CHAPTER 5

MAJOR FINDINGS

AND

CONCLUSIONS
In this Ph.D. thesis, we initially screened for anti-neoplastic compounds from plant origin. In our subsequent studies we chose anacardic acid as the lead compound for devising a strategy to develop a sequential combinatorial treatment, essentially to sensitize apoptosis resistant tumor cells to TRAIL mediated apoptosis. Next, we studied the protein and gene expression profiles of Tie-2 receptor overexpression in human tumor cell lines and human tumor biopsies and engineered a single chain antibody fragment (scFv) against the Tie2 receptor. This scFv ligand was used to study its biological effect on angiopoietin induced angiogenesis and further used as a targeting moiety for the targeted delivery of anacardic acid into the Tie2 overexpressing cancer cells such as MDA-MB-231 using PEGylated immunoliposomes. The major findings of this work carried out is summarized as follows

- In our preliminary studies to screen for potent anti-angiogenic and pro-apoptotic phytochemicals from four plant extracts, two extracts namely EA fraction of *E. jambolana* and EA fraction of *M. Paradisiaca*, having high flavonoid or phenolic content, respectively were effective in suppressing VEGF induced tumor angiogenesis as well as inducing tumor cell apoptosis *in vitro* and *in vivo*.

- Further, our studies focused on the purified phytochemical, the phenolic lipid, anacardic acid (A1) (2-hydroxy-6-pentadecylbenzoic acid), for its ability to synergize the TRAIL mediated apoptosis in cancer cells. It was found that, among the panel of 8 human tumor cell lines used, MDA-MB-231, MCF-7 and MDA-MB-435 cells were highly sensitive, Kelly and SK-N-AS were intermediately sensitive, whereas HeLa, A549 and HT-29 were highly resistant to TRAIL mediated apoptosis.

- The TRAIL mediated apoptosis in A549, HT-29 and HeLa cells, was rather more pronounced when pre-treated with A1 followed by sTRAIL and reduced the rate of proliferation upon combinatorial treatment in A549, HT-29 and HeLa cells. Suggesting the role of A1 in synergizing the action of TRAIL by sensitizing TRAIL-resistant cells to undergo apoptosis. A1-sensitized, TRAIL-mediated cell death was ascertained to be due to apoptosis assessed by the activation of caspase 3 and flowcytometric analysis and for the first time, it was shown that A1 pre-treatment followed by sTRAIL treatment up-regulates DR4 and DR5 expression at the cellular,
protein and mRNA levels in TRAIL resistant cells. Also, the TRAIL resistant cells revealed decreased expression of NFκβ, increased p53 expression and accumulation of pro-apoptotic Bax and concomitant decrease in Bcl-2 proteins in a time dependent manner, along with activation of p38 and JNK and inhibition of ERK1/2 activation. Similar results were observed in the in vivo EAT tumor model with decreased body weight, EAT cell number and secreted ascites volume and peritoneal neovasculature, in the A1-sTRAIL combinatorial therapy.

- The overexpression profile of Tie2 receptor in human tumor biopsies and cell lines was ascertained by immunohistochemistry and qPCR respectively. IHC staining of the paraffin sections of various grades of human ductal breast carcinoma tumor biopsies revealed overexpression of Tie2 receptors as the grade of cancer progressed, indicating that the Tie2 receptor overexpression is an event in the early stages of carcinogenesis. Whereas the qPCR results showed overexpression of Tie2 receptors in MDA-MB-231 and U-87MG cell lines out of the 4 cell lines tested.

- Soluble Tie2 protein was expressed and purified from Spodoptera frugiperda (Sf-21) cells using the baculovirus expression system and used in the immunization of BALB/c mice. The splenic mRNA was used to engineer the scFv cDNA, cloned into a phagemid vector and the fusion protein expressed and purified from transformed HB2151 cells. A modified dot blot assay was used to pick out the positive clones expressing the scFv-sTie2 protein. The DNA sequencing of scFv gene revealed an 823bp sequence with ORF homology to some of the scFv encoding genes and proteins from the NCBI database. The deduced amino acid sequence submitted to the online program, Iterative-Thread Assembly Refinement (I-TASSER) generated a 3D structure that was found to have good parametric scores. Next, an orientational docking of scFv-Tie-2 and Tie-2 epitopes using ZDOCK and RosettaDock molecular modeling algorithms revealed binding of the scFv in the same pocket as that of the original ligand Ang2. Further, the bioactive nature of the purified scFv-sTie2 was ascertained by the rat corneal micropocket assay that showed scFv-sTie2 could inhibit Ang2 induced corneal neovascularization.
scFv-sTie2 was further used to enhance the specificity of liposomes to the cancer cells thereby augmenting the delivery of encapsulated anacardic acid (A1) to bring about apoptosis of tumor cells and thus can be used as a useful targeting moiety in nanomedicine. The drug delivery and cytotoxicity was enhanced when PEGylated liposomes were used. Further, the dual fluorescent immunoliposomes clearly showed that they could bind to the overexpressed Tie2 receptors on the cell surface and help in liposome internalization by receptor mediated endocytosis.

The characterization of the prepared liposomal formulations showed an apparent increase in the mean size and zeta potential of the liposomes upon PEGylation and conjugation of scFv ligand onto the PEG arms.

Accordingly, this strategy of nanomedicine based cancer therapy by using scFv to target overexpressed Tie2 receptors in certain solid tumors has immense therapeutic potential as it can carry and selectively deliver a payload of a wide variety of chemotherapeutic drugs to the site of tumor growth.

Scope of the present work

- The methodology similar to our targeted therapy using immunoliposomes could be used for therapeutic purpose for different cancers.
- sTie2, a recombinant protein can be used as a ligand trap in the inhibition of angiopoietin ligand overexpressing cancers such as breast and lung cancers.
- The 96 well plate dot blot method of screening can be effectively used as an equally good alternate for ELISA and may be developed into kits for diseases.
- Fluorescently labeled sTie2 can also be used in *in vivo* imaging applications.