3.1. HYPOTHESIS

The primary purpose of this hypothesis is to overcome the resistance of melanoma cells against apoptosis. So, an apoptosis inducing chemotherapeutic drug is to be given to which the cancer cells may respond after the resistance is overcome. Dacarbazine is an alkylating agent and is the only chemotherapeutic agent approved by FDA for the treatment of melanoma. In clinical trials, the response rate achieved by dacarbazine alone was 15.3%, with most of them being partial responses (Lui et al. 2007). Even though dacarbazine shows only modest efficacy, it still continues to be the standard treatment of metastatic melanoma and also forms the base of any combination of drugs given for melanoma treatment. Any other chemotherapeutic drug hasn’t shown any significant survival benefit over dacarbazine till date (Bhatia, Tykodi, and Thompson 2009).

Another hurdle in the successful treatment of melanoma is its inherent resistance due to the over expression of survivin protein which imparts resistance to melanoma cells towards apoptosis. Since chemotherapy is supposed to kill cancer cells by inducing apoptosis, over expression of survivin ultimately leads to failure of chemotherapy (Yamanaka et al. 2011). There are certain inhibitors of survivin that are being used recently, such as, YM 155 and PKF118-310 (https://www.scbt.com/scbt/browse/Survivin-Inhibitors/_/N-1c70fyt). In addition, natural agents are also explored. Vitamin E, ardisianone, quercetin, eugenol, and curcumin are to name a few (“Natural Therapeutics Targeting Survivin | OMICS International” n.d.).

Eugenol, the main constituent of clove oil which is a herbal product, has been proved to not to be carcinogenic or mutagenic. Also, FDA has listed eugenol as a GRAS (Generally Regarded as Safe) substance when consumed orally in unburned form (Al-Sharif, Remmal, and Aboussekhra 2013). In a number of studies, eugenol is found to exert anticancer action through various effects, such as antiproliferative action, apoptotic action, and anti-angiogenic action (Al-Sharif, Remmal, and Aboussekhra 2013; Manikandan et al. 2010; Pisano et al. 2007). Numerous studies have demonstrated the promising potential of eugenol in the treatment of melanoma (Pisano et al. 2007; Ghosh et al. 2005). Al-Sharif et al. experimented and reported that eugenol was found to be toxic against breast cancer cells. The killing of cancer cells was mainly achieved by inducing the
internal apoptotic pathway and strong down regulation of E2F1 and its downstream antiapoptosis target surviving (Al-Sharif, Remmal, and Aboussekhra 2013). These results indicate that eugenol, by inhibiting survivin, may lead to overcoming of inherent resistance of the melanoma cells thereby improving the chemotherapeutic outcomes.

CD44 receptors are commonly found to be overexpressed in several types of cancers. This makes targeting CD44 receptors a good approach in order to focus the formulation to the cancer cells avoiding the healthy cells of the body (Negi et al. 2015). This can be achieved by attaching hyaluronic acid to the surface of the liposomes which in addition to targeting will also lead to long systemic circulation of the liposomes by rendering their surface hydrophilic.

We therefore hypothesize to take a chemotherapeutic agent (Dacarbazine) as base drug, and wisely combine it with Eugenol that targets the other mechanisms of aggressive resistant melanoma cells. Also, as it is found from literature that survivin protein imparts a number of cancer types including melanoma with resistance, inhibiting survivin with eugenol will improve the outcomes of the therapy. This combination of drugs is to be loaded in surface functionalized liposomes and actively targeted to the cancer cells to further improve the efficacy of the formulation and reduce the unwanted toxic effects. Targeting can be done by attaching hyaluronic acid to the surface of the liposomes which specifically recognizes the CD44 receptors which are overexpressed in melanoma cells, and also make the particle surface hydrophilic as well as cationic which aids in long circulation of liposomes.

This experiment adds to the existing knowledge about the efficiency of combinations of chemotherapeutic drugs, which can be used against aggressive cancers such as melanoma; and helps in establishing the role of anti survivin and anti angiogenic agents as novel therapeutic approaches against aggressive resistant melanomas. This work will come up with a new effective combination of anti-cancer drugs, giving new hopes for the treatment of resistant melanoma.

Hypothesis is represented in figure 3.1.
3.2. AIMS AND OBJECTIVES

The objectives of this study are:

- To design and develop functionalized nanoliposomes for combinatorial approach against melanoma.
- To develop an effective nanofomulation for melanoma patients to overcome the resistance against chemotherapy and increase the overall response rate and survival rate.
- To reduce unwanted toxicity by actively targeting the liposomes to melanoma cells.
- To study the effect of combining an anti survivin agent with the chemotherapeutic agent on the therapeutic outcomes in treatment of resistant melanoma and to assess advantages of this combination over chemotherapy.
- To screen the role of eugenol in downregulating survivin protein and consequent enhancement in the therapeutic outcome of chemotherapy when combined with anti survivin agent.
This study thus aims to overcome the resistance of melanoma cells by developing a wise combination of drugs and achieve a higher response rate in melanoma model, which is usually not achieved with the existing treatment modalities.

### 3.3. RATIONALE OF THE STUDY

Based on the literature survey, the plan was to formulate liposomes, surface functionalized with HA, and co-loaded with Dacarbazine and Eugenol.

**Choice of Nanoformulation: Liposomes**

A number of liposomal formulations are available in the market such as Doxil\textsuperscript{®} (Doxorubicin), Fungizone\textsuperscript{®} (Amphotericin-B), Novasome\textsuperscript{®} (Smallpox vaccine) and Nyotran\textsuperscript{TM} (Nystatin). Liposomes are used as drug carriers in therapeutic areas such as tumor targeting, genetic vaccination, gene and antisense therapy, immunomodulation, cosmetics, and skin care products. Liposomes are entirely biodegradable, biocompatible, flexible, non-toxic, and nonimmunogenic. They are suitable for both systemic and non-systemic administrations. Liposomes possess a lipophilic environment and aqueous “milieu interne” both in one system and are thus suitable for delivery of hydrophobic, amphipathic and hydrophilic agents (Patel et al. 2012).

**Choice of Chemotherapeutic Drug: Dacarbazine**

Patients who are not eligible for surgical removal of tumor, chemotherapy remain a reasonable base line of treatment. Dacarbazine (5-[3,3-dimethyl-1-triazenyl]-imidazole-4-carboxamide, or DTIC) is an alkylating agent that kills cancer cells by intercalation in DNA thus inhibiting cell division. Dacarbazine is the only chemotherapeutic agent approved by the FDA for treatment of melanoma. The primary purpose of dacarbazine therapy for metastatic melanoma is palliation. Despite its modest efficacy, dacarbazine continues to be the “standard treatment” of metastatic melanoma. No other therapy has yet been shown to have a significant survival benefit over dacarbazine (Bhatia, Tykodi, and Thompson 2009; Velho 2012).
Choice of Anti-Survivin agent: Eugenol

Eugenol, the main constituent of clove oil which is a herbal product, has been proved to not to be carcinogenic or mutagenic. Also, FDA has listed eugenol as a GRAS (Generally Regarded as Safe) substance when consumed orally in unburned form (Al-Sharif, Remmal, and Aboussekhra 2013). In a number of studies, eugenol is found to exert anti-cancer action through various effects, such as antiproliferative action, apoptotic action, and anti-angiogenic action (Al-Sharif, Remmal, and Aboussekhra 2013; Manikandan et al. 2010; Pisano et al. 2007). Numerous studies have demonstrated the promising potential of eugenol in the treatment of melanoma (Ghosh et al. 2005; Pisano et al. 2007). Al-Sharif et al. (Al-Sharif, Remmal, and Aboussekhra 2013) experimented and reported that eugenol was found to be toxic against breast cancer cells. The killing of cancer cells was mainly achieved by inducing the internal apoptotic pathway and strong down regulation of E2F1 and its downstream antiapoptosis target survivin. These results indicate that eugenol, by inhibiting survivin, may lead to overcoming of inherent resistance of the melanoma cells thereby improving the chemotherapeutic outcomes.

Targeting is done because:

- Targeting helps to localize, sustain, target and have an isolated interaction of the drugs with the diseased tissues.
- Targeted delivery improves efficacy while reducing side-effects.

3.4. PLAN OF WORK

- Literature review
- Conceptualization of Hypothesis
- Selection and procurement of drug and drug carrier
- Preformulation Studies
  - Identification of Drugs
  - Physicochemical characterization of drugs
  - Drug-excipient compatibility
- Analytical Method Development and Validation
Formulation Development and Optimization: Identification of suitable formulation technique, optimization of technique using Quality by Design (QbD) approach

- Formulation 1: Dacarbazine loaded Liposomes (DL)
- Formulation 2: Dacarbazine + Eugenol loaded Liposomes (DEL)
- Formulation 3: Surface Coated Liposomes of dacarbazine + eugenol (DELC)

In-vitro characterization of the optimized formulations:

- Size and size distribution
- Surface and Morphological analysis (SEM and TEM)
- Zeta potential
- Entrapment efficiency
- Drug loading
- Drug release profile
- DSC
- FT-IR

Stability Study

Hemolytic study

Cell lines studies:

- Cytotoxicity studies
- Cell uptake studies
- Proliferation assay
- Migration assay
- Apoptotic profile

In-vivo studies:

- Pharmacodynamic studies
  - Tumor Volume
  - % Tumor Growth
  - Tumor Growth Delay
  - % Tumor Growth Inhibition
  - Specific Growth Rate
Chapter 3 Hypothesis and Plan of Work

- Doubling Time
  - Biodistribution studies
  - Pharmacokinetic studies
- Summary and Conclusion