1.1. CANCER

Cancer is a broad term given to a group of diseases all involving unregulated growth of cells. Cancer originates in a normal cycle of cell division when a cell can turn cancerous and divide infinitely. Lymphatic system and bloodstream can also spread the cancer to distant parts of the body. Two types of genes are affected: Oncogenes and Tumor suppressor genes. Oncogenes are those genes that stimulate reproduction and growth of the cells. Tumor suppressor genes are those genes that obstruct the survival and division of the cells. Tumor formation can take place due to the inappropriate over-expression of normal oncogenes, formation of novel oncogenes, or because of under-expression or deactivating of tumor suppressor genes (figure 1.1). Characteristically, many genes are required to be altered in order to convert a normal cell into a cancerous one (Croce 2008).

Figure 1.1: Cancer Progression

(Source: https://en.wikipedia.org/wiki/File:Cis-Nats_and_cancer.jpg)
With over 10 million new cases per year worldwide, cancer remains a difficult disease to treat and a significant cause for morbidity and mortality. Conventional anti-cancer therapies come with a number of drawbacks and side effects. Conventional therapy for cancer includes treatment with cytotoxic drugs (e.g. doxorubicin, paclitaxel etc), radiation therapy and surgical removal of tumors. Cytotoxic drugs cause severe side effects because the mechanisms which they inhibit are common to both cancer cells and normal cells, such as ligation – relegation reaction during cell division. Thus tumor cells and normal cells both are killed by anti-cancer drugs. Bone marrow, gastrointestinal mucosa, hair follicles and gonads are among the tissues most sensitive to chemotherapy. Chemotherapeutic agents are also highly teratogenic (Nobili et al. 2006). Development of resistance is another major drawback of chemotherapy. Higher doses are required to kill resistant tumor cells which further worsen the problem of unwanted side effects. [http://www.taconic.com/wmspage.cfm?parm1=313]. Radiation therapy is successful only when tumor is localized and well defined. For surgical removal also, the tumour must be well confined. Surgery is also often not possible when the tumour is bounded by delicate and complex tissues, like brain.

The top 5 most common cancers are lung, breast, colorectal, prostate and stomach with 12.3%, 12.3%, 10.6%, 7.5% and 6.1% incidences of all cancer cases (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data).

1.2. MELANOMA

Melanin is the pigment which imparts color to skin and hair, and protects skin from the damage of ultraviolet radiations. Melanocytes are the specialized cells that are known to produce melanin. The cancerous growth of melanocytes is termed as Melanoma (Mandalà and Voit 2013). Skin cancer, including malignant melanoma and non-melanoma skin cancer, is the most common cancer in Caucasian population (Apalla et al. 2017). Melanoma is the most aggressive and deadly type of skin cancer. Though it accounts for only 4% of all skin cancers, but it causes the highest number of skin cancer-related deaths worldwide. The increase in incidence of Primary Cutaneous Melanoma in Caucasian populations has been constantly rising and is reported to double up every 10–14 years (Mandalà and Voit 2013). The highest increase is reported in men above the age
of 55 years and women of all age groups (Wouters et al. 2017). The estimated number of new cases of melanoma in 2017 is 87,110, which accounts for 5.2% of all new cancer cases. Estimated deaths in 2017 are 9,730 [National Cancer Institute. SEER Stats Fact Sheets: Melanoma of the skin. http://seer.cancer.gov/statfacts/html/melan.html. Accessed on 25th January, 2018]. This number of melanoma cases is perhaps even higher than mentioned as the National Cancer Registries has reported that the incidences are underestimated in few countries (Monshi et al. 2016; Apalla et al. 2017). Moreover, after lung cancer and breast cancer, melanoma is the third most common reason of brain metastases. Melanoma spreads to the brain in upto 75 % of melanoma patients. Brain metastases cause death in 95 % of the cases (Nicholas et al. 2013).

Till date, only four drugs have been approved for the treatment of melanoma, which are, dacarbazine, interleukin-2, ipilimumab and vemurafenib. Continuous efforts have been made to develop new drugs and new treatment strategies, but not many of them have given much hope. Dacarbazine was the first drug which was approved for melanoma treatment in 1975, and it still remains the gold standard chemotherapeutic drug against melanoma; although ipilimumab and vemurafenib have shown promising results in last few years. Recent experiments and results present the combinatorial approach as a promising one for treatment of aggressive melanomas, and so combining dacarbazine or other chemotherapeutic drugs with new agents seems to hold the potential that scientists have been looking for since decades (Velho 2012; Lu et al. 2016; Mor and Heindl 2017).

Though the better understanding of pathophysiology of melanoma has led to the development of new drugs which target specific pathways such as MAPK (mitogen-activated protein kinase) pathway, there success is limited due to the resistance of melanoma cells and higher toxicities of these drugs (Menzies and Long 2014).

1.2.1. **PREVALENCE AND EPIDEMIOLOGY**

The occurrence of melanoma has been growing among all age groups; more than 600% increase is reported in young adults between 1970 and 2009. In United States of America, melanoma is reportedly the sixth most common cancer in men and women, and the second most common cancer in women having age between 20 and 29 years
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(http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/dermatology/cutaneous-malignant-melanoma/). At current rates, 1 in 27 white men and 1 in 42 white women are expected to develop invasive melanoma in their lifetime (https://www.aad.org/media/stats/conditions). In United Kingdom (UK) also, the number of incidences are on rise (www.cancerresearchuk.org). Also, it is found to be most prevalent in age group 65-69 years (figure 1.2).

Figure 1.2: Melanoma Incidences in UK

(Source: www.cancerresearchuk.org)

Most melanomas arise in previously normal skin, and only 20% to 30% arise from pre-existing nevi. Although there are several genetic syndromes that predispose individuals to melanoma, these account for less than 15% of melanomas. These syndromes include xeroderma pigmentosa and familial atypical mole-melanoma syndrome (called dysplastic nevus syndrome) (https://www.skincancer.org/skin-cancer-information/melanoma).

In the evidence of epidemiologic studies, it is shown that solar radiation exposure is the main cause of cutaneous melanoma (Wagner et al. 2000). This relationship is further
sustained by anatomical differences in gender, race, latitude of residence, and migration. In men, the most common site of melanoma occurrence is upper back; in women, the most common sites include upper back and lower parts of legs. According to studies, people who have migrated to countries having more of ambient solar radiations have higher risk of developing melanoma than people who did not migrate. Similarly, melanoma frequency and mortality rate in whites are inversely related to distance from the equator. Differences in race are also found to exist. The lesser incidences of melanoma in highly pigmented people is a result of the protective function of melanin and smaller number of nevi (Sober et al. 2001; Matthews et al. 2017).

1.2.2. CAUSES AND RISK FACTORS

The core risk factors of melanoma are phenotype (blue eyes, fair complexion, and blond or red hair), cutaneous response towards exposure to sun (freckling, tanning inability, sunburn sensitivity), blistering sunburn history, strong recurrent sun exposure, genetic susceptibility, subtypes and amount of nevi (atypical nevi or giant melanocytic nevi), immunosuppression and history of melanoma. According to genetic investigations, 50% of familial melanomas and 25% of sporadic melanomas may be a result of mutations in the tumor suppressor protein p16. Chromosome 9p21 has been identified as the familial melanoma gene in linkage studies. Familial melanoma accounts for 8% to 12% of all melanoma cases. The inheritance mode is supposed to be polygenic. The cumulative risk of developing cutaneous melanoma in persons with a history of familial melanoma is estimated to be 50% by age 50.

1.2.3. PATHOPHYSIOLOGY

c-Kit: c-Kit is a type III transmembrane receptor tyrosine kinase. Its ligand binding causes autophosphorylation and activation of several signaling pathways, thus mediates growth, proliferation, and metastasis of cancer cells, and inhibits apoptosis (Carvajal et al. 2011). The pathways activated include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)-AKT1, and JAK-STAT signaling pathways. c-Kit mutations are reported in 39% mucosal, 36% acral, and 28% chronic sun-damaged melanomas (Curtin et al. 2006).
**RAS:** RAS signaling cascade is known to promote proliferation, survival, and invasion through two pathways, MAPK pathway and PI3K pathway. NRAS was the first component found to be activated in this pathway, and is mutated in 15–20% of all melanomas (Nikolaou et al. 2012).

**RAF:** BRAF is the most commonly mutated oncogene in melanoma (60% of all melanomas). Most prevalent mutation in BRAF is BRAFV600E which leads to activation of down-stream protein kinases (MEK and ERK) and increased proliferation of melanoma cells (Nikolaou et al. 2012).

Mutations in the gene CDKN2A within the 9p21 region have been demonstrated in familial melanomas. The CDKN2A gene is a complex gene which codes for p16 and p14arf proteins. Function of both these genes is to suppress the cellular growth. Intact p16 inhibits cyclin-dependent kinases, a critical class of enzymes whose function is to promote cellular proliferation by inhibiting the retinoblastoma protein. Therefore, intact p16 is essential to arrest the cell cycle. The p14(ARF) protein may be important in enhancing the effect of another tumor suppressor, p53 (Tsao 2000)

The MAPK and PI3K-AKT molecular signaling pathways hold critical importance in melanoma as they promote survival and proliferation of cells. Thus, discovery of abnormal proteins in these pathways has led to the development of targeted therapies for the melanoma treatment. Almost 50% of all sporadic melanoma have mutations in the BRAF gene, including a common point mutation encoding for an altered protein, BRAFV600E. The MEK gene product is one step down the pathway from BRAF and may get altered in cancer cells that are resistant towards BRAF inhibitors.

### 1.2.4. ANGIogenesis IN MELANOMA

Angiogenesis is the process of formation of new vasculature from existing blood vessels. Angiogenesis is an essential feature of all the tumors as this process provides for the ample of nutrients and oxygen, as well as helps in metastasis of tumor cells to distant tissues. Angiogenesis is found to be particularly an aggressive process in human melanomas. The pro-angiogenic ligands and their receptors and mediators are overexpressed in melanoma cells which aids in the progression and prognosis of the
disease (Danielsen and Rofstad 1998). Extensive angiogenesis of cutaneous melanomas dramatically increases the chances of melanoma related mortality. Thus, as a consequence of these facts, it is expected that inhibiting the rapid angiogenesis process will result in improved therapeutic outcomes of melanoma treatment, such as chemotherapy and radiation therapy. This has also been supported by the positive results of recently published studies. Also, inhibiting angiogenesis can be a good strategy as this process is not generally required by the normal cells of adult human beings. Therefore, one can expect to achieve a targeted therapy with minimal side effects (Mahabeleshwar and Byzova 2007). Angiogenic growth factors VEGF (Vascular Endothelial Growth Factor) and bFGF (basic fibroblast growth factor) are known to be produced in high amounts by the cancerous melanocytes. These growth factors play the key role in promoting angiogenesis in melanomas (Motl 2005; Gille 2006). Thus, turning down the production of VEGF and bFGF will result in inhibition of angiogenesis. VEGF inhibitors when given in combination with chemotherapeutic drugs are most likely to result in a clinical benefit, as while the VEGF inhibitors down regulate the process of angiogenesis, chemotherapeutic agent will be able to kill the tumor cells more effectively. Several such combinations and angiogenesis inhibitors alone are already in clinical trials to assess their effects on the therapeutic outcomes (Ribatti et al. 2010). Results of the studies performed so far have shown that angiogenesis is an appropriate process to target in order to achieve better melanoma treatment. These results therefore give a strong rationale to include an angiogenesis inhibitor in anti-melanoma treatment strategies (Ahmad Zaki et al. 2012).

1.2.5. RESISTANCE OF MELANOMA CELLS

Chemotherapy, though still being the mainstay of the treatment of melanoma and of most of the other cancers too, is now facing more and more cases of failure if used alone. Most of the chemotherapeutic agents kill the cancer cells by inducing apoptosis which is the programmed cell death of the cancer cells. But now, treatment with these agents usually results in inefficient and incomplete outcomes. This inefficiency of chemotherapeutic drugs is most commonly attributed to the intrinsic and/or extrinsic resistance of melanoma cells to systemic treatment with these agents. Human melanomas are known to have fewer pro-apoptotic molecules and altered apoptotic pathways; and are thus
characteristically resistant to anti-cancer drugs. This leads to a need to dive into the mechanisms of apoptosis resistance in order to better understand the resistance of the melanoma cells towards apoptosis inducing drugs (Helmbach et al. 2001).

Survivin is one of the several important proteins, genes and gene products which are known to impart resistance to the cancer cells. Survivin is overexpressed in a number of cancers, such as lung cancer, colon cancer etc. It acts as a regulator of both cell division and cell death. Survivin is found to be upregulated during melanocyte transformation and thus is predominantly overexpressed in melanoma cells which is mainly responsible for their resistance towards apoptosis. Survivin belongs to the IAP (inhibitor of apoptosis) family. The members of this family mainly exert their effect by inhibiting caspases directly. It has also been reported that in addition to resistance against chemotherapy, upregulation of survivin also leads to decreased survival rate and increased relapse; and suppression of survivin induces spontaneous apoptosis in melanoma cells (Yamanaka et al. 2011). Directing anti-sense oligonucleotides against survivin has been found to induce spontaneous apoptosis in melanoma in vitro models. These results suggest that targeting and inhibiting this molecule may result in overcoming of resistance and thus better control and better treatment outcomes in melanoma patients (Grossman et al. 1999; Helmbach et al. 2001; McKenzie et al. 2010).

1.2.6. TYPES OF MELANOMA

Melanomas are distinguished into subtypes based on their patterns of clinical and pathologic growth:

**Lentigo Maligna and Lentigo Maligna Melanoma**

Lentigo maligna starts as an irregular tan macule that spreads peripherally, developing multiple shades of tan and brown throughout. It occurs on sun-damaged skin in elderly fair-skinned persons. Lentigo maligna is rapidly increasing in incidence, and represents the most common form of melanoma in certain geographic areas. The lesion may progress slowly for up to 15 years in the in situ form before becoming invasive. The percentage of lentigo maligna lesions that reach invasive lentigo maligna stage is estimated to be less than 30%. Though lentigo maligna possess an extended radial growth
phase, it can be deadly when it becomes invasive. Cumulative long term sun exposure is more risky than recurrent exposure in terms of chances of lentigo malign occurence. Although lentigo maligna is becoming more common, lentigo maligna melanoma remains relatively uncommon and accounts for only 4% to 15% of all melanoma cases.

**Superficial Spreading Melanoma**

Superficial spreading melanoma is the most common subtype, constituting 70% of all melanomas fair-skinned and young people. It mostly appears in recurrently sun exposed areas having the highest density of nevus, such as upper back in men and women, and lower parts of legs in women. It is first noted as a flat or slightly raised irregularly colored patch with an asymmetric border, and it can arise in a preexisting nevus. The patch slowly expands peripherally and changes colors, revealing shades of tan, brown, blue, black, red, pink, or white over several years before become invasive. Absence of pigmentation within a superficial spreading melanoma often represents regression of the melanoma.

**Nodular Melanoma**

The second most common of all melanomas is nodular melanoma; it is the fastest growing and most aggressive melanoma. Nodular melanoma represents 10% to 15% of all melanomas. The nodular melanoma expresses as a uniform blue-black, blue-red, or pink-red nodule. Around 5% of all nodular melanomas do not have pigment (called amelanotic melanoma). The most common sites of occurrence are neck, head, and trunk. Nodular melanoma more commonly initiate in normal skin instead of preexisting lesions. Nodular melanoma is usually invasive at the time of diagnosis.

**Acral Lentiginous Melanoma**

Acral lentiginous melanoma is the least common subtype in whites but is most common in African Americans, Hispanics, Japanese, and Native Americans. Unlike lentigo maligna melanoma, acral lentiginous melanoma does not appear to develop due to sun exposure. The median age of manifestation is 65, equally in men and women. Although these melanomas spread peripherally before invading deeper, there is often a delay in
diagnosis because of the uncommon nature, with large, deep tumors at the time of presentation. Acral lentiginous melanoma usually appears as a black or brown spot of discoloration on palms or soles or under nails. Feet are the most common site in African Americans; 60% patients have subungual or plantar lesions. Sole is the most common site in all races.

**Subungal Melanoma**

Subungual melanoma is a subtype of acral lentiginous melanoma. Subungual melanomas mostly include thumb or toe and usually ascend from the matrix of nail. The Hutchinson sign, the appearance of pigmentation on the proximal nail fold as well as on the nail plate, is associated with advanced subungual melanoma.

**Mucosal Melanoma**

Mucosal melanoma is another uncommon variant of melanoma, usually developing in the mucosal tissues of the neck and head (oral and nasal cavities) and the anorectal or genital mucosa. Patients can present with bleeding or a mass lesion. Melanoma can also present in the eye, associated with the retinal pigment epithelium.

**Desmoplastic Melanoma**

Desmoplastic melanoma is a rare type. It is very aggressive having a high rate of recurrence locally. Most usually, it develops skin of the head and neck which is exposed to sun in older people. Desmoplastic melanoma is more prevalent in male then female (ratio of approx. 2 to 1). About half of this subtype develops along with lentigo maligna. Desmoplastic melanoma may clinically appear as a pigmented or skin-colored macule, papule, or nodule. Desmoplastic melanoma often invades perineurally and therefore is often symptomatic, with tingling or pain. In most cases, desmoplastic melanoma is deeply invasive (at least 5 to 6 mm thick) at the time of diagnosis.

1.2.7. SURFACE RECEPTORS IN MELANOMA CELLS

Uptake of drugs into cells after being bound to a targeting carrier is usually limited to receptor-mediated endocytosis. This receptor-mediated endocytosis could be exploited for developing site-specific and target-oriented drug delivery systems. Melanoma cells overexpress following receptors, which can be approached for targeting:

**CD44 Receptors**

CD44 or hyaluronan receptor is a transmembrane receptor associated with aggressive tumour growth, proliferation, and metastasis. Activation of CD44 receptors by a suitable ligand (such as Hyaluronic acid) is believed to increase the release of bFGF and transforming growth factor β, (TGF-β1) in melanoma cells (Negi et al. 2012). CD44 receptors have been found to be overexpressed in several melanoma cell lines such as B10-F16 and SK-MEL 28 (Mummert et al. 2003). A ligand having specific affinity for the CD44 receptors can be attached to the surface of the nanoformulations and it will take and focus most of the formulation to the melanoma cells avoiding binding to the healthy tissues. This makes CD44 receptors a suitable target to focus the nanoconstructs directly to the cancer cells, sparing the normal tissues, thus preventing the unwanted toxic side effects.

**P2X7 Receptors**

Purinergic P2X receptors are proteins of plasma membrane which are present in extensive variety of mammalian cells. There they perform as cellular sensor and enable the cells to detect and respond to extracellular adenosine triphosphate (ATP) which is an important molecule of signaling. Numerous studies and preclinical programs have shown that P2X receptors are druggable targets and selective receptor antagonism can be used as a promising approach of therapeutics. Accumulating evidence indicates that expression of P2X7 receptors have important role in proliferation, invasion and migration of the cancer cells (Jiang 2012). P2X7 receptors have been found to be overexpressed in melanoma cells (Seki et al. 2012).

**Adenosine Receptors**

Adenosine is a purine nucleoside which comprises of adenine which is attached to ribose molecule through glycosidic linkage (Görłach 2005). The extracellular adenosine exhibits its effects by four known Adenosine Receptors (AR) subtypes, namely the A1, A2A,
A2B and A3AR. All these adenosine receptors are cell surface G protein-coupled receptors and have diverse tissue-specific distributions. According to studies, activation of adenosine receptors exhibit both pro- and anti-tumorigenic effects in melanoma (Fredholm et al. 2001).

1.2.8. TREATMENT MODALITIES

The various treatment modalities used for treatment of melanoma are summarized in the following sections (figure 1.3 and 1.4).

**Figure 1.3:** Melanoma Treatment Modalities

**Figure 1.4:** Mechanism of action of different anti-melanoma agents
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Dacarbazine

Dacarbazine, also known as DTIC is an alkylating chemotherapeutic agent. Dacarbazine was approved by FDA in 1975 for the treatment of melanoma and is the only FDA approved anti melanoma chemotherapeutic drug till date. Several studies have been performed on the efficacy of dacarbazine as anti-melanoma agent, and most of them have revealed that dacarbazine, as a single agent, offers poor overall survival benefit (BHATIA et al. 2009). Serrone et al. reported median response duration of 5-6 months; objective response rate of 20%; and complete response rate of only 5% (Serrone et al. 2000). Middleton et al. reported median response duration of 6.4 months (Middleton et al. 2000) and Lui et al. reported objective response rate of 15.3% (Lui et al. 2007). Positive responses, if any, are not durable and less than 2% patients stay alive till six years after the treatment (Hill et al. 1984).

But, despite its modest efficacy, dacarbazine still continues to be the standard treatment for metastatic melanoma and forms the basis of majority of the anti-melanoma combinations. Out of the several therapies that have been tried against melanoma till date, no one has shown significant benefit over dacarbazine (BHATIA et al. 2009). Also, in a study performed on a small group of patients with metastases to the lungs, good performance status, normal blood lactate dehydrogenase enzyme levels, and long-term disease control with a good quality of life were observed after treatment with dacarbazine (Coates and Segelov 1994).

Temozolamide

Temozolamide is an analog of dacarbazine which can be administered orally. It has been extensively studied as anti-melanoma drug and as a substitute of dacarbazine for its convenience of oral intake. Like dacarbazine, it also gets converted to the active alkylating metabolite MTIC (3-methyl-[triazen-1-yl]-imidazole-4-carboxamide); though unlike dacarbazine, this conversion takes place spontaneously at physiological pH in all tissues which is the reason why this drug has good oral bioavailability. Also, temozolamide can penetrate into CNS and thus has the potential to prevent and treat melanoma brain metastasis (BHATIA et al. 2009; Velho 2012).
Middleton et al. performed a phase III trial randomized study and studied the response of temozolomide given at dose of 200 mg/m$^2$/d orally for 5 days every 4 weeks; vs dacarbazine given at dose of 250 mg/ m2/d intravenously for 5 days every 3 weeks. The effect of temozolamide was found to be statistically similar to that of dacarbazine. The median overall survival after temozolamide and dacarbazine treatment was 7.7 months and 6.4 months respectively. Overall response rate was 14% and 12% respectively for temozolamide and dacarbazine (Middleton et al. 2000).

Since temozolamide failed to show any therapeutic advantage over dacarbazine, the choice between the two drugs is made on the basis of preferred route of administration, cost factor, availability, and presence or absence of brain metastasis (Bhatia et al. 2009).

**Immunotherapy**

Melanoma cells exhibit intrinsic resistance against chemotherapy through several mechanisms (Helmbach et al. 2001), to circumvent which, researchers keep on looking for different therapeutic strategies. One such approach is potentiating the immune response against this highly immunogenic form of skin cancer (Di Franco et al. 2017). Immunotherapy improves the immune response of melanoma patients and increases the clearance of cancer cells damaged by chemotherapeutic or targeted drugs. Immunostimulators that are most commonly used in the treatment of melanoma are interleukin (IL)-2, interferon (IFN)-alpha, ipilimumab, and thymosin alpha 1 (Chen et al. 2013).

- **Ipilimumab**

Ipilimumab is a monoclonal antibody which blocks CTLA-4 receptors. CTLA-4 belongs to the immunoglobulin superfamily and is a transmembrane receptor overexpressed by T-lymphocytes (Franklin et al. 2017). It was approved by FDA as a second line treatment for melanoma in 2011 after Hodi et al. reported improved survival rates in ipilimumab treated melanoma patients, who also claimed that ipilimumab was the first agent to show a significant improvement in the survival rate in population with metastatic malignant melanoma (Hodi et al. 2010). A number of phase I/II/III studies have been conducted
with different doses of ipilimumab which have yielded higher response rate and median overall survival, also establishing a positive correlation with increasing dose of ipilimumab (Hodi et al. 2010; Robert et al. 2011).

The current approved dose of ipilimumab is 3 mg/kg every three weeks for a period of 12 weeks.

Another CTLA-4 blocker is Tremelimumab (ticilimumab), but it did not show any survival benefit over chemotherapy as revealed in a phase III trial (Franklin et al. 2017).

- **IFN-α**

IFNs are the cytokines which are released in response to the pathogens or in the presence of cancer cells. Consequent to the improved OS in the first clinical trial using high doses of IFN-α2b (Kirkwood et al. 1996), IFN-α was approved by US FDA in 1996 for the use in the adjuvant therapy for the patients that possess a high risk of reoccurrence after a melanoma resection (Di Franco et al. 2017). In another clinical trial comparing high doses to low doses of IFN, improved RFS was observed with no significant alteration in OS (Kirkwood et al. 2000). While another clinical trial comparing high doses of IFN with vaccines with the ganglioside GMK reported improved RFS as well as improved OS (Kirkwood et al. 2001).

- **Interleukin-2 (IL-2)**

IL-2 was approved by FDA in the year 1998 for the treatment of metastatic melanoma. It is a lymphokine which acts by stimulating T-cell proliferation and function (Velho 2012). Rate of complete response achieved by high dose of IL-2 is 6% while partial response rate is 10% (Atkins et al. 1999, 2000). Better results in terms of therapeutic responses have been achieved but they brought along higher rates of toxicities also; and low doses or subcutaneous administration gives poorer therapeutic responses as compared to high doses or iv administration (Keilholz et al. 1998). IL-2 has been tested in combination with chemotherapy and has shown some therapeutic benefits (table 1). Famotidine has been reported to lower the toxicity of IL-2 by enhancing the lymphokine-activated killer cell activity, while maintaining the anti-melanoma activity (Quan et al. 2012).
In melanoma, the above mentioned and many others immunocytokines have been known to be effective, but their use is limited due to the severe side effects (Chen et al. 2013).

**Topical Therapy (Imiquimod)**

Imiquimod is a topically applied agent which acts via activation of toll-like receptor 7 and 8 (TLR7/8). Activated TLR7 induces production of different cytokines such as IFN-α, IL-12 and TNF, thus activating the innate immune system (Aspord et al. 2014).

Imiquimod has been reported to be an effective and safe treatment for metastases of melanoma. Though it Doesn't cease progression, but it controls cutaneous metastases spreading from primary melanoma (Sisti et al. 2014). Moon and Spencer (Moon and Spencer 2013) have reported that topical imiquimod therapy completely cleared an invasive melanoma of 2.75 mm depth in situ (used after surgical excision of melanoma). Sue et al. (Sue et al. 2014) also reported no evidence of residual melanoma in situ when imiquimod was used after surgical excision. However, the efficiency of imiquimod remains unclear in the treatment of subcutaneous metastases, which, may even keep progressing despite complete treatment of superficial dermis (Turza et al. 2010).

**Targeted Therapy**

- **BRAF Inhibitors**

One of the causes of melanoma can be a mutation, and BRAF is found to be the most commonly mutated oncogene in melanoma, reportedly causing up to 60% of melanoma cancers. This induces the constitutive activation of the RAS-RAF-MEK-ERK signaling cascade (Mandalà and Voit 2013).

BRAF inhibitors are known to induce rapid regression of melanoma metastases, and Response Evaluation Criteria In Solid Tumors (RECIST) has reported a positive response in 50-60% patients (Menzies and Long 2014).

  - **Sorafenib**

Sorafenib is a multi-targeted tyrosine kinase inhibitor of Braf, Craf, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR),
p38, and ckit. It was the first Raf-inhibitor actively studied in melanoma patients (Mandalà and Voit 2013). Despite being studied in several Phase I/II/III trials, sorafenib has not yielded any promising results, neither as single agent, nor in combinations (Eisen et al. 2006; Mandalà and Voit 2013). Addition of sorafenib to carboplatin/paclitaxel was found to be of no benefit in terms of median progression free survival (PFS) or OS (Hauschild et al. 2009; Flaherty et al. 2010a).

- **Vemurafenib**

Vemurafenib is an orally active BRAF inhibitor. It is 30 times more selective towards mutated BRAF as compared to wild type BRAF. Vemurafenib has shown anti-proliferative activity in BRAFV600E melanoma cell lines, and it was the first BRAF inhibitor which completed phase I testing with significant clinical benefit (Flaherty et al. 2010b; Mandalà and Voit 2013). In the study, vemurafenib exhibited partial response and complete response too in the BRAF mutated melanoma patients, while no response was observed in the patients who did not carry mutated BRAF gene (Flaherty et al. 2010b). In further phase II study, vemurafenib monotherapy resulted in ORR of 53% and CR of 5%; although the side effects such as arthralgia, photosensitivity, fatigue, and alopecia were observed (Sosman et al. 2012). In a phase III trial, vemurafenib resulted in improved overall survival as compared to dacarbazine (84% vs 64%) (Chapman et al. 2011). This revelation of clinical superiority of vemurafenib over dacarbazine led to the approval of vemurafenib by FDA in 2011 and by Health Canada in 2012. The limitation of vemurafenib is the quick development of resistance by melanoma cells (Rajakulendran and Adam 2014).

- **Dabrafenib**

Dabrafenib is a highly potent BRAF inhibitor having 100 times greater selectivity for mutated BRAFV600E compared to wild type BRAF gene (Mandalà and Voit 2013). This agent was approved by FDA in 2013 (Rajakulendran and Adam 2014). In addition to objective responses in BRAFV600E mutant patients, dabrafenib has also shown significant clinical activity against brain metastasis (Falchook et al. 2012). Another randomized, controlled phase III trial in patients with BRAF V600E mutated metastatic
melanoma has claimed the efficacy of dabrafenib to be similar to vemurafenib in terms of PFS and response rates (Hauschild et al. 2012).

Major limitations in using BRAF inhibitors are the development of resistance in almost all the cases, and the toxicity associated with BRAF inhibition (Menzies and Long 2014).

- **MEK inhibitors**

There are strong evidences in favor of the concept that mutation of BRAF is associated with increased selectivity and sensitivity to inhibitors of MEK as well, which is also a gene in the same signaling cascade (Mandalà and Voit 2013).

  - **Trametinib**

Trametinib is an orally active selective inhibitor of MEK. In a phase III study comparing the efficacy of trametinib with chemotherapy (dacarbazine or paclitaxel), median progression free survival was found to be 4.8 months with trametinib, while it was only 1.5 months in the chemotherapy group (Gilmartin et al. 2011). In another study, the 6 months OS with trametinib was 81% as compared to 67% with chemotherapy (Flaherty et al. 2012). As a result of the promising outcomes of trametinib therapy, the drug was approved by FDA in 2013 for the treatment of metastatic melanoma which involve BRAFV600E or BRAFV600K mutations (Rajakulendran and Adam 2014).

**1.2.9. NANO TECHNOLOGY IN MELANOMA TREATMENT**

Just as the nanotechnology is invading every part of the biomedical sphere, treatment of melanoma is not untouched either. Nanotechnological tools enable the oncologists to specifically target the melanoma cells while offering several other advantages too, such as co-delivery of two or more drugs, dose reduction, etc. Several types of nanoparticles and nanovesicles have been explored for their applications in the treatment, either as drug carriers or as anti-melanoma agents themselves.

**Carbon Nanotubes**

Carbon nanotubes are continuous cylindrical form of one (single-wall) or more (multi-wall) layers of graphene (allotrope of carbon) with open or closed ends (Volder et al.
2013). Sobhani et al. (2017) studied the efficiency of PEGylated oxidized carbon nanotubes (O-CNT-PEG) in reducing the size of melanoma tumor after photothermal therapy. The average size of tumor in mice receiving O-CNT-PEG was greatly decreased in comparison to those which received laser therapy alone.

**Copper Nanoparticle**

Different experiments have studied the extent and mechanism of copper nanoparticles as anti-melanoma agent. Chakraborty and Basu (2017) have recently presented evidences of killing of A375 melanoma cells by copper nanoparticles due to lowering of cell membrane rigidity, DNA degradation, chromosomal condensation, cell cycle arrest in the G2/M phase, depolarization of mitochondrial membrane, and cellular apoptosis in caspase-9-mediated intrinsic pathway.

**Nanoliposomes**

Nanoliposomes offer several advantages such as easy fabrication and co-loading of different natured drugs. These and other advantages of liposomes are responsible for making them more popular in treatment of various diseases including cancer. Treatment of melanoma is no exception and liposomes are being extensively explored in this area.

**1.3. NANOLIPOSOMES**

Liposomes are vesicular, spherical colloidal particles which are composed of lipid bilayer encapsulating an aqueous core, as shown in figure 1.5. Liposomes are successful drug delivery systems in which hydrophilic drugs can be encapsulated in the aqueous core and the hydrophobic drugs can be dispersed in the bilayer of the lipid. They were discovered by Bangham and co-workers about 30 years ago. However, these liposomes were in micrometer range. Since then, liposomes have progressed commendably. In recent years, concept of nanoliposomes has been introduced where liposomes having size in nanometer range are used. Nanoliposomes, in addition to advantages of liposomes, also offer the advantages of nanoparticles. Thus, nanoliposomes are one of those very few drug delivery systems in which drugs of both the nature (hydrophilic and hydrophobic) can be
loaded in the same particle and yet the benefits of nanotechnology are also availed (Lasic and Papahadjopoulos 1995).

**Figure 1.5: Liposome**

### 1.3.1. ADVANTAGES OF NANOLIPOSOMES

Nanoliposomes possess a number of advantages, which are as follows:

- **Biodegradable and biocompatible:** Liposomes are completely biodegradable and biocompatible as they are made up of biodegradable and biocompatible lipids.

- **Non-immunogenic and non-toxic:** Liposomes usually do not trigger any immunogenic or toxic reaction in the body, that is, they are non-immunogenic and non-toxic.

- **Natural and easily available lipids:** Most of the lipids that are used for the formulation of the liposomes are natural and are easily available.

- **Drugs of different nature can be loaded:** Hydrophobic, hydrophilic, and as well as amphiphilic drugs can be loaded.

- **Co administration of drugs:** Combination of drugs having different nature (hydrophilic or hydrophobic) can be administered through same drug delivery system, i.e. liposomes.
• Protection of drug: Encapsulated drug remains protected from the external environment.

• Safety of sensitive and non-target tissues: Sensitive and non-target tissues are not exposed to the toxic drugs when drug loaded liposomes are administered in the body.

• Improved stability of drug: The drugs which are unstable or are prone to inactivation in certain conditions, such as acidic pH of stomach, remains protected and stable inside of the liposomes until they reach the target site.

• Targeting: Targeting of the potent and toxic drugs to the target tissues or cells is possible. This is done by attaching targeting ligands onto the surface onto the liposomes (active targeting), or through Enhanced Permeation Retention (EPR) effect (passive targeting).

• Reduction in dose of the drug: Since the drug is targeted to the specific site, wastage of drug at non target sites is significantly reduced. This results in the reduction in dose of the drug which is to be administered (Torchilin 2005).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Type</th>
<th>Number of Bilayer</th>
<th>Sub Types</th>
<th>Size range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Unilamellar Vesicles (ULV)</td>
<td>1</td>
<td>Small Unilamellar Vesicles (SUV)</td>
<td>20-40 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Medium Unilamellar Vesicles (MUV)</td>
<td>40-80 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Large Unilamellar Vesicles (LUV)</td>
<td>100-1000 nm</td>
</tr>
<tr>
<td>2.</td>
<td>Oligo Lamellar Vesicles (OLV)</td>
<td>2 – 10</td>
<td></td>
<td>100-1000 nm</td>
</tr>
<tr>
<td>3.</td>
<td>Multi Lamellar Vesicles (MLV)</td>
<td>More than 10</td>
<td></td>
<td>&gt;500 nm</td>
</tr>
</tbody>
</table>
1.3.2. STEALTH LIPOSOMES

Conventional liposomes can be modified also to serve other purposes, such as targeting, and to overcome the limitations of conventional liposomes, such as their early removal from blood circulation. Following are the common types of modified liposomes. The term “stealth” means cautious and surreptitious action or movement with a purpose to accomplish something which cannot be accomplished through usual route. Thus, liposomes are made stealth in order to increase their residence time in the blood circulation. Stealth liposomes are thus also known as long circulating liposomes. Conventional liposomes usually bind with opsonin proteins which are present in blood and are identified by cells of Reticulo Endothelial System (RES) as antigens (foreign bodies). Consequently, liposomes are uptaken by RES and are removed from the blood circulation. Conventional liposomes are made long circulating by coating their surface with poly ethylene glycol (PEG) or PEG derivatives, as shown in figure 1.6. This imparts hydrophilicity to the surface of liposomes, hence they escape their uptake by the cells of RES. Thus, clearance of liposomes from blood decreases and circulation time increases (Immordino et al., 2006).

![Figure 1.6: Stealth and Targeted Liposomes](https://commons.wikimedia.org/wiki/File:SolidLipidNanoparticle.jpg)
1.3.3. TARGETING LIPOSOMES

Liposomes can be modified to target a specific tissue or organ by attaching targeting ligands onto the surface (figure 1.6). Targeting ligands recognize and bind to the specific receptors that are present on the surface of target cells. These receptors are specifically present or are over-expressed on the surface of the target tissue or the diseased tissue. Thus the targeting liposomes having ligands on their surface recognize these target receptors and selectively bind to them, thereby sparing the other tissues of the body. This helps in reducing the unwanted effects of the toxic drugs and reducing the dose to be administered. For example, hyaluronic acid present on the liposomal surface binds to CD44 receptors which are over expressed in cancer cells, and folic acid can be attached to liposomes to make them preferably bind to folate receptors over expressed on diseased tissues (Forssen and Willis 1998).

1.3.4. METHODS OF PREPARATION

The classification of methods for liposomes preparation is given in figure 1.7.

![Figure 1.7: Methods of preparation of liposomes](image-url)
Chapter 1

Introduction

Solvent Dispersion

In the solvent dispersion method of liposome preparation (figure 1.7), the first step is same as that of lipid film hydration technique, that is, lipids are dissolved in a suitable organic solvent to make homogenous lipid solution. In the second step, the lipid solution is added to an excess of aqueous phase which contains the hydrophilic material(s) which are to be encapsulated in the liposomes. As a result of the interaction of organic phase and aqueous phase, the lipid molecules align themselves at the interface of the organic and aqueous phase and form a single layer. This monolayer of the phospholipids contributes to the formation of the bilayer of liposomes. Solvent dispersion technique is further classified into three methods, namely:

- Ether injection
- Ethanol injection

**Ether Injection**

In the ether injection technique, lipids are solubilized in diethyl ether or a mixture of ether and methanol. This lipid solution is injected into an aqueous solution containing the drug to be encapsulated. The addition is done using a mechanical drive at an extremely low rate, i.e., around 0.25 ml per minute. The addition has to be performed at reduced pressure and at a temperature above the boiling point of ether (55°C to 65°C). Ether is then removed under vacuum which leads to formation of liposomes.

**Advantages:**
- Ether injection method does not cause oxidative degradation of the lipids.

**Disadvantages:**
- Liposomal population formed is heterogeneous (size ranges between 70 to 200 nm).
- Compounds which are to be encapsulated are exposed to organic solvents at high temperature.
• **Ethanol injection**

Ethanol injection is another type of solvent injection method. In this method, the lipids are dissolved in ethanol to make a homogeneous solution of lipids. This ethanol solution of lipid is injected at a faster rate into a huge amount of aqueous media or the buffer under stirring which contains the drug or the compound that is to be encapsulated. As a result of the interaction of lipids in solution with the aqueous environment, the multi lamellar liposomes are formed.

**Advantages:**

- Ethanol injection method is very simple and convenient.
- The sensitive lipids are at a very low risk of degradation.
- Entrapment efficiency of the drug is comparatively higher.

**Disadvantages:**

- Dispersion of liposomes is very dilute as huge excess of aqueous media is used.
- The complete removal of ethanol is difficult.

(Akbarzadeh et al. 2013)

Figure 1.8 summarizes the steps involved in ether injection and ethanol injection method.

![Figure 1.8: Ethanol Injection and Ether Injection Method](https://www.slideshare.net/sadanand1/liposomes-17223820)
1.3.5. CHARACTERIZATION OF LIPOSOMES

**Determination of size**
Size of the liposomes is one of the most important parameters. It is the first parameter which should be determined as size of the liposomes dictates their fate in vivo, and also their mode and extent of action. There are various techniques which can be used for determining the average size and size distribution of the liposomes. To name a few of these techniques are electron microscopy, dynamic light scattering, and field-flow fractionation.

**Determination of Lamellarity**
In lamellarity determination, the number of lamellae or the lipid bilayer membranes present in the liposomes is determined. Lamellarity widely varies and depends on the type and nature of the constituting lipids and the method of preparation followed. Lamellarity is an important aspect to be analyzed as the number of lamellae has a significant effect on the encapsulation efficiency, and also on the kinetics of drug release. Number of lamellae also influences the fate of the liposomes in the body after administration.

Lamellarity of liposome is usually determined by measuring the change in visible or fluorescence signal when some lipids marker reagent is added. The results are obtained when the total signal is compared with the achieved signal after the reaction between the specified reagents and lipids marker.

**Zeta Potential**
Zeta potential is defined as a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles. Zeta potential is one of the vital parameters which are recognized to affect stability. It gives an estimate about the extent of the interaction between liposomes. High values indicate good stability of the liposome suspension, since all the liposomes having high negative or positive charge will tend to repel each other and thus no aggregation will take place. Conversely, if there is no or low charge, then flocculation will take place.

Zeta potential of liposomes is measured by using a laser which provides a light source illuminating the liposomes present within the sample cell. The incident laser beam then
passes through the centre of sample cell and then the scattered light is detected. When an electric field is applied, all the liposomes moving through the entire volume will cause fluctuation in the detected light. The frequency of the fluctuation will be proportional to the speed of the liposomes. This information is transferred to a digital signal processor and then to a computer which calculates the zeta potential.

**Encapsulation Efficiency**

The encapsulation efficiency of liposomes accounts for the amount of drug that is encapsulated in the liposomes. Determination of the encapsulation efficiency is important since it dictates the amount of the formulation that should be administered so as to deliver the therapeutic concentration of the drug.

To determine the true amount of encapsulated drug, the unencapsulated drug must first be separated from the drug loaded formulation. This separation can be accomplished by any of the various methods available. Quantification of the encapsulated material can be done by any suitable technique, including fluorescence spectroscopy, spectrophotometry, enzyme-based methods and electrochemical techniques.

**In-Vitro Drug Release**

*In vitro* drug release needs to be determined to estimate the amount of drug that will be released as a function of time inside the body. *In vitro* drug release studies are most commonly performed by using diffusion technique which involves the use of dialysis membrane.

**Stability of Liposomes**

The stability of the liposomes is one of the major parameters which need to be studied. Stability of liposomes influences their overall utility. Stability also plays important role in determining the synthesis process, administration, storage conditions and shelf life of liposomes. One of the most common stability issues that are faced by liposomes is the fusion of smaller liposomes to form bigger liposomes as bigger liposomes are more stable thermodynamically. But bigger liposomes do not serve many of the main purposes, such as targeting. Another problem is the leakage of the encapsulated drugs. Several drugs have the tendency to cross the lipid bilayer and reach the receptor compartment media. Therefore, size distribution and stability profile of the liposomes are important parameters to be studied (Vemuri and Rhodes 1995).
1.3.6. LIPOSOMAL PREPARATIONS IN MARKET

There are several liposome formulations which are already in market, and many more are in clinical trials. Marketed liposomal preparations are enlisted below in the table 1.2.

Table 1.2: Marketed Liposome Preparations

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Liposome Formulation</th>
<th>Drug</th>
<th>Route of Administration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ambisome</td>
<td>Amphotericin B</td>
<td>Intravenous infusion</td>
<td>Gilead Sciences, NeXstar Pharmaceuticals</td>
</tr>
<tr>
<td>2</td>
<td>DaunoXome</td>
<td>Daunorubicin</td>
<td>Intravenous infusion</td>
<td>Gilead Sciences, NeXstar Pharmaceuticals</td>
</tr>
<tr>
<td>3</td>
<td>Depocyt</td>
<td>Cytarabine</td>
<td>Intrathecal (intraventricular or lumbar puncture)</td>
<td>SkyePharma</td>
</tr>
<tr>
<td>4</td>
<td>Doxil/Caelyx</td>
<td>Doxorubicin</td>
<td>Intravenous infusion</td>
<td>Sequus Pharmaceuticals</td>
</tr>
<tr>
<td>5</td>
<td>Myocet</td>
<td>Doxorubicin</td>
<td>Intravenous infusion</td>
<td>Elan Pharma</td>
</tr>
</tbody>
</table>