Cancer is one of the most dreaded diseases of mankind that causes alarming mortality and morbidity in humans. Through innumerable research efforts, now it is well established that causes of the cancer are vast and diverse, but changes in life style and dietary habits can reduce the risk of this deadly disease. Thus, utilization of complementary and alternative medicines (CAM) becomes an important and promising strategy for the prevention as well as treatment of cancer. With the growing awareness about the potential role of CAM in preventing cancer and other chronic diseases, most of the researches are now focused on validating many of the ancient remedies. Both epidemiological and experimental studies suggest that enormous benefit can be derived at community level from dietary manipulation or supplementation because of their relatively low cost and little or virtually non-toxic effects. Cancer chemoprevention using dietary constituents has received growing consideration as a means of cancer control in the last few decades. It can be defined as ‘the prevention of cancer by the administration of one or more chemical entities either as individual drugs or naturally occurring constituents of the diet’. The appropriate use of a chemopreventive agent ultimately depends on the understanding of its mechanism of action at all levels, namely at the molecular, cellular, tissue and organ level. The present need in the chemoprevention has therefore been to identify/develop new molecules/agents based on their mechanism of action or to elucidate the mode of action of well-known agents. The most promising area included here is the study at molecular level as molecular targeting approaches retain (or enhance) preventive effects while reducing known
mutagenic/carcinogenic effects. These molecular targeting approaches include identification and validation of certain biomarkers namely mutations in specific genes, abnormalities in cell proliferation and alteration in gene expression, as early or intermediate end points in chemopreventive strategies.

Human diet contains a variety of naturally occurring compound having antimutagenic and anticarcinogenic properties. Garlic (*Allium sativum*) is known since ancient times for its therapeutic properties and is probably the most widely studied medicinal plant. The beneficial health effects of garlic have been attributed to its organosulfur compounds such as S-allyl cysteine, allicin, ajoene, diallyl sulfide (DAS), diallyl disulfide, and diallyl trisulfide. DAS, a lipid soluble organosulfur volatile compound from garlic, with its characteristic pungent odor is a potent antioxidant with anti-inflammatory, antimutagenic and cancer preventive properties. *In vitro* as well as *in vivo* studies from our lab and elsewhere have shown that DAS imparts chemopreventive effects against cancer in a variety of target organs such as skin, lung, esophagus, colon and liver. However, the mechanism by which DAS might exert its anticarcinogenic effects is still not fully understood. DAS has been shown to modulate the metabolism of carcinogens through various phase I and phase II detoxifying enzymes and by scavenging free radicals and blocking carcinogen bioactivation. It has also been shown to possess inhibitory effects on the proliferation of tumor cells associated with induction of apoptosis by modulating the expression of antiapoptotic (*bcl-2*), proapoptotic genes (*bax*) and the tumor suppressor gene. Therefore, present study was designed and executed to provide insight into the molecular mechanism of DAS induced tumor suppression using induction of apoptosis, induction of tumor suppressor gene expression, inhibition of oncogene expression as biomarkers in chemoprevention studies as well as its role in chemoprophylactic studies by investigating its multiple drug resistance reversal properties.

**Cancer Chemopreventive and Apoptosis Inducing Potential of Diallyl Sulfide in Mouse Skin Carcinogenesis**

During the past few decades, cancer chemoprevention by dietary constituents has received a great deal of attention as a means of effective cancer control. Studies on the tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Growing evidences from both *in vitro* and *in vivo* studies showed that suppression of apoptosis is involved in tumor promotion. Therefore, inhibition of
apoptosis is one mechanism of tumor formation and many chemopreventive agents may act through the induction of apoptosis by eliminating the defective cells, to block the carcinogenic process. Thus, induction of apoptosis is appreciated as an ideal way for the elimination of cancer cells and apoptosis inducing agents are viewed as potential agents for the prevention and/or treatment of cancer. We therefore studied the apoptosis inducing potential of DAS in DMBA induced mouse skin tumors in order to study its cancer chemopreventive mechanism.

The results of the present set of investigations revealed that DAS treatment could effectively reduce the cumulative and average number of tumors per mouse and effectively prolonged the tumor induction time of DMBA induced tumors. The animal bioassay revealed a significant delay in the onset of skin tumorigenesis in DAS supplemented Swiss mice by more than three weeks in comparison to the animals exposed to DMBA alone. The average number of tumor per mouse was found to be inhibited in groups where DAS was given one-hour prior or after the DMBA treatment, in comparison to DMBA exposed animals. An important feature of the study was that a significant percentage of animals remained tumor free in DAS supplemented groups. Further, present investigation revealed the apoptosis inducing potential of DAS in DMBA induced tumors. Flow cytometric analysis revealed appearance of sub G1 peak indicative of apoptotic population in solid tumors. Quantitation of apoptosis evaluated by the percentage of cells in the sub G1 phase revealed about 15.4% apoptotic population in DMBA treated group which was increased significantly in tumor cells of DAS+ DMBA (42.8%) and DMBA+DAS (34.6%) groups. Analysis of skin tumors revealed other characteristic features of apoptosis including the formation of DNA ladders on agarose gel, compaction of nuclear DNA and the formation of apoptotic bodies. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay of skin tumors of DAS supplemented animals showed a significant increase in the number of apoptotic bodies in comparison to the animals exposed to DMBA. Examination of nonmalignant tumors viz. focal epidermal hyperplasia, squamous cell papillomas, keratoacanthoma and malignant squamous cell carcinoma revealed a significant increase in the TUNEL positive cells in DAS supplemented tumor over positive controls (DMBA alone). These observations thus suggest that induction of apoptosis may be the major contributing factor for antitumorigenic properties of DAS.
Molecular Mechanism of DNA Damage Response and Tumor Suppression in Mouse Skin

Role of p53 and p21/waf1 in Dialyl Sulfide Mediated Tumor Suppression in Mouse Skin

The p53 tumor suppressor gene contributes to the maintenance of genomic stability by controlling cell cycle and facilitating DNA repair in response to DNA damage. Therefore, alterations in the p53 function are critical events in carcinogenesis. Thus, modulation of p53 and its effector molecules p21/waf1 levels are thought to be an effective way of intervention against cancer. The results of the present study showed a modulatory effect of DAS on the expression of tumor suppressor gene p53 (both wild and mutant type) and its effector molecule p21/waf1 in mouse skin tumors. Western blot analysis and immunohistochemical localization of the protein combined with multi-parameter flow cytometry demonstrated that DAS administration induces the expression of the wtp53 and decreases the expression of mutp53 in DMBA induced tumors. Western blotting showed enhanced levels of wtp53 in DMBA induced tumors, which were found to be significantly elevated with DAS supplementation. The densitometric analysis of blots showed an increase of 132% in wtp53 in the tumor tissue of DMBA exposed animals over untreated controls. A further increase of 40% and 31% in the wtp53 protein expression was observed in DMBA supplemented and DMBA induced tumors. The quantitative analysis of immunostained tumor sections using image analysis showed significant increase of 66.55 & 54.21% in the levels wtp53 in DAS pre treated and post treated animals. Similarly, flow cytometric analysis showed increase in the mean fluorescence intensity (MFI) for DMBA induced tumors (5.31) over normal mouse skin (1.68). A further increase in the fluorescence was recorded in the DAS supplemented groups suggesting increase in the levels of wtp53 by DAS. The MFI was 15.41 and 10.21 respectively in DAS pre and post supplemented groups.

In case of mutp53, the DMBA induced levels were found to reduce to near normal levels with DAS supplementation as evident by western blotting. The densitometry of the blots showed that in DAS pretreated tumors the expression of the mutp53 decreased by 44.9 % whereas in DAS post treated tumors the expression of the protein was decreased by 41.7% over DMBA induced tumors. The quantitative analysis of immunostained sections using image analysis showed about 53.41 and 44.26 % decrease in the mutp53 levels in DAS pre treated and post treated animals respectively. Flow cytometric analysis further confirmed these observations. The normal skin showed low expression levels of mutp53 with MFI 1.06. In DMBA induced
tumors a significant increase in the fluorescence was recorded with MFI 16.41 indicating over expression of mutp53. The FITC fluorescence was found to decrease in DAS supplemented groups with MFI 3.76 in DAS+ DMBA and 8.13 in DMBA +DAS groups, suggesting down regulation of mutp53 by DAS. The DAS exposed skin showed levels of mutp53 close to normal levels with MFI 1.65. The DMBA induced tumor showed significantly elevated levels of p21/waf1 as evident by western blotting. The DMBA induced levels of p21/waf1 were further increased in DAS supplemented groups. The quantitation of the band intensity by densitometry showed an increase of 72% in the p21/waf1 level in DMBA induced tumors. In the DAS pretreated tumors, the expression of p21/waf1 was significantly (74%) induced as compared with DMBA alone. Similarly, in the DAS post treated group, the p21/waf1 expression was 65% high over DMBA exposed group. The immunohistochemical staining of p21/waf1 levels in skin/tumor sections showed 72.86 & 61.22% percent increase in the expression of the protein in DAS pre and post treated groups over DMBA treated animals. Furthermore, flow cytometric analysis revealed a shift in the MFI from 6.34 for untreated to 22.42 for DMBA induced tumors. This shift in the MFI was further enhanced to 53.35 and 44.24 in tumors where DAS was given before and after DMBA exposure respectively. In animals were given DAS only, the fluorescence was recorded at 8.07 channel value indicating normal levels of p21/waf1 expression. These results thus demonstrate that the chemopreventive property of DAS seems to be through the p53 dependent mechanism mediated through its effector molecule p21/waf1.

Modulatory Effect of Diallyl Sulfide on p21/ras in Skin Carcinogenesis

The ras gene family members were among the first oncogenes identified and shown to have decisive roles in cell cycle and in neoplastic development. A feature of the ras coded proteins is that they must be anchored in the cell membranes to function. Specific inhibitors of this step as a means of inhibiting activity of the transforming ras protein may serve as a biomarker of the probability of tumor formation. Thus detection of mutations or altered expression of these genes could help to identify individuals at increased risk of cancer and could be utilized as a effective chemopreventive intervention strategy. The results of the present set of investigations showed a modulatory effect of DAS on the expression H-ras protein product p21/ras following single topical exposure of DMBA after 24hrs. The decrease in the levels of the protein was significant at 48 & 72 hrs of DMBA exposure. Furthermore, the
modulatory effects of DAS were also evident in DMBA induced tumors. The normal mouse skin showed levels of both cytosolic and membrane bound $p21/ras$ within the detectable limits as evident by western blot analysis. In DMBA induced tumors high levels of $p21/ras$ were found in both the fractions in comparison to controls. However, the levels of cytosolic $p21/ras$ in DAS supplemented groups were comparable to the levels of the protein in DMBA induced tumors. Whereas, an opposite trend was evident for the levels of membrane bound $p21/Ras$. A significantly diminished level of the protein was observed in the membrane fractions from DAS supplemented groups as compared to that of DMBA alone. The quantitation of $p21/ras$ levels by densitometric analysis of blots showed 11 and 15 % increase in cytosolic $p21/ras$ in DAS pre and post-treated group respectively in comparison to DMBA induced tumors. Whereas, a significant decrease in the levels of the membrane bound protein was evident in DAS supplemented groups as compared to DMBA. This decrease was 34 and 26% in tumors where DAS was given before or after the DMBA exposure respectively. The levels of both cytosolic and membrane bound $p21/ras$ in DAS exposed skin were found to be normal. The results of the immunohistochemical staining of the skin/tumor sections further lent support to observation that DAS pre and post supplementation decreased the $p21/ras$ expression by 55.82 & 46.86% respectively, thus checking the growth of neoplastic cells. The results of the flow cytometric analysis further lent support to the findings of western blotting and immunohistochemistry. A significant shift towards higher fluorescence was observed in MFI 1.21 (control) to 13.77 (DMBA) induced tumors. This shift in the fluorescence was decreased to 5.42 and 8.76 in tumors where DAS was given before and after the DMBA exposure. In DAS exposed skin, the fluorescence was recorded at 1.72 channel value indicating normal levels of $p21/ras$ expression. These results thus provide clear evidence that the membrane association of $p21/ras$ is significantly inhibited with a concomitant increase in cytosolic $p21/ras$ by DAS administration in DMBA induced mouse skin tumors. Thus, one mechanism of the growth inhibitory properties of DAS is through the suppression of development of tumors that harbor ras mutations by inhibiting the membrane association of oncogenic $p21/ras$.

**In Vitro and In Vivo Multiple Drug Reversing Potential of Diallyl Sulfide**

Multidrug resistance (MDR) mediated by the over-expression of drug efflux protein P-glycoprotein (P-gp) is one of the major obstacles to successful cancer
Molecular Mechanism of Tumor Suppression Carcinogenesis

chemotherapy. P-gp acts as an energy dependent drug efflux pump, reducing the intracellular concentration of structurally unrelated drugs. Modulators of P-gp function have been shown to restore the sensitivity of MDR to such cytotoxic drugs. Unfortunately, most of these compounds are not useful to tackle the problem of drug resistance at a clinically sustainable level because of unacceptable side effects or toxicity at doses required for effectiveness. Therefore, these limitations have spurred efforts to search for new compounds with low toxicity and high efficacy with due attention to dietary agents. An advantage of using newly identified dietary agents, as a modulator of MDR is that they enhance antitumor activity and exhibit little or no virtually no side effects.

The present study revealed the in vitro and in-vivo P-gp modulatory potential of DAS in Leukemic K562 cells and mouse liver. K562 leukemic cells were made resistant (K562/R10) towards the cytotoxicity of vinblastine (VBL) by progressive adaptation of the parental sensitive K562 cells to VBL. Cross-resistance of K562/R was found between vincristine (VCR), doxorubicin and other antineoplastic agents. A non-toxic concentration of DAS (8.75 x 10^{-3}M) enhanced the cytotoxic effects of VBL and another vinca alkaloid, VCR, time dependently in VBL resistant human leukemia (K562/R10) cells but had no effect on the parent (K562/S) cells. The results showed that DAS decreased the induced levels of P-gp in resistant cells back to the normal levels as analyzed both qualitatively and quantitatively by western blotting and immunocytochemistry. Quantitation of immunocytochemical staining showed that this inhibition of P-gp expression by DAS was 23, 54 & 79% for 24, 48 & 72 hrs respectively in K562/R10 cells. DAS had no effect on P-gp expression in parental K562/S cells. Furthermore, in-vivo combination studies showed that DAS effectively inhibited the vinca alkaloid induced P-gp over expression in mouse hepatocytes as evident by western blotting. Quantitation of immunostained tissue sections with image analysis showed that reduction in P-gp levels was up to the extent of 73% for VBL and 65% for VCR induced drug resistance. The above features thus indicate that DAS can serve as a novel, non-toxic modulator of P-gp expression and can be used as a dietary adjuvant for the reversal of MDR.