Development and Characterization of Targeted Green Nanotechnology Based Delivery System(s) for Cancer Therapy

SUMMARY
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Summary

Many efforts are being made to triumph over cancer amongst which, the use of natural constituents as chemopreventive, as well as chemotherapeutic agents, have shown a rise, because of their safety, availability and general acceptance. Inositol hexaphosphate (IP6) is as such a natural bioactive constituent of cereals, legumes etc. Various researchers have already proved that the intake of IP6 containing legumes is connected with the lowering of cancer incidences. Instead, this potent bioactive carbohydrate is not very popular in cancer therapy due to its shortcoming of fast chelation and elimination from the body, within an hour of administration. Present studies focus on the development of a suitable dosage form(s) of IP6 that may enhance its residence time, thereby increasing its chemopreventive as well as chemotherapeutic effect.

The size of nano-material is the property that makes them ideal for increasing residence time, surface area, higher drug loading, rapid commencement of therapeutic action etc. Anticancer drugs in nanoformulations display superior therapeutic index as a result of improved pharmacokinetics, distribution and accumulation of drug at the tumour site. The nano-sized system exhibited more permeability into the tumour site, as tumour sites have a leaky vasculature. Consequently, nano drug delivery systems can be successful tools for anticancer therapy. Thus, the preparation and evaluation of IP6 containing nanoformulations were explored in this research work.

Experimental section was divided into two parts: part I deals with the preparation and evaluation of IP6 loaded niosomal suspension and part II deals with the preparation and evaluation of IP6 loaded gold nanoparticles.

Identification of drug sample was performed through melting point and FTIR analysis. Melting point was found to be in the range of 22-25°C. FTIR analysis displayed the characteristic peak at 3430.3 cm^{-1} which relates to OH stretching, peaks near 1662.1 cm^{-1} was probably due to the carboxyl group and at 1061.5 cm^{-1} allocated the phosphate radical or hydrogen phosphate radical. The results were similar as reported earlier, indicating the purity of the drug sample. The standard curve of the drug was prepared by using UV-visible spectroscopy method as reported by Haug and Lantzsch. Standard solution of IP6 (100 μg/mL) was prepared and scanned in the range of 400-600 nm using distilled water as the blank. The λmax of the drug was

found to be 520 nm. Straight fit equation (y = -0.030x + 1.041) thus obtained was utilized for further quantification of IP6 in various samples.

In part I of the experiment, the IP6 loaded niosomal suspension was optimized, developed and characterized for intended topical drug delivery against skin cancer. A 2^3 (two-level, three-factor) full factorial experimental design was utilized for statistical optimization of the formulation variables, in the preparation of IP6 niosomes. For the purpose, three independent variables (cholesterol: surfactant ratio, sonication time and dicetylphosphate (DCP) concentration) and two dependent variables (particle size and encapsulation efficiency) were taken. Nine batches of different combinations were developed and evaluated.

Unilamellar niosomal vesicles were prepared from surfactant (Span 80), cholesterol and DCP, by utilizing thin film hydration method. Prepared niosomal vesicles were analysed for particle size, polydispersity index (PDI), zeta potential, encapsulation efficiency, *in-vitro* drug release, morphology and stability testing.

Particles size range was observed between 268.9±3.8 nm to 965.5±5.2 nm and the PDI value ranged from 0.212±0.18 to 0.431±0.15. The vesicles with diameter ≤ 600 nm may penetrate into deeper layers of skin and consequently raise the possibility of systemic absorption. Since a topical effect was required, therefore, the IP6 loaded niosomal formulations which showed particle size of more than 600 nm were considered further for the studies.

IP6 was efficiently incorporated into niosomal vesicles with encapsulation efficiency ranging from 11.3±2.9 to 82.9±2.6%. It was seen that the encapsulation efficiency moved parallel with particle size. Greater encapsulation was observed in larger vesicles and vice-versa. Zeta potential values were found to be in the range of -6±0.21 to -36±0.36. Batches which did not contain DCP revealed lower values and those which contained it, displayed higher values of charge, as DCP is a negative charge inducing agent. The *in-vitro* drug release of optimized formulation, at the end of 24 h was found to be 97.61±1.39%. The release kinetics of formulation was best explained by Higuchi's equation, as the plot showed the maximum linearity (R^2= 0.9627) followed by zero order (R^2 = 0.8107) kinetics. To observe the morphology of niosomes, various microscopic studies were performed and were found to be spherical in shape. IP6 loaded niosomes were tested for stability testing. Results of stability

studies showed that prepared niosomal formulation was stable till 180 days at room temperature. All the parameters were found stable during the analysis and significant degradation was not observed during storage at the temperature of 30°C±2°C and 60%±5% relative humidity. After analyzing the above mentioned pharmaceutical parameters, niosomes were further dispersed in a suspension to make them suitable for topical application.

The suspension was prepared through emulsification and analysed for pH, viscosity, texture, *in-vitro* skin permeation, irritation, *in-vitro* cell proliferation/ cytotoxicity against human cancer cell lines and *in-vivo* studies. On visual inspection, the developed niosomal suspension revealed good homogeneity. pH of the suspension was found to be 6.9±0.002 (similar to skin pH range) and its viscosity was seen to be 9650±50.0 cp with spindle number 3. Regarding the texture parameters, firmness was found to 19 g, spreadability value was 1.5 mJ and extrudability was found to be 55.6 mJ. Physicochemical parameters revealed that IP6 loaded niosomal suspension could be applied topically by application of a small amount of shear. *In-vitro* skin permeation studies were done with Swiss albino mice skin in which niosomal and plain IP6 suspensions were compared. Observation detailed that none/ negligible quantity of IP6 was left unabsorbed on the skin surface after 24 h, in the case of both, plain IP6 suspension as well as niosomal suspension of IP6. The cumulative amount of IP6 permeated from plain IP6 suspension was higher than that of niosomal suspension. The lower flux of niosomal suspension displayed prolonged drug release behaviour. Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) was used to examine the skin irritancy of the developed formulations. The irritation score was observed to be in the range of 0 to 0.42±0.03, which confirmed the formulation to be non-irritant. Studies revealed that the formulation was well tolerated. The cytotoxicity studies displayed significant changes in the tested cell lines and lead to inhibition of cell proliferation and apoptosis after the application of the niosomal formulation. IP6 pure displayed IC50 value at the concentration of 1.39 mM , whereas niosomal suspension displayed a value of 0.96 mM, i.e., niosomal suspension loaded with IP6 was significantly ($p<0.05$) more effective than pure IP6. IP6 in niosomal suspension displayed superior inhibition which may be attributed to the fact that niosomes in suspension displayed better interaction with lipid layer of the cell and hence micronized drug particles could have penetrated easily to lead to the maximum

percentage of cell death. Inhibitory effect of the niosomal suspension was seen to be in concordance with previous reports which projected that IP6 is a potential antineoplastic agent. Thus, it was considered worthwhile to evaluate the *in-vivo* efficacy of prepared formulations against DMBA induced dysregulation of markers pertaining to cellular proliferation/ differentiation and inflammation.

A short-term *in-vivo* study was performed taking Swiss albino mice as the model. 7,12-Dimethylbenzanthracene (DMBA, carcinogen) applied to induce dysregulation of proliferation markers (ODC, PCNA, COX-2 and cyclin D1) and the effect of formulation for inhibition of the dysregulation was estimated through western blotting (for protein levels) and RT-PCR (for m-RNA levels). Results revealed that IP6 loaded niosomal formulation marginalized the deleterious effect of DMBA and displayed favourable regulation towards the normal, when compared with IP6 alone and IP6 plain suspension. During the histopathological studies, the control group animals showed no alterations in the epidermal thickness. Significant hyperplasia was seen in DMBA treated skin with the increase in time. IP6 niosomal suspension displayed a better reduction in skin thickness when compared with the standard.

For part II of the experimental section, pectin encrusted gold nanoparticles containing IP6 were prepared with and without jacalin, by using reduction followed by incubation method. The inclusion of pectin as a reducing or capping agent was incited by virtue of it being a natural and nontoxic agent containing carboxylate unit which facilitated activation through jacalin. Jacalin is a lectin protein which has the ability to recognize and target tumour linked disaccharides which are over-expressed in almost all tumour cells and contain functional groups like hydroxyl and amine that facilitate pectin activation. By varying concentration of chloroauric acid (1, 2 and 3 mM), three different batches of formulations were prepared and characterised for particle size, PDI and drug loading. Particle sizes of prepared IP6-jacalin-pectin-gold nanoparticles (IJP-GNP) were found to be 67.4±2.38 nm, 128.29±3.12 nm and 283±4.94 nm respectively. Particle size increased with increasing concentration of chloroauric acid. Pectin may perform the dual function of a capping agent and may prove to be a good stabilizer. Thus, when the concentration of chloroauric acid increased without increasing concentration of pectin, then lesser reducing and capping agent may have been available which lead to an increase in size of nanoparticles. Thus, it was concluded that the particle size can be controlled by regulating the concentration of

chloroauric acid. The formulation showed PDI values of 0.24, 0.29 and 0.32 respectively. The prepared metal particles were homogenous, as revealed by PDI values were < 0.5 for each concentration. Percentage drug loaded was found to be 81.63±1.21%, 78.86±1.98% and 76.1±2.69 % respectively. Drug loading decreased with increasing concentration of chloroauric acid which may be attributed to the larger surface area of smaller metal nanoparticles which would allow attachment of an increased number of drug molecules.

Formulation made with 3mM of chloroauric acid displayed required characteristics and was taken up for further analysis. Formulation was analysed for FTIR, morphology, *in-vitro* cell line studies to estimate their potential against human colon cancer cells (HCT 15) and *in-vivo* studies in Wistar rat model. FTIR spectroscopy of pectin, chloroauric acid, IP6, jacalin and IJP-GNP was performed. All the excipients showed their characteristic peaks. In case of pectin, the peaks were observed at 2935.8 cm^{-1} and 1746.8 cm^{-1} which correspond to C-H and C=O bond vibrations. For chloroauric acid, the peaks at 1631.4 cm^{-1} and 3389.8 cm^{-1} corresponds to C-O and -OH stretches respectively. Peaks near 1654.4 cm^{-1} showed the main vibrational bands of amide groups of jacalin and the bands near 1378.8 cm^{-1} displayed vibrational mode of the COOH and C-O groups of aspartic acid, glutamic acid like amino acids, present in jacalin. Peak near 3430.3 cm^{-1} in case of IP6 relates to OH stretching. IJP-GNP revealed the shift in peaks of hydroxyl and carbonyl groups and also displayed all the characteristic peaks of IP6 and jacalin. It also exhibited the shift in peaks of amines (1655.9 cm^{-1}, 1515.0 cm^{-1} and 1203.2 cm^{-1}) and hydroxyl (3417.5 cm^{-1}) of jacalin and IP6 respectively which may be due to their involvement in complex formation. Further, the spectra of IJP-GNP displayed all the characteristic peaks of IP6 and jacalin suggesting the corona of IP6 and jacalin. The absence of the peak of gold signified the complete coverage by this corona. *In-vitro* drug release was observed to be significantly low in acidic pH, which increased after changing the pH of media to 6.8 and was found to be even higher with media containing cecal content of pH 7.4. Lesser drug release at acidic pH and higher in alkaline pH indicated that the pectin contains carboxyl groups in its structure which ionize in neutral to alkaline pH. Besides, pectin degrades in the colonic microflora. Due to the ionization/ enzymatic degradation of pectin, the corona containing jacalin and IP6 would breakdown thereby releasing the drug in this particular pH. Morphological evaluation of P-GNP through

TEM displayed spherical shape, while IJP-GNP revealed a corona surrounding the surface.

Synthesized formulations (P-GNP, IP-GNP and IJP-GNP) were evaluated for their anti-cancer activity along with IP6 (as standard) against the development of HCT-15, in a dose as well as time-dependent way by MTT assay. IJP-GNP was found to be the most effective formulation in the arrangement with the least IC50 value at the concentration of 15 µM/ml at 24 h treatment and sub IC50 value at 10 µM/ml at 48 h against standard medication IC50 value at 30 µM/ml at 24 h and sub IC50 value at 15 µM/ml at 48 h against colon cancer cells. This superior inhibition effect may be attributed to the better cellular interaction due to the presence of jacalin protein, which has the property of recognition and attachment to the cancerous cells. IP6 containing formulations did not indicate cytotoxicity against human normal colon cells (NCM460) which may be attributed to the use of natural agents. Since IJP-GNP was found to be more effective, thus it was taken up for further evaluations. In colony forming assay, the number of colonies diminished with respect to increment in the time of treatment with IJP-GNP formulation (192 colonies). The untreated HCT 15 cells were found to create an extreme of 210 colonies after 24 h. This signified a longer inhibitory effect of IP6 loaded formulations, as envisaged. Cell cycle analysis displayed the percentage of HCT cells in the G0/G1 stage for IJP-GNP. Low concentration of IJP-GNP (10 µM/ml) also displayed an induction of G1 arrest, confirming the effect of IP6 as earlier reported. Apoptosis assay was utilized in order to evaluate nuclear morphology in response to the IJP-GNP formulation. Shrunken nucleus, peripherally clumped and fragmented chromatin was observed following the treatment with the IJP-GNP formulation, indicating effective apoptosis due to the presence of IP6. In order to evaluate nuclear morphology in response to IJP-GNP treatment, DAPI staining through microscopy was performed. To further confirm our deliberations regarding cell growth inhibition associated with physiological apoptosis, AnnexinV-FITC and PI dual staining was also carried out using flow cytometer. IJP-GNP displayed time as well as dose-dependent increase of apoptotic cells and nonspecific necrotic population. The results of the quantitative measurement of ROS revealed that 10 µM/ml of IJP-GNP induced 104.43% ($p<0.05$) enhancement in ROS production as compared to control. Moreover, ROS production was increased by 160.53% ($p<0.05$) at 15 µM/ml concentration of IJP-GNP when compared to

untreated cells. Results clearly stated that IJP-GNP triggered the cells death by ROS generation. *In-vitro* studies were also correlated with the *in-silico* approach to study inter-molecular interactions between IP6, P-GNP and jacalin protein. The molecular properties and bioactivity of the leading compounds were determined by using online data server Molinspiration. The obtained values of druglikeness score showed that IJP-GNP exhibited good druglikeness score (>0.50). IP6, as well as P-GNP with jacalin protein, displayed a docking score of 4098 and 2096 respectively, as compared to the docking score of IJP-GNP complex, which displayed a docking score of 4422. Greater binding energy and binding sites were seen in complex IJP-GNP when compared with single binding of IP6, as well as P-GNP with jacalin. The docking results showed that interactive properties of IP-GNP complex with jacalin could inhibit the tumour formation activity and supported *in-vitro* studies.

During *in-vivo* studies, dimethylhydrazine (DMH) was administered subcutaneously to induce colon cancer. Animals were scrutinized for their electrocardiogram (ECG) and heart rate variability (HRV) paradigms and blood was collected through the retro-orbital plexus. The blood serum, colon tissue and colon content were collected and stored for further analysis. Colon content was estimated for pH and total acidity. Colon tissue was examined for aberrant crypt foci (ACF), morphological evaluation by SEM and histopathology, biochemical changes, western blotting and RT-PCR. Blood serum was utilized for serum metabolomics assay.

IP6, IP-GNP and IJP-GNP affected diverse parameters of ECG when given to albino Wistar rats. Depreciated ECG and HRV values were reflected in the DMH treated animals, in time as well as frequency domain. Treatment with formulations revealed significant restoration towards normal values which may be ascribed to the action of IP6. The toxic group animals displayed weight loss, decrease in pH along with an increase in total acidity and formation of aberrant crypts when compared with control. Concomitant application of IP-GNP and IJP-GNP favourably regulated the weight and pH of the treated group animals. The total acidity along with ACF was also synchronized near to normal after IJP-GNP treatment. DMH application significantly altered the biochemical parameters (protein carbonyl, malondialdehyde (MDA), GSH, SOD and catalase) in the toxic control group which were found to be significantly normalised with IP-GNP and IJP-GNP treatments.

Toxic control animals showed highest ACF count, abrupt crypts and loss of goblet cells, crypts and distorted lamina propria. Treatment with developed formulations tried to restore the normal structure of colonic mucosa. Maximum restoration of ACF count was observed by IJP-GNP, which may be attributed to the targeted delivery into the affected cells due to the presence of jacalin. The histopathological analysis displayed intact architecture of colonic mucosa in the normal control, in contrast to the toxic group animals which showed abrupt/ impaired arrangement of cells. The IJP-GNP treated groups showed maximum similarity with the normal control group which could again be a call out of targeting the required site because of jacalin.

Change in expressions of PI3K, Akt and COX-2 genes were scrutinized. Normal control was taken as the baseline (assumed with no up/ down regulation) and all comparisons were made with respect to it. Treatment with IP6, IP-GNP and IJP-GNP helped to restore the expressions of above-mentioned genes which validate the inhibitory action of IP6 for aforesaid enzymes. IJP-GNP revealed maximum restoration of protein as well as mRNA levels.

^{1}H NMR based metabolic profiling was performed to explore the biochemical changes associated with colon cancer and to further see how these changes get modulated in the rats receiving the treatment with IP6 formulation(s). Increased levels of lipoproteins, PUFAs and amino acids were seen in DMH treated animals implying rapid cellular regeneration and catabolism due to the rapid cell proliferation. The lower concentration of choline, its derivatives and serum creatine were observed which can be correlated with the cellular regeneration of tumours and increased energy consumption due to rapid cell division. Further a decreased level of glucose was also observed which indicated the increase in glucose metabolism through glycolysis to generate ATP instead of oxidative phosphorylation which enhances glucose uptake in tumour cells to meet the energy requirement of quick proliferation. All in all, the disturbed metabolic pathways as observed, lead to infer the acceleration of cell proliferation in the process of tumour formation, hyperplasia in colonic mucosa and an increase in inflammation in DMH treated rats. Almost all metabolic changes in DMH treated animals returned back towards normal after IP6 formulation(s) treatment, suggesting that the formulation(s) has potential to normalise the DMH altered metabolic changes.

Conclusion

The nanoformulations containing IP6 were developed and optimized successfully. The niosomal suspension and gold nanoparticles of IP6 were evaluated for numerous physicochemical parameters, *in-vitro* and *in-vivo* performances which exhibited the development of a consistent, stable as well as an effective delivery system for cancer therapy.

IP6 niosomal suspension was found to be non-irritant, aesthetic and had desirable properties for the topical application. Formulation proved to possess anticancer activity and induced apoptosis in SK-MEL-2 cancer cell lines. The DMBA altered expression of ODC, PCNA, COX-2 and Cyclin D1 was significantly prevented by concomitant application of niosomal formulations.

The gold nanoparticles (P-GNP, IP-GNP and IJP-GNP) were screened for the anticancer studies on the basis of the cytotoxicity observed on HCT 15 cells in a dose and time-dependent manner. Amongst all, IJP-GNP was found to be the most potent formulation with lowest IC50 value. IJP-GNP caused cell cycle arrest at the G0/G1 phase, induced ROS and subsequent apoptosis. The docking results showed that the interactive properties of IP-GNP complex with jacalin that could inhibit the tumour formation activity and supported *in-vitro* studies, which proved that the IJP-GNP inhibited the growth of colon cancer. Furthermore, *in-vivo* studies suggested that IJP-GNP displayed significant effect against progression of colon carcinogenesis by positively modulating the physiological markers, oxidative stress, inflammatory markers and hemodynamic changes.

Taken together, all these findings suggested an effective anticancer effect of the IP6 loaded formulations that raise the hope of future drug delivery strategy targeted to the numerous cancer treatments. Further studies may be performed for successive scale-up of such formulations having drugs of natural origin, from bench to bedside.