CHAPTER TWO

REVIEW OF LITERATURE
2.1 CANCER

‘Regulation’ or control of any living system is critical for its survival, growth and maintenance. From a unicellular bacterium to the highly complex multicellular forms of life, there is evidence of ‘control checkpoints’ for all the crucial cellular processes. One of the most important of such processes is proliferation. Alterations in the checkpoints may lead to ‘cancer’, a fatal disease of uncontrolled proliferation of genetically altered cells. The term cancer is derived from the Greek word ‘Karkinos’ which means crab and this name comes from the appearance of the cut surface of a solid malignant tumor, with the veins stretched on all sides. In all known cases, cancer cells are derived from the repeated divisions of a mutant cell (Dey et al., 2009). Some of these mutations may be due to the effects of carcinogens, such as tobacco, smoke, radiation, chemicals or infectious agents. A few other cancer-promoting mutations may be acquired through errors in DNA replication. Genetic alterations that render a normal cell cancerous usually arise in two classes of genes termed the oncogenes and tumor suppressors. Oncogenes, for which a gain-of-function mutation drives a cell towards cancer, are called proto-oncogenes; their mutant, hyperactive forms are called oncogenes. Most of the oncogene encoded proteins function as elements of the signaling pathways that regulate cell proliferation and survival in response to growth factor stimulation. A mutant form of this protein usually functions in a growth factor independent manner. These proteins include polypeptide growth factors, growth factor receptors, elements of intracellular signaling pathways and transcription factors. Examples of some proto-oncogenes frequently implicated in cancer are growth factors like EGF, growth factor receptors like ErbB, intracellular signaling molecules like Ras and Raf and transcription factors like myc. Tumor suppressor genes, for which a loss-of-function mutation drives a cell towards cancer. The proteins encoded by most tumor suppressor genes inhibit cell proliferation or survival and also activate DNA repair pathways. Inactivation of tumor suppressor genes therefore leads to tumor development by eliminating the regulatory proteins. Examples of some tumor suppressor genes commonly invoked in cancer include transcriptional regulatory protein pRb, cycle progression and apoptosis regulator p53 and PTEN (A lipid phosphatase that dephosphorylates PIP₃). The genetic abnormalities found in cancer cells typically activate the cancer promoting oncogenes and/or inactivate the tumor suppressor genes. Yet another class of genes involved in carcinogenesis is those which ‘sense’ and repair DNA damage and also ensure proper chromosome segregation during mitosis. These genes are also seen to be
inactivated in tumors, thereby rendering the cells highly ‘mutable’, enabling them to acquire multiple mutations (Weinberg, 1996). Along with genetic abnormalities, changes at the epigenetic level are also well known to operate in cancers. There are changes in promoter methylation-demethylation patterns in the genome of cancer cells, which is a typical phenomenon utilized to repress or activate gene expression respectively (Widschwendter et al., 2006). One of the most common examples is the promoter hypermethylation of the tumor suppressor, p16, which is found in several cancers and renders this gene inactive. There are also seen chromatin modifications, in the form of acetylation-deacetylation of lysine residues of histones, which operate in cancer cells by turning ‘on’ proliferation and pro-survival genes while turning ‘off’ the anti-proliferation (checkpoint) and pro-apoptosis genes (Weisenberger et al., 2006). Overall, cancer can be viewed at three levels (a) a disease of altered tissue behavior which recognizes that a tumor is not just a collection of cancer cells, but a complex organ whose properties reflect the interactions between genetically altered cancer cells and the host (b) a disease of altered cellular behavior, (c) a disease of genomic instability.

The process of conversion of a normal cell into a cancerous cell, termed cellular transformation, involves the accumulation of multiple genetic alterations that confer proliferation and survival benefits. For example, while normal cells fail to divide after a finite number of divisions, termed the Hayflick limit (Finkel et al., 2007), cancer cells show extensive proliferation potential. While normal cells respond to cell-cell contacts by negatively regulating proliferation, cancer cells lack such contact inhibition (Hanahan and Weinberg, 2000). Also, normal cells need exogenous growth factors to proliferate, but most cancer cells are self-sufficient in their requirement for mitogenic signals. While most normal cells need a substratum for attachment and growth, cancer cells are anchorage independent and can survive in the absence of any substratum (Hanahan and Weinberg, 2000). An abnormal growth of tissue resulting from uncontrolled, progressive proliferation generates a mass of cells which is called a tumor. Sometimes, such a mass can be self-limiting, which does not invade neighboring tissue and hence pose no danger to life. These are the benign tumors. At other times, a tumor continues to grow at its original site, breaches the basement membrane and gets into the circulatory system, whereby these cancer cells migrate to new sites in the body and initiate secondary tumors. This process is called metastasis, and such tumors are called malignant (Fidler, 2003). Thus cancer always refers to malignant neoplasms, whereas tumors can be either benign or malignant. The
cancers of the epithelium are called carcinomas which are the most common type of
cancer. Those originating in the cells of connective tissues, like bones, cartilages, blood
vessels or muscle are called sarcomas. Cancers that start in blood forming tissues such as
bone marrow are called leukemia, and those that begin in the cells of the immune system
are termed as lymphoma and myeloma. Cancers of the central nervous system that arise in
the tissues of the brain and spinal cord include gliomas and astrocytomas.

Chemotherapy, surgery and radiotherapy have remained the main attempts of cancer
treatment. Chemotherapy includes use of cytotoxic drugs to treat the cancer cells, most
often exploiting the property of rapid proliferation of the cancer cells. One of the major
limitations of the chemotherapy is the cytotoxic effects in normal cells of the body. With
succeeding generations of tumor cells, growth becomes less regulated, and tumors become
less responsive to most chemotherapeutic agents (Kornblau et al., 2006). Near the center
of some solid tumors, cell division effectively ceases, making them insensitive to
chemotherapy. Another problem with solid tumors is the fact that the chemotherapeutic
agent often does not reach the core of the tumor. Solutions to this problem include surgery
and radiation therapy. Surgical cases include those where either the tumor size is very
distinct or large. The goal of the surgery can be either the removal of only the tumor, or
the entire organ. Radiotherapy refers to the medical use of ionizing radiation for malignant
tumors. The effects of radiation therapy are localized to the region being treated. This
therapy injures or destroys cells in the area being treated (the ‘target tissue’) by damaging
their genetic material, making it impossible for these cells to continue to grow and divide.
Usually, a combination of all the three treatment regimes is used against any given cancer
for maximum benefit (Schätzlein, 2006).

2.2 CANCER CHEMOTHERAPY

Cancer chemotherapy is the use of drugs for the treatment of cancer. The drugs are
generally directed against metabolic sites essential for cell replication, DNA and RNA
synthesis. Ideally, these drugs should interfere with cellular process unique to the
malignant cells. However, currently available anticancer drugs do not specifically
recognize neoplastic cells, but rather affect all proliferating cells, both normal and
abnormal. Therefore, almost all anti-tumor agents have a steep dose response curve for
both toxic and therapeutic effects. Currently used anticancer drugs for the treatment of
breast cancer includes Antimetabolites (5-flurouracil, gemcitabine, methotrexate);
Antibiotics (doxorubicin, dactinomycin, bleomycin); Alkylating agents
(cyclophosphamide); Microtubule inhibitors (paclitaxel, vinblastine, vincristine); platinum compounds (cisplatin and carboplatin); selective estrogen receptor modulators (tamoxifen, raloxifene, fulvestrant); aromatase inhibitors (anastrozole, letrozole, exemestane); HER2 inhibitors (trastuzumab, lapatinib) and EGFR inhibitors (gefitinib). One of the inherent limitations of this approach of treatment is that these drugs also kill those normal cells that have a rapid proliferation rate. Severe vomiting, stomatitis, alopecia occur to a lesser or greater extent during therapy; myelosuppression is confined to the specific agents (cardio toxicity with doxorubicin, pulmonary fibrosis with bleomycin etc.).

2.3 MULTIDRUG RESISTANCE IN CANCER

One of the major limitations of long term chemotherapeutic treatment is the development of drug resistance; while some of the cancer cells are intrinsically resistant to various anticancer drugs, the others ‘develop’ resistance to multiple chemotherapeutic drugs upon treatment, a phenomenon known as ‘Multi Drug Resistance’ (MDR). In those cancer cells which are inherently resistant to multiple anticancer agents, on prolonged administration of the drugs, there is an increase in the expression of different classes of proteins involved in drug metabolism, detoxification and active drug efflux in addition to alteration of intracellular drug targets, like certain proteins to which the drug binds. All these mechanisms alter the effective drug concentration within a cell. These mechanisms are also known to be activated in the cancer cells which ‘acquire’ drug resistance. One of the immediate measures the cell utilizes towards drug resistance is the active efflux of the drug so as to minimize the number of molecules of the drug entering the cell. This is usually a cell membrane based mechanism, where a family of plasma membrane efflux transporters is overexpressed. These include the most common ATP-binding cassette (ABC) transporters, which have received extensive attention (Litman et al., 2001). These transmembrane protein transporters actively extrude the cytotoxic drugs by utilizing ATP. There are several subclasses of these transporters, the most well characterized in cancer being ABCB1 or MDR1 (P-glycoprotein), ABCC1 (MRP1) and ABCG2, also known as BCRP as it was discovered in a breast cancer cell line (Gottesman, 2002). These transporters are not specific to a particular class of molecules and show a wide spectrum of substrate recognition and binding. The substrates these bind to vary from ions, bile salts, anticancer drugs, microbial toxins and hydrophobic compounds, like those derived from plants.
2.4 ABC TRANSPORTERS

2.4.1 P-glycoprotein (ABCB1, MDR1)

Major mechanism of multidrug resistance in cultured cancer cells was shown to be due to the over-expression of an energy-dependent drug efflux pump, known as P-glycoprotein (P-gp) (Juliano and Ling, 1976). This efflux pump, the product of the \textit{MDR1} gene in the human (Chen et al., 1986) and the product of two different related genes, \textit{mdr}1a and \textit{mdr}1b in the mouse (Croop et al., 1989, Lothstein et al., 1989) was one of the first members to be described by a large family of ATP-dependent transporters known as the ATP-binding cassette (ABC) family (Higgins, 1992). P-glycoprotein is highly expressed in bone marrow, on the cells lining the intestine (where the function of absorption that these cells have to carry out makes them highly susceptible to toxins), at the blood brain barrier (where they play a crucial role in protecting the brain) (Schinkel, 1999), in placental trophoblasts, and several other organs which are either directly exposed to environmental toxins, like the mucosal lining along the oral cavity or whose functions involve absorption and excretion of cellular wastes, like kidney (Sparreboom et al., 1997). P-gp can detect and bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These drugs include many of the commonly used natural product anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many commonly used pharmaceuticals ranging from antiarrhythmics and antihistamines to cholesterol-lowering statins (Bogman et al., 2001) and HIV protease inhibitors (Lee and Gottesman, 1998). Levels of expression of P-gp in many different tumors are high enough to confer significant drug resistance and the presence of P-gp correlates with drug resistance in several different cancers (Lee and Gottesman, 1998). It was observed that an acquisition of drug resistance after chemotherapy is associated with increased P-gp levels (Goldstein et al., 1989). One of the direct evidences of the link between this transporter and efflux of a chemotherapeutic agent is the acute induction of P-gp in human tumors following \textit{in vivo} exposure to doxorubicin, a commonly used chemotherapeutic drug (Abolhoda et al., 1999). Clinically, it has been seen that expression of P-gp in some tumours predicts poor response to chemotherapy (Chan et al., 1991).
2.4.2 **MRP1 (ABCC1)**

Over a period of time, researchers realized that not all multidrug-resistant cells overexpress P-glycoprotein. This led to the discovery of other ABC transporters causing multidrug resistance in cancer. One of the ABC transporters that came up in this direction was the multidrug-resistance-associated protein 1 (MRP1, or ABCC1) (Wijnholds et al., 1997). MRP1 physiologically serves as a cellular defense mechanism because it is located in the basolateral side of epithelial membrane (Evers et al., 1996). It has been shown that MRP1 may protect the testicular tubules (Evers et al., 1996), cerebrospinal fluid (Wijnholds et al., 2000) and bone marrow precursor cells (Wijnholds et al., 1998). MRP1 recognizes neutral and anionic hydrophobic natural products, and transports glutathione and other conjugates of these drugs, or, in some cases such as for vincristine co-transport unconjugated glutathione (Jedlitschky et al., 1996) (Kool et al., 1999, Müller et al., 1994). MRP1 and MRP3 have been recently shown to transport methotrexate, a well known chemotherapeutic drug extending the range of compounds potentially involved in the multidrug resistance phenotype (Kool et al., 1999, Zeng et al., 1999). The discovery of MRP1 led to a search for other members of this family, resulting in the discovery of a total of 9 or 10 MRP genes, at least 6 of which have been characterized enough to indicate that they transport anticancer and antiviral compounds (MRP 1-6) (Borst et al., 1999).

2.4.3 **BCRP (ABCG2)**

Selection for mitoxantrone resistance results in multidrug-resistant cells that produce a more distant member of the ABC transporter family, ABCG2 (mitoxantrone-resistance gene) also known as BCRP (breast cancer resistance protein) or ABC-P (ABC transporter in placenta) (Doyle et al., 1998, Allikmets et al., 1998). ABCG2 is a half transporter and hence unlike MDR1 and the MRP family members, ABCG2 protein is presumed to function as a dimer (Gottesman, 2002). It is mainly expressed at the apical membranes of placental cells, in the mammary gland, intestine, colon, enterocytes, hepatocytes, erythrocytes and the human brain microvessel endothelium (Maliepaard et al., 2001, Cooray et al., 2002). It was reported that ABCG2 is involved in transport of folate (Ifergan et al., 2004) and endogenous porphyrins, thereby protecting the cells against hypoxia induced by toxic porphyrins (Krishnamurthy et al., 2004). It has a renal and hepatic secretory function as it is involved in transport of organic sulfates and bile acids respectively (Mizuno et al., 2004, van Herwaarden et al., 2003). ABCG2 can actively efflux a substantial variety of compounds ranging from fluorescent dyes to both anionic...
and cationic drugs. Reported drug substrates for this transporter mainly include mitoxantone, doxorubicin, topotecan, etoposide, prazosin, flavopiridol, Hoechst 33342 and anthracycline (Sarkadi et al., 2006). The active efflux of these different classes of chemotherapeutic drugs by ABCG2 ultimately results in the development of MDR.

### 2.4.4 Other drug transporters

Other ABC family members that have been associated with drug resistance include MDR2 gene product (Borst et al., 2000) encoding for the bile salt export protein (BSEP, ABCB11), first reported as the ‘sister of P-gp’. This protein is expressed at high levels in liver cells, and it confers low level resistance to paclitaxel (Childs et al., 1998). Another ATP transporter reported in cancer is MDR3, a phosphatidylcholine flippase that is closely related to P-gp, normally transports phospholipids into bile, but can transport paclitaxel and vinblastine out of the cell, albeit inefficiently, unless it is mutated (Smit et al., 1993). It has also been shown that Lung resistance protein (LRP) is expressed at high levels in drug-resistant cell lines and some tumors (Smit et al., 1993). Many of these transporters are expressed in the normal liver and are likely to be involved in drug disposition (Scheffer et al., 2000).

### 2.5 STEM CELLS

Stem cells are the group of cells which can reconstitute the entire tissue when required, such as, during disease or injury. Two properties of stem cells that sets them aside from the other specialized cells of a tissue are, they are able to give rise to a cell identical to itself, a property known as ‘self renewal’, and an ability to give rise to all the cells comprising the tissue, a property known as ‘multilineage differentiation’ (Fig. 2.1). By its ability to ‘self renew’, a stem cell can maintain a population of its own kind for the entire lifetime of an organism and by its ability to undergo multilineage differentiation, it ensures the maintenance of tissue homeostasis in normal and disease conditions (Smalley and Ashworth, 2003). Stem cells are of three types: totipotent cells, pluripotent cells and multipotent cells. The fertilized egg and the first 4 to 8 cells produced by its cleavage constitute the totipotent cells in an organism. Totipotent cells can give rise to all the three germ layers, as well as the extra-embryonic membranes. The pluripotent cells include the embryonic stem cells that are derived from the inner cell mass of a blastocyst. These can give rise to all the three germ layers but not the extra-embryonic membranes. Stem cells, which have now been found in multiple adult tissues and organs, have several fundamental
properties. First, they are generally very rare. For example, the long-term hematopoietic stem cells (LT-HSC) in mouse bone marrow constitute about 0.02% and the short-term HSCs (ST-HSC) are 0.10% of the total cells (Cozzio et al., 2003). Second, stem cells in their normal microenvironment (i.e., niche) rarely divide, although they possess tremendous proliferative potential (Fuchs et al., 2004). Third, stem cells can self-renew; that is, they can regenerate themselves when they divide to give rise to progenitor cells (Raff, 2003). The molecular pathways underlying the unique property of self renewal are Notch, Wnt and Hedgehog pathways. Fourth, stem cells possess multipotential, oligopotentential, or unipotential differentiation ability (Raff, 2003). Many adult stem cells also seem to have the ability to trans-differentiate into other cell types (Fialkow et al., 1991). Finally, stem cells may express unique markers or properties that can allow their enrichment and identification.

2.6 STEM CELLS IN CANCER

There are enough evidences that indicate that virtually all cancers are clonal in origin and represent the progeny of a single cell (Fialkow et al., 1991). But what still remains as a fundamental problem in cancer is which cells within the tumor have the ability to seed another tumor. The concept that not every cell within the tumor clone possesses the ability to seed another tumor comes from the following observations: In 1973, Ernest McCulloch and colleagues observed that only 1 in 100 to 1 in 10,000 murine myeloma cells had the ability to form colonies in vitro. Similarly, when several thousands of cells obtained from primary solid tumors were seeded in soft-agar, the colony forming efficiency was found to be between 0.10-0.50 percent (Kern et al., 1982). Furthermore, in vivo transplantation experiments demonstrated that autologous injection of tumor cells subcutaneously into the same patient led to a very low frequency of tumor formation. At least $10^6$ cells were required for tumor initiation in vivo which strongly suggested only a defined subpopulation of cells within the tumor have the capacity to form tumor (Southam, 1960). Subcutaneous injection of human tumor samples into mice also gave similar results (Dick, 2003). Taken together, these observations indicated that not all tumor cells within a tumor actually have the potential to either generate colonies in vitro or initiate new tumors upon transplantation in vivo. Thus, these observations raise a fundamental question that why not every cell within a tumor mass is capable of initiating a new tumor?
Two models have been proposed to explain the observed tumor cell heterogeneity, the stochastic model and the hierarchy model (Fig. 2.2). The stochastic model predicts that the tumor is homogenous and every cell within the tumor has the potential for initiating a new tumor but it depends on low probability stochastic events. According to this model, the genetic changes leading to the development and progression of malignancy are operative in all cells within the tumor. Existing therapeutic and research approaches aimed at the bulk cells of the tumor are largely based on this model. On the other hand, the hierarchy model proposes that the tumor is heterogeneous and only a limited number of cells within a tumor actually have the potential to seed another tumor (Reya et al., 2001). Emerging evidence strongly supports the hierarchy model. So, as per the hierarchy model, there is functional heterogeneity among the cells that comprise a tumor and only a few cells within the tumor possess the capability of regenerating a new tumor and these cells are termed as the tumor initiating cells (Pardal et al., 2003). Hence, most cancer cells have only limited proliferative potential and only a small subset of cancer cells has the ability to initiate new tumor growth (Fig. 2.3). According to this model, these cancer stem cells (CSCs) are biologically and functionally distinct from the bulk of tumor cells and must be specifically targeted by cancer treatments to achieve permanent cure (Velasco-Velázquez et al., 2011). They are also termed as cancer stem cells (CSCs) as they have been shown to possess stem cell-like properties. This model is supported by the recent characterization of CSCs in leukemia, breast cancer, brain tumors, pancreatic cancer, prostate cancer, ovarian cancer, colon cancer, hepatocellular carcinoma etc. Just like normal stem cells play a central role in organogenesis, cancer stem cells appear to be fueling tumorigenesis.

The concept of cancer stem cells was first firmly established experimentally in acute myelogenous leukemia (AML) (Lapidot et al., 1994). In this study, a minority of undifferentiated cells isolated from leukaemic patients proved to be the only cells capable of reconstituting tumors upon transfer into NOD/SCID mice. These cancer stem cells resemble normal haematopoietic stem cells (HSCs) in their cell surface marker expression (CD34+/CD38- phenotype), multipotency, and self-renewal properties (Bonnet and Dick, 1997). It was revealed in human leukemia that the tumor clone is organized as a hierarchy that originates from rare leukemic stem cells that possess extensive proliferative and self-renewal potential, and are responsible for maintaining the tumor clone (Bonnet and Dick, 1997). It has been reported that only a small population of the breast cancer cells were able
to induce tumor formation in the mice (Al-Hajj et al., 2003). These cells were found to express cell surface marker CD44, but low CD24 (CD44 high /CD24 low). As few as 200 CD44 high /CD24 low cancer cells were able to consistently form tumors, whereas thousands of cancer cells that had other phenotypes failed to form tumors. These tumorigenic cells behaved like cancer stem cells in that they not only gave rise to additional CD44 high /CD24 low cells, but they also gave rise to diverse populations of non-tumorigenic breast cancer cells with other phenotypes. A similar finding was subsequently made in human brain tumors in which as few as 100 CD133+ cells from human brain tumors could initiate new tumors in the brain of immunocompromised mice, while 100, 000 CD133- cells did not contain any tumor-initiating activity (Singh et al., 2004). It was also shown that multiple myeloma contains a rare subset of cells, defined by their lack of expression of CD138, that are clonogenic in vitro and tumorigenic in vivo (Matsui et al., 2004). More recently similar finding have been made for pancreatic cancer (Li et al., 2007), colon cancer (Ricci-Vitiani et al., 2006), head and neck squamous cell carcinoma (Prince et al., 2007), melanoma (Fang et al., 2005), prostate cancer (Collins et al., 2005), ovarian cancer (Zhang et al., 2008), colorectal cancer (Dalerba et al., 2007) and hepatocellular carcinoma (Suetsugu et al., 2006). It also has been shown that established cancer cell lines also contain cancer stem cells (C6 glioma, MCF7 breast, B104 neuroblastoma and HeLa adenocarcinoma) (Kondo et al., 2004, Patrawala et al., 2005). Therefore, the Hierarchy Model is a well established theory to explain the functional heterogeneity within tumors. As few as 100 cells of this small population could initiate a tumor while as many as thousands of cells of the remaining (bulk) population could not do so. In addition, these tumor-initiating cells could repopulate themselves, as well as give rise to the ‘bulk’ non-tumor initiating cells. As stated in the Hierarchy model, the small population of tumor initiating cells within multiple cancers has the intrinsic potential to give rise to all the cell types of a tumor and also to cells of its own kind, which maintains and sustains the tumor over prolonged periods (Jordan et al., 2006). These properties exactly reflect the properties of multilineage differentiation and self renewal of normal stem cells. Such striking similarities between these two cell types make it apt to call the ‘tumor initiating cells’ within the tumor as ‘cancer stem cells’ (CSCs) (Wu, 2008). With the discovery of such cells in several cancers, the existence of cancer stem cells is no longer a hypothesis, but a well established fact.
2.8 WHY RELAPSE OCCURS IN CANCER CHEMOTHERAPY?

Recent reports suggest that most cancers comprise a heterogeneous population of cells with marked differences in their proliferative potential as well as the ability to reconstitute the tumor upon transplantation (Croker and Allan, 2011). It has been evident in hematopoietic malignancy and solid tumors that only a rare population of cells with unique self-renewal and survival mechanisms called cancer stem cells drive and sustain the tumor. Cancer stem cells are biologically distinct from other cells in the tumor and are the only cells capable of initiating and sustaining tumor growth in vivo, whereas the bulk cells are not. The identification of cancer stem cells has important therapeutic implications (Greaves, 2011). Traditionally, drug therapies have been developed based on the ability of drugs to cause tumor regression in rapidly dividing cancer cells. It has now been shown that the majority of cancer cells within a tumor are non-tumorigenic (bulk cells); therapies directed against these cells would cause tumor regression. However, if therapies fail to target the tumorigenic cancer stem cells, then these cells would persist even after therapy and will be able to regenerate the tumor, resulting in tumor relapse (Fig. 2.4 A). Therefore, there are major implications for the way we study, diagnose and treat the cancer if only a rare subset of tumor stem cells drive tumor formation. Then the goal of the cancer therapy should be to identify cancer stem cells and then develop the therapies that target tumorigenic cancer stem cells (Al-Ejeh et al., 2011). Current therapeutic strategies fail to account for the potential differences in drug sensitivity or target expression between the tumor initiating cells or tumorigenic cells (cancer stem cells) and the non tumorigenic cells (Zhao et al., 2012). This new model of cancer progression is also likely to impact our understanding of the mechanisms of drug resistance. A wide variety of transporters, including members of the ABC transporter family, have been demonstrated on the normal stem cells and several of these transporters have well established roles in drug efflux. Current treatments such as chemotherapy and radiation therapy although successful at destroying rapidly proliferating cancer cells (i.e. tumor bulk) may be unable to completely eliminate the critical cancer stem cells, hence allowing them to recreate the cancer. It is therefore believed that cancer stem cells are the root cause for the malignancy and cause for relapse of the disease. Unique cancer stem cell targets could be hit with additional classes of drug compounds including small molecules from a chemical library by inducing structural modifications or screening for the activity from plant source. As a result of this type of targeted therapy, the danger of recurrence of cancer might be eliminated (Fig. 2.4
This technology and potential treatment can be used in conjunction with existing cancer treatments, targeting and destroying both cancer stem cells and bulk cancer cells. Once we eradicate the cancer stem cells, in essence we can destroy the cause responsible for treatment failure and disease recurrence, the major problems in current therapy against cancer treatment (Vira et al., 2012).

2.9 MEDICINAL PLANTS AS ANTICANCER AGENTS

Natural product secondary metabolites of plant origin play a very important role in cancer chemotherapy (Cragg and Newman, 2005). Recent reports suggest that with the analysis of anticancer drugs available in the world of 140 compounds in total, a majority (54%) is either natural products (14%), natural product derivatives (26%) or compound made by total synthesis, but modeled on natural product leads (14%) (Patel et al., 2011). Therefore, there is a significant scientific interest in discovery of anticancer drugs from plant sources. Plants have a long history of use in the treatment of cancer. Hartwell, in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used in the treatment of cancer (Hartwell, 1971). It is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, especially from medicinal plants (Cragg and Newman, 2005). The search for anticancer agents from plant sources started in the 1960s with the initiative of U.S. National Cancer Institute (NCI) and led to the discovery and development of clinically used anticancer drugs such as vinblastine and vincristine (isolated from the Madagascar periwinkle, Catharanthus roseus), cytotoxic podophyllotoxins, etoposide and teniposide (from the American mandrake or Mayapple, Podophyllum peltatum), paclitaxel (from the bark of the Pacific Yew, Taxus brevifoli), Camptothecin (from the Chinese ornamental tree, Camptotheca acuminata), Homoharringtonine (from the Chinese tree, Cephalotaxus harringtonia) and Elliptinium, a derivative of ellipticine (from a Fijian medicinal plant Bleekeria vitensis). Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing extensive laboratory investigation. Plant-derived anticancer agents in clinical development are Rohitukine or Flavopiridol (from Dysoxylum Binectariferum), Combretastatins (from Combretum Cafruml), Roscovitine (from Raphanus Sativus), Maytansine (Maytenus Serrata) and Thapsigargin (from Thapsia Garganica). Similarly, Plant-derived anticancer agents in pre-clinical developments are Bruceantin (Brucea Antidysenterica), Betulinic Acid (Betula Spp), Lapachol (Tabebuia genus), Indirubins (Indigofera Tinctoria)
Cyclopamine (*Veratrum Californicum*) *Pervilleines* *Erythroxylum Pervillei*), Silvestrol (*Aglaila sylvestre*), Triterpenoid Acids (Oleanolic And Ursolic Acid) etc. (Koul et al., 2011). In many instances, the actual compound isolated from the plant may not serve as the drug, but leads to the development of potential novel agents.

### 2.10 SESQUITERPENE LACTONES

Sesquiterpene lactones are a large and diverse group of natural compounds present in the plants especially compositae family (Heinrich et al., 1998). They are 15-carbon terpenoids consisting of three isoprene units and each isoprene unit have five carbon atoms and are oxygenated derivatives in the form of α, β and γ lactones. All sesquiterpene lactones contain α-methylene-γ-lactone ring either *cis*- or *trans*- fused to the C6-C7 or C8-C7 position of the carboxylic skeleton. Pharmacological activity described for sesquiterpene lactones mainly include anti-microbial, anti-viral, anti-inflammatory and anti-tumor (Zhang et al., 2005). In recent years, the anticancer potential of sesquiterpene lactones has attracted a great deal of interest. A recent study reveals that the covalent binding of sesquiterpene lactones to free sulfhydryl group in proteins leads to the disruption of the functions of various macromolecules. As a result, sesquiterpene lactones may interfere with key biological processes, such as cell signaling, cell proliferation, cell death or apoptosis and mitochondrial respiration, all of which constituting the molecular basis for their anticancer properties. It was also found that sesquiterpene lactones have the ability to inhibit some key enzymes (aromatase) catalyzing the hormonal synthesis (estrogen) (Zhang et al., 2005). A recent study suggests that, sesquiterpene lactone parthenolide (isolated from *Tanacetum parthenium*) induce apoptosis in primary human AML cells and was shown to specifically target the AML progenitor and leukemia stem cell populations while sparing normal hematopoietic cells (Guzman et al., 2005). Similarly, parthenolide also preferentially inhibits breast cancer stem cells (Zhou et al., 2008), arrest cell cycle progression at the G2/M checkpoint, especially at low concentrations in an invasive sarcomatoid hepatocellular carcinoma cell line (SH-J1) (Wen et al., 2002). Cynaropicrin (isolated from *Luzea cathamoites*) has been reported to induce cell cycle arrest at G1/S phase in various leukocyte cancer cell lines in addition to G2/M arrest (Cho et al., 2004). It has been reported that helenalin (isolated from *arnica* spp.) and costunolide (isolated from *Sassurea* spp.) are strong apoptosis inducers in the range of micromolar concentrations to cancer cells (Dirsch et al., 2000). Arteminolide C (isolated from *Artemisia* spp.) was found to be effective in inhibiting tumor cell growth in
a dose-dependent manner using a nude mice xenograft model (human lung and colon tumor cells) (Zhan et al., 2011). Sesquiterpene lactone, eupatoriopicrin and arctiin (isolated from *Eupatorium* and *Sassurea* spp.) are reported to have anticancer activity in animal models (Molinillo et al., 2011). The studies also suggest that, sesquiterpene lactones isolated from the plant *Inula Britannica, Viguiera Sylvatica* (Millerenolide and Thieleanin) *Centipeda min* (6-O-Angeloylenolin), *Inula viscosa* (Tomentosin and Inuviscolide) has the ability to induce apoptosis in various cancer cell lines in vitro. Accumulating evidence from both in vitro cell studies and in vivo animal cancer models has demonstrated the potent anti-cancer activity of sesquiterpene lactones. With increasing knowledge of the molecular mechanisms responsible for their anti-cancer activity, it is understood that sesquiterpene lactones are promising candidates for the development of anti-cancer drugs (Kreuger et al., 2012).

2.11 *TINOSOPRA CORDIFOLIA* (WILD.) HOOK. F. & THOMAS

*Tinospora cordifolia* is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae (Krishna et al., 2009). It is distributed throughout the tropical Indian subcontinent and China, ascending to an altitude of 300 m. The stem of *Tinospora cordifolia* is rather succulent with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey, deeply left spirally, the space in between being spotted with large rosette like lenticels. The leaves are membranous and cordate. The flowers are small and yellow or greenish yellow (Upadhyay et al., 2010). *Tinospora cordifolia* is an important medicinal plant cultivated throughout the Indian subcontinent (Fig. 2.5). Through centuries, it has been used for treating various ailments including cancer in Ayurvedic system of medicine (Thippeswamy and Salimath, 2007).

*Tinospora cordifolia* stem is bitter, stomachic, diuretic, stimulates bile secretion, enriches the blood and cures jaundice (Panchabhai et al., 2008). The extract of its stem is useful in skin diseases (Singh et al., 2003). Dry barks of *Tinospora cordifolia* has anti-spasmodic, antipyretic, anti-allergic, anti-inflammatory, anti-leprotic properties (Singla, 2010) and widely used in the treatment of diabetes mellitus (Joladarashi et al., 2012). Administration of either alcoholic or aqueous extract of *Tinospora cordifolia* decreases the blood glucose level and increases glucose tolerance in rodents (Moqbel et al., 2012). It is reported to benefit the immune system in a variety of ways (Aranha et al., 2011). The alcoholic and aqueous extracts of *Tinospora cordifolia* have been tested successfully for immuno-modulatory, (Sharma et al., 2012) anti-inflammatory and antioxidant activities
(Upadhyay et al., 2010, Pushp et al., 2011). *Tinospora cordifolia* also possess antitumor activity (Panchabhai et al., 2008). The antineoplastic activity has been evaluated in cultured HeLa cells and in Ehrlich ascites carcinoma bearing mice (Jagetia and Rao, 2006). Similarly, anti-angiogenic activity was demonstrated in angiogenesis-induced animals by studying the effect on the cytokine profile (Leyon and Kuttan, 2004a). The antimetastasis effect of a polysaccharide from *Tinospora cordifolia* was studied (Leyon and Kuttan, 2004b). Chemical constituents isolated from the stem bark of the plant are alkaloids, glycosides, sesquiterpenoids, lactones and steroids (Gupta et al., 2009).

### 2.12 WITHANIA SOMNIFERA DUNAL

*Withania somnifera* Dunal of the family Solanaceae is commonly known as ‘Ashwagandha’. *Withania somnifera* holds a position of importance similar to ginseng in China, and is an evergreen shrub, grown wild and also cultivated for medicinal use in many parts of India (Kulkarni and Dhir, 2008). It grows as a short shrub (35–75 cm) with a central stem from which branches extend radially in a star pattern (stellate) and covered with a dense matte of wooly hairs (tomentose). The flowers are small and green, while the ripe fruit is orange-red and has milk-coagulating properties. The roots of the plant are long, brown and tuberous (Joshi et al., 2010) (Fig. 2.6). It has anti-inflammatory, antistress, radiosensitizer, effective against Alzheimer's disease, antioxidant, immunomodulatory and hemopoetic properties (Tripathi et al., 2011). It is often prescribed during convalescence, for weakness and emaciation in children and the elderly and for a wide range of problems associated with old age, such as loss of energy, lack of muscular strength, poor memory, weak eyes, rheumatism, insomnia and the plant has a rejuvenating effect on the body (Uddin et al., 2012).

*Withania somnifera* has been widely regarded as the Indian Ginseng and used as an Ayurvedic medicine to promote health and longevity in India for a long time. Its efficacy in many ailments has been confirmed by various *in vitro* and *in vivo* pharmacological experiments (Uddin et al., 2012). It is known to be biologically active and exhibit anti-tumour, immunopotentiating and anti-metastatic activity (Leyon and Kuttan, 2004a). *Withania somnifera* root extract prevents DMBA-Induced squamous cell carcinoma of the skin in swiss albino mice (Prakash et al., 2002). The root of *Withania somnifera* also inhibits forestomach and skin carcinogenesis in mice (Padmavathi et al., 2005). Effect of root extracts was evaluated for the cell cycle and angiogenesis against human laryngeal
carcinoma (Mathur et al., 2006). The biologically active chemical constituents are alkaloids, steroidal lactones, saponins and withanolides (Bharti et al., 2011).

2.13 STRATEGY EMPLOYED IN THE ISOLATION OF PHYTOCONSTITUENTS WITH ANTICANCER ACTIVITY

In recent years, there has been growing interest in alternative system of medicine, especially the therapeutic use of medicinal plants. This particular interest towards medicinal plants may be due to the limitation associated with conventional medicine that they are relatively inefficient, have side effects and impart toxicity to the normal cells (Kaur et al., 2011). Moreover, the ecological awareness also suggests that ‘natural products’ are harmless. Natural compounds can be lead compounds for the development of new drugs, biomimetic synthesis development and the discovery of new therapeutic agents (Rates, 2001). Thus, it is now certain that plants are the most vital source of several compounds which possess significant therapeutic values for treatment of cancer. Different steps involved in the search of new anticancer agents from traditional medicinal plants are as follows;

2.13.1 Selection of medicinal plants

Selection of the plant can be based on the use of plants in traditional medicine in different cultures (also called ethnopharmacology review); also it is based on chemical composition uses phylogenetic or chemotaxonomic information (as certain genera and families contain compounds from a defined chemical class with known pharmacological activity). Another method of selecting a plant is that the investigator decides on a well-defined pharmacological activity and performs a randomized search, resulting in active species to be considered for further study (Sarker et al., 2006).

2.13.2 Preparation and identification of the plant material

It is important that plant collection and identification involves a professional botanist who is able to correctly identify the plant species and prepare part of the material for herbarium preservation in order to have a reference material (also called ‘voucher specimen’). Stabilization is the next step in which the plant materials are dried at ambient temperature in a shady place. The dried or stabilized plant material should then be powdered and subjected to a suitable extraction process (Sarker et al., 2006).
2.13.3 Preparation of total plant extracts using suitable solvents.

Extractions can be either “selective” or “total.” Selective extraction, typically performed when the chemical composition of the plant material is known based on chemotaxonomic information. Selective extraction is typically performed sequentially with solvents of increasing polarity. If the chemical composition is unknown a total extraction with ethanol or aqueous alcoholic mixture is employed in an attempt to extract as many compounds as possible. This is based on the ability of alcohol solvents to increase cell wall permeability, facilitating efficient extraction of large amounts of polar and medium-to-low polarity constituents (Sarker et al., 2006).

2.13.4 Screening for anticancer activity by in vitro cell culture based bioassays

The total extract first typically screened to determine the anticancer potential in cultured human cancer cells to obtain a general evaluation of anticancer activities. If the total extract is found to possess significant anticancer activity, then the further purification and isolation of the active plant extracts is carried out. Typically, the active plant extracts are sequentially fractionated with solvents of increasing polarity and each fraction and or pure compound being subjected to in vitro bioassay in cultured human cancer cells. This strategy is called bioactivity-guided fractionation or bio-assay guided isolation (Sarker and Nahar, 2012). Currently, natural product research is more emphasized on the bio-assay guided isolation (isolating the biologically active compounds) rather than trying to isolate all compounds present in any extract.

2.13.5 Fractionation

A crude natural product extract would be a cocktail of compounds. It is practically not easy to apply a single separation technique to isolate individual compounds from such a crude mixture. Therefore, the biologically active crude extract is initially separated into various discrete fractions containing compounds of similar polarities. Fractionation typically carried out by solvent partition in increasing order of solvent polarity. Commonly, non polar to medium polar to polar solvents are being used (Petroleum ether to dichloromethane to n-butyl alcohol).

2.13.6 Isolation and characterization of active compounds

The most active fraction typically subjected to classical or modern chromatography techniques. In order to purify the relatively non-polar compounds, a conventional open-column chromatography (CC) or preparative thin-layer chromatography (PTLC) or
modern chromatographic methods like preparative high-performance liquid chromatography (HPLC) or multi-flash chromatography were employed. If the target compound is polar in nature, reverse phase high-performance liquid chromatography (RP-HPLC) is most suitable (Kingston, 2011).

2.13.7 Structural decipher or elucidation

Structural decipher is considered as the end point in the identification of a bio-active compound which include conclusive structure elucidation of the isolated compound. If the target compound is known, it is often easy to compare preliminary spectroscopic data with literature data or with a standard sample. However, if the target compound is an unknown and complex natural product, a comprehensive and systematic approach involving a variety of spectroscopic techniques is required. Structure determination is typically done with ultraviolet spectroscopy (UV), infrared spectroscopy (IR), mass spectrometry (EI-MS, ESI-MS), NMR (One dimensional- 1HNNMR and 13CNMR; Two dimensional-COSY, 1H-1H, HMBC, 1H-13C, HMQC, 1H-13C and HSQC).

2.13.8 Chemical synthesis of analogues

Once the chemical structure of a bio-active (anticancer) compound is defined, total or partial synthesis can be carried out. Modulation of the biological activity and the definition of the structure-activity relationship can be established by preparation of derivatives or analogues (Jones et al., 2011).

2.13.9 Large scale isolation

After completing all these steps, large-scale isolation can be achieved by collection of such biologically active medicinal plants again. Also, partial or total synthesis can be established for the bio-active compounds.

2.13.10 Detailed pre-clinical, toxicological and clinical studies

The pre-clinical evaluation using suitable tumor model (in vitro and in vivo experiments) for molecular targets can be achieved. Similarly, clinical and toxicological trials can be established, aimed at future therapeutic use as a potential chemotherapeutic drug for the effective treatment of cancer. Fig. 2.7 shows the strategy used for the extraction and isolation of anticancer compounds from selected medicinal plants.
Figure 2.1 Hallmark properties of stem cells

Stem cells have two characteristic properties. First, ‘self renewal’ potential, by which they are capable of giving rise to a cell of its own kind and second, ‘multilineage differentiation’ potential, whereby a stem cell can give rise to all the other cell types of the particular tissue/organs via a pool of its progeny, the ‘progenitors’.

Figure 2.2 Stochastic and Hierarchy model of tumorigenesis

Stochastic model predicts that every cell within the tumor has the ability to give rise to a tumor (all red cells) and it is chance that determines which cell becomes tumorigenic. Whereas, Hierarchy model suggest that only few cells within the tumor have an inherent potential to give rise to the tumor (the red cells), while most of the cells cannot give rise to a tumor. The hierarchy model strongly supports the cancer stem cell hypothesis.
Figure 2.3 Functional heterogeneity within a tumor

Cancer stem cells have the ability to make colonies in a 3D matrix, like soft agar *in vitro* and initiate tumor formation in nude mice *in vivo*. Serial transplantation of tumor demonstrates the self renewal properties of cancer stem cells.
A. Failure of conventional therapy to target cancer stem cells. Conventional chemotherapy is effective in eliminating rapidly dividing cells. On account of their low numbers and their drug efflux property, cancer stem cells are less sensitive to these therapies. Hence while the bulk of cancer cells are killed, leading to ‘apparent’ cure of the cancer, the CSCs remain viable and active even after therapy and might contribute to re-growth of the entire tumor leading to recurrence of the cancer after a certain period.

B. Targeted chemotherapy against cancer stem cells. Therapies which can target both cancer stem cells and bulk would be more effective in eliminating cancer at the root. CSC specific drugs, which might target the drug efflux pumps and some of the CSC specific survival pathways, in combination with the conventional drugs that kill the bulk would be the ideal treatment regime for cancers. Thus targeted cancer stem cell therapy may leads to remission of the disease at the root.
Figure 2.5 *Tinospora cordifolia* (Wild.) Hook.F. & Thomas

*Tinospora cordifolia* is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. It is distributed throughout tropical Indian subcontinent, ascending to an altitude of 300 m. 

A. The leaves are membranous and cordate. B. The stem of *Tinospora cordifolia* is rather succulent with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey, deeply left spirally, the space in between being spotted with large rosette like lenticels.

Figure 2.6 *Withania somnifera* Dunal

*Withania somnifera* belongs to the family Solanaceae. The plant is commonly known as ‘Ashwagandha’ and is an evergreen shrub, grown wild and also cultivated for medicinal use in many parts of India. 

A. It grows as a short shrub (35–75 cm) with a central stem from which branches extend radially in a star pattern (stellate) and covered with a dense matte of wooly hairs (tomentose). The flowers are small and green, while the ripe fruit is orange-red and has milk-coagulating properties. B. The roots of the plant are long, brown and tuberous.
Figure 2.7 Bioactivity guided fractionation scheme

The strategy used for the extraction and isolation of anticancer compounds from the selected medicinal plants.
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