Formulation and Appraisal of Nano Drug Delivery System(s) of Bioactive Compounds for Enhanced Antineoplastic Potential

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SUMMARY

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Summary

Cancer is a major burden disease worldwide that annually takes a toll on several lives. Worldwide, the prevalence and mortality are still rising substantially despite huge attempts to address the issue. The incidence of colorectal cancer (CRC), the third largest cause of cancer mortality worldwide, is swiftly escalating in developing countries. Cancer treatment burns holes in the patient's pocket, as well as the side effects of cancer therapy worsen the quality of life of a cancer patient. Yet, thanks to modifiable risk factors like smoking, a poor diet, alcoholism, inactivity, and being overweight or obese, more than 50% of cancer incidences and deaths may be averted. It has been demonstrated that 30-40% of malignancies might be prevented with a simple diet change that included increasing fruit and vegetable intake, retaining a healthy body weight, and engaging in consistent physical activity. Also, the fight against cancer is being waged using several different strategies, one of which is an increase in the utilization of natural agents as chemopreventive and chemotherapeutic agents due to the fact that these agents are not only safe but also easily accessible and widely accepted. Natural dietary agents are known to control the genesis and course of malignancies owing to their low or no toxicity, notable multiple-site efficacy, oral ingestion, recognized mechanisms of action, competitive prices, and human acceptance.

The utilization of natural products for CRC management is very promising because diet plays a significant role in the etiology of this disease, which is triggered by the interplay of hereditary (5-10%) and environmental factors. Considering the above, there is a dire need to investigate safe natural bio-actives that aid cancer management without hampering the quality of life.

Inositol hexaphosphate/ Phytic acid (IP6) is one attractive natural phosphorylated dietary carbohydrate abundantly present in cereals, legumes, and nuts that have been an integral part of the human diet for ages. Several benefits such as antioxidant, anti-inflammatory, antidiabetic, anti-microbial, and anti-cancer as well as in the prevention of diseases such as coronary heart, neurodegenerative, and renal along with its easy accessibility, inexpensiveness, safety has made it among highly investigated drugs for disease management in recent years. Recent research indicates that IP6 has tremendous potential against different types of cancers such as colon, hepatic, breast, lung, skin, and prostate. As CRC is generally associated with unhealthy diets, intervention in diets has shown a positive effect on CRC patients. Thus, we selected IP6, which is an integral part of dietary cereals, legumes, nuts, and oils along with natural polymers- Pectin and Chitosan, to formulate a delivery system that can harness the benefits of IP6. IP6, in pure

form, fails to reach the lower git as it gets quickly absorbed after ingestion in the stomach or forms insoluble complexes and gets excreted, thereby not providing the benefits it could.

Small-sized nanoformulations are well-suited for an array of applications where an increase in residence duration, surface area, protection of loaded drug, or onset of therapeutic action is desirable. Improved pharmacokinetics, distribution, and accumulation of drugs at the tumour site contribute to the higher therapeutic index of anticancer drugs in nanoformulations. Since tumour sites have leaky vasculature, the nano-sized systems show greater permeability into the tumour site. Consequently, nano-drug delivery systems have the potential to be efficient tools in the fight against cancer. Considering this, the preparation and evaluation of IP6-loaded nanoformulations were investigated over the course of this research. Thus, in this study, we selected IP6 and natural polymers (PN-CN) and attempted formulation of a pocket-friendly, easy-to-formulate, safe, oral nanosystem that could harness the benefits of IP6 by enabling its successful reach to the lower gastrointestinal tract (GIT) (colon).

Further, the obtained NPs were modified with CE, characterized, and evaluated for anti-cancer activity in the colon (DLD-1 and HT-29) and breast (MCF7) cell lines as well as against 1, 2-dimethyl hydrazine (DMH)-induced biochemical changes, inflammation, neoplastic damage, and hyperplasia in colonic tissues of albino Wistar rats.

Initially, preformulation studies (melting point analysis and FTIR spectroscopy) were utilized in order to positively identify the drug sample. The melting point was determined to be somewhere in the range of 22-24℃. The FTIR spectrum of IP6 gave the characteristic peak at 3430.3cm^{-1} which relates to OH stretching. Peaks near 1662.1cm^{-1} were probably due to the carboxyl group and at 1061.5cm^{-1} was allocated to the phosphate radical or hydrogen phosphate radical. The fact that the results were consistent with those previously reported validates the purity of the drug.

The method of UV-visible spectroscopy reported by Haug and Lantzsch was utilized in the preparation of the standard curve of IP6. The standard solution of IP6 with a concentration 100 µg/mL, was prepared and scanned in the region of 400-600 nm with distilled water as the blank. The λ_{max} of the drug was found to be 520 nm. The straight fit equation ($y = -0.0312x + 1.0496$) thus obtained was utilized for further quantification of IP6 in various samples.

Proceeding with the IP6-loaded polymeric nanoparticle development, we selected natural polymers. As polysaccharides are very attractive candidates for the fabrication of various carriers for drug delivery owing to their low cost, biocompatibility, biodegradability, as well as convenience of chemical modifications, pectin (PN) and chitosan (CN) were opted.

PN, a plant cell wall component, present in the middle lamella is usually extracted from citrus zest or apple pomace. It is an anionic polysaccharide predominantly comprised of linear chains of d-galactopyranosyluronic acid units connected by 1, 4 α-glycosidic linkages. PN has exceptional aqueous solubility and has been exploited to target multiple proteins and drugs in several formulations like films, hydrogels, and micro/nanoparticles (NPs) due to its superior biocompatibility and biodegradability. PN-based systems were primarily employed for targeted colonic delivery, but they were also used to deliver drugs to the brain in certain instances. CN is another linear biopolymer obtained from the deacetylation of chitin found in crustacean shells. It is a biodegradable, safe, inexpensive, and easily accessible cationic polysaccharide comprising β-D-glucosamine and N-acetyl-D-glucosamine units arranged randomly. Countless drugs, particularly anticancer agents, therapeutic proteins, genes, and antigens have been investigated by employing CN-based delivery systems.

The combination of these two oppositely charged polymers employing cross-linking agents in a delivery system mutually overcomes the drawbacks of individual polymers. A higher aqueous solubility and swelling ability in an alkaline medium of PN subsequently affects the controlled release property and is decreased when combined with CN; thereby circumventing the premature release of encapsulated drug. The PN-CN combination is stable until the PN component is degraded by pectinase released by the colonic microbiota in humans. Combining PN-CN and crosslinking PN with divalent metal cations, such as Ca^{2+}, lowers the solubility, permeability, and fraction of disintegration of the polymer structures while enhancing the density and mechanical resilience of the pharmaceutical formulations, prolonging drug release. Physical crosslinking spurred on by electrostatic interactions among Ca^{2+} and the carboxyl groups accelerates the rigidity of the PN gel by producing an egg-box model thereby turning it from weak and flexible, to rigid and strong. Contrarily, CN dissolves readily in acidic environments like gastric fluid in the stomach despite having poor aqueous solubility. It retains its positive charge after protonation in an acidic environment and can interact with negatively charged molecules, which is crucial for drug transport and preventing enzymatic drug degradation in the gastrointestinal tract (GIT). Additionally, the GIT's ability to degrade drug molecules can be hampered by CN that has been crosslinked with a hardening agent.

Thus, IP6 and natural polymers (PN-CN) were selected to attempt a pocket-friendly, easy-to-formulate, safe, oral nanosystem that could harness the benefits of IP6 by enabling it to reach successfully to the lower GIT (colon).

For this, firstly an approach for NP development employing two-step crosslinking of oppositely charged PN and CN polymers was investigated for the first time. Though several studies

employing PN and CN as biomaterials for micro/nanosystem development have been reported, none of them have utilized the approach investigated in this study. The use of dual crosslinking followed by emulsification and solvent evaporation may have led to the development of safe nanosystems that could enable drug entrapment and controlled release rates in the gastric milieu. It was envisaged that two-step crosslinking of polymer networks may increase the gel strength of polymers thereby increasing their stability for colon drug delivery. Crosslinking CN with ionic and covalent crosslinkers and comparing the *in-vitro* characteristics of both-way crosslinked PN-CN-NPs and their suitability for colon drug delivery of IP6 were the major focus of the study. It was hypothesized that the two-step crosslinker-polymer interactions may reduce the water uptake (swelling) and undesirable erosion of the nanosystem until it reaches the colon. The PN-CN coacervate along with crosslinkers, when made to nanosystems, entrapped the drug and prevented its release into the upper GIT. Two sets (set I and II) of PEGylated and crosslinked PN-CN-NPs to deliver IP6 orally to the colon were formulated through a novel Polymer Blended Double Crosslinked Emulsification Solvent Evaporation (PBDCESE) method. The investigated NPs are premised on the interaction between the cationic CN (crosslinked with covalent/ionic crosslinker) with the anionic PN (crosslinked with calcium chloride (CaCl2)).

This study thoroughly investigated the effect of varying the concentration of polymeric blend (PN-CN), crosslinkers (GE/TPP), and sonication time on the *in-vitro* formulation characteristics like particle size (PS), zeta potential (ZP), entrapment efficiency (EE), drug loading (DL), cumulative drug release (CDR) and release patterns. The NPs were optimized through three-factor, three-level, Central Composite design (CCD) employing Design of Experiments (DoE) software. DoE aids in precisely selecting the optimum polymers and crosslinker concentration as well as analyzing the *in-vitro* characteristics of prepared NP sets. The optimized NPs were further evaluated for cytocompatibility and cytotoxic activity in normal macrophages (J774.2) and colon cancer cell lines (HT-29 and DLD-1) respectively. Also, cell internalization of optimized NPs was accessed in HT-29, DLD-1, and J774.2 cell lines through confocal microscopy.

Two sets of NPs: Set I (G1-G15) (IP6@GE*PN-CN-NPs) and Set II (T1-T15) (IP6@TPP*PN-CN-NPs) were formulated using the PBDCESE method. Set I consisted of GE as a crosslinker while Set II consisted of TPP as a crosslinker. Intermolecular cross-linking resulted in the formation of NPs that are efficient in entrapping and controlling the release of the drug in the colon. Additionally, the formulated NPs were stabilized via PEGylation. The blending process of PN-CN, being a simple technique, yielded a convenient drug delivery system for maximizing the lucrative attributes of the employed polymers as well as the opportunity for customizing the

properties of the resulting NPs. The technique involved in preparing NPs is simple, safe, and uses minimal organic solvents, with no temperature maintenance required, rendering it suitable for thermo-labile drugs and excipients. To enable NPs formation, the polymer concentrations were kept low to avoid bulk gel formation. Initially, PN was dissolved and $CaCl_2$ was added to it. Ca^{2+} was used as a crosslinker for PN to decrease its hydrophilicity by strengthening the PN gel network. Further, IP6 was added to the Ca-PN solution. Further, CN, pre-crosslinked with GE for set I and TPP for set II were added to Ca-PN-IP6, which was a very crucial step in NP formation as the rate of addition of crosslinked CN to the Ca-PN-IP6 greatly influenced the formation of desired NPs, therefore very slow addition rate along with continuous stirring was preferred because rapid CN addition leads to precipitation in Ca-PN-IP6 mixture, hampering NP preparation. This drug-polymer (PN-IP6-GE/TPP-CN) complex was then emulsified in a DCM solution containing 0.1%v/v Span 80 to formulate NPs. NP formation commences at this step as the aqueous polymeric phase is added to the non-aqueous phase in presence of a surfactant (Span 80). The emulsification at this step initiates the formation of spherical structures. As GE/TPP are crosslinkers for CN, it is supposed that GE/TPP crosslink the CN thereby hardening the CN network and leading to the formation of compact polymeric NPs. After complete evaporation of DCM, the NPs were further sonicated to decrease the particle size and PEGylated simultaneously by adding the poly electrolyte complex in 1% PEG solution. PEGylation, here occurred by surface adsorption of PEG on the NP surface. A total of thirty formulations (fifteen per set) were prepared to evaluate interactions between three independent variables: A, B, and C through response surface methodology (RSM) employing CCD via DoE. The responses to the experimental design were analyzed using multiple linear regression analysis to develop the model equations. ANOVA was applied to evaluate models by comparing regression coefficients and the significance of each term in polynomial equations. The envisaged quadratic model was deemed appropriate, devoid of systematic error, and thus, was implemented to optimize the NP development process for IP6@GE*PN-CN-NPs as well as IP6@ TPP*PN-CN-NPs. The overlay plot was constructed for the determination of the desired optimal region and the optimized formulation.

The formulations G1 (IP6@GE*PN-CN-NPs) and T5 (IP6@TPP*PN-CN-NPs) appeared to be the optimized formulations. G1 NPs exhibited PS of 557.2±5.027nm, EE of 77.18±2.117, and DL of 8.25±0.058 whereas T5 NPs exhibited PS of 210.6±7.93nm, EE of 87.12±2.261, and DL of 17.195±1.189. The CDR of optimized formulations G1 and T5 were found to be 93.56±0.43% & 89.68±0.38% respectively in 48h. The release of IP6 from the polymer matrix was observed to be less than 12% at pH 1.2, and less than 28% at pH 6.8 for G1 and T5 optimized NP

formulations. Fitting the obtained CDR data of the optimized G1 and T5 NPs in various kinetic models for drug release kinetics determination revealed that both followed the Korsemeyer-Peppas model of release kinetics. This was based on the highest correlation coefficient (R2) value (R2 for G1-0.971, R2 for T5-0.974). Further, to gain more insight into possible interactions between IP6 and the biopolymers used to prepare the optimized T5 NPs, the resultant spectra for pure PN, IP6, CN, PEG, IP6-loaded non-PEGylated and PEGylated NPs (T5) were examined for any possible changes in the peaks of the spectra. The observed FTIR pattern suggested chemical interactions between the drug, crosslinker, and biopolymers in the optimized T5 NPs. The TEM analysis of nanoparticles was used to characterize their shape and surface morphology. The formulated polymeric nanoparticles were found spherical with smooth surfaces, though size disparity was observed in the TEM images. TEM studies depicted particle sizes ranging between 35-260nm. The stability studies revealed the optimized formulation G1 was stable for 120 days at 25°C±5°C and 180 days at 5°C±3°C while optimized formulation T5 was stable for 180 days at both temperature conditions.

Further, for *in-vitro* cell line cytotoxicity studies, HT-29 and DLD-1 cell lines were treated for 48h with IP6, blank TPP*PN-CN-NPs, and IP6@TPP*PN-CN-NPs (T5). T5 exhibited a maximum decrease in cell viability followed by IP6 at the investigated concentration range, in contrast to the blank TPP*PN-CN-NPs. This indicated the biocompatible nature of polymers employed in NP preparation and the cytotoxic nature of drugs in colon cell lines. CLSM was used to assess the cell internalization ability of FITC@TPP*PN-CN-NPs in DLD-1, HT-29 cell lines, and Dox@TPP*PN-CN-NPs in J774.2 cell lines. The FITC@TPP*PN-CN-NPs exhibited substantial fluorescence in the cytoplasm after 6h treatment in DLD-1 as well as HT-29 cell lines indicating the cell membrane penetration abilities of the formulated NPs for intended drug action at the cellular level. The cellular uptake study performed in J774.1 cell lines revealed time-dependent uptake of PEGylated Dox@TPP*PN-CN-NPs compared to non-PEGylated-Dox@TPP*PN-CN-NPs. The PEGylated Dox@TPP*PN-CN-NPs did not show cell internalization within J774.2 cell lines after 12h treatment but after 24h treatment cell internalization was observed, indicating the stealth nature of PEGylated NPs and suggesting their utility if intended for the parenteral route.

The conventional targeting approaches propose decorating ligands on the surface of nano-carriers that enable them to identify and interact with the specific cell membrane receptors. Though this approach appears to be effective so far, the targeting effectiveness is limited by heterogeneity and variability of membrane receptors as different patients suffering from a similar disease may have a difference in the expression and distribution of receptors. Also,

receptor expression variability may occur with the progression of the disease. Therefore, for receptor-mediated targeting to fully reach clinics, seeking novel target sites to generate tailored nanocarriers is required. Membrane transporters have recently emerged as promising target areas for effective drug delivery, for instance, glucose, vitamin, ion transporters, etc. serve a crucial role in cell nourishment. Additionally, transporters may also interact with several drugs and influence their safety and efficacy. Advantageously, the inevitable function of nutrients in cell sustenance leads to low variability of transporter expression levels compared to receptors. To fulfill the tremendous nutritional requirement in the case of specific diseases such as malignancies, the expression of transporters typically elevates. Thus, transporters are a promising option for developing tumour-targeting nanocarriers.

Colon tumour cells (Caco-2, HT-29, and HCT 116) are considered to show greater expression of two transporters: the novel organic cation/carnitine transporter 2 (OCTN2, SLC22A5) and the neutral and basic amino acid transporter B (0+) ($ATB^{0,+}$, SLC6A14) than normal colon cells. This OCTN2 and $ATB^{0,+}$ expression on colon cancer cells, offers an intriguing platform to investigate the prospects of OCTN2 and $ATB^{0,+}$ targeted nanocarriers. Carnitine (b-hydroxy-c-trimethylaminobutyrate), a highly polar zwitterionic molecule that aids the transport of long-chain fatty acids through the inner mitochondrial membrane for β-oxidation, is transferred across the plasma membrane of mammalian cells primarily via the OCTN2 transporter and to a lesser extent via the ATB0, + transporter.

Therefore, the obtained IP6@TPP*PN-CN-NPs were further modified with CE as these NPs may provide dual targeting to both the transporters thereby leading to enhanced activity against colon cancer. The CE-modified NPs were then characterized and evaluated for anti-cancer activity in the colon (DLD-1 and HT-29) and breast (MCF7) cell lines. The HT-29 and MCF7 cell lines were selected for the study because they show the presence of OCTN2 and ATB0, + transporters.

The central hypothesis of this study was to attempt formulation of OCTN2 and $ATB^{0,+}$ targeted carnitine modified IP6 loaded TPP crosslinked pectin chitosan NPs for colon targeting of IP6 in a tumor cell-specific manner and explore its effects on colon and breast cancer cell lines as well as against 1, 2-dimethylhydrazine (DMH)-induced biochemical changes, inflammation, neoplastic damage, and hyperplasia in colonic tissues of albino Wistar rats.

IP6-loaded TPP crosslinked Pectin Chitosan NPs (IP6@TPP*PN-CN-NPs) and CE-modified IP6-loaded TPP*PN-CN NPs (CE-IP6@TPP*PN-CN-NPs) were prepared by using modified emulsion solvent evaporation technique. The DLS was used to determine these important NP characteristics. DLS revealed the PS of IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-

NPs to be 202.3±4.7nm and 219.3±5.5nm respectively. The PDI of the IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs was found to be 0.086±0.017 and 0.076±0.22 respectively, which is much less than 0.3, suggesting a monodisperse system in aqueous solutions. The ZP value of IP6@TPP*PN-CN-NPs was 26.97mv while the CE-IP6@TPP*PN-CN-NPs displayed a slightly higher ZP of 30.17mv indicating the presence of CE in the NPs as ionization effect of CE tends to increase the ZP. The EE of the prepared IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs NPs was found to be 87.83±0.594% & 86.931±0.783% respectively, indicating good drug adsorption on the NPs. The DL of the IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs was found to be 17.841±0.723% and 16.581±0.546% respectively, indicating good drug content within the NPs. The CDR of IP6 in 48h from the prepared IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs was determined to be 90.29±0.19% & 92.37±0.42% respectively. The release of IP6 from the polymer matrix was observed to be less than 13% at pH 1.2 and less than 27% at pH 6.8 for IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs. These low release rates in SGF and SIF could be accredited to the compact particles resulting from crosslinked PN and CN. The developed nanosystems were suited for colonic drug delivery because the maximum IP6 release rates were seen at pH 7.4. It was observed that both IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs followed the Korsemayer-Peppas model of release kinetics after fitting the acquired CDR data to several kinetic models for drug release kinetics determination. This was decided based on the maximum correlation coefficient (R2) value (R2 for IP6@TPP*PN-CN-NPs -0.967, R2 for CE-IP6@TPP*PN-CN-NPs -0.970) obtained. The release was erosion and diffusion controlled, following non-fickian or anomalous diffusion, as per the Korsmeyer-Peppas plots' diffusion exponent (n) values, which ranged from 0.5 to 1.

To confirm the presence of CE on the modified NPs and explore the probable interactions between the drug (IP6), the biopolymers (PN and CN), and CE used in the formulation of NPs, PN, IP6, CN, PEG, non-PEGylated IP6@TPP*PN-CN-NPs, IP6@TPP*PN-CN-NPs (PEGylated) and CE-IP6@TPP*PN-CN-NPs were subjected to FTIR analysis. The observed FTIR pattern, thus, suggested chemical interactions between the drug, crosslinker, and biopolymers in the IP6@TPP*PN-CN-NPs and the presence of CE in the CE-IP6@TPP*PN-CN-NPs. The NPs were investigated for TEM imaging to determine the surface morphology and shape. The CE-IP6@TPP*PN-CN-NPs were imaged using TEM. Although there was a size disparity visible in the TEM images, the developed polymeric NPs were discovered to be spherical with smooth surfaces. Particle sizes between 40nm to 240 nm were observed in TEM analyses, which were smaller than the sizes estimated by DLS because DLS reflects the

hydrodynamic diameter of NPs whereas TEM indicates the actual diameter of the NPs. The developed IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs were stable for 180 days at 25°C±5°C and at 5°C ±3°C in terms of Physical Appearance, Particle size (nm), and EE (%). No major alterations were observed in the original attributes of IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs during this period.

The cytotoxic effects of IP6, blank TPP*PN-CN-NPs, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs were evaluated in DLD-1, HT-29, MCF7, and J77.4A cell lines employing MTT assay after 24h of treatment. CE-IP6@TPP*PN-CN-NPs exhibited a maximum decrease in cell viability followed by IP6@TPP*PN-CN-NPs and IP6 at the investigated concentration range in DLD-1, HT-29, and MCF7 cell lines while no decrease in cell viability was observed in J77.4A cell lines indicating the cytotoxic and biocompatible nature of drug and drug-loaded formulation in cancer and normal cell lines respectively. The blank TPP*PN-CN-NPs exhibited no cytotoxic effect in DLD-1, HT-29, and MCF7 cell lines as well as J77.4A cell lines, indicating the biocompatible nature of polymers and drug employed in NP preparation. The drug and formulation demonstrated selective cytotoxicity for cancerous cell lines while were biocompatible in normal cell lines, however, the CE-modified NPs showed a more profound in cancer cells expressing OCTN2 transporters. CLSM was used to assess the cell internalization ability of FITC/Dox@TPP*PN-CN-NPs and CE-FITC/Dox@TPP*PN-CN-NPs in DLD-1, HT-29, and MCF7 cell lines. The FITC/Dox@TPP*PN-CN-NPs and CE-FITC/Dox@TPP*PN-CN-NPs exhibited substantial fluorescence in the cytoplasm after 6h treatment in DLD-1 as well as MCF7 cell lines indicating the cell membrane penetration abilities of the formulated NPs for intended drug action at the cellular level. The mean fluorescent intensity (MFI) of FITC@TPP*PN-CN-NPs and CE-FITC@TPP*PN-CN-NPs in DLD-1 cell lines was almost equal due to the absence of CE transporters. However, in HT-29 and MCF-7 cell lines the MFI of CE-Dox@TPP*PN-CN-NPs showed almost twice the increment as compared to Dox@TPP*PN-CN-NPs due to the presence of CE transporters (OCTN2) in these cell lines. This confirms the OCTN2-mediated uptake of CE-Dox@TPP*PN-CN-NPs in HT-29 and MCF7 cell lines.

Further, to examine the OCTN2-mediated uptake of CE-FITC@TPP*PN-CN-NPs, the qualitative and quantitative cell uptake study was assessed through flow-cytometry in HT-29 cell lines as these cell lines are reported to express OCTN2 transporter (CE transporter). The FACS results revealed 13.93% MFI of FITC@TPP*PN-CN-NPs and 51.45% MFI of CE-FITC@TPP*PN-CN-NPs in the HT-29 cell lines in 3h. Moreover, the MFI increased to 58.80% and 93.57% in FITC@TPP*PN-CN-NPs and CE-FITC@TPP*PN-CN-NPs respectively after

6h of treatment, thereby, confirming the transporter-mediated uptake of CE-modified IP6@TPP*PN-CN-NPs.

HT-29 cell lines were employed to assess the effect of IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs on cell cycle phase distribution by comparing the treatments with control cells through PI staining. CE-IP6@TPP*PN-CN-NPs showed cell cycle arrest of 82.23% in the G1 phase, 8.11% in the S phase, and 9.66 % in the G2/M phase, which was significantly higher compared to IP6@TPP*PN-CN-NPs (59.91% in G1 phase, 26.14% in S phase and 13.95 % in G2/M phase), and IP6 (44.24% in G1 phase, 36.58% in S phase and 19.18% in G2/M phase), after 24h of treatment. Flow-cytometric cell cycle analysis indicated an increase in the G1 fraction of cells treated with IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs. About 39.49% of cells in the G1/G0 phase were detected in the control, while the cultures exposed to CE-IP6@TPP*PN-CN-NPs showed an evident increase in G1/G0 phase cell population (82.23%). IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs treatment led to a significant decrease in the S phase cell population compared to the control cells. Also, the number of cells observed in the G2/M phase was decreased by IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs as compared to control cells. The study revealed that CE-IP6@TPP*PN-CN-NPs, IP6@TPP*PN-CN-NPs, and IP6 significantly arrest the cell growth in the G1 phase compared to control, and a reduction in the S phase was observed. This G1 phase arrest may probably cause mitotic slippage into the pseudo-G1 phase ultimately causing cell death. Similar results were observed in previous studies as IP6 is known to induce cancerous cell death by causing G1 phase arrest.

Following 24h of incubation, a quantitative assessment of apoptosis in HT-29 cells showed that CE-IP6@TPP*PN-CN-NPs had an early apoptotic activity of 32.65%, compared to 15.26%, 4.71%, and 2.01% in IP6@TPP*PN-CN-NPs, IP6, and control cells respectively. The CE-IP6@TPP*PN-CN-NPs significantly enhanced apoptosis as it selectively targets cancer cells due to the presence of CE transporters in HT-29 cells, compared to free IP6, and IP6@TPP*PN-CN-NPs. Flow-cytometric analysis indicated that IP6, and IP6@TPP*PN-CN-NPs, CE-IP6@TPP*PN-CN-NPs decreased the viable cell count and increased the cells in early apoptosis, more prominent effects were witnessed in CE-IP6@TPP*PN-CN-NPs treated cells. The exposure of HT-29 cells to IP6, and IP6@TPP*PN-CN-NPs, CE-IP6@TPP*PN-CN-NPs revealed the presence of about 0.80%, 1.53%, and 5.51% of cells in late apoptosis and 2.51%, 1.08%, 1.21% of necrotic cells respectively.

The rate of induced apoptosis was further confirmed by the mitochondria-dependent potential, which was found to be 1.76%, 14.23%, 38.21%, and 95.43% for control, IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs respectively.

ROS were detected and quantified using the DCFH-DA (Dichloro-dihydro-fluorescein diacetate) assay. The esterase in the cell can hydrolyze DCFH-DA to yield DCFH, but as the latter cannot escape the cell membrane, the DCFH-DA probe gets trapped within the cell. As a response, intracellular ROS levels oxidize DCFH to fluorescent DCF, enabling the ROS levels to be quantified. CE-IP6@TPP*PN-CN-NPs treated cells demonstrated enhanced ROS level generation and a mean fluorescence intensity (MFI) that was significantly higher than control cells, which may be a contributing factor to the observed higher levels of apoptosis. Following the 24h incubation period, ROS generation of IP6 and IP6@TPP*PN-CN-NPs also increased significantly in comparison to control cells. The quantitative assessment of ROS through FACS indicated augmentation in ROS generation. IP6@TPP*PN-CN-NPs showed maximum ROS generation ($p<0.05$)) followed by IP6@TPP*PN-CN-NPs ($p<0.05$) and IP6 ($p<0.05$) when compared with control (untreated cells).

As the role of OCTN2 is to transport long-chain fatty acid across the bilayer membrane, hence the binding site was more hydrophobic and had few positive and negative charge residues. Molecular docking of IP6 and CE showed that both compounds are binding well into the identified binding site. However, IP6 did not perform very well with a binding energy of -3.356 kcal/mol. On the other hand, CE could bind with a docking score of -9.356 kcal/mol. Further, to understand the stability of interactions, we performed a molecular dynamics simulation of both the docked complexes using the Desmond 2022-3 suite. 100 ns molecular dynamics of CE showed that the protein-ligand complex is stable during the simulation. All the interactions after molecular docking were conserved and were retained for more than 50% on a 100 ns MD time scale. The ionic interaction between Tyr239 and ligand was stable for more than 80%. Another H-bond was observed between carboxylic acid and Arg471 guanidium group during the molecular docking and was retained in molecular dynamics simulation. However, ionic interaction between this carboxylic group and Arg471 was lost during the simulation. Therefore, the in-silico investigation implied that IP6@TPP*PN-CN-NPs when modified with CE may get transported within the colon cells utilizing the OCTN2 transporter. This study paves the way for other drugs which can be targeted similarly.

Further, the *in-vivo* effects of prepared NPs (IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs) were evaluated in Dimethylhydrazine (DMH) treated albino Wistar rat model. DMH at a dose of 20mg/kg/week was administered subcutaneously to induce colon cancer for four

weeks. At the end of the study, 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 morphological evaluation via histopathology, oxidative stress markers, pro-inflammatory mediators, apoptotic mediators, and western blotting.

The colon content was estimated for pH and total acidity. The toxic group animals displayed weight loss, a decrease in pH along with an increase in total acidity when compared with control. Concomitant application of IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs favorably regulated the weight and pH in the treated group animals. The total acidity was also synchronized near to normal after CE-IP6@TPP*PN-CN-NPs treatment. There was a statistically significant drop in the levels of glutathione peroxidase (GSH), superoxide dismutase (SOD), and superoxide anion dismutase (catalase) in the colon tissues of DMH-treated animals ($p<0.001$), which may be related to the development of colon cancer. However, treatment with IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs restored the levels to normal. These biochemical alterations were most effectively restored by CE-IP6@TPP*PN-CN-NPs, indicating that IP6 was the most potent in this form. The histopathological analysis of normal colon tissue depicted three layers including mucosa, submucosa, and muscularis. The intact architecture of colonic mucosa was seen in the normal control group describing all three layers, in contrast to the toxic group animals which showed abrupt and disrupted arrangement of cells. The CE-IP6@TPP*PN-CN-NPs application showed maximum similarity with the normal control group which could again be a call out of targeting to the required site because of CE.

IL-6, a key cytokine in the tumor microenvironment, is unchecked in cancer and detected in elevated levels in most tumors. In the tumor microenvironment, high IL-6 levels dictate all hallmarks of cancer and multiple signaling pathways, including apoptosis, survival, proliferation, angiogenesis, invasiveness, metastasis, and metabolism. Similarly, IL-8 has various pro-tumorigenic roles in the tumor microenvironment (TME). For example, it can cause tumor cells to multiply or change into a migratory or mesenchymal phenotype. TNF-α is a pleiotropic cytokine that is involved in numerous processes such as cell death and development, oncogenesis, and immune, inflammatory, and stress response. COX-2 is implicated in inflammation-induced cancer cell proliferation, tumor growth, and metastasis. Thus, malignancies with IL-6, IL-8, TNF-α, or COX-2-dominated signaling may benefit from their blocking or decreasing their signaling alone or in combination with standard anticancer therapy. Apoptosis evasion is regarded as one of the defining characteristics of human malignancies. Caspases and other regulatory factors execute this type of cell death. The dysregulation of caspase expression and activation contributes to cancer and cancer therapy resistance.

Consequently, strategies that can cause caspase activation or disable the restraints to existing caspase activation inhibitors may be intriguing options for treating cancer. Hence, ELISA was performed to determine the anticancer potential of plain IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs by assessment in rat colon tissues for pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) and apoptotic signal initiators (caspase 3, caspase 8 and caspase 9). The concentration of pro-inflammatory markers increased in the colon tissues of toxic control group rats while the concentration of apoptotic initiators decreased. The result demonstrated that diminished caspase levels were vastly restored, and the enhanced pro-inflammatory marker concentration was observed in the treatment groups. In this study, plain IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs re-established the expression of caspase-3, -8 and -9. CE-IP6@TPP*PN-CN-NPs treatment significantly ($p<0.001$) improved the declining level of caspases-3, -8, and -9 as compared to IP6@TPP*PN-CN-NPs and plain IP6. Changes in expressions of COX-2, iNOS, PI3K, and Akt genes were scrutinized through western blot for protein levels. The expressions of COX-2, iNOS, PI3K, and Akt proteins showed a significant rise after DMH administration in the toxic control group. Treatment with IP6, IP6@TPP*PN-CN-NPs, and CE-IP6@TPP*PN-CN-NPs helped to restore the expressions of these genes.

Polymeric dual crosslinked NPs containing IP6 were successfully prepared using the novel PBDCESE method and the designed sets of NPs with a covalent crosslinker (GE) and ionic crosslinker (TPP) were optimized by employing the RSM-CCD technique. The effect of independent variables (polymer concentration, crosslinker concentration, and sonication time) on dependent variables (PS, EE, and DL) was successfully investigated to establish a good quality correlation (smaller PS, higher EE, and DL) by using the statistical design. Prepared TPP*PN-CN-NPs emerged superior to GE*PN-CN-NPs in terms of *in-vitro* NP characteristics implying that the ionic crosslinker for CN was better than the covalent crosslinker, though both sets of NPs retarded IP6 release in upper GIT. The electrovalent crosslinking with TPP however resulted in comparatively smaller and more stable NPs than covalent crosslinking with GE. The optimized blank and IP6 loaded TPP*PN-CN-NPs showed significant cytotoxicity and evident cell internalization in colon cancer cell lines while proving to be non-toxic in J774.2 cell lines as well as showed time-dependent uptake within J774.2 cell lines. The developed dual crosslinked PN-CN-NPs thereby successfully entrapped IP6 and ensured its delivery to the colon.

Further, the modification of IP6@TPP*PN-CN-NPs via CE to target OCTN2 (SLC22A5) and ATB0, + (SLC6A14) transporters was done to enable the colonic delivery of IP6 orally and to improve its targeting ability to colon cells. The CE-IP6@TPP*PN-CN-NPs showed greater

uptake within colon cells compared to IP6@TPP*PN-CN-NPs because of CE modification as CE provided transporter OCTN2 (SLC22A5) and ATB0,+ (SLC6A14) targeting ability to the NPs. The increased NP accumulation within the cancer cells leads to the enhanced activity of CE-modified IP6-encapsulated NPs in cancer cells. The enhanced uptake may be attributed to the presence of OCTN2 and ATB0,+ transporters on the cell surface which enable internalization due to CE modification, which does not happen with unmodified NPs and plain IP6. The enhanced activity of modified NPs was confirmed via other assays like MTT, CCA, ROS, apoptosis, and MMP when compared with unmodified NPs and plain IP6. DMH-induced pre-neoplastic colon cancer in albino Wistar rats confirmed a marked reduction of tumors, levels of oxidative stress markers, pro-inflammatory mediators, and an increase in apoptotic mediators on the administration of CE-IP6@TPP*PN-CN-NPs as compared to IP6@TPP*PN-CN-NPs and pure IP6. Further maximal suppression in the expression of COX-2, iNOS, Akt, and PI3K was observed in the CE-IP6@TPP*PN-CN-NPs treated group as compared to IP6@TPP*PN-CN-NPs and pure IP6 treated groups. Thus, this strategy may open avenues for enhanced targeting of drugs to the colon. Also, the results of the study confirmed that IP6@TPP*PN-CN-NPs and CE-IP6@TPP*PN-CN-NPs could constitute a promising tool for the management of DMH- induced neoplastic damage and dysregulated proliferation markers. The delivery of IP6 to the colon, orally through the prepared biopolymer NPs may open avenues for IP6 utility in colon cancer management or as a nutraceutical. Combined, these results bolster optimism for a future drug delivery approach aimed at the plethora of cancer treatments, suggesting an effective anticancer effect of the IP6-loaded formulations. Further investigation may be executed to scale up such formulations loaded with drugs of natural origins, from bench to bedside.

Even though the future is unforeseeable, we know that nanoscience and nanotechnology hold incredible potential for transforming human lives and will have far-reaching social ramifications.