Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 2181–2184

Betulinic acid and its derivatives as anti-angiogenic agents

Rama Mukherjee,[a,b,c] Manu Jaggi,[a,*] Praveen Rajendran,[a] Mohammad J. A. Siddiqui,[b] Sanjay K. Srivastava,[c,*] Anand Vardhan[b] and Anand C. Burman[a,b,c]

[a]*Divisions of Experimental Oncology, Dabur Research Foundation, 22, Site IV, Sahibabad, Ghaziabad 201 010, UP, India*
[b]*Chemical Research, Dabur Research Foundation, 22, Site IV, Sahibabad, Ghaziabad 201 010, UP, India*
[c]*Medicinal Chemistry, Dabur Research Foundation, 22, Site IV, Sahibabad, Ghaziabad 201 010, UP, India*

Received 6 January 2004; accepted 5 February 2004

Abstract—Betulinic acid (**1**) significantly caused cytotoxicity to endothelial cell line ECV304 (IC_{50} 1.26 ± 0.44 μg/mL) in a 5-day MTT assay. Novel and more potent derivatives of betulinic acid (**2**, **4**, **6**–**8**) have been synthesized with IC_{50} less than 0.4 μg/mL. The endothelial cell specificity against human tumor cell lines DU145, L132, A549, and PA-1 were determined. Further betulinic acid (**1**) inhibited TLS formation of ECV304 cells on Matrigel™ by 5.5% while its derivatives caused an inhibition of 13.1–49.2%.

1. Introduction

Betulinic acid (**1**) (3-β-hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic lupane-type triterpene that is widely distributed throughout the plant kingdom. A variety of biological activities have been ascribed to betulinic acid (**1**) including anti-inflammatory and in vitro anti-malarial effects.[1] However, betulinic acid (**1**) is most highly regarded for its anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines.[2] Betulinic acid (**1**) was previously reported to exhibit selective cytotoxicity against several melanoma-derived cell lines. However, more recent work has demonstrated that betulinic acid is cytotoxic against other nonmelanoma human tumor varieties.[3] Betulinic acid (**1**) appears to function by means of inducing apoptosis in cells irrespective of their p53 status. Because of its selective cytotoxicity against tumor cells and favorable therapeutic index, even at doses up to 500 mg/kg body weight, betulinic acid (**1**) is a very promising new chemotherapeutic agent for the treatment of HIV infection and cancer.[2]

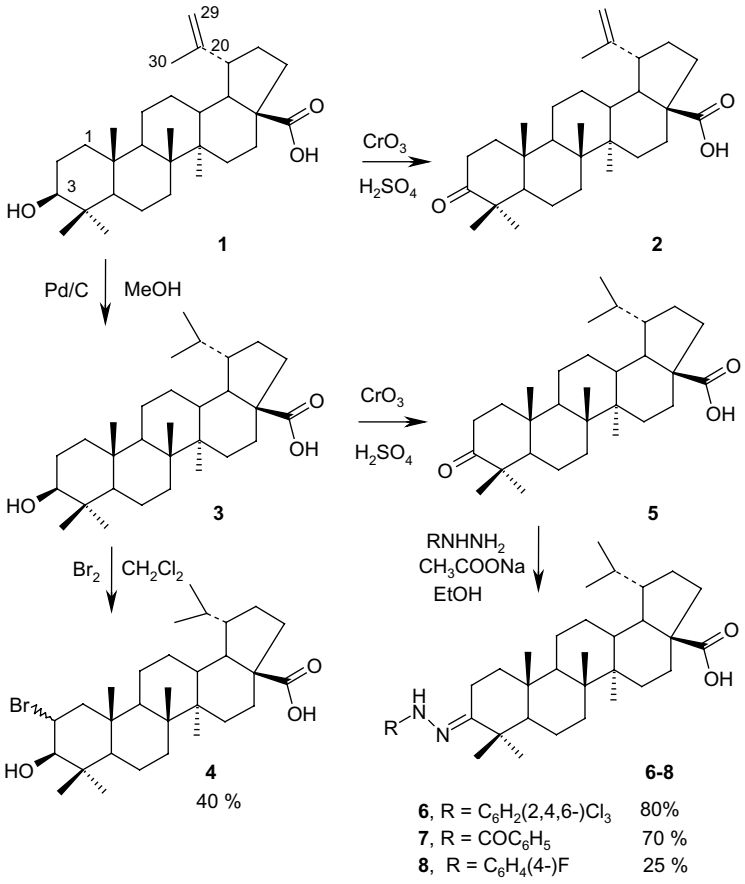
Anti-cancer agents have previously been evaluated for their anti-angiogenic potential. Epirubicin, doxorubicin, mitoxantrone, vinblastine, vincristine, and taxol have been shown to have some anti-angiogenic activity.[4–6] These experiments suggest that only a few anti-cancer agents have the ability to target both tumor cells and cells of the vasculature (endothelial cells) and are potentially effective angiogenesis inhibitors. We have investigated the potential of betulinic acid (**1**) in angiogenesis and have developed more potent betulinic acid derivatives through bioactivity-guided structure–activity studies. Here we report for the first time the anti-angiogenic activity of betulinic acid (**1**) and its potent derivatives (**2**, **4**, **6**–**8**).

2. Chemistry

Synthesis of betulinic acid derivatives (**2**, **4**, **6**–**8**)[7–9] has been described in Scheme 1. Betulinic acid (**1**) was converted to 3-oxo betulinic acid (**2**) by Jones' oxidation. Compound **1** was hydrogenated with Pd/C to furnish 20,29-dihydro betulinic acid (**3**). Compound **3** was elaborated in two ways. In first, compound **3** was brominated with liquid bromine in methylene chloride to afford 2-bromo-20,29-dihydro betulinic acid (**4**). In second, compound **3** was oxidized, as for **1**, to afford 20,29-dihydro-3-oxo betulinic acid (**5**). Compound **5** was treated with different hydrazines to yield corresponding 3-hydrazono-20,29-dihydro betulinic acid derivatives (**6**–**8**). All the compounds (**2**, **4**, **6**–**8**) were characterized by spectroscopic and analytical tools.

* Corresponding authors. Tel.: +91-120-2777901-25; fax: +91-120-27-77303; e-mail: jaggim@dabur.com

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.02.044



Scheme 1.

3. Results and discussion

Betulinic acid (**1**) is cytotoxic to endothelial cells (ECV304) in a concentration-dependant manner (Fig. 1). Increasing concentrations of betulinic acid (**1**) were tested against ECV304 cells in a 5-day MTT assay. The half-maximal cytotoxic concentration (IC_{50}) was 1.26 ± 0.44 μg/mL (~2.77 μM). Data represented is mean of three experiments. All betulinic acid derivatives (**2**, **4**, **6–8**) showed $IC_{50} < 0.4$ μg/mL (<1 μM) on ECV304 cell line. Betulinic acid and its derivatives were screened for cytotoxicity on human tumor cell lines DU145 (prostate), L132 (lung), A549 (lung), and PA-1 (ovary) and the endothelial cell specificity (ECS) was calculated as shown in Table 1. Betulinic acid (**1**) has shown low ECS against all tumor lines (ECS < 10) while derivatives **2**, **4**, **6–8** have shown moderate to high ECS against A549; compounds **6** and **8** have shown moderate ECS against L132 and compound **2** has shown moderate ECS against DU145.

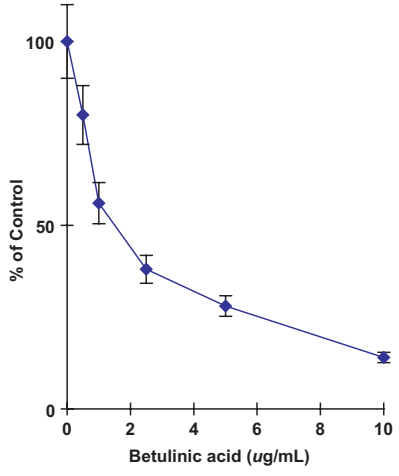


Figure 1.

Figure 2 shows the effect of betulinic acid (**1**) and derivatives (**2**, **4**, **6–8**) on tube-like structure (TLS) formation of ECV304 cells in a Matrigel™ tube formation assay. Betulinic acid (**1**) inhibited tube formation by 5.5% while the derivatives (**2**, **4**, **6–8**) screened inhibited tube formation by 13.1–49.2% as measured by tube length using image analysis. The inhibitory effect caused by the derivatives (**2**, **4**, **6–8**) was equal to or greater than betulinic acid (**1**) characterized by the inhibition of tube length, endothelial sprouting, capillary network formation, and intussusception. Experiments were in duplicates and each experiment was performed thrice.

Structure–activity relationship indicates that the cytotoxicity was increased about four to six times, after converting betulinic acid (**1**) into 3-oxo betulinic acid

Table 1. IC_{50} and ECS ratios of betulinic acid (**1**) and its derivatives (**2**, **4**, **6**–**8**) on ECV304 cells

S. no.	Compound	IC_{50} (μg/mL)	Endothelial cell specificity (ECS)[a] [IC_{50} tumor cell/IC_{50} endothelial cell]			
		ECV304	DU145	L132	A549	PA-1
1	Betulinic acid (**1**)	1.26 ± 0.44	2.2	1.0	1.1	3.2
2	**2**	0.28 ± 0.005	14.2	3.2	9.8	9.2
3	**4**	0.27 ± 0.02	7.0	3.1	11.5	7.4
4	**6**	0.21 ± 0.03	6.6	11.4	19.0	5.2
5	**7**	0.39 ± 0.07	7.7	1.0	27.7	4.3
6	**8**	0.35 ± 0.07	3.7	11.1	20.0	1.4

[a] The IC_{50} for DU145, L132, A549, and PA-1 cell lines was determined using MTT assay (data not shown) and ECS ratios calculated as described in methods.

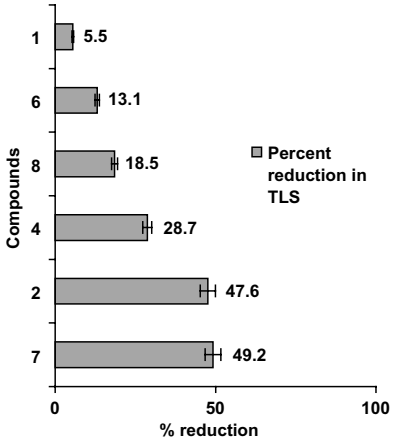


Figure 2.

(**2**), and 20,29-dihydro betulinic acid derivatives (**4**, **6**–**8**). As far as ECS is concerned, all the betulinic acid derivatives (**2**, **4**, **6**–**8**) have shown several fold higher endothelial specificity than betulinic acid (**1**). The 3-hydrazono-20,29-dihydro betulinic acid derivatives (**6**–**8**) exhibited high endothelial specificity against A549 cell line while 3-oxo betulinic acid (**2**) and 2-bromo-20,29-dihydro betulinic acid (**4**) have shown moderate specificity against DU145 and A549 cell lines, respectively. Compounds **6** and **8** had moderate specificity against L132 cell line. All betulinic acid derivatives except **8** have shown low endothelial specificity against PA-1 cell line but were better than betulinic acid (**1**). It is predicted that the high and moderate ECS compounds would cause anti-cancer effect primarily due to anti-angiogenic potential while low ECS compounds would supplement their already known anti-tumor effects. Also all the betulinic acid derivatives (**2**, **4**, **6**–**8**) have shown better anti-TLS activity than betulinic acid (**1**). 3-Oxo betulinic acid (**2**) and 3-(*N*-benzoyl)hydrazono-20,29-dihydro betulinic acid (**7**) have shown about ninefold better anti-TLS activity than betulinic acid (**1**).

3-(*N*-Benzoyl)hydrazono-20,29-dihydro betulinic acid (**7**), a potent cytotoxic agent on ECV304 cell lines, has shown high endothelial specificity as well as high anti-TLS activity. It seems that hydrazone group at position-3 and 20,29-dihydro moiety in betulinic acid (**1**) play an important role in eliciting high endothelial cytotoxicity, specificity, and anti-TLS activity.

4. Conclusion

It has been suggested, that anti-angiogenic compounds given along with standard cytotoxic drugs in combination chemotherapy regimens are more effective than cytotoxic drugs alone.[10] Long duration combination chemotherapy regimens involving cytotoxic and anti-angiogenic drugs have the drawbacks of adverse toxic effects. In such a scenario compounds such as betulinic acid derivative **7**, which possess both anti-tumor and anti-angiogenic potential could be envisioned to act effectively with acceptable toxicity profiles. Further studies are under progress to determine the in vivo efficacy of the compounds having in vitro anti-angiogenic activity. However, recognition of the anti-angiogenic potential of betulinic acid and its novel derivatives may help in designing strategies to tackle angiogenesis-dependant tumor growth.

5. Materials and methods

5.1. Chemicals

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA), Matrigel™ (Becton Dickinson, USA), DMEM (Dulbecco's modified Eagles medium), and Fetal bovine serum, FBS (Gibco BRL, USA), DMSO (Merck, India). Chemicals used in synthesis were purchased from Sigma, USA.

5.2. Cell culture

ECV304 cell line was generously gifted by Dr. Takahashi (Tokyo University, Tokyo, Japan). Human tumor cell lines DU145 (prostate), L132 (lung), A549 (lung), and PA-1 (ovary) cell lines have been procured from NCCS, Pune, India. Cell lines were grown in DMEM, containing L-glutamine and 25 mM HEPES and supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) at 37 °C, 5% CO_2, 100% humidity.

5.3. Cytotoxicity assay

Cells (10^4) were incubated with betulinic acid (**1**) or its derivatives (**2**, **4**, **6**–**8**), dissolved in DMSO (final DMSO

concn <0.1%), in triplicate wells to obtain drug concentration of 0.5–10 μg/mL. Cytotoxicity was measured after 120 h using MTT assay as described by Mosmann.[11] Each experiment was repeated thrice and mean IC_{50} values (half-maximal cytotoxicity) have been reported. ECS ratios were calculated using the formula: IC_{50} (tumor cell)/IC_{50} (endothelial cell). ECS < 10 was designated low endothelial specificity, ECS between 10 and 20 as moderate and ECS > 20 as high endothelial specificity.

5.4. Tube-like structure (TLS) formation assay

The method as described by Shinji et al. was followed.[12] Briefly, 1.5×10^4 ECV304 cells in growth medium (DMEM containing 10% FBS) were seeded on Matrigel™ (70 μL). Betulinic acid (**1**) and derivatives (**2**, **4**, **6**–**8**), solubilized in DMSO (final DMSO concn <0.1%) were added in duplicate wells at 1 μg/mL (non-cytotoxic concd at 18 h) and incubated. After 18 h the control cells start to form an intense network of tube-like structures. The total tube length was measured by image analysis and percentage inhibition of tube formation was calculated compared to controls. A qualitative assessment was performed by viewing the tube-like structures under the microscope and scoring for inhibition of endothelial sprouting, capillary network formation, and intussusception.

References and notes

1. Anderson, B.M.; Musser, H.J. WO 95/04526.
2. Cichewicz, R. H.; Kouzi, S. A. *Med. Res. Rev.* **2004**, *24*, 90.
3. Pisha, E.; Chai, H.; Lee, I. S.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W. W.; Fong, H. H. S.; Kinghorn, A. D.; Brown, D. M.; Wani, M. C.; Wall, M. E.; Hieken, T. J.; Das Gupta, T. K.; Pezzuto, J. M. *Nat. Med.* **1995**, *1*, 1046.
4. Steiner, R. *EXS* **1992**, *61*, 449.
5. Dordunoo, S. K.; Jackson, J. K.; Arsenault, A. L.; Oktaba, A. M.; Hunter, W. L.; Burt, H. M. *Cancer Chemother. Pharmacol.* **1995**, *36*, 279.
6. Burt, H. M.; Jackson, J. K.; Bains, S. K.; Liggins, R. T.; Oktaba, A. M.; Arsenault, A. L.; Hunter, W. L. *Cancer Lett.* **1995**, *88*, 73.
7. Ramadoss, S.; Jaggi, M.; Siddiqui, M. J. A.; Khanna, A. B. U.S. Patent 6,048,847, 2000.
8. Ramadoss, S.; Jaggi, M.; Siddiqui, M. J. A.; Khanna, A. B. U.S. Patent 6,214,814, 2001.
9. Jaggi, M.; Ramadoss, S.; Rajendran, P.; Siddiqui, M. J. A. U.S. Patent 6,228,850, 2001.
10. Folkman, J. Antiangiogenic therapy. In *Cancer: Principles & Practice of Oncology*, 5th ed.; Vincent, T. D., Jr., Hellman, S., Steven, R., Eds.; Lippincott–Raven: Philadelphia, 1997; pp 3075–3085.
11. Mosmann, T. *J. Immunol. Meth.* **1983**, *65*, 55.
12. Shinji, S.; Yoshiaki, S.; Hiroshi, S. *Biol. Pharm. Bull.* **1997**, *20*, 1131.