

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Faculdade de Farmácia
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derivatives as new antitumor agents

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Design, synthesis, and biological evaluation of betulinic acid derivatives as new antitumor agents

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Abstract

Chronic myeloid leukemia (CML) is a cancer that is currently treated with imatinib, however, resistance to this drug usually develops over time. Triterpenes such as betulinic acid can be analogues of triptolide, a compound recently shown to be active against CML cells resistant to imatinib. The aim of this study was to perform modifications on betulinic acid based on the structure-activity relationship of triptolide to generate new analogues, and evaluate their cytotoxicity. The main modification performed was fluorination on C-28. A total of 5 analogues were synthesized, being 3 previously described and 2 novel compounds. The MTT assay was performed using HeLa, B16F10 and HaCaT cell lines. The results show that the presence of fluorine in the molecule has an important role in increasing the antitumor activity. However, fluorination also enhanced the cytotoxicity on non-tumor cells, being betulinic acid less toxic than its tested derivatives. Considering B16F10 cells, compound **3** was the most active and selective, with IC₅₀ of 1.9 μM, being considered a promising agent for the treatment of melanoma. Further studies are needed to investigate the antitumor activity in CML cells, since the mechanism of death and activity may be different for different cell lines.

Key words: betulinic acid, antitumor, triptolide, chronic myeloid leukemia, fluorination

Introduction

Cancer is among the leading causes of death in the world (a). In 2012, around 14.1 million cancer cases were estimated in the whole world, and 8.2 million deaths caused by cancer. The most incident cancers are lung (13.0% of all diagnosed cancer cases), breast (11.9%) and prostate (7.9%). Nevertheless, other types of cancer are also of worry. Cervical cancer is responsible for 3.7% of cancer cases, melanoma for 1.6%, and leukemia cases consist of 2.5% of the total. There are four main types of leukemia: chronic myeloid leukemia, acute myeloid leukemia, chronic lymphocytic leukemia and acute lymphoblastic leukemia (c). Chronic myeloid leukemia (CML) is usually treated with imatinib, a very specific drug that inhibits an enzyme responsible for the uncontrolled proliferation of myeloid cells. However, some patients do not respond to imatinib, which may be due to a different mutation they possess, or they may develop resistance to this drug after some time of treatment. This leaves the patients with few options and in general, the disease progresses to blast crisis and death (1,2).

Recently, pre-clinical studies were carried out with triptolide (1), a compound that showed activity *in vitro* and *in vivo* against tumor cells resistant to imatinib (3). Triptolide is a diterpene present in *Tripterygium wilfordii* (4), a plant used for centuries in Chinese medicine to treat inflammation and auto-immune diseases (5). Its mechanism of action seems to be through inhibition of RNA polymerases I and II (6), and a lot has been found about its structure-activity relationship (7-10). Studies have shown that the epoxides present in the molecule are important for the antitumor activity (7), as well as the hydroxyl group at C-14, which leads to greater activity when it is in β position. Besides, when the hydroxyl group is substituted by fluorine, the activity seems to increase (7,9,10), and the toxicity is reduced (8). Nevertheless, the tridimensional structure is also important (7,9). However, triptolide shows some limitations such as water-insolubility and toxicity (9).

Triterpenes such as betulinic acid (3 β -hydroxy-lup-20(29)-en-28-oic acid) (2) have a structure similar to triptolide, as shown in Figure 1, and their antitumor activity has been described for several cancer cell lines (11-15). Betulinic acid has been reported to be selectively cytotoxic for cancer cells and not for normal cells (11,16,17). Its derivatives have been shown to induce apoptosis in cancer cells, mainly through activation of the caspase cascade (11,12), triggered by mitochondrial membrane permeabilization, without involvement of p53. This way of triggering cell death could overcome certain forms of drug resistance (11). Betulinic acid and its derivatives could be active as triptolide analogues, generating new options to solve problems such as its toxicity. Therefore, the aim of this study was to perform modifications on betulinic acid, mainly fluorination, based on the structure-activity relationship of triptolide, and evaluate the cytotoxicity of the synthesized compounds.

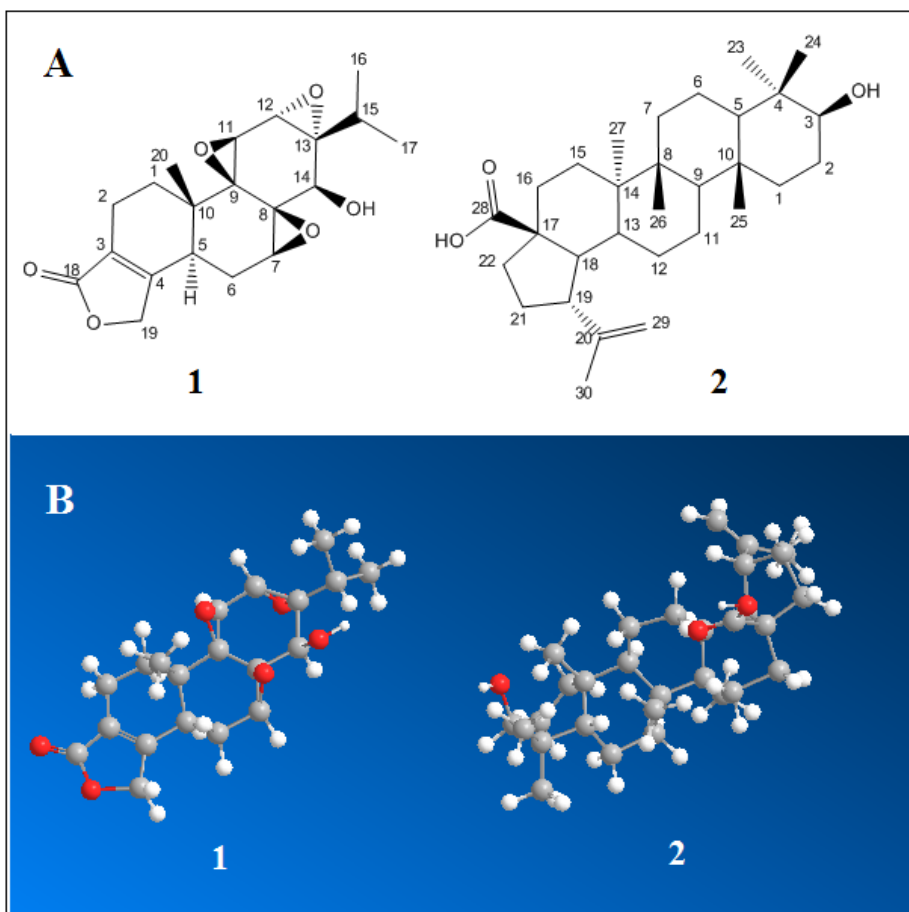


Figure 1. (A) Structures of triptolide (**1**) and betulinic acid (**2**), and (B) their 3D steric images (MM2/ChemBio3D Pro 12.0 – ChemBioOffice2008).

Materials and Methods

Plant materials

Betulinic acid (**2**) was obtained from the bark of *Platanus acerifolia*. A voucher specimen (ICN 182537) is on deposit in the Botany Department Herbarium of Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Powdered dried material (150 g) was subjected to reflux with ethanol 60% (250 mL, 2 h). After removing the ethanol under vacuum, residual aqueous phase was extracted with ethyl acetate, and the organic phase was evaporated to dryness to obtain a residue that was submitted to recrystallization. Betulinic acid was recrystallized from methanol (18,19). Yield 1.5%. Its structure was confirmed by spectroscopic methods which were in agreement with literature data (20).

Chemistry

All commercially available reagents were used without further purification unless otherwise stated. Reactions requiring anhydrous conditions were performed under a nitrogen atmosphere. Dichloromethane was dried over CaCl_2 for 24 h and then

submitted to reflux. All reactions were monitored by thin-layer chromatography on Merck silica plates GF using different solvent systems. Column chromatography (CC) was carried out on silica gel (Merck, São Paulo, Brazil, 60–230 mesh) using different gradient eluent mixtures (chloroform/cyclohexane).

Infrared spectra (IR) were recorded using a PerkinElmer FT-IR System Spectrum BX instrument. ^1H and ^{13}C -NMR spectra were obtained with a Varian Inova 400 NMR spectrometer and Varian VNMRS 75 spectrometers using tetramethylsilane (TMS) as internal standard. The solvent used for NMR analyses was CDCl_3 unless indicated. Chemical shifts are shown in parts per million (δ). High resolution mass spectra (HR-ESI-MS) were obtained on a High Resolution Microtof – TOF spectrometer. Melting points were determined using a Koffler instrument.

Synthesis

The synthetic route is presented on Scheme 1.

3-O-Acetylbetulinic acid **3** and 3-O-trifluoroacetylbetulinic acid **4**, yield 98% and 100%, respectively, were prepared from **2** by usual acylation (19,21).

General procedure for the preparation of the fluorinated derivatives (5-7)

The appropriate compound (**2-4**) (10 mmol, 1.0 Eq) was initially dissolved in distilled dichloromethane (5 mL), and then bis(2-methoxyethyl)aminosulfurtrifluoride (Deoxo-Fluor®) (11 mmol, 1.1 Eq) was added all at once. The reaction mixture was maintained at 0°C and inert atmosphere for 24 h (22). After this period, the mixture was treated with a saturated solution of NaHCO_3 (25 mL), extracted with dichloromethane and then dried over Na_2SO_4 for 1 h. The solvent was evaporated under vacuum and the crude product was purified via silica gel column chromatography using a gradient mixture of solvents (chloroform/cyclohexane), yielding pure compounds **5-7**.

28-fluor-betulinic acid (5)

Compound **5** was prepared from **2** to produce a white solid. Yield: 26%. m.p. $181\text{--}185^\circ\text{C}$. IR (ATR cm^{-1}): 3320 (O-H); 2944 (C-H); 1823 (C=O acyl halide); 1454 (C-O); 1377 (C-H); 1034 (C-F). ^1H -NMR (400 MHz, CDCl_3): δ 4.74 (brs, 1H), 4.63 (brs, 1H), 4.11 (q, $J = 7.1$ Hz, 1H), 3.17 (dd, $J = 4.8, 11.2$ Hz, 1H), 2.90 (m, 1H), 2.21 (dd, $J = 2.8, 9.6$ Hz, 1H), 2.13 (m, 1H), 2.04 (m, 2H), 1.72 (d, $J = 3.6$ Hz, 1H), 1.68 (s, 3H), 1.64 (dd, $J = 4.0, 7.6$ Hz, 2H), 1.55 (dd, $J = 3.4, 12.6$ Hz, 1H), 1.40-1.55 (m, 8H), 1.37 (s, 3H), 1.26 (m, 3H), 1.05 (m, 2H), 0.97 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.89 (dd, $J = 4.2, 12.6$ Hz, 1H), 0.82 (s, 3H), 0.75 (s, 3H), 0.67 (d, $J = 9.2$ Hz, 1H). ^{13}C -NMR (75 MHz, CDCl_3): δ 165.20 (d, $J = 281.2$ Hz); 149.13; 110.11; 78.99; 56.97; 55.32; 50.40; 49.02; 46.58; 42.42; 40.67; 38.84; 38.71; 38.47; 37.16; 35.61; 34.28; 30.89; 29.98; 29.74; 27.96; 27.36; 25.34; 20.76; 19.27; 18.24; 16.14; 15.88; 15.33; 14.70.

28-fluor-3-O-acetyl-betulinic acid (6)

Compound **6** was prepared from **3** to produce a white solid. Yield 28%. m.p. $157\text{--}167^\circ\text{C}$. IR (ATR cm^{-1}): 2946 (C-H); 1821 (C=O acyl halide); 1735 (C=O acetyl); 1540 (C=C); 1457 (C-O); 1362 (C-H); 1244 (C-O-C), 1028 (C-F). ^1H -NMR (400 MHz, CDCl_3):

δ 4.74 (brs, 1H), 4.63 (brs, 1H), 4.45 (dd, $J = 7.6, 10.4$ Hz, 1H), 2.89 (m, 1H), 2.21 (dd, $J = 2.8, 6.8$ Hz, 1H), 2.12 (m, 1H), 2.03 (d, $J = 2.0$ Hz, 3H), 2.01 (m, 2H), 1.68 (s, 3H), 1.62 (dd, $J = 1.8, 9.8$ Hz, 2H), 1.52 (m, 1H), 1.47 (d, $J = 10.0$ Hz, 3H), 1.41 (m, 3H), 1.37 (s, 3H), 1.26 (m, 3H), 1.05 (m, 1H), 0.95 (dd, $J = 1.6, 7.6$ Hz, 6H), 0.84 (s, 3H), 0.83 (dd, $J = 1.8, 3.8$ Hz, 5H), 0.78 (d, $J = 9.2$ Hz, 1H). ^{13}C -NMR (75 MHz, CDCl_3): δ 171.0; 165.21 (d, $J = 282.8$ Hz); 149.25; 110.34; 80.86; 57.20; 55.41; 50.41; 49.04; 46.64; 42.42; 40.69; 38.47; 38.40; 37.77; 37.08; 35.59; 34.22; 30.89; 29.98; 29.72; 27.90; 25.30; 23.66; 21.29; 20.77; 19.24; 18.13; 16.45; 16.19; 15.88; 14.66.

28-fluor-3-trifluor-acylated-betulinic acid (7)

Compound **7** was prepared from **4** to produce a white solid. Yield 33.5%. m.p. 166-178°C. IR (ATR cm^{-1}): 2949 (C-H); 1821 (C=O acyl halide); 1778 (C=O acyl); 1450 (C-O); 1384 (C-H); 1221 (C-O-C), 1161 (C-F). ^1H -NMR (400 MHz, CDCl_3): δ 4.75 (brs, 1H), 4.67 (brs, 1H), 4.63 (brs, 1H), 3.01 (t, $J = 9.6$ Hz, 1H), 2.90 (t, $J = 10.0$ Hz, 1H), 2.19 (m, 2H), 2.00 (m, 2H), 1.72 (s, 3H), 1.68 (s, 3H), 1.61 (m, 1H), 1.35-1.54 (m, 9H), 1.27 (m, 4H), 1.03 (m, 1H), 0.97 (s, 3H), 0.95 (s, 3H), 0.88 (s, 8H), 0.80 (d, $J = 8.0$ Hz, 1H). ^{13}C -NMR (75 MHz, CDCl_3): δ 171.89; 165.13 (d, $J = 280.1$ Hz); 157.33 (q, $J = 30.2$ Hz); 149.88; 149.20; 114.37; 110.29; 86.20; 56.97; 55.23; 50.37; 48.99; 46.62; 42.43; 40.67; 38.40; 38.25; 38.03; 37.05; 35.56; 34.12; 30.84; 29.95; 27.73; 26.89; 25.23; 23.21; 20.80; 19.34; 19.23; 18.05; 16.19; 15.86; 14.64.

Biological evaluation

For the assessment of the antitumor activity as well as cytotoxicity in non-tumor cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed for all the compounds as previously described (23).

The cell lines used were HeLa (cervical adenocarcinoma) and B16F10 (melanoma) as a screening for antitumor activity, and HaCaT (immortalized human keratinocytes) as a non-tumor cell line, for assessment of the selectivity index (SI). HeLa and murine melanoma B16F10 cells were obtained from the American Type Culture Collection (ATCC), and HaCaT cells were kindly donated by Dr. Luisa Villa (ICESP, Escola de Medicina, Universidade de São Paulo) and by Dr. Silvy S. Maria-Engler (Faculdade de Ciências Farmacêuticas, Universidade de São Paulo).

The compounds were initially dissolved in dimethyl sulfoxide (DMSO) and then with the medium. The DMSO concentration was kept below 1% which was non-toxic to the cells. Cells were treated with concentrations from each compound ranging from 1 to 100 μM , and were incubated in 37°C and 5% CO_2 for 24 h and 48 h, except for the B16F10 cells, which were incubated only for 24 h. Fifty percent inhibition concentration (IC_{50}) for the tumor cell lines (HeLa and B16F10), and fifty percent cytotoxic concentration (CC_{50}) for the non-tumor cell line (HaCaT) were determined using nonlinear regression analysis in Excel spreadsheet. Selectivity index was calculated as $\text{SI} = \text{CC}_{50}/\text{IC}_{50}$ for each tumor cell line.

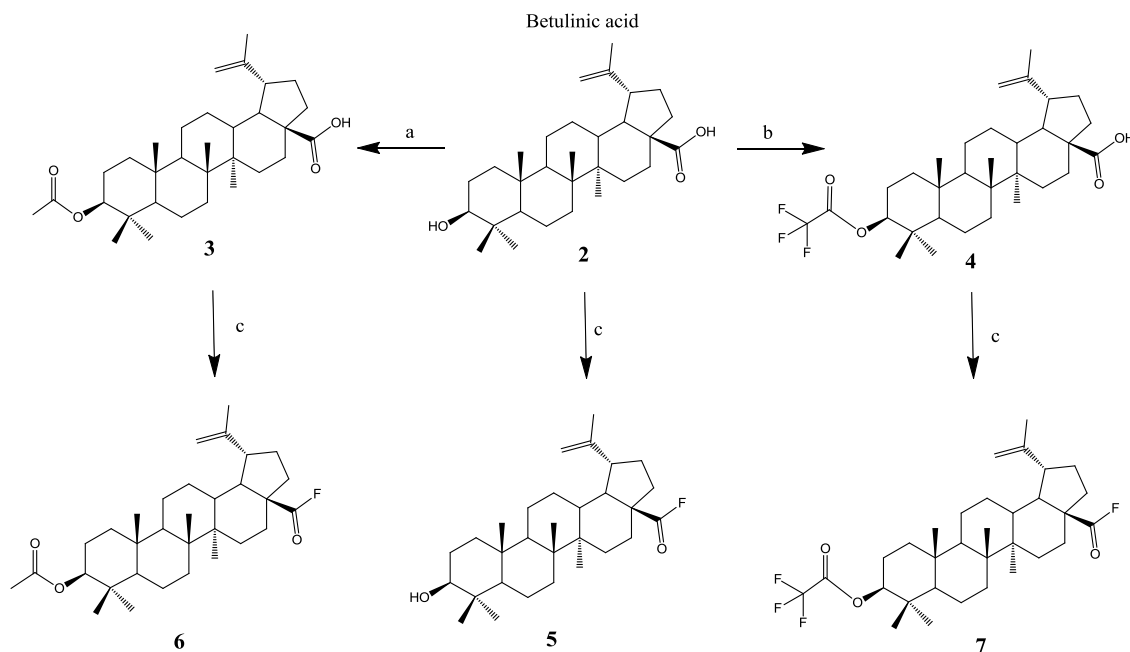
Results and discussion

Chemistry

Betulinic acid was extracted from *Platanus acerifolia* bark according to Innocente et al (18) and da Silva et al (19) and modifications on C-3 were performed to generate two derivatives, acetylated betulinic acid (**3**) and 3-fluor-acylated betulinic acid (**4**), as previously described (19,21). After these derivatives were obtained, the main modification desired was fluorination of the carboxylic acid at C-28.

Compounds **2**, **3**, and **4** were submitted to fluorination with bis(2-methoxyethyl)aminosulfurtrifluoride (Deoxo-Fluor®) to generate their fluorinated derivatives (**5**, **6**, **7**, respectively). All reactions and compounds are shown in Scheme 1.

The IR spectra showed the conversion of each carboxylic acid into the corresponding acyl fluoride through the carbonyl band, which was present at 1823, 1821 and 1821 cm^{-1} for compounds **5**, **6**, and **7**, respectively, while for their precursors this band appears around 1700 cm^{-1} . Besides, the OH absorption was not detected for compounds **6** and **7**. The ^{13}C NMR spectra of compound **5** revealed a doublet at 165.20 ppm with a coupling constant (J) 281.2 Hz, which corresponded to the coupling of the carbonyl carbon to fluorine. The same was observed for compounds **6** and **7**, with signals at 165.21 ppm ($J = 282.8$ Hz) and 165.13 ppm ($J = 280.1$ Hz), respectively. Also, a quartet at 157.33 ppm was observed for compound **7** ($J = 30.2$ Hz). This signal corresponds to the coupling of the CF_3 carbon to the three fluorine atoms.



Reagents and conditions: (a) pyridine, acetic anhydride, rt, 24 h; (b) CH_2Cl_2 , trifluoroacetic anhydride, rt, 1 h; (c) Deoxo-Fluor®, 0°C , 24 h.

Scheme 1. Synthesis of betulinic acid derivatives.

Biological evaluation

All active compounds showed cytotoxicity in a dose-response manner for all three cell lines. The main cell viability graphs are shown in Figure 2, and the IC₅₀ and CC₅₀ values are listed on Table 1.

Table 1. IC₅₀ (μM) of compounds **2-5** after 24 h and 48 h of treatment of HeLa and 24 h treatment of B16F10, and their CC₅₀ (μM) on HaCaT cells.

Compound	24 h			48 h	
	IC ₅₀ (μM)		CC ₅₀ (μM)	IC ₅₀ (μM)	CC ₅₀ (μM)
	HeLa	B16F10	HaCaT	HeLa	HaCaT
2	89	41	132	52	75
3	99	1.9	47	66	42
4	81	46	70	46	49
5	39	25	37	33	34

The IC₅₀ of betulinic acid (**2**) in HeLa cells has been previously described as 26 μM for 72 h treatment (13), which is in agreement with the results found in this study. A 24 h treatment led to IC₅₀ of 89 μM, and 48 h treatment generated IC₅₀ of 52 μM, showing that the longer the treatment time, the greater the cytotoxicity.

When looking at HeLa cells only, compound **5** is the most active, as shown by the IC₅₀ values of 39 and 33 μM after 24 h and 48 h treatment, respectively. This suggests that the presence of fluorine enhances the antitumor activity, which was also noted for triptolide (7). For betulinic acid, this enhancement in activity occurs especially with fluorination on the C-28 position. However, the cytotoxicity on the non-tumor cell line also increased in the presence of fluorine, compound **5** being the most toxic. According to a previous study, the C-28 carbonyl group is necessary for preservation of the cytotoxic property of betulinic acid (24). This importance of the carbonyl group may explain the increased activity after modification in this position. The substitution of the carboxyl group by acyl fluoride may enhance the action promoted by the carbonyl which may be essential for the cytotoxicity.

The screening on B16F10 melanoma cells was only performed with 24 h treatment, and compound **3** was the most active, with IC₅₀ of 1.9 μM, and also the most selective, with a selectivity index of 24.7, as shown in Table 2. This shows that when acting on melanoma cells, the modification on C-3 seems to have an important influence on the activity, since the acetylation on this position was responsible for a twenty times better antitumor activity. These results make compound **3** a promising agent for treating melanoma.

For B16F10 cells, fluorination at the C-28 position also enhanced the activity, since compound **5** has IC₅₀ of 25 μM, while its precursor betulinic acid (**2**) has IC₅₀ of 41 μM. The results for B16F10 cells show a better activity when comparing to the activity on HeLa cells, suggesting that the cytotoxicity of the molecules varies according to the cell line used, probably due to different mechanisms of death.

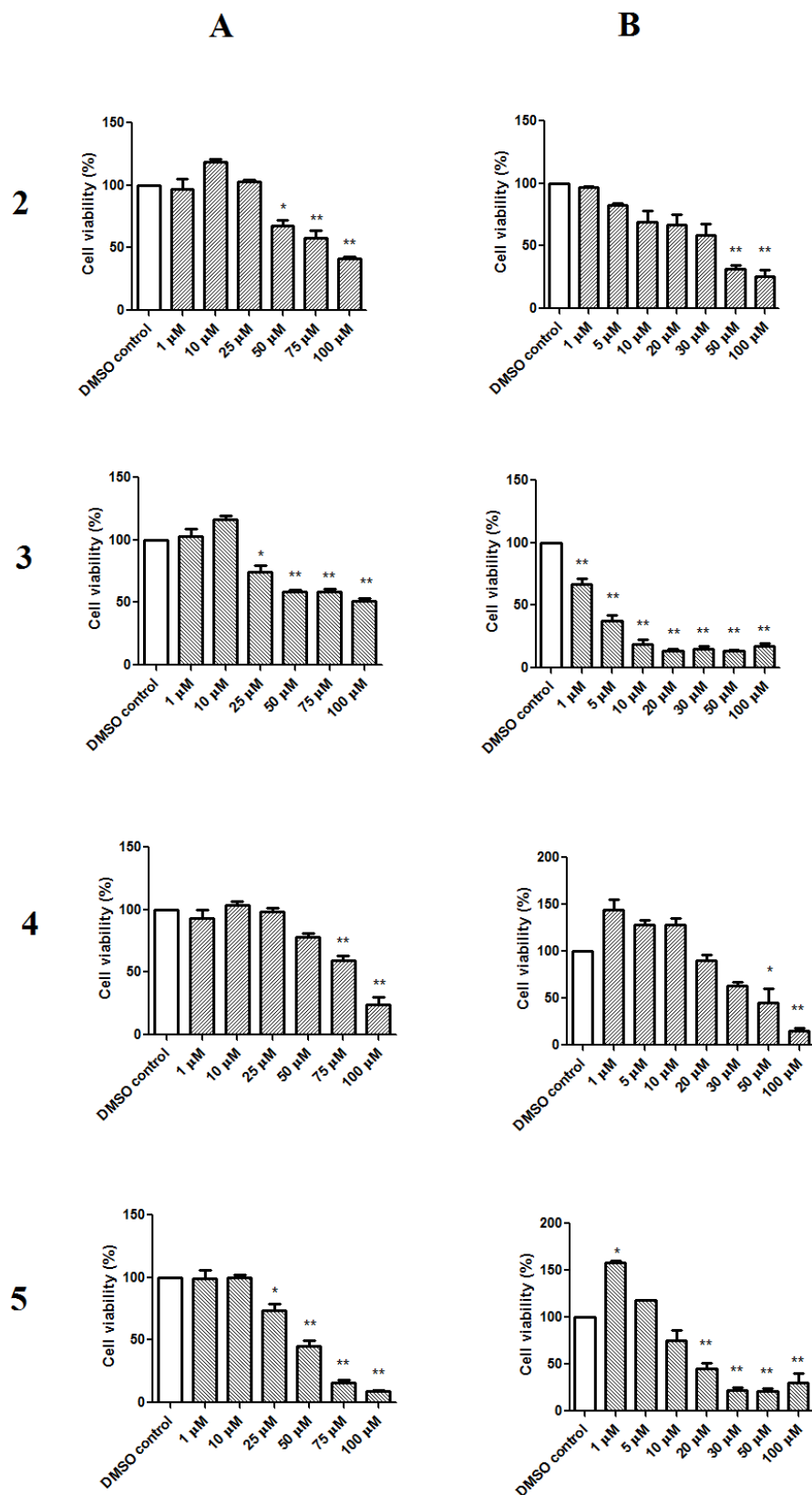


Figure 2. Cell viability of (A) HeLa cells and (B) B16F10 cells after treatment with different concentrations of the compounds **2**, **3**, **4**, and **5**, for 24 h. *P<0.01 and **P<0.001 when compared with control (One-way ANOVA followed by Tukey test). n=3 for HeLa and n=2 for B16F10.

Compounds **6** and **7** exhibited a decreased activity ($IC_{50}>100$) for both tumor lines tested, being considered inactive. This could be attributed to their low solubility in DMSO, however, even when their solubility was enhanced through addition of Tween 20®, the activity remained low. Poor solubility of betulinic acid derivatives has also led to decreased cytotoxicity in a previous study (14). Another work showed that more polar (water soluble) compounds exhibited greater cytotoxic effects than non-polar derivatives. This shows that just like triptolide, betulinic acid derivatives also have problems such as poor solubility, which have to be overcome for *in vivo* tests (25).

The selectivity index (SI) calculated by IC_{50}/CC_{50} is shown in Table 2. The results show that all molecules were more selective for melanoma B16F10 than for HeLa cells, since their IC_{50} was lower for this cell line in comparison to HeLa. The most selective compound was **3**, with a selectivity index of 24.7.

Table 2. Selectivity index (SI) comparing the tumor cell lines with the non-tumor cell line.

Compound	SI, CC_{50}/IC_{50}		
	24 h		48 h
	HeLa	B16F10	HeLa
2	1.48	3.22	1.44
3	0.47	24.7	0.64
4	0.86	1.52	1.07
5	0.95	1.48	1.03

Despite the low selectivity of betulinic acid derivatives for HeLa cells, betulinic acid (**2**) was the least toxic to non-tumor cells, with $IC_{50}>100$ μ M after 24 h of treatment. The selectivity of betulinic acid to tumor cells has already been described (16), and a study in mice showed that it had no toxic effects at concentrations up to 500 mg/kg, while doses as low as 5 mg/kg were effective to inhibit tumor development (17).

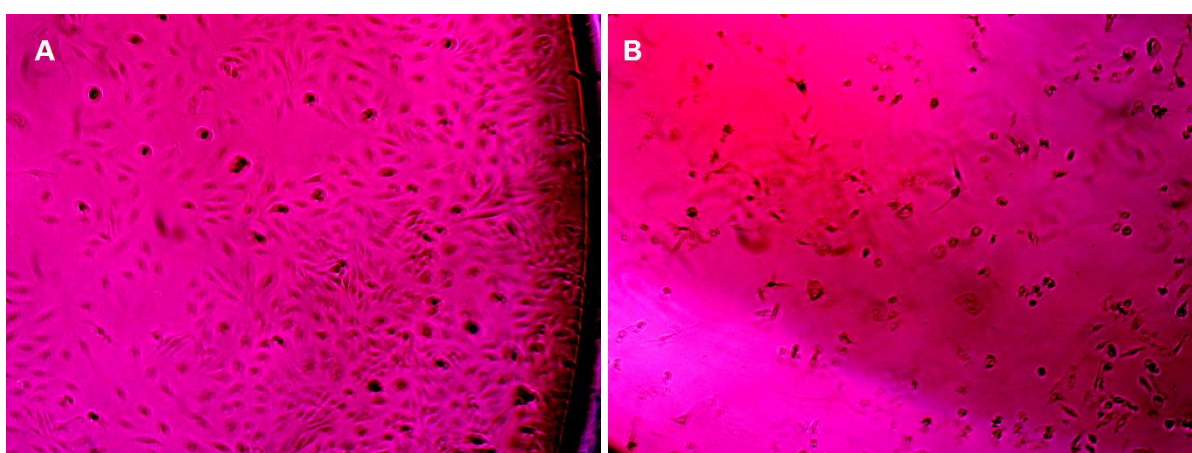


Figure 3. HeLa cells microscopy, 400 x magnification. (A) Control cells, and (B) cells treated with 100 μ M compound **5** for 24 h.

Microscopic pictures of the cells have been taken to confirm the results obtained with the MTT assay. The comparison of HeLa cells treated with 100 μ M compound **5** for 24

h with control is shown in Figure 3. The decreased presence of cells in the picture taken after treatment with compound **5** when compared with control shows that decreased viability was in fact related to the inhibition of cell proliferation.

Conclusion and Future Directions

A total of 5 compounds have been synthesized, being 2 new betulinic acid derivatives. The results suggest that fluorination increases the antitumor activity, along with the toxicity. Compound **3** was the most active to melanoma cells and the most selective of all compounds tested, being a promising agent for melanoma treatment. The IC₅₀ values on HeLa cells indicate only a moderate activity along with low selectivity for the compounds tested, however these parameters improve when looking at melanoma cells, indicating that different antitumor activity profiles are expected for different cell lines. Therefore, further studies to investigate the activity of these compounds in chronic myeloid leukemia (CML) cells are the next step. If a greater activity can be shown in CML cells, lower concentrations that are non-toxic could be used for treatment.

Since different mechanisms of death may cause the different activities observed, another perspective is the evaluation of the mechanism of death of the compounds in each cell line. Also, toxicity will be more deeply assessed through treatment of lymphocytes with the compounds, since their intended site of action is the bloodstream. Culture and treatment of fibroblasts will also be performed to assess toxicity, since this is a very common cell line used in the cited studies.

Another modification desired considering the structure-activity studied is epoxidation, which is already being carried out in our laboratory.

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Conflict of interest

All authors report no conflict of interest.

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Notes

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- c) Leukaemia Key Facts, Cancer Research UK 2013, available at: <http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/leukaemia-key-facts/uk-leukaemia-statistics>, accessed on February 17, 2014.