Assessment of Drug Delivery and Anticancer Activity of Multikinase Inhibitor in Lung Cancer

SUMMARY

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Summary and Conclusion

The present study is focused on development and assessment of polymeric nanoparticulate system designed to target lung carcinoma. The targeted delivery system is believed to deliver drug specifically to the cancer cells, resulting in high payload thus enhanced therapeutic efficacy. Such systems are capable to easily discriminate between the cancer and normal cells, thereby reducing dose related toxicity. Some xenobiotics (Capsaicin and Naringenin in current study) bestowed with antioxidant, anti-inflammatory, antiproliferative and anticancer properties. These xenobiotics were taken along with synthetic drug widely employed in lung cancer (gefitinib) in combination and co-therapy was executed, to get a synergistic effect. Also, the antioxidant property of xenobiotics reduces the oxidative stress and aided in overcoming multidrug resistance. Taking advantage of this approach the therapeutic outcome of anticancer agents can be enhanced when compared with therapy through single drugs.

The anticancer drug selected for current study was gefitinib (Gnb) as it is one of the most widely adopted drugs for treating lung cancer. Further, to improve its therapeutic potential xenobiotics capsaicin (Cap) and naringenin (Nar) were taken for co-therapy. The selected drug candidates for the present study were characterized for identification and purity by FTIR and UV spectroscopy.

Based upon the compatibility, desired biocompatible, biodegradable and ease in surface tailoring properties PCL and PLGA polymers were selected. The selected polymers were anticipated to possess capability of giving stable formulation(s), having high %EE and ability to deliver drug to the targeted site. For polymeric nanoparticles (NPs), polymer and drug ratio was selected on the basis of stability, particle size, %EE, drug loading and *in vitro* release.

For the optimization of NPs formulation(s) different factors selected included drug:polymer ratio (1:2), copolymer PVA concentration, cremophore EL concentration and stirring speed. A 3-factor 3-level central composite design (CCD) was selected for optimization of polymeric NPs which suggested 09 experimental trials with different combination of factors. All the trials for PCL and PLGA NPs were individually formulated and evaluated for response variables like, particle size, %EE, drug release and drug loading.

The experiential data for both the NPs systems were analysed mathematically, using quadratic equations and best fit model that generated best fitted equations was obtained. Response Surface Model (RSM) was implemented which best validated the relationships among selected factors and responses accompanied by interactions among factors, if any. The RSM advised significant effect of drug:polymer ratio on various responses i.e., particle size, %EE, drug release and drug loading of NPs.

Further, the design space was generated using overlay plot and optimized formulation was recognised by putting definite criteria for different response factors. The criteria applied for the NPs optimizations were fixed at the values *viz.* particle size below 250 nm, entrapment efficiency above 50%, drug release not less than 80% and drug loading above 10%.

The formulation composition optimized for PLGA NPs were drug:polymer ratio (2:1), PVA concentration (2.5%v/v), whereas for PCL NPs the composition taken was drug:polymer ratio (2:1), and PVA concentration (2.5%v/v) with a stirring speed (500 rpm) which was found suitable to yield NPs of desired characteristics. A good agreement was witnessed between predicted values and observed values of response variables for all the systems.

The targeting module folate was successfully conjugated on the surface of PLGA NPs with the help of NHS and DCC as evidenced by means of FTIR and NMR spectrum. The characteristic peaks for various groups and structural configurations were observed and confirmed through aforementioned spectra. Estimation of folate content further witnessed the folate conjugation to the PLGA NPs surface. The amount of folate attached was found to be 5.67 µM of folate/g of polymer. The physicochemical characterization of folate conjugated NPs (Gnb-PLGA-PEG-FA and Cap-PLGA-PEG-FA) revealed the particle size of 217.0±3.2nm and 213.0±5.2 nm, zeta potential -13.19±2.01mV and -14.45±0.88 mV, entrapment efficiency of 49±1.5% and 46±2.3% percent drug loading of 20.88±1.7% and 21.77±1.3%, respectively.

The biotin was successfully anchored on the surface of PCL NPs with help of NHS and DCC as denoted by characteristic peaks of FTIR and NMR spectrum. The estimation of biotin content through HABA/avidin assay, further confirmed the biotin anchoring to the surface of PCL NPs. The biotin content was found to be 39.43 µg mg^{-1} and 38.57 µg mg^{-1} for bty-Gnb and bty-Nar respectively. The physicochemical characterization of biotin

anchored NPs (bty-Gnb and bty-Nar) displayed the particle size of 201±3.2nm and 203±2.7 nm, zeta potential -21.04±1.4mV and -21.19±2.3 mV, entrapment efficiency of 44±2.9% and 43±2.3% and drug loading of 23.23±1.97% and 21.23±1.3% respectively.

The hyaluronic acid (HA) was successfully anchored on the surface of capsaicin loaded PCL NPs employing layer by layer technique (LBL). A polycationic layer of a polymer was utilized as a linker between two polyanionic layers. Chitosan (CH) was sandwiched between PCL and HA so as to form a polycationic layer for fabrication of the LBL assembly. The physicochemical characterization of HA decorated NPs (HA-PCL-CAP) displayed the particle size of 194±2.90nm, zeta potential -27.87±3.21 mV mV, entrapment efficiency of 52.89±2.19% and drug loading of 13±0.37% respectively.

The release pattern in case of PCL/PLGA NPs was found to be sustained. Approximately 85.65±3.21% and 81.43±4.32% for Gnb and Cap were released from Gnb-PLGA-PEG-FA and Cap-PLGA-PEG-FA over a period of 80 h. About 81.94±3.12% of Gnb and 80.99±4.99% of Nar were released from biotin modified PCL NPs over a period of 80 h. HA-PCL-CAP NPs exhibited sustained release behaviour with 82.43±1.93% drug release in 48 h. An insignificant change in the release profiles was observed before and after surface modification of NPs in all the three formulations, since these experiments were done *in vitro*. Further, the isobologram evidenced the synergistic effect of combination therapy. The combination index value obtained was below 0.3, which indicates a very good synergistic effect of Gnb when combined with Cap/Nar.

The folate modified, biotin modified and HA modified NPs were found to be stable during the studies performed as per ICH guidelines over a period of three months.

In vitro cell lines studies executed over A549 cell lines, displayed superiority of folate, biotin and HA decorated NPs over unmodified formulations. The supremacy of surface engineered formulation was ascertained through cell cytotoxicity assay, cell cycle analysis, cellular proliferation studies, apoptosis assay, cellular internalization, MMP and ROS studies. Further superlative response of cotherapy over individual therapy either by Gnb/Nar/Cap was evidenced by aforementioned studies in case of folate and biotin conjugated formulations.

MTT assay displayed a supressed IC_{50} value of formulation when given in combination as modified NPs. The IC_{50} values were found to be 23.5±0.76µM in cells treated with

Gnb and Cap combination (Cap@GnbPLGA-PEG-FA) and 25.4±0.32µM in case of combination treatment through Nar and Gnb (Gnb-bty-Nar) which was relatively low when compared with the IC_{50} values obtained in Gnb/Nar/Cap treated cells individually. Also, the HA decorated Cap displayed a depressed IC_{50} value of 39.74±2.11 µM when compared with unmodified Cap.

Cell cycle studies through FACS demonstrated that Cap@GnbPLGA-PEG-FA (co-therapy) arrested the A549 cells in G0/G1, S and G2/M phases respectively. While, A549 cells were preferentially arrested in G1 phase, G1phase and G0/G1 phase respectively in case of Gnb-bty-Nar co-therapy.

Cell proliferation studies revealed that the inhibition of cellular proliferation for Cap and Gnb cotherapy was much higher than individual therapy. The inhibition potential followed an order of **1**Cap@Gnb-PLGA-PEG-FA> **2**Gnb-PLGA-PEG-FA> **3**Cap-PLGA-PEG-FA> **4**Gnb-PLGA> **5**Cap-PLGA> **6**Gnb> **7**Cap. While, the order for inhibition of cellular proliferation various formulation of PCL NPs, was found in order of Gnb-bty-Nar>bty-Gnb>bty-Nar>PCl-Gnb>PCl-Nar>Gnb>Nar. The data suggested that modified formulations when given in combination displayed maximum cellular mortality when compared to individual drug formulations treated cells. Correspondingly, ROS and MMP data suggested the superiority of the HA decorated NPs, biotinylated NPs and folate anchored NPs over unmodified formulations. Also, it was demonstrated that when natural agents were given in combination, the effect obtained was superior in comparison to response obtained through individual drug. Cellular internalization studies through florescence microscopy demonstrated enhanced internalization of modified formulation in comparison to unmodified formulation.

The supremacy of modified formulations over unmodified formulation as concluded from *in vitro* cell lines data specified that surface modifications facilitated these formulations in improved cellular internalization through overexpressed receptors. Also, formulation being nanosized, possess passive targeting resulting in enhanced permeation and retention. Further xenobiotics, including phytopharmaceutical agents are expelled out from the cells due to efflux pumps present in prokaryotes and eukaryotes, leading to sub-therapeutic concentrations at desired site. However formulating them as surface modified, receptor specific, nanoformulations can lead to higher internalization into the targeted cells. Also, phytochemicals are known to act through several pathways thus a

combination of natural and synthetic drug can lead to enhanced apoptosis effected through different pathways.

In vivo studies were performed on albino wistar rats weighing 120-150 g, of either sex. The protocol for animal testing was approved through institutional ethical committee and experiments were completed in accordance to the CPCSEA guidelines for laboratory animals and ethics, Department of Animal Welfare, Government of India. Animals were randomized and divided into six groups of 12 animals each for folate conjugated and biotin conjugated NPs, while animals were divided into five groups in case of HA modified NPs. Lung cancer was induced by three consecutive i.p. injections of urethane, within a gap of 48 h over a period of one week. The development of lung tumors was initiated over a period of 8-12 weeks. During treatment period, various formulations were administered (20 mg/kg e.q. Gnb, 10 mg/kg e.q. Cap and 30 mg/kg e.q. Nar) to respective groups via intravenous tail injection at an interval of three days. The treatment was continued for a period of four weeks, followed by observation of the animals for indication of toxicity, weight loss and mortality, of animals during the study. A gap of 12 weeks was taken to develop the cancer in animals. Toxic group exhibited highest incidence of mortality, highest tumor volume and reduction in animal weight, followed by pure drug receiving group, Gnb-PLGA-PEG-FA/Cap-PLGA-PEG-FA and Cap@GnbPLGA-PEG-FA. Highest recovery was observed in the group receiving co-therapy through Cap and Gnb. In case of biotin decorated formulation reduction in animal weight declined to 46% for toxic group, 31.23% reduction for bty-Nar, 11.32% for cotherapy (Gnb-bty-Nar) group. Similarly for HA modified formulation, toxic group showed mean reduction in animal weight of 49%, PCL-CAP treated group showed 35.32% reduction, HA-PCL-CAP group displayed10.25% reduction.

The mortality incidents of animal were found to be lowest in groups receiving cotherapy, followed by biotin decorated/folate conjugated groups. Similarly the highest survival was observed in case of group receiving HA modified CAP. Toxic groups presented lowest animal survival rate, in all the three formulations (folate/ biotin/ Hyaluronic acid conjugated formulation). Likewise, trends were observed for tumor volumes and tumor incidence for all the three formulations.

The urethane-induced lung carcinoma brings about a significant imbalance in biochemical mechanisms of cells. It was clearly indicated by noticeable alteration in the

levels of oxidative stress markers like TBARS, protein carbonyl, GSH, SOD and catalase. Treatment with different formulation restored the levels of these imbalanced markers towards normal and order of activity observed was cotherapy>folate conjugated NPs> unmodified NPs>pure drug. Likewise, biotin modified co-therapy and HA modified NPs revealed highest efficiency in restoration of altered levels of oxidative stress markers towards normal.

Western blotting analysis advocated that administration of urethane to the animals triggered the up-regulation of antiapoptotic proteins such as bcl-2, and MMP-9 and down-regulation of pro-apoptotic markers such as bax, caspase-3, P-16 and caspase-9. The treatment of animal with Gnb and folate decorated formulations reinstated the level of these proteins towards normal but the effect was more intense in case of cotherapy through Cap@Gnb-PLGA-PEG-FA. Parallel trends were observed in case of cotherapy through Nar and Gnb, and therapy through HA modified formulations. Treatment of urethane administered group with various formulations resulted in down-regulation of anti-apoptotic proteins bcl-2 and MMP-9 while upregulation of pro-apoptotic proteins like BAX, caspase-9 and p16 expression was observed representing tumor inhibitory potential of all the three modified formulation. Further, highest restoration was observed with cotherapy through Gnb and Nar as well as Gnb and Cap. Concomitantly, the proteins p-16, MMP-9 and members of the bcl-2 family protein, comprising bcl-2, BAX *etc.* plays an important role in migration, invasion and metastasis of lung cancer. The outcomes of our study demonstrated that the biotin decorated NPs, folate decorated NPs and HA decorated NPs can efficiently reduce the lung tumor development in the cancer induced rats. Further, the cotherapy has proved its enhanced therapeutic potential for treating urethane-induced lung carcinoma when compared with individual therapy through either of drugs (Gnb/Cap/Nar).

Angiogenesis and cell proliferation is important characteristic feature of cancer growth. The development of carcinoma was represented through rosette shaped structured tumor cells and peribronchial cells which infiltrate the bronchial submucosa after urethane administration. The co-therapy through Cap@Gnb-PLGA-PEG-FA and Gnb-bty-Nar formulations revealed restoration of cellular architecture towards normal. Parallel results were demonstrated in HA-PCL-CAP treated animals.

The Gnb concentration accumulated in the lung carcinoma was highest in the case of the Gnb-PLGA-PEG-FA (45.17±3.23 µg/g in 4 h) followed by the Gnb-PLGA (37.43±2.91 µg/g organ in 4 h) indicating the site-specific accumulation of the folate conjugated NPs in folate over-expressed tumor cells in lung carcinoma. Further, Gnb-PLGA-PEG-FA substantially maintained greater Gnb levels in the tumor organ over a period of 48 h (21.45±1.09 µg/g tumor organ) ($p<0.001$) and 37.87±1.33 µg/g organ upto 12 h. The concentration of Gnb was found significantly lower($p<0.01$) in groups *vis-à-vis* pure Gnb (10.53±3.46 µg/g in 12 h, 3.05±0.53 µg/g in 48 h) and the Gnb-PLGA (13.32±2.34 µg/g in 12 h, 1.49±0.393 µg/g in 48 h) treated groups. In case of unmodified NPs highest Gnb concentration was detected in the liver among various organs, followed by the spleen, the heart and the kidney. Overall, Gnb accumulation was found significantly lower in aforementioned organs in folate decorated NPs treated groups than that of groups treated with unmodified formulations. Similar, results were perceived with biotinylated NPs. The highest concentration was maintained by bty-Gnb in cancer cells followed by Pcl-Gnb and Gnb. The biodistribution data further confirmed the discriminatory entry of the biotinylated NPs towards the biotin receptor that are over-expressed in diseases like lung carcinoma. Parallel results were revealed by HA decorated NPs. The maximum concentration of Cap was achieved in cancer tissue after HA-PCL-CAP administration (49.35±3.22 µg/g) followed by PCL-CAP (29.34± 1.45 µg/g) and CAP (09.21±1.02 µg/g). However, an insignificant difference in drug concentration was found when a comparison was made between co-therapy and individual therapy through modified NPs.

Further, the metabolomics study revealed restoration of elevated levels of amino acids and decreased glucose level towards normal levels in biotinylated NPs (cotherapy) treated groups. The results revealed inhibition of cell growth indicating reduced metabolism and catabolism thus it can be said that the cell death induced by co-therapy when formulated as modified NPs.

On the basis of results summarized above, ligand directed drug delivery system was found therapeutically effective and safe for regulating the cancer proliferation, in lung carcinoma. The mammalian cells stand incompetent in synthesizing some of the essential biomolecules obligatory for their growth and proliferation. Thus, these have to be taken up through some transport systems such as sodium-dependent multivitamin transporter (SMVT), high-affinity biotin transporter for biotin uptake and high affinity folate receptor for folate uptake. Also, CD44 over expressed in cancer cells that binds to its

primary ligand HA and is stated to be responsible for cellular signaling, leading to regulation of biological process within cells. Thus a swift uptake of NPs carrying such molecules is witnessed via these overexpressed receptors, delivering the drug payload. The overexpression of these receptors over cancer cell and selective uptake of such targeting modules in the cancer cells could be a probable justification for selective uptake of molecularly designed targeted NPs for lung cancer. Conjugation of targeting moiety utilizing a moderately long PEG chain linker yielded an effective ligand-receptor molecule and supports its internalization into cancer cells. Further, it has been demonstrated that co-therapy with Cap/Nar has cancer suppression and inhibitory effect. Additionally phytoconstituents have antioxidant property and are known to act through several pathways because of which multi drug resistance can also be addressed. Further, prolonged drug release maintains higher concentration of antineoplastic agents/xenobiotic leading to greater cell cytotoxicity. The combination therapy, that too as a targeted delivery, would successfully address lung carcinoma. Finally, the present investigation exhibits the prospects of co-administration of targeted module conjugated NPs as synergistic therapy to offer enhanced therapeutic efficacy and antitumor effect. In conclusion, developed nano-particulate system(s) demonstrated minimal toxicity, facilitated targeted delivery to tumor sites, with diminished access to normal cells as a result of surface modification. In light of the above studies it can be concluded that safe, natural, anticancer agents like Cap/Nar can be an alternative to synergize synthetic anticarcinogenic agents to be formulated as nanoformulations and conventional formulations for management of lung carcinoma. Further studies can be performed to recognize the effect of combination therapy on carcinomas and subsequent scale-up from bench to bedside after suitable clinical evaluations.