Monoterpenes Enhanced the Sensitivity of Head and Neck Cancer Cells to Radiation Treatment *In Vitro*

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Abstract. *Background: Perillyl alcohol (POH) elicited anticarcinogenic effects in a number of cancer models and pharmacokinetic studies in humans revealed that PA is the major circulating metabolite following POH administration. Materials and Methods: Effects of PA or POH alone, or in combination with radiation, on human head and neck squamous cell carcinoma cell lines (HNSCC), were investigated using cytotoxicity and flow cytometry assays. HNSCC cells were pretreated with 1.0 mM PA or 0.5 mM POH for 72 h before exposure to 1 or 2Gy dose of radiation. Results: Pretreatment of the cells with 1.0 mM PA or 0.5 mM POH prior to irradiation, caused the following growth inhibition: HTB-43 (50% or 71%), SCC-25 (55% or 68%), and BroTo (18% or 53%). PA and POH induced cell-cycle arrest and apoptosis. Conclusion: PA and POH have potential for use as radiosensitizers in chemo-radiation therapy of head and neck cancers and should be further studied.*

In the United States alone, over 38,000 new cases and over 11,000 deaths are estimated to occur in the year 2004 from carcinomas of the head and neck (1). Current treatment options concentrate on organ preservation using concomitant chemotherapy and radiotherapy for advanced head and neck cancer (2). Adjuvant induction chemotherapy followed by concomitant chemo-radiation, selective chemotherapy infusion, and alternating chemo-radiation therapy, using cisplatin, paclitaxel and 5-fluorouracil (or similar agents), with surgery salvage, have also been reported (3-6). Recurrent head and neck cancer continues to have poor survival rates with tumors that are frequently resistant to current chemotherapeutic agents (7). The combination of the three common chemotherapy drugs (Paclitaxel, Fluorouracil, Hydroxyurea) with twice daily radiation regimen controlled local and regional head and neck cancer in 90% of the patients (2,8). Although the study offered an important proof of concept, the particular drugs used were too toxic, with about 80% of the patients experiencing temporary severe leucopenia and thrombocytopenia. It was suggested that newer chemo-therapeutic and/or radiation protective agents may reduce such toxicity (2).

d-Limonene and perillyl alcohol (POH), naturally occurring monoterpenes synthesized in the mevalonate pathway (9), are components of the essential oils of lavender, citrus fruits, spearmint and cherries (10,11). Although the exact mechanism of action of the monoterpenes has not been fully established, several potentially important drug-related activities have been reported (13-15). Both limonene and POH are rapidly metabolized to perillic acid (PA) and dihydroperillic acid (DHPA) in humans (15,16). Pre-clinical and pharmaco-kinetic studies in humans with refractory malignancies confirmed that PA is the major circulating blood plasma metabolite following POH administration (16,17). Recently, Rajesh *et al.* (18) reported that POH was an effective radio- and chemo-sensitizer in malignant glioma cell lines. Radiosensitization was observed as early as 8 hours after treatment. In a similar study, pretreatment with POH resulted in a dose-dependent sensitization of prostate cancer cells to radiation-induced cell death (19).

Since POH is rapidly metabolized predominantly to PA, we hypothesized that the mechanism of action of POH may involve PA. We therefore investigated the effects of different concentrations of PA or POH alone, and in combination with radiation on head and neck squamous cell carcinoma cell lines (SCC-25, BroTo and HTB-43) cultured *in vitro*.

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Materials and Methods

Chemicals and reagents. Perillyl alcohol (4-isopropenyl-cyclohexenecarbinol) and perillic acid (4-isoprepenyl-1-cyclohexene-1- carboxylic acid) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phosphate-buffered saline (PBS; lacking Ca^{2+} and Mg^{2+}), penicillin (100 units/ml) and 100mg/ml streptomycin (P/S), trypsin-EDTA, DMEM/F-12, RPMI and MEM-alpha media were purchased from Invitrogen Corporation (Grand Island, NY, USA). AlamarBlue dye was obtained from Biosource Intl. Inc. (Camarillo, CA, USA). Propidium iodide (PI) and ribonuclease A (RNase A) were obtained from Sigma Chemicals (St. Louis, MO, USA). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT, USA).

Cells and cell culture. Human head and neck squamous cell carcinoma (HNSCC) of the tongue (SCC-25) and the pharynx (HTB-43) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HNSCC of the tongue cell line (BroTo) was a gift from J. Truelson, MD, (Department of Otolaryngology, University of Texas, South-western Medical Center, USA). Cells were cultured in DMEM/F-12 (SCC-25), RPMI (BroTo) or MEM-alpha (HTB-43) media supplemented with 10% FBS and 1% P/S. Cells were dislodged with trypsin–EDTA and the indicated numbers of cells were plated in tissue culture plates and allowed to adhere overnight. Stock (1.0M) solutions of POH and PA were prepared by dissolving each agent in DMSO. Treatment media were prepared by diluting the stock solution in medium to give the desired concentration and allowing to solubilize, with occasional mixing, at 37˚C for 1 h. Cells in the control groups received medium containing DMSO.

Proliferation and cytotoxicity assays

Dye-exclusion assay. Two million cells were cultured in each 100- mm tissue culture plate, allowed to adhere overnight and then treated with varying concentrations of agents for 72 h. Control cells received medium with DMSO (<0.1%; v/v) only. One set of treated and control cells were exposed to (1 or 2Gy) dose of X-irradiation (6 MV Varian Clinac 1/100, Palo Alto, CA, USA) and then incubated overnight at 37˚C in humidified 5% CO_2 atmosphere. The effect of different treatments on cell proliferation was measured by directly counting the number of cells that excluded trypan blue dye.

Colony formation assay. The colony formation assay (CFA) was performed to determine the long-term effects of monoterpene exposure on the proliferation of cancer cells as previously described (20). Cells (500/well) were cultured in 6-well tissue culture plates overnight to adhere. The following day, the culture medium was replaced with medium containing the desired concentrations of PA or POH and incubated for 72 h. The cells were then washed twice with sterile PBS, once with the complete culture medium and then incubated in fresh, complete culture medium for 14 days at 37˚C in humidified, 5% CO_2 atmosphere. Cells were observed every other day to monitor cell growth. At termination, the culture medium was removed, the cells were rinsed with PBS, stained with crystal violet (5 mg/ml in 95% ethanol) and the dye gently rinsed off. Colonies, containing at least 50 cells, were counted in each well and the cell viability was

calculated as a percentage of the number of colonies formed in the treatment group relative to the control (100%).

Combination chemo-irradiation studies. For the effects of combination treatment, cells were cultured either in 100-mm (2 million cells each) or in 6-well (500 cells/well) plates and were treated without (control) or with PA or POH. In one set of experiments, the cells were exposed to 1 or 2Gy of X-ray (6 MV Varian Clinac 1/100) while in the other, the cells were not irradiated. Following irradiation, the cells were incubated for 12 to 15 h at 37˚C in humidified, 5% CO_2 atmosphere. All the cells were harvested and used for dye exclusion or colony formation assays as described earlier.

Cell cycle analysis and apoptosis experiments. To determine whether the monoterpenes affect cell cycle progression or apoptosis, cells (500,000/well) were cultured in 6-well plates overnight at 37˚C in humidified, 5% CO_2 atmosphere. Cells were then treated with 0.5 mM POH or 1.0 mM PA for 24 or 72 h. The cells were harvested, fixed in 90% ethanol and kept at 4˚C prior to analysis. For cell cycle analysis, the cells were stained with a DNA staining solution (150 μg/ml PI, 0.1% Triton X-100, and 1mg/ml RNase A (DNase-free) in PBS, 1:1:1 by volume). The DNA contents of the control and treated cells were measured by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Twenty thousand events were acquired using CellQuest acquisition software (Becton Dickinson) and the proportion of hypodiploid (sub-G_0/G_1) events and cells in G_0/G_1, S and G_2/M- phases of the cell cycle were determined using the DNA analysis software ModFit LT 3.0 (Verity Software House, Topsham, ME, USA).

Statistical analyses. Results are expressed as Mean±SD of replicate analyses. The effectiveness of treatment methods and combination treatments were analyzed using ANOVA. Dunnett's two-sided test (at $\alpha=0.01$) was used to evaluate differences in the effects between POH, PA and the controls. The effectiveness of POH compared to control or PA was determined by Turkey's least significant difference (LSD). Differences with *p*-values less than 0.05 (*p*<0.05) were considered statistically significant.

Results

Dye-exclusion and colony formation assays were employed to test the effects of the monoterpenes alone, irradiation alone, or a combination of each monoterpene and irradiation on the HNSCC cell lines. Pretreatment with 1.0 mM PA reduced the number of viable cells by 10% in HTB-43, 19% in SCC-25 and 9% in BroTo cells respectively (Figure 1). Exposure of the cells to 2Gy dose of irradiation alone minimally reduced cell viability (Figure 1). When the cells were pretreated with 1.0 mM PA or 0.5 mM POH, and then exposed to 1Gy dose of irradiation, cell viability in each cell line was reduced as follows: HTB-43 (50% or 71%), SCC-25 (55% or 68%) and BroTo (18% or 53%), respectively (Figure 1). Similar results were obtained when the cells were pretreated with POH or PA and then exposed to 2Gy dose of irradiation (Figure 1).
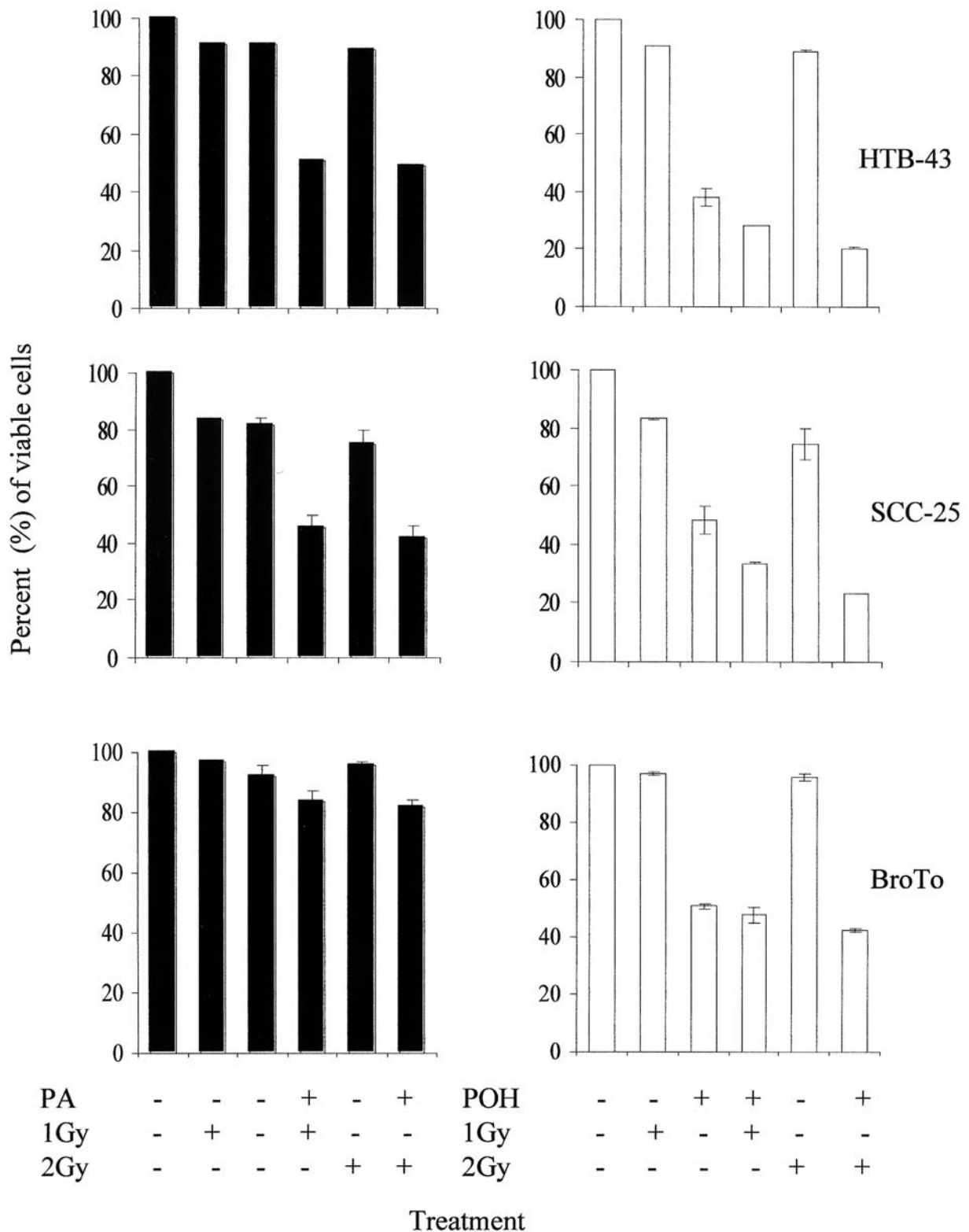
Figure 1. *Monoterpenes sensitized HNSCC cells to radiation treatment in vitro. Cells were treated with 0.5 mM perillyl alcohol (POH) or 1.0 mM perillic acid (PA) for 72 h then exposed to 1 or 2Gy of X-ray dose. Control cells, with or without monoterpenes, received no irradiation. Cells were harvested, stained with trypan blue and the proportion of viable cells relative to the control was determined. The figure represents a plot of Mean±SD (n = 3) of the percent of viable cells versus the respective treatment.*
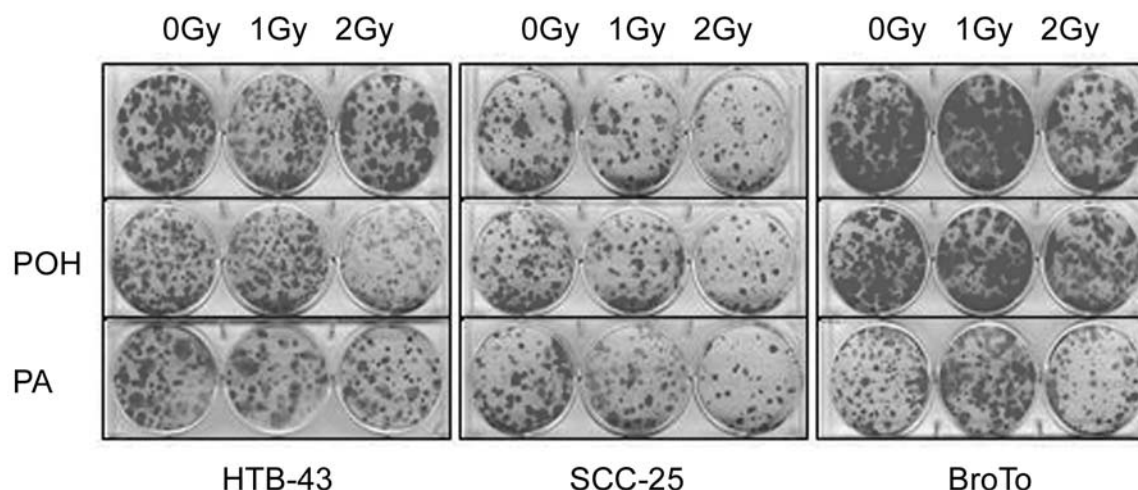
Figure 2. *Colony formation in HNSCC cells after combination treatment with monoterpenes and radiation. HNSCC cells (SCC-25, BroTo, HTB-43) were exposed to 0.5 mM perillyl alcohol (POH) or 1.0 mM perillic acid (PA) for 72 h and then exposed to 0, 1, or 2Gy dose of X-ray. Cells were harvested, washed with PBS, and cultured in 6-well plates (500/well) in complete medium for 14 days. Cells were then stained with crystal violet and colonies, containing at least 50 cells, were counted. Cell viability was calculated as a percentage of the colonies in treated group relative to that of the control groups.*

The long-term survival of cells at 14 days, when pretreated with monoterpenes followed by irradiation, was also investigated. Figure 2 shows the quantitative effect of the respective treatments on the cell lines. Fewer colonies were formed in groups pretreated with monoterpenes and exposed to irradiation compared to the controls (no treatment, radiation alone or chemotherapy alone) (Figure 2). Quantitative examination of the results (Figure 3) showed that pretreatment with monoterpenes followed by exposure to irradiation further inhibited proliferation of the HNSCC cells. The respective inhibition of proliferation in HTB-43, SCC-25 and BroTo cells were PA 35, 45 and 30% and for POH 60, 55 and 40% respectively when exposed to 1Gy dose of irradiation (Figure 3).

To determine the effects of the treatment on cell cycle progression, we performed cell cycle analysis on control and treated cells using a FACSCalibur flow cytometer (Becton Dickson) following propidium iodide staining. At 24 h, POH treatment induced a block in the G_0/G_1 phase in all three cell lines, while PA treatment blocked only BroTo cells in G_0/G_1 but had no such effect on the other cell lines (Table I). At 72 h, PA induced some G_0/G_1 arrest in all cell lines (Table I). Figure 4 shows a plot of the apoptotic index *versus* different treatments. Apoptotic index (AI) was calculated as a ratio of the mean apoptosis values in the treated groups divided by the mean apoptosis values of the respective control for each cell line. Calculation of the AI normalized the treatment effects, thereby facilitating a comparison of the response between cell lines. AI was highest for SCC-25 cells treated with POH and exposed to

2Gy dose of irradiation (Figure 4). The overall sensitivity of the cell lines were for POH: SCC-25>BroTo>HTB-43 and for PA: HTB-43≥SCC-25>BroTo (Figure 4).

Discussion

We tested a low dose (0.5 mM) of POH and a physiologically achievable level (1.0 mM) of PA as agents for use in combination chemo-radiation treatments. POH or PA alone minimally affected the viability (Figure 1) or proliferation (Figure 3) of HNSCC cells in culture. However, when the cells were pretreated with each monoterpene and then exposed to 1Gy dose of irradiation, the combination treatment with PA significantly ($p<0.05$) reduced the survival of HTB-43 and SCC-25 cells, while POH significantly ($p<0.01$) reduced survival in all the cell lines.

The reduction in viability was as a result of apoptosis induction as shown in Figure 4. Induction of apoptosis was assessed as the apoptotic index (AI), derived as a ratio of mean apoptosis values of treated cells to that of the control cells. POH, in combination with irradiation, induced higher apoptosis than PA (Figure 4), although the sensitivity of the cell lines appeared to follow similar trends: SCC-25≥HTB-43>BroTo. BroTo cells appeared less affected by monoterpenes and/or irradiation compared to SCC-25 and HTB-43 cells.

In the studies investigating the long-term effects, we found that pretreatment with monoterpenes followed by irradiation further inhibited the proliferation of the cancer cells (Figures 2 and 3). Our data confirmed that the
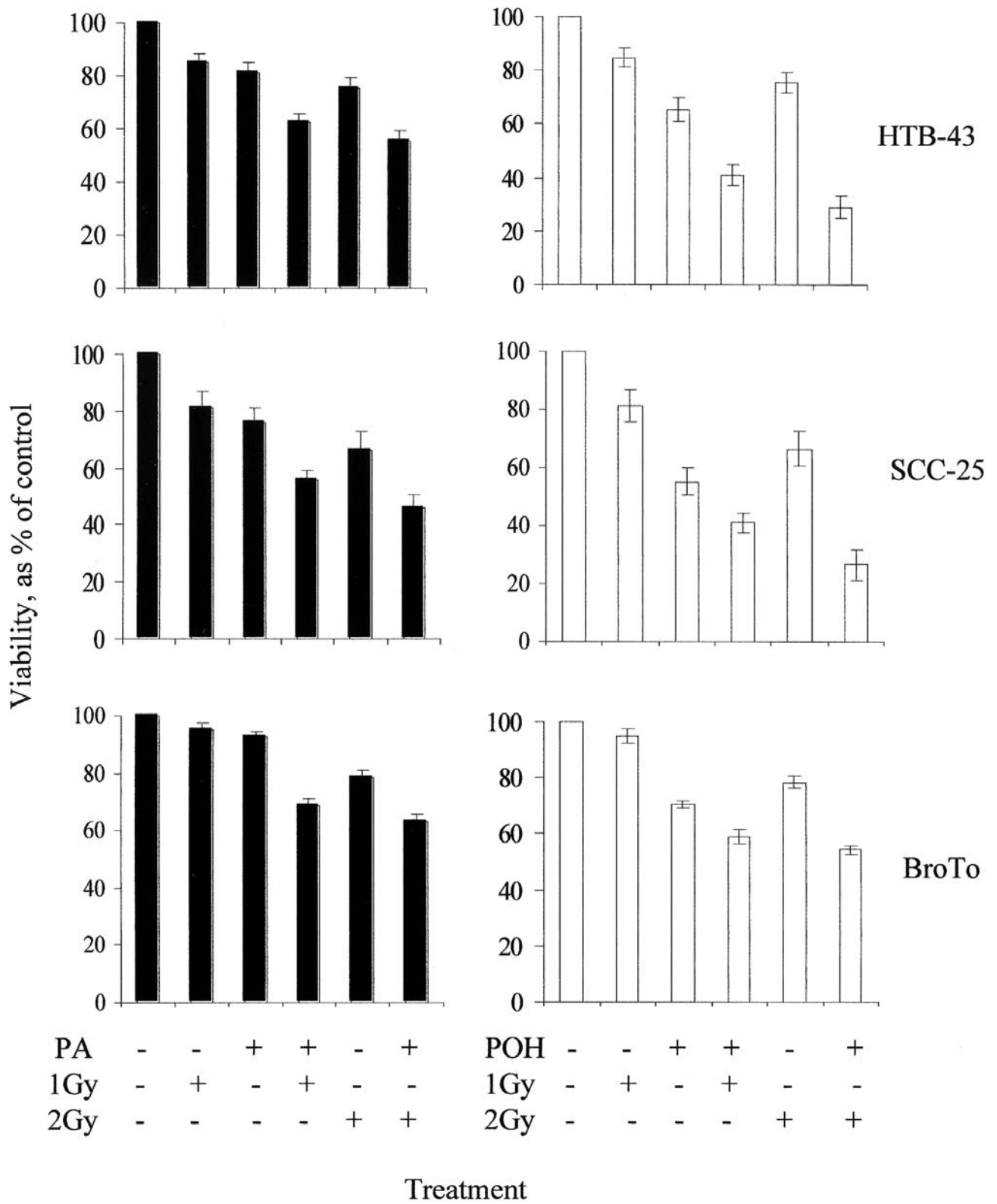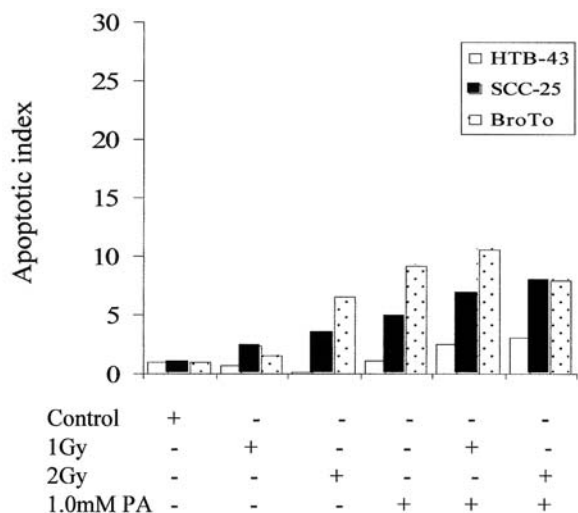
Figure 3. *Monoterpenes delayed proliferation of HNSCC cells in combination treatment with irradiation. The cells were treated as described in the legends for Figure 2.*

Tables I. *Cell cycle distribution of HNSCC cells treated with monoterpenes.*

Cell line	Treatment	24 h			72 h		
		G0/G1	S	G2/M	G0/G1	S	G2/M
HTB-43	Control	77.7 ± 0.5	9.7 ± 1.8	12.6 ± 2.3	43.5 ± 1.0	44.0 ± 1.2	12.5 ± 0.7
	0.5 mM POH	81.5 ± 1.8	0.2 ± 0.1	18.6 ± 1.3	49.5 ± 0.9	36.9 ± 1.2	13.6 ± 0.5
	1.0 mM PA	74.9 ± 1.3	14.7 ± 0.8	10.4 ± 2.0	49.4 ± 0.8	36.2 ± 0.6	13.6 ± 0.5
SCC-25	Control	46.4 ± 2.6	37.2 ± 1.4	16.4 ± 1.4	43.3 ± 2.7	37.6 ± 0.7	19.2 ± 3.5
	0.5 mM POH	60.9 ± 2.5	36.7 ± 5.2	2.4 ± 2.7	52.5 ± 1.62	39.1 ± 1.1	8.5 ±2.3
	1.0 mM PA	46.7 ± 0.5	41.0 ± 1.7	12.3 ± 2.2	62.9 ± 1.7	36.9 ± 0.7	14.6 ± 1.4
BroTo	Control	58.1 ± 1.0	26.2 ± 4.4	15.8 ± 5.4	60.9 ± 0.3	30.2 ± 0.4	8.8 ± 0.1
	0.5 mM POH	72.4 ± 2.9	17.8 ± 2.9	9.7 ± 2.5	57.4 ± 0.7	33.1 ± 0.5	9.5 ± 0.3
	1.0 mM PA	72.4 ± 5.15	21.1 ± 5.4	6.5 ± 0.3	68.0 ± 0.25	22.2 ± 0.2	9.8 ± 0.3

HNSCC cells were harvested after exposure to perillic acid (PA) or perillyl alcohol (POH) for 24 or 72 h. The cells were fixed in ethanol and stained with propidium iodide. DNA content data were acquired using BD FACSCalibur and CellQuest acquisition software (Becton Dickinson). The data were analyzed using ModFit LT version 3.0 analysis software (Verity Software House, Topsham, ME, USA). Values are Mean±SD (n=3). The table is representative of two separate experiments.

monoterpenes sensitized HNSCC cells to irradiation in combination chemoradiation protocols. These results are in agreement with the recent reports by Rajesh *et al.* (18) that pretreatment of glioma cells with POH sensitized the cells to irradiation. These authors further concluded that the mechanism of action included an enhanced induction of apoptosis in the treated cells, although they used higher radiation doses (5Gy) than in the present study (0-2Gy). Our data indicated that the monoterpenes, POH and its metabolite PA, acted as radiosensitizers in combination chemo-radiation treatment of head and neck squamous cell carcinoma cultured *in vitro*. Further studies are in progress to determine the molecular events involved in the radiosensitization.
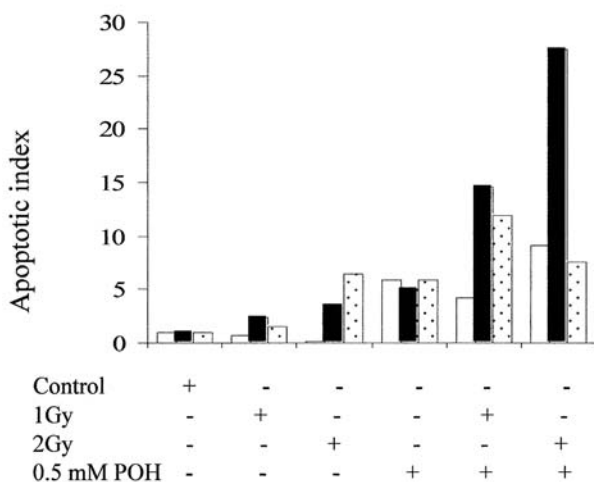
Acknowledgements

Figure 4. *Monoterpenes induced apoptosis in HNSCC cell lines. Human head and neck cancer cell lines were treated with 1.0 mM perillic acid (PA) or 0.5 mM perillyl alcohol (POH) for 72 h. At termination, the cells were harvested, washed with PBS, fixed in ethanol and stained with propidium iodide. The percentage of cells in sub-G_0/G_1 (apoptosis) was quantified using FACSCalibur flow cytometer. The figure represents a plot of the apoptotic index (AI), calculated as a ratio of the mean apoptosis values in the treated groups divided by the mean apoptosis values of the respective control for each cell line, versus the treatments.*

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