ISOLATION, STRUCTURAL MODIFICATION AND FORMULATION DEVELOPMENT OF NATURAL BIOACTIVE COMPOUNDS FOR CANCER THERAPY

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By

Vishwa Nath Shukla

DEPARTMENT OF PHARMACEUTICAL ENGINEERING & TECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY

(BANARAS HINDU UNIVERSITY)

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Summary

The research work in the present thesis was inspired by the anti-cancer potential of naturally occurring bioactive phytochemicals, nanotechnology based targeted delivery systems and chemical reactions such as click reaction used for structural modification of natural products. All standard anticancer drugs are cytotoxic and can even damage normal cells, leading to severe non-specific cytotoxicity. Therapeutic effect of any bioactive natural product molecules can be enhanced either by their semi-synthetic chemical modification or by their targeted delivery to cancerous site. The main objective of the targeted drug delivery in cancer is to deliver the anticancer drugs to the specific tumor site in the desired concentration. Nanomedicine can be employed via passive targeting (primary targeting), active targeting (secondary targeting) or both to reduce the severe non-specific toxicity of anticancer medications. It has been demonstrated that the commonly used anticancer derivatives such as paclitaxel, docetaxel etc. may increase the survival rate of cancer patients by reducing tumour volume. However, prolonged treatment of patients with anticancer drugs leads to the development of drug resistance which can decrease the overall efficacy of the treatment.

In one of our studies, 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. 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 incorporation and interaction of OLA to ALB were confirmed by FTIR, XRD, DSC, and TGA. Moreover, XPS analysis also supports the conjugation of CTX on OLA-ALB-NPs. The unconjugated formulation (OLA-ALB-NPs) and the CTX-conjugated formulation (CTX-OLA-ALB-NPs) were found to be in nanosized range. There was a maximum increase of 20 nm in particle size due to CTX conjugation. The AFM images revealed that the surface of the ALB-NPs was smooth. Based on the TEM images, both nanoparticles were spherical and had a darker zone on their inner sides, indicating that drug deposition was concentrated within the cores of the ALB-NPs. The zeta potential values of OLA-ALB-NPs and CTX-OLA-ALB-NPs were found to be -30.5 ± 2.8 mV and -33.3 ± 3.4 mV, respectively. The change in zeta potential to the negative side is assumed to be caused by the negative charge due to the dominating -COOH groups in CTX, which made the surface of the targeted NPs less positive. As a result, after antibody conjugation, the nanoparticles' zeta potential was moved to the negative side. Drug's release profile demonstrated a rapid initial release, followed by a slow and prolonged release over time. This finding also suggests that CTX conjugation had minimal effects on the drug's release profile. The fluorescence microscopy images obtained by cellular-uptake study supported that CTX-OLA-ALB-NPs internalized to a higher extent in A549 cells as compared to OLA-ALB-NPs. The accumulation of C6-CTX-ALB-NPs was much higher ($p < 0.001$) than that of C6-ALB-NPs as detected by the green fluorescence intensity of C6. The MTT test revealed that the OLA-ALB-NPs and CTX-OLA-ALB-NPs were more cytotoxic than the free-OLA. The IC_{50} values were in the order of CTX-OLA-ALB-NPs < OLA-ALB-NPs < OLA in the case of A549 cells after 24 h of incubation, suggesting CTX-OLA-ALB-NPs was the most cytotoxic.

The IC_{50} values of CTX-OLA-ALB-NPs against A549 cells were about 23 folds ($p < 0.001$), 3 folds ($p < 0.001$), lesser as compared to OLA-ALB-NPs and OLA respectively after 24 h. The cellular uptake analysis explains why OLA and its formulations have such a modest cytotoxic effect on A549 cells. Consequently, CTX-OLA-ALB-NPs outperformed OLA-ALB-NPs in terms of cytotoxicity and intracellular uptake in A549 cells. The annexin V-Alexa fluor/propidium iodide dual staining technique was used to compare the apoptosis induction in A549 cells by OLA, OLA-ALB-NPs, and CTX-OLA-ALB-NPs. According to flow cytometry, the apoptotic population was highest for CTX-OLA-ALB-NPs and significantly lower for OLA. Apoptosis was induced relatively less by OLA-ALB-NPs than by CTX-OLA-ALB-NPs. In CTX-OLA-ALB-NPs, the percentage of necrosis was lower than that in OLA-ALB-NPs or OLA. The data revealed that CTX-OLA-ALB-NPs with higher selectivity for programmed cell death had more cytotoxicity in cancer cells. It was shown in the cell cycle study that OLA might be able to stop A549 cells at the G0 or G1 phase of the cell cycle. CTX-OLA-ALB-NPs showed an improved pharmacokinetics profile, higher $t_{1/2}$ and C_{max} compared to OLA-ALB-NPs, and free OLA. As per histopathology data, CTX-OLA-ALB-NPs appeared to be safer nanoformulations.

In summary, the CTX-OLA-ALB-NPs displayed improved therapeutic responses in 24 h in the lung cancer A549 cell line. Reactive oxygen species production and a greater degree of apoptosis activation, which destroy cancer cells, contribute to enhanced efficacy. Treatment with CTX-OLA-ALB-NPs resulted in more cells being arrested in the G0/G1 phase than treatment with either free OLA or OLA-ALB-NPs.

In another research work, we developed a CTX-conjugated albumin nanoparticle containing asiatic acid (ASA) as the therapeutic candidate for EGFR targeted delivery to treat lung cancer. The underlying concept is that a nanoformulation of this kind could improve blood circulation

time and increase accumulation at tumor through both EGFR targeting and the EPR effect, which would ultimately inhibit tumor growth.

In silico docking studies with proteins like albumin, beta-lactoglobulin, and lactoferrin resulted that albumin being the appropriate polymer for ASA entrapment, and studies revealed that albumin-ASA complexes were formed through hydrogen bonding, alkyl interactions, and van der Waals interactions with a binding energy of -7.9 kcal/mol.

Initially, isolation and purification of asiatic acid were carried out, and it was characterized analytically by NMR and HRMS. Both non-targeted and targeted ALB-NPs of asiatic acid were prepared and characterized with different sophisticated techniques. Transmission electron microscopy (TEM) images revealed that both the cetuximab-conjugated nanoparticle (CTX-ASA-ALB-NPs) and the unconjugated formulation (ASA-ALB-NPs) were spherical and fell within the nano-size range. The conjugation of cetuximab resulted in a size increase of no more than 20 nm.

The AFM images showed the presence of numerous small NPs and occasional clusters of closely packed cetuximab-conjugated NPs in certain regions. The zeta potential measurements for ASA-ALB-NPs and CTX-ASA-ALB-NPs were recorded as -26.7 ± 3.5 mV and -29.5 ± 4.6 mV, respectively. The negative shift in zeta potential can be attributed to the presence of cetuximab, which increased the negative charge of the albumin COOH groups and resulted in an excess negative charge after conjugation. The study investigated the *in vitro* release of ASA under physiological and pathological conditions, revealing a higher release of ASA at pH 5.5, which mimics the tumor environment.

The molecular docking analysis revealed that ASA binds effectively with ALB, forming a 3D socket in the crystal structure of 4JK4 through two primary hydrogen bonds (GLU299 and LEU301) and numerous van der Waals bonds. This interaction exhibited a favorable binding affinity score (-7.9 kcal/mol) when considering different protein receptors acting as

nanocarriers. The results of this study indicated a reasonable correlation between the affinity scores obtained theoretically and the *in vitro* cellular uptake.

The drug release profiles of both ASA-ALB-NPs and CTX-ASA-ALB-NPs exhibited an initial rapid release followed by a delayed and sustained release, which is a typical feature of sustained-release formulations. The drug release profile was found to be largely unchanged after the conjugation of CTX with the NPs.

The results of the cytotoxicity assay demonstrated that both the ASA-ALB-NPs and CTX-ASA-ALB-NPs had higher cytotoxicity compared to free asiatic acid. After a 24 h treatment, the IC_{50} values for A549 cells indicated that the cytotoxicity order was ASA < ASA-ALB-NPs < CTX-ASA-ALB-NPs, highlighting that CTX-ASA-ALB-NPs were the most cytotoxic. The cellular uptake analysis offers insight into the cytotoxicity effect of ASA and its formulations against A549 cells. The EGFR-targeted CTX-ASA-ALB-NPs exhibited enhanced binding efficiency to cancer cells that overexpress EGFR, resulting in increased cellular uptake of ASA. To summarise, the CTX-ASA-ALB-NPs exhibited superior cytotoxic effects and intracellular uptake compared to ASA-ALB-NPs. Based on the observed cytotoxic effect of ASA and its formulations against A549 cells, we selected A549 cells for further *in vitro* experiments. The fluorescence microscopy images indicated a gradual increase in nanoparticle accumulation within A549 cells after 18 h. Throughout the experimental observation, the green fluorescence of C6 indicated that the accumulation of C6-CTX-ALB-NPs was significantly higher than that of C6-ALB-NPs at any given time.

Apoptosis is characterized by the translocation of the phosphatidylserine residue from the inner to the outer surface of the cell membrane, resulting in programmed cell death. This translocation leads to cell membrane blebbing, followed by cell shrinkage, chromatin condensation, nucleus fragmentation, and, eventually, fragmentation of the cells into apoptotic bodies. The annexin V-Alexa fluor/propidium iodide dual staining method was used to

comparatively determine the induction of apoptosis in A549 cells by ASA, ASA-ALB-NPs, and CTX-ASA-ALB-NPs. Flow cytometry analysis showed that, at the same concentration and treatment time, the apoptotic population was highest for CTX-ASA-ALB-NPs, while it was significantly lower for ASA. Therefore, the apoptosis assay also demonstrated the superior capability of CTX-ASA-ALB-NPs. The results indicate that CTX-ASA-ALB-NPs exhibit greater cytotoxicity with a higher degree of specificity toward inducing programmed cell death in cancer cells. According to the cell cycle analysis, ASA and its formulations were found to inhibit the progression of A549 cells through the G0/G1 phase.

The safety profile of ALB-NPs was also assessed by histological studies in the Wistar rats after *i.v.* treatment of ALB-NPs. Microscopical observations of H&E stained extracted sections of the lungs, liver, heart, and kidney of rats showed that the synthesized ALB-NPs were safe at the tissue level.

In another research work, we have synthesized and biologically evaluated the click chemistry-inspired triazole derivatives of natural product molecules (MDL and IPG) for breast cancer treatment. Interesting biologically active substances are synthesized from a variety of compounds isolated from natural sources. Monoterpenes have been identified as responsible for important therapeutic effects of plant extracts. Structural modifications on these pure monoterpenes were then carried out with a view to enhancing the biological activity. Starting from monoterpenes bearing an alcohol functional group, a terminal alkyne moiety was introduced, leading to the corresponding O-tethered alkynes. These alkynes were then involved in metal-catalyzed reactions leading to triazole moieties.

In this work, we attempted to evaluate the cytotoxic effect of terpenes derivatives synthesized by using click chemistry. The *in vitro* anticancer activity of the tested derivatives, evaluated against five tumor cell lines, show that the VNS10 is the most cytotoxic derivative towards breast cancer (MCF-7 cells) with an IC_{50} value of 2.38 ± 1.16 µM. All the synthesized

derivatives appeared to be safe and non-toxic on normal kidney cell lines (HEK-293). The effect of the tested derivatives on cell cycle progression was examined by flow cytometry in order to investigate the molecular mechanism of their cytotoxic activity. The results revealed that VNS10 stopped the cell cycle progression in G2/M phase.

In summary, in this study, we report that 1,2,3-triazole pharmacophores added on to a monoterpene skeleton increased the *in vitro* anticancer activity and thus should be further studied through the preparation and evaluation of a larger family of new terpenoids based on these structural features. New terpene derivatives could be the basis of a new class of anticancer compounds.

5.2. Conclusion

To conclude, the development and characterization of EGFR targeted ALB-NPs of oleanolic acid and asiatic acid were carried out and their physicochemical characterization, *in vitro* release, cellular uptake, cytotoxicity, *in vivo* pharmacokinetics, *in vivo* histopathology studies were performed.

The particle size and polydispersity of both the formulations were within acceptable limits. The zeta potential of CTX-OLA-ALB-NPs was higher (-33.3 ± 3.4 mV) than that of OLA-ALB-NPs (-30.5 ± 2.8 mV), indicating their higher stability. Similarly, zeta potential of CTX-ASA-ALB-NPs was higher (-29.5 ± 4.6 mV) than that of ASA-ALB-NPs (-26.7 ± 3.5 mV), indicating their higher stability.

The morphological assessment by TEM and AFM analysis showed that OLA-ALB-NPs and ASA-ALB-NPs were spherical and monodispersed. The XPS survey demonstrated the surface chemistry of CTX-OLA-ALB-NPs and CTX-ASA-ALB-NPs, and the result supports the presence of cetuximab on the surface of nanoparticles.

The *in vitro* drug release profile of OLA and ASA in CTX-OLA-ALB-NPs and CTX-ASA-ALB-NPs showed the pH-dependent drug release with a rapid release at pH 5.5 than pH 7.4

The C6-loaded EGFR-receptor-targeted nanoparticles of CTX-C6-ALB-NPs showed enhanced cellular uptake as compared to pure C6 and non-targeted nanoparticles in A549 cells.

The OLA-loaded EGFR-targeted ALB-NPs displayed approximately 2-fold greater cytotoxicity against A549 cells than non-targeted nanoparticles. The pharmacokinetic evaluation of both nanoparticle formulations was performed in Wistar rats, and all the nanoparticle formulations for both drugs exhibited improved pharmacokinetics compared to their respective bioactive natural moieties.

The *in vivo* histopathological evaluations of EGFR targeted nanoparticles of both drugs have demonstrated better safety in Wistar rats as compared to their bioactive natural moieties. The EGFR targeted CTX-OLA-ALB-NPs and CTX-ASA-ALB-NPs of OLA and ASA demonstrated significant enhancement of anticancer efficacy when compared to their respective bioactive natural moieties.

The results obtained from *in vivo* anticancer studies are in absolute agreement with those observed in *in vitro* toxicity studies of both the EGFR targeted formulations, showed a significant reduction in the cell number, which was measured in terms of percent cell viability in MTT assay in A549 cells.

The objective of the thesis also included the synthesis and biological evaluation of click chemistry-inspired triazole derivatives of natural product molecules for cancer treatment.

This study found compound VNS10 as the most active among all the tested cancer cell lines with IC50 values of 2.38 ± 1.16 μM. Compound VNS10 induced apoptosis and G2/M arrest in breast cancer (MCF-7) cells. VNS10 emerged as a promising compound, therefore, could serve as a lead compound for the further development and identification of 1,2,3-triazole based anticancer agents.

In order to elucidate the plausible molecular mechanism of anticancer activity and to investigate the possible binding modes of the most active compound (VNS10), molecular

docking simulations were conducted into the binding sites of molecular target; human estrogen receptor alpha ligand-binding domain in complex with 4-hydroxytamoxifen (PDB ID: 3ERT, resolution 1.9 Å) using GROMACS. The best docked poses, presumed to represent the ligand-protein interactions, were selected based on their docking scores, binding interactions with the key amino acid residues and alignment of the ligand at the active site in a relatively similar manner to the co-crystallized ligand.

Inspection of the best-docked pose of compound VNS10 indicated that it was perfectly positioned in the active site of 3ERT with binding energy score of -10.001 kcal/mol.

Among all the derivatives synthesized, VNS10 was the most potent as it not only inhibited cancerous cells but also increased ROS generation leading to onsets of apoptosis. Thus, the results of this research work and thesis have provided a new perspective on the function of terpenes and their semi-synthetic derivatives in tumour chemotherapy, and we believe that further exploration of similar terpene-based derivative drugs will reveal additional chemotherapeutic mechanisms.

5.3. Future Perspective

The focus of our future research is to develop dual-receptor-targeted and multiple-receptor-targeted nanomedicines, which can be beneficial to a large number of patients based on the receptor expression levels in specific cancer types. We also want to integrate dual-targeting nanomedicine with theranostics to improve diagnostic outcomes since it is anticipated that dual-receptor targeted nanomedicine would accumulate selectively in cancer locations. We also want to evaluate and establish the application of these targeted nanomedicine in other types of lung cancer such as squamous cell carcinoma, large cell lung carcinoma and small cell lung carcinoma after their successful development by chemical carcinogens in mice.

In future human serum albumin (HSA) can be used in place of BSA to avoid any possible immunologic response.

Furthermore, the development of nanoformulations derived from the potent lead molecules obtained from click chemistry-inspired triazole derivatives of natural product molecules can be carried out and will be further explored for enhancing their solubility, and therapeutic effect for cancer treatment. Moreover, an extensive structure-activity relationship (SAR) study can be performed for rational optimization of the lead structure. Follow-up studies will be devoted to deeply investigating the molecular mode of their cell death induction. The complete *in vivo* pharmaco-toxicological analysis will support assessing if a lead compound could be regarded as suitable for its development as effective anticancer therapeutics.