

Berberine inhibits growth, induces G₁ arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdk_i–Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP

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Chemotherapeutic approach using non-toxic botanicals may be one of the strategies for the management of the skin cancers. Here we report that *in vitro* treatment of human epidermoid carcinoma A431 cells with berberine, a naturally occurring isoquinoline alkaloid, decreased cell viability (3–77%, $P < 0.05$ – 0.001) and induced cell death (3–51%, $P < 0.01$ – 0.001) in a dose (5–75 μ M)- and time (12–72 h)-dependent manner, which was associated with an increase in G₁ arrest. G₀/G₁ phase of the cell cycle is known to be controlled by cyclin dependent kinases (Cdk), cyclin kinase inhibitors (Cdk_i) and cyclins. Our western blot analysis showed that berberine-induced G₁ cell cycle arrest was mediated through the increased expression of Cdk_i proteins (Cip1/p21 and Kip1/p27), a simultaneous decrease in Cdk2, Cdk4, Cdk6 and cyclins D1, D2 and E and enhanced binding of Cdk_i–Cdk. In additional studies, treatment of A431 cells with berberine (15–75 μ M) for 72 h resulted in a significant dose-dependent increase in apoptosis (31–60%, $P < 0.05$ – 0.001) than non-berberine-treated control (11.7%), which was associated with an increased expression of pro-apoptotic protein Bax, decreased expression of anti-apoptotic proteins Bcl-2 and Bcl-xl, disruption of mitochondrial membrane potential, and activation of caspases 9, 3 and poly (ADP-ribose) polymerase. Pretreatment of A431 cells with the pan-caspase inhibitor (z-VAD-fmk) significantly blocked the berberine-induced apoptosis in A431 cells confirmed that berberine-induced apoptosis is mediated through activation of caspase 3-dependent pathway. Together, this study for the first time identified berberine as a chemotherapeutic agent against human epidermoid carcinoma A431 cells *in vitro*, further *in vivo* studies are required to determine whether berberine could be an effective chemotherapeutic agent for the management of non-melanoma skin cancers.

Introduction

The risk of cancer is a growing health problem around the world particularly with the constantly rise in life expectancy, changes in environmental conditions, dietary habits and life-style. Among all the cancers, the incidence of non-melanoma

skin cancers, including the squamous and basal cell carcinomas, represent the most common malignant neoplasms in humans, particularly in Caucasians. It has been estimated that ~1.3 million new cases of skin cancers are diagnosed each year in the USA alone (1), which is equivalent to the incidence of malignancies in all other organs combined (2). According to current projections, one in five Americans will develop at least one non-melanoma skin cancer during their life-time. Thus cutaneous malignancies currently are a major burden on public health and healthcare expenditures. While not denying the importance of currently available sunscreens in the prevention of the risk of non-melanoma skin cancers, the use of sunscreens do not adequately protect the skin from the risk of cutaneous malignancies (3). Therefore, the development of effective chemopreventive or chemotherapeutic agents is required to address this issue.

There has been a considerable interest in the use of phytochemicals for the prevention of skin disorders including the risk of skin cancers. It has been noted that out of 121 prescription drugs in use for cancer treatment, 90 are derived from natural plant sources and ~74% of these chemotherapeutic drugs were discovered by investigating a folklore claim (4,5). Berberine is an isoquinoline alkaloid present in the roots, rhizome and stem bark of a number of important medicinal plant species (e.g. *Berberis aquifolium*, *Berberis vulgaris*, *Berberis aristata* and *Tinospora cordifolia* etc.). The potential importance of berberine is indicated by its use in the Indian Ayurvedic (6), Unani and Chinese systems of medicine since time immemorial. Berberine possesses a wide range of biochemical and pharmacological activities, viz. antidiarrheal, antiarrhythmic and antitumor activities (7–10). *Coptidis rhizoma*, containing abundant berberine, is shown to inhibit the proliferation of esophageal cancer cells (11). Berberine inhibits cyclooxygenase-2 transcriptional activity in human colon cancer cells (10,12), and preliminary studies have shown that berberine sulfate inhibits tumor promoting activity of teleocidin in two-stage chemical carcinogenesis on mouse skin (13). Berberine also inhibits DNA topoisomerase I and II in biochemical system (14,15), and in fact, several classes of compounds that inhibit eukaryotic topoisomerase I or II have antitumor activity (16). Therefore, in an effort to develop an effective chemotherapeutic drug or agent for the prevention of non-melanoma skin cancers, we attempted for the first time to examine the chemotherapeutic effect of berberine on human epidermoid carcinoma A431 cells in *in vitro* system. We show that berberine inhibits the growth, proliferation and induces apoptosis in A431 cells. Our study also provides insight into the mechanism by which berberine induces apoptosis in these cells.

Materials and methods

Chemicals and antibodies

For chemotherapeutic studies, we selected the chloro-derivative of berberine because of its greater solubility in solvents compared to its parent compound.

Abbreviations: Cdk, cyclin-dependent kinases; Cdk_i, cyclin-dependent kinase inhibitors; PARP, poly (ADP-ribose) polymerase; FACS, fluorescence-activated cell sorting; PI, propidium iodide.

We refer to berberine chloride as berberine throughout the manuscript, and it was purchased from Sigma Chemical (St Louis, MO). MTT, (3-[4,5-dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide), and all other chemicals were of analytical grade and purchased from Sigma Chemical. The primary antibodies were obtained as follows: antibodies for Bax, Bcl-2, Bcl-xL, caspase 9, cleaved caspase 9, cleaved caspase 3, anti-PARP and β -actin were purchased from Cell Signaling Technology (Beverly, MA); antibodies for cytochrome *c*, Cyclin D1, Cyclin D2, Cyclin E, Cdk2, Cdk 4, Cdk 6, Cip1/p21, Kip1/p27 and the secondary antibodies, which were horseradish peroxidase (HRP)-linked goat anti-mouse IgG and goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). The Annexin V-conjugated AlexaFluor488 Apoptosis Detection Kit and JC-1 Mitochondrial Membrane Potential Detection Kit were purchased from Molecular Probes Inc. (Eugene, OR). The pan-caspase inhibitor (z-VAD-fmk) was purchased from R&D Systems, Inc. (Minneapolis, MN). The caspase 3 Colorimetric Assay Kit was purchased from BioSource International, Inc. (Camarillo, CA). The protein assay kit was purchased from Bio-Rad (Hercules, CA) and the enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from Amersham Biosciences, (Piscataway, NJ). DMEM, fetal bovine serum, penicillin, streptomycin and trypsin/EDTA were procured from Cellgro (Herndon, VA).

Cells, culture conditions and treatments

Human epidermoid carcinoma A431 cells were purchased from the American Type Culture Collection (Manassas, VA) and normal human epidermal keratinocytes were obtained from Cell Culture Core Facility of Skin Diseases Research Centre at the University of Alabama at Birmingham, AL. The A431 cells were cultured as monolayers in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 μ g/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The normal human epidermal keratinocytes were cultured in keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Gibco/Invitrogen, Carlsbad, CA) and maintained in an incubator under the conditions as described above. In all treatments, berberine was initially dissolved in a small amount of ethanol and made up to the maximum final concentration of 0.2% (v/v) in the complete cell culture medium. The sub-confluent cells (50–60%) were treated with either varying concentrations of berberine or vehicle alone (0.2% ethanol in media) that served as control.

MTT assay for cell viability/proliferation

The effect of berberine on the cell viability/proliferation was determined using MTT assay, as described previously (17). Briefly, 1×10^4 cells/well were plated in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of berberine (0, 5, 10, 15, 25, 50 or 75 μ M) for 24, 48 and 72 h. The cells were treated with 50 μ l of 5 mg/ml MTT and the resulting formazan crystals were dissolved in dimethylsulfoxide (200 μ l). Absorbance was recorded at 540 nm with a reference at 650 nm serving as the blank. The effect of berberine on cell viability was assessed as percent cell viability compared to vehicle-treated control cells, which were arbitrarily assigned 100% viability.

Cell death assay

The trypan blue dye exclusion assay was used to determine the cytotoxic effect of berberine on the cells. Briefly, 5×10^4 cells were cultured into each well of a 6-well culture plates. After overnight incubation, the cells were treated with varying concentrations of berberine (0, 15, 25, 50 and 75 μ M) for 24, 48 and 72 h. At the desired time points, the cells were harvested, treated with 0.25% trypan blue dye and the cells that had taken up the dye were counted under a microscope using a hemocytometer. The cytotoxic effects of berberine are expressed as the mean \pm SE percentage of dead cells in each treatment group from three repeated experiments.

DNA cell cycle analysis

Sub-confluent A431 cells (50–60%) were treated with varying concentrations of berberine in complete medium for 48 h. The cells were then harvested, washed with cold phosphate-buffered saline (PBS), and processed for cell cycle analysis. Briefly, the cells (1×10^5) were resuspended in 50 μ l cold PBS to which 450 μ l cold methanol was added and the cells were then incubated for 1 h at 4°C. The cells were centrifuged at 1100 r.p.m. for 5 min, the pellet was washed with cold PBS, re-suspended in 500 μ l PBS, and incubated with 5 μ l RNase (20 μ g/ml final concentration) for 30 min. The cells were incubated with propidium iodide (50 μ g/ml) on ice for 1 h in the dark. The cell cycle distribution of the cells was then determined using FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with

CellQuest 3.3 software in the Fluorescence-activated Cell Sorting (FACS) Core Facility of the Comprehensive Cancer Center of the University of Alabama at Birmingham.

Apoptotic cell death assay by FACS

Induction of apoptosis in A431 cells caused by berberine was quantitatively determined by flow cytometry using the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Detection Kit following the manufacturer's instructions and as previously described (17). Briefly, after overnight serum starvation, A431 cells were treated with berberine (0, 15, 25, 50 and 75 μ M) for 72 h. The cells were then harvested, washed with PBS and incubated with Alexa488 and propidium iodide (PI) for cellular staining at room temperature for 10 min in the dark. The stained cells were analyzed by FACS using FACSCalibur instrument equipped with CellQuest 3.3 software. The early apoptotic cells stained with Alexa488 give green fluorescence and represented in the lower right (LR) quadrant of the FACS histogram, and the late apoptotic cells stained with both Alexa488 and PI gives red-green fluorescence are represented in the upper right (UR) quadrant. In the experiment where pan-caspase inhibitor (z-VAD-fmk) was used, the cells were pretreated with the inhibitor for 2 h before the treatment of the berberine.

Immunoprecipitation and immunoblotting

For western blot analysis the cell lysates were prepared as described previously (17). The lysates were cleared by centrifugation at 14 000 *g* for 10 min, and the supernatant fraction was used for immunoblotting. For western blotting of cytochrome *c*, mitochondria free cytosolic fraction was prepared (18). The proteins (30–50 μ g) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat dry milk in blocking buffer [20 mM Tris-buffered saline (pH 7.5) containing 0.1% Tween-20] the membrane was incubated with the desired antibody for 2 h either at room temperature or at 4°C overnight. The membrane was then incubated with appropriate peroxidase-conjugated secondary antibody, and the protein expression was detected by ECL detection system (Amersham Biosciences, Piscataway, NJ) and autoradiography with HXR-film (Hawkins Film, Oneonta, AL). To ensure equal protein loading, each membrane was stripped and reprobed with anti- β -actin antibody.

For cyclin-dependent kinase (Cdk)-inhibitor (Cdk)-Cdk binding assay, A431 cells were treated with vehicle or 75 μ M berberine for 48 h, washed with ice-cold PBS, and whole cell lysates prepared as described previously (17). Aliquots containing 200 μ g of protein were cleared with protein A/G-plus agarose beads (Santa Cruz, CA). Cip1/p21 and Kip1/p27 proteins were immunoprecipitated from whole cell lysates using specific antibodies after incubation for 8 h followed by the addition of protein A/G-plus agarose beads (50 μ l, Santa Cruz, CA) and continued incubation overnight at 4°C. Immunoprecipitates were washed, and subsequently subjected to SDS-PAGE on 12% gels followed by immunoblotting using Cdk2, Cdk4 and Cdk6 antibodies.

Mitochondrial membrane potential assay

The loss of mitochondrial membrane potential ($\Delta\Psi$ m) was quantitatively determined by flow cytometry using the lipophilic cationic probe JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) Detection Kit, following the manufacturer's instructions. Briefly, 60% confluent A431 cells were treated with berberine (0, 25, 50 and 75 μ M) for 72 h, harvested, washed with PBS buffer and 1×10^6 cells were incubated in 1 ml PBS containing 10 μ g JC-1 dye for 15 min at 37°C in the dark. Stained cells were washed, resuspended in 500 μ l PBS and used for immediate FACS analysis.

Caspase 3 activity assay

The caspase 3 activity was assayed by colorimetric protease assay ApoTarget Kit (BioSource International, CA) following manufacturer's protocol. Briefly, the cells were treated with berberine (0, 15, 25, 50 and 75 μ M) for 72 h, harvested and cell lysates were prepared following the manufacturer's protocol. Samples of the cell lysates (100 μ g per sample) were mixed with 2 \times reaction buffer and the substrate, DEVD-pNA for caspase 3, and incubated at 37°C for 2 h in the dark. The absorbance was then measured at 405 nm in a microplate reader.

Statistical analysis

The statistical significance of difference in between control and treatment groups was determined by simple ANOVA followed by Bonferroni's multiple comparison tests. A *P*-value <0.05 was considered statistically significant.

Results

Berberine decreases cell viability and induces cytotoxic effect in human epidermoid carcinoma A431 cells but not in normal human epidermal keratinocytes

The chemical structure of berberine chloride is shown in Figure 1A which is an isoquinoline alkaloid in nature and

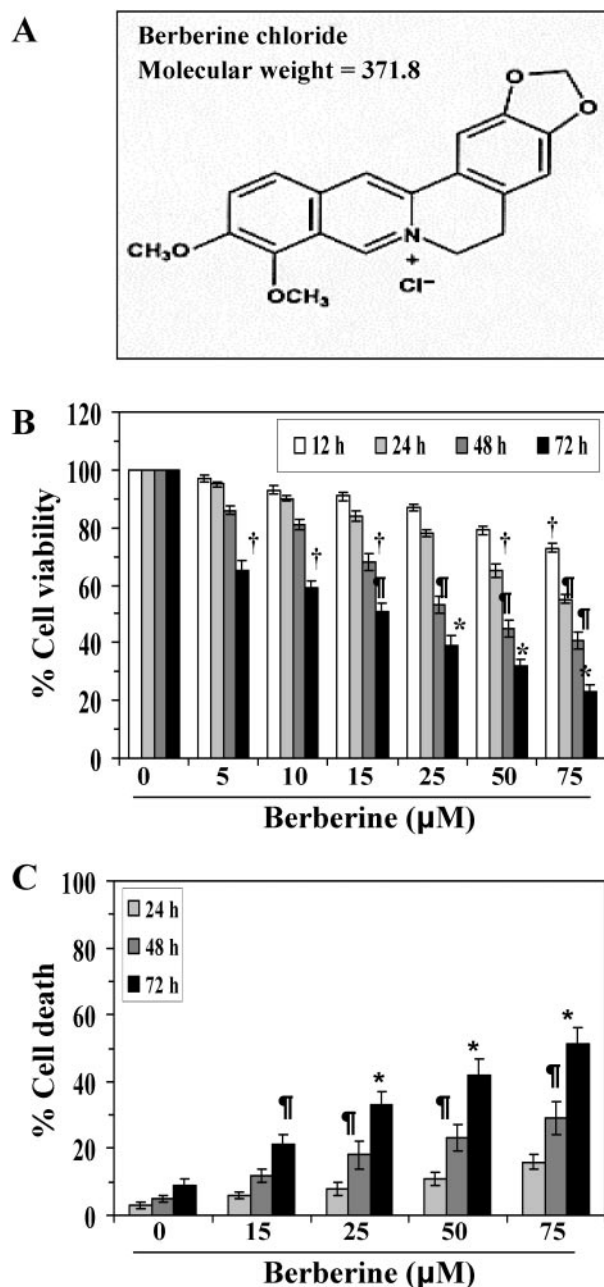


Fig. 1. Treatment of human epidermoid carcinoma A431 cells with berberine inhibits cell viability/proliferation and induces cell death in a dose- and time-dependent manner. (A), Chemical structure of berberine chloride. (B), Dose- and time-dependent effect of berberine on the cell viability/proliferation of A431 cells. Viability of the cells was determined using the MTT assay as described in the Materials and methods. The data are expressed in terms of percent of control cells (non-berberine treated) as the mean \pm SE of 10 replicates. (C), The cytotoxic effect of berberine on A431 cells was determined using the trypan blue dye exclusion assay as described in Materials and methods and is expressed in terms of percent of dead cells as mean \pm SE from three experiments. Significant difference versus control group, $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$; $^*P < 0.001$.

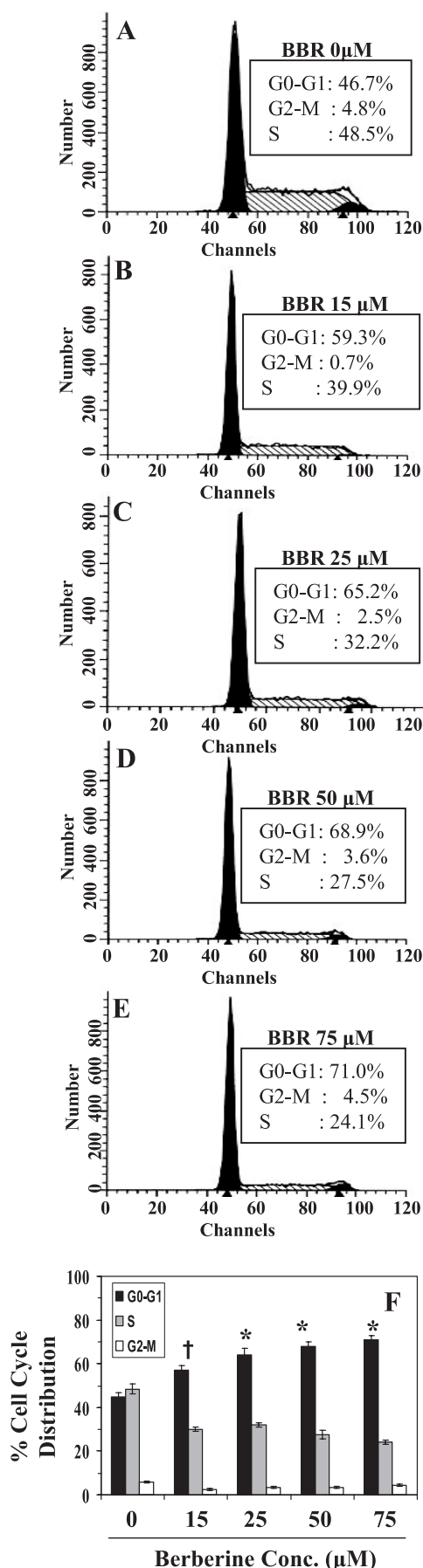
has molecular weight 371.8. As this is the first study to assess the effect of berberine on human skin cancer cells, first we determined the effect of berberine on the proliferation and cell viability of the A431 cells using MTT assay and the cytotoxic effect or cell death by using trypan blue exclusion assay. The cells were treated with varying concentrations of berberine (0, 5, 10, 15, 25, 50 and 75 μ M) for 12, 24, 48 and 72 h. The treatment of A431 cells with berberine resulted in a significant reduction in cell proliferation/viability as assessed by MTT assay. The dose-dependent reduction in viability of the cells ranged from 2 to 25% ($P < 0.05$) after 12 h, a 4–45% ($P < 0.01$) after 24 h, a 12–58% ($P < 0.05$ –0.01) after 48 h and a 35–78% ($P < 0.05$ –0.001) after 72 h of berberine treatment, as shown in Figure 1B. In additional experiments, we determined the cytotoxic effect of berberine using a trypan blue exclusion assay. Treatment of A431 cells with berberine at concentrations of 0, 15, 25, 50 and 75 μ M for 24, 48 and 72 h resulted in significant cell death (Figure 1C). As shown in Figure 1C, when compared with the control cells (non-berberine), treatment of A431 cells with berberine for 24, 48 and 72 h resulted in a 6 to 21% increase in cell death at 15 μ M, a 8–33% ($P < 0.01$ –0.001) increase in cell death at 25 μ M, a 11–42% ($P < 0.01$ –0.001) increase in cell death at 50 μ M, and a 16–51% ($P < 0.01$ –0.001) increase in cell death at 75 μ M. Further, we examined whether berberine has any cytotoxic effect on normal human epidermal keratinocytes. We did not find significant cell death in normal human epidermal keratinocytes after berberine treatment, however a marked effect on cell death (11–20%, $P < 0.07$) was observed only at the maximum concentration of berberine (75 μ M) after 48 and 72 h of treatment (data not shown), which is significantly less than the effects of the same dose of berberine on A431 cells at the same time points. Thus berberine appears to be capable of inducing cytotoxic effects on human epidermoid carcinoma cells without incurring significant cytotoxic effect on normal human epidermal keratinocytes.

Berberine induces G₁ phase cell cycle arrest in A431 cells

Based on the preliminary assays where we observed a strong growth inhibitory effect of berberine in A431 cells, we then determined the possible mechanism of anti-proliferative activity of berberine. For this purpose the effect of berberine on cell cycle progression in A431 cells was determined following 15, 25, 50 and 75 μ M doses of berberine treatment for 48 h. As summarized in Figure 2, (Panels A–E), treatment of A431 cells with berberine resulted in a significant higher number of cells in the G₁ phase at all the concentrations used: 15 μ M (59.3%, $P < 0.05$), 25 μ M (65.2%, $P < 0.001$), 50 μ M (68.9%, $P < 0.001$) and 75 μ M (71.0%, $P < 0.001$) compared to the non-berberine treated control (46.7%). As indicated in Figure 2 (Panels A–E), the dose-dependent effect of berberine on G₁ arrest in A431 cells was largely at the expense of S phase cells with a minimal change in G₂-M cell population compared with the non-berberine treated control cells (Panel A). The results of cell cycle distribution at each dose of berberine are also summarized in Panel F, which indicated the significant arrest of A431 cells in G₀/G₁ phase after berberine treatment.

Berberine increases the expression of Cdk1 (Cip1/p21 and Kip1/p27) in A431 cells

As the treatment of A431 cells with berberine induces G₁ arrest, we next assessed the effect of berberine on cell cycle



regulatory molecules involved in G₁ phase of the cell cycle progression. The Cdkis regulate the progression of cells in the G₀/G₁ phase of the cell cycle and induction of Cip1/p21 and Kip1/p27 causes a blockade of the G₁ to S transition, thereby resulting in a G₀/G₁ phase arrest of the cell cycle (19). The loss of Cdkis in human cancers leads to uncontrolled cell proliferation (20). In this context, our results revealed that treatment of human epidermoid carcinoma A431 cells with varying concentrations of berberine (0, 15, 25, 50 and 75 μM) for 48 h resulted in a dose-dependent increase in protein expression of Kip1/p27 and Cip1/p21 (Figure 3, Panel A). The berberine-induced increase in inhibitory proteins (Cip1/p21 and Kip1/p27) may have a role in a blockade of A431 cells in G₁ phase. These changes in protein expression were not due to differences in the amount of proteins loaded on the gels as the equivalent protein loading was confirmed by probing stripped blots for β-actin as shown (Panel A).

Berberine decreases the protein levels of G₁ regulatory Cdkis and cyclins in A431 cells

As studies have shown that Cdkis and cyclins play crucial roles in the regulation of cell cycle progression (21), we determined the effect of berberine on the protein levels of the Cdkis and cyclins which are negatively regulated by Cdkis (Cip1/p21 and Kip1/p27) during G₁ cell cycle progression. As shown in Figure 3 (Panel B), treatment of A431 cells with berberine resulted in a marked decrease in the expression of Cdk2, Cdk4 and Cdk6 in a dose-dependent manner at 48 h after berberine treatment. A strong reduction in Cdkis was observed at the doses of 50 and 75 μM of berberine. Moreover, the reduction in the expression of Cdk6 being more pronounced than the reduction of either Cdk2 or Cdk4. Similarly, a marked reduction in the expression of cyclins D1, D2 and E was observed in a dose-dependent manner at 48 h of treatment (Figure 3, Panel C). Equal loading of proteins was confirmed by reprobing the stripped blots for β-actin.

Berberine increases Cdk-Cdkis binding in A431 cells

Based on our observations that berberine induces the expression of Cdkis in A431 cells, and since the induction of the Cdkis has been shown to result in an increased interaction with Cdkis leading to a decrease in their kinase activity (22), we next assessed whether berberine promotes the interaction between Cdkis and Cdk. To assess this effect, Cip1/p21 and Kip1/p27 were immunoprecipitated from total cell lysates and their binding with Cdk2, Cdk4 and Cdk6 was assessed using western blot analysis. As compared to vehicle treated controls, treatment with berberine was found to enhance the binding of Cdk2, Cdk4 and Cdk6 with Cip1/p21 and Kip1/p27 (Figure 3, Panel D). These results suggest that an increased interaction between Cdkis with Cdkis plays an important

Fig. 2. Effect of berberine on cell cycle progression of human epidermoid carcinoma A431 cells. A431 cells were treated either with vehicle (0.2% ethanol in medium) or 15, 25, 50 and 75 μM doses of berberine in complete medium. After 48 h of treatment, cells were harvested and digested with RNase. Cellular DNA was stained with propidium iodide and flow cytometric analysis was performed to analyze the cell cycle distribution, as detailed in the Materials and methods. Panels A–E, Cell cycle distribution in A431 cells with the treatment of various concentrations of berberine (0, 15, 25, 50 and 75 μM). Panel F, Data from the cell cycle distribution were summarized and presented as the mean ± SE of three independent experiments. †*P* < 0.05 and **P* < 0.001 versus non-berberine treated control group. BBR, berberine.

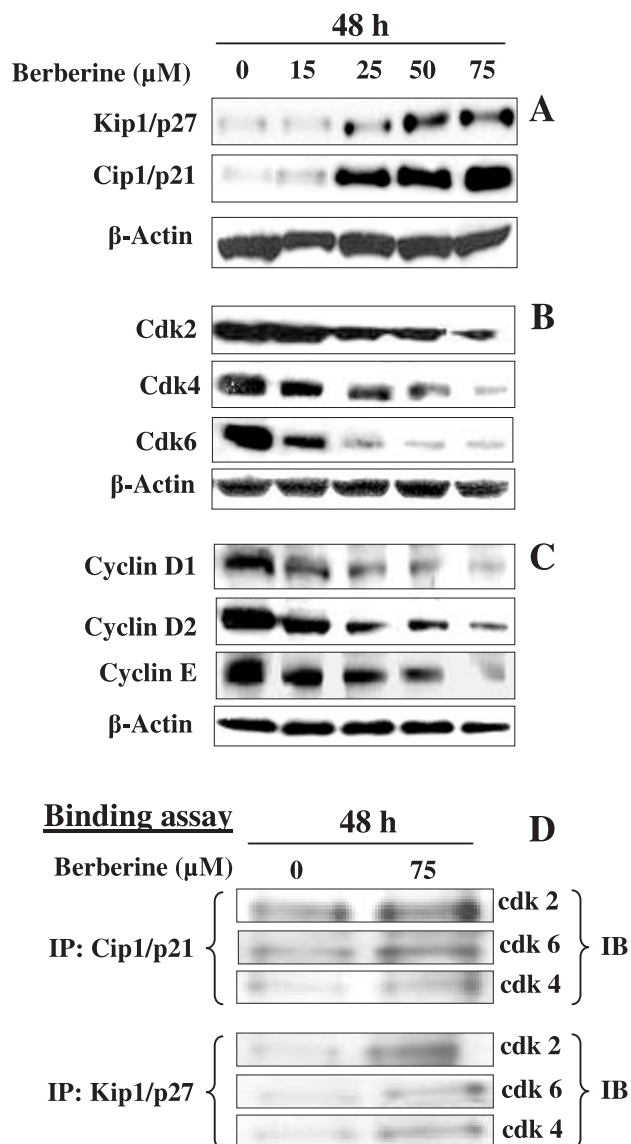


Fig. 3. Effect of berberine on G₁ phase cell cycle regulatory proteins in A431 cells. The A431 cells were treated with either vehicle (0.2% ethanol in medium) or berberine (15, 25, 50 and 75 μM) for 48 h and thereafter harvested, cell lysates prepared and then subjected to SDS-PAGE followed by Western blot analysis, as described in Materials and methods. Effect of berberine was determined on the following: (A), the expression of Kip1/p27 and Cip1/p21; (B), the expression of Cdk 2, Cdk 4 and Cdk 6; (C), the expression of cyclin D1, cyclin D2 and cyclin E. β-actin was used to verify equal loading of the samples. Representative blots are shown from three independent experiments with almost identical results. (D) In binding assay, Cip1/p21 and Kip1/p27 were immunoprecipitated using specific antibody from total protein lysates followed by SDS-PAGE and western blot analysis for Cdk2, Cdk4 and Cdk6 as detailed in Materials and methods. IP, immunoprecipitation; IB, immunoblotting.

regulatory role in the berberine-induced G₁ arrest of cell cycle progression in human epidermoid carcinoma A431 cells, possibly through their inhibition of Cdk kinase activity.

Berberine induces apoptosis in A431 cells

Further, to determine whether the loss of viability and induction of G₁ arrest in human epidermoid carcinoma A431 cells by berberine was due to the induction of apoptosis, we next treated the cells with varying concentrations of berberine and the percentage of apoptotic cells were assessed using the

Annexin V-conjugated Alexafluor488 Apoptotic Detection Kit, as described previously (17). Apoptotic cells were measured as late or early apoptotic cells, which are shown respectively in the upper right (UR) or lower right (LR) quadrants of the FACS histogram in Figure 4. Treatment of A431 cells with berberine did not induce significant apoptosis at 24 h after berberine treatment (data not shown), however, treatment of A431 cells with berberine induced significant dose-dependent induction of apoptosis at both the early and late stages of apoptosis after 72 h of berberine treatment. (Figure 4, Panels A–E). The percentages of total apoptotic cells after berberine treatments were as follows: 11.7% (0 μM, vehicle treated control), 31 (15 μM, $P < 0.05$), 39 (25 μM, $P < 0.01$), 47 (50 μM, $P < 0.001$) and 60% (75 μM, $P < 0.001$), as summarized in Panel F. These data suggest that induction of apoptosis in A431 cells after berberine treatment could be a major mechanism of berberine-caused inhibition of cell viability and/or cell death of A431 cells (Figure 1).

Berberine decreases the expression of anti-apoptotic proteins Bcl-xl and Bcl-2 while increases the expression of pro-apoptotic protein Bax in A431 cells

We next determined the possible mechanism of berberine-induced apoptosis in A431 cells. As the berberine-induced maximum cell death and apoptosis were found at 72 h after its treatment, we selected this time point for further mechanistic studies. It has been recognized that the proteins of Bcl-2 family play crucial roles in regulation of apoptosis by functioning as promoters (e.g. Bax) or inhibitors (Bcl-2 or Bcl-xl) of cell death (23–26). We therefore reasoned to determine the effect of berberine on the expression of these proteins. Using western blot analysis, we found that treatment of A431 cells with berberine resulted in a dose-dependent reduction in the levels of the anti-apoptotic proteins Bcl-xl and Bcl-2. At the same time, the level of proapoptotic protein Bax was correspondingly increased with the increasing doses of berberine under identical conditions compared with the cells that were not treated with berberine (Figure 5). Stripping and re-probing of the blots for β-actin expression confirmed equal sample loading in all the blots. Thus, these data indicated that berberine has the ability to alter the levels of pro- and anti-apoptotic proteins of Bcl-2 family in a manner that contribute to the susceptibility of A431 cells to berberine-induced apoptosis (Figure 5).

Berberine induces loss of mitochondrial membrane potential in A431 cells and subsequently enhances the release of cytochrome c

One of the mechanisms of induction of apoptosis is the loss of mitochondrial membrane potential in the cells. Loss of mitochondrial membrane potential has been linked to the initiation and activation of apoptotic cascades (27). This event is induced by variety of stimuli including the translocation of Bax from the cytosol to the mitochondria, which triggers the release of cytochrome c from the mitochondria to the cytosol (28,29). In turn, this contributes to the activation of caspases and subsequent apoptotic cell death. Therefore, first we determined whether berberine induces the loss or disruption of mitochondrial membrane potential in A431 cells. For this purpose, the A431 cells were leveled with the cationic lipophilic dye JC-1, which accumulates within mitochondria in a potential-dependent manner. On disruption of the mitochondrial membrane potential, the fluorescence emission of JC-1

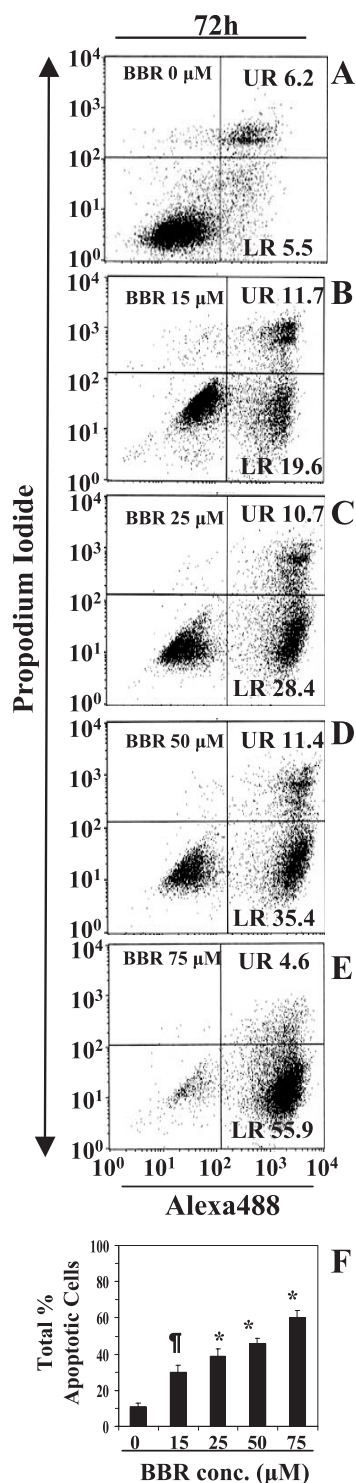


Fig. 4. Berberine induces apoptosis in human epidermoid carcinoma A431 cells in a dose-dependent manner. (A–E), A431 cells were treated with varying concentrations of berberine (0, 15, 25, 50 and 75 μM) in complete medium for 72 h then harvested for the analysis of apoptotic cells by FACS using the Annexin V-Alexa Fluor488 Apoptosis Vybrant Assay Kit following the manufacturer's protocol. The lower right (LR) quadrant of the FACS histograms indicates the percentage of early apoptotic cells (Alexa488-stained cells) and the upper right (UR) quadrant indicates the percentage of late apoptotic cells (Alexa488+propidium iodide-stained cells). (F), Total percent of apoptotic cells in each treatment group was summarized after 72 h of berberine treatment to A431 cells, and data are presented as mean ± SE of three experiments. BBR, berberine. Significant difference versus control group (non-berberine-treated), * $P < 0.001$; $^{\dagger}P < 0.05$.

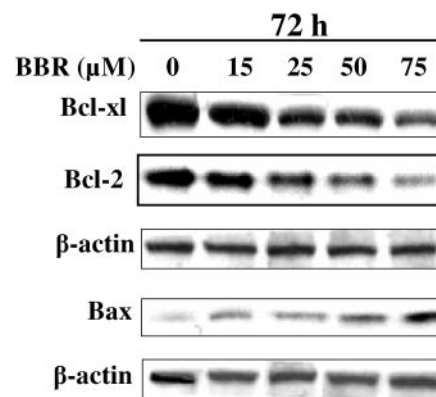


Fig. 5. Treatment of A431 cells with varying concentrations of berberine (0, 15, 25, 50 and 75 μM) for 72 h results in a dose-dependent reduction in the expression of the anti-apoptotic proteins Bcl-xl and Bcl-2, while increasing the expression of the pro-apoptotic protein Bax. After treatments for 72 h, cells were harvested, cell lysates prepared and subjected to western blot analysis to determine the expression of different proteins. β-actin was used to verify equal loading of the samples. BBR, berberine.

dye changes from orange to green. A431 cells were treated with varying concentrations of berberine (0, 25, 50 and 75 μM) for 72 h, cells were harvested, stained with JC-1 dye and analyzed using flow cytometry. As shown in Figure 6 (Panel A), treatment of A431 cells with berberine resulted in a dose-dependent increase in the number of green-fluorescence⁺ cells from 17.0% in non-berberine treated cells to 24.4, 33.9 and 46.3%, respectively by 25, 50 and 75 μM concentration of berberine treatment. These data suggest that berberine treatment to A431 cells resulted in disruption of mitochondrial membrane potential. Western blot analysis of the cytosolic fractions of the cells lysates revealed that berberine caused a dose-dependent increase in the release of cytochrome *c* to the cytoplasm thus confirming the role of berberine in the disruption of mitochondrial membrane potential. Further, to verify whether disruption of mitochondrial membrane potential by berberine results in release of cytochrome *c* from mitochondria to cytosols, the cytosolic fractions were prepared from A431 cells that had been treated with berberine (0, 25, 50 and 75 μM) for 72 h. Western blot analysis revealed that berberine resulted in a dose-dependent release of cytochrome *c* into cytosol (Figure 6B), thus confirming the disruption of the mitochondrial membrane potential on berberine treatment.

Berberine induces the activation or cleavage of caspases and poly (ADP-ribose) polymerase (PARP) in A431 cells

In a sequential manner, once the cytochrome *c* releases into cytosol, it recruits and activates procaspase 9 in the apoptosome and leads to active caspase 9 cleavage and activation and cleavage of caspase 3 (18,30–32). Activation of caspase 3 subsequently leads to apoptotic cell death through cleavage of a broad spectrum of cellular target protein, including PARP. For these reasons, we determined whether induction of apoptosis in A431 cells by berberine is mediated through the activation of procaspase 9, caspase 3 and PARP proteins. Treatment of A431 cells with berberine (0, 15, 25, 50 and 75 μM) for 72 h resulted in a dose-dependent decrease in caspase 9 while increases were observed in the cleavage of caspases 9, 3 and PARP when compared with the cells which were not treated with berberine (Figure 7A). The membranes were

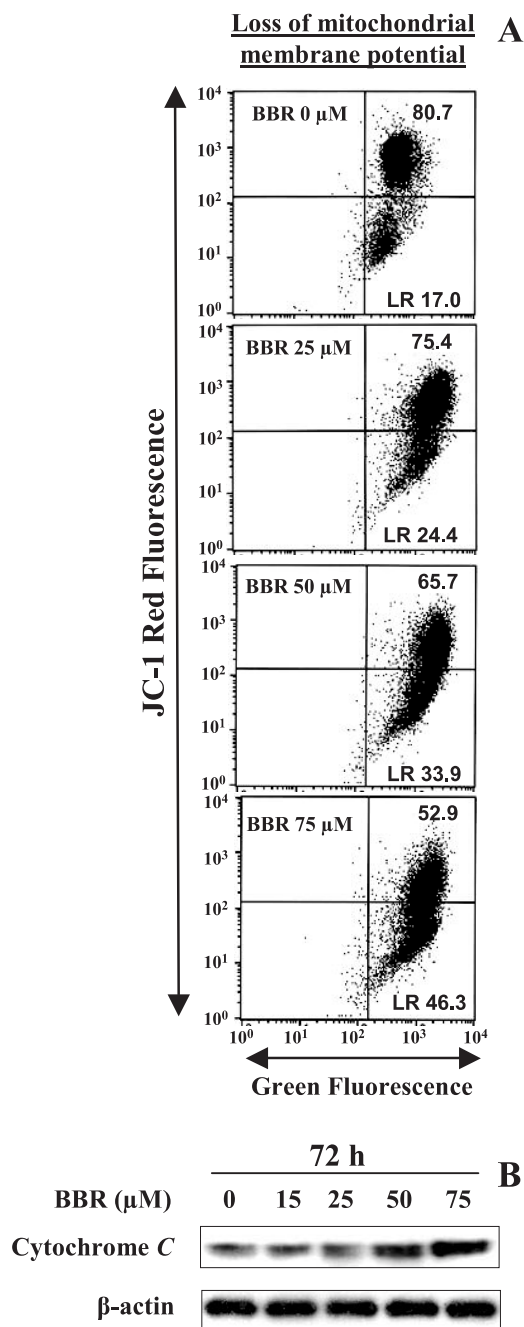


Fig. 6. Treatment of A431 cells with varying concentrations of berberine induces the loss of mitochondrial membrane potential and subsequently release of cytochrome *c*. (A), Treatment of A431 cells with berberine induces loss of mitochondrial membrane potential in a dose-dependent manner. Cells were treated with berberine (0, 25, 50 and 75 μM) for 72 h, harvested and stained with JC-1 dye probe and analyzed by flow cytometry, as described in Materials and methods. The percentage of the cells that emit only green fluorescence indicates the depolarized mitochondrial membrane, as shown in the lower right (LR) quadrant of the FACS histogram. (B), Cytosolic fractions were also prepared from the above mentioned treatment groups and subjected to western blot analysis to detect the levels of the cytochrome *c*. Representative blot is shown from three independent experiments with similar results. β-actin was used to verify equal loading of the protein samples on the gel. BBR, berberine.

also checked for equal sample loading using β-actin as a control. The berberine-induced activation of caspase 3 in A431 cells was further checked using a colorimetric caspase 3 activity assay in a similar system. Treatment of A431 cells with

berberine for 72 h resulted in a significant increase in caspase 3 activity in a dose-dependent manner than vehicle-treated A431 cells (Figure 7B), thus confirming the involvement of caspase 3 activation in apoptotic cell death of A431 cells.

Pan-caspase inhibitor (z-vad-fmk)-induced inhibition of caspases blocked berberine-induced apoptosis in A431 cells

As we observed that treatment of berberine to A431 cells increased the activation or cleavage of caspase 3, and this may involve in berberine-induced apoptosis in A431 cells, we sought to determine whether the induction of apoptosis by berberine is reduced or blocked by pan-caspase inhibitor (z-VAD-fmk) in this system. A431 cells that had been treated with berberine (50 μM), with or without z-VAD-fmk (60 μmol/l) for 72 h, were stained using the Annexin V-conjugated Alexafluor488 Apoptosis Detection Kit, as detailed previously (17), and number of apoptotic cells were analyzed using flow cytometry. As shown in Figure 7C and D, treatment of A431 cells with berberine resulted in 44.2% apoptosis compared to only 12% in non-berberine treated control cells, whereas treatment of A431 cells with berberine in the presence of pan-caspase inhibitor (z-VAD-fmk) resulted in only 16.6% apoptosis, thus the presence of pan-caspase inhibitor significantly blocked berberine-induced apoptosis (87% reduction, $P < 0.001$) in these cells. These results indicate that berberine-induced apoptosis in A431 cells is mediated primarily through the activation of caspases.

Discussion

In the present investigation, we show that berberine, which is present abundantly in *Berberis* plant species, significantly inhibits the viability, proliferation and induces cell death in human epidermoid carcinoma A431 cells (Figure 1), but this effect was not found in normal human epidermal keratinocytes under the identical conditions, except for a non-significant reduction in cell viability at higher concentrations of berberine (50 and 75 μM) and treatment of cells for a longer period of time (72 h). These data suggested that berberine may be examined as an effective chemotherapeutic agent against non-melanoma skin cancers. Further studies were performed to elucidate the mechanism of reduction in cell viability and induction of cell death in A431 cells by berberine. It has been recognized that control of cell cycle progression in cancer cells is an effective strategy to halt tumor growth (19,33) as the molecular analyses of human cancers have revealed that cell cycle regulators are frequently deregulated in most of the common malignancies (34,35). Our *in vitro* data demonstrate that treatment of A431 cells with berberine induces G₁ phase arrest of cell cycle progression indicating that one of the mechanisms by which berberine inhibits the proliferation of A431 cells is inhibition of cell cycle progression. Further molecular mechanistic studies demonstrate a marked decrease in the expressions of cyclins D1, D2 and E and Cdk2, Cdk4 and Cdk6 in A431 cells dose-dependently on berberine treatment suggests the disruption of the uncontrolled cell cycle progression of human epidermoid carcinoma cells (Figure 3) and that the berberine-induced G₁ arrest is mediated through the upregulation of cyclin-dependent kinase inhibitory proteins (Cip1/p21 and Kip1/p27) which enhances the formation of heterotrimeric complexes with the G₁/S Cdk and cyclins thereby resulting in inhibition of their activity (Figure 3, panel D).

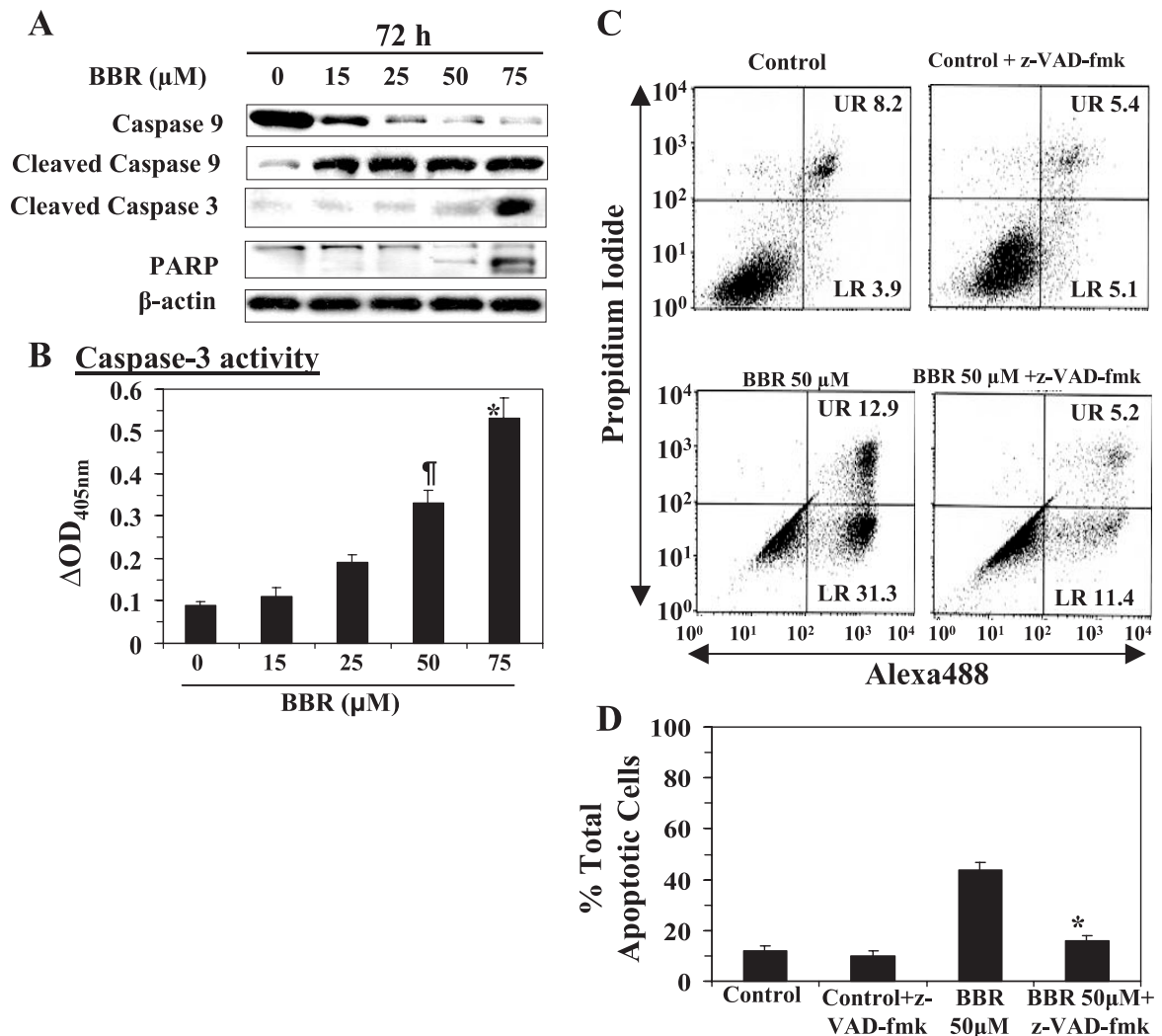


Fig. 7. Treatment of A431 cells with berberine reduces the level of caspase 9 while increases the activation/cleavage of caspases 9, 3 and PARP in a dose-dependent manner. (A), A431 cells were treated with varying concentrations of berberine (0, 15, 25, 50 and 75 μM) for 72 h, thereafter cells were harvested, cell lysates prepared and subjected to western blot analysis to detect the levels of caspases 9, cleaved caspase 9 and 3 and PARP. Equal protein loading was confirmed by probing stripped blots for β-actin as shown, and a representative blot is shown from three independent experiments with similar results. (B), The caspase 3 activity in cell lysates from the samples of Panel A was measured using a colorimetric protease assay (ApoTarget Kit). Berberine treatment to A431 cells increases the activity of caspase 3 dose-dependently. Significant difference versus non-berberine treatment group, $^{\#}P < 0.005$ and $*P < 0.001$. (C), The effect of berberine (50 μM) on apoptosis of A431 cells was determined after 72 h in the absence or presence of 60 μmol/L of the pan-caspase inhibitor (z-VAD-fmk). Percent apoptotic cells were determined by flow cytometry using Alexa488 Apoptosis Vybrant Assay Kit, as detailed in Materials and methods. The cells treated with z-VAD-fmk blocked the berberine-induced apoptosis in A431 cells. Lower right (LR) quadrant of the FACS histogram indicates the percentage of early apoptotic cells and the upper right (UR) quadrant indicates the percentage of late apoptotic cells. (D), The percentage of total apoptotic A431 cells (early + late) in different treatment groups was summarized and data are presented as mean \pm SD from three repeated experiments. Significant inhibition versus BBR alone treated cells, $*P < 0.001$. BBR, berberine.

Uncontrolled cell division depends on the activation of cyclins, which bind to Cdk to induce cell cycle progression towards S phase. Cdk kinase activity is one of the major causes of cancer progression, and their functions are tightly regulated by Cdkis, such as, Cip1/p21 and Kip1/p27 proteins. Cip1/p21 is a universal inhibitor of Cdk(s) and Kip1/p27 is commonly upregulated in response to antiproliferative signals (36). The increased expression of G₁ cyclins in cancer cells provides them an uncontrolled growth advantage because most of these cells either lack Cdkis, harbor non-functional Cdkis, or Cdkis expression is not at a sufficient level to control Cdk-cyclin activity (35,37). Consistent with these reports, the increased expressions of Cdkis together with decreased expression of cyclins and Cdkis on the berberine

treatment to A431 cells suggest that berberine might be effective as a chemotherapeutic agent for the prevention of skin cancers.

The growth arrest of cancer cells in G₁ phase provides an opportunity for cells to either undergo repair mechanisms or undergo apoptosis. In most of the advanced malignancies, cancer cells become resistant to apoptosis and/or do not respond to the cytotoxic effects of chemotherapeutic agents (35,38). We therefore examined the effect of berberine on the induction of apoptosis in A431 cells. Our flow cytometry data indicated that treatment of A431 cells with berberine resulted in a significant dose-dependent induction of apoptosis (Figure 4). Apoptosis has been characterized as a fundamental cellular activity, and plays a

crucial role as a protective mechanism against neoplastic development by eliminating genetically altered cells or those cells that have been improperly stimulated for hyperproliferation. Therefore, apoptosis is considered as a protective mechanism against the development of cancer (39). Acquired resistance towards apoptosis is considered as a hallmark of most of the cancers. Therefore, berberine seems to be a potent chemotherapeutic agent against non-melanoma skin cancers.

The key biochemical events involved in the induction of apoptosis are the upregulation of pro-apoptotic proteins and/or downregulation of anti-apoptotic protein molecules. The major apoptotic signal transduction cascade associated with programmed cell death includes the proteins of Bcl-2 family (40). The proteins of Bcl-2 family either promote cell survival (e.g. Bcl-2 and Bcl-xL) or induce apoptosis (e.g. Bax) (26,40,41). Increase in the levels of Bax and/or decrease in Bcl-2 leads to loss of mitochondrial membrane potential which is a key event in the induction of apoptosis, and involves a reduction in ATP levels, influx of ions that leads to a decrease in mitochondrial membrane potential and opening of mitochondrial permeability transition pores (41). Loss of mitochondrial membrane potential is suicidal to cells as they become bioenergetically deficient (40) and that leads to release of cytochrome *c* into the cytosol. As the level of cytochrome *c* increases in cytosol, it interacts with Apaf-1 and ATP forms a complex with pro-caspase 9, leading to activation of caspases 9 and 3 which leads to the cleavage of PARP (32,42). Consistent with these reports, in the present study, we found that treatment of A431 cells with berberine resulted in a dose-dependent decrease in the levels of anti-apoptotic proteins (Bcl-xL and Bcl-2) and simultaneously increase in proapoptotic protein Bax (Figure 5). This alteration may be responsible for the concomitant execution phase of apoptosis which included the disruption of mitochondrial membrane potential and increased release of cytochrome *c* to cytosol (Figure 6). Further, this effect of berberine leads to the dose-dependent activation or cleavage of caspases 9, 3 and PARP (Figure 7A). These data suggest that disruption in mitochondrial membrane potential and the activation of caspases on berberine treatment could be a possible mechanism of berberine-caused apoptosis in A431 cells. The involvement of a berberine-induced activation of caspase 3 and its effect on apoptosis was further confirmed using the pan-caspase inhibitor (z-VAD-fmk) in our system. The berberine-induced caspases in A431 cells was blocked by the use of cell permeable pan-caspase inhibitor (z-VAD-fmk) which resulted in a significant decrease in berberine-induced apoptosis (Figure 7C and D), confirmed the role of caspase 3 activation in the berberine-induced apoptosis.

In conclusion, our study indicates that berberine inhibits growth, induces G₁ arrest and apoptotic cell death of human epidermoid carcinoma A431 cells. We also provide mechanistic evidences that berberine-induced apoptosis in human epidermoid carcinoma cells is mediated through disruption of mitochondrial membrane potential and activation of caspase 3 pathway, although other pathways may have a role and that require further investigation. Moreover, further *in vivo* studies are required to determine whether berberine could be an effective chemotherapeutic agent for the prevention of non-melanoma skin cancers.

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