5.0 Introduction

The lack of tumor specificity is one of the most important factors for the failure of cancer chemotherapy. Tumor-targeted delivery of chemotherapeutic drugs is believed to enhance therapeutic efficacy in tumors with low systemic side effects in noncancerous tissues. Monoclonal antibodies (mAbs) against tumor antigens show a high specificity and affinity for tumor cells, which has led to the development of antibody-based cancer therapy [1-3]. Human epidermal growth factor receptor-2 (HER 2/ErbB-2/neu), a tyrosine kinase transmembrane receptor which is overexpressed in various cancers including breast, ovarian and lung cancer, is associated with poor prognosis and invasive carcinomas; [4, 5] therefore, it is a potential candidate for targeted antibody therapy [6,7]. Humanized anti-HER 2 mAb trastuzumab (Herceptin) approved for clinical use by the U.S. Food and Drug Administration, specifically binds to HER 2 receptors and mediates its antiproliferative effects on breast cancer cells; [8, 9] however, the therapeutic effects of mAb monotherapy tend to be limited [9]. Antibody-chemotherapeutic drug conjugates are reported as an alternative approach to synergize antibody-mediated cellular toxicity and chemotherapy for effective clinical translation [10-12]. For example, direct conjugation of chemotherapeutic drugs, such as geldanamycin [13, 14] and maytansinoids, [15] to trastuzumab showed improved pharmacokinetics for effective antitumor activity compared with trastuzumab monotherapy. The drug payload using this conjugation method, however, is restricted due to a significant decrease in the receptor recognition [11], immunoreactivity [16], and water solubility of the conjugates [17].

Nanoparticles, including liposomes, micelles, polymers and dendrimers, have been reported as effective drug carriers offering a high drug payload by physical incorporation or chemical
conjugation [18, 19]. Among them, dendrimers possess attractive features including (i) their nanosize range, (ii) monodispersity, (iii) rigid globular structure with high physical stability and (iv) a large number of functional groups for versatile chemical modification [20, 21].

Nevertheless, cationic PAMAM dendrimers tend to exhibit nonspecific dose-dependent interactions and cytotoxicity due to the positive charge of the peripheral amino groups [22-24]. In this regard, Baker and colleagues have reported partially acetylated PAMAM-trastuzumab conjugates for reduced nonspecific interactions [25] in HER 2-expresssing cell lines [26, 27]. Indeed, acetylated modification possibly alters (i) the physicochemical properties of the dendrimers including their water solubility, (ii) structural homogeneity and (iii) the number of peripheral amino groups available for antibody-drug conjugation [28]; therefore, precise control of the degree of acetylation is necessary.

In our study we have surface functionalized PAMAM dendrimers of G1-4 with DGA groups (DGA-G1-DGA-G4) and employed them as pH sensitive drug carrier for cisplatin [29]. Encouraged by the increased drug loading efficiency and enhanced in vitro toxicity of higher generation dendrimers, we selected DGA-G4-cisplatin in this study for targeting with herceptin. In vitro efficacy for these targeted DGA-cisplatin conjugates were further evaluated against human ovarian cancer cell lines with different levels of HER 2 expression.
5.1 Methodology

5.1.1 Synthesis of partial DGA-functionalized G4 PAMAM dendrimer

Total number of amine groups in G4 PAMAM dendrimer was partially DGA functionalized leaving the remaining groups for LC-SPDP conjugation to enable herceptin targeting. Briefly, to a G4 dendrimer solution (0.05g, 0.0035mmol) in DMSO, diglycolic anhydride (0.019g, 0.163mmol) was added drop by drop and allowed to stir for 24h at room temperature. At the end of the reaction, the solvent DMSO was allowed to evaporate and excess diglycolic anhydride were removed from the product by dialysis against MilliQ water. Partially DGA functionalized G4 PAMAM dendrimer present in the aqueous retentate was obtained by vacuum drying.

5.1.2 Synthesis of partial DGA-PAMAM-LC-SPDP

To a solution of partial DGA-G4 PAMAM dendrimer in PBS buffer (pH 7.4) sulfo-LC-SPDP dissolved in PBS was added dropwise, and the reaction mixture was allowed to stir for 3h. The unreacted reagents and byproducts were separated by ultrafiltration using a 10K MWCO Pelicon device washing initially with PBS and then with distilled water. The number of SPDP linker conjugated to DGA-G4 PAMAM was determined by pyridine-2-thione assay as described in the manufacturer’s protocol. Briefly, the DGA-G4-PAMAM-sulfo-LC-SPDP conjugate was dissolved in PBS/EDTA buffer (pH 7.4, 1 mg/mL), and the absorbance was recorded at 343 nm in comparison to a PBS-EDTA blank. To this solution, 10 µL of 15 mg/mL DTT was added and stirred for 15 min. After exactly 15 min, the absorbance at 343 nm of the reduced sample was recorded. The experiment was carried out in triplicates.
5.1.3 Synthesis of Herceptin-DGA-G4-PAMAM dendrimer

The disulfide bond on DGA-G4-PAMAM-sulfo-LC-SPDP conjugate was reduced with 10 mM DTT solution in degassed PBS-EDTA buffer (pH 7.4) at room temperature under nitrogen to provide dendrimer-thiol. This conjugate was purified by gel filtration eluting with PBS-EDTA buffer (pH 7.4) under nitrogen on a PD-10 column to remove excess reagents and byproducts and was used immediately for antibody conjugation. A thiol-reactive maleimide group was introduced in Herceptin mAb, (0.002 g, 0.00013 mmol), by reacting with sulfo-SMCC (0.0006 g, 0.00136 mmol) at room temperature for 2 h to give modified antibody (Herceptin-sulfo-SMCC). The excess reagent was removed by gel filtration on a Sephadex G-25 column. Herceptin-sulfo-SMCC was then concentrated on a microcon YM100 and immediately reacted with dendrimer thiolate (0.004 g, 0.00013 mmol) for 2 h at room temperature in PBS-EDTA buffer. After 2h, NEM (0.003 g, 0.024 mmol) was added to the reaction mixture. The final conjugate (Herceptin-DGA-G4-PAMAM) was purified by ultrafiltration (MWCO 100 000). The dendrimer antibody conjugate was characterized by SDS-PAGE, and DLS. A schematic representation of targeting DGA-G4-PAMAM with herceptin is shown in Figure 5.1
Chapter 5  Targeting studies of DGA-PAMAM-cisplatin conjugates with Herceptin and evaluation of its in vitro cytotoxicity

1. Synthesis of DGA-G4 Dendrimer Thiol

\[
\begin{align*}
\text{DGA-G4 dendrimer} & + \text{sulfo-LC-SPDP} \\
\downarrow & \\
\text{DGA-G4 dendrimer-sulfo-LC-SPDP} & \xrightarrow{\text{DTT, 2h}} \\
\downarrow & \\
\text{Reduced dendrimer thiol} & 
\end{align*}
\]

2. Conjugation of dendrimer to anti-HER 2 mAb

\[
\begin{align*}
\text{Herceptin} & + \text{sulfo-SMCC} \\
\downarrow & \\
\text{Herceptin-sulfo-SMCC} & \xrightarrow{\text{Reduced dendrimer thiol +NEM, 2h RT}} \\
\downarrow & \\
\text{Herceptin-DGA-G4 Dendrimer} & 
\end{align*}
\]

Figure 5.1 Schematic representation of Herceptin targeting with DGA-G4-PAMAM dendrimer.

Synthesis and characterization of diglycolamic acid functionalized and anti-HER 2 targeted polyamidoamine dendrimer-cisplatin nanoparticles for the treatment of cancer

[Ph.D(R.R)/197/FT/VII/2009]
5.1.4 Characterization of herceptin-DGA-G4-PAMAM dendrimer

Particle size and zeta potential of the herceptin targeted and untargeted DGA-G4 dendrimer (0.5mg/ml) were analyzed using Malvern NanoZS particle size analyzer. Further, to ensure increase in the molecular mass of the final targeted conjugate, SDS-PAGE was performed. Herceptin (100µg/mL) and herceptin-DGA-G4 dendrimer (100, 200µg/mL) were mixed with 5µl of sample buffer (20% glycerol, 0.04% bromophenol blue in Tris-HCL pH 6.8 containing 2% SDS) and the samples were individually loaded into each well and separated on 12% resolving SDS-PAGE at 100 V for 1h. Developed gels were stained with 0.025% Coomassie Blue R-250 overnight, in 40% methanol and 7% acetic acid aqueous solution. The gels were then destained in an aqueous solution containing 7% (v/v) acetic acid and 5% (v/v) methanol.

5.1.5 Conjugation of cisplatin with herceptin-DGA-G4-PAMAM dendrimer

Hydrolyzed cisplatin prepared from cisplatin using AgNO₃ (Section 4.1.1) was conjugated with herceptin-DGA-G4 dendrimer overnight. Unconjugated cisplatin was then removed by ultracentrifugation using Vivaspin™ columns with a 2 kDa MWCO membrane. DLE of herceptin-DGA-G4 dendrimer was then determined by analyzing the concentration of unbound hydrolyzed cisplatin in the filtrate by o-PDA assay.

5.1.6 In vitro cytotoxicity studies for herceptin-DGA-G4-cisplatin

5.1.6.1 HER-2 expression analysis by flow cytometry and western blot

HER-2 protein expression levels in human ovarian cancer cell lines SKOV-3 and PA-1 was evaluated by western blot and flow cytometry.

For western blot experiment, the cells (SKOV-3 and PA-1) were lysed and protein concentration was determined by Bio-Rad DC assay. Briefly, 100µg of total protein was resolved on 7.5%
polyacrylamide gel and then transferred to an Immobilon membrane (Millipore). Membranes were incubated overnight at 4°C with anti-HER 2 rabbit mAb. Blots were then washed with PBS containing 0.1% Tween 20 for 15 mins and incubated with a secondary antibody conjugated with peroxidase. Signals were detected using the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK).

For flow cytometry experiment, briefly 10^6 cells (SKOV-3 and PA-1) were suspended in 100µl of PBS solution containing 10% FBS and exposed to the primary anti-HER 2 rabbit mAb for 30 mins on ice. After washing three times, the cells were resuspended in 100µl of PBS solution with 10% FBS containing alexa fluor 488 conjugated anti-rabbit IgG monoclonal secondary antibody. The samples were then analyzed for HER 2 expression levels by flow cytometry. Unstained cells and cells treated with secondary antibody alone were used as controls.

5.1.6.2 In vitro cytotoxicity assay for Herceptin-DGA-G4-cisplatin

Cytotoxicity of the herceptin targeted and untargeted DGA-G4-cisplatin conjugates were assessed by MTT test [30] in HER 2 +ve SKOV-3 and HER 2–ve PA-1 cells. Briefly, 5×10^3 cells per well were seeded into 96 well plates and grown at 37°C overnight. Next day, the cells were treated with herceptin targeted and untargeted DGA-G4-cisplatin in the concentration range of 0.5-15µg/mL and incubated for 24 and 48h. Cisplatin in the same concentration range served as positive control. Following incubation, the medium containing nanoparticles were discarded and 20µl of MTT (5mg/mL) was added to each well and further incubated for 3-4h. Formazan crystals formed by the viable cells were then dissolved in 200µl DMSO and their absorbance was read at 570 nm. Absorbance values for untreated wells were taken as control (100% survival).
5.1.6.3 Cellular uptake: Intracellular cisplatin accumulation

To evaluate the cellular uptake of these nanoparticles, SKOV-3 and PA-1 (1 x 10^6) cells were seeded in a 35 mm culture dish and incubated for 24h, respectively. Next day the cells were treated with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin at equivalent cisplatin concentration of 50µM for 8h. Following incubation, medium was aspirated and the cells were washed thrice with PBS, tpsinized and centrifuged. The cell pellet was digested with 150µL concentrated nitric acid for 20 mins at 60°C. After diluting the samples in distilled water, intracellular Platinum (Pt) levels were measured quantitatively by ICP-OES. Results were expressed as ng Pt/ 1 x 10^6 cells.

5.1.6.4 Cellular uptake: Analysis by TEM

The intracellular translocation of herceptin-DGA-G4-cisplatin into SKOV-3 cells was studied by TEM. The cells were treated with herceptin-DGA-G4-cisplatin at 100µg/mL concentration for 12h and 24h. Following incubation, the cells were fixed in 3% glutaraldehyde and post fixed in 1% osmium tetroxide. After blocking with 2% agarose, the samples were dehydrated in a graded series of alcohol (50%-100%) and cleared by propylene oxide. Samples were then embedded in epoxy resin. For optical microscopy, 1µm thick sections were cut through ultra microtome with glass knife and stained by toluidine blue. For TEM, ultra thin sections (below 100 nm) were cut with diamond knife, stained with uranyl acetate and reynolds solution on copper grid. Sections were then observed in an electron microscope (Philips Tecnai T12 spirit by Netherland) and representative images were acquired.
5.1.6.5 Cell cycle analysis

SKOV-3 and PA-1 (1 x 10^6) cells grown in 6 well plates were treated with the DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin at IC_{50} concentration of DGA-G4-cisplatin and incubated for 24h. Untreated cells were used as control. After 24h, cells were scraped off and incubated in hypotonic PI solution (50µg/mL PI, 40µg/mL RNase A, 0.3µl/mL Nonidet P-40 in 0.1% trisodium citrate) for 10 mins. Variations in cell cycle phases after drug treatment was then analyzed on BD C6 Accuri flow cytometer.

5.1.6.6 Apoptosis assay

Apoptosis and necrosis induced by targeted and untargeted dendrimer-cisplatin conjugates were analyzed using Annexin V-PE apoptosis detection kit [31]. Briefly SKOV-3 and PA-1 (1 x 10^6) cells were seeded separately in 6 well plates and incubated for 24h. The cells were treated with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin for 18h at IC_{50} concentration of DGA-G4-cisplatin. Untreated cells were used as control. Cells were collected in new tubes after trypsinization, washed twice with ice cold PBS and resuspended in 1 ml of 1X binding buffer. To 100µl of this cell suspension 5µl of Annexin V-Phycoerythrin (Annexin V-PE) and 5µl of 7-Amino-Actinomycin (7-AAD) was added and incubated at room temperature for 15 mins. The samples were diluted with 400µl binding buffer before evaluating the percentage of apoptosis/necrosis on BD C6 Accuri flow cytometer.
5.2 Results

5.2.1 Characterization of herceptin-DGA-G4 PAMAM dendrimer

In order to improve the targeting efficacy and thereby reduce the nonspecific interactions, amine-terminated G4 PAMAM dendrimers were partially surface modified with diglycolic anhydride. The partially DGA functionalized G4 PAMAM dendrimer was purified by repeated dialysis initially using PBS (pH 7.4) and subsequently using water. In order to introduce a disulfide group on the dendrimer, heterobifunctional cross-linking agent sulfo-SPDP was conjugated to the partially DGA functionalized G4 PAMAM dendrimer to provide a protected thiol in the form of a disulfide. The extent of disulfide modification was monitored by UV spectroscopy, using the pyridine-2-thione assay as described in the manufacturer’s protocol. Briefly, DTT was added to a measured quantity of dendrimer, and absorbance of the released 2-thiopyridine at 343 nm was recorded. On the basis of this measurement, it was calculated that 1.6 SPDP linkers per mole of dendrimer was conjugated. On reduction with DTT, DGA-G4-LC-SPDP was reacted with sulfo-SMCC modified herceptin using standard protocols [32]. To minimize the presence of free antibody, a tenfold molar excess of dendrimer conjugate was used in the antibody dendrimer coupling reaction; the unreacted thiols were quenched with NEM to minimize the dimer formation due to cross-linking of dendrimer to antibodies. Free unreacted dendrimer was removed by filtration with a 100K MWCO microcon. The resultant herceptin-DGA-G4 conjugate was analyzed for its molecular mass and size using SDS-PAGE and DLS. The particle size and zeta potential of DGA-G4 increased to 68 nm and 40.8 mV respectively after herceptin conjugation (Figure 5.2).
SDS-PAGE electrophorograms of targeted and untargeted DGA-G4 PAMAM dendrimer (Figure 5.3) clearly showed that herceptin-DGA-G4-PAMAM remained stacked at the top of separating gel indicating increase in the molecular mass after conjugation of herceptin with dendrimer. Similarly, absence of bands near the molecular mass of herceptin confirms the removal of free antibody after dendrimer conjugation. The binding of cisplatin with herceptin-DGA-G4 PAMAM dendrimer and its release from the conjugate is mediated through terminal DGA groups and as determined from o-PDA assay, 53% of cisplatin loading efficiency was achieved for herceptin-DGA-G4 with the drug loading content of 582µg/mL and the amount of herceptin bound as determined by Bradford assay was 112µg/mL.

Figure 5.2 Size of Herceptin targeted DGA-G4-cisplatin determined by DLS.
Targeting studies of DGA-PAMAM-cisplatin conjugates with Herceptin and evaluation of its in vitro cytotoxicity

Chapter 5

5.2.2 Expression levels of HER 2 in SKOV-3 and PA-1 cell lines

5.2.2.1 Western blot analysis

In a first set of cell culture experiment, the expression of endogenous HER 2 protein levels in human ovarian cancer cell lines (SKOV-3 and PA-1) was analyzed by Western blot analysis. A strong expression of HER 2 protein in SKOV-3 cells was demonstrated from western blot results while in PA-1 cells the levels were below the detection limit and the results were in agreement with previous observations [33]. Vinculin served as the loading control (Figure 5.4).
Figure 5.4 HER 2 protein expression analysis in SKOV-3 and PA-1 cells by Western blotting

5.2.2.2 Flow cytometry analysis

HER 2 expression levels in these cell lines were further supported by the results from flow cytometry. From Figure 5.5 it can be seen that SKOV-3 cells overexpress HER-2 protein, as the entire cell population was shifted towards right in comparison to the unstained cells. On the contrary, incubation of PA-1 cells with anti-HER 2 mAb did not result in prominent shift in fluorescence intensity. Collectively, results from these studies confirm the appropriate conditions for the analysis of Herceptin-DGA-G4-cisplatin in HER 2+ve (SKOV-3) and HER 2 –ve (PA-1) cell lines.
Figure 5.5. HER 2 protein expression analysis by flow cytometry. A- SKOV-3 unstained and A’ stained with anti-HER 2 mAb, B- PA-1 unstained and B’ stained with anti-HER 2 mAb.
5.2.3 *In vitro* cytotoxicity of Herceptin-DGA-G4-cisplatin

The cytotoxic potential of herceptin-DGA-G4-cisplatin was tested by MTT assay in human ovarian cancer cells with different HER 2 expression status i.e. SKOV-3 (HER 2 +ve) and PA-1 (HER 2 -ve).

In SKOV-3 cells, for 24h incubation (Figure 5.6 a), herceptin-DGA-G4-cisplatin (IC_{50} 6.6µg/mL) showed better anti-proliferative activity than untargeted dendrimer-cisplatin (IC_{50} 17µg/mL) and free cisplatin (IC_{50} 20µg/mL). Similarly at 48h incubation (Figure 5.6 b), IC_{50} value for herceptin-DGA-G4-cisplatin further reduced to 4.9µg/mL in comparison to DGA-G4-cisplatin (8.5µg/mL) and free cisplatin (9.47µg/mL).

![Figure 5.6](image)

*Figure 5.6 In vitro cytotoxicity of cisplatin, DGA-G4-cisplatin and herceptin DGA-G4-cisplatin against HER 2 +ve SKOV-3 cell lines for (a) 24h and (b) 48h incubation. Values in untreated wells were taken as 100% viability. The results represent the mean ± SE (n= 3)*
To ensure that the toxicity was in a targeted manner, HER 2 non expressing cell line PA-1 was also studied (Figure 5.7). Our results showed that herceptin conjugation did not result in increased toxicity in HER 2-ve PA-1 cell line. The IC\textsubscript{50} values for PA-1 cells at 24h incubation of herceptin-DGA-G4-cisplatin, DGA-G4-cisplatin and free cisplatin was determined to be 4.4, 2.7, 2.7µg/mL respectively.

Figure 5.7 In vitro cytotoxicity of cisplatin, DGA-G4-cisplatin and herceptin DGA-G4-cisplatin against HER 2-ve PA-1 cell lines for (a) 24h and (b) 48h incubation. Values in untreated wells were taken as 100% viability. The results represent the mean ± SE (n = 3)
5.2.4 Analysis of Intracellular cisplatin accumulation

The toxicity of cisplatin nanoparticles, which is considered to be an important cellular event, is exerted only upon increased uptake and accumulation of cisplatin inside cancer cells. To examine this, SKOV-3 and PA-1 cells were incubated with herceptin targeted DGA-G4-cisplatin, DGA-G4-cisplatin and free cisplatin at equivalent cisplatin concentration of 50µM for 8h and the total Pt content was determined by ICP-OES.

Herceptin conjugation has increased the uptake of dendrimer-cisplatin nanoparticles in SKOV-3 cells resulting in 1.6 fold increased uptake in comparison to DGA-G4-cisplatin and 3 fold increased uptake in comparison to free cisplatin (Figure 5.8 a). In PA-1 cells the mean intracellular Pt accumulation were found to be less for herceptin-DGA-G4-cisplatin (1.4 fold) and DGA-G4-cisplatin (2 fold) compared to free cisplatin (Figure 5.8 b).

![Figure 5.8 Pt concentrations of (a) SKOV-3 and (b) PA-1 cells following incubation with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin at equivalent cisplatin concentration of 50 µM for 8h. The results represent the mean ± SE (n = 3). *p<0.05.](image-url)
5.2.5 Mechanism of uptake by TEM analysis

To study the uptake mechanism of herceptin-DGA-G4-cisplatin in SKOV-3 cells, TEM images were captured at 12h and 24h incubation of these conjugates (Figure 5.9). At 12h incubation, most of the nanoparticles were already internalized within the cells and found as electron dense deposits in the cytoplasm. Besides, few herceptin-DGA-G4-cisplatin nanoparticles were seen on the surface of cells undergoing the process of internalization, probably through the surface receptors for HER 2.

After 24h incubation, the nanoparticles have entered the nucleus and caused chromatin condensation, nuclear fragmentation triggering apoptosis. TEM studies clearly indicate that herceptin-DGA-G4-cisplatin nanoparticles are taken up by receptor mediated endocytosis.
Figure 5.9 TEM images of SKOV-3 cells treated with herceptin-DGA-G4-cisplatin.

(A) After 12h incubation herceptin-DGA-G4-cisplatin were internalized into the cell, and (B) after 24h the nanoparticles have entered the nucleus and caused chromatin condensation. (C) Uptake of herceptin-DGA-G4-cisplatin and higher magnification of the encircled area in c showing the uptake.
5.2.6 Effect of herceptin targeted and untargeted dendrimer-drug conjugates on cell cycle

The distribution of SKOV-3 and PA-1 cells into various phases of cell cycle after treatment with dendrimer-drug conjugates is shown in Figure 5.10 A and B. In both the cell lines, incubation with herceptin-DGA-G4-cisplatin, DGA-G4-cisplatin and free cisplatin for 24h caused significant S phase arrest of cell cycle and substantial accumulation of cells in sub G0 phase. In control group, neither S phase arrest nor sub G0 phase disruption was observed (Table 5.1 A and B).

In HER 2+ve SKOV-3 cells besides S phase arrest, a prominent difference between herceptin targeted and untargeted dendrimer-cisplatin conjugate was observed. At equivalent drug concentration, most of the cells treated with herceptin-DGA-G4-cisplatin were almost dead and started to accumulate in sub G0 phase (44.5%), where only 19% of the cells were observed in sub G0 phase for untargeted DGA-G4-cisplatin. In contrast the free cisplatin group had only 6% of cells in sub G0 phase.

In HER 2–ve PA-1 cells no significant difference between the targeted and untargeted DGA-G4-cisplatin was observed.
Chapter 5

Targeting studies of DGA-PAMAM-cisplatin conjugates with Herceptin and evaluation of its in vitro cytotoxicity

Figure 5.10 A. Effect of 24h treatment with DGA-G4-cisplatin, Herceptin-DGA-G4-cisplatin and free cisplatin on the cell cycle of SKOV-3 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sub G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.72±0.10</td>
<td>78.09±0.37</td>
<td>7.33±0.15</td>
<td>10.71±0.21</td>
</tr>
<tr>
<td>DGA-G4-cisplatin</td>
<td>19.37±0.66***</td>
<td>36.56±0.38</td>
<td>28.28±0.35**</td>
<td>11.96±0.79</td>
</tr>
<tr>
<td>Herceptin-DGA-G4-cisplatin</td>
<td>44.53±0.58***</td>
<td>29.63±0.25</td>
<td>14.73±0.41**</td>
<td>8.69±0.25</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>8.59±0.21***</td>
<td>60.32±0.28</td>
<td>20.66±0.22</td>
<td>9.68±0.27</td>
</tr>
</tbody>
</table>

Table 5.1 A. Changes in cell cycle phase distribution of SKOV-3 cells following 24h treatment with DGA-G4-cisplatin, Herceptin-DGA-G4-cisplatin and free cisplatin. Data represented as mean±SE (n=3) **p<0.01 ***p<0.001.

Figure 5.10 B. Effect of 24h treatment with DGA-G4-cisplatin, Herceptin-DGA-G4-cisplatin and free cisplatin on the cell cycle of PA-1 cells.

**Table 5.1 B. Changes in cell cycle phase distribution of PA-1 cells following 24h treatment with DGA-G4-cisplatin, Herceptin-DGA-G4-cisplatin and free cisplatin. Data represented as mean±SE (n=3) ** p<0.01 *** p<0.001.**
5.2.7 Effect of herceptin targeted and untargeted dendrimer-drug conjugates on cellular apoptosis

Changes in the symmetry of phosphotidyl serine, a characteristic feature of apoptosis were studied by measuring Annexin V-PE binding to the cell membrane by flow cytometry (Figure 5.11 and 5.12). In HER 2 expressing SKOV-3 cells dendrimer-cisplatin conjugates possessed enhanced apoptotic activity (Figure 5.11 A) with herceptin targeted DGA-G4-cisplatin causing increased cell death (72% early apoptotic cells) in comparison to DGA-G4-cisplatin (56%) and free cisplatin (23%) (Figure 5.11 B).

![Flow cytometer analysis on cell apoptosis of SKOV-3 cells incubated with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin. (A) Four different populations of cells were analyzed after treatment. Bottom left-live cells, bottom right-early apoptotic cells, top right-late apoptotic cells and top left-dead cells. (B) % of apoptosis were represented as mean ± S.E. **p < 0.01, ***p < 0.001.](image)

*Synthesis and characterization of diglycolamic acid functionalized and anti-HER 2 targeted polyamidoamine dendrimer-cisplatin nanoparticles for the treatment of cancer* [Ph.D(R.R)/197/FT/VII/2009]
Annexin V-PE binding studies were also performed in HER 2 non expressing PA-1 cell line to confirm that herceptin targeting is crucial for the enhanced toxic activity of dendrimer-cisplatin conjugate. PA-1 cells when treated with herceptin targeted DGA-G4-cisplatin and DGA-G4-cisplatin at equivalent cisplatin concentration showed similar percentage of early apoptotic cells, i.e. herceptin targeted DGA-G4-cisplatin showed 19.1% and DGA-G4-cisplatin showed 18.2% respectively while free cisplatin showed 29% apoptosis (Figure 5.12 A and B). All the results were compared with untreated control samples.

![Flow cytometer analysis on cell apoptosis of PA-1 cells incubated with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin.](image)

**Figure 5.12** Flow cytometer analysis on cell apoptosis of PA-1 cells incubated with DGA-G4-cisplatin, herceptin-DGA-G4-cisplatin and free cisplatin. (A) Four different populations of cells were analyzed after treatment. Bottom left-live cells, bottom right-early apoptotic cells, top right-late apoptotic cells and top left-dead cells. (B) % of apoptosis were represented as mean ± S.E. **p < 0.01, ***p < 0.001.
Apoptotic mode of cellular death was further confirmed by TEM. SKOV-3 cells when treated with Herceptin targeted DGA-G4-cisplatin presented a series of ultra structural changes such as chromatin condensation, chromatin crescent, intracellular vacuoles and nucleus fragmentation (Figure 5.13), all of which were characteristics of cells undergoing apoptosis.

Figure 5.13 SKOV-3 cells presenting a series of ultra structural changes of apoptosis.

Asterisks indicate nuclear condensation.

5.3 Discussion

Active drug targeting represents a promising approach for the treatment of cancer where specific expression of particular target antigens, receptors and biomarkers can be exploited. Normal cells express moderate levels of HER 2, an important target protein; whereas tumor cells of certain patients overexpress this protein. Further, HER 2 expression is seen between 5-35% of ovarian cancer patients and these patients are selected for HER 2 targeted therapy using Herceptin as a therapeutic agent [34, 35]. The concept of active targeting with herceptin may be achieved by covalent or non-covalent binding with nanocarrier systems, which ensures the specific distribution of drug into targeted tissue [36].
G4 PAMAM dendrimer with 64 amine groups on its surface provides an ample number of reactive sites for the construction of complex drug delivery systems by attaching multiple chemical moieties, such as targeting ligands, drugs, dyes, and contrast agents. Therefore, in our study, we synthesized a conjugate of herceptin with PAMAM dendrimer as the drug carrier for cisplatin. The amine-terminated G4 dendrimer was partially DGA functionalized with diglycolic anhydride and reacted with a heterobifunctional linker, sulfo-LC-SPDP to provide a 2-pyridylthiol group on the surface. This conjugate upon reduction with DTT gives a reactive thiol group that was reacted with herceptin which was modified with maleimide linker to give the final herceptin-DGA-G4-PAMAM dendrimer conjugate. The SDS-PAGE and DLS analysis of this targeted conjugate showed increase in molecular mass, size and zeta potential in comparison to DGA-G4-PAMAM. Herceptin-DGA-G4-cisplatin showed positive surface potentials relative to the untargeted DGA-G4 and this observation might be explained by the overall positive charge of herceptin in water. A similar trend was also observed by Yu Mi et al [37].

While testing the anticancer efficacy of these targeted conjugates in HER 2 +ve and –ve cell lines, herceptin-DGA-G4-cisplatin displayed enhanced toxicity against HER 2 + SKOV-3 cells than its negative counterpart PA-1. This could be mainly attributed to increased cellular uptake of these conjugates most probably through receptor mediated endocytosis, which is an essential step for many antibody targeted therapies [38]. Besides this, SKOV-3 cells are known to overexpress P-glycoprotein, encoded by multidrug resistance gene 1 (MDR 1) [39]. This protein functions as cell membrane efflux pump and effectively exports cisplatin out of the cells thereby reducing its intracellular accumulation. Herceptin targeted dendrimer-cisplatin conjugates can

*Synthesis and characterization of diglycolamic acid functionalized and anti-HER 2 targeted polyamidoamine dendrimer-cisplatin nanoparticles for the treatment of cancer*

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circumvent P-glycoprotein and enter the cell by endocytosis [40], mostly through HER 2 receptors, thereby maximizing cisplatin concentration and toxicity.

This finding was further supported by results from cellular uptake studies which showed that herceptin targeted DGA-G4-cisplatin are efficiently endocyotosed via HER 2 receptors, thus proving their enhanced cellular binding leading to higher Pt accumulation in SKOV-3 cells having overexpression of HER 2 than PA-1. The fact that HER 2-targeted therapy is responsible for inducing elicited S phase arrest and apoptotic activity in SKOV-3 cells was confirmed by cell cycle analysis and Annexin V-PE binding studies. S phase arrest observed among all the treatment groups in both the cell lines strongly supports that it is only the cisplatin released from targeted and untargeted dendrimers causes cytotoxicity. Increased apoptotic activity of cisplatin than herceptin targeted and untargeted DGA-G4 cisplatin in PA-1 cells could be mostly attributed to the fact of slow and sustained release of cisplatin from these conjugates as it is only the free cisplatin that binds with DNA and causes cell death. Collectively, it is evident that receptor mediated endocytosis via antibody targeting could be the essential reason for increased nanoparticle uptake, greater drug accumulation and enhanced cytotoxicity of herceptin-DGA-G4-cisplatin [41, 42].

5.4 Conclusion

In this study we have demonstrated that herceptin-DGA-G4-cisplatin combines the tumor targeting property of herceptin and drug delivery property of DGA-PAMAM dendrimer. Anticancer activity of herceptin-DGA-G4-cisplatin was found to be superior in HER 2 +ve SKOV-3 cells than HER 2-ve PA-1 cells, principally due to the selective HER 2 receptor targeting which in turn increases the cellular uptake and accumulation of cisplatin. Increased
intrapcellular concentration of cisplatin promotes enhanced cytotoxicity effects in terms of cell cycle arrest and apoptosis relative to untargeted DGA-G4-cisplatin and free cisplatin. Together, these results demonstrate that herceptin-DGA-G4-cisplatin have potential application in targeted drug delivery.

5.5 References


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