Abstract. Aim: The aim of this study was to prepare biotinylated PAMAM dendrimers loaded with cisplatin and to evaluate the cytotoxicity in ovarian cancer cell lines. Materials and Methods: Biotinylated and unconjugated dendrimer-cisplatin complexes were investigated for encapsulation efficiency, in vitro cytotoxic activity and cellular accumulation of cisplatin in OVCAR-3, SKOV-3, A2780 (wild-type) and CP70 (A2780/CP70, cisplatin- resistant) cells. Results: Encapsulation efficiency of cisplatin ranged from 5.33% to 21.10%. In vitro cytotoxic activity revealed that IC50 values of dendrimer-cisplatin complexes were significantly lower than that of free cisplatin in OVCAR-3, SKOV-3 and CP70 cell lines. Cellular uptake data showed highest accumulation of platinum by PAMAMG4 NH2 dendrimer complexes of cisplatin in A2780 (19.41±0.85 μg/ml) and CP70 (25.25±1.25 μg/ml) cell lines in comparison with cisplatin uptake of only 1.77±0.351 μg/ml in A2780 and 2.31±0.421 μg/ml in CP70 cells. Conclusion: In conclusion, biotinylated PAMAM dendrimers may be utilized as potential targeting agents for cisplatin delivery to ovarian cancer.

In the United States, epithelial ovarian cancer accounts for 3% of all cancers among women. About 21,880 new cases and 13,850 deaths were estimated in 2010 (1). Treatment for patients with advanced-stage epithelial ovarian cancer usually involves optimal cyto-reductive surgery and combination chemotherapy with cis-diamminedichloro-platinum (II) (cisplatin) and paclitaxel (2). Although cisplatin is very effective in treating many solid tumors including ovarian cancer, its efficacy is limited not only by tumors becoming refractory to cisplatin treatment, but also due to systemic side-effects such as nephrotoxicity and neurotoxicity (3).

In order to enhance therapeutic potential and to reduce systemic toxicity, selective targeting of cisplatin to cancer cells is of prime importance. Several target oriented nanocarriers have been recently developed. For example, when cisplatin was loaded into porous hollow nanoparticles of Fe3O4 coupled with herceptin, the IC50 was reduced three times compared with cisplatin alone (4). In another study, cisplatin release to tumor was enhanced by incorporating cisplatin into nanosterically stabilized liposomes (nSSL) followed by external application of low frequency ultrasound (LFUS), triggering cisplatin release from nSSLs (5). Single-walled carbon nanotubes (SWNT) functionalized with epidermal growth factor (EGF) have been investigated for targeted delivery of cisplatin to head and neck squamous carcinoma cells both in vitro and in vivo. A significant and rapid tumor regression was observed in mice treated with targeted SWNT-cisplatin-EGF when compared to SWNT-cisplatin (6). Several polymeric drug delivery systems have also been studied to enhance delivery of cisplatin, which include micelles, micro- or nanospheres, implants or polymer–drug conjugates (7-9). However, the strategies mentioned above involve complex administration procedures and involve carriers which are not biocompatible or have a size >100 nm, which is not desirable for intracellular delivery of cisplatin. Thus, utilizing polymers such as PAMAM dendrimers would not only improve the intracellular delivery of cisplatin but also reduce the undesirable side-effects of cisplatin such as nephrotoxicity and neurotoxicity.

PAMAM dendrimers are densely branched ‘tree-like’ macromolecules originating from an ethylenediamine core molecule. The biomimetic property i.e., the ability to interact with cellular components and the easily tailorable surface...
structure of these dendrimers makes PAMAM dendrimers interesting polymeric carriers. Surface modification of dendrimers using various ligands including small molecule ligands like vitamins (folic acid, biotin etc.), antibodies against tumor associated antigens, cell penetrating peptides have generated a wide range of target specific nanocarriers (10).

Biotin (vitamin B₇, vitamin H) belongs to a category of essential micronutrients responsible for normal cellular functions such as fatty acid biosynthesis, gluconeogenesis, growth and development. It has previously been reported that tumor cell lines such as ovarian, colorectal etc., overexpress biotin receptors along with overexpression of receptors involved in folate or vitamin B12 uptake (11). Biotin has been reported to be taken-up by sodium-dependent multivitamin transporter (SMVT), which was first reported to be present in human placenta (12). SMVT has also been shown to be expressed in other cells such as human keratinocytes, peripheral blood mononuclear, intestinal, liver and renal epithelial cells and it has been suggested that SMVT is the primary transport system responsible for biotin uptake (13). Thus, biotinylation has been used as a strategy to specifically target chemotherapeutic agents to cancer cells. Camptothecin when conjugated with biotinylated polyethylene glycol has shown enhanced cytotoxicity and apoptotic activity by caspase-dependent pathway (14). These conjugates have also shown to be 30 times more potent in resistant ovarian carcinoma cells as compared to free camptothecin, indicating the potential of biotinylation of drugs to overcome resistance to chemotherapy (14). Although direct conjugation of chemotherapeutic agents to biotin enhance their targeting ability, only small doses of drug can be delivered as only one drug molecule can be attached per biotin and the biotin–drug conjugates are small in size which can be excreted by kidney, and may be reabsorbed in proximal tubules causing undesirable accumulation in kidney. Therefore, a polymeric carrier with biotin as ligand would be a better alternative to overcome the above limitation. It was previously reported that cellular uptake of the biotinylated PAMAM dendrimers was more efficient in OVCAR-3 ovarian cancer cells when compared with HEK293T cells indicating that the biotinylated PAMAM dendrimers as potential polymeric carriers to target ovarian cancer cells (15). Moreover, the electrostatic interaction between the positive amine groups of camptothecin with carboxylate surface groups and amide core groups of PAMAM dendrimers stabilizes the dendrimer camptothecin complexes. These stabilized complexes thus enhance the targeting and delivery of camptothecin to tumor cells.

In this present study, biotinylated PAMAM dendrimers of both NH₂ and COOH surface functionalities were loaded with camptothecin and its drug-loading efficiency was determined. Dendrimer-camptothecin complexes were tested for their effect on cytotoxicity in various ovarian cancer cell lines, OVCAR-3, SKOV-3, A2780 (wild-type ovarian cancer) and a cisplatin-resistant cell line, CP70. The conjugates were also studied for hemocompatibility so as to ensure biocompatibility for human in vivo applications. In order to correlate the cytotoxicity data with cellular interaction of camptothecin-loaded biotinylated PAMAM dendrimers, an in vitro cellular uptake kinetics study was performed in all the above mentioned ovarian cancer cell lines using high performance liquid chromatography (HPLC). The uptake studies showed that dendrimers biotinylated dendrimers exhibit both charge-mediated and SMVT-mediated uptake. This study reports for the first time the presence of SMVT and its functional capability related to biotin uptake in ovarian cancer cells.

Materials and Methods

Materials. PAMAM dendrimers (both G₄NH₂ and G₃.₅COOH) were obtained from Dendritic Nanotechnologies Inc., (Mount Pleasant, MI, USA). Cisplatin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2,5dihydroxybenzoic acid (DHB), trifluoroacetic acid, nickel chloride (NiCl₂) and insulin (Recombinant Human) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Biotinylating reagents sulfo-NHS-LC-biotin (Mr 556), biotin-LC-hydrazide (Mr 371.5), EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and morpholinethiole sulfonic acid (MES) buffered saline packs were obtained from Pierce Chemical Co., (Rockford, IL, USA). Sodium diethyldithiocarbamate trihydride (DDTC), chloride, acetonitrile and methanol (HPLC grade) and cell culture media were obtained from Fisher Scientific (Pittsburg, PA, USA). Dialysis membranes were obtained from Spectrum Laboratories, Rancho Dominguez, CA, USA.

Synthesis and characterization of biotinylated PAMAM dendrimers. Biotinylation of PAMAMG₄NH₂ dendrimers was carried out as reported previously (15). Briefly, 20 mg of PAMAMG₄NH₂ dendrimer was dissolved in 2 ml of 0.1 M phosphate buffer (pH 9.0) and sulfo-NHS-LC-biotin was added at a molar ratio of 1:32 (PAMAMG₄NH₂:sulfo-NHS-LC-biotin) and the reaction mixture was stirred for 2 h at room temperature. The mixture was then dialyzed (1,000 Da MW cutoff) against de-ionized water to remove unconjugated biotin. PAMAMG₃.₅COOH was biotinylated using biotin-LC-hydrazide according to the manufacturer’s protocol. Briefly, 20 mg of PAMAM G₃.₅ COOH dendrimer was dissolved in MES buffer and to this solution EDC and biotin-LC-hydrazide were added at a molar ratio of 1:32 (PAMAMG₃.₅COOH: Biotin-LC-Hydrazide). The reaction mixture was then stirred overnight at room temperature. The final mixture was dialyzed (1,000 Da MW cut-off) for 24 h against 10% MES buffer in de-ionized water to prevent bursting of dialysis bag. The biotin-conjugated dendrimers were lyophilized (Free zone 2.5; Labconco Corporation, Kansas City, MO, USA) to yield a white product. The final product was characterized by proton nuclear magnetic resonance (1H NMR, Bruker AMX-400 spectrometer; Bruker, Rheinstetten, Germany) using D₂O as solvent at concentration of 5 mg/ml. The degree of biotinylation was calculated using Bruker MALDI-TOF (matrix assisted laser desorption/ionization–time of flight) using 2,5 dihydroxybenzoic acid in 0.1 M trifluoroacetic acid as the matrix.
Fluorescence labeling of dendrimers. Dendrimers were fluorescently labeled using fluorescein isothiocyanate (FITC) according to previously reported methods (16). In a round-bottomed flask, 20 mg of biotinylated or native PAMAM dendrimers were diluted in PBS. To this solution, 5 mg/ml solution of FITC previously prepared in acetone was added (1:1 molar ratio) and stirred overnight at room temperature. The final product was purified by Sephacryl S-300 column with acetonitrile/Tris buffer (70:30) as the elution buffer. The fractions containing dendrimer-FITC were collected and dialyzed against de-ionized water at 4°C and lyophilized before storing at 4°C for further use. Stability of the FITC-labeled dendrimers was studied at 4°C and 37°C (for five days) by determining the free FITC in the samples using Agilent 1100 high pressure liquid chromatography with fluorescent detector (Agilent Technologies, Santa Clara, CA, USA). The mobile phase used was phosphate-buffered saline (PBS, pH 7.4):acetonitrile (80:20). No second peak representing free FITC (retention time of 5.5 min) was found in the samples indicating that the labeled dendrimers were stable for five days.

Preparation of cisplatin-loaded biotinylated dendrimers. A 20 mg each of biotinylated dendrimers of both –NH2 and –COOH surface groups and their unconjugated native counterparts were dissolved in 2 ml of deionized water. In a round-bottom flask approx 14.2 mg of cisplatin was dissolved in 7.5 ml of deionized water. When the cisplatin was completely dissolved, dendrimer solution was added to cisplatin solution dropwise under stirring at room temperature. The solution was left to react for 4 h and dialyzed (3.5 kDa MW cut-off) against deionized water for 24 h. The final conjugate was lyophilized to yield a white product.

Drug loading efficiency of cisplatin-loaded dendrimers. Initially, quantitative analytical profile of cisplatin was established using a HPLC method previously reported with slight modification (17). The chromatographic system consisted of Beckman Coulter HPLC (System Gold) system, controlled by a system controller (32 Karat® workstation with PC), equipped with a 125 gradient pumping module, a 508 autosampler with a sample cooling system and a 168 photodiode array (PDA) detector. The HPLC conditions employed an Atlantis® T3 5 μm column (250 mm × 4.6 mm i.d.) (Waters, Milford, MA, USA), solvent A [water: methanol 40:30] and solvent B [acetonitrile]. The pump was set at 80:20 ratio of solvent A to solvent B. Flow rate was maintained at 1.5 ml per minute and the sample volume was 50 μl with a detection wavelength of 254 nm. An aliquot of 1 ml of test sample was first incubated with 150 μl of freshly prepared 10% (w/v) DDTC in 0.1 N NaOH solution in a water bath for 5 min at 4°C. The mixture was separated into aqueous (top) and chloroform (bottom) layers by centrifugation. A 50 μl aliquot of the chloroform layer was injected into the HPLC. For estimation of cisplatin in cell extracts, 20 μl of NiCl2 (300 μg/ml) was added as an internal standard prior to incubation with DDTC. The limit of detection for cisplatin with this method was 0.1 μg/ml and the method resulted in a linear regression (R²=0.9958) for range of 1 μg/ml to 100 μg/ml of cisplatin.

Cell culture. NIH OVCA3 cells (Passage # 32-45) were obtained from American type culture collection (ATCC, Manassas, VA, USA) and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution. SKOV-3 cells (passages 32-46) were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution. A2780 and CP70 cell lines were obtained as kind gift from Steve W Johnson, Fox Chase Cancer Center, Philadelphia, PA, USA; cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution and 0.25 units/ml insulin. All the cells were maintained at 37°C in a humidified incubator containing 5% CO2.

In vitro cytotoxic activity by MITT assay. Cytotoxicity of cisplatin-loaded biotinylated dendrimers was performed in four different ovarian cancer cell lines in NIH OVCA-3, SKOV-3, A2780 and CP70. A total of 5x10⁴ cells in 200 μl of medium per well were plated in a 96-well plate. Following incubation overnight, the medium was replaced with media containing cisplatin-loaded dendrimers and only cisplatin at different concentrations (0.1-200 μM) in separate wells. After 24 h of incubation, the medium was removed and the cells were washed with ice cold PBS three times. A volume of 50 μl of MTT at a concentration of 5 mg/ml was added in each well. Following incubation for 4 h, formazan crystals formed were dissolved in 150 μl of dimethylsulfoxide and absorbance was measured at 590 nm with 650 nm absorbance as the background using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The IC₅₀ value for each cisplatin containing sample was calculated using nonlinear regression curve in GraphPad Prism 5.03 software. The logarithmic concentrations of cisplatin samples were plotted against the percentage viability for curve fitting. Viability of the cells exposed to cisplatin samples was expressed as a percentage of the viability of cells grown in the absence of cisplatin samples.

Hemocompatibility of cisplatin-loaded dendrimers. Human red blood cells (RBC) with adenine-saline added and leukocytes reduced were obtained as a kind gift from Coastal bend blood center, Corpus Christi, Texas. RBCs were separated by centrifugation at 4,000 rpm for 5 min at 4°C. RBCs were washed three times with physiological saline and resuspended in saline to obtain an RBC suspension at 2% (v/v) hematocrit. RBC suspension thus prepared was used within 24 h of preparation.

A 100 μl volume of 2% (v/v) RBC suspension was added to 100 μl of cisplatin-loaded dendrimers with concentrations ranging from 1 μM to 1 mM in a 96-well plate. 0.1% Triton X-100 served as the positive control. Incubation of RBC suspensions with cisplatin-loaded dendrimers and Triton X-100 was performed at 37°C for 12 h under gentle shaking (100 rpm) on a laboratory shaker. The RBC suspensions were centrifuged at 1,500 rpm for 10 min and a 100 μl of the supernatant was then carefully pipetted out and assayed for the released hemoglobin by measuring the absorbance at 540 nm and the percentage of hemolysis was calculated.

Gene expression analysis of SMVT using reverse transcription-polymerase chain reaction (RT-PCR). Presence of SMVT in ovarian cancer cell lines SKOV, OVCA-3, A2780 and A2780/CP70 was determined using RT-PCR. Total RNA from the above cell lines was isolated with RNA easy mini kit (Qiagen Sciences, MD, USA). The concentration of RNA was quantified using Nanodrop 1000 UV-Vis spectrophotometer (Nanodrop Technologies, Inc, Wilmington, DE, USA). The first strand of cDNA was synthesized using 3 μg of RNA with RevertAid® premium first strand cDNA synthesis kit (Fermentas Inc., Glen Burnie, MD, USA) per the manufacturer’s protocol.
Purity of the total RNA for DNA contamination was assessed by performing no reverse transcriptase control experiments with PCR using GAPDH primers provided in RevertAid® premium first strand cDNA synthesis kit (Fermentas Inc.). The cDNA thus obtained was diluted at 1:100 ratio with de-ionized water for PCR of SMVT. The sequences for the forward and reverse human SMVT (Gene bank accession # NM_021095) primers were designed using PrimerQuestSM software (Integrated DNA technologies, Iowa, USA) and were 5’-CTGTCCGTGCTGGCCCTGGGC-3’ and 5’-GACCCAGGCAATTAGGAGGAGC-3’ respectively. Primers were obtained from Integrated DNA Technologies. PCR was performed using 50 μl of reaction volume containing 2 μl of 1:100 diluted cDNA, 0.2 mM MgCl₂, 0.5 μM primers, and 2.5 units of Taq DNA polymerase (Fermentas Inc.). The reaction was run for 35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed through 1% agarose gel containing ethidium bromide and the images were captured using Quantity one® software (Bio Rad, Hercules, CA, USA).

Functional capability of SMVT in biotin uptake in ovarian cancer cells. To investigate the functional capability of SMVT receptors in biotin uptake in ovarian cancer cells, uptake of FITC-conjugated biotinylated dendrimers was performed in OVCAR-3 cells. Briefly, 1x10⁵ cells per well were seeded in 12-well plates 24 h prior to experiment and the cells were grown at 37°C with 5% CO₂ in an incubator. The cells were then incubated with excess of biotin (1 mM) to saturate the SMVT receptors for 30 min prior to treatment with different concentrations of biotinylated dendrimers and incubated at 37°C for 1 h. The cells were washed with PBS, trypsinized and suspended in 200 μl of 2% fetal bovine serum solution in water. The cells were analyzed for intracellular fluorescence intensity and corrected for background fluorescence of control cells using flow cytometer (Beckton Dickinson Inc., CA, USA) and the data was processed using Cell-Quest Pro software.

Cellular uptake and accumulation of cisplatin. Platinum content was quantitatively estimated using the HPLC method as described earlier. OVCAR-3, SKOV-3, A2780 and CP70 cells (5x10⁴) were seeded in 6-well plates. After 24 h, the cells were treated with 50 μM of cisplatin containing dendrimers. After 1 h and 6 h exposure to the Pt containing dendrimers, the drug-containing medium was removed and cells were washed with ice-cold PBS three times. Cells were trypsinized and suspended in 200 μl of PBS and then lysed by sonication for 30 s at 40 amplitude using a probe sonicator (Vibra Cell, Sonics & Materials Inc., USA) before analysis. Lysates were spiked with 20 μl of NiCl₂ (300 μg/ml) as an internal standard followed by incubation with DDTC for 30 min in a 37°C water bath. The samples were chilled on ice and cisplatin was extracted with 1 ml of chloroform. After separating the chloroform layer from the aqueous layer by centrifugation, 50 μl of chloroform layer was injected into HPLC. The ratio of peak areas of platinum to internal standard was calculated to assess the cellular uptake of cisplatin.

Statistical analysis. Statistical analyses were performed by Graph Pad Instat®, La Jolla, CA, USA and evaluated for statistically significant differences by Student’s t-test. P≤0.05 was considered statistically significant.

### Results

**Synthesis of biotinylated PAMAM dendrimers.** Biotinylation of PAMAMG₃NH₂ and PAMAMG₄COOH dendrimers resulted in a high yield fluffy white fibrous solid (>85%). ¹H NMR data revealed the presence of biotin ring juncture protons at 6=4.4 and 4.6 ppm which were absent from the parent PAMAM dendrimers, however, the other characteristic peaks of the dendrimer (2.6-3.6 ppm) were observed in both the parent and biotinylated PAMAM dendrimers. The extent of biotinylation was quantified using MALDI-TOF spectroscopy. For PAMAMG₃NH₂ and PAMAMG₄COOH, MALDI-TOF depicted 22 and 19 biotin molecules attached respectively.

**Drug-loading efficiency of biotinylated dendrimers.** Estimation of cisplatin loading efficiency of biotinylated dendrimers by HPLC revealed that the percentage of cisplatin encapsulated as calculated by platinum content ranged from 5.33% to 21.10%. The percentage of encapsulation for cisplatin-loaded biotinylated dendrimers is presented in Table I. Biotinylated PAMAMG₄NH₂ showed the highest loading of cisplatin, whereas biotinylated PAMAMG₃COOH showed the lowest.

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**Size, zeta potential, short-term in vitro stability and in vitro release profile in physiological pH (7.4) of the dendrimer–cisplatin conjugates were investigated (data not shown).** Dendrimer cisplatin conjugates showed a positive zeta potential with size range of 20-40 nm and >90% of cisplatin was retained in all dendrimer–cisplatin conjugates after 24 h when incubated at 37°C. The dendrimer–cisplatin complexes released cisplatin until 96 h, with release being rapid from dendrimers with carboxylate surface functionality (>40%) when compared with amine surface functionality (>16%).

**In vitro cytotoxic activity.** Cytotoxic activity of free cisplatin, cisplatin encapsulated in biotinylated and native PAMAM dendrimers was studied by MTT assay. To understand the effect of dendrimer–cisplatin conjugates on cisplatin resistance, the ovarian cancer-resistant cell line CP70 was used and results were compared with its wild-type sensitive A2780 cell line and other ovarian cancer cell lines (OVACR-3 and SKOV). Figure
Figure 1. Effect of dendrimer–cisplatin complexes on cell viability of various ovarian cancer cells determined by MTT assay. Percentage of cell viability of cisplatin loaded dendrimers on various ovarian cancer cell lines as determined by MTT assay. Cells were treated with dendrimers for 24 h at 37˚C followed by thorough washing with ice-cold PBS for three times prior to addition of MTT. The four graphs represent four cell lines as follows: A: OVCAR-3, B: SKOV-3, C: A2780, D: CP70.

Figure 2. Hemolysis induced by various concentrations (1 μM to 1 mM) of dendrimer–cisplatin complexes after 12 h incubation in a 2% (v/v) RBC suspension. The released hemoglobin was measured spectrophotometrically at 540 nm, and the percentage of hemolysis was calculated by considering hemolysis of 0.1% Triton X-100 as 100%. p<0.05 for all concentrations of PAMAMG₄NH₂ dendrimers when compared with rest of the dendrimers.

Hemocompatibility of the biotinylated dendrimers. Effects of cisplatin-loaded biotinylated PAMAM dendrimers on hemolytic activity were compared with their native counterparts and the results are shown in Figure 2. All the cisplatin-loaded dendrimers caused hemolysis in a concentration-dependent manner. The hemolytic activity profile observed was as expected, cationic...
dendrimers (PAMAMG₄NH₂ and biotin-PAMAMG₄NH₂) showed greater hemolytic activity than their anionic counterparts (PAMAMG₃.₅COOH and biotin-PAMAMG₃.₅COOH). Interestingly, cisplatin-loaded biotinylated dendrimers showed significantly less hemolytic activity than native dendrimers, for both cationic and anionic surface dendrimers. For example, among cationic dendrimers at 1 mM concentration, biotinylated dendrimers (biotin-PAMAMG₄NH₂) showed approximately 7.5 times lower hemolysis than native dendrimer (PAMAMG₄NH₂). Similarly, among anionic dendrimers at 1 mM concentration, biotinylated dendrimers (biotin-PAMAMG₃.₅COOH) showed approximately 6.8 times lower hemolysis than native dendrimer (PAMAMG₃.₅COOH). A similar pattern was observed at other concentrations (1 μM, 100 μM, 500 μM) also.

**Cellular uptake in presence of excess of biotin.** The FACS data revealed that pretreatment with excess biotin significantly reduced uptake of biotinylated dendrimers. The cellular uptake of the dendrimers at 0.1 μM without pretreatment of biotin was 90.23±4.2% whereas in the presence of biotin it was reduced to 78.24±0.19%. However, at higher concentrations of the dendrimer (1 μM and 10 μM) presence of biotin did not affect the extent of uptake of biotinylated dendrimers.

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**Table II. IC₅₀ (μM) values of cisplatin-loaded PAMAM dendrimers.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>OVCAR-3</th>
<th>SKOV</th>
<th>A2780</th>
<th>CP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
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<td>69.66±5.67*</td>
<td>26.99±2.11</td>
<td>171.9±0.9*</td>
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<td>25.88±2.34</td>
<td>69.46±2.2</td>
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<td>47.98±0.97</td>
<td>39.15±1.19</td>
<td>47.56±1.0</td>
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</table>

*IC₅₀ values of cisplatin are statistically significant from dendrimer-conjugated cisplatin (p<0.05). #This value is not statistically significant from cisplatin.

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**Figure 3.** Cellular accumulation of cisplatin from various dendrimer–cisplatin complexes measured in OVCAR-3, SKOV-3, A2780 and CP70 cells (5x10⁴) after exposure of 1 h and 6 h. The concentration of all dendrimers was 50 μM equivalent of cisplatin. The cisplatin content was estimated using HPLC. Each value represents the mean±S.D (n=4).
SMVT expression in ovarian cancer cells. A single band representing amplified DNA was detected in normal HEK293T cells and ovarian cancer cells SKOV, OVCAR-3, A2780 and A2780/CP70 as shown in Figure 4. The volume analysis report generated using Quantity One® revealed the following volume intensities per mm²: 80801.7, 62276.8, 83445.6, 214023.4, 161734.1 for HEK293T, SKOV, OVCAR-3, A2780 and A2780/CP70 cells respectively.

Cellular uptake of cisplatin-conjugated dendrimer by ovarian cancer cells. Cellular uptake of cisplatin by ovarian cancer cells A2780 and CP70 were evaluated using HPLC. The amount of platinum accumulation in cells and amount of platinum present in culture medium after 1 h and 6 h of incubation of cisplatin is presented in Figure 3. Results show that intracellular concentrations of cisplatin were significantly higher with native PAMAM dendrimers with amine surface
groups after 6 h incubation. Interestingly, the intracellular platinum concentrations measured after 1 h of incubation revealed that there was no significant uptake of cisplatin by any of the dendrimer complexes of cisplatin. The uptake of cisplatin complexes of amine terminated native PAMAM dendrimer was higher in CP70 cells (25.25±1.25 μg/ml) when compared with A2780 cells (19.41±0.85 μg/ml). Moreover, there was no significant difference in uptake between A2780 (10.48±0.98 μg/ml) and CP70 (8.18±1.32 μg/ml) cells.

Discussion

Biotinylation has been an attractive targeting approach to target chemotherapeutic agents to cancer cells. Biotinylation strategy has been used in many polymeric and liposomal delivery systems to enhance targeting capability of delivery system. Recently, Tseng et al. used biotinylated-EGF-modified gelatin nanoparticles to enhance cisplatin accumulation in lung cancer cells through inhalation (18). Similarly, immunoliposomes made of poly (ethylene glycol)-phospholipid (bio-PEG-diestearoylphosphatidylethanolamine)-containing monoclonal antibodies raised against transferrin receptors were biotinylated to target transferrin receptors of skeletal muscle (19). Amine terminated PAMAM dendrimers were conjugated with folic acid-PEG (polyethylene glycol) to target 5-fluorouracil to tumor cells (20). It was previously reported that biotinylated dendrimers may be exciting nanocarriers for sodium multivitamin transport (SMVT) receptor-mediated targeting (15). In present study, PAMAMG₄NH₂ and PAMAMG₃.₅COOH dendrimers were conjugated to biotin and cisplatin was encapsulated into these dendrimers. The cisplatin-biotinylated dendrimer complexes were then evaluated for in vitro anti cancer activity and cellular uptake by ovarian cancer cells and compared with cisplatin complexes of respective native PAMAM dendrimers.

Although antibodies against ovarian cancer antigen (CA125) have been used as ligands to target ovarian cancer, their immunogenicity, macromolecular structure, instability, poor tumor penetration are their significant limitations. Biotin, which is a smaller molecule (Mr 244.32) when conjugated to dendrimer, not only enhances the tumor penetration but also overcomes the limitations of antibody therapeutics.

Biotinylation of PAMAMG₄NH₂ and PAMAMG₃.₅COOH was carried out using sulfo-NHS-LC-biotin and biotin-LC-hydrazide respectively. PAMAMG₄NH₂ and PAMAMG₃.₅ COOH were chosen as these generations have greater cellular uptake in cancer cells with minimal toxicity as shown in previous studies (15). Sulfo-NHS-LC-biotin is a water-soluble amine reactive biotin reagent which is a derivative of D-biotin containing a spacer arm off the valeric acid side chain, terminating in an NHS ester. The NHS ester end reacts with amine groups of dendrimers to form stable amide-bond derivatives. Similarly, biotin-LC-hydrazide is a hydrazine derivative of D-biotin, of its valeric acid carboxylate. Hydrazides can be coupled with carboxylic acid groups by using the carbodiimide reaction. The carboxylate group of dendrimers is first activated by carbodiimide to an O-acylisourea intermediate; via nucelophilic addition this intermediate reacts with biotin-LC-hydrazide to form a stable covalent bond. Both these reagents have a 6-aminocaproic acid spacer which provides greater length between dendrimers and bicyclic biotin rings which in turn provides more efficient interaction with the target. NMR spectroscopy of biotinylated dendrimers revealed the presence of two biotin ring juncture protons. MALDI-TOF spectroscopy data showed the extent of biotinylation for both PAMAMG₄NH₂ and PAMAMG₃.₅COOH. The HABA assay has been used previously for quantification of extent of biotinylation, but results suggest that its accuracy is limited to lower generations of dendrimers. This may possibly due to steric hindrance caused by a large number of biotins in a highly biotinylated molecule, which blocks the HABA dye from binding to biotin (21). The extent of biotinylation was approximately equal for both PAMAMG₄NH₂ (22 molecules) and PAMAMG₃.₅COOH (19 molecules). Earlier studies indicated that as the number of biotin groups attached increased with increase of generations (from G₁ to G₄), their cellular uptake was also improved. However, the number of biotins attached to dendrimers mentioned above was the maximum attainable number and further increase in biotinylation was not achievable.

To reduce the systemic toxicity of cisplatin, several polymer–drug conjugates were prepared (22-24). One important criterion for a polymer to form complexes with cisplatin is availability of suitable ligating groups for platinum, for example, nitrogen donors such as amines, or oxygen donors such as carboxylate or hydroxyl groups (25). PAMAM dendrimers are one such class of polymers which have inherent carboxylate and amine ligating groups. Initially, Malik et al. reported that cisplatin was able to form complexes with carboxylic acid terminated PAMAM dendrimers and those complexes were found to accumulate within tumor tissue following intravenous injection into xenograft model (26). In this study, cisplatin was encapsulated into biotinylated dendrimers with amine and carboxylate surface functionalities along with encapsulation into their native counterparts. Initially, dendrimers were reacted with cisplatin using 0.9% sodium chloride as reaction medium. But, the encapsulation efficiencies were very low (~0.1-2% data not shown) because high chloride ion concentration inhibits hydrolysis of cisplatin and this in turn inhibits the reactivity of cisplatin to dendrimers. When the reaction medium was replaced with water from 0.9% NaCl, encapsulation efficiency increased (~10-11%) drastically, indicating that fast hydrolysis is required for high ligand
exchange. Biotinylated PAMAMG₄NH₂ dendrimer showed the highest encapsulation efficiency where as biotinylated PAMAMG₃.₅COOH exhibited low encapsulation efficiency (Table I). Interestingly, biotinylated and native dendrimer complexes of cisplatin with carboxylate surface groups were readily soluble in water but similar complexes with amine surface groups were soluble only after 30 s of vortexing indicating cross-linking phenomenon.

The IC₅₀ values of biotin-PAMAMG₄NH₂, PAMAMG₄NH₂, biotin-PAMAMG₃.₅COOH and PAMAMG₃.₅COOH against OVCAR-3 were 2.5-, 2.1-, 2.9- and 3.08-fold lower than those of free cisplatin, respectively. Similarly, the IC₅₀ against OVCAR-3 were 2.5-, 2.1-, 2.9- and 3.08-fold lower indicating cross-linking phenomenon.

Surface groups were soluble only after 30 s of vortexing complexes of cisplatin with carboxylate surface groups were developed as carriers of amino acids with 12 transmembrane domains. It was reported earlier that expression of SMVT in cancer cells is enhanced due to increased requirement for nutrients by rapidly growing cancer cells (11). The bands at 400 bp in agarose gel revealed that SMVT is expressed in normal as well as all the ovarian cancer cells tested in the study. The 400 bp band corresponds to the size of the fragment between the forward (923-943) and reverse (1333-1313) primer positions. However, from volume analysis reports, it is evident that expression of SMVT is higher in A2780 and A2780/CP70 type of ovarian cancer cells. Results indicate that SMVT expression levels depend on the nature of the cell line and in some cancer cell lines it is overexpressed. Therefore, identification of SMVT expression enables the determination of the suitability of the biotinylated dendrimers for drug targeting.

The results clearly indicate that amine terminated cisplatin loaded dendrimers, both biotinylated and native, show greater hemolytic activity than their carboxylate terminated counterparts. This can be explained by the fact that surface of a normal erythrocyte is negatively charged due to the presence of glycolipids and some glycated integral and peripheral proteins (27). Thus, cationic dendrimers, by virtue of electrostatic attraction, get into the proximity of red blood cells and thus increase hemolysis. The most important finding of this study is that a significant reduction of hemolysis was observed when both cationic and anionic dendrimers were biotinylated. This result is in concordance with data from cytotoxicity studies. Similar to the reduction in cytotoxicity, the reduction in hemolytic activity can also be attributed to bulky aromatic rings of biotin shielding the surface charge of dendrimers. Thus, biotinylated dendrimers can be valuable carriers as they are able to reduce the hemolysis, an important attribute for in vivo application.

SMVT, also known as SLC5A6, was discovered to be transporter for water-soluble vitamins such as biotin and pantothenic acid. The hSMVT gene is located on chromosome 2p23 and SMVT receptor is a protein of 635 amino acids with 12 transmembrane domains. It was reported earlier that expression of SMVT in cancer cells is enhanced due to increased requirement for nutrients by rapidly growing cancer cells (11). The bands at 400 bp in agarose gel revealed that SMVT is expressed in normal as well as all the ovarian cancer cells tested in the study. The 400 bp band corresponds to the size of the fragment between the forward (923-943) and reverse (1333-1313) primer positions. However, from volume analysis reports, it is evident that expression of SMVT is higher in A2780 and A2780/CP70 type of ovarian cancer cells. Results indicate that SMVT expression levels depend on the nature of the cell line and in some cancer cell lines it is overexpressed. Therefore, identification of SMVT expression enables the determination of the suitability of the biotinylated dendrimers for drug targeting.

Cellular accumulation of cisplatin as measured by Pt content by HPLC in A2780 and CP70 cells revealed that in both cell lines the uptake was negligible at 1 h. This might be due to the delayed uptake of dendrimers by ovarian cancer cells. However, after 6 h the cellular uptake data revealed that in both these cell lines PAMAMG₄NH₂ showed highest uptake amongst all other dendrimers. Interestingly, the uptake was higher in the CP70 cell line (25.25 μg/ml) when compared with the cisplatin-sensitive A2780 cell line (19.41 μg/ml), presumably due to sensitization of ovarian cancer cells for cisplatin therapy. The higher cellular uptake of PAMAMG₄NH₂ might be attributed to the high cationic surface property of PAMAMG₄NH₂. Biotin PAMAMG₄-NH₂ showed uptake next to unconjugated PAMAMG₄NH₂. This reduction in uptake by biotinylated dendrimers was due to the bulky aromatic rings of biotin reducing the cationic charge of dendrimers.

Biotinylated PAMAM dendrimers of amine and carboxylate surface groups were developed as carriers of cisplatin to enhance its therapeutic effects and reduce its side effects. Cisplatin was successfully encapsulated into the biotinylated dendrimers of both surface functionalities. Dendrimer–cisplatin complexes were more effective than free cisplatin in all ovarian cancer cell lines, including cisplatin-resistant cell lines. The cellular uptake data revealed accumulation of cisplatin in ovarian cancer cell line with PAMAMG₄NH₂ exhibiting the highest uptake. This is probably due to the dominance of charge-mediated uptake over SMVT mediated uptake. Biotinylation of dendrimers thus reduces the non-specific charge-mediated uptake and increases specific SMVT-mediated uptake, as well as also increasing the in vivo biocompatibility, as seen with reduction in hemotoxicity with biotinylated dendrimers.
Results suggest that biotinylated PAMAM dendrimers may be potential drug carriers for cisplatin targeting to ovarian cancer. Future studies will include elucidation of mechanism of enhanced anticancer activity and study the application of these nanocomplexes for antibody-based pretargeting strategy of cisplatin in a murine ovarian cancer model.

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