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 produce
melanin. The cancerous growth of melanocytes is termed as Melanoma. 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.

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

Advanced malignant melanoma has a poor prognosis and chemotherapy is most of the
times not effective because of the resistance of melanoma cells. According to several
studies, the mechanisms of resistance in melanoma are different from hematological
tumors. The inherent resistance of melanoma cells is reported to be due to a protein
named survivin. Survivin protein is a member of the inhibitor of apoptosis (IAP) family,
and exerts its effects by directly inhibiting caspases. Several cancer types, including
melanoma, have been reported to overexpress survivin. Survivin protects cancer cells
from apoptosis, which is supposed to be induced by chemotherapy in order to kill cancer
cells. Survivin is also known to play an essential role in angiogenesis by promoting the
expression of the vascular endothelial growth factor (VEGF) in cancer cells. It is reported
that inhibiting the function of survivin in melanoma cells can spontaneously cause
apoptosis, impairing the growth of the tumor. Downregulation of survivin has also been
found to inhibit migration, metastasis, and proliferation of cancer cells, both in vitro and
in vivo. The fact that it is overexpressed in most cancer cells, but hardly expressed at all
in any normal tissue, makes it an attractive target for targeted anti-cancer therapies. Hence, combining an anti-survivin agent with Dacarbazine, which is the standard anti-melanoma chemotherapeutic drug, is most likely to result in overcoming of resistance leading to better outcomes of therapy.

In addition to survivin, melanoma cells express other surface receptors also, such as, CD44, P2X7 and Adenosine receptors. Cellular uptake of drugs bound to a targeting carrier is mostly restricted to receptor-mediated endocytosis. This receptor mediated endocytosis could be exploited for designing site-specific and target-oriented delivery systems. 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. 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.

Keeping the above facts in mind, we hypothesized to take a chemotherapeutic agent (Dacarbazine) as base drug, and wisely combine it with another drug (Eugenol) that targets the resistance mechanism of melanoma. From previous studies, eugenol is found to possess both anti angiogenic and anti survivin effect. This combination of drugs was to be loaded in liposomes and targeted actively to the melanoma cells to further improve the efficacy of the formulation and reduce unwanted toxic effects. This study adds to the existing knowledge about the efficiency of combinations of drugs, which can be used against aggressive cancers such as melanoma, and helps in establishing role of anti survivin agent as a novel therapeutic approach against aggressive resistant melanomas.

Lipid (S100) was provided by Lipoid, Germany. Dacarbazine was a generous gift sample from Intas Pharmaceuticals, Ahmedabad. Eugenol and Hyaluonic Acid were purchased from Sigma, USA. After procurement of drugs and excipients, preformulation studies
Chapter 9  
Summary and Conclusion

were carried out. Dacarbazine and eugenol were subjected to identification tests such as melting point determination and UV spectrophotometry. Partition coefficients of both the agents were determined and found to be in close proximity with the reported values. Solubility of both agents was determined in different solvents.

Afterwards, a novel analytical method based on absorptivity measurements was developed for the simultaneous estimation of dacarbazine and eugenol. Since the combination of dacarbazine and eugenol could not be analyzed by HPLC (because dacarbazine produces active metabolites) or LCMS (because eugenol is a volatile liquid), we developed a UV spectroscopic method based on absorptivity calculation for analysis of in-vitro sample. This method was robust and reproducible. PBS (pH 7.4) : Propylene glycol (9:1) was found to be a suitable solvent media and hence was selected for drugs release study. Ethanol was found to be suitable for drugs loading determination as both drugs as well as lipid could be dissolved in it. Thus, UV method was developed for these solvents. The developed method was appropriately validated and was found to be accurate and precise. Finally, the method was later applied for the determination of loading and release rate of the drugs from the liposomes.

For the synthesis of liposomes, solvent injection method was followed using ethanol as the organic solvent. Lipid and cholesterol were dissolved in ethanol. Then the eugenol was dissolved in this organic phase because of its lipophilic nature. Separately, dacarbazine was dissolved in distilled water, which constituted the aqueous phase. This aqueous phase was then kept on stirring, and the organic phase was rapidly injected into it using a syringe. This method produces smaller-sized liposomes with comparatively higher encapsulation efficiencies of the entrapped drug. According to a theory known as bilayer planar fragments (BPFs) theory, the lipids which are dissolved in ethanol precipitate at the phase boundary of water and ethanol (organic solvent), resulting in the formation of BPFs. When the organic solvent is completely diffused in the external aqueous phase, vesicle formation takes place consequent to self-assembly of BPFs.

QbD (Quality by Design) is a scientific and systematic approach for the development of pharmaceutical formulations. It involves defining and taking into consideration all the
parameters that critically affect the final quality and performance of the formulation. QbD can be focused by defining the aspects of the quality that are needed to be optimized. Thus, critical quality attributes (CQAs) of the formulation were optimized by following QbD approach. QbD is more economical and time effective method and thus has replaced hit and trial method for formulation optimization. DOE (Design of Experiment) is an integral part of QbD approach which involves use of the software to generate “structured” data tables. The software enables us to obtain graphical interpretation of the results and effect of each parameter on the CQAs of the formulation. Central composite design (CCD) was chosen because it generates better factorial design. Also, CCD can be used for working on factors as less as two in number.

At first level, only dacarbazine loaded liposomes were prepared and optimized in order to find out the optimum value of lipid concentration and water:ethanol ratio (dependent variables / Response). CQAs (Independent variables / Factors) selected were size of the liposomes and entrapment efficiency of the drug as both these are crucial parameters greatly influencing the performance of the formulation. After putting minimum and maximum values of the independent variables in CCD statistical experimental design, the software suggested 20 runs with 5 center points. These 20 formulations were prepared and analyzed. Software generated polynomial equations which could predict the effect of individual factors (independent variables) as well as combinations of factors on the responses (dependent variables). The size ranged from 65±2.12 nm to 113.56±3.21 nm. The size of the liposomes increased with increase in lipid concentration and drug concentration and decreased with increase in water:ethanol ratio. Values of entrapment efficiency ranged from 11.14±3.21% to 31.67±1.24%. Entrapment efficiency was most effected by lipid concentration, followed by drug concentration and water:ethanol ratio. As the lipid concentration increased, more number and larger liposomes were formed, thus higher was the entrapment efficiency. Also, when the drug concentration was increased, more drug was entrapped in the liposomes. On the other hand, greater water:ethanol ratio led to smaller liposomes which resulted in lesser drug entrapment. Optimum formulation of dacarbazine loaded liposomes was identified by numerical optimization by setting the constraints on dependent variables, size (minimum), and
entrapment efficiency (maximum). The software suggested 41 solutions, out of which the one having highest desirability factor was selected. The selected formulation suggested 13.168 mg/ml of lipid concentration, 5 as water:ethanol ratio, and 3 mg/ml of drug concentration. The predicted size of the optimized formulation was 69.093 nm, and the obtained size was 74.06±1.6 nm; the predicted entrapment efficiency of the optimized formulation was 25.436 %, while the obtained entrapment efficiency was 24.19±1.64 %. So, the predicted and obtained values were in close proximity.

For the second level, the optimum values of lipid concentration (13.168 mg/ml) and water:ethanol ratio (5) were already known from the results of first level. Here, the two independent variables, dacarbazine concentration and eugenol concentration, were optimized. The dependent variables were size and ratio of entrapped eugenol to entrapped dacarbazine. Size was constrained at minimum. Ratio of entrapped eugenol to entrapped dacarbazine was constrained at maximum. It is because dacarbazine is a very potent anti-cancer drug having very low anti-cancer dose (2-4.5 mg/kg), while eugenol is an herbal agent with high anti-cancer dose as compared to dacarbazine. Since both the drugs are to be entrapped in the same formulation, the ratio of entrapped eugenol to entrapped dacarbazine should be as high as possible so that maximum amount of eugenol is administered when anti-cancer dose of dacarbazine is given to the animals (through liposomes). When the minimum and maximum values of independent variables were put in CCD statistical experimental design, the software suggested 13 runs with 5 center points. The formulations were made and analyzed. Same as first level, polynomial equations were generated to predict the effect of individual factors and combinations of factors on dependent variables. The size of optimized formulation ranged from 100.28 ± 2.74 nm to 178.81 ± 6.72 nm. The size of the liposomes was more influenced by eugenol concentration. Dacarbazine concentration and eugenol concentration both had positive effect on size of the liposomes. Thus, size of the liposomes increased with increase in any of the drugs concentration. Values of ratio of entrapped eugenol to entrapped dacarbazine ranged between 1.5 ± 0.17 and 3.24 ± 0.21. Dacrabazine concentration had a negative effect and eugenol concentration had a positive effect on entrapped eugenol:dacarbazine; while dacarbazine concentration has a more pronounced effect. Optimum formulation of
dual loaded liposomes was identified by numerical optimization by setting the constraints on dependent variables, size (minimum), and eugenol:dacarbazine (maximum). The software suggested 15 solutions, out of which the one having highest desirability factor was selected. The selected formulation suggested 1 mg/ml of dacarbazine concentration, and 6.882 mg/ml of eugenol concentration. The predicted size of the DEL synthesized by the suggested formula was 120.889 nm, and the size obtained was 124.90 ± 3.72 nm. The predicted eugenol:dacarbazine value was 2.873, while it was practically observed to be 2.906 ± 0.83. So the predicted and obtained values were found to be in agreement.

Surface Functionalization of optimiaed liposomes was done with hyaluronic acid (HA). To coat HA, which is negatively charged, on the surface of liposomes through ionic interaction, cationic liposomes were first formulated by including cetyl tetra ammonium bromide (CTAB) in the organic phase during formulation. Different concentrations of hyaluronic acid (HA) solution were used to coat the optimized cationic liposomes, and the size of the final coated liposomes was assessed in each case to determine the optimum concentration of HA solution to be used. 0.01% concentration was selected as the optimum concentration of HA solution. The liposomes coated with 0.01% HA solution were later analyzed by TEM and SEM to visualize and confirm significant coating of HA.

The formulations prepared and further analyzed were Blank Liposomes (BL), Dacarbazine loaded Liposomes (DL), Dacarbazine and Eugenol loaded Liposomes (DEL), and HA coated Dacarbazine and Eugenol loaded Liposomes (DELC).

Blank liposomes (BL) had a size of 54.50 ± 1.73 nm, which increased to 74.06 ± 1.6 nm when dacarbazine was loaded (DL). When both the drugs were loaded (DEL), the size further increased to 124.90 ± 3.72 nm. After surface functionalization of the optimized dual loaded liposomes (DELC), the size remarkably increased to 159.15 ± 3.62 nm. PDI, which is a measure of the size distribution of the nanoparticles, was within suitable range for all the formulations.

Zeta potential of BL was found to be -9.97 mV, which indicates moderate stability of the liposomes. The zeta potential of DEL was found to be -8.70, which is nearly same as that
of the blank liposomes. Since both the drugs are entrapped inside the liposomes, the surface charge remaine more or less unaltered. Zeta potential of cationic liposomes was 27.7 mV. After coating of liposomes with HA, the zeta potential was -12.8. Here, the negative charge is supposed to be due to the carboxylic groups of HA present on the surface of the liposomes. This increase in the negative charge of the liposomes indicates towards successful coating of the liposomes with the electronegative HA.

Surface morphology was observed by TEM and SEM. It could be seen in the TEM images, the blank liposomes were hollow structures bounded by thin lipid bilayer. However, the DEL had drug entrapped in the core, as well as in the lipid bilayer which was evident by the thickening of the bilayer. In the image of the DELC, the surface of the liposomes was irregular which confirmed the coating of liposomes by HA.

SEM images were also in agreement with the TEM images, showing somewhat smaller and regular structure of blank liposomes, bigger structures of DEL, and bigger with irregular surface of DELC. The sizes revealed by TEM and SEM analysis were also in agreement with the results of particle size analysis by zeta sizer. Also, SEM and TEM images revealed no aggregation of the liposomal structures.

In DEL, the dacarbazine loading was found to be 19.874 ± 0.738 %, while that of eugenol was found to be 48.37 ± 1.74 %.

The entrapment efficiency of dacarbazine in DEL was calculated as 18.80 ± 0.963 %. The entrapment efficiency of eugenol in DEL was calculated as 38.84 ± 2.48 %.

In DELC, the dacarbazine loading was found to be 15.272 ± 0.751 % and Eugenol loading was found to be 44.392 ± 0.85 %.

The entrapment efficiency of dacarbazine in DELC was calculated as 17.49 ± 1.04 %. The entrapment efficiency of eugenol in DELC was calculated as 36.94 ± 0.94 %.

The release of drugs was determined for DEL and DELC. The release was found to be more sustained from DELC, indicating towards the role of HA-coating in slowing down the release of drugs. From DEL, in 12 hours, 63.6 ± 2.04 % of dacarbazine and 77.36 ±
2.74 % of eugenol was released, while form DELC, only 44.24 ± 2.55 % of dacarbazine and 68.62 ± 3.20 % of eugenol was released in 12 hours. In 24 hours, the cumulative released amount was found to be 78.46 ± 4.7 % of dacarbazine and 89.21 ± 3.64 % of eugenol from DEL, and 61.78 ± 3.74 % of dacarbazine and 81.73 ± 2.10 % of eugenol from DELC. 98.36 ± 1.21 % dacarbazine and 99.73 ± 0.18 % eugenol was released in a span of 72 hours from DEL. 84.67 ± 2.64 % dacarbazine and 97.1 ± 1.78 % eugenol was released from DELC in 72 hours. Thus, the release of both the drugs from DEL and DELC was found to be sustained, but DELC showed more sustained drugs release, owing to HA-coating on the surface.

Liposome were found to be fairly stable when stored at 4°C for 4 weeks in lyophilized form as they did not show any remarkable increase in size or PDI; neither did they show significant reduction in their drug content. This implies that, in the lyophilized form and under suitable storage conditions, formulated liposomes were able to retain their size without any leakage or leaching of drugs.

For the cytotoxicity assessment of the developed formulation, first the cytotoxicity of DEL was compared with DS (Dacarbazine Solution) and DL in SK-MEL-28 cells. As was known that eugenol inhibits survivin protein thereby inducing apoptosis and inhibiting angiogenesis, SK-MEL-28 cells were chosen because of the fact that they overexpress survivin protein. The different concentrations of dacarbazine (in each formulation) tested on SK-MEL-28 cells were 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 12.5 µg/ml, and 16.25 µg/ml. DS produced a cytotoxicity of only 25.91 ± 1.060 % at concentration of 16.25 µg/ml, while DL at the same concentration of dacarbazine produced cytotoxicity of 35.89 ± 1.109 %. In contrast to these, DEL produced cytotoxicity of 76.96 ± 0.351 %, which was more than the double of the cytotoxicity of DL. This clearly suggests that eugenol is quite capable of potentiating the effect of dacarbazine on melanoma cells. This also indicates that combining eugenol with dacarbazine may allow significant reduction of dacarbazine dose during chemotherapy. Since dacarbazine is a cytotoxic drug, reduction in its dose implies reduction in unwanted toxicity on normal body cells.
After SK-MEL-28 cells, B16F10 cells were employed to assess the therapeutic superiority of final formulation (DELC) over DS, DL and DEL. B16F10 melanoma cells are also known to overexpress survivin and CD44 receptors. Here the concentrations (of dacarbazine) tested were 0.05 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml. Lower concentrations were tested this time because more potent action was expected from DELC. At concentration of 0.05 µg/ml, DS produced cytotoxicity of 3.60 ± 0.055 %, while DELC produced cytotoxicity of 35.72 ± 0.466 %. At concentration of 0.5 µg/ml, cytotoxicity of DS was only 10.20 ± 0.288 %, whereas DELC produced 95.08 ± 0.310 % cytotoxicity. So, our final coated liposomes of Dacarbazine and Eugenol showed enhancement of more than 900% in cytotoxicity as compared to that of Dacarbazine Solution at the same concentration of dacarbazine (0.5 µg/ml) in both the formulation.

However, the question arises here that wether the superior effect of our formulation is simply due to the synergistic effect of dacarbazine and eugenol, or actually due to inhibition of resistance mechanism by the eugenol. To find out the answer, we assessed the cytotoxicity of liposomes of only dacarbazine (DL), and liposomes of only eugenol (EL) and compared the additive cytotoxicities with that of DEL. The additive values of individual cytotoxicity of DL and EL was less than the cytotoxicity of DEL at every concentration tested. This indicated that addition of eugenol has contributed more than synergism and has inhibited survivin thus suppressing the resistance of melanoma towards dacarbazine.

Apoptosis assay was performed on SK-MEL-28 cells using Annexin V kit. The concentration of dacarbazine in all the formulations was 10 µg/ml. In DS treated cells, the percentage of viable, early apoptotic, late apoptotic and necrotic cells were 78.2 ± 0.1, 6.66 ± 0.115 %, 8.43 ± 0.057 % and 6.66 ± 0.057 % respectively. In the cells treated with DL, the percentage of early apoptotic, late apoptotic and necrotic cells were 17.2 ± 0.1 %, 23.2 ± 0.1 % and 5.3 ± 0.2 % respectively, while viable cells were 54.53 ± 0.057%. The viability of cells decreased to 38.3 ± 0.1 % when treated with DEL. Early apoptotic, late apoptotic and necrotic cells in the DEL treated group were 6.36 ± 0.057 %, 39.23 ± 0.057 % and 16.1 ± 0 %. While performance of DLC was slightly inferior to DEL, it performed
much better than DL. This can be owed to better uptake of liposomes by melanoma cells due to HA coating and thus indicates towards importance of surface functionalization. Finally, DELC caused maximum late apoptosis (45.16 ± 0.057 %) and necrosis (19.0 ± 0.1 %) out of all the formulations because of better uptake of the liposomes which contained both dacarbazine and eugenol.

Migration and Proliferation assays were performed in EA.hy926 cells. EA.hy926 cell line, which is derived as the hybrid of primary human umbilical vein cells (HUVECs) and the continuous human lung carcinoma cell line A549, is presently the best characterized macro-vascular endothelial cell line.

In migration assay, inhibition of migration of cells by the formulations was studied. It was performed by wound healing assay, where a small linear scratch (representative wound) was created in the middle of the well having confluent monolayer of cells by gently scraping with sterile 200μl micropipette tip. Photomicrographs of the scratch were taken at 0 hour (Initial time point), 24 hours, 48 hours and 72 hours. The concentrations of DS, DL and DEL tested were 0.1 μg/ml and 1 μg/ml, while concentrations of DLC and DELC tested were 0.1 μg/ml. At 1 μg/ml, DS showed a migration inhibition of 44.13 ± 0.152 % at 24 hours, which was quite significant, but inhibition drastically decreased to only 7.43 ± 0.057 % and 4.3 ± 0.1 % at 48 hours and 72 hours respectively. In case of DL (1 μg/ml), inhibition was 55.66 ± 0.115 %, 45.43 ± 0.152 % and 37.73 ± 0.057 % at 24 hours, 48 hours and 72 hours respectively. This indicates towards somewhat sustained release of dacarbazine from DL as the decrease in performance was not as steep as DS. In DEL (1 μg/ml) treated group, there was a remarkable migration inhibition of 116.96 ± 0.057 %, 144.43 ± 0.115 % and 139.13 ± 0.152 % at three different time points. Here comes the role of eugenol which has contributed significantly in inhibiting the migrating ability of the cells. As the % inhibition has increased from 24 hours to 48 hours, and then only slightly decreased at 72 hours, this indicated towards more sustained release of the drugs. In DLC (0.1 μg/ml) treated group, inhibition was 93.13 ± 0.115 %, 88.03 ± 0.57 % and 76.7 ± 0.1 %. Here, the action was sustained for 72 hours but not as much as in case of DEL (inhibition has decreased rather than increasing). This shows that Eugenol plays a more significant role (than HA coating) in sustaining the release of dacarbazine.
Inhibition in the cells treated with our final formulation DELC (0.1 µg/ml) was the highest; 116.86 ± 0.057 %, 118.26 ± 0.115 %, and 133.53 ± 0.0115 %. It should be noted here that DELC gave similar results as DEL at a concentration one tenth of it. This indicated that surface coating has improved the performance of the formulation by around ten times. Also, the inhibition continuously increased for 72 hours which implies most sustained action out of all the formulations. It is because of the presence of both, eugenol and HA coating. One interesting point noted here is that DLC performed better than DEL (comparing results of 0.1 µg/ml). This unexpected behavior can be attributed to the fact that EA.hy926 cells overexpress CD44 on the cell surface and HA reportedly binds to EA.hy926 cells. This must have resulted in better uptake of DLC as compared to DEL which does not have HA coating to bind to CD44 receptors.

Proliferation assay was performed to study and compare the anti-proliferative activity of the formulations. Concentrations tested were 0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml, and 10 µg/ml. DS at dacarbazine concentration of 10 µg/ml reduced the viability of cells to 77.31 ± 0.47, while DL reduced it to 58.83 ± 0.610 %. In DEL treated group, the cell viability was 34.46 ± 0.643 %. Viability of cells treated with DLC was 27.08 ± 0.605 % which was little less than DEL because of the same reason as stated above. Most interestingly, the DELC left only 6.14 ± 0.618 % cells viable. Thus the anti-proliferative performance of DELC was much more significant than DS, which is the conventional way how the dacarbazine is administered.

Since liposomes were coated with HA to favor their uptake in melanoma cells, it was necessary to assess and compare the cellular uptake of the uncoated and coated formulations. For this purpose, rhodamine dye was used. Rhodamine is a fluorescent agent and thus can be traced using flow cytometry. Uncoated (Rh-L) and Coated (Rh-L-C) Rhodamine Liposomes were prepared and tested in B16F10 cells for uptake. Rhodamine Solution (Rh-S) showed negligible uptake in the cells, while Rh-L showed higher uptake. This enhanced uptake of Rh-L in comparison to Rh-S can be owed to Enhanced Permeation and Retention (EPR) effect. However, Rh-L-C showed even higher uptake in the melanoma cells. In addition to EPR, HA coating must have contributed to
the significantly higher cellular uptake of coated liposomes, supporting the important role of surface functionalization in targeted therapy of melanoma.

Since, HA also imparts hydrophilicity to the liposomes, it was anticipated that the liposomes would be long-circulating and avoid phagocytosis by the macrophages. In order to ascertain this, cellular uptake was also assessed in RAW 264.7 cells. RAW 264.7 cells are a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice. This cell line is a commonly used model of mouse macrophages for the study of cellular responses to microbes and their products (https://www.invivogen.com/raw). Results revealed that uptake of uncoated liposomes by RAW 264.7 cells was almost 200% more than that of coated liposomes. Thus, HA coating could successfully reduce the macrophage (non target cells) phagocytosis of the liposomes and enhanced the accumulation in cancer cells (target cells).

To check the suitability of the formulation for intra venous administration, hemolytic test was performed. The results of the study showed that DELC caused 1.709 % hemolysis. Hence, it can be suggested that the formulated liposomes are minimally toxic to the RBCs.

Finally, in-vivo studies were performed to confirm the superior abilities of DELC as anti-melanoma treatment. B16F10 cells were used to induce melanoma in C57/BL6 mice by subcutaneous injection in the right flank region.

For pharmacodynamics study, animals were divided into 4 groups (Group I: Control; Group II: DS; Group III: DLC; Group IV: DELC). The three formulations taken were- Dacarbazine Solution (DS), Dacarbazine Liposomes Coated (DLC), and Dacarbazine and Eugenol Liposomes Coated (DELC). Dacarbazine liposomes were also coated (DLC) and included in the experiment so as to enable us to do a fair comparison between ‘dacarbazine alone’ and ‘dacarbazine + eugenol’ therapies. Corresponding therapeutic dose of dacarbazine in mice came out to be 24 mg/kg. A sub-therapeutic dose of 10 mg/ml of dacarbazine was selected for the experiments to be able to obtain a clear difference between the effects of DS, DLC and DELC. Thus, all the formulations were given equivalent to 10 mg/kg of dacarbazine
In addition to measuring tumor volumes at definite points of time, following parameters were also calculated: % Tumor growth, Tumor Growth Delay, % Tumor Growth Inhibition, Specific Growth Rate (SGR), and Doubling Time.

Dosing was started when tumor volume in all the animals reached 100-200 mm$^3$, and continued for 10 days once daily. A significant difference in the tumor volumes could be seen among the 4 groups at the end of the treatment. Group I which was the control group and received only normal saline had mean tumor volume of $2473.24\pm1693.59$ mm$^3$ at day 7. Since tumor volumes had exceeded the 2000 mm$^3$ mark, the animals of group I were sacrificed on day 7 on ethical grounds. Such fast growth of tumors in control group indicates towards extremely aggressive nature of B16F10 cells. Group II received DS and since a sub therapeutic dose was given, not a very significant anti-tumor effect was observed with mean tumor volume being $1741.46\pm582.88$ at day 7 and $2574.84\pm783.81$ at day 9. On ethical grounds, the animals of group II were sacrificed on day 9. Group III received DLC and though the amount of dacarbazine was same, the mean tumor volume of this group was significantly lesser than Group II ($1874.03\pm705.83$ on day 9 and $2303.06\pm790.24$ mm$^3$ on day 11). Here, the enhancement in anti-tumor effect can be attributed to two factors; first is the passive targeting by EPR effect which would have resulted due to the encapsulation of the dacarbazine into the nano sized liposomes, and second, coating of the liposomes surface with HA which increased the accumulation of the dacarbazine loaded liposomes in the tumor by active targeting to the CD44 receptors overexpressed by melanoma cells.

The mean tumor volume of Group IV which received DELC was $1237.16$ mm$^3$ on day 11 which was least of all the groups. In addition to the effects exhibited by DLC, DELC showed even better anti-melanoma effect due to the inclusion of eugenol. These results support our hypothesis that in presence of eugenol, dacarbazine could perform better due to the inhibition of survivin and resistance.

**Percent tumor growth** was also calculated at each time point. Though the values decreased in all the groups throughout, it was least for DELC administered mice (group IV) at every time point.
Specific growth rate was calculated for all the four groups and was obviously found highest for the control group (0.445±0.104). The specific growth rate of DS group was lower than the control (0.34±0.756). When DLC was administered, specific growth rate was further decreased (0.2813±0.050) owing to the effect of targeting by HA. Finally, it was found to be least in DELC group (0.2186±0.039) further supporting the improved anti-melanoma effects of including eugenol in the formulation.

Doubling time of control, DS, DLC and DELC groups was 1.557±0.578, 2.038±0.483, 2.687±0.65 and 3.27±0.729 respectively. Hence, when doubling time in DS group was hardly 25% more than control group, it was more than 100% increased in DELC group.

Tumor growth delay was calculated by determining the difference in time taken by the control and test groups to reach 700 mm$^3$ of tumor volume. The tumor volume was specified as 700 mm$^3$ because this is the least volume of the tumor attained by any of the animals (one animal in the DELC group had 700 mm$^3$ tumor volume even at the end of the treatment). Value of tumor growth delay was 0.58 days for DS group, while it was 1.50 days and 3.83 days for DLC and DELC groups respectively. Thus, the tumor growth was delayed by more than 6 times in DELC group as compared to DS group.

Percentage of tumor growth inhibition was calculated and was found to be 29.58%, 49.89% and 65.06% for DS, DLC and DELC groups respectively. Hence, the tumor growth inhibition was more than twice in DELC group as compared to DS.

Histological analysis of tumor and organs of the animals was performed after the completion of pharmacodynamics study. Animals were sacrificed and sections of their tumor, lungs, and liver were collected and fixed in 10% formalin. The tissues were then decalcified in 10% EDTA (ethylenediaminetetraacetic acid). Afterwards, tissues were embedded in paraffin, sectioned and stained with haematoxylin-eosin and Masson’s trichrome. Slides were prepared from the tissue sections, and observed under a light microscope. Evaluation and comparisons between the histology of tissue from different treatment groups was done.
**Tumor** of control group showed live polygonal melanoma cells. Minimal necrotic cells were observed, while tumors of DS and DLC groups showed more necrotic cells than control group. Tumor cells of DELC group were mostly in necrotic phase, as evident by the microscopic images.

In the **lungs** of control group, cancer like cells could be clearly seen, indicating towards metastasis of melanoma. In the lungs of DS and DLC groups, few areas having cancer cells were observed. The overall anatomy of lungs was distorted. However, the lungs sections of DELC group showed absolutely normal anatomy. This supports the anti-metastatic property of DELC.

Histology of **liver** sections was studied as dacarbazine is reported to cause hepatotoxicity. In the control group, hepatocytes were found to be normal, blood cells in large amounts were seen in central vein and portal vein. In DS, severe hemorrhage and clumping of hepatic cells was observed at several places. Some clumping and hemorrhage was also observed in DLC group, however, it was less severe than DS group. Interestingly, the liver sections of DELC group showed perfectly normal hepatocytes and clean veins. This proves that when dacarbazine is administered as solution, it reaches to liver and causes damage to the liver, but encapsulating it in HA coated liposomes prevents the hepatotoxicity up to a significant extent.

Liposomes were surface coated with HA with an intention to achieve more accumulation of liposomes in tumor and less in other organs. Not only to obtain better therapeutic outcomes, this is also important to reduce unwanted toxicity of the drug in other organs. Dacarbazine is known to cause severe hepatic toxicity, and thus we needed to check if our formulation could help with reducing its concentration in liver. Therefore, Indocyanine Green (ICG) loaded liposomes were prepared and concentration of ICG was determined in tumor and in major organs including liver, lungs, kidney, heart and spleen after 1, 6, and 12 hours of administration to study the biodistribution of our formulation. ICG was selected for this purpose because ICG is majorly taken up by liver to be excreted in bile in the unconjugated form. Hence, concentration of ICG in liver would give a better estimate of availability of dacarbazine to liver when given in free and in
liposomal form. When ICG was given in solution form (ICG Sol), 79.62 ± 5.47 % of ICG was present in liver after 1 hour of administration and less than 4% was present in blood. Other organs and tumor had very less amount of ICG (2% - 5%). This indicates that when given in solution form, the drug is freely available to be taken up by the liver and shows no specific accumulation at the tumor site. After 6 hours and 12 hours, concentration of ICG was further decreased in all organs including tumor. In liver also, the amount of ICG was reduced to 44.67±3.32 % and 13.74±1.93 % at 6 hours and 12 hours respectively. This indicates that ICG was excreted from the body. ICG, when given in uncoated liposomes (ICG-Lip), showed 18.2 ± 2.72 %, 21.54 ± 1.7 %, and 36.55 ± 4.75 % accumulation in liver at 1, 6 and 12 hours respectively. As the ICG was released from the liposomes, it was taken up by the liver. In comparison to ICG sol, ICG-Lip showed more accumulation of ICG in spleen. This may be the result of the RES (Reticuloendothelial system) uptake of ICG-Lip by macrophages. In tumor also, ICG was present in comparatively greater amounts (8.5 ± 1.72 %, 26.79 ± 1.75 % and 11.51±2.53 % at 1, 6 and 12 hours respectively). This can be owed to the passive targeting of ICG-Lip to the tumor by EPR effect. Finally, when ICG was given in coated liposomes (ICG-Lip-HA), it reached liver in very less amounts (4.32 ± 0.65 %, 6.56 ± 0.83 % and 11.63 ± 2.81 % at 1, 6 and 12 hours respectively). In tumor, ICG content was 9.51±1.45 % after 1 hour. This low amount can be due to the sustained release of ICG from ICG-Lip-HA. However, after 6 hours, ICG concentration in tumor reached 45.56 ± 3.2 %, and it still was 42.83 ± 4.3 % even at the end of 12 hours, indicating towards prolonged stay of DELC at the tumor site. All other organs showed negligible presence of ICG at all the time points.

HA, along with active targeting, also gives a hydrophilic surface to the liposomes. Hence, the liposomes were expected to be long circulating and to stay longer in blood circulation in comparison to free drug. It has also been established that longer the particles stay in the systemic circulation, greater is the chance of accumulation in the tumor. Hence, to ascertain longer circulation of our HA coated liposomes in blood, the concentration of ICG was determined in the blood at different time points after i.v. injection of ICG Sol, ICG-Lip, and ICG-HA-Lip. Percent amount of ICG in ICG Sol group was only 3.98%, 1.87% and 0.34% at the end of 1 hour, 6 hours and 12 hours respectively. The reason for extremely low concentration of ICG in blood at even as early as 1 hour is that the plasma half life of ICG is
only 3-4 minutes. Thus, when present in free form, it is readily removed from the blood by liver. In the form of ICG-Lip, amount of ICG was 52.54 %, 20.65 % and 12.12 % at 1 hour, 6 hours and 12 hours respectively. A sharp decrease in the can be seen from 1 hour to 6 hours. This implies that the ICG-Lip was sharply drained off the blood in 6 hours and was further decreased till 12 hours. In contrast, in the ICG-Lip-HA group, the amount of ICG in blood was 84.63 %, 35.74 % and 29.73 % after 1 hour, 6 hours and 12 hours respectively, which is much greater than ICG-Lip group. This shows that HA has worked efficiently towards making the liposomes long circulating.

Our results of biodistribution and pharmacokinetic study are in accordance with the previously reported results where researchers have concluded that anchoring HA to the surface of the nanoparticles promote their longer stay in the systemic circulation. According to them, this long circulation is the result of hydroxyl residues of HA that grant the nanoparticles a hydrophilic surface which in turn reduces the attachment of circulating serum opsonins to the nanoparticles and their subsequent clearance by the RES.

CONCLUSION

Keeping the highly resistant and aggressive nature of melanoma in mind, dacarbazine and eugenol loaded liposomes were successfully developed for a combinatorial approach against melanoma. The QbD approach enabled us to synthesize the said anti-melanoma formulation with optimum parameters in the most logical manner. Applying this QbD approach at two levels further made the process easier to reproduce, and more cost-effective. Surface functionalization of the formulation made the entire therapy more targeted to spare normal body cells from unwanted toxicity. In-vitro characterization of the nanoliposomes ascertained the utility of the QbD application. This work is a good example and illustration of the successful application and value of the QbD approach in the development of effective pharmaceutical nanoformulations. The performance of the formulation as an anti-melanoma agent was assessed by cell line studies. Combining eugenol with dacarbazine resulted in much higher anti-melanoma activity of the formulation. This enhancement is supposed to be due to the inhibition of the anti-apoptotic protein survivin, which is overexpressed in the melanoma cells, and makes
them resistant towards apoptosis. Including Eugenol has supposedly resulted in downregulation of survivin protein, consequent to which, dacarbazine could perform its function to its maximum potential. In addition to increased apoptosis and cytotoxicity, this combination also promises to inhibit the metastatic potential of the melanoma. Further, HA coating was found to enhance the uptake of the liposomes in B16F10 cells and reduce the phagocytic engulfment by macrophages. Pharmacodynamics study showed significant difference in the tumor volumes after 10 days of the treatment with DELC and Dacarbazine Solution (DS). Results of biodistribution study supported the results of cell uptake study by revealing more concentration of drug from DELC in blood and tumor, and considerably less amount in the liver. Histological analysis of tumor sections showed that there was significantly more necrosis in the DELC treated group. In lungs, metastasis could be seen in the control and dacarbazine solution group, but not in DELC group. Liver toxicity was also not observed in the DELC group, while hemorrhage was seen in DS group. DELC was also found to be safe for blood cells and thus suitable for i.v. administration. Conclusively, combining eugenol with the dacarbazine results in better therapeutic outcomes in the treatment of melanoma and thus can be a hope against this resistant, aggressive and deadly cancer. Thus, the combination of dacarbazine and eugenol holds the promise of overcoming the resistance of melanoma cells and challenges of anti-melanoma therapies.

**FUTURE PERSPECTIVES**

To be able to apply this approach clinically for the better treatment of melanoma patients, different concentrations of eugenol should be combined with different amounts of dacarbazine and analyzed to assess the extent of its action and find out the combination offering best benefit-to-risk ratio. Clinical studies will further enable the researchers to ascertain the superiority of this combination over chemotherapy alone and take this to the commercial level for the betterment of the melanoma patients.