blue), in spite of being labor and time consuming. The estimated toxicity of tested compounds was highly variable depending on the bioassay, even with those involving the use of mammalian cells (CHO-K1 and J774 cell lines). The *V. fischeri* bacteria test (Microtox) has been found to be correlated with other tests in higher organisms.[30] However, in our case, no relationship was found.

Under our conditions, antileishmanial activity of betulin derivatives against the promastigote stage was dependent on the parasite species tested. Some derivatives that showed good activity against *L. infantum* were only moderate or weakly active against promastigotes of the *L. donovani* line used and vice versa. On the other hand, the lack of correlation between the results against promastigotes and amastigotes points to the need for using at least the intracellular stage in macrophages as the screening method to identify lead compounds against *Leishmania*. This need has been recognized,[25,31,32] and is possibly critical for reducing the attrition rates of drug discovery.

Moreover, in our earlier study with *L. donovani* axenic amastigotes (strain MHOM/SD/1962/1S-Cl2d), derivatives **2** and **6** were totally inactive,[21] whereas these compounds significantly inhibited the proliferation of amastigotes in macrophages. Thus, in any anti-*Leishmania* drug screening, infected macrophages should be preferred for identifying potentially useful drugs.

In conclusion, we found that all tested betulin derivatives showed inhibitory activity against *L. donovani* and *L. infantum* promastigotes. Toxicity for mammalian macrophages was variable and generally higher than that of betulin, which probably excludes the majority of these compounds as potential antileishmanial products. However, compound **16** strongly ($>$70%) inhibited multiplication of the intracellular stages—those actually causing the disease in the infected hosts. Its activity against the two *Leishmania* species, and IC_{50} value in the micromolar range without significant toxicity for macrophages, points to the interest of this derivative. The pharmacology of this compound is not known, but structurally related betulinic acid, after i.p. injection, reached a peak serum concentration of 4 μg (ca 6.01 μM),[33] a value close to the estimated IC_{50} for **16**. If bioavailability of the tested derivative were comparable, then **16** would be a promising lead for chemotherapy of leishmaniasis, which would suggest further studies of the disease in animal models using mice or hamsters.

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