European Journal of Medicinal Chemistry 44 (2009) 2468–2472

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biological evaluation of abietic acid derivatives

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ARTICLE INFO

Article history:
Received 9 December 2008
Received in revised form
12 January 2009
Accepted 15 January 2009
Available online 24 January 2009

Keywords:
Abietic acid
Cytotoxicity
Antimycotic activity
Antiviral activity

ABSTRACT

A series of C18-oxygenated derivatives of abietic acid were synthesized and evaluated for their cytotoxic, antimycotic, and antiviral activities. In general, the introduction of an aldehyde group at C18 did improve the resultant bioactivity, while the presence of an acid or alcohol led to less active compounds.

1. Introduction

Diterpene resin acids are important defense compounds of conifers against potential herbivores and pathogens [1]. The biological activity of natural abietane acids has been reviewed [2]. Antimicrobial, antiulcer and cardiovascular activities are the most representative for this class of diterpenoids. Abietic acid, an abietane diterpenoid, has shown antiallergic [3], anti-inflammatory [4], phytoalexin-like [5], and anticonvulsant activities [6]. Despite the diverse biological properties of this family of compounds, little effort has been made for the biological study, specially of derivatives.

As a part of our research program towards the discovery of bioactive terpene compounds, we were interested in confirming the results reported in a recent patent on the use of abietic acid and derivatives as antitumor agents [7]. We describe here the syntheses of a number of derivatives of commercially available (−)-abietic acid (**1**) (Fig. 1) [8], and the results of preliminary evaluation of their cytotoxic, antimycotic and antiviral activities. In this study, an oxygenated moiety (such as methyl ester, alcohol, or aldehyde) was introduced into the lipophilic abietane skeleton. In this context, simple and sequential modifications were performed in the molecule of abietic acid **1**. Compound **1** and **7** derivatives with different functional groups at C18 and isomeric double bonds were tested. All the compounds were easily obtained in good yield, by standard or reported chemical procedures. Some of the synthesized compounds have been isolated as natural products, in particular, abietinol (**3**) and abietinal (**4**) [9], however, no reports have appeared on their biological activities.

2. Results and discussion

2.1. Chemistry

The synthesis of the C18-functionalized abietanes used in this work begins with the preparation of the required methyl ester **2** from commercial (−)-abietic acid, following a reported procedure (Scheme 1) [10]. Thus, abietic acid **1** was esterified by treatment with lithium hydroxide and methyl sulfate to give ester **2** in quantitative yield. Reduction of ester **2** with $LiAlH_4$ in dry tetrahydrofuran at reflux gave alcohol (abietinol) **3** in quantitative yield. Oxidation of **3** was carried out by treatment with PCC on alumina [11]. This oxidation afforded aldehyde (abietinal) **4** in 40% yield.

Next, the conjugated double bonds present in ester **2** were isomerized using a known protocol [10] with few modifications (Scheme 1). Thus, methyl abietate (**2**) was treated with 33% HBr in acetic acid and the resulting bromo derivatives were heated in DMF at 80 °C in the presence of lithium hydroxide to afford compound **6** in 56% overall yield. Ester **6** was saponified with KOH in aqueous methanol to give acid **5**. Reduction of **6** with $LiAlH_4$ in dry

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doi:10.1016/j.ejmech.2009.01.014

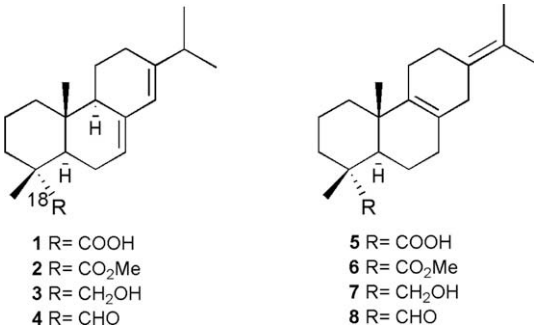


Fig. 1. Chemical structures of tested abietanes.

tetrahydrofuran at reflux gave alcohol **7** in 86% yield. Finally, oxidation with PCC on alumina [11] of **7** afforded aldehyde **8** in 40% yield.

2.2. Biological evaluation

Firstly, the abietanes **1**–**8** were tested in vitro for antimycotic activity. All compounds did not show antimycotic activity against *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis*, *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* in concentrations below 100 μg/mL except abietane **4** with MIC value of 50 μg/mL against *A. fumigatus* (data not shown). The introduction of an aldehyde group at C18 led to a compound with anti-*A. fumigatus* activity in the series of abietanes **1**–**4**. MIC values for the two reference antifungal drugs, amphotericine B and itraconazole (Sigma, New Jersey, USA), used as positive controls, were within the established values for the AFST-EUCAST and CLSI-M38-A protocols.

Next, the abietanes **1**–**8** were tested in vitro for potential antitumor and cytotoxic activities determining the concentration of the compound that induces 50% killing (CC_{50}) of the HeLa tumor line, and the Vero cell line, respectively (Table 1). All these compounds produced a dose-dependent inhibition on the growth of the HeLa tumor cell line and Vero cell line, with R^2 (coefficient of linear regression) >0.8. The abietane **4** showed in vitro potential antitumor activity with CC_{50} value of 5.6 ± 0.5 μg/mL. However, the abietane that showed the highest potential antitumor activity on the HeLa tumor line and the lowest cytotoxic on non-cancerous cells (Vero cell line) was the abietane **2** with CC_{50} values of 3.6 ± 1 μg/mL and 49.4 ± 3 μg/mL, respectively. This means that the abietane with the highest selectivity index (SI) was methyl abietate (**2**) (SI = 13.7). Selectivity refers to the ability of a compound to recognize its target without interacting with other targets. Nowadays SI values are evaluated for any biological activity because it can be therapeutically relevant for designing and synthesizing new drugs. Ideally a compound selectivity index of 100 to a 1000 or more is ideal but in some instances lower values can provide sufficient selectivity to derive a good therapeutic index. Recently, an SI value of 14.3 on cell lines is indicative of potential antitumor activity for biopharmaceutical use [12]. Specifically, according to SI value the order of activity in the series abietanes (**1**–**4**) at position C18 was found to be ester > aldehyde > acid > alcohol. On the other hand, the antitumor activity data in the series of abietanes (**5**–**8**) indicated a significant reduction of activity dependent of the isomerization of the two double bonds.

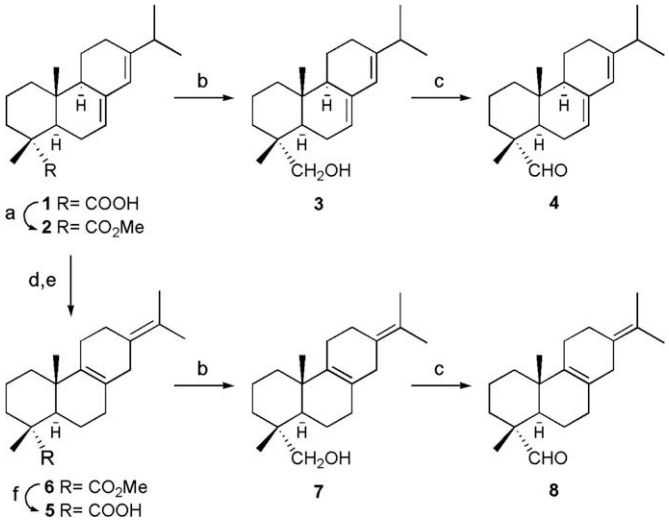
Table 1
Cytotoxic activity of abietane diterpenes determined by the MTT technique expressed as CC_{50} (μg/mL).[a]

Compound	Cell lines[b]				
	HeLa		VERO		
	CC_{50}	$R^{2,c}$	CC_{50}	R^2	SI[d]
1	14.9 ± 0.6	0.99	52.5 ± 5.6	0.92	3.5
2	$\mathbf{3.6 \pm 1}$	0.87	$\mathbf{49.4 \pm 3}$	0.98	**13.7**
3	5.2 ± 0.5	0.97	12.7 ± 2.4	0.76	2.4
4	5.6 ± 0.5	0.95	31.5 ± 6.5	0.80	5.6
5	53.1 ± 4.6	0.95	43.9 ± 4.7	0.94	0.8
6	46.6 ± 1.3	1	19.2 ± 1.8	0.92	0.4
7	19.7 ± 2.1	0.91	20.2 ± 1.5	0.95	1.0
8	33.4 ± 6.6	0.81	55.3 ± 2.2	0.99	1.6
Vincristine	0.05 ± 0.01	0.95	1.1 ± 0.2	0.95	22

Numbers in bold belong to the most potent compound.
[a] 50% Cytotoxic concentration in 48 h.
[b] HeLa, human cervix epitheloid carcinoma ATCC CCL-2; Vero, *Cercopithecus aethiops* African green monkey kidney ATCC CCL-81.
[c] R^2: coefficient of linear regression.
[d] SI, selectivity index is defined as VERO CC50 over HeLa CC50.

Gigante and co-workers [13] prepared different catechols from abietic acid and evaluated their antitumoral activity. All compounds produced a dose-dependent inhibition on growth of the tumor cells evaluated. The order of activity in the catechol series with different groups at position C18 was ester > acid ≥ alcohol. The results reported by Gigante and co-workers are consistent with our results because introduction of a methyl ester group at C18 also enhances the resultant antitumor activity.

The antiviral activity of the abietanes against herpes simplex virus type 1 (HSV-1) was determined using a modified end-point titration technique (EPPT) (Table 2) [14]. The abietanes (**2**–**5**) reduced the HSV-1 replication with values below 100 μg/mL. The abietane that showed the highest antiviral activity was abietinol (**3**). According to Vlietinck et al. [15], only the compounds with reduction factor (R_f) of the viral titer over 1×10^3 (R_f: ratio of the virus titer in the absence over virus titer in the presence of the tested compound) show relevant antiviral activity. Abietinol (**3**) was found to be slightly active against HSV-1 over infected confluent monolayers of HeLa cells with R_f value of 1×10^1 at a concentration of 6.25 μg/mL. Gigante and co-workers reported the effect of the catechol methyl 11,12-dihydroxyabieta-8,11,13-trien-18-oate on HSV-1 at concentration of 15 μM [13]. These results are consistent with our results because the methyl abietadien-18-oate (**2**) showed anti-HSV activity. However, the replacement of the ester by a hydroxyl group at C18 enhances the antiviral activity.



Scheme 1. Reagents and conditions: (a) LiOH, Me_2SO_4, DMF; (b) $LiAlH_4$, THF; (c) PCC/Al_2O_3, DCM; (d) HBr, AcOH; (e) LiOH, DMF; (f) KOH, MeOH, H_2O.

Table 2
Cytotoxicity and anti-HSV-1 activity of abietane diterpenes on HeLa Cells determined by the end-point titration technique.

Compound	CC_{100} (μg/mL)[a]	Viral reduction factor[b]	Antiviral activity (μg/mL)[c]
1	>100	NA	–
2	50	$10^{0.5}$	6.25
3	25	10^{1}	6.25
4	>50	$10^{0.5}$	12.5
5	>100	10^{1}	100
6	100	NA	–
7	100	NA	–
8	>100	NA	–
Heparin	>100 U.I./mL	10^{2}	0.5 U.I./mL
Acyclovir	>600	10^{4}	6.0

[a] Minimal toxic dose that detached 100% of the cell monolayer.
[b] Ratio of the virus titer in the absence over virus titer in the presence of the tested compound.
[c] Maximal nontoxic dose that showed the highest viral reduction factor. NA: no activity.

3. Conclusions

In conclusion, we have prepared and tested several abietanes for their antitumor, antifungal and antiviral activities in vitro. In general, the isomerization of the double bonds present in abietic acid led to less active compounds. In the series of abietic acid derivatives (**1**–**4**), the compound with an aldehyde group presented both antifungal and antitumor activities. Methyl abietate presented the highest cytotoxicity and abietinol presented a weak antiviral activity. These results encourage us to continue our research of this series by synthesizing additional abietane-type derivatives with the aim of obtaining biologically more potent compounds.

4. Experimental

4.1. Chemistry

Optical rotations were determined using a 5-cm path-length cell, using dichloromethane as solvent (concentration expressed in g/100 mL). $[\alpha]_D$-Values are given in 10^{-1} deg cm^2/g. NMR spectra were recorded on a 300 MHz spectrometer with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$ as solvent unless otherwise described. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. *J* values are given in hertz. Mass spectra (MS) were run by electron impact (EI) at 70 eV. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F-254 in 0.25 mm-thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating on a hotplate. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). All non-aqueous reactions were carried out in an argon atmosphere in oven-dried glassware. Commercial reagent grade solvents and chemicals were used as received unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure.

4.1.1. Methyl abietadien-18-oate (methyl abietate, *2*)

A solution of abietic acid (**1**) (75%, 68.7 g, 0.17 mol) in DMF (280 mL) was treated with LiOH (20 g, 2.8 equiv.) and stirred for 20 h. Then, Me_2SO_4 (110 mL, 7 equiv.) was added and stirred overnight. The following day the mixture was poured into water and extracted with hexane. The extract was washed with NaOH 0.1 N and brine, dried, and concentrated. Purification of the residue by flash chromatography, using hexane-ethyl acetate (from 7:3 to 6:4) as eluent, provided methyl abietate **2** (54 g, 100%) as an orange oil: $[\alpha]^{20}_D$ −92.0 (c 0.69); ^1H NMR (300 MHz) δ 5.77 (1H, s), 5.36 (1H, m), 3.62 (3H, s), 2.21 (1H, m), 1.25 (3H, s), 1.01 (3H, d, $J = 6.9$), 0.99 (3H, d, $J = 6.9$), 0.82 (3H, s); ^{13}C NMR (75 MHz) δ_C 179.0 (s), 145.3 (s), 135.5 (s), 122.3 (d), 120.6 (d), 51.8 (d), 50.9 (d), 46.6 (s), 45.1 (d), 38.3 (t), 37.1 (t), 34.9 (q), 34.5 (s), 27.5 (t), 25.7 (t), 22.5 (t), 21.4 (q), 20.8 (q), 18.1 (t), 17.0 (q), 14.0 (q); HRMS (EI) *m/z* 316.2422 $[M]^+$, calcd for $C_{21}H_{32}O_2$: 316.2402.

4.1.2. Abietadien-18-ol (abietinol, *3*)

A suspension of $LiAlH_4$ (3.10 g, 82 mmol) in tetrahydrofuran (100 mL) was stirred as abietic acid (**1**) (70%, 5 g, 11.6 mmol) in tetrahydrofuran (50 mL) was added. The mixture was refluxed for 15 h, then it was cooled to 0 °C and 3.1 mL of H_2O, 3.1 mL of 15% NaOH and 9.3 mL of H_2O were added sequentially and carefully. The resulting white solid was filtered off and washed with ethyl acetate. The extract was concentrated and purified by chromatography eluting with hexane-ethyl acetate (7:3) to give 3.3 g (100%) of pure alcohol **3** as a white solid: mp 81–83 °C; $[\alpha]^{20}_D$ −132.0 (c 0.33); ^1H NMR (300 MHz) δ 5.75 (1H, s), 5.37 (1H, br s), 3.31 (1H, d, $J = 11.0$), 3.07 (1H, d, $J = 11.0$), 0.99 (3H, d, $J = 6.8$), 0.98 (3H, d, $J = 6.8$), 0.84 (3H, s), 0.80 (3H, s); ^{13}C NMR (75 MHz) δ_C 144.9 (s), 135.4 (s), 122.4 (d), 120.9 (d), 71.8 (t), 50.7 (d), 43.4 (d), 38.8 (t), 37.3 (s), 35.6 (t), 34.7 (d), 34.5 (s), 27.4 (t), 23.7 (t), 22.6 (t), 21.3 (q), 20.7 (q), 18.1 (t), 17.6 (q), 14.1 (q); HRMS (EI) *m/z* 288.2440 $[M]^+$, calcd for $C_{20}H_{32}O$: 288.2453.

4.1.3. Abietadien-18-al (abietinal, *4*)

A solution of alcohol **3** (400 mg, 1.39 mmol) in DCM (7 mL) was treated with PCC on alumina [11] (4 g). After being stirred for 2 h, the mixture was filtered through silica eluting with fresh DCM. Then, the filtrate was concentrated and chromatographed on silica eluting with hexane-ethyl acetate (7:3) to give 160 mg of aldehyde **4** as a colorless oil: $[\alpha]^{20}_D$ −55.0 (c 0.63); ^1H NMR (300 MHz) δ 9.21 (1H, s), 5.78 (1H, s), 5.35 (1H, br s), 2.21 (1H, m), 1.14 (3H, s), 1.02 (3H, d, $J = 6.8$), 1.00 (3H, d, $J = 6.8$), 0.85 (3H, s); ^{13}C NMR (75 MHz) δ_C 206.2 (d), 145.5 (s), 135.6 (s), 122.2 (d), 120.0 (d), 50.5 (d), 49.0 (s), 42.4 (d), 38.3 (t), 34.8 (d), 33.8 (s), 32.9 (t), 27.4 (t), 25.4 (t), 22.5 (t), 21.3 (q), 20.8 (q), 17.2 (t), 14.3 (q), 14.1 (q); HRMS (EI) *m/z* 286.2320 $[M]^+$, calcd for $C_{20}H_{30}O$: 286.2297.

4.1.4. Methyl abieta-8,13(15)-dien-18-oate (*6*)

Methyl abietate (**2**) (45 g, 0.14 mol) was dissolved in a 33% HBr solution in acetic acid (120 mL) and stirred for 20 h. Then, the reaction was quenched with H_2O and the supernatant was decanted, the resulting solids were washed with additional H_2O and decanted again. The solid was dried under reduced pressure to leave the corresponding crude dibromo derivative (66 g). The crude bromo derivative was mixed with LiOH (10 g, 0.23 mol) and DMF (200 mL) was added. The mixture was heated at 80 °C for 16 h, then, it was poured into H_2O and extracted with hexane. The combined extracts were washed with brine, dried, and concentrated. The resulting residue was chromatographed on silica (9:1 hexane–EtOAc as eluant) to give 25 g (56%) of the isomerized ester **6** as an orange oil. The ^1H NMR and EIMS data agree with those reported in the literature [10].

4.1.5. 8,13(15)-Abietadien-18-oic acid (*5*)

A mixture of ester **6** (200 mg, 0.63 mmol), KOH (85%, 800 mg, 14 mmol), H_2O (2 mL) and methanol (12 mL) was refluxed for 16 h. Then, additional KOH (85%, 400 mg, 7 mmol) was added and reflux continued for 3 days more. After this time, the reaction mixture was then cooled, poured into aqueous HCl (1.2 M, 20 mL) and extracted three times with DCM. The organic extract was dried over $MgSO_4$ and concentrated under reduced pressure to give the crude acid **5** (190 mg, 99%) as a foam: $[\alpha]^{20}_D$ + 155.0 (c 0.2); ^1H NMR (300 MHz)

δ 1.67 (3H, s), 1.64 (3H, s), 1.20 (3H, s), 0.99 (3H, s); ^{13}C NMR (75 MHz) δ_C 185.8 (s), 138.0 (s), 128.2 (s), 126.1 (s), 120.7 (s), 47.4 (s), 45.9 (d), 36.9 (s), 36.7 (t), 35.2 (t), 34.7 (t), 31.6 (t), 27.5 (t), 24.8 (t), 21.6 (t), 20.1 (q), 19.6 (q), 19.4 (q), 18.1 (t), 16.1 (q); HRMS (EI) *m/z* 302.2250 $[M]^+$, calcd for $C_{20}H_{30}O_2$: 302.2246.

4.1.6. 8,13(15)-Abietadien-18-ol (**7**)

A suspension of $LiAlH_4$ (2.10 g, 58 mmol) in tetrahydrofuran (45 mL) was stirred as ester **6** (3.1 g, 9.8 mmol) in tetrahydrofuran (15 mL) was added. The mixture was refluxed for 15 h, then it was cooled to 0 °C and 2.1 mL of H_2O, 2.1 mL of 15% NaOH and 6.3 mL of H_2O were added sequentially and carefully. The resulting white solid was filtered off and washed with ethyl acetate. The extract was concentrated and purified by chromatography eluting with hexane-ethyl acetate (5:5) to give 2.4 g (85%) of pure alcohol **7** as a colorless oil: $[\alpha]^{20}_D + 55$ (c 4.3); ^{1}H NMR (300 MHz) δ 3.42 (1H, d, $J = 11.0$), 3.16 (1H, d, $J = 11.0$), 1.66 (3H, s), 1.64 (3H, s), 1.01 (3H, s), 0.80 (3H, s); ^{13}C NMR (75 MHz) δ_C 138.7 (s), 128.3 (s), 125.8 (s), 120.6 (s), 72.2 (t), 45.2 (d), 37.5 (s), 37.4 (s), 35.7 (t), 35.0 (t), 34.7 (t), 31.8 (t), 27.5 (t), 25.0 (t), 20.1 (q), 19.6 (q), 19.6 (q), 18.6 (t), 18.2 (t), 17.4 (q); HRMS (EI) *m/z* 288.2460 $[M]^+$, calcd for $C_{20}H_{32}O$: 288.2453.

4.1.7. 8,13(15)-Abietadien-18-al (***8***)

A solution of alcohol **7** (600 mg, 2.1 mmol) in DCM (8 mL) was treated with PCC on alumina [11] (6 g). After being stirred for 2 h, the mixture was filtered through silica eluting with fresh DCM and ethyl acetate. Then, the filtrate was concentrated and chromatographed on silica eluting with hexane-ethyl acetate (8:2) to give 240 mg of aldehyde **8** as a colorless oil: $[\alpha]^{20}_D + 155.0$ (c 0.8); ^{1}H NMR (300 MHz) δ 9.22 (1H, s), 1.67 (3H, s), 1.65 (3H, s), 1.08 (3H, s), 1.02 (3H, s); ^{13}C NMR (75 MHz) δ_C 206.5 (d), 138.0 (s), 128.0 (s), 126.2 (s), 120.8 (s), 49.6 (s), 44.0 (d), 36.3 (s), 35.1 (t), 34.6 (t), 31.9 (t), 31.3 (t), 27.4 (t), 24.8 (t), 21.2 (t), 20.0 (q), 19.6 (q), 19.4 (q), 17.3 (t), 13.8 (q); HRMS (EI) *m/z* 286.2250 $[M]^+$, calcd for $C_{20}H_{30}O$: 286.2297.

4.2. Biological assays

4.2.1. Antifungal assay

The antifungal activity of abietanes **1**–**8** was evaluated following the Clinical and Laboratory Standards Institute M38-A protocol (CLSI) [16] for filamentous fungi and the standard method proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) [17] for fermentative yeasts. *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (CECT 11901), *C. albicans* (ATCC 10231), *A. flavus* (ATCC 204304) *A. fumigatus* (ATCC 204305), *A. terreus* (CDC 317), and *A. niger* (ATCC 10124), were used to evaluate antifungal activity. Briefly, seven serial dilutions of the compounds were dispensed into 96-well flat-bottomed microdilution plates in duplicate at final concentrations between 100 μg/mL and 2 μg/mL. Amphotericine B (Sigma Chemical Co., MO, USA) and itraconazole (Sigma Chemical Co., MO, USA) were used as positive controls at a range of 0.031–16 μg/mL. The plates were frozen at −70 °C until required. The inoculum size for microdilution plates were $0.5–2.5 \times 10^5$ and $0.4–5 \times 10^4$ CFU/mL for yeast and filamentous fungi, respectively. For the AFST-EUCAST method, the Minimal Inhibitory Concentrations (MICs) were determined after 24 h of incubation and is defined as the lowest concentration that resulted in a 90% reduction of growth. For the CLSI M38-A method, the MICs were determined after 48 h of incubation and are defined as the lowest dilution that resulted in total inhibition of visible growth. MIC results were expressed as range and geometric mean (GM) of triplicates of each compound tested three times against each of the fungal species in different assays.

4.3. Antitumor activity and cytotoxicity

The cell lines used were human cervix epithelioid carcinoma cells (HeLa cell line ATCC CCL-2) and *Cercopithecus aethiops* African green monkey kidney cells (Vero cell line ATCC CCL-81). Herpes simplex virus type 1 (HSV-1) was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HSV-1-infected HeLa cell cultures. Cells were grown in MEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 mg/mL of streptomycin, 20 mg/mL of *l*-glutamine, 0.14% $NaHCO_3$, and 1% each of nonessential amino acids and vitamin solution. The cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

The antitumor activity on HeLa cells and cytotoxic activity on Vero cells have been carried out using in vitro assay on cell growth and tetrazolium-dye (MTT) cytotoxicity assay, according to the protocol reported by us [18], which was used with a few modifications. Cell monolayers were trypsinized and washed with culture medium and then plated at 1.5×10^4 cells per well for HeLa, and 1.25×10^4 cells per well for Vero cells in a 96-well flat-bottomed plate. After 24 h of incubation, each diluted compound was added to the appropriate wells and the plates were incubated for further 48 h at 37 °C in a humidified incubator with 5% CO_2. Vincristine was used as positive control. The CC_{50} for each compound were obtained from dose–effect curves for linear regression methods and CC_{50} values are expressed as the mean ± S.E.M. of at least four dilutions by quadruplicate.

4.4. Antiviral assays

The antiviral activity against HSV-1 has been carried out on HeLa cells using end-point titration technique, according to the protocol reported by us [14], which was used with a few modifications. Twofold dilutions of the compounds and viral suspension (one infection dose, 1 D.I.) were mixed and incubated for 0.5 h at 37 °C before they were added on confluent monolayer cells and incubated again at 37 °C in humidified 5% CO_2 atmosphere for 36 h. Acyclovir and Heparin sodium salt were used as positive controls.

Acknowledgments

Financial support from the Spanish Ministry of Science and Education, under a "Ramón y Cajal" research grant, and also from the Generalitat Valenciana (project GV/2007/007) is gratefully acknowledged. L.B.-G. thanks the financial support from Antioquia University of Colombia and COLCIENCIAS, Bogotá, Colombia (Grant RC 432-2004).

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