ABSTRACT SUMMARY
A novel multifunctional nano-therapeutic system based on dendrimers was developed characterized and the activity was tested in prostate cancer cells. Poly(amide)amine (PAMAM) dendrimer was conjugated to docetaxel (DTX) using hemi-succinate derivative of docetaxel. The conjugation was characterized using various analytical methods. The conjugate was further complexed with eIF4E siRNA and the activity was tested in prostate cancer cells. Results showed that 3-4 molecules of DTX is conjugated to dendrimer and the conjugate can form a complex with siRNA at 5:1 N/P ratio. The conjugates showed higher activity than the free drug.

INTRODUCTION
Prostate cancer is the second leading cause of death in men in the United States. However most prostate cancer (PC) patients progress to advanced castration-refractory prostate cancer (CRPC) within a few years of androgen ablation therapy. Although docetaxel (DTX) has shown improved survival in CRPC patients, resistance to treatment severely limits patient survival. The treatment induced overexpression of cell survival and anti-apoptotic proteins leads to drug resistance. In this regard eukaryotic translation initiation factor 4E (eIF4E) plays a critical role in regulating the translation of mRNAs that encode several proteins involved in cell growth and survival. eIF4E levels are commonly elevated in advanced PC and is associated with reduced patient survival. Combined delivery of eIF4E siRNA and DTX -carrier can result in reversal of drug resistance and synergistic activity in advanced prostate cancer. To this end the goal of this study was to develop a multifunctional delivery system using polyamidoamine (PAMAM) dendrimer. Since dendrimer has a large number of surface functional groups, DTX can be covalently attached to slowly release the drug in the cell, while siRNA can be electrostatically complexed with rest of the functional groups to release it rapidly in the cell leading to enhanced efficacy. As a first step to realize this goal, a synthetic method was developed to conjugate DTX to dendrimer and then the conjugate was used to complex the siRNA. Various techniques were used to characterize the multifunctional nanotherapeutic system and the preliminary anti-cancer activity was tested in prostate cancer cells.

EXPERIMENTAL METHODS
DTX was covalently conjugated to fourth generation PAMAM dendrimer using hemi-succinate derivative of DTX (DTX-Suc). Briefly, 500 mg DTX was reacted to 1.5 equivalent succinic anhydride in presence of pyridine. The reaction was carried out up to 3-4 days. The resulted mixture was then purified through flash chromatography. In the second step, PAMAM dendrimer was conjugated to DTX-Suc. Briefly, 140 mg of DTX-Suc was added with 88.55 mg (5 eq) of N-hydroxy Succinimide (NHS) and 35.42 mg EDC in presence of dimethyl sulphoxide (DMSO) with constant stirring for 4-6 hours. After 4-6 hours, 100 mg of PAMAM dendrimer was added and the reaction was carried out for three days with constant stirring at room temperature. The resultant conjugate was purified by dialysis and lyophilized. The conjugate was characterized by FTIR, 1H NMR, ESI mass, MALDI-TOF. In vitro release studies were carried out in culture media (DMEM + 10% Fetal Bovine Serum (FBS) and 1% streptomycin/penicillin), and PBS 7.4 pH buffer with 1% tween 80. The conjugate was then complexed with eIF4E siRNA at different N/P ratios (5:1, 10:1 and 20:1). The complex was characterized using 1% agarose gel electrophoresis. The anti-proliferative activity of the conjugates was then tested in prostate cancer (PC3) cells using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. Briefly, PC3 cells were seeded in 96 well plates at cell density of 10,000 cells/well and cultured overnight. The following day, cells were treated with serial dilutions of DTX, DTX-PAMAM and DTX-PAMAM-siRNA. After 36 hours of treatment, the treated solution was removed and the cell viability was tested by MTT assay. siRNA was complexed to PAMAM and PAMAM-DTX in serum free media.

RESULTS AND DISCUSSION
DTX was conjugated to the G4 PAMAM dendrimer through amide bond between the primary amines on the dendrimer surface and NHS ester of DTX. 1H NMR of DTX-PAMAM displayed chemical shift (δ) at 7.2 ppm
which confirmed the presence of aromatic nucleus of DTX. Additional peaks between δ 1-2 ppm confirmed the formation of amide bond. FT-IR spectra showed aromatic C=C bending and stretching at around 1652.33 cm⁻¹ (data not shown). Additionally an intense peak at 1465.4 cm⁻¹ confirmed the presence of amide bending peak in the conjugate. MALDI –TOF spectra of DTX PAMAM conjugate (Fig. 1) displayed the molecular ions peaks at 16390.017 [M⁺] and 8148.847.415 [M²⁺] while in case of PAMAM dendrimer, the peaks were found at 13603.832 [M⁺] and 6745.482 [M²⁺]. The results show that 3-4 DTX molecules are conjugated to one molecule of dendrimer.

In vitro release studies conducted, showed that 40.7±0.38 and 45.9±1.2% DTX was released over 12 hrs in cell culture media and PBS 7.4 buffer, respectively (Fig. 2). Gel electrophoresis results showed that DTX-dendrimer conjugate was able to form complex with eIF4E siRNA at N/P ratio of 5:1 or higher (Fig. 3), while in absence of DTX, dendrimer-siRNA complex was formed at 1:1 N/P ratio or higher (data not shown).

As seen from MTT assay in Fig 4. The drug conjugates showed enhanced activity in prostate cancer cells. As expected the free siRNA did not show any activity (data not shown). The DTX-dendrimer-siRNA complex showed slightly higher activity than DTX-dendrimer conjugate. However, further studies in resistant prostate cancer cells may show a much higher activity for the siRNA-dendrimer-DTX conjugate due to the overexpression of eIF4E.

CONCLUSION
A multifunctional nanotherapeutic system was developed by conjugating DTX to dendrimer and complexing the resultant conjugate to eIF4E siRNA. The conjugate had 3-4 molecules of DTX and the conjugate formed a complex with siRNA at N/P ratio of 5:1. The conjugates exhibited higher anti-cancer activity than free DTX and free siRNA. Future studies will focus on optimizing the number of DTX and siRNA molecules in the dendrimer and testing the conjugates in drug resistant prostate cancer cells.