

Iberin induces cell cycle arrest and apoptosis in human neuroblastoma cells

UNMESH JADHAV¹, RAVESANKER EZHILARASAN¹, STEVEN F. VAUGHN²,
MARK A. BERHOW² and SANJEEVA MOHANAM¹

¹Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine, Peoria, IL 61605;

²National Center for Agricultural Utilization Research, USDA, Peoria, IL 61604, USA

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Abstract. Epidemiological studies have indicated that increased consumption of cruciferous vegetables is associated with a statistically significant reduction in the risk for cancers. The major bioactive agent in these vegetables is a class of sulfur-containing glycosides called glucosinolates. Isothiocyanates, derivatives of glucosinolates, have been shown to possess anticancer properties in a variety of tumor cell lines. In this study, we evaluated the antiproliferative, cell cycle modulation and proapoptotic effects of isothiocyanate iberin in human neuroblastoma cells. Treatment of neuroblastoma cells with iberin resulted in a dose- and time-dependent inhibition of growth, increased cytotoxicity, and G₁ or G₂ cell cycle arrest depending upon cell type. The iberin-induced cell cycle arrest in neuroblastoma cells was associated with inhibition of expression of Cdk2, Cdk4, and Cdk6 proteins. Fluorescence microscopic analysis of DNA-staining patterns with DAPI revealed an increase in apoptotic cell death in iberin-treated cells as compared with control cells. FLICA staining showed that iberin induced apoptosis, and this apoptotic induction was found to be associated with the activation of caspase-9, caspase-3, and PARP. These findings suggest that the anticancer efficacy of iberin is mediated via induction of cell cycle arrest and apoptosis in human neuroblastoma cells and has strong potential for development as a therapeutic agent against cancer.

Introduction

The increased incidence of cancer in the general population has led investigators to search for compounds having an efficient suppressive effect against cancer. Considerable epidemiological evidence shows that diets high in vegetable and fiber lead to a low cancer risk and confer protection from various forms of cancer (1-3). Consumption of cruciferous vegetables especially the genus *Brassica* (broccoli, cabbage, brussels sprouts, kale, cauliflower, etc.) has been reported to reduce the risk of human cancer (3-6). Cruciferous vegetables contain a group of sulfur-containing secondary metabolites termed glucosinolates, and the chemopreventive benefit of these vegetables is attributed to their relatively high glucosinolate content (7-9).

Isothiocyanates are hydrolysates derived from glucosinolates and have recently been the focus of intense interest for their anticarcinogenic activities and potential use in the chemoprevention of cancer. Several mechanisms for the activities of isothiocyanates in cancer chemoprevention have been proposed including inhibition of phase I carcinogen activating enzymes, induction of phase II carcinogen detoxification enzymes and induction of cell cycle arrest and apoptosis (10-13). Previous studies indicate that natural isothiocyanates such as sulforaphane and phenethyl isothiocyanate possess strong antitumor activities *in vitro* and *in vivo* (14-19). Iberin, a sulfoxide analogue of sulforaphane, is a naturally occurring member of the isothiocyanate family of cancer chemopreventive agents. There are few studies on iberin in comparison to those on sulforaphane. Iberin increased glutathione S-transferase and quinone reductase activities in the urinary bladder of rats demonstrating protective effects against chemical carcinogenesis (20). Iberin also upregulated thioredoxin reductase 1 expression in human MCF cells suggesting a role in the maintenance of redox in cell homeostasis (21). However, the anticancer effects of iberin on tumor cells have not been investigated in detail.

Neuroblastoma is an aggressive childhood cancer of the peripheral nervous system arising from neural crest sympathoadrenal progenitor cells (22). Despite recent advances in combination therapy, prognosis for patients with stage IV neuroblastoma is poor (23) and so there remains a need for more effective, less cytotoxic treatments. Therefore, developing an effective treatment strategy is important. Isothiocyanates

Correspondence to: Dr Sanjeeva Mohanam, Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine, Peoria, IL 61605, USA
E-mail: smohanam@uic.edu

Abbreviations: CDK, cyclin-dependent kinase; DAPI, 4',6-diamino-2-phenylindole; FLICA, fluorochrome labeled inhibitor of caspases; LDH, lactate dehydrogenase; MTT, 3-(4,5 dimethyl-2 thiazolyl)-2,5 diphenyl-2H tetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide

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demonstrate antitumor properties in adult cancer models and negligible toxicity in normal cells, but little is known about the effect of these agents on pediatric cancers. We investigated the effects of iberin on the proliferation, apoptosis, and cell cycle alterations of human neuroblastoma cells.

Materials and methods

Reagents. Iberin was isolated from *Lesquerella fendleri* seedmeal as described previously (24). DMSO, 4',6-diamino-2-phenylindole (DAPI), phenylmethylsulfonyl-fluoride (PMSF), propidium iodide (PI) and 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. CytoTox 96 non-radioactive cytotoxicity assay kit was purchased from Promega. Fluorochrome labeled inhibitor of caspases (FLICA) was from Immunochemistry Technologies. Cdk2, Cdk4, and Cdk6 antibodies were from Biomeda. Antibodies for caspase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling. Anti- β -actin antibody was obtained from Abcam. Reagents for electrophoresis and Western blotting were obtained from Fisher and Amersham Bioscience, respectively.

Cell culture. The human neuroblastoma SK-N-AS, SK-N-SH and SK-N-BE(2) cell lines were obtained from American Type Culture Collection (Rockville, MD) and were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml) and maintained at 37°C in a 95% air/5% CO₂ humidified incubator. SK-N-BE(2) has a MYCN amplification and non-functional mutant p53 whereas SK-N-SH expresses wild-type p53 with a low MYCN copy number. Neuroblastoma cells were treated with iberin at indicated concentrations or with an equal volume of DMSO (final concentration <0.1%).

Cell proliferation assay. Cells were plated at a density of 1×10^5 cells/well in microtiter plates and treated with different concentrations of iberin for indicated time periods as mentioned. Then 20 μ l of 5 mg/ml MTT in PBS, was added to each well and allowed to incubate for 4 h. After incubation, 100 μ l of DMSO was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader. The results were presented as the percentage of cells treated with vehicle DMSO.

Cytotoxicity assay. To assess cell cytotoxicity, LDH leakage was determined in the extracellular cell-culture medium. Cells were plated in microtiter plates and were treated with different concentrations of iberin for specified time intervals. The cell-free supernatant was obtained by centrifugation (400 x g) for 10 min and was used to determine the activity of LDH leaked through cell membranes. LDH activity was measured using the CytoTox 96 non-radioactive cytotoxicity assay kit according to the manufacturer's recommended protocol.

DNA cell cycle analysis. Cells treated with or without iberin were harvested, washed once with PBS, and fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature. Cells were pelleted, washed once with PBS, and re-suspended

in a PI solution (50 μ g/ml PI, Sigma; 0.1 mg/ml RNase A in PBS, pH 7.4) for 30 min in the dark. Flow cytometry analysis was performed (25) using FASCalibur flow cytometry system (Becton Dickinson, San Jose, CA). Forward light scatter characteristics were used to exclude the cell debris from the analysis. The percentages of cells within the G₁, S, and G₂/M phases of the cell cycle were determined by analysis with the program CellQuest.

Apoptosis assay. In the initial phase of apoptosis, the caspases become activated and the FLICA binds to these activated caspases. Human neuroblastoma cells were treated with different concentrations of iberin for 24 h. After 24 h the cells were stained with FLICA following the manufacturer's instructions.

Nuclear staining with DAPI. Cells were treated with or without iberin, washed twice with PBS and then fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with DAPI solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed via a fluorescence microscope.

SDS-PAGE and Western blot analysis. Cells were treated with different concentrations of iberin for 24 h. The controls were treated with DMSO. For Western blot analysis, cells were extracted in a buffer solution containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium fluoride, 1 mM PMSF and 10 μ g/ml aprotinin on ice for 20 min. Samples were subjected to SDS-PAGE and separated proteins were transferred onto membranes followed by the blocking of membranes with 5% nonfat milk powder (w/v) in Tris-buffered saline (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 1 h at room temperature or overnight at 4°C. Membranes were probed for the protein levels of Cdk2, Cdk4, and Cdk6 using specific primary antibodies followed by the appropriate peroxidase-conjugated secondary antibody, and visualized by an enhanced chemiluminescence detection system. Similarly, for apoptotic molecules, caspase-9, caspase-3, and PARP were probed using their specific primary antibodies followed by appropriate secondary antibody and enhanced chemiluminescence visualization. Membranes were stripped and re-probed with β -actin antibody as a protein loading control.

Statistical analysis. Statistical significance of the experimental results was determined by the Student's t-test. For all analyses $p < 0.05$ was accepted as a significant probability level.

Results

Iberin inhibits growth of human neuroblastoma cells. Our aim was to investigate whether iberin treatment imparts an antiproliferative effect against neuroblastoma cells, as this is the first study assessing the effect of iberin in human neuroblastoma SK-N-AS, SK-N-SH and SK-N-BE(2) cells. To assess the biological activity of iberin in terms of cell growth, neuroblastoma cells were treated with 1-, 2.5-, 10- and 25- μ M doses of iberin for 24 and 48 h. Iberin showed a

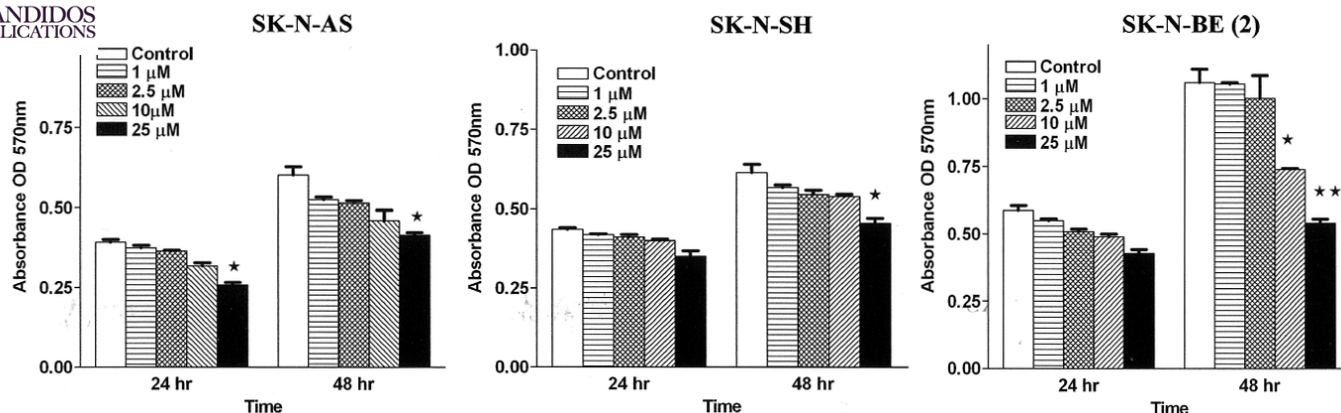


Figure 1. Growth inhibitory effects of iberin on SK-N-AS, SK-N-SH and SK-N-BE(2) cells. To assess the effect of iberin on cell growth, human neuroblastoma SK-N-AS, SK-N-SH and SK-N-BE(2) cells were treated with either DMSO vehicle control or 1-, 2.5-, 10- and 25- μ M doses of iberin. After 24 and 48 h of treatment, viable cells were scored using metabolic dye-based MTT assay. Data represent mean \pm SE of three independent experiments; each assayed in duplicate. Significant difference from vehicle control, * p <0.05; ** p <0.01.

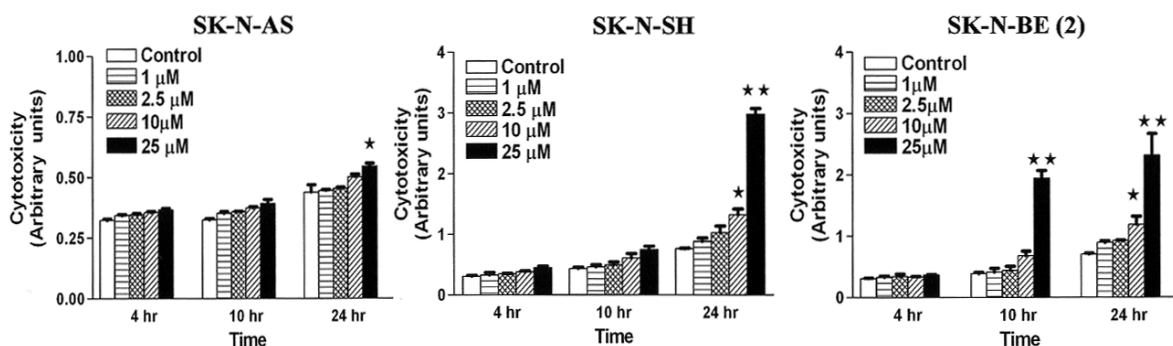


Figure 2. Cytotoxicity of iberin on human neuroblastoma SK-N-AS, SK-N-SH and SK-N-BE(2) cells. Cells were cultured in complete medium, and treated with either DMSO vehicle control or 1- to 25- μ M doses of iberin. After the indicated treatment times, LDH released into the medium was measured using a cytotoxicity assay kit as described in Materials and methods. Data represent mean \pm SE of three independent experiments; each assayed in duplicate. Significant difference from vehicle control, * p <0.05; ** p <0.01.

strong time-dependent inhibition of growth at a 25- μ M concentration in SK-N-AS and SK-N-SH at 24 and 48 h respectively whereas there was significant reduction at a 10- μ M concentration in SK-N-BE(2) cells. As shown in Fig. 1, iberin treatment resulted in a dose-dependent inhibition of cell growth compared to vehicle-treated controls. Iberin treatment also resulted in time-dependent inhibition of cell growth, and this effect was more pronounced at 48 h post-treatment (Fig. 1). These data suggest that iberin is a potent isothiocyanate in inhibiting the growth of neuroblastoma cells.

Cytotoxicity. LDH release assay was used to evaluate cytotoxicity. An increase in the number of plasma membrane-damaged cells results in an increase in LDH activity in the culture supernatant. To evaluate the cytotoxic effects of iberin, neuroblastoma cells were exposed to varying concentrations of iberin for 24 h, and the LDH activity was measured in the medium. The results showed a concentration-dependent LDH release from the iberin-exposed tumor cells. As shown in Fig. 2, treatment of SK-N-SH and SK-N-BE(2) cells with doses of 10 μ M of iberin for 24 h resulted in a significantly

increased LDH release compared to that of DMSO-treated vehicle controls. However, significant cytotoxic effects were found in SK-N-AS cells only at a 25- μ M dose of iberin for 24 h. These results indicate that iberin exposure causes significant damage to the plasma membrane of the neuroblastoma cells.

Induction of apoptosis. Apoptosis is a controlled form of cell death. To assess whether the cytotoxic effects of iberin might be mediated by apoptosis, we treated neuroblastoma cells with iberin under similar condition as in other studies, and then analyzed the cells by fluorescence microscopy following DAPI and FLICA staining. Within 24 h of treatment of 10 μ M of iberin, SK-N-AS cells clearly exhibited significant morphological changes and chromosomal condensation, which are indicative of apoptotic cell death (Fig. 3A). Such results imply that the cytotoxic action of iberin was due to its ability to induce apoptosis. It was shown previously by others (26) that the proportion of cells reactive to FLICA was strongly correlated with the fraction of apoptotic cells identified by the presence of nuclear fragmentation. The detection of activated caspases by application of FLICA was

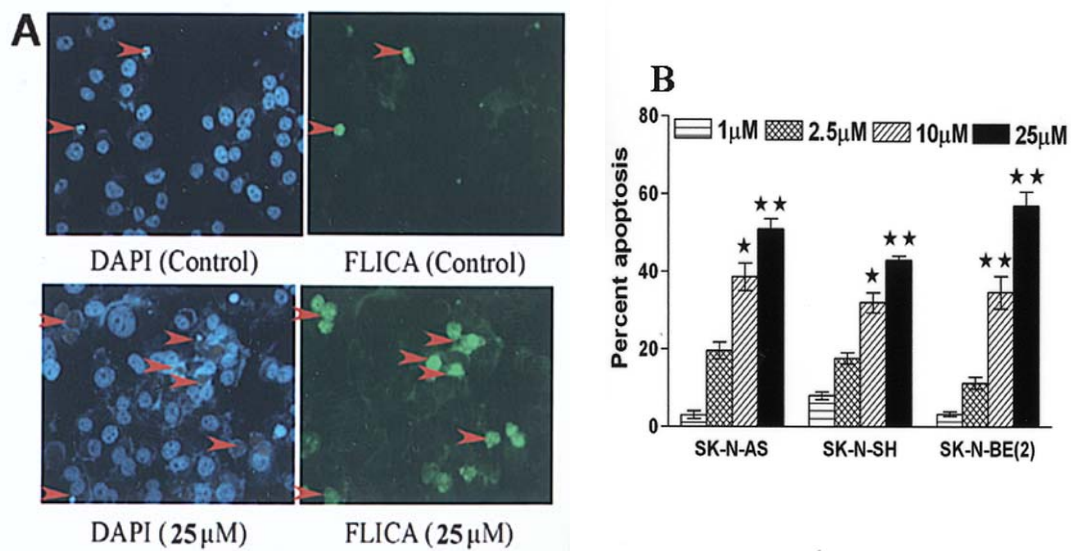


Figure 3. Effect of iberin on induction of apoptosis in human neuroblastoma cells. (A) The human neuroblastoma SK-N-AS cells were exposed to 25 μ M of iberin for 24 h. An equal volume of vehicle (DMSO) was added to the controls. To detect activation of caspases and chromatin condensation/fragmentation, cells were stained with FLICA and DAPI respectively as described in Materials and methods, and subsequently apoptotic cells were quantified by fluorescent microscopy. (B) Percentage of apoptotic cells are shown and each column represents the mean \pm SE of the data obtained by FLICA staining from three independent experiments. Significant difference from vehicle control, * p <0.05; ** p <0.01.

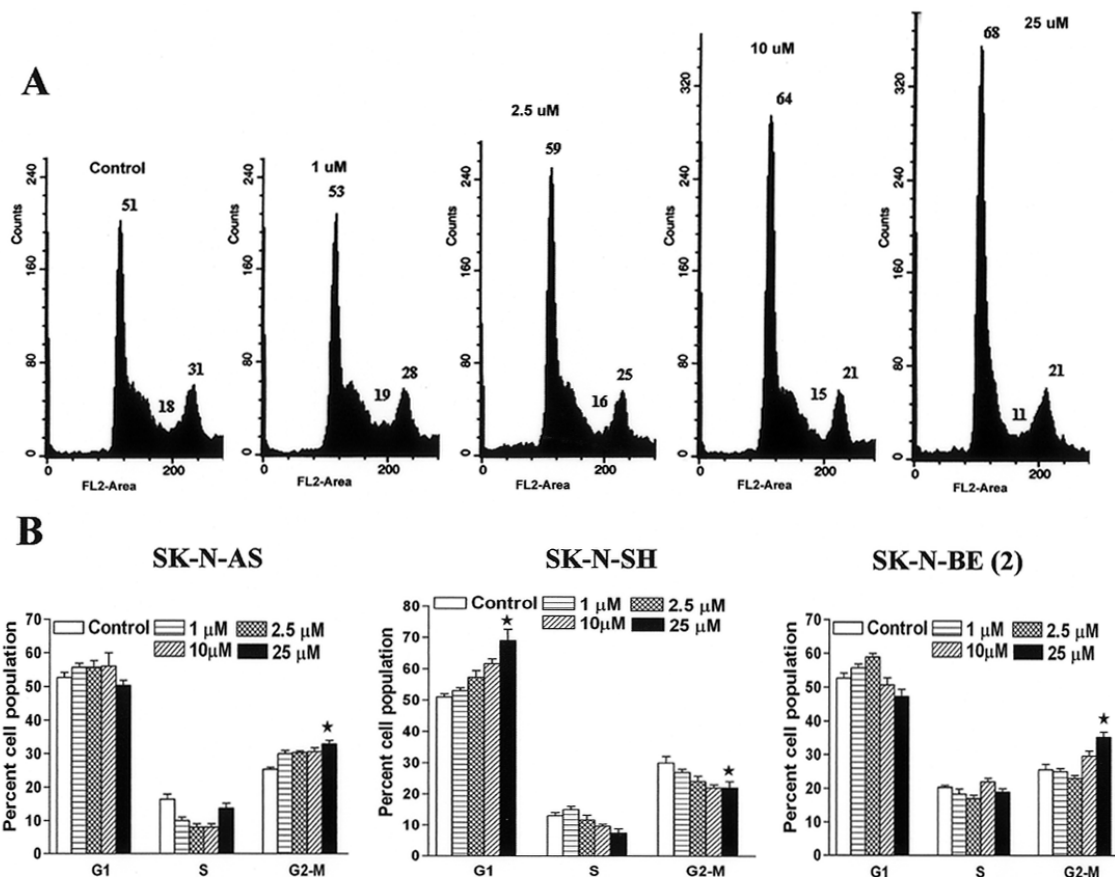


Figure 4. Effect of iberin on cell cycle progression in SK-N-AS, SK-N-SH and SK-N-BE(2) cells. Cells were cultured in complete medium, and treated with either DMSO vehicle control or 1- to 25- μ M doses of iberin. After 24 h, cells were collected, washed with PBS, and then cellular DNA was stained with propidium iodide as detailed in Materials and methods. The percentage of cells in G1, S and G2-M phases was analyzed by flow cytometry. (A) PI fluorescence pattern for cell cycle distribution of SK-N-SH cells for different treatments of iberin. (B) The percentage of cell cycle distribution data for each treatment group of SK-N-AS, SK-N-SH and SK-N-BE(2) cells. Three independent experiments were performed and the mean \pm SE are presented. Significant difference from vehicle control, * p <0.05; ** p <0.01.

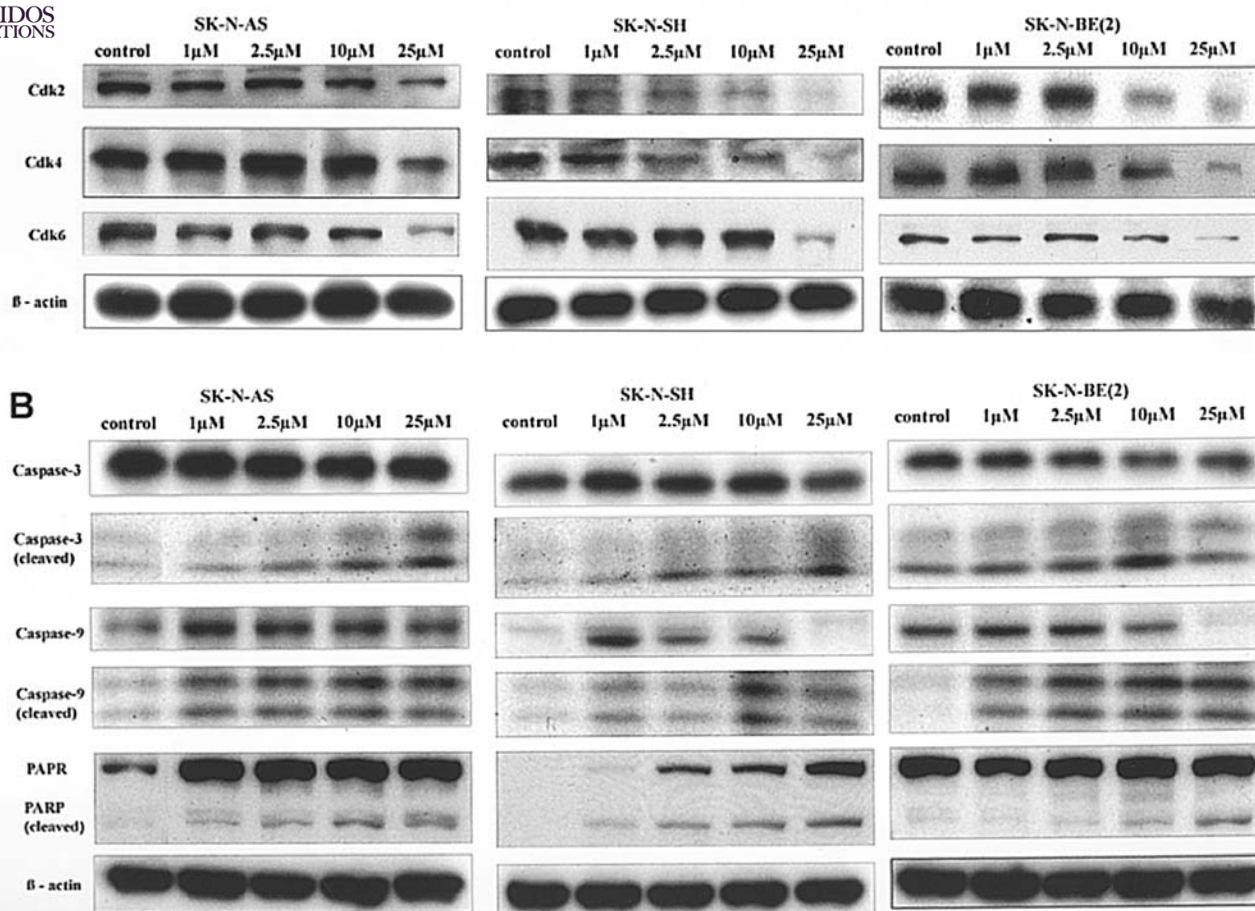


Figure 5. (A) Effect of iberin on cell cycle regulators in human SK-N-AS, SK-N-SH and SK-N-BE(2) neuroblastoma cells. Cells were treated with DMSO or 1- to 25- μ M doses of iberin for 24 h. At the end of treatment time, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes were probed with anti-Cdk2, Cdk4, and Cdk6 antibodies followed by the appropriate peroxidase-conjugated secondary antibodies, and visualized by an enhanced chemiluminescence detection system. The same blots were stripped and re-probed with antibody against β -actin. (B) Apoptotic effect of iberin on SK-N-AS, SK-N-SH and SK-N-BE(2) neuroblastoma cells. Tumor cells were cultured in complete medium, and treated with either DMSO vehicle control or 1- to 25- μ M doses of iberin for 24 h. At the end of treatments, cell lysates were prepared and SDS-PAGE and Western blot analysis were performed for caspase-9, caspase-3, and PARP using specific antibodies as described in Materials and methods. Membranes were also stripped and re-probed with anti- β -actin antibody for protein loading correction.

performed with fluorescence microscopy. As shown in Fig. 3B, a 10- μ M concentration of iberin effectively induced apoptosis in 30-35% of cell population following 24 h of treatment in neuroblastoma cells compared with controls; iberin treatment at a 25- μ M dose resulted in >40% apoptotic cells following 24 h of treatment. In the present study a strong correlation was also seen between the percentage of cells labeled with FLICA and those labeled with DAPI exhibiting nuclear fragmentation (Fig. 3A). These findings demonstrate that iberin activates the induction of apoptosis in human neuroblastoma cells.

Induction of cell cycle arrest. The inhibition of deregulated cell cycle progression in tumor cells is an effective strategy to control tumor growth (27). To assess whether iberin-induced growth inhibition of cells is mediated via alterations in cell cycle, we evaluated the effect of iberin on cell cycle distribution. We performed cell-cycle analysis with growing neuroblastoma cells followed by treatment with varying concentrations of iberin for 24 h. As summarized in Fig. 4B, treatment of SK-N-SH cells with iberin for 24 h resulted in a

significantly higher number of cells in the G₁ phase at the following concentrations: 1 μ M (53%), 2.5 μ M (59%), 10 μ M (64%) and 25 μ M (68%, $p < 0.05$), compared with the vehicle-treated control (46%). Similar observations were obtained from the analysis of the effects of iberin treatment on cell cycle progression of SK-N-BE(2) cells. Although, the 10- and 25- μ M doses of iberin did not induce G₁ arrest, there was a significant accumulation of cells in the G₂/M phase at a 25- μ M dose ($p < 0.05$) in SK-N-BE(2). Remarkably, unlike the SK-N-SH cells, the fraction of SK-N-AS cells in the G₁ phase was not affected by iberin at any of the concentrations evaluated, but at a 25- μ M concentration they showed an increase in G₂/M cell population at 24 h of treatment. These results suggest that the inhibition of deregulated cell cycle progression could be one of the molecular events associated with the selective anticancer efficacy of iberin in neuroblastoma cells.

Downregulation of protein levels of G₁ regulatory Cdks. Based on the above findings indicating that iberin causes cell cycle arrests in neuroblastoma cells and the cell cycle is

controlled by the expression and activation of several cyclins and Cdks, we asked whether their expression levels changed after cell exposure to iberin. Total cell lysates were prepared following iberin treatment of neuroblastoma cells at 1-, 2.5-, 10- and 25- μ M doses for 24 h, and the cell lysates were assayed for Cdk2, Cdk4, and Cdk6 using immunoblot analysis. As shown in Fig. 5A, compared with control, iberin treatment resulted in almost complete inhibition in Cdk2 in SK-N-SH cells; however, the inhibitory effect of iberin in SK-N-AS and SK-N-BE(2) was of lower magnitude. In terms of its effect on Cdk4, iberin caused a decrease in Cdk4 levels in tested neuroblastoma cell lines; it had a profound effect on the reduction of the Cdk4 level in SK-N-SH cells at 2.5 μ M, in SK-N-BE(2) cells at 10 μ M and in SK-N-AS at 25 μ M in comparison to control cells following 24 h of treatment (Fig. 5A). Iberin also decreased the expression of Cdk6 levels in SK-N-AS cells; however, it exhibited an effect at 25 μ M in SK-N-SH and SK-N-BE(2) cells. These results suggest that the suppressive effects of iberin on the growth of neuroblastoma cells are partly caused by downregulating the levels of specific Cdks. Taken together, these results suggest that alterations in the levels of cell cycle regulators by iberin play a major role in its effect on human neuroblastoma cells in terms of cell cycle arrest and cell growth inhibition together with possible apoptosis induction.

Role of caspase activation in iberin-induced apoptosis. Caspase-3 activation and PARP cleavage are characteristic indicators of apoptosis. Based on the above results showing the induction of apoptosis by iberin, we determined the effect of iberin on the activation of caspase-9 and caspase-3 following 24 h of treatment. The cleavage of caspases is directly related to their activation status. Treatment of neuroblastoma cells with iberin (0, 2.5, 10, and 25 μ M) for 24 h caused an increase in cleaved caspase-9 and -3, which were very prominent at a 25- μ M dose of iberin (Fig. 5B). We also assessed PARP cleavage, a nuclear protein that is specifically cleaved by activated caspases. Consistent with the cleavage of caspases, iberin also caused a strong increase in PARP cleavage (Fig. 5B) when compared with the cells which were not treated with iberin; equal protein loading was confirmed by probing the same membrane with β -actin antibody. Taken together, these results show that iberin induced apoptosis in a dose-dependent manner, and that apoptosis is mediated by caspase activation.

Discussion

Conventional chemotherapy of advanced malignant tumors has done little to improve the treatment outcomes in human patients. Prevention and therapeutic intervention by dietary phytochemicals is a newer approach in cancer management (28). Different epidemiological studies have indicated that diet and cancer are closely associated and people who consume higher amount of fruits and vegetables have a lower risk of various types of cancers (29,30). Previous studies have revealed that isothiocyanates are potent inducers of the expression of enzymes implicated in the detoxification of a variety of chemical carcinogens, and are highly effective in chemically induced cancers in animals (14,31,32). Isothio-

cyanates are known to inhibit the growth of cancer cells and to induce apoptosis (6,15,33) but the mechanisms are still only partially understood. The isothiocyanate iberin, a sulforaphane sulfoxide analog, has been reported to exhibit some biological effects (12,20) but its anticancer mechanism is still elusive. In this study, we demonstrated that the possible roles of iberin on the human neuroblastoma cells were i) to decrease the percentage of viable cells in a dose- and time-dependent manner, ii) to arrest the cell cycle via the down-regulation of Cdks, and iii) to induce apoptosis via activation of caspase-3 and caspase -9 followed by cleavage of PARP.

Previous studies showed that iberin demonstrated chemopreventive effects in laboratory animals (12,20) and this takes place by the induction of phase II enzymes, which function in carcinogen detoxification. In the present study, the strong growth inhibitory activity of iberin compared to sulforaphane in cultured human cancer cells prompted us to study its mechanism of action (16). The results from the present study indicate that iberin inhibits human neuroblastoma cell proliferation in a concentration- and time-dependent manner. It is noteworthy that the range of effective doses of iberin (1-25 μ M) in neuroblastoma cells is comparable with that shown to be active by sulforaphane in other tumor cell lines such as medulloblastoma (34), colon (35) and prostate cancer cells (36). Moreover, it has been demonstrated that iberin was \sim 2 times more effective than sulforaphane in human myeloid leukemia HL60 cells and its drug-resistant sublines (16). It seems that these doses of iberin may have significant antitumor effects in other tumor cell lines and may correspond to clinically achievable pharmacological concentrations of the drug.

In this study, we demonstrated that iberin is cytotoxic to neuroblastoma cells and that cytotoxicity was the result of iberin-induced apoptosis via changes of cell cycle and caspase cascade activation. There are several reports that isothiocyanates induce cell arrest in the G₁ or G₂/M phase depending upon molecular targets of different signaling pathways (37-39). To better understand the inhibitory effect of iberin on the proliferation of neuroblastoma cells, we tested whether iberin had the capacity to block cell cycle progression by flow cytometric analysis of PI-stained cells. These studies showed significant changes of cell cycle distribution in SK-N-AS, SK-N-SH and SK-N-BE(2) cells following iberin treatment at 24 h. Iberin induced G₂/M accumulation after 24 h of treatment in SK-N-AS and SK-N-BE(2) cells similar to the effects of sulforaphane in colon and prostate cancer cells (38,39). In SK-N-SH, the proportion of cells in the G₁ phase was increased and those in the S phase decreased after 24 h of treatment at all tested concentrations. It has been shown that sulforaphane induced a G₂/M cell cycle arrest at 15 μ M (40); and at higher doses (>25 μ M), a G₁ cell cycle arrest in HT-29 cells (41). Our data demonstrated that the iberin-induced distribution of cells in the cell cycle changed depending on the histotype of the neuroblastoma cells. Moreover, these findings suggest, as is common with therapeutic agents, that the response can vary depending upon the properties of the particular tumor cell type that is being targeted.

The results of The DAPI and FLICA staining assays indicated that marked apoptosis occurred in all three tested



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neuroblastoma cell lines following iberin treatment. Neuroblastomas can acquire a sustained high-level drug resistance during chemotherapy. p53 mutations are rare in primary neuroblastomas, but a loss of p53 function could play a role in multidrug resistance (42). Advanced neuroblastoma frequently relapses, and it is possible that p53 mutations develop later. Many chemotherapeutic agents act via p53 and the presence of a mutation, a deletion or functional inactivation of p53 often renders the tumor cells resistant towards chemotherapeutic treatment (43). Therefore, compounds, which are able to induce apoptosis in cancer cells independent of p53 are of special interest. The essential role of p53 for the induction of apoptosis in Jurkat cells by sulforaphane was suggested previously (17). As expected, the p53-dependent apoptosis pathway may be involved in the mechanisms of iberin-induced apoptosis since SK-N-AS and SK-N-SH cells have normal p53. However, the SK-N-BE(2) cell line that was used in this study had impaired p53 expression (44). These observations are in accordance with other studies. Sulforaphane was also reported to induce apoptosis in p53-deficient or mutant p53-expressing human cancer cell lines (36,45). Our results suggested that p53 is not the only mediator of apoptotic effects of iberin in neuroblastoma cells. Thus, we conclude that iberin induces neuroblastoma cell apoptosis by both p53-dependent and p53-independent pathways and may have a significant therapeutic effect for cells in neuroblastoma tumors. To note, iberin is a potent inducer of apoptosis in *MYCN* amplified SK-N-BE(2) cells. The data presented in this paper provide the first evidence that the isothiocyanate iberin causes apoptosis in human neuroblastoma cell lines and we demonstrated that apoptosis induced by iberin was independent of p53 and N-Myc alterations.

Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle regulatory proteins including Cdks provides an important mechanism for the inhibition of growth. Isothiocyanates have been shown to be capable of blocking cell cycle progression through the inhibition of multiple Cdk activity (46,47). The exact molecular targets of iberin are currently unknown. Cell cycle arrest occurs by loss in the activity of Cdks and we tested the hypothesis that iberin will impart antiproliferative effects through Cdk machinery. We next investigated by Western blotting analysis the effects of iberin on the expression of Cdks in neuroblastoma cells, the major regulators of the cell cycle. The results from the immunoblotting analyses demonstrated that iberin affected the intracellular protein levels of Cdk2, and Cdk6; however, the levels of Cdk4 protein were downregulated in a concentration-dependent manner. Our data therefore indicate that iberin has specific mechanisms for inducing cell cycle arrest.

Apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including neuroblastoma (48,49), since impaired apoptosis is involved in the pathogenesis of cancer. Thus, apoptosis is an emerging therapeutic target of bioactive agents of diet (50). Apoptosis involves the activation of members of the caspase family of cysteine proteases in a hierarchical cascade, with caspases functioning as triggers and executioners of the apoptotic

process. This may be regulated by various mitochondrial apoptogenic mediators. Caspase-3 is a major executioner protease, responsible for initiating the apoptotic program and it is activated via cleavage by other caspases including caspase-9 (51,52). The implication of caspase-3 in the apoptotic mechanism has been described previously in other systems (51). To explore the possible mechanisms of iberin-induced apoptosis, the expression and activation of caspase-3, caspase-9, and PARP were examined by Western blotting. The activation of caspase-9 and caspase-3 has been recognized as a hallmark of mitochondrial cell death in a variety of different cell types (53). Iberin produced the cleavage of procaspase-3 and caused specific cleavage of the caspase-3 substrate PARP, indicating specific evidence of apoptosis. Taken together, these findings indicate that iberin-induced apoptosis of human neuroblastoma cells is mediated via caspase activation and associated events.

To our knowledge, ours is the first systematic study to demonstrate the direct, selective antiproliferative and proapoptotic effects of iberin against human neuroblastoma cells. In conclusion, our data demonstrate that *MYCN* amplified or unamplified neuroblastoma cells with normal or functionally defective p53 can be induced to undergo apoptosis by relatively low concentrations of iberin and that this response is preceded by cell cycle arrest associated with caspase activation. This raises the possibility that iberin, at physiologically attainable concentrations, may have chemopreventive and even therapeutic potential for human neuroblastoma. Our present findings warrant further iberin *in vivo* efficacy studies in preclinical human neuroblastoma cancer models.

Acknowledgments

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