Involvement of mitochondrial and B-RAF/ERK signaling pathways in berberine-induced apoptosis in human melanoma cells

Ana Burgeiro[a,d], Consuelo Gajate[a,b], EL Habib Dakir[a], Janny A. Villa-Pulgarín[a,c], Paulo J. Oliveira[d] and Faustino Mollinedo[a]

The natural isoquinoline alkaloid berberine exhibits a wide spectrum of biological activities including antitumor activity, but its mechanism of action remains to be fully elucidated. Here, we report that berberine induced apoptosis in human melanoma cells, through a process that involved mitochondria and caspase activation. Berberine-induced activation of a number of caspases, including caspases 3, 4, 7, 8, and 9. Pan-caspase inhibitor, z-VAD-fmk, and caspase-8 and caspase-9 inhibitors prevented apoptosis. Berberine also led to the generation of the p20 cleavage fragment of BAP31, involved in directing proapoptotic signals between the endoplasmic reticulum and the mitochondria. Treatment of SK-MEL-2 melanoma cells with berberine induced disruption of the mitochondrial transmembrane potential, release of cytochrome *c* and apoptosis-inducing factor from the mitochondria to the cytosol, generation of reactive oxygen species (ROS), and a decreased ATP/ADP ratio. Overexpression of *bcl-x*$_L$ by gene transfer prevented berberine-induced cell death, mitochondrial transmembrane potential loss, and cytochrome *c* and apoptosis-inducing factor release, but not ROS generation. *N*-acetyl-L-cysteine inhibited the production of ROS, but did not abrogate the berberine-induced apoptosis. Inhibition of extracellular signal-regulated kinase (ERK) phosphorylation, by using the mitogen-activated protein kinase/ERK kinase inhibitor PD98059, and reduction of B-RAF levels by silencing RNA induced cell death of SK-MEL-2 cells, and diminished the berberine concentration required to promote apoptosis. These data show that berberine-induced apoptosis in melanoma cells involves mitochondria and caspase activation, but ROS generation was not essential. Our results indicate that inhibition of B-RAF/ERK survival signaling facilitates the cell death response triggered by berberine. *Anti-Cancer Drugs* 22:507–518 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:507–518

Keywords: apoptosis, berberine, B-RAF, extracellular signal-regulated kinase signaling, melanoma, mitochondria, SK-MEL-2

[a]Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas - Universidad de Salamanca, [b]Unidad de Investigación, Hospital Universitario de Salamanca, [c]APOINTECH, Centro Hispano-Luso de Investigaciones Agrarias (CIALE), Parque Científico de la Universidad de Salamanca, Salamanca, Spain and [d]Center for Neurosciences and Cell Biology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal

Correspondence to Dr Faustino Mollinedo, Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas - Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca, E-37007 Salamanca, Spain
Tel: +34 923 294806; fax: +34 923 294795;
e-mail: fmollin@usal.es

Received 15 August 2010 **Revised form accepted** 31 December 2010

Introduction

Berberine, also known as Natural Yellow 18 (5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo (5,6-*a*) quinolizinium), is a benzyl tetra isoquinoline alkaloid extracted from *Hidrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (turmeric tree) [1]. Berberine has a remarkable spectrum of biological activities including anti-inflammatory [2], antimicrobial [3], hypolipidemic [4], hypoglycemic [5], antiviral [6], and antidepressant effects [7]. Berberine also proved to be an effective in-vitro and in-vivo anti-tumor agent against a wide variety of human cancer cells including tongue squamous carcinoma [8,9], nasopharyngeal carcinoma [10], breast cancer [11], hepatoma [12], prostate cancer [13], osteosarcoma [14], lung cancer [15], gastric carcinoma [16], neuroblastoma [17], and brain cancer [18,19] cells. Berberine also exerts cytostatic activity against murine melanoma cell lines [20,21], this action is attributed to selective mitochondrial accumulation and dysfunction [22]. Assays on isolated mitochondria showed that berberine acted on the adenine nucleotide translocator to induce the mitochondrial permeability transition [23], and promoted oxidative stress by interfering with a site located at or close to complex I [22]. However, the ability of berberine to prompt apoptosis in melanoma cells remains to be elucidated.

Melanoma is the most dangerous type of skin cancer. Metastatic malignant melanoma has a very poor prognosis with a low survival rate, being refractory to current therapies [24,25]. Therefore, search for new therapeutic approaches is needed. The incidence and mortality rates of

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0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32834438f6

this type of cancer increase every year. Natural compounds with potential cytotoxic and/or cytostatic effects on melanoma could be a good therapeutic option, as many phytochemical-based compounds are considered as relatively nontoxic, inexpensive, and available as oral forms [26].

Berberine has been reported to promote G_1 and G_2 cell cycle arrest in distinct cell lines [16,21,27–29]. In this study, we report that berberine induces apoptosis in human melanoma cells through a caspase-dependent and mitochondrial-dependent mechanism. In addition, we have found that B-RAF/extracellular signal-regulated kinase (ERK) signaling plays a major role in the susceptibility of melanoma cells to undergo berberine-induced apoptosis, which could be of potential therapeutic value.

Materials and methods

Materials

Berberine hemisulfate salt was obtained from Sigma-Aldrich (St Louis, Missouri, USA) and prepared in dimethyl sulfoxide. The total volume of vehicle dimethyl sulfoxide in the distinct assays was less than 0.1–0.5%, which had negligible effects in all the experiments. Propidium iodide, ribonuclease A, bovine serum albumin, *N*-acetyl-L-cysteine (NAC), and 3,3,9-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] were from Sigma-Aldrich. Pan-caspase inhibitor, z-VAD-fmk, was from Alexis Biochemicals (Lausen, Switzerland). Caspase-8 inhibitor, z-IETD-fmk, caspase-9 inhibitor, z-LEHD-fmk, and mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, PD98059, were from Calbiochem (Gibbstown, New Jersey, USA). Acrylamide/bisacrylamide, ammonium persulfate, and *N,N,N′,N′*-tetramethylethylenediamine were obtained from Bio-Rad (Hercules, California, USA). Hoechst 33342 and dihydroethidine (DHE) were from Molecular Probes (Carlsbad, California, USA). Lipofectamine 2000 reagent was from Invitrogen (Eugene, Oregon, USA). RPMI-1640, fetal bovine serum (FBS), antibiotics, L-glutamine, sodium pyruvate, nonessential amino acids, and 0.25% trypsin-EDTA were purchased from Gibco (Carlsbad, California, USA).

Cell culture

Human melanoma cell lines SK-MEL-2, SK-MEL-5, SK-MEL-28, and MALME-3M, obtained from the American Type Culture Collection (Manassas, Virginia, USA), were grown in RPMI-1640 supplemented with 15% heat-inactivated FBS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 1% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml of streptomycin at 37°C in 5% CO_2 humidified air. Cells were periodically tested for mycoplasma infection and were found to be negative.

Analysis of cell cycle and apoptosis by flow cytometry

Quantitation of apoptotic cells was determined by flow cytometry as the percentage of cells in the sub-G_1 region (hypodiploidy) in cell cycle analysis as described earlier [30], using a FACScalibur flow cytometer (Becton Dickinson, San Jose, California, USA).

Western blotting in cell extracts

Cells at approximately 70% of confluence (approximately 1.0×10^6) were lysed in 60 μl of 25 mmol/l Hepes (pH 7.7), 0.3 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.1% Triton X-100, 20 mmol/l β-glycerophosphate, and 0.1 mmol/l sodium orthovanadate supplemented with protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Fifty micrograms of protein extract were run on SDS-polyacrylamide gels, transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (GE Healthcare, Princeton, New Jersey, USA), blocked with 5% (w/v) powder defatted milk in Tris-buffered saline with Tween 20 (50 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 0.1% Tween 20) for 60 min at room temperature, and incubated for 1 h at room temperature or overnight at 4°C with the following specific antibodies: anti-17 and 19 kDa active caspase-3 rabbit polyclonal antibody (1 : 750 dilution), and 1C12 anti-57 kDa caspase-8 mouse monoclonal antibody, which also detects the cleaved p43 and p18 subunits (1 : 500 dilution) (Cell Signaling Technology, Beverly, Massachusetts, USA); N-15 anti-50 kDa caspase-4 goat polyclonal antibody, which also detects the active cleaved p20 subunit (1 : 250 dilution), C-15 anti-31 kDa BAP31 goat polyclonal antibody, which also detects the 20 kDa cleavage product (1 : 500 dilution), F-7 anti-84 kDa anti-B-RAF mouse monoclonal antibody (1 : 750 dilution), and anti-42 and 44 kDa phosphorylated ERK mouse monoclonal antibody (1 : 750 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA); anti-35 kDa caspase-7 mouse monoclonal antibody, which also recognizes the cleaved p11, p20, and p32 subunits (1 : 750 dilution), C2.10 anti-116 kDa poly(ADP-ribose) polymerase mouse monoclonal antibody, which also detects the 85 kDa cleavage product (1 : 750 dilution), and anti-26 kDa Bcl-X rabbit polyclonal antibody (1 : 250) (BD Biosciences, San Jose, California, USA), and anti-47 kDa caspase-9 rabbit polyclonal antibody, which also recognizes the cleaved p10, p17, and p38 subunits (1 : 250 dilution, Calbiochem); AC-15 anti-42 kDa β-actin mouse monoclonal antibody (1 : 5000 dilution, Sigma). Secondary antibodies were anti-mouse biotinylated (GE Healthcare), anti-rabbit biotinylated (GE Healthcare), and anti-goat horseradish peroxidase (Santa Cruz Biotechnology). When secondary biotinylated antibodies were used, membranes were subsequently incubated with streptavidin–horseradish peroxidase conjugate (GE Healthcare). Signals were detected using an ECL kit (GE Healthcare).

Analysis of mitochondrial protein release

Release of proapoptotic proteins from the mitochondria to the cytosol was analyzed by western blot as described earlier [31]. In brief, cells (3×10^6) were harvested,

washed with ice-cold PBS, and gently lysed for 30 s in 50 µl of an ice-cold buffer containing 250 mmol/l sucrose, 1 mmol/l EDTA, 25 mmol/l Tris (pH 6.8), 0.05% digitonin, 1 mmol/l DTT, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 mmol/l phenylmethylsulfonyl fluoride. Lysates were centrifuged at 13 000 $\times g$ for 3 min at 4°C. Supernatant contained the cytosolic fraction, free of the mitochondria, and the pellet contained mostly nuclei and mitochondria. Pellets were resuspended in 50 µl of the above ice-cold buffer and spun down at 13 000 $\times g$ for 10 s at 4°C to sediment nuclear fraction, and the supernatants were centrifuged at 13 000 $\times g$ for 3 min at 4°C. These resulting pellets were enriched in the mitochondria and were resuspended in 20 µl of the above ice-cold buffer. Fifty micrograms of proteins of cytosolic fraction were electrophoresed by SDS-15% polyacrylamide gels, transferred onto a hybond ECL nitrocellulose membranes, blocked with 5% (w/v) powder defatted milk in Tris-buffered saline with Tween 20 for 60 min at room temperature, and incubated overnight at 4°C with the specific antibodies given below: anti-15 kDa cytochrome *c* mouse monoclonal antibody (1 : 500 dilution, BD Biosciences); anti-67 kDa apoptosis-inducing factor (AIF) rabbit polyclonal antibody (1 : 500 dilution, BD Biosciences); and AC-15 anti-42 kDa β-actin monoclonal antibody (1 : 5000 dilution, Sigma). Then, the blots were incubated with biotinylated anti-mouse and anti-rabbit inmunoglobulin antibodies (GE Healthcare), followed by incubation with streptavidin–horseradish peroxidase conjugate (GE Healthcare). Signals were developed using an ECL kit (GE Healthcare).

Quantification by high-performance liquid chromatography of intracellular adenine nucleotides

An acid extraction procedure was used to evaluate the intracellular concentration of adenine nucleotides as described earlier [22]. Adenine nucleotides were separated by reverse-phase high-performance liquid chromatography. The chromatographic apparatus used was a Beckman-System Gold (Beckman Coulter, Fullerton, California, USA), consisting of a 126 Binary Pump Model and a 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100RP-18 (5 µm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mmol/l phosphate buffer, pH 6.5, and 1% methanol was performed with a flow rate of 1.1 ml/min. The required time for each analysis was 5 min. Quantification was achieved by using ATP, ADP, and AMP standard curves. Energy charge was calculated as [ATP + 0.5(ADP)/ATP + ADP + AMP].

Flow cytometry analysis of mitochondrial transmembrane potential and oxidative stress

Dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and generation of reactive oxygen species (ROS) were evaluated by incubating cells in PBS for 40 min at 37°C with 20 nmol/l $DiOC_6(3)$ (green fluorescence) and 2 µmol/l DHE (red fluorescence after oxidation), respectively, followed by analysis on a FACScalibur flow cytometer as described earlier [30].

Confocal microscopy

SK-MEL-2 cells in glass-bottom dishes were incubated with 75 µmol/l berberine for 24 h. DHE (2 µmol/l) and Hoechst 33342 (2 µg/ml) were added to the cell culture 40 and 30 min before the end of drug treatment, and then the cells were analyzed by confocal microscopy using a Leica laser scanning confocal microscope (Bannockburn, Illinois, USA). The antifading reagent SlowFade Gold (Invitrogen) was used to preserve fluorescence signal intensity.

Cell transfection with *bcl-x$_L$*

SK-MEL-2 cells were transfected with 8 µg of pSFFV-Neo expression vector containing the human *bcl-x$_L$* open reading frame driven by the long terminal repeat of the splenic focus-forming virus (pSFFV-Bcl-X_L) [32], using Lipofectamine reagent (Invitrogen) and according to the manufacturer's instructions. As a control, transfection was performed with empty pSFFV-Neo plasmid. Transfectants were selected by growth in complete culture medium supplemented with 800 µg/ml G418 (Sigma, St Louis, Missouri, USA), and then grown in the presence of 250 µg/ml G418. Stable transfectants were achieved after approximately 30 days. The different clones were isolated and monitored for Bcl-X_L expression by western blot, using the anti-26 kDa Bcl-X rabbit polyclonal antibody (1 : 250 dilution, BD Biosciences).

siRNA-mediated downregulation of B-RAF

Three 21-base B-RAF-specific siRNA sequences, from v-*raf* murine sarcoma viral oncogene homolog B1, locus ID 673, were synthesized by Applied Biosystem/Ambion (Foster City, California, USA): siRNA1 (sense: 5′-CAGU UGUCUGGAUCCAUUUtt-3′, antisense: 5′-AAAUGGAU CCAGACAACUGtt-3′), siRNA2 (sense: 5′-GCAUAAUC CACCAUCAAUAtt-3′, antisense: 5′-UAUUGAUGGUGG AUUAUGCtc-3′), siRNA3 (sense: 5′-CAGAGGAUUUUA GUCUAUAtt-3′, antisense: 5′-UAUAGACUAAAAUCCU CUGtt-3′). For siRNA-mediated downregulation of B-RAF, SK-MEL-2 cells were seeded at 10^5 cells/well in complete cell culture medium in six-well plates on the day before transfection. Cells were then transfected in 1 ml RPMI-1640 culture medium in the absence of serum and antibiotics with 20 nmol/l B-RAF-specific siRNA1, siRNA2 or siRNA3, or 20 nmol/l scramble siRNA (control siRNA) with 10 µg/ml lipofectamine (Invitrogen) according to the manufacturer's instructions. After 5 h, 1 ml of RPMI medium containing 10% FBS was added, and the cells were cultured for 48 h with or without the drug, and then the cells were harvested and lysed for analysis. The siRNA2 sequence showed the highest efficiency in downregulating B-RAF.

Flow cytometry detection of intracellular proteins

Cells were fixed and permeabilized using the Fix and Perm cell permeabilization kit (CALTAG Laboratories, Burlingame, California, USA) according to the manufacturer's instructions. Cells were then incubated with specific antibodies (antiphosphorylated ERK antibody, 1 : 100 dilution; anti-B-RAF, 1 : 100 dilution) for 1 h at room temperature, washed with PBS, stained for 1 h with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) at 1 : 100 dilution in PBS and analyzed in a FACScalibur flow cytometer.

Statistical analysis

Data are expressed as means ± standard deviation of the number of experiments indicated in the respective figure legends. Multiple comparisons were made using one-way analysis of variance followed by a Bonferroni post-hoc test. Statistical comparisons between the two experimental groups were made using Student's *t*-test. Significance was accepted at a *P* value of less than 0.05.

Results

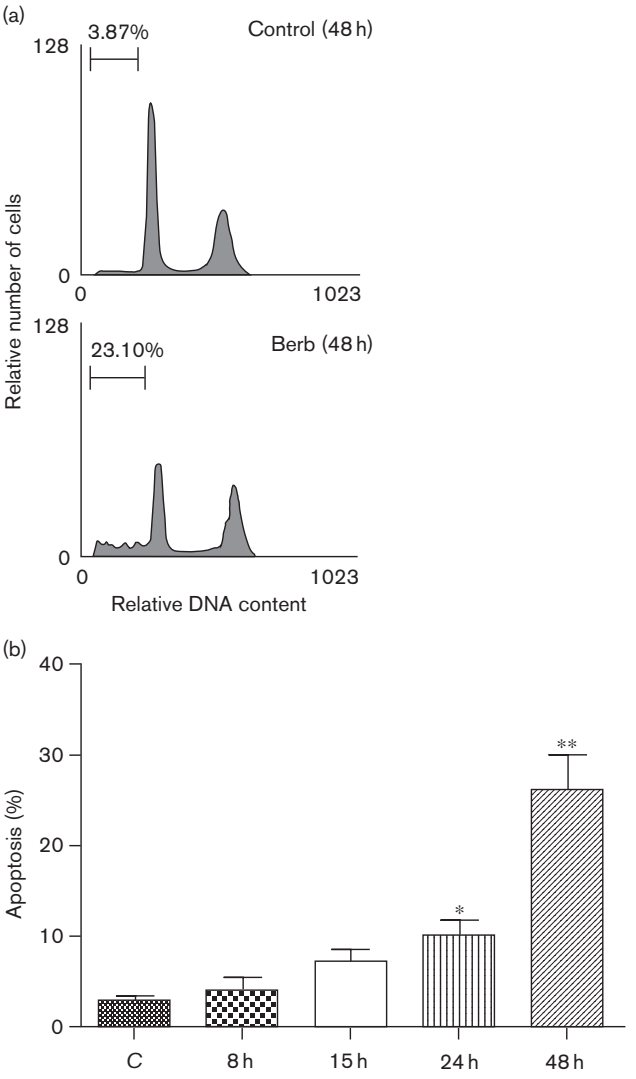
Berberine induces caspase-dependent apoptosis in human melanoma cells

We observed that the treatment of human metastatic SK-MEL-2 melanoma cells with berberine at 10, 25, or 50 μmol/l for 24 and 48 h failed to elicit a significant cell death (data not shown). However, treatment of SK-MEL-2 cells with 75 μmol/l berberine for 48 h resulted in the induction of apoptosis, as assessed by the presence of cells in the sub-G_1 region (hypodiploidy) in cell cycle analysis (Fig. 1a), in a time-dependent manner (Fig. 1b). We also noticed that 75 μmol/l berberine induced apoptosis in additional human melanoma cell lines after 48 h incubation including (percentage apoptosis in parentheses): SK-MEL-5 (37.28 ± 9.20%), SK-MEL-28 (17.65 ± 5.10%), and MALME-3M (30.72 ± 7.35%).

Berberine-induced apoptosis of SK-MEL-2 cells involved a time-dependent activation of caspases 3, 4, 7, 8, and 9, as assessed by the appearance of the respective cleaved active caspases (Fig. 2a). Drug-induced cell death was abrogated by preincubation of SK-MEL-2 cells with the pan-caspase inhibitor, z-VAD-fmk, (Fig. 2b), thus indicating the involvement of caspase activation in berberine-induced apoptosis. In addition, caspase-8 inhibitor, z-IETD-fmk, and caspase-9 inhibitor, z-LEHD-fmk, also inhibited apoptotic response (Fig. 2b). Interestingly, berberine treatment led to the generation of the p20 caspase–cleavage fragment of BAP31 (Fig. 2a), an integral protein of the endoplasmic reticulum that is a caspase-8 substrate [33] and links proapoptotic signals between the endoplasmic reticulum and the mitochondrion [34].

Time-lapse videomicroscopy (Supplementary Fig. S1) shows clearly that SK-MEL-2 cells stop dividing in the presence of berberine, and undergo typical morphological changes of apoptosis (blebbing, cell shrinkage, rounding up).

Fig. 1

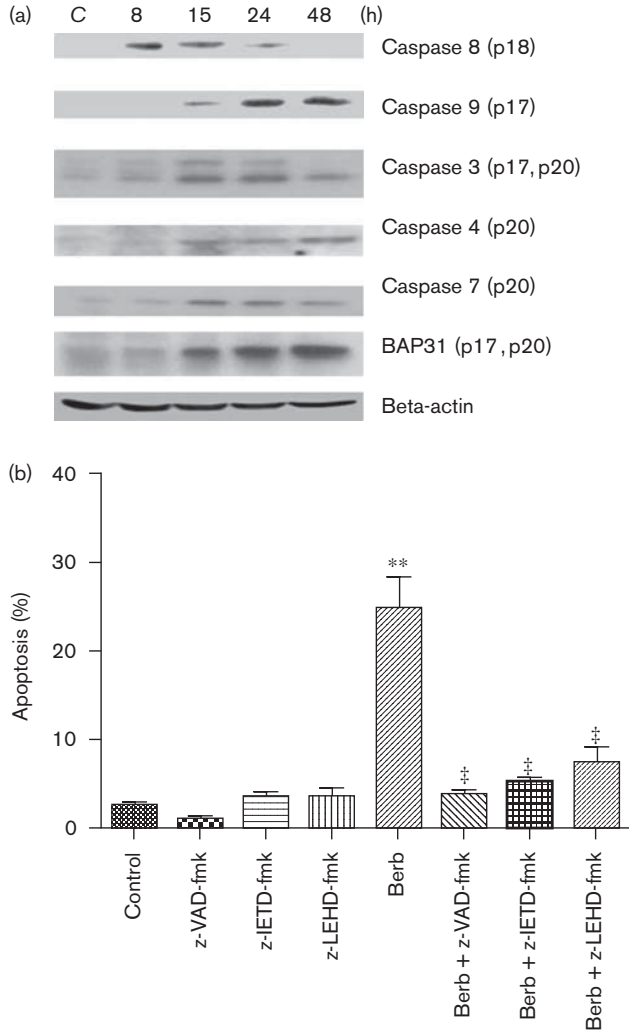


Induction of apoptosis by berberine in SK-MEL-2 cells. (a) Representative histograms of untreated control and 75 μmol/l berberine (Berb)-treated cells, incubated for 48 h, and analyzed for cell cycle by flow cytometry. The proportion of cells in the sub-G_1 region, representing apoptotic cells, is shown. (b) Time-course of 75 μmol/l berberine-induced apoptosis. Data shown are means ± standard deviation of five independent experiments. Asterisks indicate values that are statistically different from untreated control cells at *P* value less than 0.05 (*) and *P* value less than 0.01 (**).

Berberine induces $\Delta\Psi_m$ disruption, cytochrome *c* release, and ROS generation

Berberine treatment led to ROS generation in SK-MEL-2 cells, as detected by flow cytometry and confocal microscopy, using DHE (nonfluorescent) that becomes ethidium (red fluorescence) after its oxidation by ROS (Fig. 3a). Mitochondrial depolarization was also detected in berberine-treated cells by using the cationic probe

Fig. 2



Caspase activation in berberine-treated SK-MEL-2 cells. (a) Untreated control cells (*C*) or cells treated with 75 μmol/l berberine for the indicated times were analyzed by western blot using specific antibodies for the proteins shown on the right. The active caspase forms and cleaved BAP31 are shown. Beta-actin was used as a loading control. Blots are representative of three experiments performed. (b) SK-MEL-2 cells were preincubated without (control) or with 50 μmol/l of z-VAD-fmk, 100 μmol/l of z-IETD-fmk, or 100 μmol/l of z-LEHD-fmk for 1 h, and then incubated in the absence or presence of 75 μmol/l berberine (Berb) for 48 h, and analyzed by flow cytometry to evaluate apoptosis. Untreated control cells were run in parallel. Data shown are means ± standard deviation of three independent experiments. $^{**}P$ value less than 0.01 vs. untreated control; $^{\ddagger\ddagger}P$ value less than 0.01 vs. Berb.

$DiOC_6(3)$ (Fig. 3b) or tetramethylrhodamine methylester (data not shown), which accumulate into the polarized mitochondria as a function of $\Delta\Psi_m$. Following a time-course, we detected disruption of $\Delta\Psi_m$ after 8 h incubation (Fig. 3b), which was accompanied by the onset of apoptosis (Fig. 1b). We found that both $\Delta\Psi_m$ disruption and the trigger of apoptosis were rather simultaneous (Fig. 3a, 1b, and data not shown). Berberine also induced cytochrome *c* and AIF release from the mitochondria to the cytosol (Fig. 3c), and decreased the ATP/ADP ratio and the cellular energy charge (Fig. 3d).

Bcl-X_L overexpression prevents cell death induced by berberine

As Bcl-X_L preserves mitochondrial integrity [30,35,36], we examined the effect of overexpressing Bcl-X_L in SK-MEL-2 cells to further analyze the role of mitochondria in the antitumor effect of berberine on melanoma cells. We stably transfected SK-MEL-2 cells with pSFFV-*bcl-x*$_L$ (SK-MEL-2-Bcl-X_L), containing the human *bcl-x*$_L$ open reading frame, or with control pSFFV-Neo plasmid (SK-MEL-2-Neo). SK-MEL-2-Neo cells behaved as nontransfected SK-MEL-2 cells in all the parameters studied. Western blot analysis showed that SK-MEL-2-Neo cells expressed low levels of endogenous Bcl-X_L, whereas a high expression of this protein was observed in SK-MEL-2-Bcl-X_L cells (Fig. 4a, inset). SK-MEL-2-Neo cells underwent apoptosis after treatment with berberine. However, overexpression of Bcl-X_L by gene transfer in SK-MEL-2 cells totally blocked apoptosis after treatment with 75 μmol/l berberine for 48 h (Fig. 4a), although an increase in the percentage of cells in the G_2 phase of cell cycle was observed (data not shown). Bcl-X_L overexpression also abrogated the release of cytochrome *c* and AIF from the mitochondria (Fig. 4b), and $\Delta\Psi_m$ dissipation (Fig. 4c). Altogether, these results further show the involvement of the mitochondria in berberine-induced apoptosis. The oxidative stress response induced by berberine was slightly decreased upon Bcl-X_L overexpression, but this inhibition was not statistically significant ($P>0.05$) (Fig. 4d), indicating that the ROS generation was not crucial for berberine-induced apoptosis.

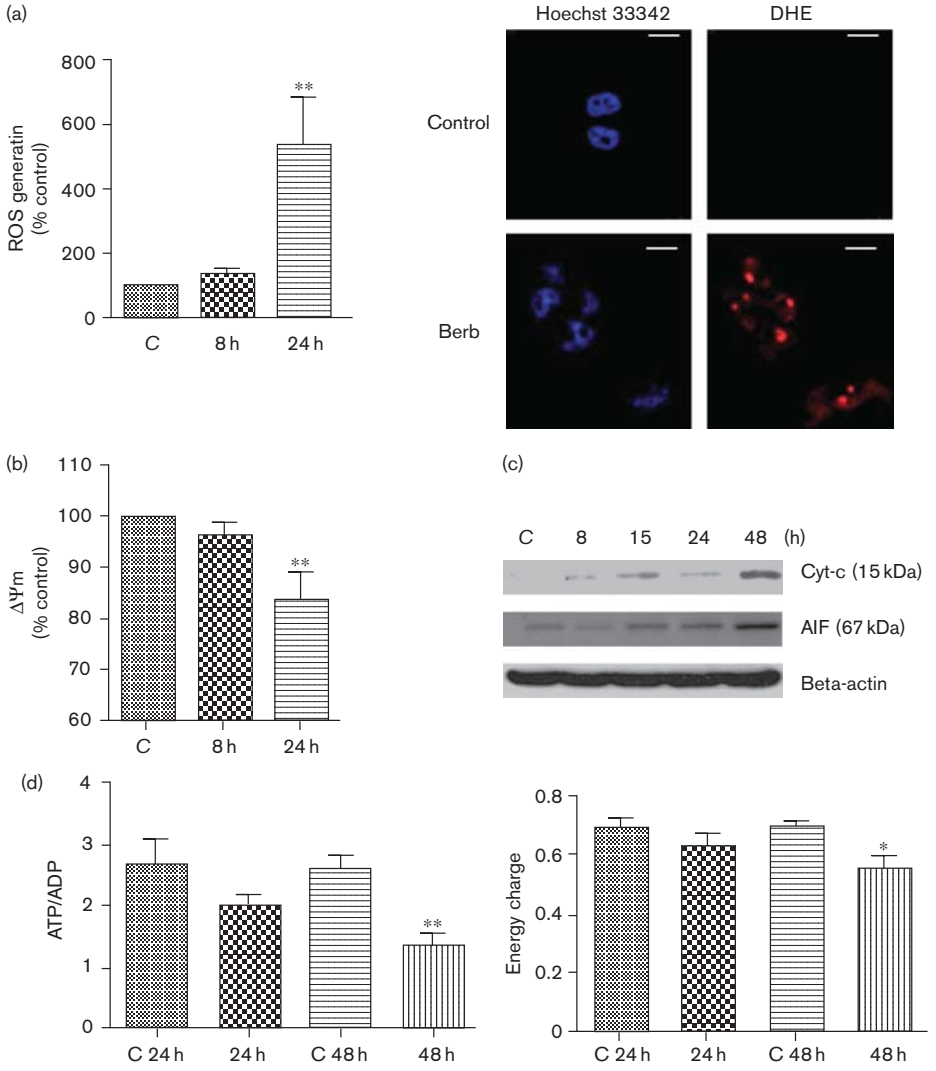
Inhibition of ROS does not prevent berberine-induced apoptosis

The above data suggested that although berberine prompted the generation of ROS, these were not involved in berberine-induced apoptosis. This conclusion was further supported by the fact that incubation of SK-MEL-2 cells with the antioxidant NAC inhibited ROS generation (Fig. 5a), but it did not prevent $\Delta\Psi_m$ disruption (Fig. 5b) and apoptosis (Fig. 5c) after berberine treatment.

Effect of MEK/ERK inhibition on berberine-induced cytotoxicity

Constitutive activation of the MEK-ERK signaling pathway is often found in melanomas [37], and this activated signaling route has been suggested to be involved in the resistance of melanoma to treatment [38]. On these grounds, we used the selective and cell-permeable MEK inhibitor, PD98059, which inhibits ERK activation, to assess the involvement of ERK signaling in berberine-induced apoptosis. Incubation of SK-MEL-2 cells with 50 μmol/l PD98059 downregulated phosphorylated-ERK level, as assessed by immunofluorescence flow cytometry

Fig. 3



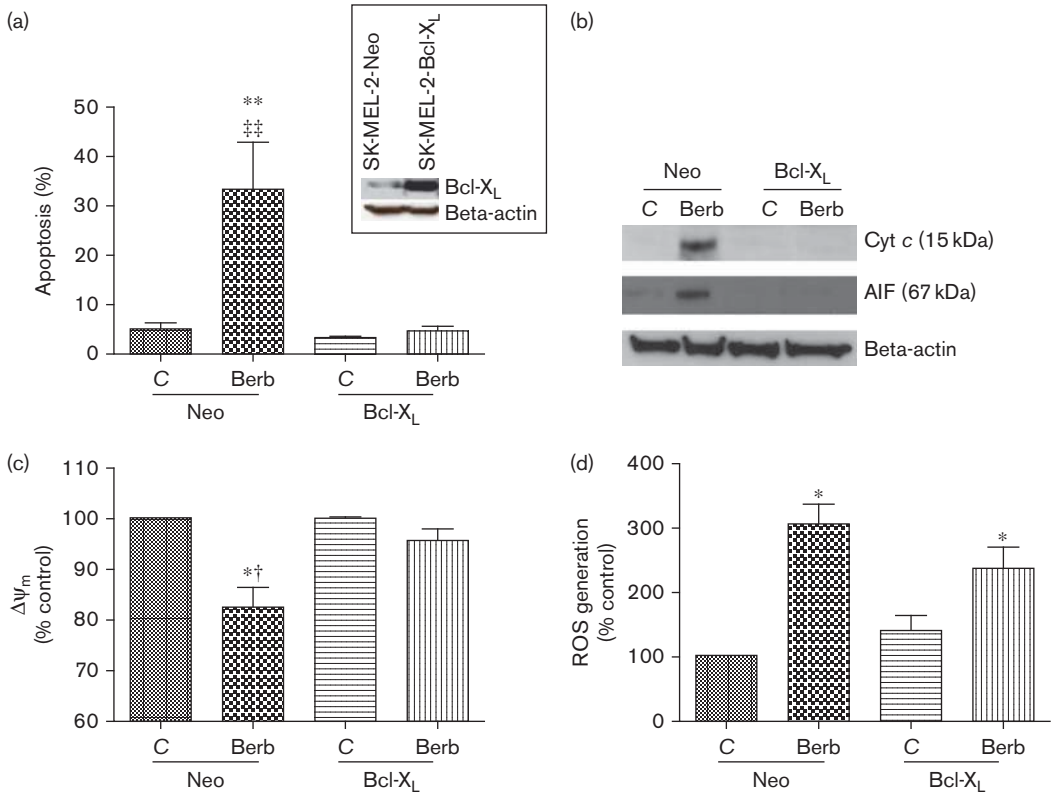
Berberine induces mitochondrial dysfunction in SK-MEL-2 cells. (a) Left, cells were treated with 75 μmol/l berberine for 8 and 24 h, and then analyzed for reactive oxygen species (ROS) generation, using dihydroethidine (DHE) and flow cytometry. (a) Right, cells were incubated for 24 h in the absence (control) and in the presence of 75 μmol/l of berberine (Berb), and then DHE oxidation (red fluorescence) by ROS generation was examined by confocal microscopy. Hoechst 33342 stained nuclear DNA (blue fluorescence). Bar, 25 μm. (b) Cells were treated with 75 μmol/l berberine for 8 and 24 h, and then analyzed for mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipation using $DiOC_6(3)$. (c) Cells treated with 75 μmol/l berberine were harvested at the indicated times and cytosolic proteins were analyzed by immunoblotting with anti-cytochrome *c* (Cyt *c*) and anti-apoptosis-inducing factor (AIF) antibodies. Beta-actin served as an internal control for equal protein loading in each lane. Untreated control cells (C) were run in parallel. (d) Effects of berberine on ATP/ADP ratio (left) and energy charge (right) in SK-MEL-2 human melanoma cells, subsequent to incubation of cells for 24 and 48 h in the absence (C24h, C48h) and in the presence of berberine (24h, 48h). Data shown are means ± standard deviation of three to five independent experiments, or are representative of three experiments performed. **P* value less than 0.05 vs. untreated control, ***P* value less than 0.01 vs. untreated control.

(data not shown). Interestingly, SK-MEL-2 cells became more sensitive to berberine after PD98059 pretreatment. MEK/ERK inhibition promoted a weak apoptotic response in SK-MEL-2 cells, and the percentage of apoptotic cells was highly increased subsequent to incubation with berberine at a concentration of 20 μmol/l, which by itself, in the absence of MEK/ERK inhibition, was unable to elicit cell death (Fig. 6a). The high increase in sensitivity to berberine-induced cell death upon PD98059 pretreatment was also assessed by poly(ADP-ribose) polymerase breakdown (Fig. 6b).

Effect of B-RAF silencing on berberine-induced cytotoxicity

B-RAF gene is mutated in approximately 70% of malignant melanomas, and has been suggested as a therapeutic target for melanoma [39]. Silencing B-RAF, by using siRNA, downregulated B-RAF expression (Fig. 7a) and

Fig. 4



Bcl-X_L overexpression prevents berberine-induced cell death, mitochondrial transmembrane potential ($\Delta\Psi_m$) loss and release of mitochondrial apoptogenic proteins in SK-MEL-2 cells. (a) Bcl-X_L overexpression abrogated berberine (Berb)-induced apoptosis (75 μmol/l berberine, 48 h), measured as the percentage of cells in the sub-G_1 region of cell cycle. Inset, Bcl-X_L protein expression in SK-MEL-2-Neo and SK-MEL-2-Bcl-X_L cells was analyzed by SDS-PAGE of 40 μg cell extract protein and immunoblotting. Beta-actin was used as a control for protein loading. (b) SK-MEL-2-Neo and SK-MEL-2-Bcl-X_L transfected cells were untreated (*C*) or treated with 75 μmol/l berberine (Berb) for 48 h, and cytosolic fractions were analyzed by immunoblotting with anti-cytochrome *c* (Cyt *c*) and anti-apoptosis-inducing factor (AIF) antibodies. Immunoblotting for β-actin was used as an internal control for equal protein loading in each lane. (c) SK-MEL-2-Neo cells (Neo) and Bcl-X_L-transfected SK-MEL-2 cells (*Bcl-X_L*) were treated with 75 μmol/l of berberine for 24 h, and then analyzed for $\Delta\Psi_m$ dissipation using $DiOC_6(3)$. (d) Effect of Bcl-X_L overexpression on berberine-induced reactive oxygen species (ROS) generation. SK-MEL-2 cells (Neo) and Bcl-X_L-transfected SK-MEL-2 cells (*Bcl-X_L*) were treated with 75 μmol/l berberine for 24 h, and then analyzed for ROS generation using dihydroethidine. Untreated control cells (*C*) were run in parallel. Data shown are means ± standard deviation of four independent experiments, or are representative of three experiments performed. Values that are statistically different from untreated control cells at *P* less than 0.05 (*) and at *P* value less than 0.01 (**), and from berberine-treated cells at *P* value less than 0.05 (†) and *P* value less than 0.01 (††) are indicated.

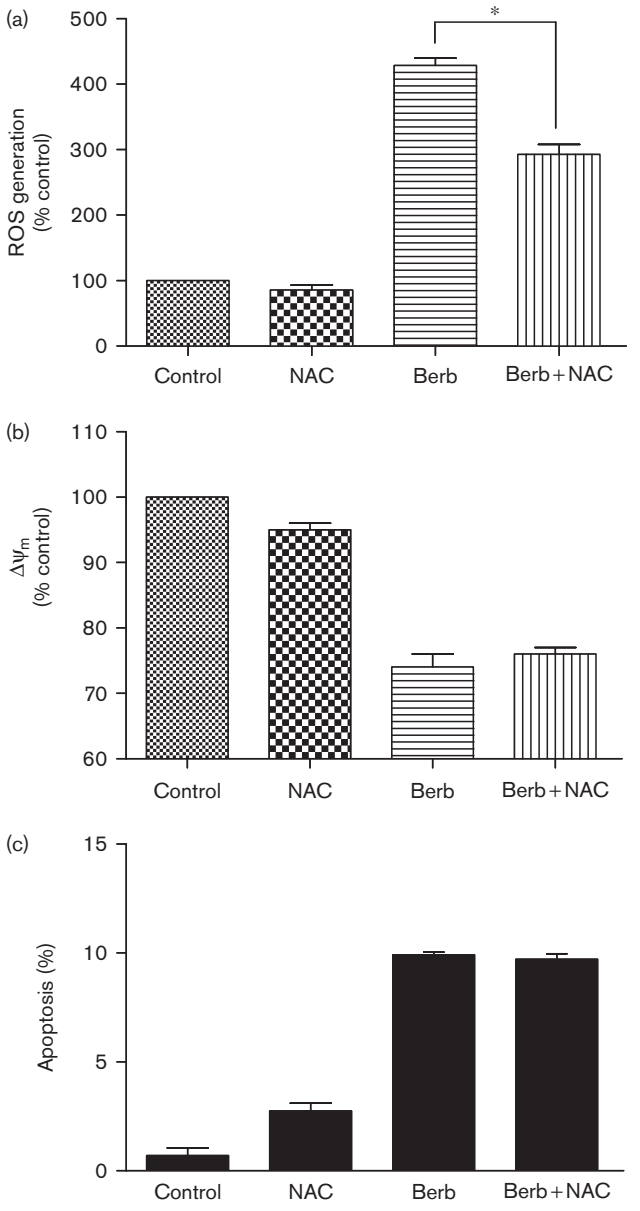
promoted apoptosis (Fig. 7b and c). B-RAF silencing potentiated the apoptotic response induced by 20 μmol/l berberine, a concentration that was unable to induce significant apoptosis in the absence of B-RAF silencing (Fig. 7b and c).

Discussion

The data reported here show that berberine induces apoptosis in human melanoma cells in a mitochondrial-dependent and caspase-dependent manner. The induction of apoptosis by berberine involved activation of the intrinsic mitochondrial signaling pathway, as assessed by $\Delta\Psi_m$ dissipation, cytochrome *c*, and AIF release from the mitochondria, and the blockade of these processes by Bcl-X_L overexpression. The intrinsic mitochondrial signaling pathway leads eventually to the cleavage of procaspase 9, initiating an enzymatic reaction cascade that results in the execution of apoptosis [40]. Here, we report that berberine induced caspase-9 activation, and inhibition of this caspase activity prevented apoptosis. Altogether, these data indicate that the mitochondria play a key role in berberine-induced apoptosis. Earlier studies have shown that berberine accumulated in the mitochondrion [21,22]. Thus, mitochondria are mainly involved in the antitumor action of berberine. In this regard, Patil *et al.* [41] have recently reported a correlation between the induction of apoptosis and an increase in the levels of cytoplasmic cytochrome *c* and caspase-9 activity in MCF-7 breast cancer cells, after berberine treatment, further supporting a major involvement of mitochondria in the antitumor mechanism of action of berberine.

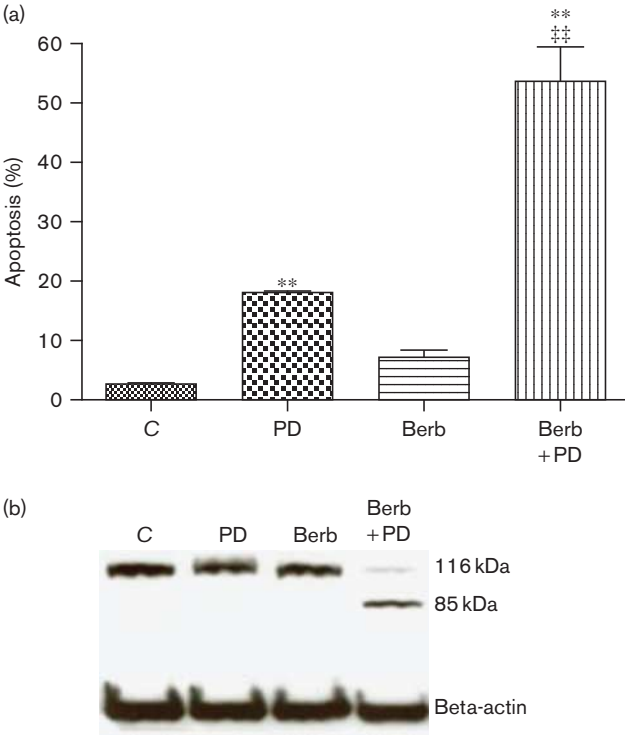
The results reported here also indicate that berberine treatment promoted oxidative stress. Bcl-X_L overexpression

Fig. 5



N-acetyl-L-cysteine (NAC) does not inhibit apoptosis. SK-MEL-2 cells were preincubated with or without 10 mmol/l NAC for 2 h, and then incubated in the absence or presence of 75 μmol/l of berberine (Berb) for 24 h, and analyzed for (a) reactive oxygen species (ROS) generation, (b) mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption, and (c) apoptosis. Untreated control cells were run in parallel. Data shown are means ± standard deviation of three independent experiments. *P value less than 0.05.

Fig. 6

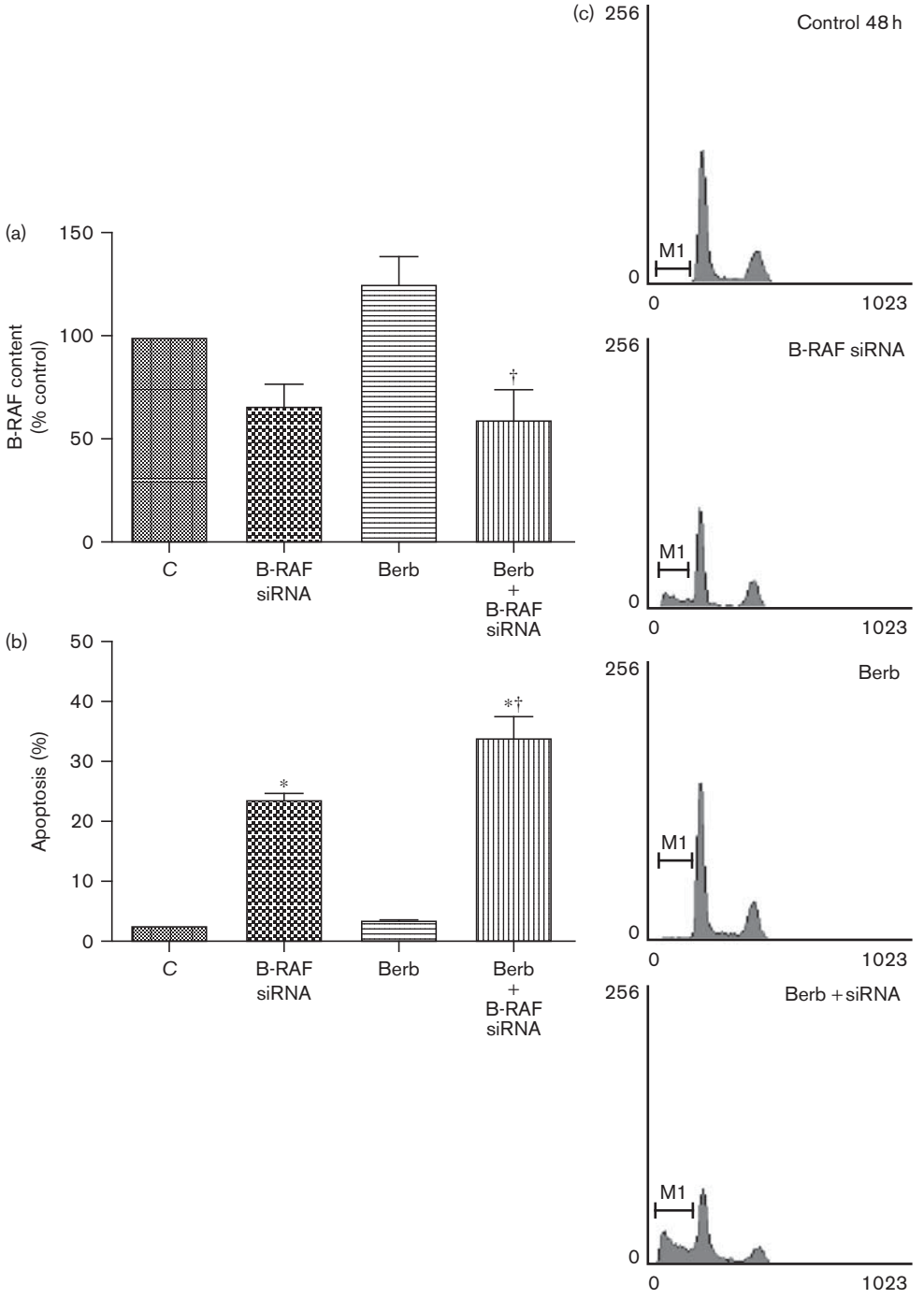


Extracellular signal-regulated kinase inhibition potentiates berberine-induced apoptosis in SK-MEL-2 cells. SK-MEL-2 cells were preincubated with 50 μmol/l PD98059 (PD) for 1 h, and then incubated in the absence or presence of 20 μmol/l of berberine (Berb) for 48 h, and analyzed for (a) apoptosis and (b) poly(ADP-ribose) polymerase cleavage. The 116 kDa intact form and the 85 kDa cleavage product of poly(ADP-ribose) polymerase are indicated. Beta-actin was used as a loading control. Untreated control cells (*C*) were run in parallel. Data shown are means ± standard deviation of three to five independent experiments, or are representative of three experiments performed. **P value less than 0.01 vs. untreated control cells, $^{\dagger\dagger}P$ value less than 0.01 vs. berberine-treated cells (Berb).

abrogated berberine-induced $\Delta\Psi_m$ loss and, cytochrome *c* and AIF release from the mitochondria, but was unable to inhibit the production of ROS in a statistically significant manner. This suggests that the production of ROS might be, at least in part, because of a direct effect of berberine on the respiratory chain. In this context, berberine has been shown to inhibit mitochondrial complex I and increase complex I-dependent generation of ROS [21,22]. As Bcl-X_L overexpression abrogated berberine-induced cell death, but not ROS generation, our data suggest that oxidative stress is a side effect of berberine action, and is not essential for the apoptotic response induced by this drug. Furthermore, our data showing that the ROS scavenger NAC inhibited berberine-induced ROS production but not berberine-induced apoptosis, further confirmed that ROS generation was not involved in the apoptotic response triggered by berberine.

Our results provide novel insights into the mechanism of berberine-mediated antimelanoma activity. Thus, the data reported here suggest that induction of apoptosis is the major effect that underlies berberine antitumor action, at least in melanoma cells. Nevertheless, berberine treatment required both a high drug concentration and rather protracted incubation times to promote apoptosis. Thus, the use of different drug concentrations and cell types may explain the distinct effects on cell cycle and

Fig. 7



Potentiation of berberine-induced apoptosis by B-RAF silencing. SK-MEL-2 cells were transfected with B-RAF siRNA or scramble control siRNA (*C*), and then incubated in the absence or presence of 20 μmol/l berberine (Berb) for 48 h, and analyzed for B-RAF expression by immunofluorescence flow cytometry (a) and for apoptosis (b). Data shown are means ± standard deviation of three independent experiments. **P* value less than 0.05 vs. untreated control cells, †*P* value less than 0.05 vs. berberine-treated cells (Berb). A representative experiment of apoptosis determination by flow cytometry is shown in (c), showing the potentiation of berberine-induced cell death by B-RAF siRNA. M1 indicates the sub-G_1 region of the cell cycle profile, representing apoptotic cells.

apoptosis observed after berberine treatment [16,21,27–29]. Our results showed that berberine promoted G_2 arrest when apoptosis was abrogated by Bcl-X_L over-expression. This indicates that berberine affects the cell cycle, and this action is more evident when the apoptotic response was blocked.

These data also show that berberine induces cleavage of BAP31, a polytopic integral membrane protein of the endoplasmic reticulum that is part of a complex that contains Bcl-2/Bcl-X_L and procaspase 8 [33]. This leads to the generation of the proapoptotic caspase-8-derived p20 fragment of BAP31, which enhances mitochondrial *c* release into the cytosol [34], and acts as a linker between the endoplasmic reticulum and the mitochondria during apoptosis. Thus, it might be envisaged that the endoplasmic reticulum stress could amplify or contribute to the above berberine-induced mitochondrial-mediated apoptotic response. In this regard, inhibition of caspase 8, which acts on BAP31, inhibited berberine-induced apoptosis; and caspase 4 and caspase 7, which are associ ated with the endoplasmic reticulum [42], were activated after berberine treatment.

A number of different signaling pathways and processes involved in cancer have been reported to be affected by berberine in mammalian cells, including p38 mitogen-activated protein kinase [43], nuclear factor-κB [44–46], and invasion mediated by urokinase-type plasminogen activator and metalloproteinases 2 and 9. In addition, berberine has been reported to activate the aryl hydrocarbon receptor (AhR) in hepatoma cells [47]. AhR has been suggested to predispose hepatocytes to Fas(CD95)-mediated apoptosis [48], and it has also been shown to have a proliferative and antiapoptotic action [49], playing a putative role in tumor progression. Here, we have found that the MEK inhibitor, PD98059, potentiated berberine-induced apoptosis in melanoma cells. However, this inhibitor has also been reported to be an antagonist of AhR at concentrations commonly used to inhibit MEK [50]. Taken together, it could be envisaged that berberine may affect a plethora of signaling routes with opposing effects on cancer development. On these grounds, it could be hypothesized that berberine antitumor action might be enhanced by combining berberine with additional agents that prevent activation of cancer cell survival signaling pathways.

The RAS/RAF/MEK/ERK survival signaling pathway is constitutively activated in melanomas, and thus exerts several key functions in melanoma development and progression [51]. Constitutive activation of MEK-ERK signaling is often found in melanomas [37,52], and activating B-RAF mutations are present in 66% of melanomas, most of them (approximately 90%) involving codon 600 (earlier denoted codon 599) [53,54]. Thus, the RAS/RAF/MEK/ERK pathway may represent a promising therapeutic target in melanomas. We found that ERK inhibition or B-RAF silencing induced a weak apoptotic response in melanoma cells, which was dramatically increased by subsequent berberine treatment. Interestingly, once ERK was inhibited, berberine was able to induce a high apoptotic response in melanoma cells at lower concentrations. Similar results, although at a lower extent, were obtained with the combined action of B-RAF silencing and berberine. Thus, inhibition of RAS/RAF/MEK/ERK signaling seems to be crucial to improve the apoptotic effects of berberine, hence highlighting the importance of B-RAF/ERK signaling in the survival and treatment regimens of melanoma cells. SB-590885, a potent B-RAF kinase inhibitor, has been shown to exert a rather modest decrease in melanoma tumor growth in xenograft assays [55]. In our hands, the antimelanoma activity of berberine was much further potentiated by ERK inhibition than by B-RAF silencing. On these grounds, inhibition at the end of the RAS/RAF/MEK/ERK signaling cascade leads to a higher potentiation of the apoptotic response triggered by berberine in melanoma cells. Although the results reported here have been obtained using human cell lines, which are not often representative of their parent tumors, combi nation therapy between ERK signaling inhibitors and berberine might be of potential interest in the treatment of melanoma.

Conclusion

The data reported here show the involvement of mitochondria and caspase activation in berberine-induced apoptosis in melanoma cells, and point out the importance of B-RAF/ERK signaling in the survival and treat ment regimens of melanoma cells. Inhibition of B-RAF/ERK survival signaling facilitates the cell death response triggered by berberine, providing novel insights into the mechanism of berberine-mediated antimelanoma activity. A putative involvement of endoplasmic reticulum stress is also considered to contribute to berberine-induced apoptotic response.

Acknowledgements

This work was supported by Grants from the Fundaçao para a Ciencia e Tecnologia (FCT) (Ministerio da Ciencia, Tecnología e Ensino Superior of Portugal, PTDC/QUI-QUI/101409/2008) to P.J.O., the Ministerio de Ciencia e Innovación of Spain (SAF2008-02251, and RD06/0020/1037 from the Red Temática de Investigación Cooperativa en Cáncer, Instituto de Salud Carlos III, cofunded by the Fondo Europeo de Desarrollo Regional of the European Union) to F.M., the Fondo de Investigación Sanitaria and European Commission (PS09/01915) to C.G., and the Junta de Castilla y León (GR15-Experimental Therapeutics and Translational Oncology Program, and Biomedicine Project 2009 to F.M.; and Biomedicine Project 2010–2011 to C.G.). A.B. is recipient of a predoctoral fellowship from the Portuguese FCT (SFRH/BD/32943/2006). C.G. is supported by the Ramón y Cajal Program from the Spanish Ministerio de Ciencia e Innovación.

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