Effects of Betulinic Acid Alone and in Combination with Irradiation in Human Melanoma Cells

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Recently, betulinic acid was identified as a highly selective inhibitor of human melanoma growth and was reported to induce apoptosis in these cells. We have investigated the growth-inhibitory properties of this compound alone and in combination with ionizing radiation in a panel of established human melanoma cell lines as well as in normal human melanocytes. Betulinic acid strongly and consistently suppressed the growth and colony-forming ability of all human melanoma cell lines investigated. In combination with ionizing radiation the effect of betulinic acid on growth inhibition was additive in colony-forming assays. Betulinic acid also induced apoptosis in human melanoma cells as demonstrated by Annexin V binding and by the emergence of cells with apoptotic morphology. The growth-inhibitory action of betulinic acid was more pronounced in human melanoma cell lines than in normal human melanocytes. Notably, despite the induction of apoptosis, analysis of the expression of Bcl-2 family members in betulinic-acid-treated cells revealed that expression of the anti-apoptotic protein Mcl-1 was induced. Furthermore, the antiproliferative action of betulinic acid seemed to be independent of the p53 status. The properties of betulinic acid make it an interesting candidate, not only as a single agent but also in combination with radiotherapy. We conclude that the strictly additive mode of growth inhibition in combination with irradiation suggests that the two treatment modalities may function by inducing different cell death pathways or by affecting different target cell populations. ***Key words: apoptosis/Mcl-1/p53. J Invest Dermatol 114:935–940, 2000***

Although patients with malignant melanoma have an excellent prognosis when treated at an early stage, the management of advanced melanoma is still a major problem that is not satisfactorily solved by current treatment regimens, such as chemotherapy and/or immunotherapy. The role of adjuvant therapy in melanoma has been discussed recently (Barth and Morton, 1995; Lee *et al*, 1995). Although some authors believe that melanoma should be regarded as a radioresistant tumor, the majority of experimental evidence does not support this view (Geara and Ang, 1996; Jenrette, 1996; Overgaard *et al*, 1996). The effect of radiotherapy may be enhanced, however, in combination with substances that act as response modifiers or radiosensitizers. In human melanoma, for example, a combination of radiotherapy with hyperthermia has been found to be of benefit (Overgaard *et al*, 1996; Chelvi and Ralhan, 1997).

The observation that betulinic acid is a highly selective substance active against human melanoma cells *in vitro* and in a mouse model (Pisha *et al*, 1995) led us to investigate the effects of betulinic acid alone and in combination with radiotherapy in a panel of human melanoma cell lines. This substance is of special interest, as, at least in the above-mentioned mouse system, even at high doses no evidence of acute or chronic toxicity is found. To our knowledge, clinical trials with betulinic acid have not been completed to date. Betulinic acid and its derivatives also inhibit human immunodeficiency virus type 1 entry, presumably after a postbinding step (Evers *et al*, 1996; Kashiwada *et al*, 1996; Soler *et al*, 1996; and references therein). In addition to its activity against human melanoma cells, betulinic acid inhibits the growth of neuroectodermal tumor cells, where it has the potential to induce apoptosis (Fulda *et al*, 1997; Schmidt *et al*, 1997). It is of interest to note in this context that both neuroectodermal cells and melanocytes are derived from the neural crest. Regarding the mode of action of betulinic acid, little is known about the antiproliferative and apoptosis-inducing mechanisms. In neuroectodermal cells, the effect of betulinic acid was demonstrated to be accompanied by caspase activation. Overexpression of Bcl-2 and Bcl-X_L in these cells inhibited the effects of betulinic acid on the mitochondrial membrane potential, protease activation, and DNA fragmentation. (Fulda *et al*, 1997). Betulinic acid may also induce apoptosis through direct effects on mitochondria (Fulda *et al*, 1998). The therapeutically promising characteristics of betulinic acid regarding specificity and mode of action make it an interesting candidate for further research. As no effective treatment for melanoma in advanced stages is currently available we investigated the effects of this substance alone and in

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combination with γ irradiation on a panel of benign and malignant cells of melanocytic origin.

MATERIALS AND METHODS

Fetal bovine serum (heat inactivated), Dulbecco's modified Eagle's medium, and other cell culture reagents were purchased from Gibco BRL (Grand Island, NY). Medium and growth factors for normal human melanocytes were obtained from Clonetics Corporation (Remagen, Germany). Protease inhibitors were obtained from Boehringer Mannheim (Mannheim, Germany). Enhanced electroluminescence (ECL) Western blotting reagents were obtained from Boehringer Mannheim. Betulinic acid was obtained from Calbiochem.

Cell lines and culture Normal human epidermal melanocytes (adult and neonatal, fourth passage) were obtained from Clonetics Corporation (CellSystems Biotechnologie, Germany). Cells were cultured in melanocyte growth medium (MGM-2, serum-free) containing phorbol 12-myristate-13-acetate (10 μg per ml), hydrocortisone (0.5 μg per ml), insulin (5 μg per ml), human fibroblast growth factor B (1 μg per ml), and gentamycin sulfate and amphothericin-B (both at 50 μg per ml) supplemented with bovine pituitary extract. The melanoma cell line A375 was obtained from ATCC. The melanoma cell line 518A2 was a kind gift of Dr. Peter Schrier, University of Leiden, and has been described recently (see Jansen *et al*, 1997, and references therein). The human melanoma cell lines MES20 and MES21 were generated from melanoma metastases and characterized in our laboratory (Selzer *et al*, 1998). The cell lines Neo II-tr and Neo-IV-tr were established from normal primary human neonatal melanocytes in this laboratory by E. Selzer. The melanoma cell lines used for this study as well as the cell line Neo-II/IV-tr were maintained in Dulbecco's modified Eagle's medium (4500 mg glucose per liter) supplemented with 10% fetal bovine serum (Gibco BRL).

Determination of apoptosis: DNA fragmentation and Annexin V binding assay

DNA fragmentation DNA fragmentation was determined by agarose-gel electrophoresis as described previously (Selzer *et al*, 1998).

Fluorescence-activated cell sorter (FACS) analysis Cells were harvested by trypsin ethylenediamine tetraacetic acid (EDTA) treatment, fixed in ethanol, and analyzed on an EPICS-Elite flow cytometer after propidium iodide staining as recently described by Wei *et al* (1998).

Annexin V binding assay Apoptotic cell death of individual cells was scored by Annexin V fluorescein isothiocyanate (FITC) binding according to the manufacturer's protocol (Bender, Austria). Briefly, cells were harvested by trypsin-EDTA, washed in phosphate-buffered saline (PBS), and adjusted in calcium binding buffer to a cell density of 1.5×10^6 cells per ml. Cells were incubated for 10 min with Annexin V-FITC and propidium iodide (final concentrations 1 μg per ml), washed in PBS, and placed on glass slides for analysis by fluorescence microscopy (Zeiss, Jena, Germany).

Western blot analysis and immunodetection Cell extracts were prepared in lysis buffer containing 0.14 M NaCl, 0.4 M triethanolamine, 0.2% sodium deoxycholate, 0.5% Nonidet p-40, supplemented with 1 mM phenylmethylsulfonyl fluoride, 8.0 μg per ml aprotinin and 1 mM orthovanadate. The amount of soluble proteins was quantified by means of a modified Bradford analysis (Bio-Rad, Richmond, CA). Total lysates (20 μg per lane) were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Bound antigen was visualized with the ECL detection system from Boehringer Mannheim. These procedures were performed as suggested by the manufacturers. Anti Mcl-1, bak, bad, bax, and p53 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Conditions for determination of survival and colony-forming assays In growth inhibition experiments, cells in exponential growth phase were treated with betulinic acid at the concentrations indicated. In brief, cells were maintained in standard medium, harvested, and plated at equal densities (750,000 cells in 5 cm tissue culture dishes). Cells were exposed to betulinic acid 18 h after plating. The survival experiments with betulinic acid were performed as previously described, with modifications (Pisha *et al*, 1995). Betulinic acid or dimethylsulfoxide controls were added to the cells at the indicated concentrations. Determination of survival was performed 72 h after treatment by counting cell numbers in four independent dishes per dose point. The control dishes were handled during the experiments exactly as the treated cells. Cell survival experiments were performed at least in triplicate for each cell line.

Clonogenic assays In the clonogenic survival experiments, subconfluent cells were treated with betulinic acid and then irradiated. Cells were then trypsinized and plated after appropriate dilution onto 5 cm culture dishes. Plating efficiencies for the different cell lines used were in the range 0.3–0.85. Between 200 and 2000 cells were seeded per dish in order to obtain between 100 and 300 single well separated colonies per

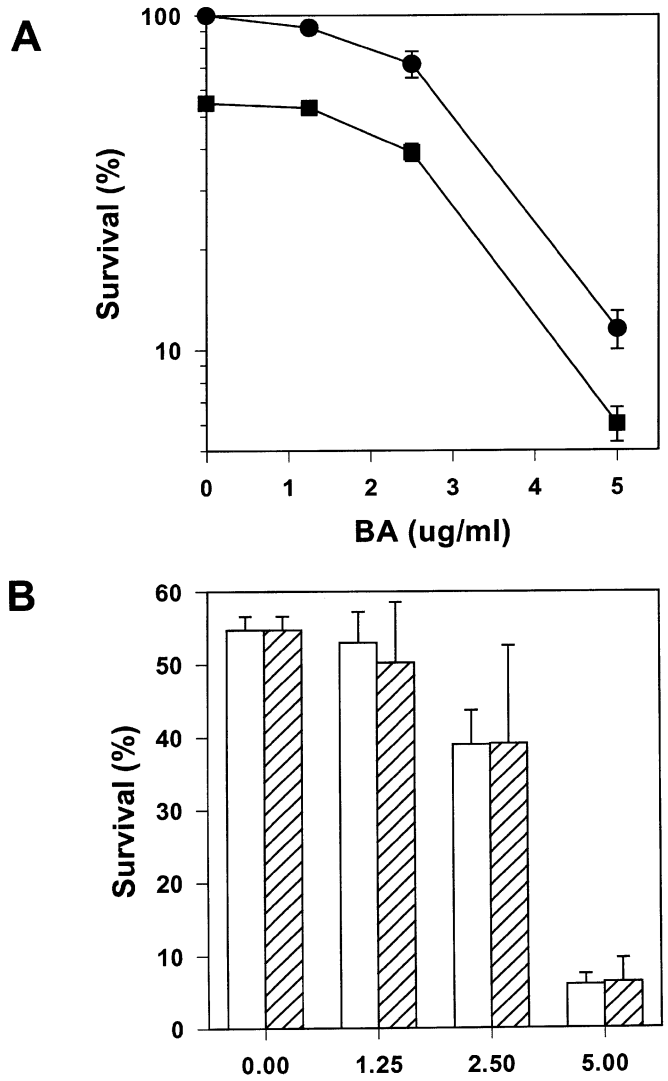

Figure 1. Effect of betulinic acid and radiotherapy on survival. (*A*) Survival of melanoma cell lines after treatment with betulinic acid alone or in combination with irradiation as assessed by inhibition of colony-forming ability. The survival curve of four melanoma cell lines (MES20, MES21, A375, and 518A2) is shown in the presence of betulinic acid alone (●) or in combination with γ irradiation (■) at the indicated concentrations. Irradiation for all data points was a single dose of 2 Gy. Survival data are pooled and given as means together with the corresponding standard error of the mean. The colony-forming experiments were performed in triplicate for each cell line used. For details of colony-forming experiments, see *Materials and Methods*. (*B*) Graphical analysis of the combined effect of radiotherapy and betulinic acid on survival of melanoma cell lines. Open bars represent data obtained from survival experiments as measured by colony inhibition in the presence of the indicated concentrations of betulinic acid and after 2 Gy irradiation. The hatched bars represent the predicted (calculated) amount of growth inhibition if the effect was assumed to be purely additive. The survival experiments were performed as described under *Materials and Methods*. Data are given as means together with standard deviations. This analysis shows no synergy for the combined treatment.

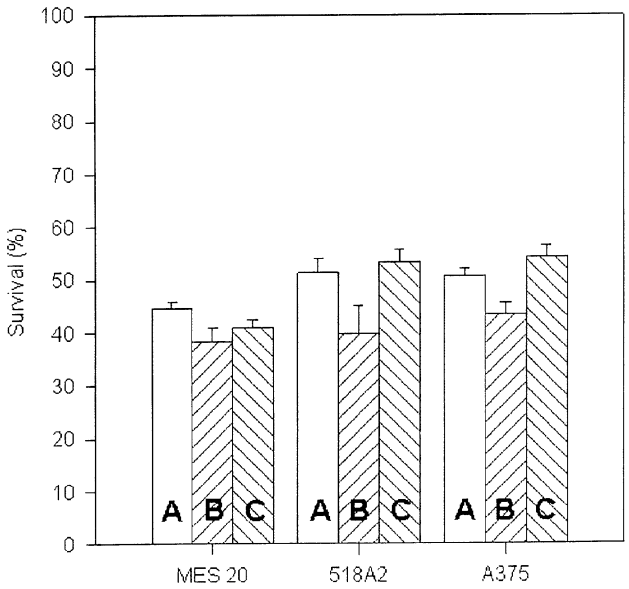

Figure 2. Influence of the timing of the exposure to betulinic acid on survival. This figure shows the results of addition of betulinic acid 8 h before irradiation (*A*), simultaneously (*B*), and 8 h after irradiation (*C*). Survival was measured by colony formation in three different melanoma cell lines. The concentration of betulinic acid used for the experiments was 2.5 μg per ml. Irradiation was performed with a dose of 2 Gy. The data presented are the results of three independent experiments (means and SEM) and are given in percentage survival (of the untreated control cells).

dish. After 8–12 d culture plates were washed, fixed in 95% ethanol, and stained with 0.1% crystal violet. Colonies containing more than 50 cells were scored as clonogenic survivors. Six dishes were counted per betulinic acid concentration investigated. The colony-forming experiments were performed three times with each cell line and for each type of treatment. Cells were irradiated with a conventional radiation source with 250 kV X-rays (dose rate at 4 Gy per min) generated by a Stabilipan machine (Siemens).

RESULTS

The following experiments were conducted in order to evaluate the response of melanoma cells to betulinic acid alone or in combination with irradiation. In addition to the melanoma cell lines we also investigated the effects of betulinic acid on normal human melanocytes during early passages as well as on permanent cell lines generated directly from normal human melanocytes (cell lines Neo-II-tr and Neo-IV-tr).

Analysis of the survival of melanoma cells after treatment with betulinic acid alone and in combination with irradiation We initially compared the clonogenic survival curves of four different melanoma cell lines (ME20, ME21, 518A2, and A375) after treatment with betulinic acid alone or in combination with irradiation (results are shown in **Fig 1*A***). The dose used for irradiation in these experiments was deliberately chosen to be in the range used in clinical radiotherapy (2 Gy). The concentrations of betulinic acid in the medium were between 1 and 5 μg per ml. The surviving fraction obtained after treatment of the cells with betulinic acid alone was multiplied by the measured surviving fraction obtained after irradiation of the cells with a dose

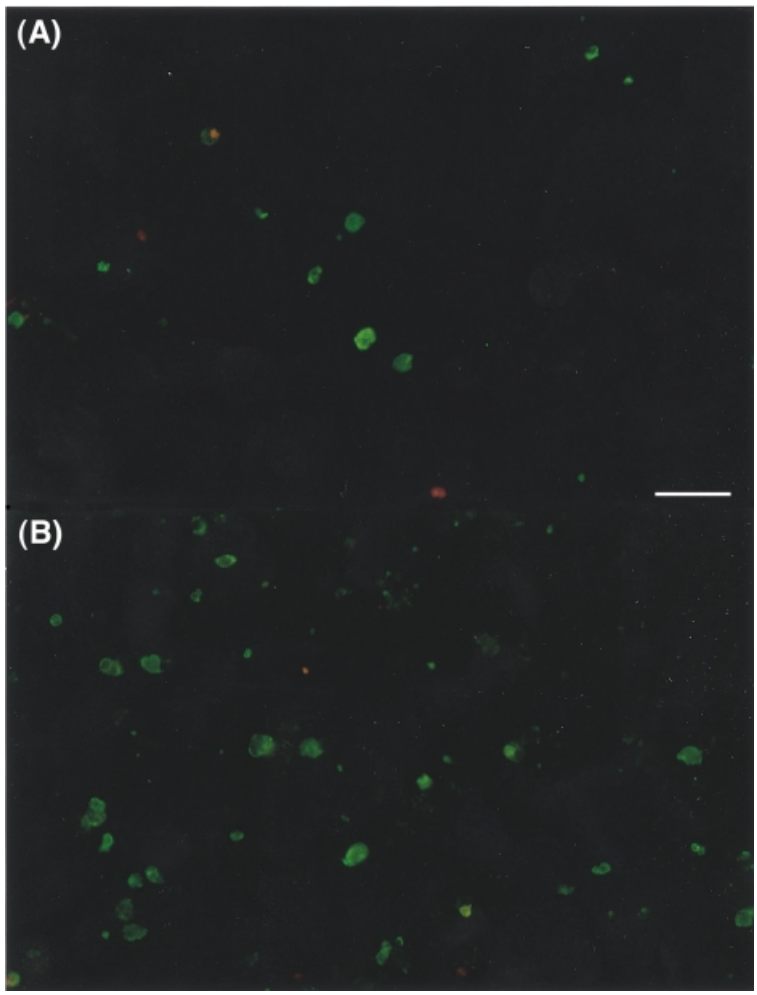

Figure 3. Betulinic acid induces apoptosis in melanoma cells. AnnexinV-propidium iodide staining and fluorescence microscope visualization (×400) of melanoma cells (for a reference see Jansen *et al*, 1995) incubated for 24 h in the presence (*A*) or absence (*B*) of 10 μg betulinic acid per ml. In the control group (*a*) 7.1% (SD ±3.4%) of the cells stained positively (green staining) for Annexin V compared with 20.3% (SD ±4.7%) in the betulinic-acid-treated group (*b*). *Scale bar*: 50 μm.

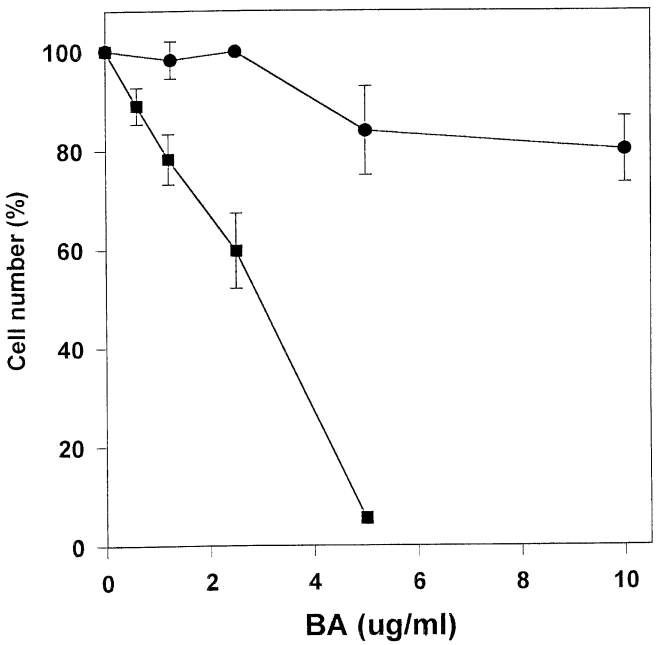

Figure 4. Growth analysis of human melanocytes and melanoma cell lines after treatment with betulinic acid reveals that normal melanocytes are less susceptible to inhibition by betulinic acid. For these experiments, three human melanocytic (neonatal) primary cell cultures and four different melanoma cell lines (MES20, MES21, A375, and 518A2) were used. In short, on the first day betulinic acid was added to the cell cultures (during the exponential growth phase) to the final concentrations indicated. Cell numbers were counted after 3 d. Data are given as means (plus standard deviations) of cell counts from the pooled data obtained from the experiments performed with normal melanocytes (●) and melanoma cell lines (■).

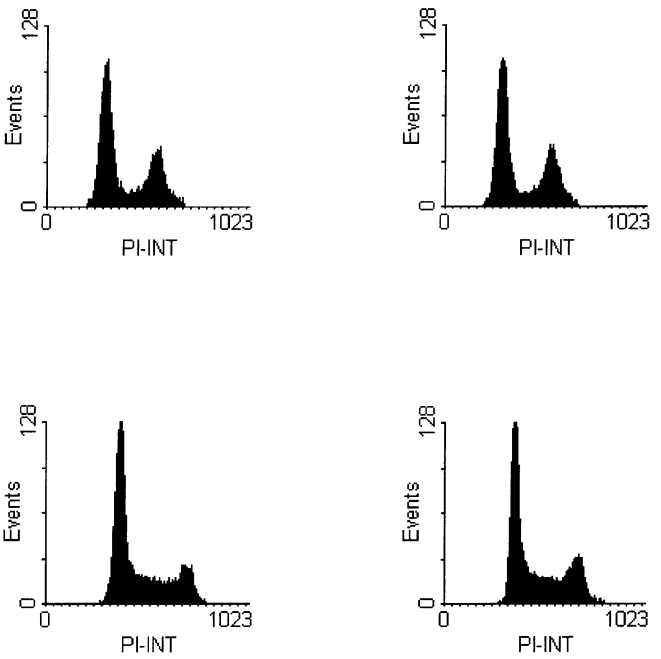

Figure 5. DNA histograms of betulinic-acid-treated normal melanocytes and of a melanoma cell line. Melanoma cell line MES20 (*lower panel*) and normal melanocytes (*upper panel*) were treated with betulinic acid (5 μg per ml) for 8 h. After 8 h, cells were harvested and DNA histograms were determined as indicated in *Materials and Methods*. The melanoma cell line contains a significantly higher S-fraction than the normal melanocytes.

of 2 Gy. Therefore this product represents the expected survival if the two treatments are independent of each other and contribute to the survival in a purely additive manner. The hatched bars in **Fig 1**(***B***) represent this calculated amount of growth inhibition. Analysis of these data revealed that betulinic acid and irradiation have an additive effect on the inhibition of colony-forming ability. The additive effect of betulinic acid in combination with irradiation was also investigated with regard to the timing of the administration in three (MES20, 518A2, A375) melanoma cell lines. The timing of the addition of betulinic acid in combination with irradiation had no major impact on the outcome of the colony-forming tests, further indicating that the two treatments function independently of each other (see **Fig 2**). Furthermore, we have found no significant effects of irradiation on the extent of betulinic-acid-induced apoptosis by FACS analysis (data not shown). The potential implications of these observations regarding the therapeutic usage and mode of action of the two types of treatment are discussed below.

Induction of apoptosis in normal melanocytes and melanoma cells by betulinic acid It was recently shown that betulinic acid inhibits growth in human melanoma cells by inducing apoptosis (Pisha *et al*, 1995; Rieber and Strasberg-Rieber, 1998). We tested this hypothesis but we were unable to demonstrate a typical DNA laddering by conventional DNA-gel electrophoresis in ethidium-bromide-stained gels. We therefore thought to demonstrate apoptosis in betulinic-acid-treated melanoma cells by the Annexin V binding assay. In contrast to melanoma cell lines, primary human melanocytic cell lines showed only a slight statistically insignificant increase in staining with Annexin V after treatment with betulinic acid (data not shown). In contrast to normal melanocytes, melanoma cells displayed intensive Annexin V staining and typical apoptotic morphology. Whereas in the untreated group of three human melanoma cell lines (MES20, MES21, and 518A2) the average rate of apoptotic cells was 8.6% (SD ±2.0%), cells incubated in the presence of 10 μg betulinic acid per ml displayed an average apoptotic rate of 15.8% (SD ±2.4%). A representative photograph of Annexin V binding to apoptotic melanoma cells after treatment with betulinic acid is shown in **Fig 3**.

Effects of betulinic acid on the growth of normal primary human melanocytes We have compared the effects of betulinic acid on primary human normal melanocytes with the effects on cell lines established from melanoma metastases. The data presented in **Fig 4** show that the growth of normal human melanocytes measured 3 d after addition of betulinic acid at the indicated concentrations is less affected than the growth of human melanoma cells. These data indicate that betulinic acid has a more pronounced effect on melanoma cells than on normal melanocytes grown in cell culture and these results are in agreement with the lack of induction of apoptosis by betulinic acid assessed by the Annexin V assay in normal melanocytes.

We have further investigated the effects of betulinic acid on cell cycle distribution in normal melanocytes and in melanoma cell lines. Cellular DNA content was determined after propidium iodide staining by flow cytometry. The DNA histograms of untreated and betulinic-acid-treated melanocytes and melanoma cells revealed no significant differential effect between these cell types that might provide an explanation for the different growth-inhibitory effects (**Fig 5**).

Expression of Mcl-1 and other Bcl-2 family members after treatment with betulinic acid Because induction of apoptosis is often associated with changes in the expression pattern of proteins, we investigated the expression of Bcl-2 homologs after treatment with betulinic acid in melanocytes. We have recently shown that melanoma cells express the Bcl-2 family members Bcl-2, Bax, Bak, Bad, Mcl-1, and Bcl-$_X$ (Selzer *et al*, 1998). With the exception of Bcl-2 (for references see Jansen *et al*, 1998; Selzer *et al*, 1998), the specific

role for these proteins in human melanocytes has not been elucidated and cell-type-specific differences from other cellular systems regarding function cannot be excluded. We found that betulinic acid rapidly induced (within a few hours) the expression of the anti-apoptotic protein Mcl-1 in melanoma cells. One typical experiment with a time course analysis of the induction of Mcl-1 by betulinic acid is shown in **Fig 6**. Densitometric scanning analysis of this experiment demonstrated a nearly 2-fold rapid induction of Mcl-1 protein expression compared with control cells. The induction of protein expression was sustained and could be demonstrated up to 48 h after addition of betulinic acid. The data obtained by densitometric analysis of Western blots of Mcl-1 expression measured in five different cell lines (MES20, MES21, 518A2, A375, and Neo-II-tr) after 24 h treatment with betulinic acid showed a nearly 3-fold induction of Mcl-1 (mean 3.17, SD ±1.12). Given the cell-growth-inhibitory effect of betulinic acid this is an unexpected finding as one would expect the induction of pro-apoptotic proteins under treatment with an apoptosis-inducing substance. We have further investigated the expression of Mcl-1 and p53 under different cytotoxic treatments. As is shown in **Fig 7**(*a*), irradiation but not betulinic acid induced the expression of p53 in the MES20 cell line. By contrast, the expression of Mcl-1 was not induced by irradiation but was induced by betulinic acid in the same cells, as well in another melanoma cell line (518A2). The expression of Mcl-1 was not modulated by treating MES20 and 518A2 melanoma cells with cisplatin, even at a concentration that stabilized the expression of p53 at higher levels in the same cells (**Fig 7***b*). It should be noted that the 518A2 cell line contains a mutation in p53 and therefore expresses elevated basal levels of p53, whereas no evidence for expression of p53 under normal conditions was seen in the other cell line (P. Schrier, unpublished observation). In neuroectodermal cells, Bax and Bcl-xs were found to be upregulated under treatment with betulinic acid (Fulda *et al*, 1997). We were unable to repeat this finding for Bax and other Bcl-2 family members in two of our cell lines, despite a clear induction of Mcl-1 under the same conditions (see **Fig 7***c*). No evidence for expression and/or induction of Bcl-xs could be found in these cell lines by Western blotting (not shown), a finding that is consistent with previously published observations (Selzer *et al*, 1998).

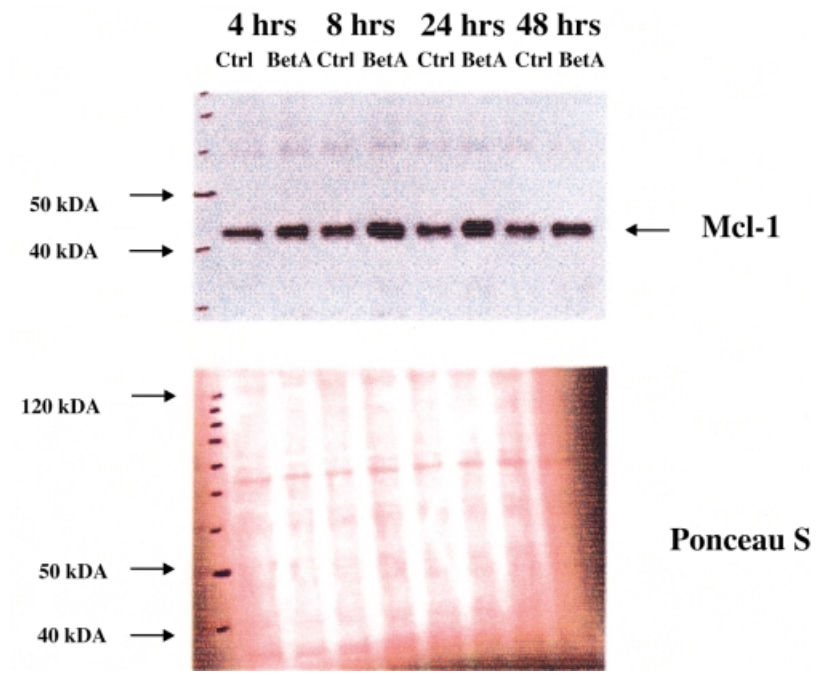

Figure 6. Western blot analysis of Mcl-1 protein expression after treatment with betulinic acid – time course analysis in one cell line (Neo II-tr). The positions of the molecular weight markers (10 kDa protein ladder) and of Mcl-1 are indicated on the left. Betulinic acid was added to a final concentration of 5 μg per ml in the cell culture medium to cells in the exponential growth phase. Protein lysates were prepared and electrophoresis was carried out as described in *Materials and Methods*. After 4, 8, 24, and 48 h of cell culture the cells were analyzed for the expression of Mcl-1. As a loading control the Ponceau S stained membrane used for the Mcl-1 blot is shown in the figure below. A time course analysis with another transformed cell line (line Neo-IV-tr; the induction of Mcl-1 by betulinic acid after 24 h is shown in **Fig 7***c*) showed nearly identical results (not shown).

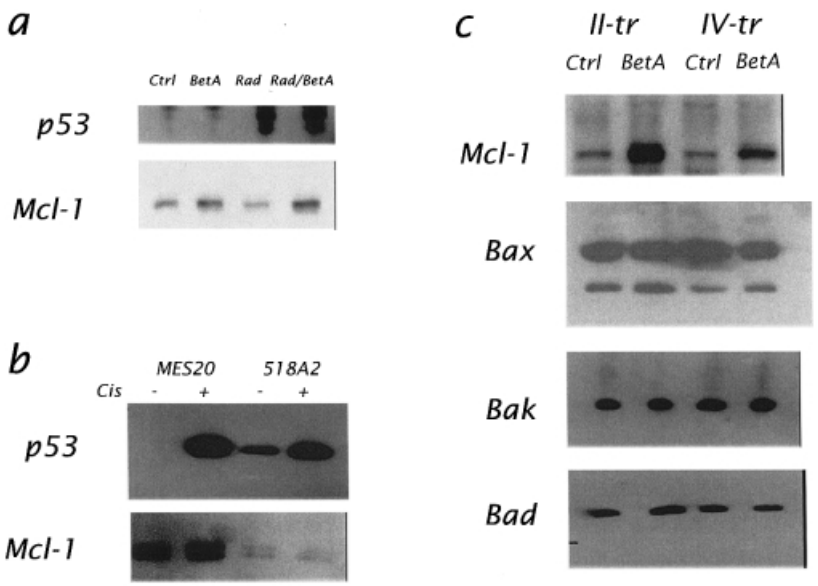

Figure 7. Analysis of Bcl-2 family members and p53 protein expression in melanoma cell lines. Mcl-1 and p53 protein expression in controls (Ctrl), betulinic acid (BetA), and in irradiated (Rad) melanoma (MES20) cells. Mcl-1 and p53 protein expression after 18 h in two different melanoma cell lines (MES20 and 518A2) in the presence or absence of cisplatin (20 μM). Mcl-1, Bax, Bak, and Bad protein expression in two cell lines (Neo II-tr and Neo IV-tr) in the presence or absence of 5 μg betulinic acid per ml.

DISCUSSION

In conclusion, our data suggest that betulinic acid might be useful in the treatment of patients suffering from melanoma. This hypothesis is based on the following observations. Betulinic acid consistently inhibited the growth of all melanoma cell lines tested. The effect of betulinic acid on melanoma cell lines is also stronger than the growth-inhibitory effect of betulinic acid on primary melanocytes. The effects of betulinic acid in combination with irradiation on survival are clearly additive as measured by the colony formation test. If the cell death pathways of these two treatments overlapped, then we could expect that they would interfere with each other, possibly leading to nonadditive effects. All our data reported here indicate that betulinic acid and irradiation most probably differ in their mode of action, consistent with the purely additive manner of growth inhibition. The two treatments may induce cell death through a different mechanism and/or betulinic acid and irradiation may affect different types of susceptible cell populations.

A feature of betulinic acid of potential clinical importance is suggested by the recent observation that its cytotoxicity is enhanced by low pH values in the culture medium. The pH in many tumors is lower than in normal tissues (Noda *et al*, 1997). Noda *et al* also found that the activity of this substance was higher against resting cells than cells that are in a growth phase. This might be of additional benefit in the combined treatment of tumors with radiotherapy and betulinic acid, as resting cells are regarded as less sensitive against ionizing radiation than dividing cells.

We have also tested the expression of bcl-2 homologs after treatment with betulinic acid. Notably, one member of this class of protein that was strongly regulated after treatment with betulinic acid was Mcl-1. This is an interesting finding, as this protein is known to inhibit apoptosis. Although, to our knowledge, it has not been directly shown that Mcl-1 has a functional role in melanocytic cells, it might be speculated that the induction of this protein by betulinic acid reflects an adaptive response of melanocytes against a cytotoxic stimulus. It is possible that a fraction of the cell population is protected against the effects of betulinic acid by overexpressing Mcl-1. We have found that betulinic acid does not induce the expression of p53 in the MES20 melanoma cell line, although induction of p53 has recently been described in two other melanoma cell lines (Rieber and Strasberg-Rieber, 1998). Recently, in neuroectodermal tumors, induction of apoptosis by betulinic acid was found to be p53-independent, and this may also be the case in our cell lines, as the 518A2 cell line containing a mutated p53 was sensitive to treatment with betulinic acid. This question is certainly of interest and will be further investigated.

Our data clearly show that the combination of these treatments has an additive effect. Due to the above-mentioned beneficial properties of betulinic acid, including its higher activity against resting cells (Noda *et al*, 1997), betulinic acid might target tumor cell populations that are especially resistant to ionizing radiation. Furthermore, although additional clinical studies are clearly needed, the cell type specificity of betulinic acid suggests that this substance might be used in melanoma therapy together with radiotherapy without enhancing side-effects on normal melanocytic and nonmelanocytic tissues.

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