Notes

Sulforaphane Induces Cell-Cycle Arrest and Apoptosis in Cultured Human Lung Adenocarcinoma LTEP-A2 Cells and Retards Growth of LTEP-A2 Xenografts in Vivo

Hao Liang,[†] Baitang Lai,[‡] and Qipeng Yuan*,[†]

State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China, and Cell Biology Department, Beijing Thoracic Tumor Research Institute, Beijing 101149, People's Republic of China

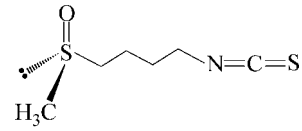
Received April 11, 2008

Sulforaphane (**1**), a glucosinolate-derived isothiocyanate found in the cruciferous vegetable broccoli, is considered an anticarcinogenic component. In the present study, the proliferation and apoptosis induction in human lung adenocarcinoma LTEP-A2 cells by **1** was investigated. Compound **1** caused G_2/M-phase arrest ($p < 0.05$) and increase of apoptotic cell fraction ($p < 0.05$) in a time- and dose-dependent manner. Intraperitoneal injection of **1** significantly inhibited growth of LTEP-A2 xenografts in nude mice, and 9 days after tumor cell implantation with 100 mg/kg intraperitoneal injection of **1**, the average tumor weights in **1**-treated mice was >70% lower than that of the control mice.

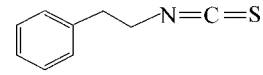
Cruciferous vegetables contain compounds associated with protection against cancer. It has been shown that the cancer chemopreventive effects of cruciferous vegetables are related to their unique content of glucosinolates.[1] When vegetables are ground or chopped, the enzyme myrosinase (thioglucoside glucohydrolase, EC3.2.3.1) and glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of these glycoside molecules, producing glucose, sulfate, and a diverse group of aglycon products. The resultant aglycons then undergo nonenzymetic, intramolecular rearrangement to yield isothiocyanates, thiocyanates, or nitriles. Glucosinolates are not bioactive until they have been enzymatically hydrolyzed to a chemically related isothiocyanate.[2]

Sulforaphane (4-methylsulfinybutyl isothiocyanate, **1**), an isothiocyanate derived from glucoraphanin (4-methylsulfinylbutyl glucosinolate), has received much attention over the past decade. Initially studied as an inducer of phase II enzymes, **1** has been shown subsequently to possess anticarcinogenic activities.[3−5] Several different potential mechanisms of action have been proposed for the role of **1** in cancer chemoprevention.[5−9] Sulforaphane may modulate carcinogen metabolism by induction of phase II detoxification enzymes and inhibition of cytochrome P-450-dependent monooxygenase and histone deacetylase.[6−9] Compound **1** also induces cell-cycle arrest and apoptosis in human colon cancer and breast cancer cell lines and inhibits tumor formation.[10−13] It was also found that the induction of cytoprotective enzymes by sulforaphane in animal systems occurs at lower concentrations, followed by an anti-inflammatory reaction at higher concentrations[14] and then by the induction of apoptosis and cell-cycle arrest at even higher concentrations. Thus, this substance appears to be an important chemoprotective agent.[15]

Previous studies have shown that phenethyl isothiocyanate and sulforaphane (**1**) and their *N*-acetylcysteine conjugates inhibit lung adenoma formation induced by tobacco carcinogens in A/J mice at the post-initiation stage.[16] Moreover, phenethyl isothiocyanate (**2**) and **1** can induce apoptosis in human non-small lung cancer A549 cells by a mechanism of direct covalent binding to cellular proteins.[17] It has also been found that caspase-3 is a key regulator of apoptosis in response to combined sulforaphane and tumor necrosis factor-related apoptosis-inducing ligand in human lung adenocarcinoma A549 cells through down-regulation of ERK and Akt.[18] Furthermore, it has been reported that **1** was most effective in the inhibition of lung metastasis induced by B16F-10 melanoma cells.[19]



1



2

In the present study, the inhibitory effects of sulforaphane (**1**) and phenethyl isothiocyanate (**2**) were evaluated on human lung adenocarcinoma LTEP-A2 cells. Then, the effects of **1** were studied on cell-cycle progression and apoptosis induction in LTEP-A2 cells. Finally, compound **1** was investigated for its effects on the growth of nude mice bearing LTEP-A2 xenografts.

The effect of sulforaphane (**1**) on the proliferation of LTEP-A2 cells was determined by a trypan blue dye exclusion assay, and the results are shown in Figure 1. The results revealed a concentration-dependent inhibition in survival of LTEP-A2 cells upon exposure to different concentrations (6.25, 12.5, 25, or 50 μM) of **1** at the time determined. The IC_{50}, as measured by the number of viable cells in cultures 24 h after the addition of **1**, was seen at a concentration of 6.25 μM. When the cells were treated with concentrations of **1** for different times (3, 24, 48, or 72 h), a time-dependent inhibition in survival could be observed. Thus, treatment with **1** caused a time- and dose-dependent reduction in LTEP-A2 cell number. Figure 1 shows the survival curve of LTEP-A2 cells at different time points and after treatment with different concentrations of phenethyl isothiocyanate (**2**). When the harvested cells were counted, the numbers of treated cells decreased only in a dose-dependent manner, and the number of viable cells following a 24 h exposure to 12.5 and 25 μM **2** was reduced by 12% and 81%, with

* To whom correspondence should be addressed. Tel: +86 10 6443 7610. Fax: +86 10 6443 7610. E-mail: yuanqp@mail.buct.edu.cn.
[†] Beijing University of Chemical Technology.
[‡] Beijing Thoracic Tumor Research Institute.

10.1021/np800233q CCC: $40.75
Published on Web 10/15/2008

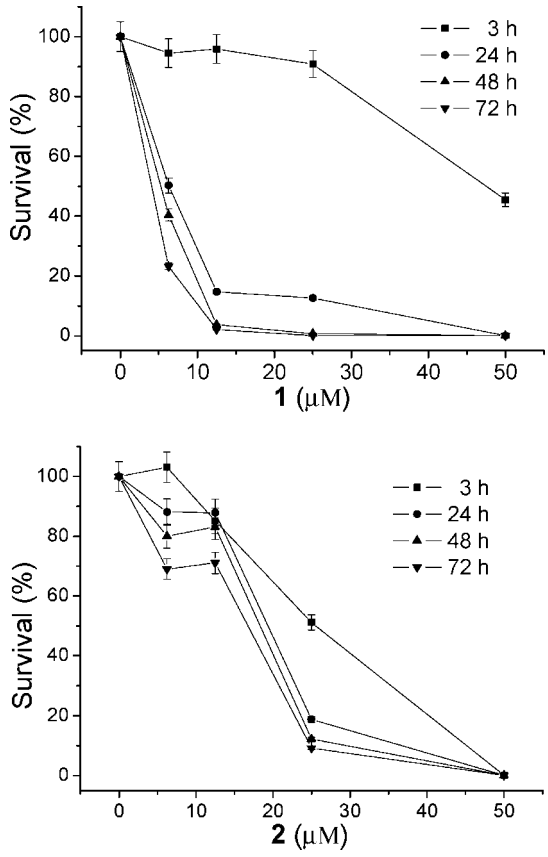


Figure 1. Effects of sulforaphane (**1**) and phenethyl isothiocyanate (**2**) treatment on the proliferation of LTEP-A2 cells.

the IC_{50} being about 20 μM. Compound **1** was significantly more effective as an inhibitor of lung tumor cell growth than **2**. The data presented herein indicate that **1** was highly effective in suppressing the proliferation of human LTEP-A2 lung adenocarcinoma cells in culture. Compared with reported results, marked differences in the resistance of the cell lines to **1** and **2** were observed. The IC_{50} values for **1** were 3.8, 10.0, and 95.0 μM in lymphoblastoid cells,[20] prostate cancer cells,[21] and human colon adenocarcinoma cells,[22] respectively. By contrast, the IC_{50} values for **2** were 5.1, 7.0, and 23.2 μM in chronic lymphocytic leukemia cells,[23] human colon adenocarcinoma cells,[22] and ovarian cells,[24] respectively. These results indicate that side-chain changes in the structure of isothiocyanates could have a significant impact on their anticancer activity.

To elucidate the rate at which **1** altered cell-cycle progressions, this was determined at several time points. Table 1 summarizes the effects of sulforaphane on the cell-cycle distribution of LTEP-A2 cells. These effects were particularly apparent following treatment with **1** at 6.25 and 50 μM. The immediate effects (3 h), observed at drug concentrations of 6.25 and 50 μM, appeared primarily as an slight increase in the proportion of cells in the G_2/M phase of the cell cycle (from 15.3 to 18.1%) accompanied by a slight compensatory decrease in G_1 phase cells (from 59.3 to 55.6%). Longer exposure (48 h) to concentrations of 6.25 and 50 μM led to a significant decrease in the proportion of G_2/M cells, while the percentage of cells in the S phase increased from 25.2 to 42.5%. Prolonged (72 h) exposure of LTEP-A2 cells to **1** appeared as a decrease in G_1-phase cells (from 45.7 to 26.5%), a loss of G_2/M-phase cells (from 24.0 to 8.8%), and a significant increase in the proportion of S-phase cells (from 29.4 to 64.7%). Moreover, these observations imply that the effects of **1** were time- and dose-dependent. Apoptosis was also observed at a 50 μM concentration of **1** and at later stages during the treatment (at 48 and 72 h). This was manifested by the appearance of cells with a decreased DNA content, identified as cells with DNA values below that of G_1 cells ("sub-G_1 cells"). Thus, as an example, after 3 h of treatment with 50 μM **1**, only 0.88% of apoptotic cells were found. This fraction increased significantly to 6.13 and 31.9% after 24 and 48 h of treatment, respectively (Table 1). These data indicate that target cells start to die as late as 24 h after treatment with **1** by an apoptosis-related mechanism. Sulforaphane induced apoptosis in a time- and dose-dependent fashion. The results of the present study indicate that the antiproliferative activity of **1** against LTEP-A2 cells is due to its ability to induce apoptosis.

Cultured cancer cells are valuable tools for rapid screening of potential anticancer agents as well as for the elucidation of the mechanism of activity. Prior to clinical trials, however, it is essential that the in vivo efficacy of potential anticancer agents is determined in a suitable animal model.[25] Therefore, a study was conducted to determine whether administration of **1** affects the growth of LTEP-A2 xenografts in nude mice. The effect of intraperitoneal injection of **1** on the growth of LTEP-A2 xenografts is shown in Figure 2. As can be seen from this figure, this caused a significant inhibition of LTEP-A2 xenograft growth. At the lower doses, **1** also inhibited the incidence of lung adenocarcinoma, but the decrease of average tumor weights was not statistically significant. Nine days after starting treatment with 50 mg/kg intraperitoneal injection of **1**, the average tumor weights in control and SFN-treated mice were 578 $\pm$ 25 and 350 $\pm$ 27 mg, respectively, reflecting a 40% reduction in tumor weight in the group treated with **1**. Similarly, 9 days after tumor cell implantation with 100 mg/kg **1**, the average tumor weight in **1**-treated mice (138 $\pm$ 13 mg) was >70% lower than that of control mice. Body weights of the control and treated mice were recorded to determine if administration of **1** causes weight loss. However, the average body weights of the control and **1**-treated mice did not differ significantly throughout the treatment protocol in both experiments (data not shown). Oral administration of sulforaphane (50 mg/kg, 3 times/week) significantly inhibited growth of human prostate cancer PC-3 xenografts in nude mice, reflecting a >50% reduction in tumor volume.[25] Daily sulforaphane injections (66 mg/kg/day for 3 weeks) in severe combined immunodeficient mice with human pancreatic cancer PANC-1 tumors resulted in a decrease of mean tumor volume by 40%.[26] We now show that growth of established LETP-A2 tumor xenografts was suppressed at a similar sulforaphane dose.

Sulforaphane (**1**) is a naturally occurring cancer chemopreventive isothiocyanate found as its glucosinolate precursor in the cruciferous vegetable broccoli. The present cell culture and xenograft studies suggest beneficial properties of **1**, in inhibiting the growth of tumors, arresting the cell cycle, and enhancing apoptosis. Our data indicate that the LTEP-A2 human lung cancer cell line is highly sensitive to growth inhibition by **1**. The simultaneous appearance of G_2/M arrest and apoptosis clearly indicated that cell death is a primary direct effect due to treatment with **1**, and the growth inhibition of LTEP-A2 cells produced by this compound resulted from a combination of apoptosis and cell-cycle interference in which G_2/M arrest is a key event. More importantly, it has been demonstrated that the growth of LTEP-A2 xenografts in nude mice was inhibited significantly on intraperitoneal injection of **1**. In conclusion, the results of the present study strongly argue for systematic preclinical and clinical evaluations of sulforaphane (**1**) for its activity against human lung cancer.

Experimental Section

Chemicals. Sulforaphane (**1**) was obtained as described previously, and its purity (>98%) was assessed by HPLC analysis, MS, and NMR spectroscopy.[27] Phenethyl isothiocyanate (**2**) (>99%) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Lung adenocarcinoma LTEP-A2 cells, obtained from the Department of Cell Biology, Beijing Thoracic Tumor Research Institute, were grown in suspension and propagated in RPMI 1640 medium supplemented with 20% heat-inactivated bovine serum, streptomycin 100 μg/mL, and penicillin 100 U/mL (all obtained from

Table 1. Effects of Sulforaphane (**1**) on Cell Cycle and Apoptosis of Human Lung Adenocarcinoma A2 Cells

		cell-cycle progression			
concentration (μM)	treatment time (h)	G_0/G_1	S	G_2/M	apoptosis (%)
6.25	0	63.0 ± 3.15	19.8 ± 0.99	17.2 ± 0.86	1.25 ± 0.06
	3	59.3 ± 2.97	25.4 ± 1.27	15.3 ± 0.77	1.41 ± 0.07
	24	50.4 ± 2.52	29.2 ± 1.46	20.3 ± 1.02	0.96 ± 0.05
	48	52.8 ± 2.64	25.2 ± 1.26	22.0 ± 1.10	0.77 ± 0.04
	72	45.7 ± 2.29	29.4 ± 1.47	24.0 ± 1.20	1.13 ± 0.06
50	0	65.8 ± 3.29	20.1 ± 1.01	14.1 ± 0.71	1.20 ± 0.06
	3	55.6 ± 2.78	26.4 ± 1.32	18.1 ± 0.91	0.88 ± 0.05
	24	55.4 ± 2.77	19.3 ± 0.97	25.3 ± 1.27	6.13 ± 0.31
	48	52.1 ± 2.61	42.5 ± 2.12	5.40 ± 0.27	31.9 ± 1.60
	72	26.5 ± 1.33	64.7 ± 3.23	8.80 ± 0.44	22.3 ± 1.12

Sigma, St Louis, MO). To maintain exponential growth, the cultures were divided every third day by dilution to a concentration of 1×10^5 cells/mL.

Cell Treatment and Cytotoxicity Test. A 5 mL aliquot of the complete cell-culture medium containing increasing concentrations of **1** or **2** (6.25, 12.5, 25, or 50 μM) was prepared. Exponentially growing LTEP-A2 cells at a concentration of 400 000 cells/mL were subsequently added. For each experiment, treatment was performed in three independent experiments and separate cultures were set up for each treatment. Cell concentrations were measured as a function of time by counting trypan blue-excluding cells on cell aliquots removed from culture at the designated times (3, 24, 48, and 72 h). Results were calculated as viable cells in **1**- or **2**-treated cultures relative to controls. IC_{50} values, the drug concentration causing cell toxicity by 50% following a 24 h exposure, were calculated by interpolation from dose−response curves.[28] All data were determined as means ± SD (n = 3), and Fisher's exact test was adopted for statistical evaluation of the results.

Flow Cytometric Measurement of Cell Proliferation. Flow cytometry was performed using a FACStar$^+$ flow cytometer (Becton Dickinson, Sunnyvale, CA) equipped with an argon laser (Innova 90, Coherent Radiation, Palo Alto, CA) operating at 488 nm (500 mW) for excitation of ethidium bromide. The preparation of samples for measurement of the cell-cycle distribution of nuclei by DNA content was performed according to a two-step method reported elsewhere.[29,30] Briefly, cultures were centrifuged for 5 min at 800g and treated with 1 mL of solution I (584 mg/L NaCl, 1000 mg/L Na citrate, 25 mg/L ethidium bromide, 10 mg/L RNase, and 0.3 mL/L Nonidet P-40). Then, after about 1 h, 1 mL of solution II (15 g/L citric acid, 0.25 M sucrose, and 40 mg/L ethidium bromide) was added and the samples were briefly vortexed. Ethidium bromide fluorescence and forward scatter and side scatter of nuclei in suspension were recorded for cell nuclei in the list mode. For each sample, 10 000 events were registered. The fraction of cells in the different compartments of cell cycle was calculated as described by Schreiber et al.[31] All data are means ± SD (n = 3), and Fisher's exact test was adopted for statistical evaluation of the results.

Determination of Apoptosis. Apoptosis induction in sulforaphane (**1**)-treated LTEP-A2 cells was assessed by flow cytometric analysis of cells with sub-G0/G1 DNA content following staining with propidium iodide. For analysis of cells with sub-G0/G1 DNA content, cells (5×10^5 cells) were seeded into T75 flasks, and allowed to attach overnight. The medium was replaced with fresh complete medium containing **1**. Following incubation for 3, 24, 48, or 72 h at 37 °C, floating and attached cells were collected, washed with phosphate-buffered saline, and fixed with 70% ethanol. Fixed cells were then treated with 80 mg/mL RNase A and 50 mg/mL propidium iodide for 30 min and analyzed using a flow cytometer.[32] All data are means ± SD (n = 3), and Fisher's exact test was adopted for statistical evaluation of the results.

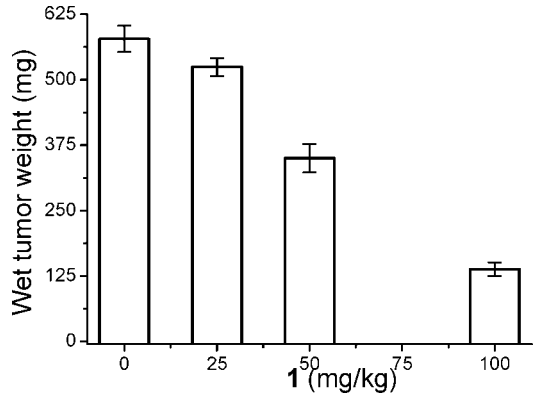


Figure 2. Effect of intraperitoneal injection of sulforaphane (**1**) on wet weight of LTEP-A2 tumor xenografts in nude mice.

Xenograft Assay. Male or female athymic mice (6-week-old) were purchased from the Institute of Materia Medica (Shanghai, Chinese Academy of Sciences) and maintained in accordance with the Institutional Animal Care Use Committee guidelines. LTEP-A2 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA), and a 0.1 mL suspension containing 10^6 cells was injected subcutaneously in both the left and right flank of each mouse. Mice were randomized into four groups of 8 mice/group (2 tumors/mouse). Experimental animals were treated with intraperitoneal injection of sulforaphane (**1**) (25, 50, or 100 mg/kg, 3 times/week) beginning the day of tumor cell implantation. Control mice received an equal volume of the vehicle. Statistical significance of difference in wet tumor weight or body weight between control and treated mice was assessed by the Student's t test.

Acknowledgment. The authors are grateful to Professor Y. Liu (Vegetables and Flowers Institute, China Academy of Agriculture Science) for broccoli seeds. This research was supported by National Natural Science Foundation of China (20806005) and the Yong Scholars Fund of Beijing University of Chemical Technology (QN0809).

References and Notes

(1) Fahey, J. W.; Zalcmann, A. T.; Talalay, P. *Phytochemistry* **2001**, *56*, 5–51.
(2) Rouzaud, G.; Rabot, S.; Ratcliffe, B.; Duncan, A. J. *Br. J. Nutr.* **2003**, *90*, 395–404.
(3) Zhang, Y.; Talalay, P.; Cho, C. G.; Posner, G. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2399–2403.
(4) Zhang, Y.; Kensler, T. W.; Cho, C.; Posner, G. H.; Talalay, P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *9*, 3147–3150.
(5) Gerhäuser, C.; You, M.; Liu, J.; Moriarty, R. M.; Hawthorne, M.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Cancer Res.* **1997**, *57*, 272–278.
(6) Langouët, S.; Furge, L. L.; Kerriguy, N.; Nakamura, K.; Guillouzo, A.; Guengerich, F. P. *Chem. Res. Toxicol.* **2000**, *13*, 245–252.
(7) Barcelo, S.; Gardiner, J. M.; Gescher, A.; Chipman, J. K. *Carcinogenesis* **1996**, *17*, 277–282.
(8) Myzak, M. C.; Karplus, P. A.; Chung, F. L.; Dashwood, R. H. *Cancer Res.* **2004**, *64*, 5767–5774.
(9) Myzak, M. C.; Hardin, K.; Wang, R.; Dashwood, R. H.; Ho, E. *Carcinogenesis* **2006**, *27*, 811–819.
(10) Fahey, J. W.; Talalay, P. *Food Chem. Toxicol.* **1999**, *37*, 973–979.
(11) Gamet-Payrastre, L.; Lumeau, S.; Gasc, N.; Cassar, G.; Rollin, P.; Tulliez, J. *Anticancer Drugs* **1998**, *9*, 141–148.
(12) Jackson, S. J.; Singletary, K. W. *J. Nutr.* **2004**, *134*, 2229–2236.
(13) Jackson, S. J.; Singletary, K. W. *Carcinogenesis* **2004**, *2*, 219–227.
(14) Heiss, E.; Herhaus, C.; Klimo, K.; Bartsch, H.; Gerhäuser, C. *J. Biol. Chem.* **2001**, *276*, 32008–32015.
(15) Zhao, B.; Seow, A.; Lee, E. J.; Poh, W. T. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 1063–1067.

(16) Conaway, C. C.; Wang, C.; Pittman, B.; Yang, Y.; Schwartz, J. E.; Tian, D.; McIntee, E. J.; Hecht, S. S.; Chung, F. *Cancer Res.* **2005**, *65*, 8548–8557.
(17) Mi, L.; Wang, X.; Govind, S.; Hood, B. L.; Veenstra, T. D.; Conrads, T. P.; Saha, D. T.; Goldman, R.; Chung, F. L. *Cancer Res.* **2007**, *67*, 6409–6416.
(18) Jin, C.; Moon, D.; Lee, J.; Heo, M.; Choi, Y. H.; Lee, C.; Park, Y.; Kim, G. *Carcinogenesis* **2007**, *28*, 1058–1066.
(19) Thejass, P.; Kuttan, G. *Life Sci.* **2006**, *78*, 3043–3050.
(20) Misiewicz, I.; Skupinska, K.; Kowalska, E.; Lubinski, J.; Kasprzycka-Guttman, T. *Acta Biochim. Pol.* **2004**, *51*, 711–721.
(21) Cho, S. D.; Li, G.; Hu, H.; Jiang, C.; Lu, J. *Nutr. Cancer* **2005**, *52*, 213–224.
(22) Bonnesen, C.; Eggleston, I. M.; Hayes, J. D. *Cancer Res.* **2001**, *61*, 6120–6130.
(23) Trachootham, D.; Zhang, H. ; Zhang, W.; Feng, L.; Du, M.; Zhou, Y.; Chen, Z.; Pelicano, H.; Plunkett, W.; Wierda, W. G.; Keating, M. J.; Huang, P. *Blood*, in press.
(24) Satyan, K. S.; Swamy, N.; Dizon, D. S.; Singh, R.; Granai, C. O.; Brard, L. *Gynecol. Oncol.* **2006**, *103*, 261–270.
(25) Singh, A. V.; Xiao, D.; Lew, K. L.; Dhir, R.; Singh, S. V. *Carcinogenesis* **2004**, *25*, 83–90.
(26) Pham, N.; Jacobberger, J. W.; Schimmer, A. D.; Cao, P.; Gronda, M.; Hedley, D. W. *Mol. Cancer Ther.* **2004**, *3*, 1239–1248.
(27) Liang, H.; Li, C.; Yuan, Q.; Vriesekoop, F. *J. Agric. Food Chem.* **2007**, *55*, 8047–8053.
(28) Sun, S. Y.; Yue, P.; Shroot, B.; Hong, W. K.; Lotan, R. *J. Cell Physiol.* **1997**, *173*, 279–284.
(29) Nüsse, M. ; Beisker, W.; Kramer, J.; Miller, B. M.; Schreiber, G. A.; Viaggi, S.; Weller, E. M.; Wessels, J. M. In *Methods in Cell Biology*; Darzynkiewicz, Z., Crissman, H. A., Eds.; Academic Press: San Diego, 1994; Vol. 42, pp 149−58.
(30) Fimognari, C.; Nüsse, M.; Hrelia, P. *Environ. Mol. Mutagen.* **1999**, *33*, 220–223.
(31) Schreiber, G. A.; Beisker, W.; Braselmann, H.; Bauchinger, M.; Bögl, K. W.; Nüsse, M. *Int. J. Radiat. Biol.* **1992**, *62*, 695–709.
(32) Xiao, D.; Srivastava, S. K.; Lew, K. L.; Zeng, Y.; Hershberger, P.; Johnson, C. S.; Trump, D. L.; Singh, S. V. *Carcinogenesis* **2003**, *24*, 891–897.

NP800233Q