CHAPTER 1

GENERAL INTRODUCTION

AND

REVIEW OF LITERATURE
Cancer or malignant neoplasm is a grave disease. Contrary to the prevailing understanding, cancer is not just a single disease, instead is a conglomerate of diverse diseases that require assorted treatment modalities for a cure. Beneath the complexity of cancer prevails an intricate set of events which compel all cancer cells and their progeny towards aberrant proliferation, metastasis and destruction of the neighboring tissue matrix augmented by the accumulation of oncogenic mutations that disrupt normal cellular functions, promote abnormal cell proliferation, sustained angiogenesis and evasion of apoptosis.

Among all the prominent causes of human deaths, cancer takes the second position surmounting to ~ 8.8 million deaths worldwide in 2015, which is expected to rise to 13.1 million by 2030 (Ferlay et al., 2015). Considering the pathological traits of human malignancies (Figure. 1.1) such as growth signaling self-sufficiency, resistance to growth inhibitory signals, evasion of apoptosis, immortality, enduring angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2011) and increased cytokine production (Cavallo et al., 2011), various strategies of cancer treatments can be adopted (Figure. 1.2).

The conventional first line of treatment is surgery, which is acceptable only for non-hematological tumors and the complete surgical eviction of the tumor is impossible in case of a metastasized cancer. The second line of treatment by radiation and chemotherapy affect even the normal cells by either damaging their DNA or inhibiting cell division respectively since they lack tumor cell-specificity and have severe side effects. Occasionally, secondary cancer can arise from these treatments if the normal cell’s repair mechanism fails. Consequently targeted therapies focus on the mutated,
crucial and overexpressed proteins in a cancer cell. For example, in cancer such as acute promyelocytic leukemia (APL), that is caused by the fusion between the promyelocytic leukemia (PML) and the retinoic acid receptor alpha (RARα) genes, results in a leukemic phenotype due to the presence of the PML-RARα fusion protein, which can be treated by using all-trans retinoic acid that degrades the fusion protein (Wang and Chen et al., 2008). However, the majority of the cancers have multiple genetic changes that continue to accumulate over time along with epigenetic changes due to DNA methylation leading to chromosomal instability and silencing of tumor suppressor genes, which can be treated using inhibitors of DNA methylation as a targeted therapy (Rodriguez-Paredes and Esteller, 2011). Hormonal therapy, however, maintains tumor dormancy instead of curing cancer until the cancer cells become resistant to hormone treatment. Drugs that inhibit tumor angiogenesis can also be used in cancer treatment. However, their action not only affects tumor cells but also the normal cells as they rely on the same angiogenic factors. Thus targeting a single factor will not suffice in cancer therapy.

Another alternative means of cancer therapy involves the use of monoclonal antibodies or peptides that bind to over-expressed cell surface proteins that are specific to cancer cells which can be exploited to deliver cytotoxic drugs to the tumor. But the limitations of this approach are the steadily changing expression of cell surface proteins that may either be downregulated or lost altogether and the instability of the targeting protein. Also, cancer treatment based on immunotherapy such as vaccines and antibodies requires a longer time frame to see observable effects and hence is used as an adjuvant cancer treatment (Gerritsen and Sharma, 2012).

**Figure 1.2. The well known cancer treatment strategies.** Various strategies are adopted for the treatment of cancer. But no single strategy is powerful enough to inhibit cancer. The tumor mass in the middle was adapted from (Junttila and Sauvage, 2013).
Thus novel treatment modalities that target the ‘hallmarks of cancer’ are permitted by the US Food and Drug Administration (FDA) with many more in the pipeline (Table 1.1.). For instance, trastuzumab a monoclonal antibody against epidermal growth factor receptor (EGFR) that is regularly upregulated in almost all cancers can help in internalization and degradation of the EGFRs, thus inactivating EGFR signaling thereby restricting tumor cell proliferation and inducing apoptosis (Hynes, 2016).

<table>
<thead>
<tr>
<th>cancer hallmark</th>
<th>cellular target</th>
<th>agent</th>
<th>name</th>
<th>product/company</th>
</tr>
</thead>
<tbody>
<tr>
<td>self-sufficiency in growth signalling</td>
<td>epidermal growth factor receptor (EGFR)</td>
<td>inhibitor</td>
<td>gefitinib</td>
<td>Iressa/AstraZeneca</td>
</tr>
<tr>
<td></td>
<td>tyrosine kinase-type cell surface receptor (HER2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insensitivity to growth inhibitory</td>
<td>proto-oncogene MYC (via estrogen receptor)</td>
<td>inhibitor</td>
<td>ICI 182780</td>
<td>Fulvestrand/AstraZeneca</td>
</tr>
<tr>
<td>signals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>evasion from apoptosis</td>
<td>tumour suppressor p53/ E3 ubiquitin-protein ligase MDM2</td>
<td>inhibitor</td>
<td>JNJ-26854165, RG7112</td>
<td>JNJ-26854165/Janssen Research &amp; Development, RG7112/Hoffmann-La Roche</td>
</tr>
<tr>
<td>limitlessness of replicative potential</td>
<td>telomerase</td>
<td>inhibitor</td>
<td>imetelstat</td>
<td>GRN163/Geron</td>
</tr>
<tr>
<td>sustained angiogenesis</td>
<td>vascular endothelial growth factor (VEGF)</td>
<td>monoclonal antibody inhibitor</td>
<td>bevacizumab</td>
<td>Avastin/GeneTech/Roche/Axitinib/Pfizer, Inc</td>
</tr>
<tr>
<td>tissue invasion and metastasis</td>
<td>hepatocyte growth factor receptor (HGF)</td>
<td>inhibitor</td>
<td>tivantinib</td>
<td>ARQ197/ArQule</td>
</tr>
<tr>
<td></td>
<td>cMET</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Examples of targeted cancer therapeutics developed to target the hallmarks of cancer. Table lists the small molecule inhibitors and monoclonal antibodies that are FDA approved or undergoing clinical trials.

Further, agents that selectively kill cancer cells constitute proteins derived from viral sources such as E4orf4, NS1 and apoptin (Noteborn 2009). The tumor necrosis factor alpha related apoptosis inducing ligand (TRAIL), Human Alpha-lactalbumin Made LEthal to Tumor cells (HAMLET) and the melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24), all these endogenous proteins bind to their cognate receptors and induce apoptosis.

For the selective and targeted removal of tumor cells, a combination of agents those which induce apoptosis, inhibit angiogenesis, induce cell senescence, along with inhibitors of cell signaling, cell cycle or HIF-1α (hypoxia inducible factor-1) may be used. Also, along with chemotherapy, inducers of death receptors and tissue-specific differentiating agents can also be used. Theoretically, strategic and combinatorial application of these drugs may effectively contain cancer (Blagosklonny, 2004).
The medical community with regard to modern anticancer therapy is forked into one group asserting the use of purified or synthesized chemical compounds and another favouring alternative cancer therapy such as those based on natural sources, including plant extracts (Zulkipli, 2015). Medicinal plant-based drug discovery research has yielded pharmacologically important cancer therapeutics. Natural compounds isolated from medicinal plants continue to be a steady source of lead molecules for anticancer treatment. Several natural product-based drugs (~49%) have been introduced into the market for cancer treatment (Newman, D and Cragg 2012).

Furthermore, drug delivery efficacy and the therapeutic index of anticancer drugs can be enhanced significantly using nanocarriers such as liposomes, which have become the quintessential model of drug delivery and various approved products such as Ambisome® (Gilead, Foster City, CA, USA), DaunoXome® (Gilead) and Myocet® (Cephalon, Frazer, PA, USA) are now available for clinical use (Cheng and Allen 2010). Additionally, the liposomal phospholipid bilayer surface can be easily modified to incorporate polyethylene glycol arms to obtain PEGylated liposomes such as the PEGylated liposomal doxorubicin (Doxil/Caelyx®, Alza/Johnson & Johnson, New Brunswick, NJ, USA) that have longer circulation times in vivo, resulting in increased concentration of the drug delivered to the site of the tumor. Actively targeted liposomes can be prepared to minimize the off-target effects by conjugating monoclonal antibodies, peptides, small molecule ligands or antibody fragments on the liposomal surface using the PEG arms. For clinical development, the single chain variable fragment (scFv) is the adept choice of targeting agents over monoclonal antibodies or other antibody fragments owing to factors such as decreased immunogenicity, and better pharmacokinetics/biodistribution profiles.

With this background, this study was designed to study the antineoplastic properties specifically focusing on anti-angiogenesis and pro-apoptosis using plant extracts from the seeds, roots, and leaves of E. jambolana Lam, M. paradisiaca L and C. indica Wight & Arn, respectively. Anacardic acid (A1) purified from leaves of Anacardium occidentale was adopted in our combinatorial treatment strategy, essentially as a means of sensitizing TRAIL-resistant cancer cells to undergo TRAIL-induced apoptosis through the targeted delivery of A1 into the apoptosis-resistant cancer cells using anti-Tie2 scFv tagged PEGylated immunoliposomes.
1.1. Angiogenesis

Angiogenesis and vasculogenesis are the processes of genesis of new blood vessels and are crucial steps in the embryonic development, normal homeostasis, and in the etiology of many human diseases such as cancer. The tumor growth is promoted and metastasis is facilitated by angiogenesis resulting in dissemination of tumor cells (Figure 1.3.). In 1971, Judah Folkman’s work shed light on the significance of tumor angiogenesis, which showed that depriving a tumor of its own blood supply would shrink and kill the tumor. Endothelial cells (ECs) are the basic building blocks of blood and lymphatic vessels that form their innermost layer, which in turn is surrounded by the vascular basement matrix made of smooth muscles and pericytes. However only the ECs make up the capillaries and the vessel stabilizing signal is provided by the pericytes (Jeltsch et al., 2013). Under normal physiological conditions, quiescent ECs are activated by the angiogenic switch, which is a conglomerate of various regulatory factors that regulate angiogenesis by sustaining an accurate balance between the activators and inhibitors whereas, in case of tumor angiogenesis, the neovasculature is abnormal, complex, leaky and tortuous in nature (Gacche and Meshram 2014).

Figure 1.3. Series of events that occur during the process of tumor angiogenesis resulting in the growth of blood vessels that feed the growing tumor. Tumor angiogenesis illustration source: The Angiogenesis foundation.
1.1.1 The VEGF and VEGFR pathway

Neo-angiogenesis is an intricate series of events initiated by physiological stimuli, such as hypoxia that upregulates the expression of vascular endothelial growth factor (VEGF). Initially, VEGF was reportedly called vascular permeability factor (VPF), a factor isolated from tumor secretions (Senger et al., 1983). Later, VEGF was described as a potent angiogenesis inducing EC mitogen (Leung et al., 1989). The family of VEGF comprises of 5 ligands (VEGF-A, B, C, D and placental growth factor, (PlGF) and their receptor tyrosine kinases (VEGFR1, VEGFR2 and VEGFR3), (Figure 1.4.). The homodimeric VEGF ligands are secreted as glycoproteins in various isoforms because of alternative exon splicing (Tischer et al., 1991; Houck et al., 1991) and post-translational modifications that include VEGF_{121}, VEGF_{145}, VEGF_{162}, VEGF_{165}, VEGF_{183}, VEGF_{189}, VEGF_{206} (Xiong et al., 1998; Vempati et al., 2014). The ligand VEGF-A binds to VEGFR2 and R1, VEGF-B and PlGF bind to VEGFR1, VEGF-C and D bind to VEGFR3. VEGFR2 is an essential mediator of VEGF-induced angiogenesis and hence is targeted therapeutically (Millauer et al., 1993; Kowanetz & Ferrara, 2006). Expression of VEGF is induced by the HIF-1α consequent to hypoxia (Brogi et al., 1996). Simultaneously, hypoxic conditions also trigger expression of matrix metalloproteinase (MMP) such as MMP9 that break down the extracellular matrix (ECM) around the ECs and helps in EC migration (Carmeliet, 2003).

![Figure 1.4. Illustration of VEGF ligands, their receptor types and their specificities. The family of VEGF ligands include VEGF-A, B, C, D, E and PlGF, that bind to VEGFR-1, R-2 and R-3, the three different receptor tyrosine kinases, respectively. VEGFR-1 can additionally be expressed in a soluble form with only the extracellular domain. Source of illustration Cross et al., 2003.](image-url)
Concurrent to the upregulation of VEGF, there is another set of events that result in destabilization of the cell-cell interactions between the ECs and the pericytes through the angiopoietin pathway culminating in further exposure of ECs to the action of VEGF. Thus the ECs proliferate and migrate in presence of high VEGF levels forming a column of cells which ultimately fuses to form a tubular blood vessel. However, a strong angiogenic response is initiated upon VEGF-A binding to VEGFR2 (Waltenberger et al., 1994), whereas it is weak when VEGF-A binds to VEGFR1 resulting in suppression of excess angiogenesis (Ferrara, 2004). The pericyte cells are recruited onto the new vasculature, re-establishing the pericyte-EC interactions, forming functional vasculature (Carmeliet and Jain, 2011), in which the angiopoietin pathway plays an important role.

1.1.2 The Ang-Tie2 pathway

Supplementing the VEGF-VEGFR signaling in angiogenesis is the angiopoietin signaling pathway comprising of three human growth factors/ligands called angiopoietins (Ang), namely Ang1, Ang2 and Ang4 (Ang3 is an Ang4 mouse ortholog) (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999) along with their cognate receptors that are EC-specific, type I transmembrane receptor tyrosine kinases Tie-1 and Tie2 which regulate angiogenesis and vascular morphogenesis (Ramsauer and D'Amore, 2002). The inactivating mutation studies in mouse embryo uncovered the significance of Tie2 receptor in vascular system, which lead to embryonic death (Dumont et al., 1994; Sato et al., 1995). On the contrary, the activating mutation studies of Ang1 ligand showed antagonistic results (Suri et al., 1998 and Thurston et al., 1999). The most widely studied human Tie2 ligands are the Ang1 and Ang2. Ang1 is the predominant Tie2 agonist which upon binding to Tie2, initiates dimerization and the tyrosine residues in the intracellular domain is autophosphorylated promoting EC survival and vessel stability (Matilde and Agrawal, 2008). However, Ang2 is the known antagonist of Ang1, which was ascertained by the gain of function studies of Ang2, that showed identical vascular defects as that of the mice with impaired Ang1 (Maisonpierre et al., 1997) and also upon binding of Ang2 to Tie2, there was no phosphorylation of Tie2. Thus Ang2 competes and restricts binding of Ang1 to the Tie2 (Reiss et al., 2007). As a result, the pericytes and EC interaction is destabilized and the exposed EC are sensitized and start proliferating in presence of VEGF. Thus, Ang2 potentiates VEGF-induced angiogenesis resulting in augmented EC migration and sprouting (Figure. 1.5.).
On the contrary, enhanced Ang2 expression in absence VEGF brings about the regression of vessels since the exposed ECs suffer apoptotic cell death (Lobov et al., 2002). Another obscure and intriguing aspect of Ang2 is its ability to moderately activate the Tie2 receptor in the deficiency of Ang1 (Yuan et al., 2009). Similarly, Ang2 is seen to serve as a Tie2 agonist under certain conditions in epidermoid, colon and prostate cancers (Daly et al., 2013).

Furthermore, various studies have shown that VEGF or phorbol myristate acetate (PMA) can induce the cleavage of the extracellular domain of Tie2 receptor to create a small soluble form of Tie2 (sTie2) (Findley et al., 2007; Onimaru et al., 2010), that is implicated in the of Ang-Tie signaling regulation. The presence of sTie2 has been shown to be present in normal human serum, elevated in coronary artery disease, congestive heart failure and renal cell carcinoma (Harris et al., 2001; Chung et al., 2003; Chong et al., 2004).

Figure 1.5. The angiopoietins control the endothelial cell activities that effect tumor vasculature. Although Ang1 and Ang2 both bind to Tie2 receptor, Ang1 stimulates normalization and integrity of blood vessels. Conversely, Ang2 is an antagonist to the Ang1/Tie2 signaling; binding of Ang2 to Tie2 results in destabilization of blood vessels, increased vessel permeability, and stimulation of new vessel sprouting.

Source: Amgen oncology educational resource.
1.1.3. Anti-angiogenic cancer therapy

1.1.3.1. Anti-VEGF based strategies

The targeting of tumor angiogenesis as a strategy for cancer therapy was pioneered by Judah Folkman in 1971 (Folkman, 1971). This major finding initiated widespread research on inhibition of tumor angiogenesis and more and more preclinical and clinical data have been accumulated exponentially (Gacche et al., 2014). A total of 10 different anti-angiogenic drugs have received approval by the FDA to date. Bevacizumab and ziv-aflibercept are approved for use as anti-VEGF agents, while axitinib, cabozeatinib, pazopanib, sorafenib, sunitinib and regorafenib are the small-molecule RTK inhibitors (Jain et al., 2014) (Figure 1.6).

The humanized monoclonal antibody Bevacizumab binds to VEGF and sequesters it from circulation thus preventing the interaction with the VEGFR. Various other strategies for inhibition of VEGF signaling have been investigated such as the use of soluble receptors and ribozymes, and combinatorial strategies to inhibit RTKs (Bergers et al., 2003; Fischer et al., 2008). Aflibercept (VEGF-Trap), a soluble form of VEGF receptor that is a fully humanized fusion protein of the VEGF-binding domains of VEGFR1 and VEGFR2 with the antibody Fc fragment, inactivates different members of the VEGF family (Holash et al., 2002; Dupont et al., 2005).

Figure 1.6. Common mechanisms of action for ziv-Aflibercept, bevacizumab, ramucirumab, and VEGFR activity of RTK inhibitors. Source of illustration (Clarke and Hurwitz, 2013)
Albeit reports suggest that anti-angiogenic therapy prolongs the survival of cancer patients, it is inadequate and unsatisfactory because of the temporary and moderate efficacy of the anti-angiogenic drugs in the clinical setting. Also, these therapeutics present off-target toxicity (Gacche et al., 2014) and tend to expedite tumor invasion and metastasis post withdrawal of treatment (Griffioen et al., 2012).

**1.1.3.2. Non-VEGF based anti-angiogenesis strategies**

The targeting strategy for Ang–Tie system involves the use of RTK inhibitors to target Tie2 along with Ang1 and Ang2 traps. Till date, just a handful of compounds have reached clinical studies such as CEP-11981, an effective Tie2 and pan-VEGFR inhibitor for the treatment of patients with advanced solid tumors. CE-245677, an inhibitor of the tyrosine kinase receptor A (TRKA) that also inhibits Tie2 was discontinued because of severe side effects as a result of TRKA inhibition (Williams, 2008). The two Angiopoietin traps that sequester excess Angs are the peptibody, AMG-386 that binds to both Ang ligands (Oliner et al., 2004) and PF-4856884 (also known as CVX-060), a bispecific CovX-Body selectively binds Ang2 (Huang et al., 2008).

**Figure. 1.7. Regulation of angiogenesis by angiopoietins.** (a) Perivascular Ang1 maintains vascular integrity. (b) Hypoxia induces expression of VEGF that initiates angiogenesis and Ang2 induces migration of endothelial cells. (c) Interaction of the transmembrane receptor Delta-like 4 (DLL4) with the ligands of the Notch family leading to movement of sprouting tip towards the sources of angiogenic factors. (d) Some therapeutic intervention points.
Angiogenesis is also regulated by the interaction of the transmembrane receptor Delta-like 4 (DLL4) with the ligands of the Notch family. Hence inhibition of Notch and Jagged are potential strategies for anti-angiogenic therapy (Benedito et al., 2009).

In the angiogenic sprouting of ECs, the leading cell called the “tip cell” leads the formation of neo vasculature by sensing the gradient of VEGF by chemotaxis secreted by hypoxic tumor tissue. VEGFR3 and DLL4 are overexpressed in the tip cell while Jagged and Notch are overexpressed in the trailing stalk cells along with paracrine signaling between the two cells (Tammela et al., 2008; Suchting and Eichmann 2009). Thus the pharmacological intervention of tip cells has anti-angiogenic benefits (Figure 1.7).

The hypoxia-inducible factors (HIFs), the transcription factors regulated by hypoxia that initiates angiogenic gene expression as in tumors can also be inhibited as a strategy for anti-angiogenic therapy. Since the inhibition of HIF activation is difficult to control using conventional drugs, targeting HIF-1α using a locked antisense oligonucleotide, EZN-2968 is currently under clinical investigation (Patnaik et al., 2009).

Primary tumors have been reported to suppress the growth of metastases by expressing two natural angiogenic inhibitors namely angiostatin and endostatin (Kisker et al., 2001) both of which exhibit potent antitumor activity in experimental cancers in mice (Prox et al., 2003).

Angiogenesis by EC proliferation critically depends on the enzyme methionine aminopeptidase that can be inhibited by a fungal compound called Fumagillin, previously used as an amebicide. Chemical analog of Fumagillin named TNP-470 has shown potent metastasis inhibition abilities in human xenografts (Kruger and Figg, 2000). Clinical trials of this compound are ongoing.

One other alternative strategy for attacking tumor angiogenesis is by the use of Vascular disrupting agents (VDAs), the drugs that particularly disrupt the existing tumor vasculature by specifically damaging the endothelial linings of tumor blood vessels and ceasing oxygen and nutrients to the tumor, leaving the normal vasculature unaffected (Gridelli et al., 2009, Hinnen and Eiskens, 2007, Tozer et al., 2005; O’Hanlon et al., 2005). Combretastatin A4 phosphate (zybrestat, fosbretabulin) (Cooney et al., 2005) and vadimezan (ASA404) have shown promising responses in clinical study however VDAs are known to cause cardiovascular toxicity (Subbiah et al., 2011).
1.2. Apoptosis

The word “apoptosis” meaning “leaves falling from a tree” is derived from the Greek word “απόπτωσις” (“apo” = “away” and “ptosis” = “fall”). Apoptosis or process of programmed death of cells is necessary to maintain cellular balance in the body and is involved in organ development, tissue regeneration, immune response and tumor suppression by the removal of unnecessary, infected, irreparable and damaged cells without affecting the normal cells. The morphological hallmarks of apoptosis include shrinkage of cell, blebbing of cell membrane, chromatin condensation, nucleosomal fragmentation and formation of apoptotic bodies. The membrane blebbing feature of apoptosis has been shown to facilitate removal of the dying cells by the cells of the immune system such as phagocytes before the lysis and release of their cytoplasmic contents, which is not the feature of other forms of cell death (Savill and Fadok, 2000).

Various causes, such as cellular stress by starvation or heat shock, cytotoxic drugs, DNA damage, radiation, or infection can induce apoptosis (Gonzalvez and Ashkenazi, 2010). The abnormal signaling of apoptosis is responsible for diseases such as metabolic disorders, autoimmune diseases and cancer wherein apoptosis is suppressed. However in neurodegenerative disorders such as Alzheimer’s and Huntington’s diseases, apoptosis is uncontrolled (Gonzalvez and Ashkenazi, 2010).

Figure 1.8. Apoptosis induction pathway. Apoptosis is accomplished by two different pathways, the extrinsic and the intrinsic pathways that are initiated either by pro-apoptotic receptors (death receptor mediated) or by intracellular BCL2 proteins (mitochondria mediated), respectively. Illustration source: Genentech BioOncology.
There are essentially two pathways through which apoptosis is accomplished namely the extrinsic and the intrinsic pathways (Figure 1.8), that are initiated either by pro-apoptotic receptors (death receptor mediated) or by intracellular BCL2 proteins (mitochondria-mediated), respectively. Also, both the pathways can be simultaneously activated with a crosstalk between them (Qiao and Wong, 2009). Eventually both the pathways lead to the activation of cysteine aspartate proteases called caspases.

1.2.1. The Extrinsic pathway

The extrinsic apoptotic pathway is activated by the binding of extracellular protein ligands (death ligands) to the cell surface receptors (death receptors) containing the death domains and organization of the death-inducing signaling complex (DISC) (Figure 1.9). In humans, the extrinsic pathway is involved in the immune response regulation along with maintenance of the immune cells repository by the pro-apoptotic ligands expressed by cytotoxic-T lymphocytes and natural killer cells (Gonzalvez and Ashkenazi, 2010). The tumor necrosis factor-α (TNF-α), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand /apoptosis ligand 2 (TRAIL/Apo2L) are the death ligands that belong to the TNF superfamily (Gonzalvez and Ashkenazi, 2010). Also belonging to the TNF-receptor superfamily are the transmembrane death receptors (DR) with extracellular domain that is cysteine-rich and a cytoplasmic “death domain” (DD) with a unique ~80 amino acid sequence (Hotchkiss et al., 2009).

![Figure 1.9. The extrinsic apoptosis signaling cascade. The activation of extrinsic pathway is by the death receptors, once it binds to its ligand including TNF-TNFR1, FasL-Fas and TRAIL-DR4 or -DR5. Illustrations adapted from (Jin and El-Deiry, 2005).]
There are six known DRs namely TNF-R1 (receptor of TNF-α), Fas receptor (receptor of FasL), DR3 (receptor of TL1A), DR4 and DR5 (receptors of TRAIL) and DR6 (receptor of N-APP (an amino-terminal fragment of the amyloid precursor protein) implicated in the pathology of Alzheimer) (Gonzalvez and Ashkenazi, 2010). Ligand binding to death receptors oligomerizes the receptors which changes the intracellular receptor-DD conformation and recruits the death signal adaptor proteins such as FADD (Fas-associated death domain) for Fas or TRADD (TNFR-associated death domain) for TNFR1 (Scott et al., 2009). Along with the oligomerized receptors and their DDs the recruited FADD forms a multi-protein death-inducing signaling complex (DISC), followed by the binding of caspase-8 and -10 to the FADD, activating them by autocatalytic cleavage (Hotchkiss et al., 2009). Further, caspase-8 and -10 activate the effector caspases-3, -6 and -7 ultimately leading to apoptosis (Gonzalvez and Ashkenazi, 2010). The extrinsic pathway is regulated by the cellular FLICE-inhibitory protein (c-FLIP) recruited by the DISC. c-FLIP has structural homology with caspase-8 and -10 and thus competes for binding to the FADD and blocks caspase activation (Henriquez et al., 2008). Further, TRADD can either interact with FADD to initiate apoptosis or with TRAF2 to inhibit apoptosis by NF-κB signaling (Hsu et al., 1996).

1.2.1.1. TRAIL-induced apoptosis

One exceptional feature of TRAIL compared to the other pro-apoptotic family members such as TNF-α, Fas ligand and TRAIL is its selective ability to induce apoptosis in tumor cells, leaving normal cells unaffected (Gonzalvez and Ashkenazi, 2010). Hence, since its discovery in 1995, TRAIL has become an excellent agent for cancer therapy (Gonzalvez and Ashkenazi, 2010). Human Apo2L/TRAIL is a 281 amino acid, type 2 transmembrane glycoprotein, upon cleavage from the cell surface forms a soluble ligand (Gonzalvez and Ashkenazi, 2010). The extrinsic pathway is activated by the binding of the homotrimeric form of TRAIL to the pre-assembled death receptor trimers on the cell surface (Gonzalvez and Ashkenazi, 2010). TRAIL binding leads to assembly of receptors into high-molecular-weight complexes (Gonzalvez and Ashkenazi, 2010). Apart from the agonistic TRAIL receptors DR4/TRAIL-R1 and DR5/TRAIL-R2, there are two antagonistic TRAIL receptors that also belong to the TNFR superfamily namely the decoy receptors DcR1/TRAIL-R3 and DcR2/TRAIL-R4, devoid of the functional DD (Jacquemin et al., 2010).
Furthermore, there is a third decoy receptor called osteoprotegerin, a soluble protein that binds TRAIL is involved in bone metabolism.

The cell surface deficiency of DRs is as a result of the internalization of the DRs after TRAIL binding by either clathrin-mediated or dynamin-dependent endocytosis. TRAIL, however unlike TNF and FasL, does not require internalization to induce an apoptotic signaling cascade which has acute significance on apoptotic signaling wherein the endocytosis leads to acquired TRAIL resistance, upregulation of apoptosis inhibitors, prevention of continuous caspase activation and inhibition of a mitochondrial response (Twomey et al., 2015). Furthermore, TRAIL is also able to activate kinase signaling cascades such as IKK (inhibitor of κB kinase) thus activating NF-κB, c-Jun N-terminal Kinase (JNK) and of p38 MAPK pathways (Figure 1.10) (Varfolomeev et al., 2005) leading to pro-survival and pro-proliferation signals and transcription of anti-apoptotic factors such as c-FLIP and XIAP (Gonzalvez and Ashkenazi, 2010).

**Figure. 1.10. TRAIL induced apoptosis pathway.** TRAIL after trimerization binds to five different receptors but only two, the death receptors 5 and 4 (DR5, DR4) contain a cytoplasmic DD allowing homotypic interaction with the DD within FADD. Source of illustration (Gonzalvez and Ashkenazi, 2010).
CHAPTER 1

General Introduction

The normal cells show limited expression of DR4 and DR5 whereas it is high in certain solid tumors (Dimberg et al., 2012). DR4 and DR5 activation using agonistic antibodies as well as recombinant human TRAIL (rhTRAIL) are being studied in phase II clinical trials (Gonzalvez and Ashkenazi, 2010). Additionally, the problem with TRAIL-based cancer therapy is that of resistance to TRAIL monotherapy (Dimberg et al., 2013).

1.2.1.2. Resistance to TRAIL mediated apoptosis

Resistance to TRAIL mediated apoptosis is one of the strategies used by tumor cells to evade apoptotic cell death. Various cancer cells, including colon and lung, become resistant to TRAIL by the loss of expression of functional DR4 and/or DR5 on the cell surface (Jin et al., 2004; Zhang et al., 2008) or also through upregulation of DcRs was initially proposed to be the cause for resistance to the apoptotic signal by competing with functional DRs (DR4 and/or DR5) (Marsters et al., 1997; Pan et al., 1997). DcR1 prevents assembly of the DISC and DcR2 inhibits initiator caspase activation, inhibits DR4 co-recruitment with DR5 (Merino et al., 2006), promotes NF-kB activation (Degli-Esposti et al., 1997) and also trigger cell survival and proliferation signaling via AKT activation (Lalaoui et al., 2011). The other flaws in the pathway include inactivation of caspases by overexpression of c-FLIP, loss of proapoptotic and upregulation of antiapoptotic Bcl-2 family members, upregulation of inhibitor of apoptosis (IAP) proteins XIAP/survivin/clIAP-1/clIAP-2, ineffective transport of receptors to the cell surface (Dimberg et al., 2013). Further, the Mcl-1 expression is usually upregulated in cancers which is responsible for the TRAIL-resistant phenotype (Clohessy et al., 2006). Mcl-1 protein accumulation was increased in colon carcinoma cells (HT-29 and HCT116) upon TRAIL treatment and silencing Mcl-1 by siRNA followed by TRAIL treatment significantly enhanced the apoptosis (Vaculová et al., 2004; 2009). Further, TRAIL-induced ERK activation has been shown to promote apoptosis resistance. Melanoma cell lines showed sensitization to TRAIL-induced apoptosis when the ERK1/2 activation was blocked (Zhang et al., 2003). TRAIL-induced apoptosis was enhanced by an MEK1/2 inhibitor (U0126) post combinatorial treatment in the human epithelial colon cancer cells (HT-29, HCT116) (Vaculová et al., 2006; 2009). Phosphorylation of ERK1/2 and p38 MAPK signaling cascades is required to sensitize colon cancer cells to TRAIL mediated apoptosis (Do et al., 2014). ERK1/2 signaling
pathway can also sensitize human colon cancer cells bearing RAS mutations to TRAIL-mediated apoptosis (Drosopoulous et al., 2005). Inhibition of PI3K/Akt signaling cascade also resulted in potentiation of TRAIL-mediated apoptosis in HT-29 cells (Vaculová et al., 2006). c-Myc is another regulator that potentiates TRAIL-mediated apoptosis by upregulating DR5 cell surface levels in vitro and in vivo (Wang et al., 2004). Furthermore, DR4 and DR5 upregulation can be activated by reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs) and p53 (Jung et al., 2005). Several reports have shown that MAPKs, such as ERK 1/2, p38, and JNK mediate upregulation of DRs (Shenoy et al., 2009; Prasad et al., 2011).

1.2.2. The intrinsic pathway

The mitochondrial or the intrinsic apoptotic pathway is initiated after mitochondrial outer membrane permeabilization (MOMP) by intracellular stress such as DNA damage, cytoskeletal damage, heat shock, cell detachment, ROS outbreak, hypoxia, irradiation and chemotherapeutic assault (Hotchkiss et al., 2009). Pro-apoptotic proteins such as cytochrome c, second mitochondrial activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO), apoptosis inducing factor (AIF), endonuclease G or Omi/HtrA2 are released from inner mitochondrial membrane space into the cytoplasm upon changes in mitochondrial membrane potential (Figure 1.11.) (Henriquez et al., 2008).

Figure 1.11. The intrinsic apoptosis pathway is initiated inside cells. The main event is the Bcl-2 family mediated mitochondrial outer membrane permeabilization (MOMP), leading to cell death via the discharge of apoptosis inducing molecules. Illustration adapted from (Jin and El-Deiry, 2005).
MOMP is regulated by the members of BCL2 protein family (Figure 1.12.), that are divided into anti-apoptotic proteins that promote cell survival (A1, BCL2, BCLW, BCLXL and MCL1), pro-apoptotic proteins that initiate apoptosis (BAX, BAK, BCLXS and BOK) and BH3-only death proteins that initiate apoptosis (BAD, BID, BIK, BIM, BMF, HRK, NOXA and PUMA) (Kelly and Strasser, 2011).

The interaction of BCL2 proteins amongst each other is regulated by the anti-apoptotic and BH3-only proteins, whereas pro-apoptotic BCL2 proteins initiate BAX and BAK proteins that create pores by inserting into mitochondrial membrane leading to MOMP. The cytochrome c binds to the adaptor protein Apaf-1 (apoptotic protease-activating factor-1), activating the assembly of a multimeric protein complex called apoptosome triggering proteolytic cleavage of procaspase-9 to activated caspase-9 (Qiao and Wang, 2009). Caspase-9 further activates effector caspases such as caspase-3. Also the efflux of Smac/DIABLO inactivates IAPs, thereby escalating caspase activation (Qiao and Wang, 2009). Another caspase-independent pathway of apoptosis induction by the mitochondria is by the efflux of AIF and endonuclease G after MOMP which translocates into the nucleus bringing about condensation of chromatin and fragmentation of DNA (Fulda and Debatin, 2006). Further, Omi/HtrA2 is capable of apoptosis induction both by caspase-dependent (inhibiting endogenous inhibitors of caspases) as well as caspase-independent apoptosis (Fulda and Debatin, 2006).

1.2.3. Cross-talk among extrinsic and intrinsic pathways

The apoptosis initiated by the extrinsic pathway may also require the activation of intrinsic pathway which is determined by the cell type (Gonzalvez and Ashkenazi, 2010). The activation of caspases by the extrinsic pathway initiates apoptosis in type I cells, while type II cells require further activation of the mitochondrial pathway as an amplification circuit (Falschlehner et al., 2009). The crosstalk between the two pathways
is by the BH3-only protein Bid that is cleaved to truncated Bid (tBid) by caspase-8 or -10, that later translocates into the mitochondria, activating either Bax or Bak and ultimately inducing MOMP and thus release of pro-apoptotic factors (Levine et al., 2008, Falschlehner et al., 2009). Most cancer cells could undergo apoptosis in a type II manner.

1.3. Apoptosis regulation

1.3.1. The family of IAP

In humans the family of IAPs (Inhibitor of Apoptosis) plays an important role in the inhibition of apoptosis as a reaction to varied apoptotic stimuli (Hunter et al., 2007) and promotes survival signaling pathways and proliferation, frequently overexpressed in cancer and are good targets for cancer therapy (Fulda and Vucic, 2012).

IAP family comprises of eight anti-apoptotic proteins that include the X-linked inhibitor of apoptosis protein (XIAP), survivin and cellular inhibitor of apoptosis protein 1 and 2 (cIAP-1, cIAP-2) (Qiao et al., 2009) with their conserved BIR (baculoviral IAP repeat) domain (Gonzalvez and Ashkenazi, 2010) (Figure 1.13a.).

- The sole inhibitor of caspases that acts downstream of the intrinsic pathway is the XIAP which inactivates caspases-3, -7 and -9 (Fulda and Vucic, 2012). The rest of the IAPs block the assembly of apoptosis signaling complexes.
- The smallest member of the IAP family, Survivin has a single BIR domain that is involved in cell division and inhibition of cell death (Altieri, 2008). It is highly upregulated in human tumors but not in normal adult tissues and involved in cancer progression and drug resistance (Pennati et al., 2008).
- Smac/DIABLO, an endogenous IAP antagonist released from mitochondria binds to XIAP to block XIAP-mediated inhibition of caspases (Fulda and Vucic, 2012).
- cIAP-1/2 in turn binds to Smac/DIABLO to inhibit its binding to XIAP (Kim and Choi, 2010). cIAP-1/2 is also involved in NF-κB signaling pathways.

1.3.2. The MAPK (mitogen-activated protein kinases) pathways

Basic cellular processes such as cell differentiation, cell proliferation, migration and apoptosis are all regulated by the MAPK pathways activated in response to extracellular signals. MAPKs are serine-threonine kinase modules comprising of three major subgroups in humans namely the extracellular signal-regulated kinase (ERK), the
c-Jun N-terminal kinase (JNK) and the p38 MAPK (Figure. 1.13b.) (Hu and Kong, 2004; Dhillon et al., 2007). Each of the cascades includes three tiers of kinases: a MAPK kinase kinase (MAP3K) which activates a MAPK kinase (MAP2K) by phosphorylating, which in turn phosphorylates a MAPK. Ultimate, the activated MAPK phosphorylates substrate proteins including transcription factors such as p53, c-myc and c-jun (Kim and Choi, 2010). Development and progression of cancer by events like tumor cell proliferation, tumor angiogenesis and apoptosis require the activation of MAPKs wherein the ERK pathway is known for its proliferation signalling, activated by growth factors and mitogens (Dhillon et al., 2007) and the signal transduction is mediated by members of the Ras family (Kim and Choi, 2010). In solid tumors, ERK signalling pathway is constitutively active since K-Ras and B-Raf are frequently mutated. The ERK pathway is known to promote tumor invasion by the degradation of extracellular matrix by upregulating the expression of MMPs (Kim and Choi, 2010).

JNK and p38 pathways generally mediate anti-proliferative and pro-apoptotic effects. They are stress-activated MAPK pathways which are activated by the proinflammatory cytokines such as TNF-α and IL-1β, and also by metabolic, oxidative, hypoxic, genotoxic and pharmacological stress (Kim and Choi, 2010). Once activated, JNK can bind to and phosphorylate p53, leading to an increased p53 transcriptional activity and stability. p53 is also activated by the p38 MAPK and is involved in apoptotic signaling. There are many chemotherapeutic agents that induce apoptosis by activating p38 (Dhillon et al., 2007).

Figure 1.13. (a) Inhibitor of apoptosis protein (IAP) family the structure of the eight mammalian IAPs with their known functional domains (Obexer and Ausserlechner, 2014). (b) Mitogen-activated protein kinase (MAPK) signaling pathways (Kim and Choi, 2010).
1.3.3. The tumor suppressor p53

The “guardian of the genome”, protein p53 is the tumor-suppressor protein encoded by the TP53 gene (Hotchkiss et al., 2009) that triggers cell cycle arrest or apoptosis to preserve the integrity of the genome by the elimination of irreparably damaged cells in response to DNA damage, cellular stress by radiation, chemical agents or ROS and thus inhibiting neoplasia (Brady and Attardi, 2010). Upon p53 transcriptional activation by phosphorylation, it upregulates pro-apoptotic genes of both the extrinsic and the intrinsic pathway such as PUMA, NOXA, BAX and FAS at the transcriptional level and represses the expression of anti-apoptotic genes such as BCL-2 (Brady and Attardi, 2010; Eisenberg et al., 2009). Furthermore, p53 can directly promote MOMP by interactions with the members of the Bcl-2 family (Brady and Attardi, 2010). Many human cancers have mutated TP53 gene (Brady and Attardi, 2010). Loss of p53 provides immense growth advantage to tumor cells by evading apoptosis (Eisenberg et al., 2009).

1.3.4. The NF-κB transcription factor

The Nuclear Factor-κB (NF-κB) family of eukaryotic transcription factors is involved in the cellular responses to infection and injury. But in cancer, its regularly activated constitutively promoting tumor cell proliferation, survival, angiogenesis and metastasis and thus is a potential target for cancer chemotherapy (Brown et al., 2008).

NF-κB in the cytoplasm are inactive since they are bound to their inhibitors (inhibitor of κB, I-κB). Once separated from the inhibitor by the degradation of I-κB, they translocate to the nucleus to activate transcription. The activated NF-κB transcription factors assemble by the dimerization of five different subunits (RelA (p65), c-Rel, RelB, p50 and p52) (Brown et al., 2008). In cancer, activation of the NF-κB signaling leads to suppression of apoptosis since NF-κB induces the transcription of anti-apoptotic genes such as Bcl-xL, c-FLIP, XIAP and cIAP-1 (Dhillon et al., 2007). NF-κB and JNK signaling have opposing roles in apoptosis, as NF-κB signaling inhibits oncogene-induced apoptosis, while JNK activation promotes apoptosis. However, there are also reports of a pro-apoptotic role of the NF-κB, wherein the overexpression of the subunit c-Rel leads to the expression of DR4, DR5, Fas and FasL and inhibition of cIAP-1, cIAP-2 and survivin (Chen et al., 2003).
1.4. Plant based natural products in cancer therapy

Natural products are small molecules that are produced as secondary metabolites for the survival of plants. These molecules exhibit molecular structures called “privileged scaffolds” that have high-affinity to bind to multiple targets (e.g. proteins) and bring about biological effects. Natural products have been an inexhaustible resource for anticancer drug discovery and a variety of natural products have been identified as potent anticancer drugs. New technologies such as high-throughput screening has accelerated natural product drug discovery which were once considered to be outdated by pharmaceutical companies in the 1990’s (Bailly 2009).

Currently available cancer chemotherapeutics have restricted potential in terms of their toxicity to normal cells, drug inefficacy, expensive production costs and development of drug resistance (Aggarwal, et al., 2007(a)), all have compelled us to explore the multifaceted approach for the primary prevention of this disease (Aggarwal and Shishodia, 2006). According to a recent review, 49% of cancer therapeutic drugs are either natural products or their derivatives (Newman and Cragg, 2012). About 19 natural product-based drugs have been approved, among which five of these drugs namely, temsirolimus, trabectedin, ixabepilone, everolimus and romidepsin, have been developed for cancer treatment between 2007 and 2009 (Mishra and Tiwari, 2011). Overall, it has also been suggested that less than one-fifth of the ring systems found in natural products are represented in current synthesized chemical drugs. Development of new small molecules from natural sources is based on the discovery of bioactive secondary plant metabolites that target tumor angiogenesis and induce apoptosis in cancer cells.

Plant polyphenols, catechins, flavonoids, terpenes, tannins, alkaloids and polyacetylenes are well characterized for the presence of a wide variety of anti-tumor properties with respect to inhibition of tumor angiogenesis (Lu et al., 2016a), induction of apoptosis and inhibition of cell proliferation (Lee et al., 2013). Compounds such as taxol, camptothecin, and combretastatin have been reported to have potent anti-angiogenic properties (Fan et al., 2006). Further, anti-angiogenic effects through VEGF signalling inhibition have been reported from dietary functional foods such as genistein from soybean, epigallocatechin gallate from green tea and resveratrol from red grapes.
Curcumin (Diferolelyl methane), a phenolic compound derived from rhizome of curcuma species in pre-clinical cancer research has shown inhibition of carcinogenesis by inhibition of proliferation, angiogenesis and metastasis in cancers including colorectal, pancreas, gastric, and prostate. It also is a chemo-sensitizer that potentiates action of other drugs like doxorubicin in treating multi-drug resistant and chemotheraphy-resistant cancers (Qi et al., 2010), by blocking the anti-apoptotic NF-KB signaling pathway (Hemaiswarya and Doble, 2006) (Figure 1.14). A mixture of phenolic compounds from the rhizome of Ginger (Zingiber officinale), induce cytotoxic activity through apoptosis in cancer cells (Brahmbhatt et al., 2013). Resveratrol, a polyphenolic phytoalexin found in grape skins, peanuts, berries induces apoptosis in HL60 cells and T47D cells through CD95 signaling-dependent apoptosis (Wheat and Currie, 2008). Genistein, a phytosterol flavonoid induces apoptosis in human promyelocytic HL-60 leukaemic. It inhibits tyrosine kinases, angiogenesis and arrests cell cycle in G2/M phase (Wheat and Currie, 2008).

Quercetin induces apoptosis by ROS mediated intrinsic pathway due to its ability to act as a pro-oxidant and to induce GSH depletion and release of cytochrome-c to the cytosol and activating of caspase9 (Gibellini et al., 2010).

7-hydroxystaurosporine (UCN-01) is an alkaloid that is currently in clinical trials, has recently been shown to be a potent Chk1 (a serine-threonine kinase) inhibitor (Zhao et al., 2002). It synergizes the cytotoxic action of cisdiaminedichloroplatinum II and enhances apoptosis in ovarian cancer cells. Also, UCN-01 in combination with 5-fluouracil potentiates apoptosis (Sigmond et al., 2004).

Figure 1.14. Molecular targets of curcumin and reveratrol (Aggarwal et al., 2007b).
1.4.1. Potentiation of TRAIL induced apoptosis using plant compounds

As a consequence of the problem of acquired resistance to TRAIL induced apoptosis in certain cancers, an alternative approach would be the combinatorial therapy of TRAIL with other anti-cancer drugs for an effective therapeutic strategy to achieve better efficacy compared to monotherapy. The main intention of combinatorial TRAIL therapy is sensitization of TRAIL resistant cells or synergizing the activity of TRAIL. Consequently, researchers are looking for TRAIL sensitizers to overcome resistance to TRAIL. Variety of chemical compounds and natural products are known to be potent sensitizers, capable of restoring TRAIL signaling pathway in TRAIL resistant cancer cells. These compounds include inhibitors of histone deacetylases (Lauricella et al., 2012), inhibitors of proteasome (Kahana et al., 2011) or cannabinoids (Pellerito et al., 2010).

These combinatorial therapeutic strategies notably initiate TRAIL mediated apoptosis as a consequence of endoplasmic reticulum (ER) stress, leading to expression of DR4 and/or DR5 (Moon et al., 2013; Yamaguchi and Wang 2004; Abdelrahim et al., 2006) (Figure 1.15.). Reactive oxygen species (ROS) is produced owing to ER stress (Moon et al., 2012), which in turn triggers other signaling pathways such as DNA damage mediated activation of p53 promoting expression of DR5 by extrinsic pathway (Kannappan et al., 2010) or via intrinsic pathway through p53 upregulated modulator of

![Figure 1.15. Signaling pathway of TRAIL-drug combinatory therapy that help in synergizing the apoptosis inducing ability of TRAIL or in sensitizing resistant cells to TRAIL mediated apoptosis by upregulating the death receptors (Refaat et al., 2014).](image-url)
apoptosis (PUMA), Bax pro-apoptotic proteins and phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) (Sung et al., 2010; Park et al., 2013). CCAAT-enhancer-binding protein homologous protein (CHOP) is another downstream checkpoint that is activated via MAPKs such as p38/ERK that sequentially upregulate DR5 transcription (Woo et al., 2013), induce Bim, a pro-apoptotic protein (Ghosh et al., 2012) or inhibit the Bcl-2 and Mcl-1 survival proteins (Sung et al., 2010). DR5 can also be upregulated by c-Jun N-terminal kinases (JNKs), through the Sp1-dependent cascade and decrease the expression of Bcl-2 and Mcl-1 (Sung et al., 2012).

1.4.2. Bioactive plant compounds evaluated in the present study

The present study was conducted to validate the effectiveness of complementary and alternative medicine by evaluating the anti-angiogenic and pro-apoptotic properties of four plant extracts; ethyl acetate (EA) and n-butanol (NB) fractions from seeds of Eugenia jambolana Lam (Myrtaceae), EA fraction from the roots of Musa paradisiaca L (Musaceae) and EA fraction from leaves of Coccinia indica Wight & Arn (Cucurbitaceae), since pharmacologically important bioactive organic compounds such as phenols, flavonoids, tannins, saponins, and terpenes have better solubility in ethyl acetate and n-butanol. Although previous studies have reported the anti-hyperglycemic and anti-oxidative bioactivities of these plant sources (Chatterjee et al., 2009), various other studies have reported a myriad of biological activity that also includes a potent anticancer activity (Baliga, 2011; Pekamwar et al. 2013; Nadumane and Timsina 2014).

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid) (A1), a polyphenolic lipid purified from the cashew-nut shells and leaves of Anacardium occidentale, has been shown to be involved in the inhibition of numerous enzymes, including histone acetyltransferase (HAT), phosphatidylinositol-specific phospholipase-C, tyrosinase, xanthine oxidase, tissue factor VIIa, lipoxygenase, and cyclooxygenase (COX), prostaglandin endoperoxide synthase and the expression of nuclear factor-KB (NF-KB) as well as the activation of aurora kinase A. It is also a mitochondrial uncoupler of oxidative phosphorylation. A1 exhibits antitumor activity and sensitizes tumor cells to ionizing radiation (Sung et al., 2008, Hemshekhar et al., 2012). Recently, A1 has been shown to upregulate p53 transcription factor (Tan et al., 2012).
1.5. Targeted drug delivery using immunoliposomes

1.5.1. Liposomes

British haematologist Alec D Bangham first described liposomes to be self-assembling lipid vesicles comprised of a single or multiple bilayers of phospholipids (Shailesh et al., 2009) with an aqueous core (Figure. 1.16.) Phospholipids spontaneously start forming spherical vesicles encasing a central aqueous medium, when they are taken in a solution provided their concentration is higher than the critical micelle concentration (CMC).

Phospholipids (PLs) are the commonly used lipids, notably phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine. PLs vary in their mixture of fatty acid chains with different chain length and degree of saturation (Gomez and Fernandez, 2006). The size of liposomes varies starting with 30 nm to several microns, depending on the composition phospholipids, and superficial attributes that can be modified as per the requirement.

Liposomes formed of a single lipid bilayer are called unilamellar liposomes, subdivided further into small unilamellar vesicles (SUVs) ranging from 20 to 100 nm and large unilamellar vesicles (LUVs) that are larger than 100 nm. If the LUVs are above 100 nm to the size of a cell, they are called the giant unilamellar vesicles (GUV), containing a large aqueous core that can be used to entrap hydrophilic drugs (Jeroska and Orwar, 2008). However, multilamellar vesicles (MLVs) comprise of two or multiple lipid bilayers with aqueous medium in-between. Different liposomal formulations require different preparation methodologies. The table below lists the most widely used methods and their resulting sizes.
1.5.2. Liposomal Delivery Systems

Using the liposomal technology as a delivery system has many applications starting from medicine; in vaccine development (Betz et al., 2001, Cevc and Blume, 2001), delivery of diagnostic agents (Erdoğan et al., 2008; Torchilin et al., 1997), chemotherapeutic drugs (Lukyanov et al., 2004; Lee et al., 2002), delivery of DNA (Torchilin et al., 2003), in textile industries (as carrier of dyes) (Barani and Montazer, 2008) and also in food industry (as nutritional supplements or to deliver other molecules) (Meure et al., 2008).

Of all the applications, liposomes are extensively used as drug delivery modules to deliver therapeutic agents (i.e., doxorubicin, paclitaxel or topotecan) (O’Brien et al., 2004), hormones such as parathyroid hormone and growth hormone (Fleisher et al., 1995; Fukunaga et al., 1991) and enzymes such as elastase and beta glucorinedase (Meers, 2001; Fonseca et al., 2002). There are many liposomal formulations available in the. Few of which are given in Table 1.2.

<table>
<thead>
<tr>
<th>Bioactive Agent</th>
<th>Trade Name</th>
<th>Company Name</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>Ambisome</td>
<td>Gilead Sciences</td>
<td>Fungal &amp; Protozoal infections</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>Depocyte</td>
<td>Pacira</td>
<td>Meningitis</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>DaunoXome</td>
<td>Gilead Sciences</td>
<td>HIV-related Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Myocet</td>
<td>Zeneus</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>IRIV Vaccine</td>
<td>Epaxal, Inflexal V</td>
<td>Berna Biotech</td>
<td>Hepatitis A, Influenza</td>
</tr>
<tr>
<td>Morphine</td>
<td>Depodur</td>
<td>Skye Pharma,Endo</td>
<td>Postsurgical analgesia</td>
</tr>
<tr>
<td>Verteporfin</td>
<td>Visudyne</td>
<td>QLT,Novartis</td>
<td>Age-related macular degeneration, Pathologic myopia, Ocular histoplasmosis</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Doxil/Caelyx</td>
<td>Ortho Biotech, Schering-Plough</td>
<td>HIV-related Kaposi’s sarcoma, Metastatic breast cancer, Metastatic ovarian cancer</td>
</tr>
</tbody>
</table>

Table 1.2. Drugs available as formulations of liposomes

Research is ongoing for the development of improved liposomal delivery systems for treatment of cancers that include leukemia, Kaposi’s sarcoma and breast cancer (Lorusso et al., 2004; Ralf et al., 2005). Doxorubicin encapsulated liposomes (trade name: Doxil) are used in treatment of Kaposi’s sarcoma, metastatic ovarian cancer and also in multiple myeloma. Further, studies have confirmed that pegylated liposomal doxorubicin is equally effective compared to free drug in various tumor models (Siegal et al., 1995; Williams et al., 1993). However, the disparity among pegylated liposomal doxorubicin (PEG-LD) and free doxorubicin was revealed by the pharmacokinetics
studies that showed that PEG-LD had better area under the curve (AUC), had reduced clearance rates with lower distribution volumes (Woking et al., 1994) and thus came to be known as “stealth liposomes” (Figure 1.17.). Liposomal cisplatin used in treatment of mouse pancreatic cancer had comparatively lower toxicity to that of free cisplatin but with similar rate of response. Lipoplatin (liposomal cisplatin) has been designated with a status ‘Orphan Drug’ for treatment of pancreatic carcinoma (Stathopoulos et al., 2005).

**Figure. 1.17. Different modifications on liposomes** (A) Plain liposome, (B) Stealth liposome with a protective polymer of PEG, (C) Ligand-targeted PEGylated liposomes. (D) Stimulus-sensitive immuno-targeted liposomes with mAbs attached to long-chain PEG via stimuli-sensitive bonds (Koshkaryev et al., 2013)

### 1.5.3. Fate of Liposomes *in vivo*

Following systemic administration of liposomes either through nasal aspirations, skin or intravenous routes, they get concentrated in the reticuloendothelial system (RES) that includes organs such as liver, spleen and lungs (Chrai et al., 2002). Liver takes up the majority of the liposomes, thus decreasing their half-life. Also, the liposomes *in vivo* are either endocytosed by macrophages and neutrophils, or fuse to normal cells dispensing their payload into their cytosol or do not release their payload because of adsorption onto surface of cells. Opsonization by opsonins (serum proteins) of the liposomes marks them to be phagocytosed by the macrophages. Immunoglobulins, fibronectins and β-2-macroglobulins are some of the opsonizing agents (Jesorka and Orwar, 2008) (Figure 1.18).

The retention and circulation time of the liposomes *in vivo* is determined by their bulk, lipid content, its charge, solvation, rigidity of lipid bilayers, sensitivity to pH changes, the opsonin binding kinetics and liposomal surface modifications such as attaching monosaccharides or antibodies (Shehata et al., 2008). Also in general, larger the liposomes, the faster they are eliminated from the circulation.
Another important aspect that affects structural stability of liposome is the optimum lipid composition used in the preparation of liposomes. The natural or synthetic phospholipid derivatives widely used are egg phosphatidylcholine (EPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) that are chosen depending on the phase transition temperature (Small, 1984). DSPC, in comparison to others has the highest stability and retains the integrity of the liposomes in a broad range of temperature (Sulkowski et al., 2005). The nature of the drug decides its ability to be loaded into liposomes i.e. if it is hydrophilic or hydrophobic since hydrophilic drugs are encapsulated in the aqueous medium of the liposome while the hydrophobic drugs are incorporated between the lipid bilayer.

Cholesterol is one of the major constituents that helps to sealing the gaps as a result of incomplete filling up by different lipids and proteins that stabilize the liposomal membrane.

Furthermore, the charge on the surface of liposomes can affect their fate in vivo. Liposomal surface charges can be altered using either negatively or positively charged phospholipids. Lipids such as phosphoglycerol, phosphoserine, phosphatidic acid and PEGylated phosphoethanolamine can be used to prepare anionic liposomes (Moghimi and Szebeni, 2003). However, cationic liposomes have a half-life smaller than neutral ones (Sou and Tsuchida, 2008).

Figure 1.18. Clearance of pegylated and nonpegylated liposomes via the reticuloendothelial system (RES) in the liver and spleen. Illustration (Zamboni, 2005).
On the other hand cationic liposomes can be formulated for gene or DNA transfer using positively charged lipids such as 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), 1,2-dioleoyl-3-dimethylammoniumpropane (DODAP) or dimethyldioctadecyl ammonium bromide (DDAB) (Gao and Huang, 1995; Lv et al., 2006).

1.5.4. Strategies to extend half-life and Efficacy of liposomal drug delivery

Just by altering the liposomal formulation using cholesterol and saturated phospholipids cannot completely address the problems of opsonization and subsequent clearance by RES. Therefore various strategies are to be adopted to surpass these hindrances by conjugating the liposomal exterior to form a barrier using an inert molecule. Surface modification with peptides, antibodies, and polymers has shown to lengthen the retention time in vivo (Shehata et al., 2008).

However liposomal surface modification using polyethylene glycol (PEG) with favorable characteristics such as biocompatibility, solubility, non-toxicity, low immunogenicity and also adequate excretion behavior is the most important development that eventually lead to development of long circulating stealth liposomes (Garbuzenko et al., 2005; Dadashzadeh et al., 2010). Also, other streic protector molecules such as polymers of polyacrylamide, polyvinyl alcohol, and polyvinylpyrrolidone have been used in the formulation of stealth liposomes (Takeuchi et al., 2001).

Due to the reduced uptake by macrophages and RES, PEGylated liposomes could accumulate passively within the tissues and organs by passive targeting (Jesorka and Orwar, 2008), that contributes to minimal side effects and toxicity and improved liposomal stability since the PEG arms avoid self aggregation.

1.5.5. Targeted liposomes

Besides increasing the liposomal circulation and clearance time, they have also been made to aim at a particular location in the body as a treatment modality for cancer, based on the structural and functional differences between cancerous and normal cells which can be exploited for targeted therapies.
1.5.5.1. Immunoliposomes

Liposomal targeting could be accomplished using targeting ligands such as monoclonal antibodies or their fragments like single-chain fragment variable (scFv), receptors, growth factors, peptides (for cell to cell interactions), glycoproteins or carbohydrates (Jesorka and Orwar, 2008). Attaching distinct ligands onto the liposomal surface promotes liposome binding to specific target cells leading to endocytosis, ultimately delivering the drug payload inside the cells (Chrai et al, 2002).

The target for the immunoliposomes on the tumor cells or tumor vasculature should be present in high density, and preferably non-existing on normal tissue. Acknowledging this strategy, immunoliposomes are engineered along with ligands for specific binding to the tumor, its extracellular matrix or the tumor-associated vasculature (An et al., 2016; Murphy et al., 2008; Accardo et al., 2014). The generalized concept of extravasating liposome and target interaction is shown (Figure 1.19a and b).

![Figure 1.19](image)

**Figure 1.19. (a) Structural and design strategies for liposomal drug delivery.** Liposome surface can be modified to endow stealth capabilities through PEGylation, receptor-mediated endocytosis using various targeting ligands. **(b) Liposome targeting** (A) Extravasation of liposomes due to leaky tumor vasculature, (B) tumor cell specific binding of targeted liposomes. (C) Internalization after binding (D) Drug release post binding and internalization.

The number of antigens available for binding to the targeted immunoliposomes decides its therapeutic efficacy (Hosokawa et al., 2003). Incongruity of antigen/target presence in the tumor could hinder efficacy since the nearby normal cells without the antigen may also be damaged due to the bystander effect sometimes resulting in unwanted cytotoxicity (Allen, 2002). Further, using an immunoliposomes tagged with internalizing ligands that initiate receptor-mediated endocytosis are pharmacologically better than using the non-internalized ligands since they internalize the drug and/or carrier resulting in concentrated drug delivery (Kirpotin et al., 2006).
Various formulations of liposomal chemotherapeutics such as liposomal doxorubicin, liposomal vincristine and liposomal irinotecan are approved by the US FDA for lymphoblastic leukemia and metastatic pancreatic carcinoma. Currently under phase II clinical trial is the HER2-targeted liposomal doxorubicin for breast cancer (Miller et al., 2016). In humans, liposomes tagged with an anti-transferrin receptor antibody have been used to deliver a wildtype p53 gene sequence to restore the correct function of p53 (Senzer et al., 2013). Polymeric nanoparticles containing docetaxel are being used to target the prostate-specific membrane antigen (PSMA) in phase I trials for prostate cancer (Hrkach et al., 2012; Von Hoff et al., 2016). Additionally, cellular adhesion molecules or growth-factor receptors have been targeted using ligands including folate, RGD and transferrin, respectively. Due to their lack of specificity but ubiquitous expression, they are used in imaging and few therapeutic strategies (van Dam et al., 2011; Singh et al., 2009).

1.6. Antibody based tumor targeting

Of late, monoclonal antibodies (mAbs) are in the limelight for their undeniable use in the clinics for treating life threatening diseases like cancer. Generally, IgG antibodies comprises of two highly conserved identical heavy and light chains (Padlan 1994). The complete antibody consisting of heavy and light chains can be split into four domains of the heavy chains and two of light chains. The amino-terminal of both the domain is highly variable and is called the variable domain. The antigen-binding site or the paratope comprises the variable domains of the heavy and the light chain called the complementarity-determining regions (CDR). However, the heavy chain variable region essentially determines the binding specificity. The other conserved domain of the heavy chain is called the constant domain (Fc region) which is crucial for the immune cell recruitment.

In vivo, the Fc receptors on macrophages and other immune cells bind to the Fc region of mAbs, leading to uptake and removal of the mAbs from circulation (Ternant and Painaud, 2005). Also, binding of Fc can activate secondary signals such as activation of mast cells, leading to degranulation (Daeron et al., 1980).

mAbs used in cancer treatment such as trastuzumab (anti-HER2 mAb,) blocks the tumor growth cell signaling pathways, moreover it also triggers antibody
dependent cell-mediated cytotoxicity (ADCC) (Glennie and Van de Winkel, 2003). Also, the murine mAb used earlier were immunogenic in humans and were found to form human anti-mouse antibodies (HAMA) and were promptly removed from circulation (Thorpe et al., 2003; Adams and Weiner, 2005). Although it was found that the Fc mediated activation of ADCC or complement-dependent cytotoxicity (CDC) of murine mAbs was not effective in humans (Qu et al., 2005), some murine mAbs such as muromonab (Orthoclone OKT3®) and ibritomomab (Zevalin®) are authorized for clinical usage. To decrease the immunogenicity of mAbs, chimeric mAbs (Morisson et al., 1984), humanized mAbs (Jones et al., 1986) or completely human mAbs (Baskar et al., 2009) are being developed. Yet, completely human mAbs could elicit an immune response thus reducing its therapeutic potential (de Vries et al., 2007).

1.6.1. Single chain fragment variable (scFv) antibody

In spite of their immense uses in medicine, mAbs have a few pitfalls that include their large size (150 kDa, 14.2 x 8.5 nm) (Sarma et al., 1971), a long half-life of several days (Lipman et al., 2005) which leads to immune reactions rendering it ineffective. Therefore small antibody fragments such as single chain fragment variable (scFv) and Fab’fragments are thought to be less immunogenic since they are devoid of the Fc domain and do not elicit the HAMA response.

Fab’s are the fragments of a mAb that are monovalent comprising of a portion of the heavy and the light chain (Figure 1.20a.) whereas, single chain Fv fragments are the portions of antibodies comprised of only the V_H and V_L domains linked together via a flexible polypeptide linker (Figure 1.20b.). The potential benefits of using scFv fragments over the complete antibodies for liposome targeting include longer circulating time compared to mAb-tagged liposomes by evading the immune system, lower production cost, as scFv fragments are produced using prokaryotic expression (Kipriyanov and Moldenhauer, 1997), selection of scFv with the desired specificity by phage display technology (Pini and Bracci, 2000), the choice of tag based protein purification and downstream processing (Lindner et al., 1997) and capability to engineer fully human scFv fragments that inhibits cross immunogenic reactions (Pavlinkova et al., 2001).
1.6.2. Production of scFv antibodies

Construction of scFv antibodies till recently has been primarily using hybridoma technology using the immunized mice splenocytes (Clackson et al., 1991; Finlay et al., 2006) and human B lymphocytes (Marks et al., 1991; Zhang et al., 2006). scFv, a noncovalent heterodimer is composed of the V<sub>H</sub> and V<sub>L</sub> domains that can be later used to construct the recombinant scFv DNA. The steps involved include mRNA isolation from hybridoma, cDNA synthesis, PCR amplification of specific genes coding V<sub>H</sub> and V<sub>L</sub> fragments using the cDNA as template, linking the V<sub>H</sub> and V<sub>L</sub> PCR products using a linker DNA sequence, followed by screening for the correct scFv-antigen binder by bio-panning, a technique developed by McCafferty (McCafferty et al., 1990) and coworkers in which the phage recombinants display the scFv antibodies on their tips that can be picked by affinity selection. This method has the capability of in vitro selection of scFv from large repertoire of variable domains bypassing the conventional and tedious hybridoma technology.

scFv antibodies are regularly expressed in prokaryotic expression systems and extracted from either the periplasmic space or the inclusion bodies. Typically, a leader sequence is usually engineered into the scFv construct to aid in the transport of the synthesized scFv to the nonreducing periplasmic space that promotes correct folding of the protein (Guisez et al., 1993; Seddon et al., 1994).

Figure 1.20. Graphical representations of whole antibody (IgG1) and various fragments. (a) Fab fragments of native antibody. (b) Engineered fragments of antibody. (Richards et al., 2017).
1.6.3. Coupling of scFv to liposomes

Complete antibodies or scFv fragments can be conjugated to the exterior of liposomes with the help of the distal PEG arm. A variety of modifications to the PEG arms allow the coupling of targeting ligands, that includes hydrazide-PEG (Hz-PEG) (Zalipsky, 1993), pyridyl-dithio propionylamino-PEG (PDP-PEG) (Allen et al., 1995), maleimide-PEG (Mal-PEG) (Kirpotin et al., 1997), cyanuric acid-PEG (cyanur-PEG) (Bendas et al., 1999) and p-nitrophenylcarbonyl-PEG (Torchilin et al., 2001).

The Mal-PEG based coupling is the widely used, highly efficient coupling method in which antibodies are coupled post thiolation, to the liposomes formulated with Mal-PEG-polyethylene-glycol-distearoylphosphatidylethanolamine (Mal-PEG-DSPE) by the formation of thio-ether bonds. The immunoliposomes coupled to scFv fragment by MAL-PEG method have identical circulation time compared to that of uncoupled PEGylated liposomes (Cheng et al., 2008). Usually a cysteine residue is engineered at the C terminus of the scFv to aid in conjugation with proper orientation on the immunoliposomes (Marty et al., 2006).

1.6.3.1. Post-insertion technique

Besides conventional antibody fragment coupling onto the derivatized Mal-PEG-DSPE lipids containing liposomes, another method called the post-insertion technique can be adopted to prepare immunoliposomes (Ishida et al., 1999). In this method initially, ligands, complete antibodies or their fragments are coupled to micelles of derivatized PEG-lipid, similar to conventional coupling followed by incubation of the conjugated antibody-PEG-lipids with the pre-formed liposomes, either empty or with a drug payload, which results in insertion of the conjugated PEG-lipids into the outer liposomal membrane. Both methods of immunoliposome preparation seem to have similar properties of cell binding, drug release rate and pharmacokinetics/biodistribution (PK/BD) (Iden and Allen, 2001).

1.6.4. Targeted drug delivery using scFv antibodies

Besides the clinically used immunoliposomes mentioned previously, currently, 13 ligand-targeted nanomedicines have been moved into clinical trials (Table 1.3.), which include lipid and polymer-based delivery vehicles, a retroviral vector and bacterially-derived minicells.
There are many more novel pre-clinical immunoliposomes designed to deliver the cytotoxic drug payload (Shargh et al., 2016) that includes delivery of siRNA, small molecules and other ligands. siRNA to inhibit the expression of chemokine receptor 4 (CXCR4), a protein involved in cell survival and cancer metastasis was encapsulated into immunoliposomes coupled with anti-HER2-scFv containing 9 residues of arginine (9R) at its C-terminal was used to selectively target the HER2 overexpressing BT-474 mammary carcinoma cells, although it failed to be effective in MDA-MB-231 cells since they do not express HER2 (Jiang et al., 2015). A fusion peptide of 9R and scFv of nimotuzumab (anti-EGFR mAb), was developed to target EGFR overexpressing lung cancer cells for targeted delivery of siRNA to EGFR-overexpressing cells to inhibit MET, EGFR and KRAS gene expression to overcome tyrosine kinase inhibition resistance (Lu et al., 2016b). Polyethylene glycol–poly (D,L-lactide) (PEG–PLA)-based nanoparticles conjugated with anti-HER2-scFv antibody was used to selectively deliver siRNA to inhibit Plk1, a kinase enzyme involved in cell division to HER-2 overexpressing BT474 breast cancer cells but not in MDA-MB-231 cells (Dou et al., 2014).

A fusion peptide of scFv of cetuximab and a truncated form of protamine was used to deliver the siRNA to inhibit the human wings apart-like (hiWAPL) gene expression in HeLa cells as a treatment modality for cervical cancer (Zhang et al., 2014).

Table 1.3. List of ligand-targeted nanomedicines under clinical evaluation.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Company</th>
<th>Approx. size (nm)</th>
<th>Payload</th>
<th>Ligand</th>
<th>Target</th>
<th>Clinical indication</th>
<th>Clinical phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light-based nanomedicine</td>
<td>MeBioPharm</td>
<td>50-200</td>
<td>Oxaliplatin</td>
<td>Protein</td>
<td>Transformin receptor</td>
<td>Metastatic gastric, gastro esophaegus junction, empaglifloz adenocarcinoma</td>
<td>Phase II</td>
</tr>
<tr>
<td>SCT-53</td>
<td>SynerGene Therapeutics</td>
<td>50</td>
<td>p53 plasmid DNA</td>
<td>Antibody fragment (scFv)</td>
<td>Transformin receptor</td>
<td>Solid tumors</td>
<td>Phase Ib</td>
</tr>
<tr>
<td>SCT-94</td>
<td>SynerGene Therapeutics</td>
<td>50</td>
<td>Rb514 plasmid DNA</td>
<td>Antibody fragment (scFv)</td>
<td>Transformin receptor</td>
<td>Solid tumors</td>
<td>Phase I</td>
</tr>
<tr>
<td>MM-302</td>
<td>Merckinack Pharmaceuticals Lipidex</td>
<td>75-110</td>
<td>Doxorubicin</td>
<td>Antibody fragment (scFv)</td>
<td>ErbB2 (HER2)</td>
<td>Breast cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>Liposomat-MM</td>
<td>University Hospital Basel 213-101</td>
<td>85</td>
<td>Melanoma antigen and 491b</td>
<td>Melanoma vaccine</td>
<td>Single domain antibody (sAb) fragment (VH)</td>
<td>DC-SIGN</td>
<td>Phase I</td>
</tr>
<tr>
<td>Anti-EGFR 216-236</td>
<td>to-BB Technologies</td>
<td>140</td>
<td>Doxorubicin</td>
<td>Protein</td>
<td>Antibody fragment (Fab/2)</td>
<td>Glutathione transporters</td>
<td>Phase II</td>
</tr>
<tr>
<td>MCC-465</td>
<td>Mitsubishi Pharma Corporation</td>
<td></td>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td>Not characterized</td>
<td>Phase I</td>
</tr>
<tr>
<td>Polymers-based nanomedicine</td>
<td>BIND Biosciences</td>
<td>100</td>
<td>Docetaxel</td>
<td>Small molecule</td>
<td>Prostate specific membrane antigen</td>
<td>Solid tumors</td>
<td>Phase II</td>
</tr>
<tr>
<td>CALAA-01</td>
<td>Calando Pharmaceuticals Selecta Biosciences</td>
<td>50-70</td>
<td>RRM2 siRNA, Nucleotide, T-helper cell peptide, TLR agonist</td>
<td>Protein</td>
<td>Antibody fragment (Fab/2)</td>
<td>Antigen presenting cells</td>
<td>Solid tumors</td>
</tr>
<tr>
<td>SEL-068</td>
<td>Eshbina/EDV, Inc</td>
<td>250</td>
<td>Paclitaxel</td>
<td>Antibody</td>
<td></td>
<td>EGFR</td>
<td>Solid tumors</td>
</tr>
<tr>
<td>Retroviral vector</td>
<td>Euphus Biotechnologies</td>
<td>100</td>
<td>Cytosidal dominant negative cyclin G1 DNA construct</td>
<td>Small molecule</td>
<td>Collagen</td>
<td>Sarcoma, osteosarcoma, pancreatic cancer</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

Table 1.3. List of ligand-targeted nanomedicines under clinical evaluation.

There are many more novel pre-clinical immunoliposomes designed to deliver the cytotoxic drug payload (Shargh et al., 2016) that includes delivery of siRNA, small molecules and other ligands. siRNA to inhibit the expression of chemokine receptor 4 (CXCR4), a protein involved in cell survival and cancer metastasis was encapsulated into immunoliposomes coupled with anti-HER2-scFv containing 9 residues of arginine (9R) at its C-terminal was used to selectively target the HER2 overexpressing BT-474 mammary carcinoma cells, although it failed to be effective in MDA-MB-231 cells since they do not express HER2 (Jiang et al., 2015). A fusion peptide of 9R and scFv of nimotuzumab (anti-EGFR mAb), was developed to target EGFR overexpressing lung cancer cells for targeted delivery of siRNA to EGFR-overexpressing cells to inhibit MET, EGFR and KRAS gene expression to overcome tyrosine kinase inhibition resistance (Lu et al., 2016b). Polyethylene glycol–poly (D,L-lactide) (PEG–PLA)-based nanoparticles conjugated with anti-HER2-scFv antibody was used to selectively deliver siRNA to inhibit Plk1, a kinase enzyme involved in cell division to HER-2 overexpressing BT474 breast cancer cells but not in MDA-MB-231 cells (Dou et al., 2014).

A fusion peptide of scFv of cetuximab and a truncated form of protamine was used to deliver the siRNA to inhibit the human wings apart-like (hiWAPL) gene expression in HeLa cells as a treatment modality for cervical cancer (Zhang et al., 2014).
The siRNA mediated inhibition of viral nucleoprotein mRNA of H5N1 virus infected cells was achieved by conjugating humanized anti-HA scFv to liposomes to target the hemagglutinin expressing H5N1 infected cells (Khantasup et al., 2014).

A fusion peptide of a short collagen binding peptide (TKKTLRT) and a cetuximab-based scFv was used to target the EGFR overexpressing A-431 melanoma cells. The fusion peptide selectively induced apoptosis and inhibited cell proliferation in vitro (Liang et al., 2015).

For efficient delivery of doxorubicin to cancer cells and to inactivate the endosome degradation, an immuno-virosome fusion peptide of anti-PAP (placental isozyme of alkaline phosphatase) scFv and a portion of sendai virus F-protein was able to selectively deliver doxorubicin to the PAP-expressing cells (Kumar M et al., 2015).

Apart from these few strategies mentioned for drug targeting, there are many other cell specific surface markers expressed on tumor cells that can be exploited to target the anticancer agents selectively to tumor cells. Furthermore, instead of targeting only the cancer cells in a tumor, it would be a much effective strategy to simultaneously target both the tumor cells and its neovasculature which are disorganized, immature, tortuous, and hyperpermeable. The most widely used angiogenic marker for targeted drug delivery to the tumor vasculature is the Integrin alphaVbeta3 (C C Kumar et al., 2000; R O Hynes 2002). Other potential targets expressed both on the tumor cells and on the tumor vasculature include the VEGFR, aminopeptidase N and Tie2 receptor (H.F. Dvorak et al., 1995; Pastorini et al., 2003; Jones et al., 2001). Among these, Tie2 is one of the highly conserved endothelium-specific receptor tyrosine kinase overexpressed during tumor angiogenesis (Partanen and Dumont, 1999) in the endothelial cells near the site of the tumor (Willam et al., 2000) and it has also been shown to be overexpressed in certain cancer such as leukemia, gastric tumor, breast tumor and gliomas (Martin et al., 2008).

In this study we report the development of a novel antibody fragment, scFv-sTie2 as a targeting ligand for liposomes to target the Tie2 expressing cancer cells. scFv-sTie2 conjugated PEGylated immunoliposomes were shown to bind specifically to Tie2 positive cells resulting in improved drug delivery.
1.7. Rationale and scope of the proposed topic of PhD thesis

As apoptotic means of cell death causes minimal tissue damage, cancer therapy based on apoptosis is the focus for developing new antitumor therapy. A complete perception of the molecular signaling cascades involved in apoptotic cell death along with other combinatorial strategies is required for a definitive and multifaceted attack on cancer. The probable remedial approach ranges from careful suppression of anti-apoptotic pathways, tumor angiogenic pathways, exploitation of resistance to drugs, addiction to oncoproteins, unrestrained cell cycles, hypermitogenic and hypoxic features of cancer cells. These coinciding and integral strategies depend on the prudent use of combinatory drug formulations aimed at their respective targets.

For the selective and positive removal of cancer cells, a combination of both apoptosis-inducing and angiogenesis inhibiting agents may be used (Tiwari, 2012). Since therapeutic agents may affect tumor as well as normal cells, controlled and targeted delivery of these agents enable the use of higher doses for increased therapeutic efficacy. Targeted drug delivery involves the association of a drug with a carrier system such as PEGylated-immunoliposomes that can actively target only the cancer cells by coupling the targeting antibodies on the liposomal surface. PEGylated-immunoliposomes have been shown to have increased circulation half-life and better drug delivering capabilities.

With this background, this study was strategized to evaluate neoplastic effects of extracts of plant, to develop a sequential combinatorial treatment, essentially to sensitize apoptosis resistant cancer cells to TRAIL induced apoptosis, using the phytochemical anacardic acid and finally to develop a tumor targeted drug delivery system using anti-Tie2 scFv tagged immunoliposomes.

The study was conducted with the following objectives:

1. Studies on anti-neoplastic activity of plant extracts and mechanism of sensitization to TRAIL induced apoptosis by anacardic acid.

2. Production of single chain variable fragment (ScFv) antibody against sTie2 and its effect on angiopoietin induced angiogenesis.

3. Studies on ligand targeted liposomes for bioactivity.