Chapter 8: Summary and Conclusions

Cancer is a major killer disease in most developed and underdeveloped countries. It is a growing public health menace, and more than six million new cases of this disease are reported every year. All cancers cell genotypes are manifestation of six essential alterations in cell physiology such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis and evasion of programmed cell death (apoptosis). Evasion of apoptosis is hallmark of all cancer and it has been accepted as the predominant mechanism of drug-induced cell death in preclinical experimental models and in clinically sensitive tumors. So targeting it provides a bull eye oriented approach in novel anticancer drug development. Nature has provided a rich storehouse of herbal remedies to cure all ailments of humankind. Plant products have been a source of medicinal agents since ancient time and the introduction of active agents derived from nature into the cancer armamentarium has changed the natural history of many types of human cancer as exemplified by introduction of paclitaxel, vincristine, vinorelbine, teniposide, camptothecin. Their dominant role is evident from the fact that approximately 60% of anticancer compounds are either natural products or natural product derivatives. A golden triangle consisting of Ayurveda, modern medicine and science will converge to form a real discovery engine that can result in newer, safer, cheaper and effective therapies (CSIR theme). Experimental agents derived from natural products are offering us a great opportunity to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of their action.

Sensing the importance and anti-cancerous properties of plants, the present study entitled “To establish the mechanism of action and develop some plant based products as novel anticancer agents” was carried out to elucidate the molecular mechanism of action of apoptosis induced by a novel lignan composition AP9-cd from Cedrus deodara and a triterpenediol (TPD) from Boswellia serrata for its therapeutic applications in cancer. The review of literature on AP9-cd and Boswellia serrata have described in-depth the pharmacological profile of their activities from ancient traditional knowledge and current scientific investigations. This dissertation is an attempt in elucidating the molecular mechanism of action and describing other preclinical data so as to bring them to a level of novel anti-cancer therapeutic leads.
Several problems are encountered mostly with anticancer compounds, such as normal tissue toxicity, poor specificity, low systemic bioavailability, high incidence of multi-drug resistance (MDR) phenotype and drug permeation barriers at tumor site which involves active efflux of drug. All these type of problems lead to compromised clinical outcomes even though an anticancer drug has strong *in vitro* efficacy. Therefore there is always a need to develop a suitable drug carrier system for a better drug delivery at the site of action with minimum exposure to normal cells. Problems related to cancer chemotherapy are reported to be partially overcome by delivering them using nanoparticulate system. Therefore, a nanoparticles drug delivery system involving solid lipid nanoparticles of both the drugs/agents was designed and evaluated for *in vitro and in vivo* anticancer activity.

AP9-cd, a standardized lignan composition from *cedrus deodara* consisting of (-)-wikstromal, (-)-matairesinol and dibenzyl butyrolactol, showed cytotoxicity in several human cancer cell lines. An attempt was made in this study to investigate the mechanism of cell death in human leukemia Molt-4 and HL-60 cells. It inhibited Molt-4 cell proliferation with 48-h IC_{50} of ~15 μg/ml, increased sub-G0 cell fraction with no mitotic block, produced apoptotic bodies and induced DNA ladder formation. It is interested to note that AP9-cd is a synergistic composition, as individual components have lower cytotoxic effect as compare to AP9-cd. Flow cytometric analysis of annexinV-FITC/PI stained cells showed time-related increase in apoptosis and post-apoptotic necrosis. All these biological end-points indicated cell death by apoptosis. AP9-cd induced cell death involved any early generation of reactive nitrogen or oxygen species which may consequently activate effector caspases leading to DNA fragmentation and activation of apoptotic pathways. Massive nitric oxide (NO) formation within 4h was found to be initial events with subsequent late appearance of peroxides in cells. The source of indigenous nitric oxide was found to be through inducible nitric oxide synthase (iNOS). Persistently high NO formation and peroxides formation are either metabolized further to reactive oxygen species or increasing proportions of NO utilized to the formation of other lethal reactive nitrogen oxide species (RNOS). The biological consequences of such interactions can produce activation of both intrinsic and extrinsic apoptotic pathway and suppression of nuclear transcriptional factor NF-kB. AP9-cd significantly induces apical death receptors such as Fas, DR4, TNF-R1 and suppression of NF-kB. Early generation of NO may
further disrupts the balance of Bcl-2 family member proteins and may affect mitochondrial membrane potential, $\Psi_{\text{int}}$. Despite above effects, AP9-cd induced release of cytochrome c from mitochondria, drastically decrease Bcl-2/ Bax ratio and loss of mitochondrial membrane potential in HL-60 cells that finally activate caspases. AP9-cd activates caspase-8,-9 and -3 in both HL-60 and Molt-4 cells. Caspase-9 activation is solely because of mitochondrial dependent release of apoptotic factors whereas activation of caspase-8 and death receptor suggests the involvement of mitochondrial independent (extrinsic) pathways mediated through cell surface death receptors. Caspase-3 plays a pivotal role in programmed cell death via proteolytic cleavage of poly (ADP-ribose) polymerase which can be activated though both mitochondrial dependent and independent pathways. Further, caspase-3 activation correlated with NO generation that was partially impaired by nitric oxide synthase (NOS) inhibitors and ascorbate suggesting a role of pro-oxidant species in caspase-3 activation. The present studies have provided a deeper insight into the mechanism of action of AP9-cd induced apoptosis in cancer cells for the first time. AP9-cd produced no cytotoxicity in primary rat hepatocyte culture as well as in mice peritoneal macrophages. AP9-cd has a very high LD$_{50}$ value (1000 mg/kg) in comparison to podophyllotoxin (15 mg/kg).

Physico-chemicals characteristics of AP9-cd such as partition coefficient (log P) and water solubility were not excellent. A low solubility, a faster metabolic rate and a not very high log P (approx. 3) indicate the need for appropriate formulation development so as to achieve improved and sustained bioavailability and thus it was packaged suitably into a lipophilic system like Solid lipid nanoparticles (SLNs). Solid lipid nanoparticles (SLNs) of AP9-cd were successfully formulated through micro-emulsion method involving compritol 888 ATO, lecithin soya and tween-80. Particle size of AP9-cd SLNs was in the range of 100-200nm and they were round, regular and solid and there was a 40% increase in size after six months stability study. AP9-cd loaded SLNs have more cytotoxic and anti-tumor potential than the parent drugs. AP9-cd-SLNs have significantly lower IC$_{50}$ value, higher apoptotic potential with no cytotoxicity on normal cells. The in vivo fate of the AP9-cd and its SLNs was also evaluated in Ehrlich ascites tumor (EAT) model. AP9-cd causes 51% tumor regression at 300mg/kg b.wt. intra-peritoneal in EAT model, whereas AP9-cd-SLN in vivo potential was significantly higher (58%) than the AP9-cd in EAT bearing mice.
So the method and composition of the formulation was up to the marks and it successfully increased the apoptotic and anti tumor potency of AP9-cd at same doses. The approach of drug development from plant depends on the selection of a suitable plant. There are several ways in which this will be done including traditional knowledge of whole plant or its constituents, serendipity, hit and trial or a combination of several factors. Among these the most common and successful strategy is selection of plant material on the basis of traditional knowledge in different cultures. Based on this knowledge we had chosen next chemical entity from gum resin of Boswellia serrata. Gum resin extracts of Boswellia species have been traditionally used to treat various chronic inflammatory disorder and cancer. Based on the ethno pharmacological knowledge of gum resin of Boswellia species we isolated pentacyclic triterpenediol from Boswellia serrata, which exists in nature as an isomeric mixture of 3α, 24-dihydroxurs-12-ene and 3α, 24-dihydroxyolean-12-ene (TPD or SS-176, RRL code). Some of our preliminary studies on TPD show its cytotoxicity potential against a panel of human cancer cell lines. It was therefore our interest to find out in-depth the mechanism of action of cell death by TPD, particularly in the induction of programmed cell death (apoptosis) in HL-60 cells. TPD induced apoptosis measured by various biological end points such as DNA fragmentation, apoptotic body’s formation and annexin-V binding in HL-60 cells, while no such effect in terms of DNA fragmentation and damage was observed in human PBMC and monkey kidney normal cell line CV-1. Early events which cause apoptosis were early generation of reactive nitrogen or oxygen species (RNOS). Nitric oxide plays an important role in regulating electron transport chain and its overproduction cause inhibition of electron transport chain, which may lead to apoptosis and cell cytotoxicity. The production of NO by TPD in HL-60 cells is presumably through iNOS induction. It is also reported that increases intracellular levels of ROS induce p21 in both normal fibroblasts and in p53-negative cancer cells. TPD up-regulation of p21 and its inhibition by NAC in HL-60 cells, suggests pro-oxidant role of TPD. Both the ROS and iNOS inhibitors had a protective effect on the DNA damage induced by TPD, suggesting the pro-oxidant effect of TPD in cancer cells. NO and ROS production by TPD further produce loss of mitochondrial membrane potential, ΔΨm, which was inhibited substantially by cyclosporine-A indicating involvement of mitochondrial PTP in the release of proteins. Further, TPD induces activation of caspase-8,-9 and -3 in HL-60 cells.
which were inhibited significantly by ascorbate, NAC and sMIT. TPD more aberrantly induce caspase-9 activation, which is solely regulated by mitochondrial release of apoptotic protease activating factor-1 (Apaf-1) and cytochrome c, suggesting the role of mitochondrial dependant apoptosis pathway. TPD also induced the caspase-8 activation and cell surface receptors such as TNF-R1 and DR-4 in HL-60 cells, which was significantly inhibited by NAC and sMIT, suggesting again the involvement of pro-oxidant effect of TPD in the extrinsic signaling pathway of apoptosis in HL-60 cells.

We further investigated the effect of TPD on the level of Bcl-2 family proteins. Normally Bcl-2 level is much higher in cancer cell and TPD induced cleavage of Bcl-2 in HL-60 cells, suggesting that RNOS oxidatively modified Bcl-2 protein to a Bax like protein through proteolytic cleavage by caspase-3. Bcl-2 cleavage by TPD causes the opening of mitochondrial transpermeable pore and brings about Bax translocation from cytosol to mitochondria and consequent release of small molecules like AIF, Smac/DIABLO, apoptosis inducing factor (AIF) and cytochrome c from mitochondria, which impairs mitochondrial functions and brings the cells to a “point of no return” to enter apoptosis via activation of caspases. AIF is translocated to the nucleus and directly induce chromatin condensation and DNA fragmentation, without the help of caspases. TPD induce Smac/DIABLO not only promotes the activation of procaspase-3 by neutralizing the activity of IAP such as survivin but also down regulates the expression of survivin. So, by inhibiting the survivin, TPD reinforces the caspase-3 activity and finally enhances apoptosis. When caspase-3 was activated by TPD, ICAD (inhibitor of caspase activating DNase) got cleaved, resulting in the release of CAD, which causes DNA fragmentation in nuclei. Activated caspase-3 could utilize poly-ADP ribose polymerase (PARP), a DNA repair enzyme as its substrate as a result a cleaved product of PARP was observed in our studies. Both the PARP cleavage and inhibition of ICAD was substantially protected by the NAC and sMIT. So these findings suggest that TPD caused early NO and ROS production are responsible for the induction of apoptosis through both intrinsic and extrinsic apoptotic pathway.

We further asked if TPD is able to induce apoptosis in cervical carcinoma HeLa cells because there is rapid increase in the occurrence and mortality rate of cervical carcinoma. Current anticancer drugs for cervical cancer were mostly ineffective.
because of the presence of multi-drug resistance receptors in cervical cancer and they have maximum 5-year survival rate. Moreover, they have poor expression of p53 and PUMA (p53 up-regulated modulator of apoptosis) proteins with over expression of oncoproteins E6 and E7, which disarm cervical cancer cells to evade apoptosis. Therefore, there is an urgency to develop novel anticancer leads for management of cervical cancer.

Our results show that TPD kills HeLa cells by activation of apoptotic signaling with biological end-points such as DNA fragmentation, AnnexinV binding and increase in sub-G0 DNA fraction. TPD induced early generation of reactive nitrogen or oxygen species (RNOS), which altered the levels of apoptosis related proteins. Continuous and overproduction of RNOS cause loss of mitochondrial membrane potential and alteration of transcriptional factors such as p53 and p21. The tumor suppressor p53 is a transcription factor that plays a pivotal role in controlling cell cycle checkpoint regulation, DNA repair, transcription, and induction of apoptosis. The level of p53 is very low in HeLa cells and TPD remarkably increase its expression. Induction of p53 by TPD tilts HeLa cells on the road to apoptosis. Over-expression of p53 induces PUMA and p21 (a cyclin-dependent kinase inhibitor) level. PUMA is a member of the Bcl-2 family and a potent inducer of apoptosis mediated by p53. PUMA interacts with Bcl-2 and down-regulate its expression, increased translocation of Bax to mitochondria, loss of mitochondrial membrane potential, release of cytochrome c and activation of executioner caspases. Activation of caspase-9 and -3 were significantly inhibited by various RNOS inhibitors such as ascorbate, NAC and sMIT indicating that RNOS responsible for the apoptosis induce by TPD in HeLa cells. TPD induce apoptosis through the intrinsic pathway because there was no activation of death receptor machinery as well as caspase-8. Moreover, caspase-3 activation was mediated by caspase-9 not through caspase-8. This was confirming by completely blocking the caspase-3 activity by the addition of caspase-9 inhibitor. TPD is also able to modulate other targets such as NF-kB, survivin and PARP which are judiciously over-expressed in all cancer cells. The down-regulation of NF-kB by TPD inhibits the activity of survivin and enhance caspases activity that lead to apoptosis.

Once the usefulness and mechanisms of action of TPD in both HL-60 and HeLa cells are established the next step would be to improve its therapeutic efficacy (reduction in the side effects and dose) by appropriate pharmaceutical formulation development for
controlled and targeted release. TPD have low solubility, a faster metabolic rate and a not very high log P (approx. 3). These properties of TPD indicate the need for appropriate formulation development to improved bioavailability and thus it was packaged suitably into a lipophilic system like Solid lipid nanoparticles (SLNs). TPD-SLNs were successfully formulated through micro-emulsion method involving compritol 888 ATO, lecithin soya and tween-80 with 75% drug entrapment efficiency. Particle size of TPD loaded SLNs was in the range of 180nm and they were spherical, regular and solid and there was a small increase in size after six months stability study (22%) and the system was indicated to more or less stable upon storage. SEM and TEM studies indicated that SLNs to be regular solid spherical particles. Both TPD and its SLNs were evaluated for the \textit{in vitro} and \textit{in vivo} anti-tumor activity. TPD loaded SLNs showed significantly higher cytotoxic/ anti-tumor potential than the parent drugs. TPD-SLNs have less IC\textsubscript{50} value (9pg) in HL-60 cells; they induced DNA fragmentation at low dose (20pg), enhanced the apoptotic cell population up to 50% as compare to TPD. Blank SLNs i.e. SLNs without TPD did not have any cytotoxic effect on the cancer as well as normal cells. When TPD and TPD loaded SLNs were evaluated in Sarcoma-180 solid tumor model the effect obtained with TPD loaded SLNs was significantly higher than the TPD (12 times) at 10 mg/kg b. wt. The increase in bioavailability could be due to overcome of reticulo-endothelial system (RES), liver bypass and evade metabolism therein. It was reported that a particle size of <200nm helps to overcome of reticulo-endothelial system (RES).

Acute and sub-acute toxicity of a drug describes its safety window and useful in future clinical data support. TPD have no toxic effects on gross general behavior of animals and had no any toxicological symptoms and mortality at the limit test dose level of 1000 mg/kg body weight. Acute oral median lethal dose (LD\textsubscript{50}) of TPD in male mice was more than 1000mg/kg body weight orally and 400mg/kg/ b.wt./ i.p. The safety window of TPD was much high and it specifically target cancer cells.

\textbf{Conclusion:}

In conclusion it may be suggested that both AP9-cd and TPD have strong apoptotic and anti-tumor potential. They induce cell death through the early massive generation of reactive nitrogen and oxygen species, which are responsible for the activation of apoptotic machinery in cancer cells. TPD and AP9-cd are successfully formulated in to solid lipid nanoparticles (SLNs) dosage form and found to be more cytotoxic,
Summary and conclusions

apoptotic and anti-tumor than the parent drug \((in \, vitro \, and \, in \, vivo)\). Both the drugs and their SLNs were showed no cytotoxicity in normal cells. The safety window of both the compounds is broad and has significant anti-tumor potential in tumor bearing mice.

Therefore both these agents look drug like molecules and fulfill the criteria for successful development of novel anti-cancer chemotherapeutic agents. These studies nevertheless provide important information about the pro apoptotic nature of TDP and AP9-cd, prospecting these candidates for developing into a potential anti-cancer therapeutic. However, a well-designed multi-dose long term chronic animal studies followed by human clinical trials in appropriate cancer types are required for their establishment as novel anti-cancer therapeutics.