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ORIGINAL ARTICLE

Anti-leishmanial activity of betulin derivatives

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Leishmanicidal activity of 24 derivatives of naturally occurring and abundant triterpenes belonging to the lupane series, betulin, betulinic acid and betulonic acid, is described in this study. The easily modified positions of the lupane skeleton, the hydroxy groups of C-3 and C-28, as well as the carbon–carbon double bond C-20–C-29 were used as a starting point to prepare a library of triterpenoid derivatives for bioactivity studies. The compounds were evaluated against *Leishmania donovani* axenic amastigotes on a microplate assay at 50 μM. GI_{50} values of the most effective compounds were evaluated, as well as their cytotoxicity on the human macrophage cell line THP-1, and anti-leishmanial activity against *L. donovani*-infected THP-1 macrophages was determined. Betulonic acid was the most potent derivative, yielding a GI_{50} value of 14.6 μM. Promising and distinct structure–activity relationships were observed, and these compounds can be regarded as significant lead molecules for further improvement and optimization.
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INTRODUCTION

Leishmaniases are diseases caused by protozoan parasites that affect millions of people in more than 88 countries worldwide. These parasites are transmitted by female sand flies belonging to the genus *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively. Leishmaniasis causes three main forms of clinical disease: (1) visceral leishmaniasis, the most severe form, is usually fatal if not treated and affects internal organs such as the liver, spleen and bone marrow; (2) mucocutaneous leishmaniasis, a chronic form, causes extensive destruction and disfiguration of the nasopharynx region; and (3) cutaneous leishmaniasis, the mildest form, is usually self-healing within a few months to years, causing scarring at the site of the lesion(s). First-line drugs include pentavalent antimony (Sb^v) compounds, pentamidine or amphotericin B. All these drugs are administrated by injection and require clinical supervision or hospitalization because of the possibility of severe side effects. However, parasite resistance to Sb^v drugs has resulted in the discontinued use of these compounds in some endemic regions for visceral leishmaniasis.[1] Liposomal amphotericin B shows reduced toxicity, but is prohibitively expensive for use in less-developed countries. Recently, miltefosine, an alkylphospholipid derivative and the first orally administered drug, has been approved for use in India. However, the teratogenic effects of this drug prevent its use in pregnant women,[2,3] and parasite resistance is easily generated in the laboratory.[4] As such, there is an urgent need for the development and testing of new compounds for the treatment of all clinical forms of leishmaniasis.

Betulin **1** (lup-20(29)-ene-3β,28-diol) is an abundant naturally occurring triterpene found in the plant kingdom (Figure 1). It is the principal extractive (up to 30% of dry weight) of the bark of white-barked birch trees (*Betula* sp.).[5] This pentacyclic triterpene can be converted into betulinic acid **2**,[6] which has shown anti-inflammatory,[7] antimalarial[8] and especially cytotoxic activity against several tumor cell lines by inducing apoptosis in cells.[9,10] Some betulin derivatives have also shown remarkable anti-human immunodeficiency virus activity with new mechanisms of action.[11,12] Structure–activity relationship studies and pharmacological properties of betulin and its derivatives have been reviewed recently.[13]

Previously, dihydrobetulinic acid **3** was examined as a new lead compound for anti-leishmanial therapy.[14] It was shown that it targeted DNA topoisomerases I and II by preventing DNA cleavage and formation of an enzyme–DNA complex, which ultimately induced apoptosis in *Leishmania donovani* promastigotes and amastigotes in infected macrophages with an IC_{50} value of 2.6 and 4.1 μM, respectively. Parasitic burden in golden hamsters was reduced by 92% after a 6-week treatment with dihydrobetulinic acid **3** (10 mg kg^{-1} body weight). In another study, in which leishmanicidal inhibition activity of a plethora of natural products was screened, betulinic acid **2** isolated in small quantities from *Betula platyphylla* var. *japonica* was found to be weakly active against *Leishmania major* promastigotes, the extracellular form of the parasite, with an IC_{50} value of 88 μM.[15] It was also noted that in triterpenes with ursane, oleanane or lupane skeletons, a carboxyl substituent was required for anti-leishmanial activity. In a related study, it was shown that a rare natural product, betulin aldehyde **4**, obtained from *Doliocarpus dentatus* (Aubl.) showed *in vitro* activity against *Leishmania amazonensis* amastigotes in infected macrophages, reducing infection by 88% at 136 μM and by

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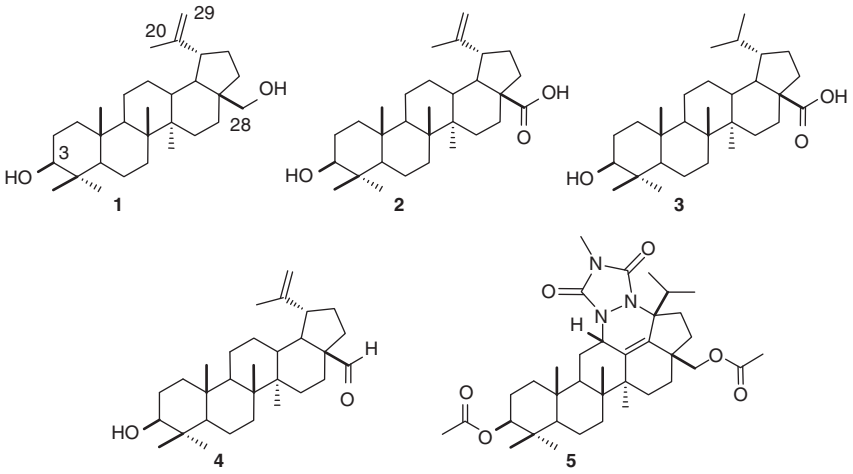


Figure 1 Chemical structures of betulin **1**, betulinic acid **2**, dihydrobetulinic acid **3**, betulin aldehyde **4** and betulin heterocycloadduct between 3,28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine **5**.

58% at 68 μM.[16] At these doses, **4** also showed some toxicity against peritoneal macrophages, with survival indices of 70 and 80%, respectively. Previously, we studied anti-leishmanial activity of heterocyclic betulin derivatives, in which the heterocycloadduct between 3,28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine **5** was the most effective derivative with a GI_{50} value of 8.9 μM against *L. donovani* amastigotes.[17] These results prompted us to investigate more closely the anti-leishmanial activity of 24 betulin derivatives that have been chemically modified in positions C-3, C-28 and C-20–C-29 of the lupane skeleton.

RESULTS AND DISCUSSION

We found that betulin **1** (isolated from *Betula* sp.) has moderate anti-leishmanial activity against *L. donovani* axenic amastigotes, showing 35% inhibition at 50 μM in a microplate assay (Table 1). Acetylation, esterification or etherification of the hydroxy groups at C-3 or C-28 in most cases retained anti-leishmanial activity. We observed that 28-*O*-Cinnamoylbetulin **6** was totally inactive and 28-*O*-nicotinoylbetulin **7**, 28-*O*-tetrahydropyranylbetulin **8**, 28-*O*-chrysanthemoylbetulin **9** and betulinyl-28-*O*-carboxymethoxycarvacrolate **10** were only slightly active. Only 28-*O*-(*N*-acetylanthraniloyl)betulin **11** and 28-*O*-bromoacetylbetulin **12** showed improved anti-leishmanicidal activity (59 and 86% inhibition at 50 μM, respectively), compared with **1**. In addition, 3-*O*-acetylbetulin **13** had similar anti-leishmanial inhibition activity compared with the starting material betulin **1**, whereas 3,28-di-*O*-acetylbetulin **14** and 3,28-di-*O*-levulinoylbetulin **15** were totally inactive.

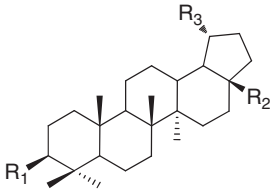
Oxidation of **1** seems to have a beneficial effect on anti-leishmanial activity. Betulin aldehyde **4** displayed improved anti-leishmanial activity with a 64% inhibition at 50 μM. Betulinic acid **2** possessed moderate anti-leishmanial activity with a 40% inhibition at 50 μM. 28-*O*-Acetyl-3-oxobetulin **16** and betulonic aldehyde **17** showed moderate anti-leishmanial activity similar to the starting material **1**, but betulonic acid **18** had remarkable anti-leishmanial activity with a 98% inhibition at 50 μM. Reduction of the carbon–carbon double bond of betulonic acid **18** to the corresponding dihydrobetulonic acid **19** decreased anti-leishmanial activity to 72% at 50 μM. Furthermore, methylation of betulonic acid **18** to methyl betulonate **20** decreased the inhibition activity at 50 μM to 40%. L-aspartyl amide of betulonic acid **21** showed reduced leishmanicidal activity compared with betulonic acid **18,** with a 69% inhibition at 50 μM. Vanillyl betulonate **22** was totally inactive.

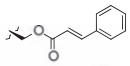
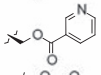
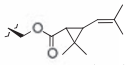
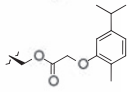
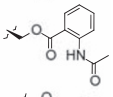
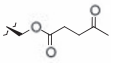
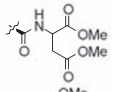
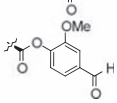
Removal of the C-3 hydroxy group of **1** resulted in 3-deoxy-2,3-didehydrobetulin **23**, the anti-leishmanial activity of which diminished to 13% at 50 μM. Oxime derivatives **24** and **25** showed good leishmanicidal activities at 50 μM, with 69 and 73% inhibition, respectively. Moreover, betulin derivative **26** with a nitrile group at C-28 showed good anti-leishmanial activity with a 63% inhibition at 50 μM.

Derivatives (**12**, **18**, **19**, **21** and **25**) that showed the best anti-leishmanial activity on microplate assay at 50 μM against *L. donovani* axenic amastigotes were selected for further investigations: GI_{50} values, cytotoxicity to the macrophage cell line THP-1 and anti-leishmanial activity against the *L. donovani*-infected macrophage cell line THP-1 were evaluated. Betulonic acid **18** showed the best GI_{50} value of 14.6 μM on microplate assay against *L. donovani* axenic amastigotes, followed by L-aspartyl amide derivative **21** and oxime derivative **25**, with GI_{50} values of 21.2 and 22.8 μM, respectively (Table 1). 28-*O*-Bromoacetylbetulin **12** and dihydrobetulonic acid **19** had moderate GI_{50} values of 34.9 and 56.0 μM, respectively. Cytotoxicity of derivatives **12**, **18**, **19**, **21** and **25** was tested against the macrophage cell line THP-1 at concentrations of 50, 25 and 12.5 μM (Table 2). Betulonic acid **18** showed cytotoxicity against the THP-1 cell line at all test concentrations. Dihydrobetulonic acid **19** and oxime derivative **25** showed cytotoxicity against the THP-1 cell line at 50 and 25 μM, but at 12.5 μM concentration, cytotoxicity of **19** and **25** was reduced to 22.0 and 13.6%, respectively. L-aspartyl amide derivative **21** and 28-*O*-bromoacetylbetulin **12** were nontoxic to macrophage cell line THP-1 at all test concentrations.

Finally, anti-leishmanial activity of compounds **12, 19**, **21** and **25** was tested against *L. donovani*-infected macrophage cell line THP-1, with concentrations that showed $<30\%$ cytotoxicity to the THP-1 cell line (Table 3). In all cases, anti-leishmanial activity was reduced when compared with that in the corresponding microplate assay with *L. donovani* axenic amastigotes. L-aspartyl amide derivative **21** and 28-*O*-bromoacetylbetulin **12** showed good anti-leishmanial activity at 50 μM, inhibiting 53 and 56% of the intracellular parasites, respectively (compared with 69 and 86% inhibition using axenic amastigotes in the microplate assay, respectively). At 25 μM, 28-*O*-bromoacetylbetulin **12** still had the best activity of the compounds examined showing 34% inhibition, dihydrobetulonic acid **19** and L-aspartyl amide derivative

Table 1 Anti-leishmanial activities at 50 μM on microplate assay and GI_{50} values for the most potent synthetic betulin derivatives against *Leishmania donovani* axenic amastigotes



Compound	R_1	R_2	R_3	*Inhibition (%) at 50 μM*	GI_{50} *(μM)*
1	OH	CH_2OH	CH_3-C=CH_2	35.0	
6	OH		CH_3-C=CH_2	0.0	
7	OH		CH_3-C=CH_2	8.8	
8	OH		CH_3-C=CH_2	10.5	
9	OH		CH_3-C=CH_2	13.4	
10	OH		CH_3-C=CH_2	16.6	
11	OH		CH_3-C=CH_2	59.2	
12	OH		CH_3-C=CH_2	86.0	34.9
13	OAc	OH	CH_3-C=CH_2	37.4	
14	OAc	CH_2OAc	CH_3-C=CH_2	0.0	
15			CH_3-C=CH_2	0.0	
4	OH	CHO	CH_3-C=CH_2	64.3	
2	OH	CO_2H	CH_3-C=CH_2	39.8	
16	O=	CH_2OAc	CH_3-C=CH_2	40.6	
17	O=	CHO	CH_3-C=CH_2	46.2	
18	O=	CO_2H	CH_3-C=CH_2	97.6	14.6
19	O=	CO_2H	CH_3CHCH_3	72.1	56.0
20	O=	CO_2Me	CH_3-C=CH_2	40.1	
21	O=		CH_3-C=CH_2	69.3	21.2
22	O=		CH_3-C=CH_2	0.0	
23	-	CH_2OH	CH_3-C=CH_2	13.2	
24	OH	CH=NOH	CH_3-C=CH_2	69.1	
25	=NOH	CH=NOH	CH_3-C=CH_2	72.9	22.8
26	OAc	CN	CH_3-C=CH_2	62.7	
Positive control[a]				95	
Negative control[b]				0.0	

Abbreviation: DMSO, dimethyl sulfoxide.
[a]Amphotericin B (1 μM).
[b]Culture medium+DMSO.

21 were only weakly active at this concentration. Finally, at 12.5 μM concentration, oxime derivative **25** showed the best anti-leishmanial activity with a 52% inhibition, whereas L-aspartyl amide derivative **21** was totally inactive and the rest showed only weak activity.

We have shown that by simple chemical modification, anti-leishmanial activity of ubiquitous naturally occurring triterpene, betulin, can be improved considerably. It is possible to derive relatively potent anti-leishmanial compounds with low micromolar GI_{50} values. In

Table 2 Cytotoxicity of the most potent synthetic betulin derivatives on macrophage cell line THP-1

	Inhibition of growth (%)		
Compound	*50 μM*	*25 μM*	*12.5 μM*
12	0.0	0.0	0.0
18	85.3	77.7	38.2
19	80.2	30.0	22.0
21	0.0	14.0	3.6
25	61.4	55.2	13.6

Table 3 Anti-leishmanial activities of the most potent synthetic betulin derivatives against macrophage cell line THP-1 infected with *Leishmania donovani*

	Inhibition of growth (%)		
Compound	*50 μM*	*25 μM*	*12.5 μM*
12	56.3	34.4	17.8
19	nt	20.6	14.3
21	53.3	16.0	0.0
25	nt	nt	51.5

Abbreviation: nt, not tested because the toxicity to the THP-1 cell line was >30% at that concentration.

general, carbonyl or carboxyl groups at C-3 or C-28 have a beneficial effect in anti-leishmanial inhibition activity, and these compounds can be regarded as significant lead molecules for further improvement and optimization. Further studies are required to develop more potent betulin derivatives with leishmanicidal properties, and with no toxicity in macrophage cell lines or in human host cells. Moreover, thorough early ADME, biological mechanism and animal studies are required to evaluate anti-leishmanial activity *in vivo*.

EXPERIMENTAL SECTION

Chemical syntheses of betulin derivatives screened in this study for anti-leishmanial activity are described in detail elsewhere.[18] Anti-leishmanial activities of betulin derivatives were screened using a fluorescent viability microplate assay with *L. donovani* (MHOM/SD/1962/1S-Cl2d) axenic amastigotes and alamarBlue (resazurin, AbD Serotec, Oxford, UK) as described previously.[19–21] Initial screening was carried out by assessing the inhibition of amastigote growth at 50 μM of betulin derivative. All compounds were tested at least twice in triplicate. Complete medium, both with and without dimethyl sulfoxide, was used as negative controls (0% inhibition of amastigote growth). The most potent betulin derivatives from initial screening were selected for further investigation. For these compounds, the GI_{50} value (concentration for 50% growth inhibition) was also determined, as well as screening for activity on infected macrophages. The latter assay was carried out as previously described using the retinoic acid-treated human macrophage cell line THP-1 infected with *L. donovani* expressing the luciferase gene (*Ld*:pSSU-int/LUC) at a 3:1 parasite:macrophage ratio.[17,22] Compounds (at 50, 25 and 12.5 μM) to be tested were added for 48 h, and luminescence was determined after adding a luciferase substrate and measuring in a microplate reader. Amphotericin B was included as a positive control on each plate and resulted in >90% inhibition at 1 μM. The effect of compounds on THP-1 cells alone was assessed using the alamarBlue viability assay.

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