Sigma-2 receptor ligand anchored telmisartan loaded nanostructured lipid particles augmented drug delivery, cytotoxicity, apoptosis and cellular uptake in prostate cancer cells

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ABSTRACT
Recently, the anticancer activity of telmisartan (TEL) has been discovered against prostate cancer. Nevertheless, despite favorable therapeutic profile, poor aqueous solubility and suboptimal oral bioavailability hamper the anticancer efficacy of TEL. Therefore, in this investigation, sigma-2 receptor ligand, 3-(4-cyclohexylpiperazin-1-yl) propyl amine (CPPA) anchored nanostructured lipid particles of telmisartan (CPPA-TEL-NLPs) were engineered using stearic acid for targeting prostate cancer, PC-3 cells. The mean particle size of TEL-NLPs was measured to be 25.4 ± 3.2 nm, significantly (p < 0.05) lower than 32.6 ± 5.3 nm of CPPA-TEL-NLPs. Correspondingly, the zeta-potential of TEL-NLPs was measured to be −15.4 ± 2.3 mV significantly (p < 0.05) higher than −9.6 ± 2.7 mV of CPPA-TEL-NLPs. The encapsulation efficiency of CPPA-TEL-NLPs was estimated to be 72.7 ± 4.3%, significantly (p < 0.05) lower than 77.5 ± 5.4%, displayed by TEL-NLPs. In addition, FT-IR and PXRD confirmed the molecular encapsulation of the drug in amorphous state. In vitro drug release study was conducted to determine the drug delivery potential of tailored nanoparticles. TEL-NLPs released 93.36% of drug significantly (p < 0.05) higher than 85.81%, released by CPPA-TEL-NLPs in 24 h. The IC_{50} of CPPA-TEL-NLPs was measured to be 20.3 μM significantly (p < 0.05) lower than 36.3 μM presented by TEL-NLPs in PC-3 cells. In contrast, CPPA-TEL-NLPs displayed the IC_{50} of 41.3 μM, significantly (p > 0.05) different from 43.4 μM, exhibited by TEL-NLPs in PNT-2 cells. We elucidated that CPPA-TEL-NLPs entered the PC-3 cells via receptor mediated endocytosis pathway and thus exhibited superior cytotoxicity, apoptosis and greater extent of cellular uptake in PC-3 cells. In conclusion, CPPA-TEL-NLPs may be a promising nanomedicine and warrant further in vivo investigations for gaining clinical success.

Introduction
Prostate cancer is one of the leading cancers in men both in India and United States. According to recent trends, the incidences of prostate cancer are increasing in India by 1% every year. Treatment modalities for prostate cancer include surgery, radiation therapy and chemotherapy. However, drug resistance owing to activation of androgen receptors (ARs), expression of efflux pumps, establishment of alternative survival pathway such as Akt/PI3K, MAPK/ERK or JAK/STAT3 and cross-talk between cancer cells and surrounding microenvironment limited the drug uptake in tumor vasculature.

Recently, the anticancer activity of telmisartan (TEL) has been discovered against prostate cancer. TEL attenuated the prostate cancer by augmentation of apoptosis following the pathways of activation of caspases, inactivation of p38 MAPK and down-regulation of ARs. Moreover, TEL is effective against human hormone-refractory prostate cancer (PC-3 cells, absence of ARs androgen independent, ARs – ve) cells, androgen-independent prostate cancer (DU-145 cells, ARs – ve) cells and androgen-dependent prostate cancer (LNCaP cells, presence of ARs androgen dependent, AR + ve) cells. TEL displayed the in vivo anticancer potential against prostate cancer in transgenic rat model at the dose of 2 or 10 mg/kg/day for 12 weeks. Nevertheless, despite favorable therapeutic profile, poor aqueous solubility (6.58 × 10^{-4} mg/ml) and suboptimal oral bioavailability in rodents (absolute bioavailability ~67%) and humans (absolute bioavailability ~43%) hamper the anticancer efficacy of TEL. In addition, dose-dependent side effects are also associated with TEL treatment like renal dysfunction, myocardial infarction and cardiac dysfunction. This may be attributed to rapid distribution of TEL in liver, gall bladder, kidney, small intestine and heart. Consequently, physicochemical, structural and biological interactions of TEL with cellular receptors like ARs and peroxisomes proliferator activated receptors (PPARs), located in visceral organs also largely influence the biodistribution pattern. This entails the administration of TEL in a safe homing device, which could selectively deliver the high pay load in prostate cancer cells as compared to normal cells.

Nanostructured lipid particles (NLPs) of biodegradable lipids like stearic acid, palmitic acid and tripalmitin have been widely investigated for drug delivery to tumor vasculature. Particularly, NLPs below 100 nm have displayed high cellular uptake in cancer cells and surmounted the poor physicochemical and pharmacokinetic elements of cytotoxic drugs. In addition, certain fatty acids like stearic acid are safe to administer through parenteral route of administration and have already been approved by US FDA for human consumption. NLPs exhibited several merits over the
other nanoparticle forms, because only physiological compounds (lipids, fatty acids, oleic acid present in natural oils) are used for preparation. Moreover, NLPs can also be scaled up at the industrial level owing to its high stability.

Sigma receptors (σ) are highly over-expressed on a variety of human tumor cells including non-small cell lung cancer, melanoma, breast cancer and prostate cancer. Previous reports indicated that the sigma receptor ligand, anisamide effectively mediated the selective delivery of liposomal doxorubicin in prostate cancer cells, both in vitro and in vivo. Correspondingly, haloperidol, a sigma receptor ligand has also shown improved cellular uptake in MCF-7 breast cancer cells by more than ~10 folds as compared to unmodified liposomes. However, neither anisamide nor haloperidol distinguished between sigma-1 or sigma-2 receptor subtypes with respect to binding. Sigma-2 receptors have shown several potential advantages over sigma-1 receptors including higher density of sigma-2 receptors vis-à-vis sigma-1 receptors on tumor tissues or cultured cancer cells, and a correlation between sigma-2 receptor expression and tumor proliferation has been established both in vitro and in vivo, but no such correlation was monitored for sigma-1 subtype. Hence, sigma-2 receptor ligand mediated drug delivery cargo effectively delivered a high payload in tumor cells as compared to normal cells.

The 3-(4-cyclohexylpiperazine-1-yl) propyl amine (CPPA) is a synthetic, small, and pure sigma-2 ligand, whereas aptamers, antibodies, peptides have been scrutinized as targeted ligands though owing to high molecular weight possess lower diffusivity in cancer cells. In addition, CPPA bearing cyclohexyl piperazine moiety showed commendable affinity (Ki ~1 nM) for sigma-2 receptors. The utility of CPPA for targeting sigma-2 receptors has already been established both in vitro and in vivo. Therefore, in this investigation, CPPA anchored TEL loaded stearic acid NLPs (CPPA-TEL-NLPs) were prepared for targeting human prostate cancer cells (PC-3, sigma-2 receptor +ve/presence of sigma-2 receptors and ARs –ve) and compared with unmodified nanoparticles (TEL loaded NLP (TEL-NLPs)) in terms of enhanced drug delivery, apoptosis and cellular uptake. Further, various analytical, spectral and biological techniques were employed to determine the therapeutic potential of both CPPA-TEL-NLPs and TEL-NLPs, respectively.

Materials and methods

Materials

TEL was a kind gift sample from Swiss Garnier Life Sciences, India. 1-Cyclohexyl piperazine was purchased from Sigma-Aldrich (St. Louis, MO). N-3 (bromopropyl) phthalimide was purchased from Alfa Aesar (T Nagaru, Chennai) India. Hydrazine hydrate, N-hydroxy succinimide and N,N-Dicyclohexyl carbodiimide was purchased from Loba Chemie (Colaba, Mumbai) India. Stearic acid was obtained from Thomas Baker (Lohar Chawl, Mumbai, India). All other chemicals used were of highest analytical grade.

Cell culture and mediums

Human prostate cancer cell line (PC-3) and human normal prostate epithelium cell line (PNT-2) were maintained separately in 95% air and 5% CO₂ at 37°C using Dulbecco’s Modified Eagle’s Medium (DMEM) (Biologicals, Kfar Saba, Israel) supplemented with 5% fetal calf serum. All experiments were performed with asynchronous populations in exponential growth phase (24 h after plating).

Synthesis of sigma-2 receptor ligand, CPPA

The sigma-2 receptor ligand, CPPA was prepared by Gabriel synthesis. In brief, a mixture of N-(3-bromobutyl)-phthalimide (15 mM), 1-cyclohexylpiperazine (10 mM) and K₂CO₃ (20 mM) in ethanol (100 ml) was refluxed for 3 h. The reaction mixture was then filtered and solvent was evaporated to obtain a crude product (Compound 1) which was then used without further purification. A solution of compound 1 (free bases, 1.2 mM) and hydrazine monohydrate (0.05 ml, 15 mM) in dichloromethane (30 ml) was stirred at room temperature. Consequently, white and gelatinous precipitates were formed. Precipitates were then collected by filtration and washed with excess of water. Furthermore, anhydrous Na₂SO₄ was used to dry the organic layer, and solvent was evaporated for obtaining CPPA and purified using column chromatography (silica gel, CHCl₃:MeOH 95:5). The synthesis of CPPA was confirmed by ¹H NMR spectroscopy using BRUKER DPX 300 MHz spectrophotometer (Bruker, Billerica, MA). In addition, molecular weight of CPPA was validated by using mass spectroscopy (WATERS, Q-TOF MICROMASS, LC-MS) (Waters Corporation, Milford, MA).

Conjugation of CPPA to stearic acid

The CPPA was conjugated to stearic acid by covalent coupling technique. In brief, stearic acid (100 mg), dicyclohexylcarbodiimide (DCC, 145 mg, 2 mM) and N-hydroxy succinimide (NHS, 80 mg, 2 mM) were added to anhydrous tetrahydrofuran (THF, 5 ml) and then reaction mixture was stirred at room temperature for 4 h. The activated stearic acid was reacted with a solution of CPPA (79 mg, 1.1 mM) in anhydrous THF (5 ml) and stirred at room temperature overnight. The solvent was evaporated and precipitates were filtered off. The free stearic acid and free CPPA from the precipitates were removed by column chromatography (silica gel, CHCl₃:CH₂OH; 95:5). The conjugation of CPPA with stearic acid was confirmed by fourier-transforms infrared spectroscopy (FT-IR, PerkinElmer, Waltham, MA). Samples were prepared using KBr pellets (5 mg sample in 200 mg KBr) at a force of 40 psi for 5 min with a hydrostatic press. Spectra were recorded in the range of 4400–400 cm⁻¹ at a resolution of 4 cm⁻¹. In addition, mass spectroscopy of purified CPPA-conjugated stearic acid was carried out using WATERs, Q-TOF, Micromass (ESI-MS) spectrometer with electron multiplier detector equipped with GC-MS data system. The probe available for solid samples was used in this study. Electron ionization mass spectrum was obtained at ionizing energy value of 70 and 12 eV, with ionization current of 60 μA and vacuum of 10⁻⁶ torr.

Quantitatively, the amount of CPPA conjugated to stearic acid was also determined. In brief, a calibration curve of CPPA was prepared in dimethyl sulfoxide in the concentration range of 20–100 μg/ml at 215 nm by using a UV/Visible Spectrophotometer (1800, Shimadzu, Kyoto, Japan). The amount of free CPPA obtained in filtrate after precipitation of CPPA stearic acid conjugate was calculated from the linear regression equation of calibration curve (Y = 0.009x + 0.020 R² = 0.993). The conjugation efficiency was calculated from the following formula:

\[
\text{Conjugation efficiency} = \left( \frac{\text{Amount of ligand added} - \text{Amount of free ligand}}{\text{Amount of ligand added}} \right) \times 100
\]
**Determination of partition coefficient of TEL**

The partition coefficient (K) of TEL was determined in lipid-water binary system. Briefly, 100 mg of TEL was suspended in a mixture containing 10 ml of water and 1 g of hot melted stearic acid in a separating funnel. The mixture was maintained at 70 °C to prevent the solidification of stearic acid. The mixture was then shaken for 30 min. The concentration of TEL in aqueous phase was determined by passing through 0.22 μm membrane filter (MDI, Ambala, India). The absorbance of the filtrate was measured at 298 nm using a UV–Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan). The experiment was carried out in triplicate (n = 3) and averaged.

\[ K = \frac{C_s}{C_a} \]

where K represents the partition coefficient, Cs denoted to concentration of drug in stearic acid phase and Ca was denoted to concentration of drug in aqueous phase.

**Preparation of TEL-NLPS**

TEL-NLPS were prepared by solvent diffusion method. In brief, 120 mg of stearic acid and 10 mg of TEL were dissolved in a mixture of 6 ml of ethyl alcohol and 6 ml of acetone. Next, this organic phase was dispersed in 100 ml of distilled water, maintained at 70 °C and constantly stirred for 30 min by employing a magnetic stirrer. Finally, the nanoparticle suspension was cooled at room temperature and lyophilized (Lark technology, New Delhi, India) to get the fine powder of TEL-NLPS. Correspondingly, CPPA conjugated stearic acid was employed for fabricating the CPPA-TEL-NLPS. NLPS without incorporation of drug were also prepared for comparative studies.

**Labeling of fluorescent dye to nanoparticles**

Fluorescent labeled TEL-NLPS and CPPA-TEL-NLPS were prepared, by adding 5 mg/ml of rhodamine 6G in organic phase during preparation of nanoparticles. The labeling efficiencies were evaluated by dissolving each formulation in PBS (pH ~ 7.4) till the concentration reached to 0.10 mg/ml. Next, the fluorescence intensity of labeled nanoparticles was measured at λex = 553 nm and λem = 574 nm by using a fluorometer (Perkin Elmer LS-5B, Cambridge, UK). A standard solution of 0.005–0.015 mg/ml of rhodamine 6G was prepared by diluting 10 mg/ml of methanolic solution of dye with PBS, pH ~ 7.4. Labeling efficiency was estimated as the ratio of weight of rhodamine 6G (%) to the weight of TEL-NLPS or CPPA-TEL-NLPS.

\[ y = 0.724x + 0.037R^2 = 0.990 \]

**Characterization of nanoparticles**

**Particle size and zeta potential**

Particle size samples were dispersed separately in phosphate buffer saline (PBS, pH ~7.4) before analysis. Malvern Nano ZS (Malvern Instruments, Worcestershire, UK) was used to determine the particle size and zeta-potential. A 150 mV electric field was applied to measure the electrophoretic velocity of nanoparticles. All measurements were carried out in triplicate (n = 3).

**Transmission electron microscopy (TEM)**

Particle shape and surface topography were examined by transmission electron microscopy (TEM, FTI Tecnai F20). Briefly, an aqueous suspension of nanoparticles was individually drop casted onto a carbon coated copper grid, and the grid was air dried at room temperature before loading it into the microscope which was maintained at a voltage of 80 kV.

**Fourier-transforms infrared (FT-IR) spectroscopy**

FT-IR was performed to address the issue of any chemical incompatibility between drug and excipients according to the specifications as mentioned earlier. In brief, spectrum of TEL, NLPs, physical mixture of TEL and NLPs as well as TEL-NLPS was recorded.

**Powder X-ray diffraction pattern (PXRD)**

The polymorphic state of the drug in lipid matrix was confirmed by X-ray diffractometer (X’Pert PRO, Panalytical Company, Almelo, The Netherlands) using Ni-filtered, Cu Kα-radiation, voltage of 60 kV and current of 50 mA. The scanning rate was 1°/min over 10° to 60° diffraction angle (2θ) range. The crystal lattice of TEL, NLPs, physical mixture of TEL and NLPs, and TEL-NLPS was accounted.

**Encapsulation efficiency and drug loading capacity**

The encapsulation efficiency and drug loading capacity were determined by dissolving separately 50 mg quantity of each sample in 10 ml of 0.05 M NaOH. Both samples were left undisturbed for 72 h at room temperature. Subsequently, samples were ultra-centrifuged (Sorvall Ultra-centrifuge) at 40 000 rpm for 2 h and the supernatant liquids were filtered off using 0.22 μm membrane filter (MDI, Ambala, India). The absorbance of the TEL was measured at 298 nm by using a UV–Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan). All measurements were carried out in triplicate (n = 3). Encapsulation efficiency and drug loading capacity were calculated by using the following formulas:

\[ \% \text{Encapsulation efficiency} = \frac{\text{Amount of drug recovered}}{\text{Amount of drug added}} \times 100 \]

\[ \text{Drug loading capacity} = \frac{\text{Amount of drug recovered}}{\text{Amount of nanoparticles}} \]

**In vitro drug release**

In vitro drug release experiment was executed using dialysis membrane technique. In brief, 46.48 mg of TEL-NLPS (~40 mg TEL) and 50.6 mg of CPPA-TEL-NLPS (~40 mg TEL) were separately added to a dialysis bag (12 kDa pore size). The bags were then suspended separately in 900 ml of PBS (pH~7.4, without serum) and 900 ml of PBS (pH ~ 7.4) containing 20% normal goat serum, maintained at 37 °C and 100 rpm, as recommended for dissolution testing of parenteral products. At different time intervals, 5 ml of the sample was withdrawn and simultaneously replaced with fresh dissolution medium to maintain the sink conditions. The drug concentration was measured at 298 nm by using a UV–Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan).
**Therapeutic efficacy testing of TEL-NLPs and CPPA-TEL-NLPs in prostate cancer cells**

**In vitro cytotoxicity assay**

MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was employed for in vitro cytotoxicity assay. Briefly, 6 × 10⁴ PC-3 cells (Sigma-2 receptor positive and ARs, –ve cells) or PNT-2 cells (Sigma-2 receptor negative) were seeded in 200 μl of serum DMEM medium contained in each well of a 96-wells microtitre plate. After 24 h of incubation period, the medium was replaced with serum free-DMEM. Consequently, seeded PC-3 or PNT-2 cells were incubated with a gradient concentration of TEL, TEL-NLPs, CPPA-TEL-NLPs and NLPs equivalent to 20–100 μM of TEL for 72 h. At the end of treatment, 0.5 mg/ml of MTT dye was added to each well and the plates were incubated for another 4 h at 37 °C. After cell lysis, formazan crystals were obtained, which were solubilized in 100 μl of dimethyl sulfoxide. The absorbance was measured at 570 and 630 nm as reference wavelength by ELISA Reader (Tecan, Männedorf, Switzerland).

**Apoptosis assay**

A fluorimetric caspase-3 apoptosis assay kit was employed to analyze the apoptosis induced by TEL, TEL-NLPs, CPPA-TEL-NLPs and NLPs. The assay was performed as per the manufacturer’s instructions (Merck Millipore, Billerica, MA) at a concentration ~20 μM of TEL for 48 h in cell culture dishes of 35 × 15 mm. The PC-3 cells were collected in a pellet form at the end of treatment and re-suspended in 50 μl of cell lysis buffer (100 μl of 10 mM EDTA + 250 μl of 50 mM tris-HCl + 250 μl of 0.5% SDS + 250 μl of 0.5 mg/ml Proteinase K + 4150 μl Water). The buffer was incubated on ice for 10 min. Subsequently, the cells were centrifuged at 3000 × g for 5 min, and the supernatant was transferred to a 96-wells plate to which 50 μl of the reaction buffer (50 mM/L PIPES, pH ~7.4, 10 mM/L EDTA, 0.5% CHAPS) containing 10 mM/L dithiothreitol and 5 μl of the respective substrate was added. The plate was incubated at room temperature for 1 h, and the fluorescence was measured in a fluorometer (Spectra Fluor, Tcanc, λex ≈ 400 nm and λemi ≈ 505 nm). The protein content was measured using BCA protein assay kit. The experiment was performed in triplicate (n = 3).

**In vitro cellular uptake assay: quantitative and qualitative analyses**

The cellular uptake assay was performed both quantitatively and qualitatively. In brief, 2 × 10⁵ PC-3 cells were seeded in Lab-Tek II Chamber slide™ system (Nalge Nune, Penfield, NY). Using PBS (~7.4), dosing solutions of rhodamine 6G labeled TEL-NLPs and CPPA-TEL-NLPs (25–100 μM of TEL) were prepared and diluted with DMEM. The cell monolayers were rinsed thrice and pre-incubated with 1 ml of DMEM at 37 °C for 1 h. Cellular uptake was initiated when 1 ml of DMEM was replaced with 1 ml dosing solution of rhodamine 6G labeled TEL-NLPs and CPPA-TEL-NLPs, respectively. The slide chamber was then incubated for 5 h at 37 °C. Subsequently, the experiment was ceased by washing the cell monolayers thrice with ice cold PBS (pH≈7.4) and lysing the cells with 1 ml of 0.5% v/v Triton X-100 (25 mM tris-HCl pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 5% glycerol). Cell-associated fluorescence was measured using a fluorometer (Spectra Fluor, Tcanc, λex = 553 nm, λemi = 574 nm). Consequently, cellular uptake of CPPA-TEL-NLPs in PC-3 cells was also accounted in the presence of various inhibitors such as 10 μg/ml filipin (inhibitor of caveolae mediated endocytosis), 20 μg/ml brefeldin A (inhibitor of Golgi apparatus), 100 mM/L monensin (an antiporter of Na+/H+), 10 mM/L NH₄Cl, 20 μg/ml chlorpromazine (inhibitor of clathrin coated pits), 450 mM/L sucrose (inhibitor of clathrin coated pits), 25 μM/L cytochalasin D, 0.1% w/v sodium azide (energy depleter) and 33 μM/L nacodazole (Macropinocytosis inhibitor). The protein content of the cell lysate was measured using BCA protein assay kit.

After 5 h of incubation period, the DMEM medium was removed and the plates were washed thrice with sterile PBS (pH≈7.4). Finally, the plates were fixed with 4% paraformaldehyde, and cover slips were individually mounted on clean glass slides with fluoromount-G mounting medium (Southern Biotechnology, Birmingham, AL) and scanned under confocal laser scanning microscope (CLSM) at λex = 553 nm, λemi = 574 nm. Then 4,6-diamidino-2-phenylindole (DAPI) dye was used for nucleus staining.

**Statistical analysis**

Results were expressed as the mean±S.D. for n = 3 by using column statistics in GraphPad Prism04 (San Diego, CA) Software. Unpaired ‘t’ test was used to calculate the statistical significant difference between mean values of two groups. One-way and two-way ANOVA test was used for the comparison between different groups.

**Results**

**Design, synthesis, preparation and characterization of CPPA-TEL-NLPs**

We have designed, synthesized and prepared CPPA-TEL-NLPs, primarily by synthesizing CPPA followed by its conjugation with stearic acid for tailoring CPPA-TEL-NLPs (Scheme 1(A,B)). TEL exhibited the partition coefficient (K) of 2.024 that indicated its lipophilic nature. CPPA was synthesized by the reaction of 1-cyclohexylpiperazine with N-protected-3-bromopropyl amine viz. phenethylamine derivative in dry alkaline conditions that was later deprotected using hydrazine hydrate (Scheme 1(A)). Mass spectrum exhibited the M⁻¹ peak at 226 which is equivalent to the molar mass of CPPA (Figure 1). Eventually, the carbonyl group of stearic acid was activated by using DCC and NHS, in the presence of anhydrous THF. This was followed by the addition of CPPA in anhydrous THF that consequently lead to the formation of CPPA conjugated stearic acid (Scheme 1(B)). The conjugation of CPPA with stearic acid was confirmed FT-IR spectroscopy (Figure 1) and mass spectroscopy (Supplementary Figure 1).

The FT-IR spectrum revealed a distinguished peak at 1637 cm⁻¹, which affirmed the presence of –CONH bond. Correspondingly ¹H NMR also confirmed the conjugation step. ¹H NMR spectrum predicted the presence of protons of the alkyl chain and amine group of propyl amine that served as a linker between stearic acid and CPPA (Figure 1). Primary amine group is a preferred substituent for assuring a high affinity for sigma-2 receptors and high sigma 1: sigma 2 selectivity. Hence CPPA possesses high affinity for sigma-2 receptors owing to the presence of primary amine group. Quantitatively, 58.6% of CPPA was conjugated to stearic acid.

**Measurement of particle size, zeta potential and surface topography**

The mean particle size of TEL-NLPs was measured to be 25.4 ± 3.2 nm, significantly (Unpaired t test, p < 0.05) lower than...
Correspondingly, the zeta-potential of TEL-NLPs was measured to be 15.4 ± 2.3 mV significantly (Unpaired \( t \) test, \( p < 0.05 \)) higher than 9.6 ± 2.7 mV of CPPA-TEL-NLPs (Figure 2(A,B) and Table 1). The surface topography of TEL-NLPs and CPPA-TEL-NLPs was compared to envisage the coating of sigma-2 receptor ligand on the surface of TEL-NLPs (Figure 2(A,B)). TEM micrographs indicated that both TEL-NLPs and CPPA-TEL-NLPs were spherical in shape and in this way, we can predict favorable cellular uptake of nanoparticles in cancer cells.

### Encapsulation efficiency and drug loading capacity analyses

The encapsulation efficiency of CPPA-TEL-NLPs was measured to be 72.7 ± 4.3%, significantly (Unpaired \( t \) test, \( p < 0.05 \)) lower than 77.5 ± 5.4% displayed by TEL-NLPs (Table 1). Furthermore, drug loading capacity was measured to be 8.6 and 7.9 mg per 10 mg of nanoparticles for TEL-NLPs and CPPA-TEL-NLPs, respectively.

### FT-IR analysis

FT-IR spectra were recorded to scrutinize the impact of lipid on the chemical stability of drug. The stretching frequencies of TEL,
TEL-NLPs, physical mixture of TEL and NLPs as well as TEL-NLPs have been recorded and presented in Table 2. The spectrum of TEL exhibited the specific peaks at 3065 cm$^{-1}$ (aromatic C–H stretch), 2977 cm$^{-1}$ (aliphatic C–H stretch), 1687 cm$^{-1}$ (carboxylic acid), 1623 cm$^{-1}$ (aromatic C–C bending and stretching), 1467 cm$^{-1}$ (C–H bend), 1386 cm$^{-1}$ (OH bending and C–O stretching of carboxylic acid) and 746 cm$^{-1}$ (1,2 di substituted benzene ring vibrations), respectively. The spectrum of NLPs displayed peaks at 2964 cm$^{-1}$ (aliphatic C–H stretch), 2908 cm$^{-1}$ and 2840 cm$^{-1}$ for –CH$_3$/CH$_2$ groups and 1697 cm$^{-1}$ for –OC=O. Respectively, these peaks were almost unchanged in the physical mixture. The spectrum of TEL-NLPs exhibited peaks at 2914 cm$^{-1}$ (aliphatic C–H stretch) and 2851 cm$^{-1}$ for CH$_3$/CH$_2$ groups and moreover on 1698 cm$^{-1}$ for –OC=O without any significant shift. Hence, FT-IR spectra evinced that TEL persisted its characteristics in the lipid matrix and further encapsulation did not impact the chemical stability of the drug.

PXRD analysis

PXRD analysis was made to record the polymorphic state of TEL in the lipid matrix. The XRD pattern of TEL demonstrated the sharp and intense peaks, revealing its crystalline structure (Figure 3). In contrast, the peaks presented by NLPs were diffused and of low intensities. Correspondingly, the physical mixture of TEL and NLPs displayed a mixture of sharp peaks with diffused peaks.
Subsequently, the diffused peaks displayed by TEL-NLPs were of little intensities indicating the amorphous state of TEL in lipid matrix.

**In vitro drug release**

In vitro drug release study was conducted to determine the drug delivery potential of tailored nanoparticles in presence and absence of serum. TEL-NLPs released 93.36% of drug significantly (Two-way ANOVA test, \( p < 0.05 \)) higher than 85.81%, released by CPPA-TEL-NLPs in PBS (pH=7.4, without serum) at 24 h (Figure 4(A)). On the other hand, TEL-NLPs released 97.23% of the drug with no significant difference (Two way ANOVA, \( P > 0.05 \)) from 95.61%, released by CPPA-TEL-NLPs in PBS (pH=7.4, with 20% serum) at 24 h (Figure 4(B)).

**CPPA-TEL-NLPs exhibited enhanced cytotoxicity in PC-3 cells in comparison to TEL-NLPs and TEL alone**

The cytotoxicity of TEL, TEL-NLPs, CPPA-TEL-NLPs and NLPs was measured against PC-3 and PNT-2 cells. The IC\textsubscript{50} of CPPA-TEL-NLPs was measured to be 20.3 \textmu M significantly (Unpaired t test, \( p < 0.05 \)) lower than 36.3 \textmu M presented by TEL-NLPs in PC-3 cells (Figure 5(A)). On the other hand, the IC\textsubscript{50} of TEL and NLPs was >100 \textmu M. In contrast, CPPA-TEL-NLPs displayed the IC\textsubscript{50} of 41.3 \textmu M significantly (Unpaired t test, \( p > 0.05 \)) not different from 43.4 \textmu M, exhibited by TEL-NLPs in PNT-2 cells.

**Discussion**

Advances in the delivery of targeted drug delivery systems involve first line organ targeting to third line sub-cellular organelles targeting. Moreover, targeting nanotherapeutics to intracellular

![Figure 3](image-url). PXRD of TEL, NLPs, physical mixture of TEL and NLPs as well as TEL-NLP.

![Figure 4](image-url). In vitro drug release of TEL-NLPs and CPPA-TEL-NLPs carried out in (A) phosphate buffer saline (PBS, pH=7.4, without serum) and (B) PBS (pH=7.4, with 20% serum). TEL-NLPs released 93.36% of the drug significantly (Two way ANOVA test, \( p < 0.05 \)) higher than 85.81%, released by CPPA-TEL-NLPs at 24 h in PBS (pH=7.4). Moreover, TEL-NLPs released 97.23% of the drug with no significant difference (Two way ANOVA, \( p > 0.05 \)) from 95.61%, released by CPPA-TEL-NLPs in PBS (pH=7.4, with 20% serum) at 24 h.

**CPPA-TEL-NLPs exhibited enhanced apoptosis in PC-3 cells**

Next, the apoptotic activity was assessed by Caspase-3 cleavage and expressed in terms of mean fluorescence intensity and percent cellular uptake. CPPA-TEL-NLPs induced greater extent of apoptosis (One way ANOVA, \( p < 0.05 \)) in comparison to TEL-NLPs and TEL in PC-3 cells (Figure 5(B)).

**CPPA-TEL-NLPs exhibited enhanced cellular uptake both quantitatively and qualitatively in PC-3 cells**

We examined and visualized both quantitative and qualitative internalization of rhodamine 6G labeled CPPA-TEL-NLPs and TEL-NLPs in PC-3 cells by tracking nanoparticles using fluorimetry (Figure 5(C,D)). After incubation of a gradient concentration of CPPA-TEL-NLPs and TEL-NLPs separately with PC-3 cells for 5 h, we observed the concentration dependent internalization of nanoparticles. The highest mean fluorescence intensity and cellular accumulation was measured to be 82.9% for CPPA-TEL-NLPs, significantly (Two way ANOVA test, \( p < 0.05 \)) higher than 52.6% of TEL-NLPs. In this way, CPPA-TEL-NLPs displayed \(~\text{1.57} \text{fold}\) higher cellular uptake in comparison to TEL-NLPs in PC-3 cells.

Moreover, cellular uptake of CPPA-TEL-NLPs was also measured in the presence of various inhibitors to elucidate the mechanism of internalization. Inhibitors like filipin, brefeldin, monensin, NH\textsubscript{4}Cl, chlorpromazine, and sucrose significantly (Unpaired t test, \( p < 0.05 \)) reduced the cellular uptake to 48.8%, 60.5%, 62.3%, 44.9%, 56.3% and 46.9%, respectively, in PC-3 cells in comparison to 82.9% displayed by the CPPA-TEL-NLPs alone (Figure 5(C)). The qualitative CPPA-TEL-NLPs and TEL-NLPs internalization in PC-3 cells was further examined and visualized by CLSM (Leica, Biomed, Bad Bergzabern, Germany). After incubation for 5 h, the high intensity intracellular fluorescence spots (Red fluorescence of nanoparticles) were observed clearly in cultured PC-3 cells. Representative CLSM images exhibited that CPPA-TEL-NLPs were homogenously distributed into the cytoplasm of PC-3 cells nearby nucleus in comparison to TEL-NLPs (Figure 5(D)).
compartments is associated with site specific delivery, minimization of multidrug resistance (MDR) and enhanced therapeutic index. Targeting ligands then penetrate the cancer cell compartments through over-expressed extracellular receptors and release the drug payload. In this series, biodegradable polymeric nanoparticles or drug conjugates ranging between 10 and 200 nm have gained lot of attention in cancer nanobiology owing to self-stability and flexibility of encapsulating both hydrophilic and lipophilic drugs with high encapsulation efficiency. Subsequent to this, both low molecular weight synthetic ligands (galactose, glucose and mannose) and high molecular weight protein (antibodies) ligands can be anchored over the surface of nanovectors. TEL has shown high therapeutic efficacy in prostate cancer in both in vitro and in vivo settings. However, poor physicochemical, bio-pharmaceutical and pharmacokinetic limitations deliver a suboptimal therapeutic concentration at the target site. Therefore, in this study, we have synthesized and confirmed the preparation of CPPA conjugated stearic acid (Scheme 1(A,B)) that later used in customizing of CPPA-TEL-NLPs for targeting sigma-2 receptor positive human prostate cancer, PC-3 cells. The synthesis of CPPA ligand and its conjugation with stearic acid was confirmed by 1H NMR, mass spectroscopy and FT-IR spectroscopy (Figure 1). It is reported that particle size, shape and surface characteristics of targeted nanotherapeutics greatly influence the cellular accumulation of therapeutic drugs. Hence, nanoparticles have been widely accepted and validated for their small size and large surface area, which make them superior for delivery of anticancer drugs in comparison to other drug delivery systems. Zeta potential is a vital tool for understanding and predicting the long-term stability of the nanoparticles. The magnitude of the zeta potential of nanoparticles greater than $+25 \text{mV}$ or less than $-25 \text{mV}$ typically have high degrees of stability. This may be contributed to the existence of repulsive forces between the particles that prevent agglomeration process. However, neutral particles have been recognized intrinsically more stable due to the absence of interparticulate (both attractive and repulsive) interactions. In addition, surface charge on nanoparticles also determines their blood circulation time, metabolism, excretion and immunological consequences. The cationic nanoparticles are internalized more rapidly by the anionic cell membranes instead of neutral and anionic nanoparticles. The plasma proteins bind to the surface of the nanoparticles and form a soft corona that initiates the process of elimination of nanoparticles by presenting them to the mononuclear phagocyte system (MPS) inside the liver and spleen. In contrast, neutral nanoparticles survive in blood circulation for a longer period of time as opsonization processes are not equally activated as like for charged nanoparticles. Therefore, depending upon the composition of nanoparticles, a negative zeta potential was observed for TEL-NLPs owing to the presence of $\text{O-C} = \text{O}^-$ functional group in stearic acid and TEL. However conjugation with
sigma-2 receptor ligand reduced the zeta potential of CPPA-TEL-NLPs efficiently toward neutral values. Therefore, a highly stable and efficacious dosage form may be predicted in biological assays. Continuation to this, shape also directly influences the cellular uptake of nanoparticles.24 The cellular endocytosis of nanoparticles >100 nm followed the order of nanorods > nanospheres > nanocylinders and nanocubes.25,26,36 In spite of this, nanoparticles were endocytosed more hastily in comparison to nanorods when nanoparticles size was below 100 nm.25,26 Hence, the particle size (<100 nm), zeta potential (negative surface charge) and shape (spherical) of the tailored formulations, TEL-NLPs and CPPA-TEL-NLPs were found to be favorable for targeting and administration through intravenous route (Figure 2(A,B) and Table 1). Moreover, high encapsulation efficiency and drug loading capacity of both TEL-NLPs and CPPA-TEL-NLPs (Table 1) also enhanced the opportunities for overwhelming the poor physicochemical and pharmacokinetic limitations. Next, FT-IR and XRD techniques were employed to characterize any chemical and polymorphic transitions of the encapsulated drug in lipid matrix. FT-IR spectra confirmed that there was no alteration in the chemical stability of TEL in lipid encapsulated drug in lipid matrix. FT-IR spectra confirmed that there was no alteration in the chemical stability of TEL in lipid encapsulated drug in lipid matrix (Table 2). In addition, PXRD substantiated the crystalline to amorphous state transformation of TEL in lipid matrix (Figure 3).

Generally, owing to inconsistent structural geometry, the amorphous phase involves minimal energy and thus renders maximum bioavailability to drugs.23,57 Following this, in vitro release study of TEL from TEL-NLPs and CPPA-TEL-NLPs was conducted in PBS (pH ~ 7.4) in presence and absence of serum (Figure 4(A,B)). Coating of CPPA ligand remarkably reduced the release of TEL in PBS (pH ~ 7.4). Though, TEL is a lipophilic drug and carries limited solubility in water (6.58 x 10^-4 mg/ml). However, amorphization of TEL in nanovectors promoted the solubility and dissolution in aqueous buffer. Furthermore, addition of serum in dissolution medium also encouraged the release of TEL owing to interaction between serum proteins and lipid nanovectors. In last, the therapeautic potential of TEL-NLPs and CPPA-TEL-NLPs was investigated using in vitro cytotoxicity, apoptosis and quantitative and qualitative cellular uptake assays in human prostate cancer, PC-3 cells. The CPPA-TEL-NLPs outstandingly reduced the IC50 to ~1.78 folds lower than the IC50 displayed by TEL-NLPs in PC-3 cells (Figure 5(A)). In contrast, owing to lipophilic nature and lower solubility of TEL at pH ~7.4 consequently elevated the IC50 > 100 µM for free drug. Hence, the lower IC50 of CPPA-TEL-NLPs against PC-3 cells may be contributed to greater endocytosis of nanoparticles. However, CPPA-TEL-NLPs did not exhibit the analogous results in PNT-2 cells that might be due to lower expression of sigma-2 receptors in PNT-2 cells.24 Correspondingly caspase-3 cleavage based apoptosis assay22 also exhibited somewhat analogous results (Figure 5(B)). Extension to this, mechanism of penetration of CPPA-TEL-NLPs in PC-3 cells was elucidated using fluorescent microscopy (Figure 5(C,D)). The CPPA-TEL-NLPs were remarkably endocytosed in PC-3 cells in comparison to TEL-NLPs, expressed in terms of enhanced fluorescence intensity and percent cellular uptake, respectively. Receptor mediated endocytosis is a key mechanism for ligand modified nanoparticles in cancer therapy.24 Our cellular uptake data also demonstrated the involvement of receptor mediated endocytosis of CPPA-TEL-NLPs in PC-3 cells (Figure 5(C)). Ammonium chloride, chlorpromazine and sucrose being the inhibitors of clathrin-coated pits remarkably reduced the cellular uptake of CPPA-TEL-NLPs in PC-3 cells. More interestingly, filipin, an inhibitor of caveolae mediated endocytosis also partially shut down the cellular uptake process. In support, monensin and brefeldin also diminished the cellular uptake and displayed the involvement of acid endosomes and Golgi apparatus. Hence, these inhibitors indicating the role of receptor mediated endocytosis (clathrin-dependent/caveolae-mediated) in cellular internalization of CPPA-TEL-NLPs in PC-3 cells (Figure 5(C)). Furthermore, CPPA-TEL-NLPs (red fluorescence) by following the receptor mediated endocytosis pathway distributed more intensely nearby nucleus (blue staining), whereas accumulation of TEL-NLPs was slightly far away from the nucleus (Figure 5(D)). Hence, we suppose that CPPA-TEL-NLPs would have followed the specific mechanism for intracellular delivery of the drug while TEL-NLPs would have followed the mechanism of passive diffusion. Previous reports convinced that sigma-2 receptor ligand conjugated nanocomposites bind to the receptor and enter the cell. Moreover, sigma-2 receptor is also expressed intracellulary on cell organelles like mitochondria and endoplasmic reticulum. Hence, our findings indicate that CPPA-TEL-NLPs would have enter the PC-3 cells via receptor mediated endocytosis (clathrin-dependent/caveolae-mediated) pathway and subsequently would have switch on multiple apoptosis pathways including mitochondria mediated cell death mechanism. This would have ultimately contributed to the synergistic cytotoxicity and apoptosis induced by the CPPA-TEL-NLPs in comparison to TEL-NLPs and free TEL in PC-3 cells.

Conclusion
In conclusion, sigma-2 receptor ligand, CPPA anchored nanostructured lipid particles of TEL (CPPA-TEL-NLPs) prepared by solvent diffusion method have shown promising chemotherapeutic potential in human prostate cancer, PC-3 cells in comparison to unmodified, TEL-NLPs. Further receptor mediated internalization of CPPA-TEL-NLPs in PC-3 cells switch on multiple apoptosis pathways. This consequently indicated the utility of targeted nanomedicine in the treatment of prostate cancer. Therefore, CPPA-TEL-NLPs warrant further in vivo tumor regression study to scale up the technology for clinical translation.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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