Preface

Cancer is the leading cause of death worldwide and is responsible for nearly 10 million deaths in 2020 as per the GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer. An estimated number of 19.3 million new cancer and nearly 10.0 million cancer deaths occurred in 2020, worldwide. The global cancer burden is expected to rise by 47% from 2020, with 28.4 million cases in 2040. As per the American Cancer Society, in 2023, 1,958,310 new cancer cases and 609,820 cancer deaths are projected to occur in the United States. The number of cancer cases in India is projected to go up from 14.6 lakh in 2022 to 15.7 lakh in 2025, according to the Indian Council of Medical Research-National Cancer Registry Programme (ICMR-NCRP). It is one of the most serious global health issues, which is largely caused by environmental exposures such as radiation, pesticides, fertilizers, etc., pollutants in the environment, lifestyle modifications, cancer-causing infections including the human papillomavirus (HPV) and hepatitis, poor diets, and also due to some forms of high-dose chemotherapy and radiation therapy. The biological capabilities of cancer that are acquired during their multistep development are called hallmarks of cancer. They include sustaining proliferative signaling, resisting cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, reprogramming energy metabolism, and evading immune destruction.

Lung cancer is the second most common cancer worldwide. It arises from abnormal epithelial cells in the airways of the lungs, and it is the major cause of cancer-related deaths worldwide. Smoking and tobacco consumption are the major risk factors for lung cancer.

At present, the clinical options for cancer treatment predominantly consist of conventional modalities, such as surgery, chemotherapy, and/or radiation therapy. Unfortunately, resistance to conventional cancer therapies is a frequent occurrence and often serves as the primary cause

of tumour relapse, posing a significant obstacle to preventing the progression of the disease to an advanced stage. Due to the high toxicity, limited tumour specificity, and growing resistance of current anticancer agents, our research has been driven to explore novel and more effective therapeutic molecules, with a particular focus on their targeted delivery to lung cancer cells. Natural product-based compounds can have the capabilities to counter the issues of toxicity, ineffectiveness, and invasiveness of current therapeutic approaches. Natural products are being explored as a source of potential anticancer drugs. In the current era of anticancer drugs, more than 70% are direct derivatives from natural sources, structurally modified compounds, or inspired by nature.

In this direction, we explored the triterpenoid class of compounds to select the therapeutic molecule. Oleanolic acid (OLA) and Asiatic acid (ASA) are pentacyclic triterpenoids that occur naturally in several plants used for food and medicine. Preclinical outcomes have proved the anticancer effects of both molecules as well as other diverse beneficial effects.

It has been shown that both molecules have potential as an anticancer agent, although their use has been restricted due to their limited bioavailability and poor absorption when administered orally. Nanoparticle-based delivery of anticancer agents offers numerous advantages in cancer treatment, such as improved pharmacokinetics, targeted tumour cell delivery, reduced side effects, increased efficacy, and enhanced safety.

Protein nanoparticles, such as albumin-based nanoparticles (ALB-NPs), offer several distinct characteristics, including biodegradability, targetability, non-immunogenicity, lower cytotoxicity, and biocompatibility. During the studies, albumin (ALB) was chosen as the nano-drug delivery carrier after *in silico* molecular docking analyses. Our rationale behind selecting ALB as a drug delivery carrier was also based on the following features (i) It is highly abundant in plasma (35-50 mg/mL), has great stability, less immunogenicity, has excellent tumour-homing ability, and has a biological half-life of around 19 days (ii) ALB-NPs demonstrated the

enhanced permeability effect (EPR) due to their lower size (approximately 7.2 nm) which led to the accumulation of the NPs in cancerous cells.

Previous reports have shown that ALB is more abundant around cancerous tissues compared to normal tissues, as cancerous cells utilize ALB as a source of nutrition and energy within the tumor microenvironment. Previous studies have reported that ALB preferentially interacts and binds with albondin, which is a membrane-associated gp60 protein expressed on endothelial cell surfaces. This interaction facilitates the active internalization and transportation of ALB. Furthermore, the research findings demonstrate that ALB exhibits interactions with and binding to SPARC (secreted acidic protein rich in cysteine), which is over-expressed in various cancer cells.

Thus, small anticancer compounds conjugated or entrapped within ALB-NPs are simultaneously transported to the cancerous environment. The present research indicates that the utilization of ALB-NPs as a carrier for small anticancer compounds enables their targeted delivery to the cancer environment. The conjugation or entrapment of these compounds within ALB-NPs facilitates their simultaneous transportation to the cancerous site.

The pharmacokinetics of drugs has been significantly improved through the utilization of non-ionic surfactants, including D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), tween 80, and others, in combination with nanocarriers. TPGS is a widely recognized formulation stabilizer, emulsifier, and permeation enhancer that has received approval from the US FDA. As an amphiphilic derivative of natural vitamin E, it is well-established in the scientific literature. Furthermore, TPGS plays a crucial role in preventing opsonization while also enhancing the stability and prolonging the half-life of NPs in the bloodstream.

In addition, surface modification of NPs with TPGS can significantly increase their *in vivo* blood-circulating time and enhance tumor accumulation.

Nanocarriers are commonly employed to improve therapeutic outcomes by modifying the cellular uptake mechanism of small bioactive molecules. However, nanocarriers labelled with monoclonal antibodies targeting specific receptors found in tumours can attain the desired level of specificity needed for effective tumour treatment. The targeted delivery of anticancer molecules has the potential to address several challenges, including drug resistance, poor bioavailability, poor target specificity, and unwanted side effects. In light of these considerations, we have focused our efforts on targeting the Epidermal growth factor receptor (EGFR), whose overexpression is associated with lung cancer.

Many human malignancies, including non-small cell lung cancer (NSCLC), are overexpressed with transmembrane receptor tyrosine kinase, namely, EGFR. Cetuximab (CTX) is a monoclonal antibody with a chimeric structure that exhibits a strong binding affinity to the extracellular domain of the EGFR. This antibody is composed of both mouse and human components and belongs to the IgG1 class. Notably, its affinity for EGFR is considerably higher than that of epithelial growth factor. As a result, CTX can be utilized as a targeting ligand to functionalize NPs at substantially lower doses.

In one of our research works, OLA incorporated CTX conjugated ALB nanoparticles were developed not only to improve the therapeutic potential of OLA but also to reduce its *in vivo* toxicity for lung cancer therapy. To this day, there has been no mention of OLA being delivered via CTX functionalized ALB-NPs.

Elevation of therapeutic potential has been achieved by site-specific EGFR targeted delivery of OLA to treat lung cancer. Initially, OLA was isolated and characterized analytically. *In silico* docking studies with proteins like albumin, beta lactoglobulin and lactoferrin resulted that albumin to be the appropriate polymer for OLA entrapment and studies revealed that albumin-OLA complexes were formed through hydrogen bonding, alkyl interactions and van der waals interactions with a binding energy of -8.2 kcal/mol.

The successful integration and interaction of OLA with ALB were validated using FTIR, XRD, DSC, and TGA techniques. Additionally, XPS analysis further confirms the attachment of CTX to OLA-ALB-NPs. Both the unconjugated formulation (OLA-ALB-NPs) and the CTX-conjugated formulation (CTX-OLA-ALB-NPs) exhibited nanoscale dimensions. The conjugation of CTX resulted in an increase in particle size by up to 20 nm. AFM images showed that the surface of the ALB-NPs was smooth. TEM images indicated that both types of nanoparticles were spherical and exhibited a darker area on the inside, suggesting that the drug was predominantly deposited in the cores of the ALB-NPs. The zeta potential measurements for OLA-ALB-NPs and CTX-OLA-ALB-NPs were -30.5 ± 2.8 mV and -33.3 ± 3.4 mV, respectively. The shift towards a more negative zeta potential is likely due to the presence of anionic groups in CTX, which made the surface of the targeted nanoparticles less positive. The drug's release profile from prepared nanoformulations demonstrated a rapid initial release, followed by a slow and prolonged release over time. Fluorescence microscopy images from the cellular uptake study demonstrated that CTX-OLA-ALB-NPs were internalized more extensively in A549 cells compared to OLA-ALB-NPs. The green fluorescence intensity of C6 indicated that the accumulation of C6-CTX-ALB-NPs was significantly greater ($p < 0.001$) than that of C6-ALB-NPs. The MTT assay showed that both OLA-ALB-NPs and CTX-OLA-ALB-NPs exhibited higher cytotoxicity than free OLA.

The analysis of cellular uptake provides insight into the modest cytotoxic effect of OLA and its formulations on A549 cells. As a result, CTX-OLA-ALB-NPs demonstrated superior cytotoxicity and intracellular uptake compared to OLA and OLA-ALB-NPs in A549 cells. The annexin V-Alexa Fluor/propidium iodide dual staining method was employed to assess apoptosis induction in A549 cells by OLA, OLA-ALB-NPs, and CTX-OLA-ALB-NPs. Flow cytometry results showed that the apoptotic population was highest in cells treated with CTX-OLA-ALB-NPs and significantly lower in those treated with OLA. OLA-ALB-NPs induced

apoptosis to a lesser extent than CTX-OLA-ALB-NPs. These findings indicate that CTX-OLA-ALB-NPs, with their higher selectivity for programmed cell death, exhibited greater cytotoxicity in cancer cells. The cell cycle study demonstrated that OLA has the potential to arrest A549 cells in the G0 or G1 phase. CTX-OLA-ALB-NPs exhibited an enhanced pharmacokinetic profile, with a longer half-life (t1/2) compared to OLA-ALB-NPs and free OLA. Histopathology data indicated that CTX-OLA-ALB-NPs are safer nanoformulations.

Inspired by the anticancer potential of natural product-based compounds, we re-explored the triterpenoid class of compounds to select another therapeutic molecule for lung cancer therapy as part of another research work. In this direction, we explored Asiatic acid (ASA), also known as 2α,23-Dihydroxyursolic acid, which is a pentacyclic triterpenoid that occurs naturally in several plants used for food and medicine, such as Centella Asiatica, Shorea robusta, etc. Preclinical outcomes have proved the diverse beneficial effects of Asiatic acid, including neuroprotective, anti-inflammatory, cardioprotective, and antitumor activities, etc.

Despite exhibiting promising anti-proliferative activity, ASA's hydrophobicity and low aqueous solubility significantly limit its potential for widespread therapeutic use as an anticancer agent. For safe delivery of the therapeutic molecule to cancerous cells, we utilized nanoparticle-based drug delivery systems (DDS) due to their potential as an alternative therapy, including target/receptor-specific drug delivery, prolonged circulation, enhanced bioavailability, controlled and sustained drug release, reduced side effects, and the ability to deliver anticancer drugs with targeting agents for improved therapeutic effects.

We also aimed to optimize and develop biocompatible EGFR-targeted ALB-NPs that were loaded with Asiatic acid (ASA), a triterpenoid molecule derived from plants. In this study, we developed a cetuximab (CTX)-conjugated albumin nanoparticle-containing ASA as the therapeutic candidate for EGFR-targeted delivery to treat lung cancer. The underlying idea is that such a nanoformulation can enhance blood circulation time and increase tumor

accumulation through EGFR targeting and the EPR effect, leading to cancer growth inhibition. Initially, ASA was isolated and purified, and its analytical characterization was performed using NMR and HRMS. TEM images showed that both the cetuximab-conjugated nanoparticles (CTX-ASA-ALB-NPs) and the unconjugated formulation (ASA-ALB-NPs) were spherical and within the nanosize range. The conjugation with cetuximab resulted in a size increase of no more than 20 nm.

AFM images revealed many small nanoparticles (NPs) and occasional clusters of closely packed cetuximab-conjugated NPs in specific areas. Zeta potential measurements for ASA-ALB-NPs and CTX-ASA-ALB-NPs were found to be -26.7 ± 3.5 mV and -29.5 ± 4.6 mV, respectively. The study examined the *in vitro* release of ASA under both physiological and pathological conditions, showing an increased release of ASA at pH 5.5, which simulates the tumor environment.

The drug release profiles of ASA-ALB-NPs and CTX-ASA-ALB-NPs demonstrated an initial burst release followed by a slower, prolonged release, which is characteristic of sustained-release formulations. The overall drug release pattern remained largely unaffected by the conjugation of CTX to the nanoparticles.

The cytotoxicity assay results indicated that both ASA-ALB-NPs and CTX-ASA-ALB-NPs exhibited greater cytotoxicity than free ASA. Cellular uptake analysis provides insights into the lower cytotoxic effect of ASA compared to its nanoparticle formulations against A549 cells. The EGFR-targeted CTX-ASA-ALB-NPs showed improved binding efficiency to cancer cells with high EGFR expression, leading to increased cellular uptake of ASA. Thus, CTX-ASA-ALB-NPs demonstrated superior cytotoxic effects and intracellular uptake compared to ASA-ALB-NPs. Based on these cytotoxicity results, A549 cells were chosen for further *in vitro* experiments. Fluorescence microscopy images showed a steady increase in nanoparticle accumulation within A549 cells over 12 hours. Throughout the observation period, the green

fluorescence of C6 indicated that the accumulation of C6-CTX-ALB-NPs was significantly greater than that of C6-ALB-NPs at all observed time points.

Apoptosis involves the translocation of phosphatidylserine residues from the inner to the outer surface of the cell membrane, initiating programmed cell death. This process results in membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, and, ultimately, the formation of apoptotic bodies. To compare the induction of apoptosis in A549 cells by ASA, ASA-ALB-NPs, and CTX-ASA-ALB-NPs, the annexin V-Alexa Fluor/propidium iodide dual staining method was employed. Flow cytometry analysis revealed that, at identical concentrations and treatment durations, CTX-ASA-ALB-NPs induced the highest level of apoptosis, while ASA showed significantly lower apoptotic activity. Consequently, the apoptosis assay confirmed the superior efficacy of CTX-ASA-ALB-NPs. These findings suggest that CTX-ASA-ALB-NPs have enhanced cytotoxicity and higher specificity for inducing programmed cell death in cancer cells. Cell cycle analysis indicated that ASA and its formulations inhibited the progression of A549 cells through the G0/G1 phase. Additionally, the safety profile of ALB-NPs was evaluated through histological studies in Wistar rats following intravenous administration. Microscopic examination of H&E-stained tissue sections from the lungs, liver, heart, and kidneys of the rats revealed that the synthesized ALB-NPs were safe at the tissue level. The newly developed CTX-ASA-ALB-NPs, with their low toxicity and high therapeutic efficacy, present a promising nanomedicine option for effectively treating lung cancer.

This thesis also includes research works focussed on semi-synthesis derivatization of bioactive compounds of plant origin of novel or known structures as lead to produce entities of higher activity and/or lower toxicity.

Natural products, primarily secondary metabolites from plants, microorganisms, and animals, continue to play a significant role in modern drug discovery. Terpenoids, known for their vast

diversity and numerous biological activities, have garnered considerable interest. Utilizing terpenoids and their derivatives for breast cancer treatment appears to be a promising strategy. 1,2,3-triazoles have been considered a promising moiety for the preparation of drugs against cancer. Despite being a synthetic scaffold, it showed great viability and functionality in embedded natural hybrid molecules.

Recognizing the pressing need for potent and safer anticancer agents in clinical practice and considering the pharmacophoric affinity of terpenes towards receptors overexpressed in cancer, a novel class of triazole-tethered monoterpene hybrid molecules was designed. Inspired by the significance of the incorporation of 1,2,3-triazole functionality in the natural scaffold and in continuation of the search for potent anticancer agents, we used a hydroxyl group-containing natural bioactive precursors, including monoterpenes, for the modification.

Furthermore, the cytotoxic effects of the synthesized derivative compounds were assessed using *in vitro* experiments and molecular docking studies. This research offers a novel perspective on discovering effective anti-cancer agents through the structural modification of natural products.

The semi-synthetic derivatives were subjected to *in vitro* anticancer evaluation by using MTT assay. From the IC_{50} values, it was found that most of the compounds retained anticancer activity with improved cytotoxicity as compared to parent compounds. The results of these experiments also showed that most of the synthesized compounds inhibited MCF-7, MDA-MB-231, A549, SiHa, and BL60 cells in a concentration-dependent manner. The most potent among all derivatives were subjected to detailed anticancer studies such as apoptosis assays, cell cycle analysis, etc.

The study revealed that compounds VNS10 and VNS15 provide a suitable core to be exploited for further structure-activity relationship (SAR) and *in vivo* studies to develop potent anticancer agents.