Perillyl alcohol and perillic acid enhanced the sensitivity of head and neck cancer cells to radiation treatment in vitro

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PERILLYL ALCOHOL AND PERILLIC ACID ENHANCED THE SENSITIVITY OF
HEAD AND NECK CANCER CELLS TO RADIATION TREATMENT IN VITRO

by

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A thesis submitted in partial fulfillment
of the requirements for the

Master of Science in Biochemistry
Chemistry Department
College of Sciences

Graduate College
University of Nevada, Las Vegas
August 2004
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Entitled

Perillyl Alcohol and Perillic Acid Enhanced the Sensitivity of Head and Neck Cancer Cells to Radiation Treatment In Vitro

is approved in partial fulfillment of the requirements for the degree of

Master of Science in Biochemistry

Examination Committee Chair

Dean of the Graduate College
ABSTRACT

Perillyl alcohol and perillic acid enhanced the sensitivity of head and neck cancer cells to radiation treatment in vitro

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Limonene and perillyl alcohol are the naturally occurring monoterpenes found commonly in plant fruits and vegetables. Perillyl alcohol (POH) elicited anticarcinogenic effects in a number of cancer models and pharmacokinetic studies in humans revealed that perillic acid (PA) is the major circulating metabolite following POH administration. Effects of these monoterpenes combined with and without radiation on human head and neck squamous cell carcinoma cell lines (HNSCC) were measured using cytotoxicity and flow cytometry assays. HNSCC cells were pretreated with 1.0 mM PA or 0.5mM POH for 72 hr before exposure to 1Gy or 2Gy radiation dose. Pretreatment with 1.0mM PA or 0.5mM POH sensitized the cells to radiation, causing growth inhibition in HTB-43 (50% and 71%), SCC-25 (55% and 68%) and BroTo (18% and 53%) cells, respectively. PA induced cell-cycle arrest in G0/G1 phase, caused cell death via apoptosis and a reduction in p53 protein in SCC-25 cells.
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ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor Dr. J. Abiodun Elegbede for his help, stimulating suggestions and encouragement for the past two years throughout my research thesis work. His constructive criticism, patience and guidance have been my motivating factors allowing me to accomplish this research work successfully. It was a great pleasure to me to conduct this thesis under his supervision.

Secondly, I would like to express my appreciation to other members of my examination committee Dr. Bryan Spangelo, Dr. Ronald Gary and the graduate college representative Dr. Susan Meacham, for their constructive comments during my research and thesis writing. I would also like to thank Dr. S. Carper and Dr. Bhowmik for their thoughtful suggestions and support. My thanks also go to Mr. John Adebayo and Nicole Stevens for their technical support and Aruna for statistical analysis of my research. My sincere gratitude to Dr. Beau J. Toy of the Radiation Oncology Centers of Las Vegas and Henderson for allowing me to use their radiation facility and for his suggestions and technical support.

My appreciation also goes to my fellow colleagues in our research group, Daniel E. Ezekwudo, Lakshmi Yeruva, Chinelo Nwosu, Pamela Ogba and Qais Naziri for their presence in the lab and support which assisted me throughout my research. Thanks to Dr. Isaac Achoba who is another milestone in my life. I would like to thank my parents Mrs. Rebecca S. Hena and Mr. Samaila Hena, my guardians Mr. and Mrs. Gana Malgwi, and my darling wife Azira, for their unwavering love, support and prayers. This work was
supported in part by grants from the UNLV Planning Initiative Award and ACS#IRG-103719 (to JAE).
CHAPTER 1

INTRODUCTION

1.1 Purpose of the study

The rate of occurrence of head and neck squamous cell carcinomas (HNSCC) "account for about 13,000 of all cancer deaths" in the United States (Cancer Facts and Figures 2004). Currently, combination therapy, drug treatments followed by radiation elicit higher survival rates and more effective in preserving of organs in the body than other cancer treatment (Kies et al., 2001; Vokes et al., 2003). This treatment option cure rate is higher than the cure rate for the well known traditional method of treating head and neck cancer using surgery with radiation therapy. Treatment of local and regional head and neck squamous cell carcinoma, in combination with therapy using drugs like fluorouracil, paclitaxel and hydroxyurea with radiation control local and regional head and neck cancer better than either radiation alone or drugs (Kies et al., 2001; Vokes et al., 2003). Though chemo-radiotherapy showed a better cure rates for treating this type of cancer with increased survival than other forms of therapy, the drugs commonly tested were toxic to the body causing low level of white blood cells (i.e. leucopenia and thrombocytopenia) (Kies et al., 2001; Vokes et al., 2003). Therefore, there is need to develop new agents that will be less toxic to the body and increase survival rates (Kies et al., 2001; Vokes et al., 2003).
The naturally occurring plant monoterpenes are constituents of many fruits and vegetables commonly consumed by humans namely, oranges, spearmint, cherries and others (Alonso et al., 1992; Kjonaas and Croteau, 1983). The monoterpenes, limonene and perillyl alcohol (POH), were reported to elicit anticarcinogenic effects in a number of animal studies (Elegbede et al., 1984, 1986, and 1993) and are currently undergoing clinical trials in the US and Britain (Ripple et al., 2000; Murren et al., 2002; Chow et al., 2002). Pharmacokinetic studies of POH in humans revealed that the major circulating metabolite is perillic acid (PA) (Ripple et al., 1998; Ripple et al., 2000; Murren et al., 2002; Chow et al., 2002). This implies that PA may be involved in the mechanism of action of POH. The goal of this research is to investigate if PA will sensitize head and neck cancer cells to radiation effects when used in combination chemo-radiation treatment \textit{in vitro}.

1.2 Research questions

The mechanism of action of the monoterpenes has not been fully established, it has been reported that monoterpenes are involved in regulation of some cellular activities in cell growth, including the up-regulation of mannose-6-phosphate/insulin-like growth factor II receptors (Jirtle et al., 1993), increased tissue growth factor receptors (Jirtle et al., 1993) and the inhibition of prenylation of small GTP-binding proteins (Crowell et al., 1991; Kawata et al., 1994). Other reported activities include the induction of phase I and phase II detoxification enzymes (Elegbede et al., 2002), expression of Bak and programmed cell death via apoptosis (Stayrook et al., 1997). This research work will
elucidate the possible mechanism of action of PA as a radio-sensitizer of HNSCC cells in culture. This work will attempt to answer some of the following research questions:

i) Will PA sensitize head and neck cancer cells to radiation in chemo-radiation treatment?

ii) Will PA cause cell cycle arrest?

iii) Will the radio-sensitization effect of PA be due to cell death via apoptosis?

iv) Will PA affect the expression of p53 protein levels?

1.3 Significance of the study

For many years research has been done on the risk factors associated with cancer and on factors possibly associated with the prevention of cancer development. The identification of possible agents commonly found in the food we eat that may be helpful in preventing cancers shows great promise (Hakim et al., 2000). Progress in HNSCC treatments has involved combination therapies that include naturally occurring substances in foods. Treatment of HNSCC using drug alone was reported to minimally increase the response rate. However, due to low rates of survival and high levels of toxicity with the common chemotherapeutic drugs and low cure rates with traditional methods (Landis et al., 1998), efforts have been redirected at the use of drugs that are less toxic in combination with radiation to produce better cure rates (Rajesh et al., 2003). Synthetic drugs such as fluorouracil, hydroxyurea or paclitaxel are generally very toxic which underlies the necessity for developing newer agents that are less toxic for chemoradiotherapy (Vokes et al., 2003). Since monoterpenes are found naturally in plants commonly consumed by humans, are less toxic to the body they have the potential to
serve as radio-sensitizers in the combination chemo-radiation treatment of HNSCC cells 
\textit{in vitro}. Since there is no information in the treatment of HNSCC using PA in 
combination with radiation, this research will broaden our knowledge regarding the effect 
of PA as a new agent with less toxicity in the treatment of HNSCC in chemo-radiation 
treatment \textit{in vitro} and possible mechanism of inhibition.

1.4 Hypothesis

Since PA is found to be the major circulating plasma metabolite in humans after the 
administration of POH, we therefore hypothesized that PA may be affecting cancer cell 
cycles, thereby sensitizing the cells to irradiation effect. To test this hypothesis, we 
proposed to investigate and confirm that pretreatment of head and neck cancer cells with 
PA will sensitize the cells to radiation effects.
CHAPTER 2

LITERATURE REVIEW

Another group of plant compounds that received attention as chemopreventive agents in cancer therapies are the monoterpenes. Monoterpenes are naturally occurring nonnutritive plant compounds that are synthesized via the mevalonate pathway in plants (Chayet et al., 1977). These compounds function physiologically in plants as chemorepellents and chemoattractants against insects and exhibit distinctive fragrances observed in many plants (as reviewed by McGarvey and Croteau 1995). d-limonene (Fig. 1, p.7), the parent monoterpane synthesized through the mevalonate pathway serves as the precursor for the synthesis of other oxygenated monoterpenes including perillyl alcohol, POH (Fig.1, p.7), menthol, perillylaldehyde, and carveol (as review by McGarvey and Croteau 1995). D-limonene and POH are the two naturally occurring monoterpenes (Fig.1, p.7) found in the essential oils of citrus fruits as well as caraway, lavender, spearmint and cherries (Alonso et al., 1992; Kjonaas and Croteau, 1983).

Food commonly consumed by humans contains a large variety of compounds that either serve as blocking or suppressing agents in the inhibition of tumors in the body (Wattenberg, 1983 and 1985). These are often plant compounds (carotenes, phenols, methylated flavones and plant sterols) which may either have preventive or blocking activity against cancer. One of the earliest studies of this kind was reported by Wattenberg (1983). Wattenberg studied the blocking effects of some green coffee beans
compounds on neoplasia. One basic role of blocking agents is the ability to induce phase I and phase II detoxification enzymes in the liver that prevent toxicity and tumor development. Induction of glutathione S-transferase activity is being used as a measure for determination of blocking effect of kahweol palmitate and cafesol palmitate isolated in the green coffee beans. Wattenberg’s group used rats induced with the chemical carcinogen 7,12-dimethylbenz(a)anthracene and found that kahweol palmitate and cafesol palmitate obtained from these coffee beans increased the activity of glutathione S-transferase which led to inhibition of mammary neoplasia (Wattenberg, 1983).

Monoterpenes effects on human skin squamous cell carcinomas have been recently studied. Hakim et al. (2000) conducted a cohort study in an Arizona population commonly known for growing and consuming fresh citrus fruits. They compared the history of squamous cell carcinoma of the skin and citrus consumption in the population using a questionnaire for assessment of citrus consumption prepared with citrus pulp and peels supplemented as food among the participants. The predominant types of citrus prepared in this cohort study were grapefruits, orange and orange juice with grapefruit having the highest percentage. Based on their findings there was no positive “association” between citrus consumption and squamous cell carcinoma of the skin in the subjects (Hakim et al., 2000). However, the level of citrus peel intake and SCC was found to be significant due to the high concentration of limonene found in citrus peel compared with other citrus content which has low concentration of d-limonene (Hakim et al., 2000).

In rat, Crowell (1992) reported the systematic availability of the metabolites perillic acid (PA) and dihydroperillic acid, (DHPA) (Fig.1, p.7) after administration of d-
limonene. Further study was done in vitro on the inhibition effect of PA, on a murine fibroblast cell line (NIH3T3). Rats were fed 1 g limonene per kg body weight in corn oil. The rats were sacrificed through ether inhalation while blood and tissue samples were collected for analyses of limonene, PA and DHPA. The levels of limonene, PA and DHPA were detected using gas chromatography at low concentrations in samples collected after 1 hr of feeding, i.e., in lung (0.48 μmol/g), serum (0.62 μmol/g) and in kidney (1.09 μmol/g). In contrast, higher concentrations were found in adipose (2.14 μmol/g) and mammary gland tissues (1.10 μmol/g) (Crowell et al., 1992).

Comparison of the effects of limonene or PA on cell proliferation of murine fibroblast cells showed that PA is more effective than limonene after 4 days exposures while limonene minimally inhibited the viability of cells while 3 mM PA caused complete inhibition of cell growth (Crowell et al., 1992).

Figure 1 Structures of the monoterpenes.
2.2 Chemopreventive activities of monoterpenes

Monoterpenes have been reported to possess either blocking or suppressing effects against many different cancer models. In 1994, using orange peel oil containing about 90-95% d-limonene, Crowell et al used fasting men and women (23-55 yr) and were asked to refrain from taking citrus fruits or soft drinks for a period of 48 hr. Each person was given 100mg per kg d-limonene in a semisolid custard-like food containing 5% orange. Blood was then drawn from each subject before and twice ingestion after 4 and 24 h. The level of toxicity of the plasma metabolites was monitored in all participants using capillary gas chromatography. After 4 hr post-ingestion the level of PA (35μM) was predominant, followed by DHPA (33μM) and limonene-2-ol (16μM) after 4 hr post-ingestion (Crowell et al., 1994b). No intrasubject change in d-limonene and metabolites was observed in samples analyzed after the intake of limonene but four out of the seven participants suffered from mild eructation while others reported mild satiety and slight fatigue (Crowell et al., 1994b).

In 1984, Elegbede et al. determined the effects of d-limonene in rats fed for a week before induction of tumor by DMBA. When rats were treated with limonene for one week and subsequently treated with DMBA for a period of 27 weeks it caused a decrease in mammary tumor development compared to the control groups (Elegbede et al., 1984). Elegbede et al. (1986) studied the effects of orange oil and d-limonene in mouse skin tumors in two-stage skin carcinogenesis induced by 7,12-dimethylbenza[a]-anthracene (DMBA) given in 0.2 ml of acetone. The control group received 0.2 ml acetone while the orange oil and d-limonene were administered topically or by dietary intake. Testing started on the 7th day and lasted for another one-week period in which the tropically
tested mice were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA), orange oil and d-limonene all in acetone mixture. Before the 10th week post-initiation, they weight the treated animals and during the initial 4 weeks, they monitored the diet intake and observed that no palpillomas developed in the treated mice given either orange oil or d-limonene after initiation with DMBA. In animals that were induced with DMBA followed by TPA, the rate of papilloma development was observed to be higher than their first observations and that high rate of papilloma occurrence was result of the effectiveness of orange oil compared to d-limonene (Elegbede et al., 1986). In another study using 3 day old female Wistar-Furth rats Elegbede et al (1993), used limonene (5%) and sorbrerol (1%) to study the blocking effect when DMBA is used to initiates carcinogenesis. The liver enzymes glutathione transferase (GST) and uridine diphosphoglucuronosyl transferase (UDPGT) function in the liver to prevent carcinogen interaction with DNA. They used the level of detoxification enzymes to measure the effectiveness of limonene intake. Both the limonene and sobrerol were reported to increase the activity of GST and UDPGT in rat livers (Elegbede et al., 1993). Another observation was that both limonene and/or sorbrerol diets significantly (P<0.05) caused the level of non-protein sulphydryls to be elevated compared to the glutathione levels observed earlier (Elegbede et al., 1993).

To further elucidate the chemopreventive activities of monoterpenes Haag and Gould (1994) used 70 Wistar-Furth rats to investigate the preventive effect of administering POH on rats given DMBA to initiate papilloma development. “Complete regression was defined as the nonpalpability of the tumor for 3 weeks. Partial regression was defined as half or less of the original diameter of the tumor induced via DMBA that was palpable”
(Haag and Gould 1994). After first mammary tumor induction, 2.5% POH caused a complete regression of the tumor in 22 out of 27 rats after 3 weeks compared to control group which had only 9 out of 29 showing complete tumor regressions (Haag and Gould 1994). In a second study using 215 rats treated with chemical carcinogen DMBA, they also reported that 1% POH only showed significant level of tumor regression and the redifferentiation of tumor cells observed may have been the POH administered (Haag and Gould, 1994).

Mills et al. (1995) used Fischer 344 rats treated with diethylnitrosamine (DEN) to investigate the effect of POH on carcinogenesis. After 19 weeks of daily replacing the POH, they found out that the mean liver tumor weights of the rats treated with POH were about 10-fold less compared with control animals. To elucidate the possible means of action in the liver tumor, they confirmed that POH induced apoptosis, caused increased levels of manose-6 phosphate/insulin growth factor and transforming growth factor β type I, II and III receptors in all the treated groups (Mills et al., 1995).

2.3 Biosynthesis of monoterpenes from mevalonate pathway

There are two distinct pathways that plants used for the biosynthesis of monoterpenes. One pathway involves the condensation reaction occurring in the cytosol of two acetyl-CoA via the mevalonate pathways to form isopentenylpyrophosphate (IPP) which is the precursor of isoprenoids. The other pathway occurs in the plant plastid involves the condensation of pyruvate and D-glyceraldehyde-3-phosphate catalyzed to yield IPP. This reaction is catalyzed by a transferase (as reviewed by McGarvey and Croteau, 1995).
Figure 2. Synthesis of 5-phosphomevalonate from the condensation of two acetyl-CoA units via the mevalonate pathway (Adapted from McGarvey, D. J. and Croteau, R. 1995).
Figure 3. Synthesis of geranyl pyrophosphate from 5-phosphomevalonate, precursor molecule for monoterpenes synthesis (Adapted from McGarvey, D. J. and Croteau, R. 1995).
Figure 4. Cyclization of geranyl pyrophosphate to form the naturally occurring monoterpenes limonene and perillyl alcohol (Adapted from McGarvey, D. J. and Croteau, R. 1995).
The synthesis of monoterpenes starts with the condensation reaction of two acetyl-CoA units catalyzed by acetyl-CoA acetyltransferase to form acetoacetyl-CoA (Goldstein and Brown, 1990). The HMG-CoA synthase catalyzed the next reaction involving the addition of another acetyl-CoA unit to form β-hydroxyl-β-methylglutaryl-CoA (HMG-CoA) (Fig.2, p.11). The next step which is “a rate limiting step in cholesterol biosynthesis in animals” (as reviewed by McGarvey and Croteau, 1995), the HMG-CoA undergoes a reduction reaction in the presence of NADPH catalyzed by HMG-CoA reductase to form mevalonate (Fig.2, p.11). Mevalonate is then phosphorylated by the kinase enzyme called mevalonate kinase to form 5-phosphomevalonate which undergoes another phosphorylation reaction catalyzed by phosphomevalonate kinase, to form 5-pyrophosphomevalonate (Fig.2, p.11). This compound is further decarboxylated by the action of pyrophosphomevalonate decarboxylase to form isopentenylpyrophosphate, IPP (Fig.3, p.12) (as reviewed by McGarvey and Croteau, 1995). The IPP is polymerized with its isomer 3, 3-dimethylallyl diphosphate by prenyltransferases to form the monoterpenes precursor geranylpyrophosphate (GPP) (Fig.3, p.12). Limonene synthase catalyzes the cyclization of geranylpyrophosphate via a dephosphorylation reaction to form limonene (Fig.4, p.13). Limonene is catalyzed by limonene 7-monooxygenase to form perillyl alcohol (oxygenated hydroxyl product) in the presence of NADPH (Fig.4, p.13).

2.4 Mechanism of action

Chemopreventive agents may have either cancer blocking or suppressing activity against different types of tumors (Wattenberg 1983 and 1985). Blocking effects occurred
during the initiation phase of carcinogenesis to prevent the interaction of the chemical carcinogens with DNA via modulating carcinogen metabolism to less toxic forms later eliminated in the body. The suppressing chemopreventive agents act differently by preventing the development of initiated cells during the promotion phase of carcinogenesis (Wattenberg, 1985). The mechanism of tumor regression by monoterpenes still remains unclear but several cellular activities have been reported to be related with monoterpenes. These mechanisms are thought to include induction of cell cycle arrest or blocking of the G₁ phase, programmed cell death via apoptosis (Shi et al., 1997) and activation of phase I and phase II detoxification enzymes (Matlzman et al., 1991; Austin et al., 1988). Other effects include the inhibition of the post-translational modification of the covalent attachment of farnesyl or geranylgeranyl groups to the carboxylic terminus of GTP-binding proteins involved in signal transduction (Clarke et al., 1992; Crowell et al., 1994). Elegbede et al. (2003), using human carcinoma cell lines (BroTo and A549), reported the effect of POH and perillaldehyde (PALD) administered \textit{in vitro} for 12 to 24 hr. POH or PALD caused a dose-dependent inhibition of cell growth on BroTo and A549 cells (Elegbede et al., 2003). They found that 1.0 and 1.2 mM POH caused 50% inhibition in BroTo and A549 cells while concentration of 3.2 and 3.0 mM for PALD was reported after 24 hr (Elegbede et al., 2003). The mode of cell death was via apoptosis when cells were treated with POH, while PALD related cell death was thought to be through a possible pathway that is yet unknown (Elegbede et al., 2003). The effect of POH was reported to be protective against colon cancer and other types of cancers by induction of the detoxification enzymes in liver (Maltzman et al., 1991). They
reported that 5% limonene diet increases the activities of cytochrome p450 family members and epoxide hydratase (Maltzman et al., 1991).

Reddy et al. (1997) studied the effect of POH on rats induced with the carcinogen azoxymethane (AOM). They treated rats with AOM after being fed either POH/kg body weight or saline for two weeks and the rats remain on POH or the vehicle for 55 weeks. The percentage of control rats with invasive colon adenocarcinomas was observed to be greater than the treated group (Reddy et al., 1997). Another observation found was that the rate of occurrence and diversity of noninvasive colon adenocarcinomas in these rats was unaffected (Reddy et al., 1997). For some unknown reason, they discovered that the 2g POH diet significantly (p<0.001) inhibited the incidence of total colon adenocarcinomas and small intestinal adenocarcinoma (Reddy et al., 1997). However, the apoptotic index was increased by about 5 to 6-fold when rats were given either 1g or 2g POH per body weight diet compared to the control groups (Reddy et al., 1997).

Stayrook et al. (1997) studied the treatment of malignant hamster pancreatic ductal epithelial cells and nonmalignant hamster pancreatic ductal epithelial cells with thymidine incorporation of POH. From the data acquired, POH caused apoptosis in malignant cells treated with varying concentrations of POH. The increase in the apoptotic populations increased proportionately with increase concentration of POH causing higher rates of apoptosis (Stayrook et al., 1997). The population of cells that undergo apoptosis in nonmalignant cells was minimal compared to the malignant cells and POH also caused a two-fold increase in Bak expression in malignant hamster pancreatic ductal epithelial cells while its expression remained unaltered in non-malignant cells (Stayrook et al., 1997).
Several human clinical trials conducted have revealed that PA is the most abundant circulating plasma metabolite followed by DHPA after administration of POH in humans (Ripple et al., 1998; Ripple et al., 2000; Murren et al., 2002). Ripple et al. (2000) conducted a phase I clinical trial of POH administered to subjects with incidence of advanced refractory malignancies. The administration of POH to these subjects was done four times per day in a continuous manner at 3 different doses of 800mg/m$^2$, 1200mg/m$^2$ and 1600mg/m$^2$. They administered POH to subjects in soft gelatin capsules containing 250mg of POH and 250mg of soya bean oil. They observed an increased in the levels of PA and DHPA in the plasma to occur at 1-2.5 hr and 2-3.5 hr respectively after post-ingestion of POH (Ripple et al., 2000). The level of plasma metabolite as reported in their work of 1998 for PA was found to be predominant than DHPA (Ripple et al., 2000). After 29 days of POH administration, they noticed that the level of PA or DHPA in the plasma was decreasing with time and the maximum tolerated dose of POH to be 1200mg/m$^2$ (Ripple et al., 2000). Chow et al. (2002) using healthy individuals to determine the systemic availability of PA when administered with a single dose of Mediterranean-style lemonade rich in limonene. They started taking blood samples from subjects before given them light breakfast containing limonene in the form of Mediterranean-style lemonade over a time period of 10-20 minutes. Blood samples after completion of the lemonade intake, were again drawn at different time points and analyzed the level of limonene in juice and plasma level of PA. Rapid increased in the plasma level of PA after 1 hr consumption was observed and declined rapidly as a function of time with the half life of PA ranging from 0.82 to 1.84 hr (Chow et al., 2002). PA levels were undetected after 24 hr in all the samples collected (Chow et al., 2002). Brown et al.
(2000) investigated the effects of epidermal growth factor receptor (EGFR) in combination with either cisplatin or 5-fluorouracil (5-FU) on the growth rate of human squamous cell carcinoma (SCC) SCC-25 and BroTo cell lines in vitro. They reported that EGFR are found on the surface of many different tumor cell lines with IgG monoclonal antibodies (Mabs). When they treated the cells with cisplatin or 5-FU and then exposed to different concentrations of epidermal growth factor receptor (EGFR Mab), the cell growth of SCC-25 cells was significantly (p < 0.001) decrease compared to the cells treated with cisplatin alone (Brown et al., 2000). They also reported that both BroTo and SCC-25 showed significant inhibition with EGFR Mab and 5-FU compared with 5-FU alone (Brown et al., 2000).

In a study to determine the possible involvement of PA in cell proliferation, Ferri et al. (2001) investigated the possible mode of action of PA on rat arterial smooth muscle cells (SMC). When cells were treated with 1-3 mM concentrations of PA for up to 72 hr, PA caused a time- and concentration-dependent inhibition of SMC cells and a decrease in cholesterol biosynthesis in the cells. The incorporation of mevalonate, farnesol or geranylgeraniol did not influence the inhibition effect exerted by PA (Ferri et al., 2001). To find out whether the prenyl transferases are the possible target for PA, the incorporation of radiolabelled farnesol or geranylgeraniol into the cellular proteins was blocked by PA in the cells. With this result, their concluded was that PA inhibits cell growth via prevention of prenylation of proteins in the SMC cells and the concentrations of PA (1-3 mM) that significantly (p < 0.01) inhibited SMC cell growth was in correlation with 0.47 mM plasma concentration that was achieved by Ripples et al. 2000 (p.88) in their clinical studies using POH on patients with malignancy (Ferri et al., 2001).
2.4 Combination chemoradiotherapy

It was reported that the sensitivity of radiation exposure on cell growth in different types of cancer cells is dependent on which phase of the cell cycle the cells are in and the effect of radiation sensitivity can be improved when used in combination with other chemotherapeutic agents (Rotman and Aziz 1998). In 1993, Gordon and his group reported that many chemotherapeutic agents may stop the repair of radiation damage by cancer cells which implies the possibility of synergistic or additive action between chemotherapeutic agent and radiation when used in combination chemo-radiation therapy (Gordon et al., 1993). Combination treatment using drugs and radiation has been tested in clinical practice in malignant tumors (Rotman and Aziz 1998 and Vokes et al., 2000). Many randomized clinical trials were performed with drugs and radiation which indicated that certain chemotherapeutic agents have the ability to inhibit radiation resistance seen in other types of cancer cells thereby improving sensitization to radiation effect (Kim et al., 2000).

Kies et al., (2001) studied the use of combining drug and radiation in the treatment of head and neck cancer and reported that the intense radiation exposure and chemotherapy drugs, when delivered together in concurrent combination treatment was highly successful at controlling the local spread of head and neck cancer in subjects with this advanced disease (Kies et al., 2001). They reported that when they combined the known chemotherapeutic drugs (paclitaxel, fluorouracil and hydroxyurea) with a prolonged twice daily radiation treatment, the outcome was a successful management of the local and regional spread of head and neck cancer in about 90% of the patients (Kies et al., 2001). The overall survival rate of 55% observed at three years, which they said was
encouraging, but the treatment could not prevent distant metastasis which occurs in 20% of patients (Kies et al., 2001). Even though they maintained that the study was an improvement in treatment of head and neck cancer, the particular drugs used were too toxic to the patients which led to death of other patients. In all the subjects used for this studied, 81% was reported to experience temporary severe drops in white blood cells and platelets, which is common with chemotherapy and a severe constrained to eat normally for at least a year (Kies et al., 2001).

In a current study by Vokes et al., (2003) combination chemo-radiation therapy was performed with patients with stage IV head and neck cancer. They administered 6 doses of carboplatin and paclitaxel weekly followed by twice daily radiation dose at 1.50 Gy per fraction for 5 cycles. They observed high response rate and reduction in distant failures rates among the subjects (Vokes et al., 2003) and concluded that a treatment approach which caused high survival rates and progression with preservation of organ in a population of stage IV patients with advanced locoregionally carcinoma of the head and neck cancer has been elucidated (Vokes et al., 2003). A phase II trial was carried out using chemoradiotherapy in order to achieve locoregional control of head and neck cancer with high survival rate and preservation of organ. Patients were treated with cisplatin administered every 28 days, followed by 5 days infusional fluorouracil and 12 hr oral administration of hydroxyurea for 11 doses before 5 days twice daily radiation exposure at 1.5 Gy per fraction. Subjects with locoregional advanced stage IV of the head and neck cancer were selected specially those with cancer of the larynx, oral cavity, paranasal sinuses, cervical esophagus and pharynx. It was reported that treatment using chemo-radiotherapy results in a locoregional control rate that exceeds 90% among the
subjects (Vokes et al., 2003). Their conclusion was that using agent that are less toxic to subjects in combination therapy, toxicity can be avoided when used in future studies and, possibly, radiation protective agents (Vokes et al., 2003).

The HNSCC account “for approximately 45,000 new cases and 13,000 deaths per year in United States” (Cancer Facts and Figures 2004). Most of these cancers begin in the oral cavity and the traditional treatment option for locally advanced HNSCC is surgery followed by intensive radiation regimen. However, these treatment modalities are associated with high morbidity with low survival rates (Stupp et al., 1987). “The way a particular head and neck cancer response to any treatment options depends on the primary site of occurrence in the body. For instance, cancers that initiate on the vocal cords behave in a differently manner than do those that arise in the larynx indicating different response rates” (Al-Sarraf M., 1988; Landis et al., 1998). Squamous cell carcinoma is the most common type of head and neck cancer which arises in the cells that line the inside of the oral cavity, throat and nose. The uncommon types of head and neck cancers include sarcomas salivary gland tumors and lymphomas (Landis et al., 1998). The three main types of treatment options for head and neck cancer was reported to include radiotherapy, surgery and chemotherapy. The primary treatment option for head and neck cancer are radiation therapy or surgery, or combination of the two; with chemotherapy sometimes used as an adjuvant (Landis et al., 1998). The best treatment option for a particular head and neck cancer depends on the time of diagnosis, site of incidense and the extent of the tumor developed (Landis et al., 1998). Recent findings in using drugs and radiation indicate that combination of chemotherapy given at the same
time with radiation therapy exhibited a more effective treatment option with higher survival rates than either single traditional treatment modality (Vokes et al., 2003).

Rajesh et al., (2003a) first tested the importance of POH as sensitizer in radio- and chemo-sensitization treatment using malignant glioma cell lines. Concentration of 0.5 mM POH and 5.5 Gy dose of radiation were used in combination treatment on glioma cells to test the effect of pretreatment of POH as radio-sensitizer. They pretreated cells with POH at intervals of 2 and 24 hr and then irradiated the cells with 5.5 Gy and the cells were immediately replated for 2 weeks in tissue culture plate with fresh medium. After incubation, the earliest onset of radio-sensitization by POH was observed after 8 hr of treatment (Rajesh et al., 2003a). Cell cycle analysis on glioma cell line (T98G) treated with POH caused a dose and time-dependent increase in sub-G0/G1 cell population and a block at G2/M phase of the cell cycle (Rajesh et al., 2003a). Cells treated with 1 mM POH exhibited increased in early apoptotic population positive for Annexin V only. Their data indicated that apoptosis and radio-sensitization of glioma cells by POH was via the expression of Fas ligand and Fas receptor (Rajesh et al., 2003a). In another study, Rajesh et al. (2003b) using prostate cancer cells, cells were treated with 0.1-0.5 mM POH for a period of 72 hr and then exposed to different doses of radiation. They observed a significant reduction of the cell growth in DU145 and PC-3 cells when treated with concentrations between 0.7 and 1 mM POH (Rajesh et al., 2003). Pretreatment of these cells with POH also elicited a dose- and time-dependent sensitization of the prostate cancer cells to radiation (Rajesh et al 2003b). The authors then treated DU145 and PC-3 cells with POH for 72 hr in the presence and absence of 5.5 Gy dose of radiation. They reported an increase in the population of cells in sub-G0/G1 and early apoptotic cells.
positive for Annexin V (Rajesh et al., 2003b). They also reported an increase in the 
$G_2/M$ phase and a decrease in the $G_0/G_1$ phase of the cell cycle which appeared to 
precede the onset of apoptosis in both DU145 and PC-3 (Rajesh et al., 2003b). Treatment 
with POH also caused an increased expression of cyclin D1 (Rajesh et al., 2003b). One 
interesting finding was that POH did not alter the level of Fas receptor expression in all 
the prostate cancer cells (Rajesh et al., 2003b).
CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

Perillyl alcohol (4-isopropenyl-cyclohexenecarbinol) and perillic acid (4-isopropenyl-1-cyclohexene-1-carboxylic acid) were procured from Aldrich Chemical Co. (Milwaukee, WI). Phosphate buffered saline (PBS; lacking Ca^{2+} and Mg^{2+}), penicillin (100 units/ml) and 100μg/ml streptomycin (P/S) and trypsin-EDTA were purchased from Invitrogen Corporation (Grand Island, NY). AlamarBlue dye was obtained from Biosource Intl. Inc. (Camarillo, CA). Propidium iodide (PI) and ribonuclease A (RNase A) were obtained from Sigma Chemicals (St. Louis, MO). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). DMEM/F-12, RPMI and MEM-α media were purchased from Invitrogen Corporation (Grand Island, NY). Standard diluent buffer (contains 15 mM sodium azide), p53 standard, rabbit anti-p53 (detection antibody containing 15 mM sodium azide), anti-rabbit IgG-Horseradish peroxidase (HRP) containing 3.3 mM thymol, HRP diluent (contains 3.3 mM thymol, wash buffer concentrate (25x), stabilized chromogen (tetramethylbenzidine) and stop solution were procured from BioSource International Inc. (California, USA). BCA protein assay reagent kit was obtained from Pierce (Rockford, IL).
3.2 Cells and cell culture

Human squamous cell carcinoma of the tongue, SCC-25 procured (ATCC Rockville, MD), BroTo (a gift from J. Truelson, MD, Department of Otolaryngology, University of Texas, South-western Medical Center) and human squamous cell carcinoma of the pharynx HTB-43 (ATCC Rockville, MD) were used in these studies. T-Lymphocytes cells, Jurkat cells (a gift from Dr. S. Carper), was also used as control cell line for Annexin-V flow cytometry. Cells were cultured in DMEM/F-12 (SCC-25), RPMI (BroTo), MEM-α (HTB-43) or RPMI 1640 (Jurkat) 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 (1M) solutions of POH and PA were prepared by dissolving each agent in dimethyl sulfoxide (DMSO). Treatment media were prepared by diluting the stock solution in medium to give the desired concentration and allowed to solubilize at 37°C for 1 hr with occasional mixing. Cells in the control groups received medium containing DMSO. Cells treated with various concentration of each agent were incubated in humidified, 5% CO₂ atmosphere at 37°C.

3.3 Dye-exclusion assay

Two million cells were cultured in each of 100mm tissue culture plates and allowed to adhere overnight. Cells were then treated with varying concentrations of agents for 72 hr. Control cells received medium with DMSO (<0.1%; v/v) only. Control, PA- and POH-treated groups were exposed to 1Gy or 2Gy dose of irradiation source (X-ray, 6MV Varian Clinac1/100 Palto Alto, CA) and were incubated overnight at 37°C in humidified 5% CO₂ atmosphere. Cells were harvested with trypsin-EDTA and centrifuged at 840
rpm (Allegra™ 6KR Centrifuge, Palo Alto, CA). Live cells were counted on a hemocytometer. The effect of different treatments on cell proliferation was measured by directly counting the number of live cells that excluded trypan blue dye.

3.4 Mitochondrial dehydrogenase assay

A modified method for the determination of mitochondrial dehydrogenase activity in live cells was used according to the manufacturer's instruction. The alamarBlue assay incorporates the REDOX indicators that both fluoresce and change color (from blue to red) due to the chemical reduction of the growth medium resulting from cell proliferation (Fields and Lancaster 1993.). Cells (5000 cells/well) were plated in 96-well plates and allowed to adhere overnight. The following day, the culture medium was replaced with medium (100 μl) containing the desired concentration of POH or PA and incubated at 37°C in humidified CO₂ atmosphere for 24, 48 or 72 hr. At the end of the exposure period, the treatment medium was replaced with 100μl of fresh medium and 10μl of the alamarBlue dye in each well and the cells incubated at 37°C in 5% CO₂ atmosphere for at least 4hr. Fluorescence was measured after excitation at 530nm wavelength and emission at 590nm wavelength using GENios fluorescence plate reader (PHENIX Research Product, Hayward, CA). Cytotoxicity was assessed by comparing the fluorescence intensity data from calculating the viable cells in the treatment group relative to the control groups (100%).
3.5 Clonogenic assay

Cells (2 x 10⁶) cultured overnight in 100mm tissue culture plate were similarly treated with or without 1.0 mM PA or 0.5 mM POH for 72 hr and exposed to 1Gy or 2Gy doses of radiation as described earlier (p.25) and clonogenic or colony formation assay (CFA) was performed as described by Elegbede et al. 2003 (see Ref.17, p.84) to determine the long-term effects of the monoterpenes on the proliferation of cancer cells. Cells were washed twice with sterile PBS and the complete culture medium after incubation. Cells (500/well) were then cultured in 6-well tissue culture plates. Fresh, complete culture medium was then added to each well and the cells were incubated for 10-14 days at 37°C in humidified, 5% CO₂ 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 (5mg/ml in 95% ethanol) and the dye gently rinsed off and allowed to dry. 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%).

3.6 Combination Chemo-irradiation studies

One thousand cells were plated in 96-well tissue culture plates overnight for cells to adhere. The culture medium was replaced with fresh 200µl culture medium. Cells were then irradiated with varying doses of X-ray, from 0, 1, 2, 3, 5 and 8Gy (6 MV Varian Clinac 1/100, Palto Alto, CA). After radiation, cells were then incubated overnight at 37°C in humidified 5% CO₂ atmosphere. Treatment medium was replaced with fresh completes medium (100µl) and incubated for 5 days before performing alamarBlue assay.
as described earlier (p.26). For 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, cells were exposed to 1 or 2Gy of X-ray (6 MV Varian Clinac 1/100, Palo Alto, CA) while in the other set the cells were not irradiated. Following irradiation, the cells were incubated for 12 to 15 hr at 37°C in humidified, 5% CO$_2$ atmosphere. All the cells were harvested and used for dye exclusion or colony formation assays and cell cycle analysis as described (p.26 and p.28).

3.7 Cell cycle analysis

To determine whether the monoterpenes affect cell cycle or apoptosis, cells (5 x 10$^5$/well) were cultured in 6-well plate overnight at 37°C in humidified, 5% CO$_2$ atmosphere. Cells were then treated with 1.0 mM PA or 0.5 mM POH for 24 hr or 72 hr. After incubation (as described by Elegbede et al., 2003, p.85), cells were harvested, fixed in 90% ethanol and kept at 4°C prior to analysis. For cell cycle analysis, the cells were stained with the DNA staining solution (150µg/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 following propidium iodide staining using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Twenty thousand events were acquired 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).
3.8 Apoptosis assay

Cells (1 x 10^6 cells/plate) were plated overnight and treated with 0.5 mM POH or 1.0 mM PA for 72 hr. Both control and treated cells were harvested after incubation and washed with cold PBS at 400x g for 5 min. Pellets were resuspended in cold PBS and counted. According to the manufacturer’s instruction, the numbers of cells was adjusted to approximately 500,000 cells per ml before pelleting at 400x mg for 5 min. Pellets were then washed in 2.0 ml x 1 Annexin-V Binding buffer (BD PHARMINGEN, San Jose, CA) at 500 x mg for 5 min. The pellets were treated with Annexin-V-FITC conjugated (BD PHARMINGEN, San Jose, CA) and incubated in the dark for 15 min. The treated mixture contains 5 μl of Annexin V conjugated to approximately 500,000 cells in 1 μl fetal bovine serum. The volume of cells with Annexin V conjugated mixture was then adjusted up with addition of 450 μl of x 1 Annexin-V binding buffer, (each tube contains cells-conjugate mixture of approximately 500 μL). Acquisition to discriminate between apoptotic and necrotic or dead cells was done by staining the cells-conjugate mixture with 10 μL PI solution (500 μg/ml) (BD PHARMINGEN, San Jose, CA).

Acquisition was done on BD’s FACS Calibur cytometry on the FL1 (Annexin) and FL3 (PI) channels with threshold on FSC and Duplet Discriminating Module (DDM) set at FL1. The level of shift in events distribution in the treated cells stained with Annexin-V only and Annexin-V-PI populations in comparison to untreated controls is indicative of degree of effectiveness of the treatment agent(s). Numeric quantitations of these events are accomplished with population gating.

We further visualized apoptotic cells using detection system from Phoenix Flow System Inc. according to the manufacturer’s instructions. Cells (HTB-43, SCC-25 or
Jurkat T-lymphocyte, were fixed with formaldehyde to retain (crosslink) fragmented DNA within apoptotic cells. The 3'OH termini of DNA strand breaks were labeled with BrdUTP in the reaction catalyzed by the terminal deoxynucleotidyl transferase (TdT); cellular DNA was counterstained with PI and DNA content was measured using 488 nm laser excitation in FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). To calculate the percentage of cells in the respective phases of the cell cycle, the DNA content frequency histograms were deconvoluted using the MultiCycle software (Phoenix Flow Systems, San Diego, CA).

3.9 Cell lysate preparation

Cells were plated and allowed to stay overnight. The following day, the medium was aspirated off and cells were treated with 0.5 mM POH or 1.0 mM PA for 72 hr. Cells were then exposed to 1Gy or 2Gy dose of irradiation and incubated overnight. Cells were harvested and transferred into a 15 ml centrifuge tube. The cells were then centrifuged at 840 rpm for 5 minutes and media was aspirated off. The pellets were resuspended and then washed three times with 5 ml of cold PBS. The pellets were resuspended in 1ml of PBS and were transferred into a microfuge tube and then centrifuged at 13,000 rpm (BECKMAN Microfuge® R Centrifuge, Palo Alto, CA) for 10 minutes at 4°C and stored at -80°C for protein analysis. Cells were thawed and lysed in cell extraction buffer (containing protease inhibitor and PMSF) for 30 minutes on ice while vortexing at 10 minutes interval (according to Pierce BCA protein assay, Rockford, IL). The extracts are then transferred into microcentrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C. Aliquoted the clear lysate into clean microcentrifuge tubes and assayed.
3.10 Determination of p53 protein in HNSCC cells.

The protein concentration of each sample was determined using BCA protein assay according to the manufacturer’s instruction. Different dilutions of bovine serum albumin (BSA) standards were prepared (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000µg/ml) and 25µl each of prepared BSA standards or samples (10µl of cell lysate and 15µl diluent PBS) were then pipetted into 96-well microtiter wells and 200µl of BCA working reagent was then added into all wells and incubated for 30 minutes at room temperature and the absorbance was measured at 570 nm wavelength using ELISA plate reader. Protein concentration of each sample was then calculated from the absorbance reading. The p53 protein was determined and quantified using Biosource p53 ELISA according to the manufacturers instructions. Standard diluent buffer (100µl) were added to zero wells in p53 antibody-coated wells and well(s) reserved for chromagen blank was left empty. Standards, samples or controls (100µl) were then pipetted into their appropriate reserved wells. The plate was covered and incubated for 2 hr at room temperature. The extracts were diluted in cell extraction buffer (5µl sample into 95µl). After incubation, the solution was aspirated off from the wells and each well was washed 4 times with wash buffer. One hundred microliter of anti-p53 (detection antibody) solution into each well except the chromogen blank(s) and mixed gently. The plates were covered and incubated for 1 hr at room temperature. The solution was again aspirated from each well and each well washed again 4 times with wash buffer. Anti-rabbit IgG-HRP (100µl) working solution was transferred into each well except the chromogen blank(s) before incubating for 30 minutes at room temperature. Solutions were again aspirated off from the wells and washed 4 times with wash buffer. Standard chromogen (100µl) to each well
including the chromogen wells and plates were further incubated for 30 minutes at room temperature in the dark. The stop solution (100μl) was then added into each well and mixed gently until solution change from blue to yellow. The absorbance was read on GENios plate reader at 450 nm wavelength against a chromogen blank. Absorbance of each sample was calculated to quantify the level of p53 protein (according to manufacturer’s instruction using BioSource p53 (Total) ELISA assay).

3.11 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 α = 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-value less than 0.01 (p<0.01) were considered statistically significant. The combination treatment act with significant difference (ANOVA at α =0.01) in each cell line (HTB-43, SCC-25 and BroTo) and method while both treatment factors (monoterpenes and radiation) are significant on the rate of effectiveness (ANOVA at α = 0.01). Comparison with control, both PA and POH are significantly different from control (Dunnetts two sided test at α = 0.01). Comparison of compounds with each other, POH results in the highest rate of effectiveness than PA in all the cell lines (Turkey’s LSD ay α = 0.01). Comparison of radiation, 1Gy of radiation results in significantly better effectiveness than no radiation in combination treatment (ANOVA at α = 0.01).
CHAPTER 4

FINDINGS OF THE STUDY

4.1 Analysis of results

The cytotoxicity effect of PA and POH was determined by alamarBlue assay in HNSCC cells exposed for 24, 48 and 72 hr time point. Treatment with 1.0 mM PA and 0.5 mM POH caused a dose- and time-dependent inhibition with greater effect observed after 72 hr on HTB-43 (19% and 51%), SCC-25 (24% and 63%) and BroTo (31% and 40%) cells respectively (Fig. 5, p.38).

To determine the effect of radiation on cell proliferation, cells were exposed to numerous dose of radiation from 1Gy to 8Gy. A dose dependent inhibition on cell proliferation was observed with 1Gy as follows: HTB-43 (20%) > SCC-25 (8%) > BroTo (5%) respectively (Fig. 6, p.39). Drastic inhibition was seen with 8Gy dose (Fig.6, p.39).

Radio-sensitization was designed with 1.0 mM PA and 0.5 mM POH. Dye-exclusion and colony formation assay were employed to test the effects of either monoterpenes alone or irradiation or combination of both on these 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. Exposure of these cell lines to 2Gy dose of irradiation alone minimally reduced the viability of these cells (Fig. 7, p. 40). When the cells were pretreated with 1.0 mM PA or 0.5 mM POH and exposed to 1Gy dose of radiation the viability of each cell line was reduced as follows: HTB-43 (50% and 71%), SCC-25...
(55% and 68%) and BroTo (18% and 53%) respectively (Fig. 7, p. 40). Similar results were obtained when the cells were pretreated with POH or PA and then exposed to 2Gy dose of irradiation (Fig. 7, p.40).

The long-term survival of cells from 12 to 14 days, when pretreated with monoterpenes followed by irradiation was also investigated. Figure 8 (p.41) 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 either the control (no treatment, radiation alone or chemotherapy alone) (Fig. 8, p.41). SCC-25 cells formed the fewest colonies of all the cell lines (Fig.8, p.41). Quantitative examination of the results (Fig. 9, p.42) 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, 30%) and for POH (60, 55, and 40%) respectively when exposed to 1Gy of irradiation (Fig.9, p.42).

To determine the effects of the treatment on the cell cycle progression, we performed cell cycle analysis on treated cells using a FACSCalibur flow cytometry (Becton Dickson, San Jose, CA) following propidium iodide staining. At 24 hr, POH treatment induced a block in the $G_0/G_1$ phase in all the three cell lines at 24 hr while PA treatment blocked only BroTo cells in $G_0/G_1$ but no such effect on the other cell lines (Table 1, p.43). At 72 hr, PA induced some $G_0/G_1$ arrest in all cell lines (Table1, p.43). Figure 10 (p.44) 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 respective control for each cell line. Calculation of the
AI normalized the treatment effect for each cell line, thereby facilitating a comparison between them. AI was highest for SCC-25 cells treated with POH and exposed to 2Gy dose of irradiation (Fig. 10, p.44). The overall sensitivity of the cell lines were for POH: SCC-25>BroTo>HTB-43 and for PA: HTB-43>SCC-25>BroTo respectively (Fig. 10, p.44).

To confirm PA or POH induced cell death, both Annexin V (AV) and propidium iodide (PI) staining by flow cytometry was performed with HTB-43, SCC-25 and BroTo cells exposed to 1.0mM PA and 0.5 mM POH for 72 hr exposure. Jurkat T-Lymphocytes cells were used as control cell line. The lower left quadrant of the cytograms shows the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for FITC-Annexin V binding and showing PI uptake. The lower right quadrant represents the apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating Annexin V binding and cytoplasmic membrane integrity. The percentage of cells single positive for FITC-annexin V binding after treatment with 1.0 mM PA or 0.5 mM POH was 5.9 and 17.9% for Jurkat cells (Fig.11, p. 45), 38.8 and 49.8% for HTB-43 (Fig.12, p.46.), SCC-25 having 13.8 and 23.8% (Fig.13, p.47) and for BroTo cells 1.5% and 0.6% (Fig.14, p.48). We observed high percentage of late apoptotic cells, double positive for both FITC-annexin V/PI in BroTo cells compare to early apoptotic cells (Fig.14, p.48).

To discriminate apoptotic cells from necrotic cells, we further visualized apoptotic cells using detection system from Phoenix Flow System Inc. The percentage of live and apoptotic cells were quantified using the MultiCycle software. Our data showed that in the control cell line (Jurkat cells) only 0.6% and 0.7% of cells were apoptotic compared
to live cells (Fig.15, p.49) while for HTB-43 the percentage of apoptotic cells for PA and POH treatment was 77.2 and 63.5% respectively (Fig.16, p.50). In SCC-25 cells treatment with 1.0 mM PA and 0.5 mM POH for 72 hr resulted in 62.3 and 78.0% of apoptotic cells (Fig.17, p.51).

4.2 Initial attempt to quantify p53

When the DNA of a cell is damage due to either radiation or agent, the tumor suppressor protein, p53 is activated for repair process. The p53 gene is the most commonly mutated gene in human cancers especially in head and neck cancers. In 1996, Kiuru et al. and colleagues affirm that since p53 is highly mutated in HNSCC, this will probably account for their sensitivity or resistance to either radiation or agent. In an attempt to detect or quantify the level of p53 in HNSCC cells, HTB-43, BroTo, and SCC-25 cells were treated with PA or POH alone or radiation alone or combination of both. We identified and quantified the p53 protein level in HNSCC cells using the BioSource p53 (Total) immunoassay called Enzyme Linked-Immuno-Sorbent Assay (ELISA) by referring to a standard curve (plotted in Fig.18, p.53). With HTB-43 and BroTo cells override was observed, meaning that the fluorescence signal exceeded the range for detection by the instrument. This was probably due to high level of p53 proteins expressed in these two cell lines compared to SCC-25 cells. With SCC-25, the result indicated that the level of p53 in the control was higher than the treated group (Table 2 and Fig.18, p.52 and p.53). However, these results may not be reliable. Observed protein levels should be proportional to the number of cells. We expect the protein in the control group to be higher than in the treatment groups because cell number should be greater in
the control than treated groups due to cell death caused by the agent (for example, see Figure 7, p.40). But we observed that the protein level in the treatment groups was higher than the control, suggesting that some type of error may have occurred during this experiment. Furthermore, it could be that SCC-25 cells express very little or no p53. Min et al. (1994) reported that the squamous cell carcinomas of the tongue, SCC-25 have p53 levels that are not easily detected. They also reported a p53 mutation observed in SCC-25 due to deletion at codon 209 of two base pairs (Min et al., 1994). There is need for further investigation of the p53 protein using western blotting technique, and repeating the ELISA. In addition to SCC-25, it may be desirable to look again at more diluted samples from HTB-43 and BroTo cells, which appear to have high concentrations of p53.
Figure 5. Inhibition effects of monoterpenes on human head and neck cancer cells. Cells were plated in tissue culture plates and exposed to equimolar concentrations of PA or POH for 24, 48 and 72 hr. AlamarBlue assay was used as described in the text to measure the cytotoxicity of each agent. Values represent Mean ± SD (n=4).
Figure 6. The response of head and neck squamous cell carcinoma cells to radiation exposure. HTB-43, SCC-25 and BroTo cells were plated separately in tissue culture plate and exposed to different doses of irradiation from 1 to 8Gy and allowed to stay overnight. Treatment medium and cells were incubated for 5 days. At termination, the effect of radiation exposure was determined by alamarBlue assay as described in the text. Graph represent Mean ± SD (n=4).
Figure 7. Monoterpenes sensitized HNSCC cell lines to radiation treatment in vitro. Cell lines were treated with 1.0 mM PA or 0.5 mM POH for 72 hr then exposed to 1 Gy or 2 Gy of X-ray dose. Control cells with or without monoterpenes received no irradiation. Cells were harvested, stained with trypan blue dye and proportion of viable cells relative to the control was determined. The graph represent Mean ± SD (n=3) of % viability versus treatment regimen.
Figure 8. Colony formation in HNSCC cells after combination treatment with monoterpenes and radiation. HNSCC cells (SCC-25, BroTo, HTB-43) were exposed to 0.5mM perillyl alcohol (POH) or 1.0mM perillic acid (PA) for 72 hr and then exposed to 0, 1, or 2Gy X-ray. Cells were harvested, washed with PBS, and cultured in 6-well plates (500/well) with complete medium for 14 days. Cells were stained with crystal violet and colonies, containing at least 50 cells, were counted. Cell viability was calculated as percentage of the colonies in treated group relative to that of the control groups.
Figure 9. Monoterpenes delayed proliferation of HNSCC cells in combination treatment with irradiation. The cells were treated as described in the legends for Fig.8 (P.41).
### Tables 1. Cell cycle distribution of HNSCC cells treated with monoterpenes. HNSCC Cells were harvested after exposure to perillic acid (PA) or perillyl alcohol (POH) for 24 or 72 hr. The cells were fixed in ethanol and stained with propidium iodide. DNA content data were acquired using BD FACSCalibur and CellQuest acquisition software (Beckon Dickinson, San Jose, CA). The data were analyzed using ModFit LT version, 3.0 analysis software (Verity software House, Topsham, ME). Values are Mean ± SD (n=3). The table is representative of two separate experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>24 hr</th>
<th>72 hr</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$G_0/G_1$</td>
<td>$S$</td>
</tr>
<tr>
<td>HTB-43</td>
<td>Control</td>
<td>77.7 ± 0.5</td>
<td>9.7 ± 1.8</td>
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<td></td>
<td>0.5 mM POH</td>
<td>81.5 ± 1.8</td>
<td>0.2 ± 0.1</td>
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<td></td>
<td>1.0 mM PA</td>
<td>74.9 ± 1.3</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>SCC-25</td>
<td>Control</td>
<td>46.4 ± 2.6</td>
<td>37.2 ± 1.4</td>
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<tr>
<td></td>
<td>0.5 mM POH</td>
<td>60.9 ± 2.5</td>
<td>36.7 ± 5.2</td>
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<tr>
<td></td>
<td>1.0 mM PA</td>
<td>46.7 ± 0.5</td>
<td>41.0 ± 1.7</td>
</tr>
<tr>
<td>BroTo</td>
<td>Control</td>
<td>58.1 ± 1.0</td>
<td>26.2 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>0.5 mM POH</td>
<td>72.4 ± 2.9</td>
<td>17.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>1.0 mM PA</td>
<td>72.4 ± 5.15</td>
<td>21.1 ± 5.4</td>
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</table>
Figure 10. Monoterpenes induced apoptosis in HNSCC cell lines. Human head and neck cancer cell lines were treated with 1.0 mM perillic acid (PA) and 0.5 mM perillyl alcohol (POH) for 72 hr. At termination, cells were harvested and washed with PBS. Cells were fixed in ethanol, washed and stained with propidium iodide and percentage of cells in sub- G0/G1 phase of the cell cycle was quantified using flow cytometry. Figure represent apoptotic index (AI) calculated as a ratio of the mean apoptosis values in the treated groups divided by the mean apoptosis of respective control for each cell lines. Values represent two separate experiments.
Figure 11. Contour diagram of FITC-Annexin V /PI flow cytometry of Jurkat cells. Control and treated cells were exposed for 72 hr, harvested and washed with 5 ml wash buffer. Treated cells with FITC-Annexin V conjugate and stained with PI. Acquisition was done with FACSCalibur flow cytometry. Lower left quadrants of each point show the viable cells, which exclude PI and negative for FITC-Annexin V binding. The upper right quadrants (DP) contain non-viable cells positive for FITC-Annexin V binding and PI uptake. The lower right quadrants (SP) represent the apoptotic cells, single positive for FITC-Annexin V and negative PI.
Figure 12. HTB-43 cells contour diagram of FITC-Annexin V/PI flow cytometry treated for 72 hr. Control and treated cells were harvested and washed with 5 ml wash buffer. Treated cells with FITC-Annexin V conjugate and stained with PI. Acquisition was done by flow cytometry. Lower left quadrants of each point show the viable cells, which exclude PI and negative for FITC-Annexin V binding. The upper right quadrants (DP) contain non-viable cells positive for FITC-Annexin V binding and PI uptake. The lower right quadrants (SP) represent the apoptotic cells, single positive for FITC-Annexin V and negative PI.
Figure 13. Contour diagram of FITC-Annexin-V/PI flow cytometry of SCC-25 cells exposed for 72 hr. Control and treated cells were harvested and washed with 5 ml wash buffer. Cells treated with FITC-Annexin-V conjugate and stained with PI. Acquisition was done using flow cytometry. Lower left quadrants of each point show the viable cells, which exclude PI and negative for FITC-Annexin V binding. The upper right quadrants (DP) contain non-viable cells positive for FITC-Annexin V binding and PI uptake. The lower right quadrants (SP) represent the apoptotic cells, single positive for FITC-Annexin V and negative PI.
Figure 14. BroTo cells Contour diagram of FITC-Annexin V/PI flow cytometry of treated for 72 hr. Untreated and treated cells were harvested and washed with 5 ml wash buffer. Cells were treated with FITC-Annexin -V conjugate and stained with PI. Acquisition was done by flow cytometry. Lower left quadrants of each point show the viable cells, which exclude PI and negative for FITC-Annexin V binding. The upper right quadrants (DP) contain non-viable cells positive for FITC-Annexin V binding and PI uptake. The lower right quadrants (SP) represent the apoptotic cells, single positive for FITC-Annexin V and negative PI.
Figure 15. Jurkat cell death analysis using BrdUTP labelling. Control and treated groups were exposed for 72 hr, then harvested and washed with 5 ml of wash buffer. Incubated cells in DNA labelling for 1 hr at 37°C in water bath. After incubation, cells were rinsed twice with 1.0 ml rinsed buffer and resuspended cell pellet in 0.1 ml of Fluorescein-PRB-1 Antibody solution for 30 minutes in the dark. Add 0.5 ml of propidium iodide/RNase A solution and incubated for more 30 minutes and cells distribution analyzed was conducted using flow cytometry. M1 and M2 represents live and apoptotic cells.
Figure 16. HTB-43 cell death analysis exposed for 72 hr using BrdUTP labelling. Untreated and treated group were harvested after treatment and washed with 5 ml of wash buffer. Incubated cells in DNA labelling for 1 hr at 37°C in water bath. After incubation, cells were rinsed twice with 1.0 ml rinsed buffer and resuspended in 0.1 ml of Fluorescein-PRB-1 Antibody solution for 30 minutes in the dark. Added 0.5 ml of propidium iodide/RNase A solution and incubated for another 30 minutes. Cells were analyzed using flow cytometry. M1 and M2 represents live and apoptotic cells.
Figure 17. Cell death analysis of SCC-25 cells exposed for 72 hr using BrdUTP labelling. Control and treated cells were harvested after treatment with agent and washed with 5 ml of wash buffer. Cells were incubated in DNA labelling for 1 hr at 37°C in water bath. After incubation, cells were rinsed twice with 1.0 ml rinsed buffer and resuspended in 0.1 ml of Fluorescein-PRB-1 Antibody solution for 30 minutes in the dark. Added 0.5 ml of propidium iodide/RNase A solution and incubated for another 30 minutes. Cells were analyzed using flow cytometry. M1 and M2 represents live and apoptotic cells.
Table 2. Reduction of p53 protein level in SCC-25 cells in culture. Cell samples and standards were added to microtiter wells coated with p53 antibody and incubated for 2 hours. Aspirated solution in wells washed 4 x with wash buffer and pipetted 100 µl anti-p53 detection antibody in to wells except chromagen blanks. Covered plate and incubated for 1 hour. Aspirated off solution, washed wells 4x with wash buffer and added 100 µl anti-rabbit IgG-HRP working solution and incubated for 30 minutes. Washed and added standard chromagen to all wells, incubated for 30 minutes in the dark at room temperature. Added stop solution to each well and read the absorbance at 450 nm. Table represents average of duplicate reading for p53 protein level.

<table>
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<tr>
<th>Treatment</th>
<th>p53 pg/5µl</th>
<th>Protein ug/5µl</th>
<th>pg/µg protein</th>
<th>% of p53 level</th>
<th>% reduction of p53 level</th>
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<tr>
<td>Control</td>
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<td>6.83</td>
<td>54.63</td>
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<td>0.5 mM POH</td>
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<td>19.5</td>
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</tr>
<tr>
<td>1.0 mM PA</td>
<td>69.34</td>
<td>12.49</td>
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<tr>
<td>1Gy</td>
<td>59.58</td>
<td>8.95</td>
<td>6.66</td>
<td>12.2</td>
<td>87.8</td>
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<tr>
<td>2Gy</td>
<td>53.64</td>
<td>15.06</td>
<td>3.56</td>
<td>6.5</td>
<td>93.5</td>
</tr>
<tr>
<td>1Gy/PA</td>
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<td>21.36</td>
<td>2.55</td>
<td>4.1</td>
<td>95.9</td>
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<td>2Gy/PA</td>
<td>78.15</td>
<td>14.53</td>
<td>5.38</td>
<td>9.8</td>
<td>90.2</td>
</tr>
</tbody>
</table>
Figure 18. The expression of p53 protein in SCC-25 cells following chemo-radiation treatment with monoterpenes: Added 100μl (5μl sample into 95μl) of sample in appropriate microtiter wells and incubated for 2 hrs. Pipetted 100μl of anti-p53 into each well after washing with buffer. Covered and incubated plate for 1 hr. Washes 4x with buffer and adds 100μl of anti-rabbit IgG-HRP working solution except blank well and incubates for 30 minutes. Wash 4x with buffer and added 100μl of standard chromagen and incubated in dark for 30 minute. Add 100μl of stop solution and Read absorbance at 450 nm. Figure represents values of the treated groups compared with standard values.
5.1 Discussions of results

During cell growth, the metabolic activity results in a chemical reduction of the alamarBlue dye from blue to red indicating cell viability. Continued cell proliferation maintains a reduced environment (change of dye color to red) while inhibition of growth maintains an oxidized environment (no change in dye color, blue). Reduction related to cell growth causes the alamarBlue dye to change from oxidized form to reduced form. Fluorescence intensity is proportional to the number of viable cells and comparison of this intensity in treated cells to that of the untreated control is used as a measure of cytotoxicity of PA or POH on cell growth. In this present study, treatment of HNSCC cells to either 1.0 mM PA or 0.5 mM POH inhibited the proliferations of cells in a time-dependent manner with drastic effect observed after 72 hr exposure in all the cell lines (Fig.5, p.39).

We have tested a low dose (0.5 mM) of POH and physiological achievable level (1.0 mM) of PA as agents for use in combination chemo-radiation treatments. POH or PA alone minimally affected the viability (Fig.7 and 9, p.41 and 43) or proliferation (Fig.9, p.43) of HNSCC cells in culture. However, when the cells were pretreated with each monoterpenes and then exposed to 1Gy dose of irradiation, the combination treatment with PA significantly (p<0.01) reduced the survival of HTB-43 and SCC-25.
cells (Fig. 7 and 9, p.40 and 42) as well as that of BroTo cells (p<0.01). The reduction in viability was as a result of apoptosis induction as shown in Figure 10 (p.45). Induction of apoptosis was assessed as 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 (Fig. 10, p.45) although the sensitivity of the cell lines appears to take 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 (Fig. 10, p.45)

In the studies investigating the long-term effects, we found that pretreatment with monoterpenes followed by irradiation inhibited the proliferation of the cancer cells (Figs. 8 and 9, p.42 and 43). Our data confirm the radio-sensitization effects of the monoterpenes. These results are in agreement with recent reports by Rajesh et al (2003) showing that pretreatment of glioma cells with POH sensitized the cells to irradiation. These authors further concluded that the mechanism of action included enhanced apoptosis induction in the treated cells, although they used higher irradiation doses (5Gy) compared to the dose used in the present study.

To confirm apoptosis induction by the monoterpenes, FITC-Annexin -V/PI staining flow cytometry was performed with head and neck cancer cells, Jurkat cells serving as the control cells. In the early stages of apoptosis one of the changes that occur at the cell surface involves the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the external surface of the cell. Annexin-V being a Ca $^{2+}$ dependent phospholipids binding protein binds to PS with high affinity at the external surface, hence it is used to measured cells that undergo early apoptosis. We report that
pretreatment with 1.0 mM PA or 0.5 mM POH increased the cell population of both necrotic and apoptotic cells (p.46-49) confirming the observation seen with increased in apoptotic index in HNSCC cells. For cells that are apoptotic, the plasma membrane remains intact, but necrotic cells release its content leading to shrinking of plasma membrane. Figures 11-17 (p.46-49) shows the results of applying annexin V assay to indicate the number of apoptotic and necrotic cells in relation to time elapsed after 72 hrs treatment with PA or POH (p.46-49). We confirm that pretreatment of cells prior to radiation by monoterpenes results into induction of apoptosis (Fig.17 and 18, p.52-53).

5.2 Conclusions and recommendations for further study

In a biological system, when radiation is absorbed, free radicals are produced that cause the breakage of the nucleic acids and other macromolecules, causing DNA damage. Many drugs have been reported to have the properties of inhibiting the repair of the DNA damage in cancer cell caused by radiation exposure (Rosenthal et al., 1998). Our data indicated that the monoterpenes acted as radiosensitizers through the induction of cell death via apoptosis in combination chemo-radiation treatment of head and neck cancer cells in vitro. Our data also indicated that the monoterpenes POH as well as its metabolite PA acted as radiosensitizers in combination chemo-radiation treatment of head and neck squamous cell carcinoma in vitro.

As the knowledge of the molecular events surrounding the cause of cancer has advanced, attention has been center on developing novel agents with new mechanisms of action. Since this was the first research work to elucidate the radio-sensitization of
perillic acid in head and neck cancer cells, further studies on the sensitization effect of the monoterpenes will help in elucidating the mechanism of action. This will include

(i) the effects of higher radiation doses
(ii) the effects of fractionated doses
(iii) comparative effects of alternating and concomitant chemo-radiation protocols
(iv) the role of monoterpenes in the expression levels of p53 and other related signaling proteins like bcl-2, p21 in downstream of p53 regulation
5.3 Definition of terms

<table>
<thead>
<tr>
<th>Terms</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Apoptosis</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Death of cells through injury, disease or treatment.</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>Cell cycle arrest</td>
<td>The halt in a series of events involving the growth, replication, and division of a eukaryotic cell</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor protein</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Cancerous cells that affect the middle layer of the skin</td>
</tr>
<tr>
<td>Inhibition</td>
<td>The reduction or of a biochemical reaction, due to interaction with a chemical agent</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Continuous development of cells</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray is the unit of radiation dose</td>
</tr>
<tr>
<td>G0-Phase</td>
<td>Resting period of cells</td>
</tr>
<tr>
<td>G1-Phase</td>
<td>Growth and preparation of the chromosomes for replication</td>
</tr>
<tr>
<td>G2-Phase</td>
<td>Preparation for cell division</td>
</tr>
<tr>
<td>M-Phase</td>
<td>Mitosis or cell division</td>
</tr>
<tr>
<td>S-Phase</td>
<td>Synthesis of DNA</td>
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APPENDIX

BIOACTIVE PLANT COMPOUNDS INHIBITED THE PROLIFERATION AND INDUCED APOPTOSIS IN HUMAN CANCER CELL LINES, IN VITRO

Daniel Samaila, Daniel E. Ezekwudo, Kidist K. Yimam and J. Abiodun Elegbede

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Acknowledgement: This research was supported by a grant from the 2002-2003 UNLV Planning Initiative Award to JAE. BRIN Medical Student Summer Research Fellowship Award supported KKY during 2003 summer.

Running Title: Plant Compounds and Cancer Treatment

Key Words: Plant Compounds, Monoterpenes, Jasmonates, Human Cancers

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SUMMARY:

Several of the current chemotherapeutic drugs (vinblastine, methotrexate, taxol, etc) were first identified in plants. In developing countries, the practice of medicine still relies heavily on plant and herbal extracts for the treatment of human ailments. Current interests in phytochemicals as agents for the treatment of human cancers were rekindled because these compounds occur naturally and are less toxic than synthetic agents. Monoterpenes occur naturally in a wide variety of plants while the jasmonates are a family of plant stress hormones. We studied the effects of the naturally occurring monoterpenes, perillyl alcohol (POH) and its major human metabolite, perillic acid (PA), on human head & neck (BroTo and SCC-25), and human lung carcinoma (HTB-182 and CRL-5928) cell lines. We also studied the effects of the jasmonates on human prostate adenocarcinoma (PC-3 and HTB-81) cell lines in culture. Both classes of compounds inhibited the proliferation of cancer cells. POH was more effective than PA at reducing the proliferation of human head & neck and lung squamous cell carcinoma cells. The jasmonates also inhibited the proliferation of prostate cancer cells. Differential potencies were observed between the various members of the jasmonate family. The monoterpenes and the jasmonates should be further investigated for their effects on human cancer cell lines.
INTRODUCTION:

The effects of several plant compounds on animal cells have been studied. Plant compounds such as carotenoids, lycopene, indoles, thiocyanates and others exhibit antioxidant properties in animal cells while the carotenoids and vitamin E also serve as nutrients. Other plant compounds such as tea polyphenols, indoles and monoterpenes showed chemopreventive activity in a number of human cancers (1-3).

Monoterpenes are a diverse class of isoprenoid compounds synthesized by plants through the mevalonate pathway (4). d-Limonene (LIM) and perillyl alcohol (POH) are naturally occurring monoterpenes commonly found in the essential oils of citrus fruits, lavender, spearmint, cherries and others (5,6). Both LIM (7-9) and POH (10) elicited chemotherapeutic effects against mammary tumors causing the complete regression of more than 80% of established DMBA- or NMU-induced mammary carcinomas. In vivo, both POH and LIM are readily metabolized to the major metabolites, perillic acid (PA) and dihydroperillic acid (DHPA), in experimental animals (11). Pre-clinical and pharmacokinetic studies of limonene (12,13) and POH in humans (14,15) showed that human metabolites of both agents (16) are similar to the metabolic profile observed in experimental animals (11). Although the mechanism of the anti-tumor action of the monoterpenes is not yet known, several potentially important drug-related activities have been observed (17-21).

The jasmonates, first isolated from the jasmine plant, are a class of plant stress hormones consisting of cis-jasmone (CJ), jasmonic acid (JA), and methyl jasmonate (MJ) respectively (22). JA is involved in the intracellular signaling response by plants to injury while MJ causes the induction of a proteinase inhibitor, which accumulates in response to
wounding or pathogenic attacks (23). In plants, this response is termed the hypersensitive response (HR) and results in the formation of a zone of dead cells around the infection site, the synthesis of salicylic acid and the accumulation of antimicrobial agents, such as pathogenesis-related proteins and phytoalexins (24,25). The layers of dead cells that surround the site of pathogen entry are thought to function as a physical barrier that inhibits further proliferation and spread of the pathogen.

Available reports indicate that the jasmonates may also have effects on animal cells in culture. Jasmone and analogs, for example, inhibited the proliferation and induced cell death in lymphoblastic leukemia (MOLT-4) and breast cancer cells cultured in vitro, but did not affect normal human blood lymphocytes or erythrocytes (26). JA and MJ caused apoptotic death, increased caspase-3 activity and induced mitochondrial membrane depolarization in these cell lines (26).

We postulated that these plant compounds might inhibit the proliferation of human cancer cells in vitro. We, therefore, investigated the effects of the monoterpenes (POH and PA) on the proliferation of human squamous cell carcinoma of the head & neck (BroTo; SCC-25) and lung (HTB-182; CRL-5928) cell lines. We also studied the effects of the jasmonates on prostate adenocarcinoma (PC-3; HTB-81) cell lines cultured in vitro. The effects of these agents on human lung fibroblast, normal (HFL-1), cultured in vitro were also evaluated. In this report, we present preliminary findings showing that these bioactive plant compounds inhibited the proliferation and induced cell death in human cancer cells in culture.
MATERIALS AND METHODS:

Chemicals and Reagents:

The monoterpenes (POH and PA) were obtained from Aldrich Chemical Company (Milwaukee, WI). The jasmonates (CJ, JA, MJ) were purchased from Sigma Chemical Company (St. Louis, MO). Cell culture media (RPMI, EMEM, DMEM, Ham’s F12 and Ham’s F12-K) were obtained from ATCC (Manassas, VA). Phosphate buffered saline (PBS, lacking Ca^{2+} and Mg^{2+}), penicillin (100 units/ml) and streptomycin (100μg/ml) (P/S) were obtained from Invitrogen Corporation (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). All other reagents and chemicals were of the purest grade and procured from reputable vendors.

Cells and Cell Culture:

Human squamous cell carcinoma of the tongue (SCC-25), human squamous cell carcinoma of the lung (HTB-182 and CRL-5928), human prostate adenocarcinoma (PC-3 and HTB-81) and human lung fibroblast, normal, (HFL-1) cell lines were purchased from ATCC (Manassas, VA). Human squamous cell carcinoma of the tongue (BroTo) cell line was a gift from J. Truelson, MD, Department of Otolaryngology, University of Texas-Southwestern Medical Center. Cells were cultured in appropriate media supplemented with 10% FBS and 1% P/S. Cells were dislodged with trypsin-EDTA, adequate amounts, as specified in each study, were plated and allowed to adhere onto tissue culture plates overnight. The respective monoterpene (POH, PA) or jasmonates (JA, CJ, MJ) was dissolved in DMSO to give a 1.0M stock solution. Cisplatin was mixed directly with sufficient medium to achieve the desired concentration. Treatment solutions were
prepared by diluting the stock solution with the appropriate medium, with occasional mixing at 37°C for at least 1 hr, before use. Cells were treated by replacing the growth medium with medium containing the desired concentration of each agent and incubated at 37°C in humidified, 5% CO₂ atmosphere. Cells in control wells received either medium only (for cisplatin and 5-FU controls) or medium containing DMSO (≤0.3% v/v; for jasmonate and monoterpene controls). At stated time points, effects of the agents were determined as described below.

**Cytotoxicity Assays:**

**Mitochondrial Dehydrogenase (MTT) Assay:**

The effects of the different agents on cancer cell lines were studied using the MTT assay (27), an assay based on the reduction of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to purple formazan crystals by the mitochondrial dehydrogenase enzyme. Specifically, cells (5,000 cells/well) were plated in 96-well plates and allowed to adhere overnight. Cells were treated with the agents or the appropriate Control medium for up to 72 hr. After exposure to the agent for a specified period of time, the treatment medium was replaced with 100µl of fresh medium and 10µl of MTT (5mg/ml) and the plate incubated at 37°C in humidified 5% CO₂ atmosphere for 3 hr. The reaction was stopped and the formazan crystals solubilized with 100µl of the stop solution (0.1N HCl in isopropanol). The optical density of the solution was read at 570nm against a reference at 630nm on an ELISA plate reader (Packard Instruments, Meridan, CT). Cell viability was calculated as a percentage of the control cells (100%).
Colony Formation Assay:

The long-term effects of the agents on the treated cells in culture were assessed using the colony formation assay (CFA) as previously described (28). Specifically, cells (200-500 cells/well) were plated in 6-well plates and allowed to adhere overnight. The following day, the control or the treatment medium (with the desired concentration of an agent) was added to appropriate wells and incubated for the desired time period. Cells were washed twice with 1X PBS and once with the fresh medium. Fresh medium was then added to each well and the cells were incubated for 10–14 days. The plates were viewed under the microscope every other day to monitor cell growth. At termination, the medium from each well was removed by aspiration, the cells washed with 1X PBS and the colonies stained with crystal violet (0.5% in 95% ethanol) for 5 min and the dye was gently rinsed off. Colonies, each containing at least 50 cells, were counted and the cell viability or drug effectiveness was assessed based on the number of colonies formed in the treatment group relative to the control (100%).

Flow cytometry analysis for cell cycle distribution:

The DNA content of treated cells was assessed by flow cytometry following propidium iodide (PI) staining as previously described (28). Cancer cells (5 x 10^5) were plated in a 60mm diameter tissue culture plate overnight to adhere. The cells were then treated with varying concentrations of POH, PA or MJ for the specified period of time. Following treatment, cells were harvested, washed, re-suspended in 100μl of ice-cold PBS, fixed with 90% ethanol and kept at 4°C prior to flow analysis. To determine the
cell cycle distribution and to quantitate apoptotic cells with subdiploid DNA content, cells were stained with DNA staining solution (100 μg/ml PI, 0.1% Triton X-100, and 1 mg/ml RNase A (DNase-free) in PBS lacking Ca^{2+}, Mg^{2+}; 1:1:1 by volume) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). PI fluorescence was linearly amplified and both the area and width of the fluorescence pulse were measured. Twenty thousand events were acquired and the percentages of hypodiploid (apoptotic; sub-G_0/G_1) events and of cells in G_0/G_1, S and G_2-M phases were determined using the DNA analysis software ModfitLT version 3.0 (Verity Software, Topsham, Maine).

Statistical Analyses:

Results were expressed as Mean ± SD of replicate analyses accompanied by the number of independent experiments. Data analyses were performed by one-tailed t-test. Differences at p<0.05 or better were considered statistically significant.

RESULTS:

Effects of monoterpenes on squamous cell carcinomas:

The effects of the monoterpenes (POH and PA) on the proliferation of human head & neck squamous cell carcinoma and human lung fibroblast (HFL-1) cells in culture were evaluated using MTT and CFA techniques. The concentration of POH that inhibited proliferation by 50% (IC_{50}) was calculated (Fig 1) to be 625μM (BroTo), 800μM (HFL-1) and 930μM (SCC-25). BroTo cells appeared more sensitive to POH than SCC-25 and HFL-1 cells. PA was not as effective as POH against the cell lines.
though at concentrations greater than 750μM, PA inhibited proliferation of SCC-25 cells by greater than 30% (Fig 1B). POH (Fig 1A) was more effective than PA (Fig 1B) at reducing cellular proliferation. We evaluated the long-term effects of cell exposure to the monoterpenes using colony formation assay technique. Exposure to POH (500μM or 1000μM) for 24 hr significantly (p < 0.05) reduced the proliferation of SCC-25 and BroTo cells. HFL-1 cells also appeared very sensitive to POH. Figure 2B shows the effect of PA on these cell lines. Similar to earlier observations (Fig 1B), SCC-25 cells appeared more sensitive to PA than either the BroTo or HFL-1 cells. Exposure to 1000μM PA inhibited the proliferation of SCC-25 cells by >25% (Fig 2B).

We also examined the effects of the monoterpenes on human lung squamous cell carcinoma cell lines (CRL-5928 and HTB-182). Both POH and PA reduced the proliferation of CRL-5928 cells in dose- and kinetic-dependent manners (Fig 3). The 72-hr IC$_{50}$ for POH was 750μM compared to 1800 μM for PA (Fig 3). Similar responses were observed with HTB-182 (data not shown). We also compared the effects of POH (500μM), PA (1000μM), cisplatin (2.5μg/ml) and 5-FU (100μg/ml) on the human squamous cell carcinoma (HTB-182) cell line treated for 24, 48 or 72 hr using mitochondrial dehydrogenase assay technique (Fig 4). We found that at 48 hr, the monoterpenes reduced cellular proliferation better than in the groups treated with cisplatin or 5-FU (Fig 4). The relative efficacy of these agents, at the stated concentrations and 48 hr exposure, were POH>PA>cisplatin>5-FU (Fig 4). The response to POH at 72 hr (Fig 4) is not clear.

Effects of the jasmonates on human prostate cancer cells:
We also evaluated the effects of the plant stress hormones on human prostate adenocarcinoma (PC-3; HTB-81) cell lines cultured in vitro. Figure 5 shows the comparative effects of the jasmonates on human adenocarcinoma cell lines treated for 72 hr. The IC$_{50}$ values were MJ (1.8mM) and CJ (2.25mM). An IC$_{50}$ value was not achievable for JA (Fig 5A). PC-3 cells exhibited differential sensitivities to the three jasmonates with MJ being more effective than CJ or JA at reducing the proliferation of the cancer cells (Fig 5A). At concentrations greater than 2.0mM, MJ significantly inhibited proliferation of PC-3 cells better than CJ (p<0.05) or JA (p<0.005). MJ also significantly (p< 0.005) inhibited the proliferation of HTB-81 prostate cancer cells better than CJ or JA (Fig 5B) at 1mM concentration or higher.

**Plant compounds induced cell cycle arrest and sub-G$_0$/G$_1$ populations in cancer cells:**

In preliminary experiments, we studied the effects of POH, PA and MJ on cell cycle distribution and subdiploid DNA content of cancer cells. POH caused cell cycle arrest in the G$_0$/G$_1$ phase and an increase in the hypodiploid content of SCC-25 and BroTo cells (Table 1A) after 24 hr exposure. PA also caused cell cycle arrest in the G$_0$/G$_1$ phase in BroTo cells but not in SCC-25 cells and slightly increased the sub-G$_0$/G$_1$ content compared to the control (Table 1A). Table 1B shows that MJ (at 2mM) caused cell cycle arrest at G$_0$/G$_1$ phase in both PC-3 and HTB-81 cell lines in a time-dependent fashion. The cell cycle arrest was accompanied by increased proportion of sub-G$_0$/G$_1$ population in both cell lines (Table 1B). These preliminary data will be further studied to confirm the induction of apoptosis.
DISCUSSION AND CONCLUSION:

We used biochemical, cytotoxicity and flow cytometric assays to evaluate the potential effects of 2 major plant compounds on the proliferation of several human cancer cell lines in culture. We found that POH and, to a lesser extent PA, the major circulating metabolite of the monoterpenes, reduced the proliferation of human head & neck squamous cell carcinoma SCC-25 (Fig 1), human lung squamous cell carcinoma cell lines CRL-5893 (Fig 3) and HTB-182 (Fig 4) in culture. Our data suggest that the mechanism for the proliferation inhibition by these compounds may involve cell cycle arrest and the induction of apoptosis. Ongoing and further investigations will confirm these effects.

Several reports have indicated that naturally occurring monoterpenes (d-limonene and perillyl alcohol) are present in human diets (13,29,30) though only the metabolites of these compounds have been detected in blood plasma following consumption of the monoterpenes (12-16). Since the monoterpenes are protective against a number of different cancers (7-10,17,20) and are currently being studied in clinical trials (12,14,15), we postulated that the mechanism of action of these compounds would involve the circulating metabolites. Furthermore, recent reports indicated that PA, at doses (1-3.5mM) higher than physiologically achievable levels, inhibited the proliferation of human colon cancer cells by the up-regulation of p21\(^{Waf1/Cip1}\) protein and down-expression of cyclin D1 and cdk4 proteins (31). PA also inhibited the proliferation of smooth muscle cells via the inhibition of protein prenylation (32). In cultured rat lens, however, PA appeared to induce opacification of the cultured lenses (33) but via a mechanism other than the inhibition of isoprenylation. Our results, showing cell cycle...
arrest and the inhibition of proliferation of these cancer cells at physiologically achievable PA concentrations, are in agreement with these reports (30-33).

When we tested the plant stress hormones on human prostate adenocarcinoma cell lines, we found that MJ was a more potent inhibitor of the proliferation of the cancer cells in culture than CJ or JA. Although CJ showed some activity against PC-3 cell line (Fig 5A), it did not elicit any inhibitory effect on HTB-81 cells at the concentrations tested. JA was the least effective, not showing any effect on either cell line (Fig 5). These results are in agreement with the reports by Fingrut and Flescher (26) who tested the jasmonates on human leukemia (MOLT-4) and breast cancer cell lines. These investigators found that MJ was a more potent inhibitor of proliferation of the cells than JA. They concluded that the mechanism of action of MJ appeared to be via the induction of apoptosis (26). Our preliminary flow cytometry data following the propidium iodide staining and DNA analysis of cells treated with POH, PA or methyl jasmonate (Table 1) indicated that the inhibition of proliferation by these plant compounds and metabolite might be through cell cycle arrest and the induction of apoptosis.

The role of plant compounds on the etiology of human diseases is currently being investigated intensively. Evidence show that as humans include more plant products in their diets, the potential for the consumption of several plant compounds that may be protective against human diseases increases. Current efforts to identify, characterize and understand the mechanism of action of these bioactive plant compounds are in the right direction. Further studies are in progress to delineate the mechanism(s) of action of the monoterpenes and the jasmonates, and to assess their potential effects on human cancer cells.
Table 1: Effects of bioactive plant compounds on cell cycle distribution and hypodiploidy in head & neck squamous cell carcinoma (1A) and prostate adenocarcinoma (1B) cell lines in vitro.

1A: Effect of 24 hr exposure to POH and PA on BroTo and SCC-25 cell lines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BroTo</th>
<th>SCC-25</th>
</tr>
</thead>
</table>
|           | \(G_0/G_1\) | S | \(G_2-M\) | sub- | \(G_0/G_1\) | S | \(G_2-M\) | sub-
|           |            |       |            | \(G_0/G_1\) |            |       |            | \(G_0/G_1\) |
| Control   | 54.0       | 33.2  | 12.8       | 0.33  | 46.1       | 38.2  | 15.7       | 0.28  |
|           | ±7.1       | ±5.0  | ±2.1       | ±0.1  | ±4.5       | ±2.7  | ±1.8       | ±0.1  |
| 0.5mM POH | 76.5       | 12.0  | 11.5       | 2.87  | 65.7       | 23.9  | 10.5       | 9.55  |
|           | ±2.9       | ±2.5  | ±0.4       | ±1.1  | ±0.5       | ±0.9  | ±0.3       | ±0.2  |
| 1.0mM PA  | 70.7       | 18.5  | 10.8       | 1.36  | 45.4       | 41.7  | 12.8       | 0.54  |
|           | ±3.1       | ±4.7  | ±1.6       | ±0.1  | ±0.5       | ±0.8  | ±0.5       | ±0.1  |

1B: Effect of methyl jasmonate (MJ) on PC-3 and HTB-81 cells in culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC-3</th>
<th>HTB-81</th>
</tr>
</thead>
</table>
|           | \(G_0/G_1\) | S | \(G_2-M\) | sub- | \(G_0/G_1\) | S | \(G_2-M\) | sub-
|           |            |       |            | \(G_0/G_1\) |            |       |            | \(G_0/G_1\) |
| 24 hr     | Control   | 51.9  | 27.0  | 21.2       | 0.11  | 53.8       | 30.0  | 16.2       | 0.33  |
|           | ±0.3      | ±0.4  | ±0.7      | ±0.0  | ±0.5      | ±0.6  | ±1.1      | ±0.3  |
|           | 2.0mM MJ  | 46.3  | 30.8  | 22.9       | 0.12  | 40.6       | 32.9  | 26.5       | 1.02  |
|           | ±0.4      | ±0.1  | ±0.3      | ±0.1  | ±3.0      | ±0.3  | ±2.7      | ±0.7  |
| 48 hr     | Control   | 53.8  | 26.9  | 19.3       | 0.15  | 81.0       | 9.9   | 9.1        | 0.15  |
|           | ±1.5      | ±1.6  | ±0.1      | ±0.0  | ±1.4      | ±0.5  | ±0.9      | ±0.0  |
|           | 2.0mM MJ  | 73.0  | 5.5   | 21.5       | 1.63  | 51.8       | 20.7  | 27.5       | 4.0   |
|           | ±1.0      | ±1.8  | ±0.8      | ±2.0  | ±6.9      | ±0.6  | ±6.2      | ±1.0  |
| 72 hr     | Control   | 70.7  | 13.2  | 16.2       | 0.14  | 87.8       | 5.6   | 6.6        | 0.18  |
|           | ±0.3      | ±0.6  | ±0.8      | ±0.1  | ±0.5      | ±0.6  | ±0.1      | ±0.0  |
|           | 2.0mM MJ  | 48.2  | 28.2  | 23.7       | 0.3   | 48.1       | 31.3  | 20.6       | 8.8   |
|           | ±0.3      | ±0.1  | ±0.2      | ±0.2  | ±4.2      | ±6.3  | ±2.1      | ±3.4  |

Cancer cells (5 x 10^6) were plated in 60mm diameter tissue culture plates and treated with POH, PA or MJ for the specified period of time and analyzed on a FACSCalibur flow cytometer as described in the text. Values are Mean ± SD of triplicate analyses. Sub-\(G_0/G_1\) = % Apoptosis or hypodiploid population.
FIGURE LEGENDS:

Figure 1: Effect of the monoterpenes on human head and neck cancer (SCC-25; BroTo) and human lung fibroblast (HFL-1) cells in culture. Cells were exposed to varying concentrations of perillyl alcohol (POH) or perillic acid (PA) for 24 hr and the effects assessed by mitochondrial dehydrogenase assay as described in the Methods section. Cytotoxicity was measured as cell viability. Cell viability was calculated as a percentage of untreated Control cells (100%). Figure represents a plot of the log value of % viability versus concentration of monoterpenes. Values are Mean ± SD (n=6); result is representative of 3 separate experiments.

Figure 2: Monoterpenes caused long-term inhibition of proliferation of cells in culture. Human cancer (SCC-25; BroTo) or human fibroblast (HFL-1) cells were exposed to equimolar concentrations of perillyl alcohol (POH) or perillic acid (PA) for 24 hr and the effects determined by colony formation assay as described in the Methods section. The colonies (containing ≥50 cells/colony) formed were counted and the cell viability was calculated as a percentage of the untreated Control groups (100%).

Figure 3: Cytotoxic effects of monoterpenes on human lung cancer cell line CRL 5928. Cells were exposed to varying concentrations of perillyl alcohol (POH) or perillic acid (PA) for 24, 48 or 72 hr and the effect evaluated as described for Figure 1.

Figure 4: Sensitivity of lung squamous cell carcinoma cells to different chemotherapeutic agents. Human lung cancer (HTB-182) cells were exposed to perillyl alcohol (POH),
perillic acid (PA), cisplatin or 5-fluorouracil (5-FU) for 24, 48 or 72 hr and the viability
determined using the MTT assay technique as described for Figure 1.

**Figure 5:** Comparative effects of the jasmonates on human prostate cancer cells in
culture. Human prostate adenocarcinoma cell lines PC-3 or HTB-81 were treated with
varying concentrations of jasmonic acid (JA), cis-jasmone (CJ) or methyl jasmonate (MJ)
for 72 hr and the cytotoxicity determined as described for Figure 1.

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Figure 1: Effects of monoterpenes on human head & neck cancer and fibroblast cells in culture

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Figure 2: Monoterpenes caused long-term inhibition of proliferation of cancer cells in culture

![Graph showing viability of cancer cells at different concentrations of POH and PA](image)

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Figure 3: Monoterpenes exhibited cytotoxic effects against human lung cancer (CRL 5928) cells in culture.
Figure 4: Sensitivity of lung squamous cell carcinoma (HTB-182) cells to different chemotherapeutic agents

Surviving fraction as % of Control

Exposure time, hr

- □ 500uM POH
- □ 1000uM PA
- □ 2.5 ug/ml Cisplatin
- □ 100 ug/ml 5-FU

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Figure 5: Comparative effects of the jasmonates (JA, CJ, MJ) on cultured human prostate cancer (PC-3; HTB-81) cells.
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