CHAPTER 4
SYNTHESIS AND BIOLOGICAL CHARACTERIZATION OF DRUG-DENDRIMER CONJUGATES
4.1: INTRODUCTION

The emerging role of dendritic macromolecules for anticancer therapies has highlighted the advantages of these well-defined materials as the newest class of macromolecular delivery devices. These delivery systems enhance the therapeutic effect while reducing or preventing toxic side effects associated with conventional chemotherapeutic drugs (Yang W et al. (2009)). They can be employed either by the enhanced permeability and retention (EPR) strategy or as polymeric platforms with the help of a targeting moiety for selective delivery (Yang W et al. (2009)).

Dendrimers induces passive targeting effects reducing the non-specific toxicity of the carried drugs. This effect is called enhanced permeability and retention effect (ERP effect). This effect exploits the abnormalities of tumour vasculature called hypervascularization, extensive production of vascular permeability factors stimulating extravasation within tumor tissues, and lack of lymphatic drainage. The macromolecular anticancer drugs when administered intravenously (i.v.) escape renal clearance due to their large size. Being unable to penetrate through tight endothelial junctions of normal blood vessels, their concentration builds up in the plasma rendering them long plasma half-life and selectively accumulate in the tumor tissues due to its abnormal vascular nature. Due to the lack of efficient lymphatic drainage in the solid tumor, the concentration of the drug will build up in the tumor reaching several folds higher than that of the plasma. Indeed, this selective high local concentration of macromolecular anticancer drugs in tumor tissues has proven superior in therapeutic effect with minimal side effects in both preclinical and clinical settings. (Greish K. (2010)).

Dendrimers are also capable of specific targeting to carry drugs towards specific cells by grafting the target molecules onto the dendrimer surface termini. This approach is called active targeting approach. Several ligands including antibodies, folic acid, single chain antibodies have been conjugated to the delivery systems for specific targeting.
Delivery of drugs occur via a conjugated cleavable linker like amides, esters, hydrazones, etc. that are activated by disease-specific signals (on-demand drug delivery), such as chemical/oxidation, changes in the surrounding pH or external stimuli, such as magnetic fields, light or specific enzymes (self-immolative approach) (Shmilovici A.et.al(2010)). Haag et.al have recently analyzed stimuli-responsive polymeric nanocarriers, including dendrimers, dendrons etc., for the controlled transport of active compounds (drugs, peptides, genes, etc (Fleige E et.al( 2012)).

This chapter deals with the synthesis and biological characterization of

1) Non targeted conjugates of mPEG dendrimers and acetal PEG dendrimers using lupeol and doxorubicin as model drugs.

2) Targeted conjugates of acetal PEG dendrimers using doxorubicin as model drugs.

Figure 4.1: Schematic representation of different drug-targeting approaches
(Adapted from S. Parveen et al (2012))
4.2: MATERIALS AND METHODS

4.2.1: Materials
Succinic anhydride, Folic acid, N,N-Dicyclohexylcarbodimide (DCC) were purchased from Sigma Aldrich. Triethyl amine (TEA) was purchased from Pfister. Aurasynthesis, doxorubicin hydrochloride injection from Pfizer and 4-(dimethylamino pyridine) (DMAP) was purchased from merck. N-Hydroxysuccinimide (NHS), Tetra Hydrofuran (THF), Dimethyl sulphoxide (DMSO) and Trifluoroacetic acid (TFA) was purchased from Sisco research laboratories (SRL), thiazol tetrazolium blue (MTT reagent) was purchased from sigma Aldrich.

4.2.2: Synthesis of drug-dendrimer conjugate

4.2.2.1: Coupling of Lupeol to the mPEG (G2) (Cl)4 :
3 mg of lupeol dissolved in 1ml of methanol was added to 20 mg of mPEG (G2) (Cl)4 dissolved in 10 ml of anhydrous toluene containing 60 mg of sodium bicarbonate. The solution was stirred at room temperature for 72 hr, filtered and concentrated.

4.2.2.2: Coupling of lupeol to acetal PEG(G2)(Cl)8 :
4 mg of lupeol dissolved in 1ml of methanol was added to 20 mg of acetal PEG(G2)(Cl)8 dissolved in 10 ml of anhydrous toluene containing 60 mg of sodium bicarbonate. The solution was stirred at room temperature for 72 hr, filtered and concentrated.

4.2.2.3: Coupling of doxorubicin to mPEG(G2.5)(OH)4
Synthesis of succinylated PEG dendrimer from mPEG(G2.5)(OH)4 (mPEG(G2.5)(COOH)4)
0.1mmol of succinic anhydride was dissolved in 20ml THF containing 0.1mmol of DMAP. This solution was kept at 0°C-4°C for 30mins. A solution of 0.019mmol of mPEG(G2.5)(OH)4 dissolved in 45ml of THF containing 0.1mmol of TEA was added dropwise to the above solution. The mixture was then stirred at 0°C-4°C for 2hr and at room temperature for overnight. At the end of the reaction, the solution was dried and then dissolved in dichloromethane and filtered to remove unreacted succinic anhydride. The
solution was then concentrated and precipitated using cold diethyl ether. The precipitated product was dried under vacuum.

**Conjugation of doxorubicin to mPEG(G2.5)(COOH)₄ i.e. (mPEG(G2.5)-Doxorubicin conjugate).**

The 0.012mmol of succinylated mPEG(G2.5)(COOH)₄ dendrimer was dissolved in 2ml DMSO containing 0.13mmol of NHS, 0.041mmol of DCC, and 0.072mmol of TEA. To this solution 0.06mmol of doxorubicin is added under nitrogen atmosphere and stirred overnight at 25°C-30°C. At the end of the reaction time the reaction mixture was dialysed against water for 4hr with three water changes to remove the unreacted free doxorubicin. This product was obtained as lyophilised powder.

**4.2.2.4: Coupling of doxorubicin to acetal(G2.5)(OH)₈**

**Synthesis of succinylated PEG dendrimer from acetal(G2.5)(OH)₈ (acetal(G2.5)(COOH)₈)**

0.8mmol of succinic anhydride was dissolved in 20ml THF containing 0.72mmol of DMAP. This solution was kept at 0°C-4°C for 30mins. A solution of 0.009mmol of acetal(G2.5)(OH)₈ dissolved in 45ml of THF containing 0.72mmol of TEA was added drop wise to the above solution. The mixture was then stirred at 0°C-4°C for 2hr and at room temperature for overnight. At the end of the reaction, the solution was dried and then dissolved in dichloromethane and filtered to remove unreacted succinic anhydride. The solution was then concentrated and precipitated using cold diethyl ether. The precipitated product was dried under vacuum.

**Conjugation of doxorubicin to acetal(G2.5)(COOH)₈ i.e. (acetal-PEG(G2.5)-Doxorubicin conjugate)**

The 0.2⁴mmol of succinylated acetal(G2.5)(COOH)₈ dendrimer was dissolved in 2ml DMSO containing 0.11mmol of NHS, 0.019mmol of DCC, and 0.072mmol of TEA. To this solution 0.007mmol of doxorubicin is added under nitrogen atmosphere and stirred overnight at 25°C-30°C. At the end of the reaction time the reaction mixture was dialyzed against water for 4hr with three water changes to remove the unreacted free doxorubicin. This product was obtained as lyophilized powder.
4.2.2.5: Synthesis of targeted acetal PEG(G2.5)-doxorubicin conjugate with folic acid as the targeting moiety:

Conversion of acetal group of acetal(G2.5)(COOH)₈ to active aldehyde group for coupling to folic acid.

0.2g of succinylated acetal(G2.5)(COOH)₈ dendrimer was refluxed with 8ml of TFA:H₂O(1:1) at 65°C-70°C for 20hr. The solution was concentrated and extracted using dichloromethane. The combined organic layers were concentrated and precipitated using diethyl ether. The precipitate can dried under vacuum. The reaction was monitored for the complete conversion of acetal group to aldehyde group using TLC.

**Conjugation of folic acid to activated acetal(G2.5) dendrimer (FA-PEG(G2.5)-(COOH)₈)**

6mg (0.013mmol) of folic acid was added to 2ml of sodium bicarbonate buffer (pH 9.5) containing 20mg activated aldehyde activated acetal(G2.5) dendrimer. The solution was stirred for 2hr at 25°C-30°C for 2 hr. To this solution 80ul of 0.5M sodium cyanoborohydride was added in dark and was stirred for overnight. At the end of the reaction time, the reaction mixture was dialysed against water for 4hr, three water changes to remove unreacted free folic acid. The final product was obtained as lyophilized powder.

**Conjugation of doxorubicin to FA-PEG(G2.5)-(COOH)₈ i.e. (FA-PEG(G2.5)-Doxorubicin conjugate.**

The 0.2⁴mmol of FA-PEG(G2.5)-(COOH)₈ dendrimer was dissolved in 2ml DMSO containing 0.11mmol of NHS, 0.019mmol of DCC, and 0.072mmol of TEA. To this solution 0.007mmol of doxorubicin is added under nitrogen atmosphere and stirred overnight at 25°C-30°C. At the end of the reaction time the reaction mixture was dialyzed against water for 4hr with three water changes to remove the unreacted free doxorubicin. This product was obtained as lyophilized powder.

4.2.3: IN VITRO DRUG RELEASE STUDIES

Drug release experiments were conducted using dialysis method. Typically, 1.0 ml of the doxorubicin-dendrimer conjugate in concentration of 1mg/ml in phosphate buffer saline was sealed in a dialysis bag. The dialysis bag were
immersed in 10 mL of the same buffered media and was continuously rotated with stirring rate of 50 rpm at 37 °C. At regular time intervals 1ml of the sample was withdrawn equivalent volume of the solution was added to the dissolution vessel. The amount of doxorubicin released was calculated spectrophotometrically. The study was done under two different pH conditions i.e. pH 5.5 and pH 7.4 at 37°C.

4.2.4: BIOLOGICAL CHARACTERIZATION

4.2.4.1: Cell culturing and growth
Human prostate cancer cells (PC-3 cells) and human colon cancer cells (HCT-15) were obtained from NCCS, Pune, India. The cells were grown in RPMI 1640 medium supplemented with 10% of bovine fetal serum and antibiotics (100 IU of penicillin/100 mg/mL of streptomycin). The cells were maintained in a 25 cm² T-flask and incubated at 37°C under 5% CO2 in a humidified atmosphere. The cell viability was measured by staining the cells with trypan blue and counted in a haemocytometer under a light microscope.

4.2.4.2: Determination of Cytotoxicity
The cytotoxicity assay was performed in a 96-well microtitration plate. Cells were seeded at a density of approximately 5*1000 cells/well. The plate was then incubated at 37°C, 5% CO2, and 90% humidity for 24 h. Subsequently different concentrations of free drug, non-targeted drug-dendrimer conjugates and targeted drug-dendrimer conjugates were added to the appropriate wells.. Negative control wells with no compound were also included. The assay was performed in triplicates.

4.2.4.2: MTT Assay
The cytotoxicity was quantitatively estimated by a non-radioactive, colorimetric assay system using a tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil-tetrazolium bromide (MTT). MTT was dissolved in phosphate-buffered saline at 5 mg/mL. MTT solution was then added directly to all appropriate microtitre plate wells (10 μL per 100 μL medium) containing cells and complete growth medium, with or without the tested compound. The
plate was then incubated for 4 h at 37°C to allow MTT to metabolize to formazan. Subsequently, the supernatant was aspirated and 100 μL of DMSO was added and mixed thoroughly to dissolve the dark blue formazan crystals. The optical density (OD) was measured absorbance at 570nm and 620nm as reference was recorded in an ELISA plate reader (FLUO star OPTIMA).
4.3: RESULTS AND DISCUSSION

Synthesis of lupeol-dendrimer conjugates

Coupling of Acetal PEG(G2)(Cl)₈ with Lupeol

Figure 4.2: Coupling of the lupeol isolated from aloe vera to acetal PEG(G2)(Cl)₈ dendrimer by reacting lupeol to the acetal PEG(G2)(Cl)₈ dendrimer dissolved in toluene in the presence of NaHCO₃, stirred at room temperature for 72 h
Coupling of the purified lupeol from aloe vera to the mPEG(G2)(Cl)₄ dendrimer:

Figure 4.3: Coupling of the lupeol isolated from aloe vera to mPEG(G2)(Cl)₄ dendrimer by reacting lupeol to the mPEG(G2)(Cl)₄ dendrimer dissolved in toluene in the presence of NaHCO₃, stirred at room temperature for 72 h.
The preparation of lupeol-dendrimer conjugates was done using G2 dendrimers containing active chloride groups at the periphery. The chloride groups present on the dendrimer were reacted to the hydroxyl group of the lupeol. The reactions were done in the presence of NaHCO₃ to provide the basic condition required for the reaction and thus neutralize the released HCl. Figure 4.2 and figure 4.3 shows the overall reaction for the synthesis of acetal PEG(G2)-lupeol conjugate and mPEG(G2)-lupeol conjugate respectively. Excess of lupeol was added to each reaction in order to achieve better conjugation. The un-reacted lupeol was removed by simple dialysis. The conjugation of lupeol to the polymer was confirmed by FTIR. The spectra showed a complete disappearance of C-Cl stretch was observed. The peak due to olefinic moiety present on the lupeol was observed at 1654 cm⁻¹. The amount of lupeol per gram of the polymer was calculated UV/Visible spectrophotometry. The conjugation done in this case is a direct conjugation i.e. the drug is directly attached to the carrier system and the release of drug is not possible. Permanent attachment of the drug moiety is generally used only when the drug exhibits activity in the attached form.

Figure 4.4: FTIR spectra of mPEG(G2)-lupeol conjugate
Synthesis of targeted and non targeted doxorubicin-dendrimer conjugates:

Coupling of Doxorubicin was conjugated mPEG(G2.5)(OH)₄

![Diagram of synthesis process]

Figure 4.5: Overall synthesis of mPEG(G2.5)–doxorubicin conjugate.

Step 1: 4-(dimethylamino pyridine) (DMAP)/ Triethyl amine (TEA)
Step 2: N,N-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), Triethyl amine (TEA), DMSO, Doxorubicin.
Coupling of Doxorubicin was conjugated acetal PEG(G2.5)(OH)₈

Figure 4.6: Overall synthesis of acetal PEG(G2.5)–doxorubicin conjugate.

Step 1: 4-(dimethylamino pyridine) (DMAP)/ Triethyl amine (TEA)
Step 2: N,N-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), Triethyl amine (TEA), DMSO
The synthesis of doxorubicin-dendrimer conjugate was done using mPEG(G2.5) and acetalPEG(G2.5) dendrimers containing hydroxyl groups on the periphery. The hydroxyl groups on the periphery were first activated by succinic anhydride to form active acid group on the periphery. Succinic anhydride has a five-atom cyclic structure that is highly reactive toward nucleophiles, including hydroxyl and amine groups. Nucleophilic attack at one of the carbonyl groups opens the anhydride ring, forming a covalent bond with that carbonyl and releasing the other to create a free carboxylic acid group.

DMAP is a commonly used catalyst used to accelerate the acylation of alcohols and amines. Hydroxyl groups react with the acylated catalyst in the rate-determining second step to form an ester linkage, along with the deactivated catalyst. TEA is a strong base which is utilized to recover the deactivated catalyst (Klotz, I. M.(1967), Xu, S et.al.(2005)).

The second step involved the conjugation of the activated dendrimer to the amine group of the doxorubicin. It is known that DOX has an amino group, which is not the active site for DOX effectiveness. DOX can be conjugated in the presence of NHS and DCC, through amide bonding (Ding W. and Guo L. (2013)). In brief, DCC reacts with the carboxylic group to form an active O-acylisourea intermediate that can be easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. The intermediate formed in this case is unstable in aqueous solutions and lead to the regeneration of carboxylics and release of N-substituted urea. However the use of NHS in the reaction improves the efficiency of the reaction by producing stable intermediates. DCC couples NHS to carboxyls, forming an NHS ester that is more stable than the O-acylisourea intermediate while allowing for conjugation to primary amines at physiological pH. The FTIR studies were carried to confirm the presence of amide linkage between the COOH group present on the polymer and the NH$_2$ group of the doxorubicin. The most significant peak in the spectra was due to the presence of –CONH linkage. The carbony (C=O) and the amine (N-H) groups present in the
amide linkage exhibited at 1618 cm\(^{-1}\) and 1598 cm\(^{-1}\) respectively. The amount of doxorubicin conjugated per gram of the polymer was calculated using UV/Visible spectrophotometry (Yang SJ et al. (2010), Pan L et al. (2001)).

Figure 4.6: FTIR spectra of mPEG(G2.5)(OH)\(_4\)
Synthesis of Folic acid-dendrimer-Doxorubicin conjugate for targeted delivery:

**Figure 4.7:** Overall synthesis of Folic acid-dendrimer-doxorubicin conjugate

**Step 1:** Trifluoro acetic acid/Water (1:1)
**Step 2:** Folic acid/ sodium bicarbonate (pH 9.5), Na(CN)BH4
**Step 3:** N,N-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), Triethyl amine (TEA), DMSO

Succinylated PEG dendrimer with eight COOH groups at the periphery

TFA/H2O (1:1) Succinylated PEG dendrimer with eight COOH groups on one side and aldehyde(CHO) group on the other side

Folic acid
Sodium Bicarbonate buffer(pH:9.5)/Na(CN)BH4

Doxorubicin
DCC / NHS
TEA, DMSO

Folic acid-Dendrimer-Doxorubicin Conjugate
For the preparation of targeted delivery systems folic acid was used as the targeting moiety. The first step for synthesis was to de-protect the acetal group by converting it to a more reactive aldehyde group. The oxidation was done in the presence of TFA:H₂O. The presence of aldehyde group was identified by chemical analysis using silver mirror test. The active aldehyde group on the dendrimer was reacted to the amine group of folic acid in presence of sodium bicarbonate buffer with pH 9.5. The reaction involves condensation of the carbonyl compound and amine to give a carbinolamine, followed by dehydration and subsequently loses one molecule of water in a reversible manner by alkylimino-de-oxo-bisubstitution, to form the imine. Presence of sodium cyanoborohydride (NaBH₃CN) helps in reduction of the unstable imine intermediate to an amine product (Baxter E.W. and Reitz A.B. (2002)). The FTIR spectra of FA-PEG(G2.5)-Doxorubicin conjugate was studied to confirm the amide linkage between COOH group of the polymer and the NH₂ group of the doxorubicin. Also peak due to the amine linkage i.e. C=N bond formed between the CHO group of the polymer with the NH₂ group of the folic acid. The significant peak due to the C=N bond was observed at 1687 cm⁻¹ along with the peaks due to the amide linkage between the doxorubicin and the polymer at 1629 cm⁻¹ and 1598 cm⁻¹ due to carbony (C=O) and the amine (N-H) groups respectively.

![Figure 4.8: FTIR spectra of FA-PEG(G2.5)-Doxorubicin conjugate.](image-url)
Biological characterization

The validation of the synthesized drug-dendrimer conjugates for the increased therapeutic efficacy of the conjugated drug when compared to the free drug was done using MTT assay. The study was done in two different cell lines i.e. human prostate cancer cell line (PC3) and human colorectal cancer cell line (HCT-15).

Initially a study to check the cell viability in presence of dendrimer alone was conducted using MTT assay. Generation 2 dendrimers containing chloride groups on the periphery were used for the study. The results showed that both mPEG(G2) and acetal- PEG(G2) dendrimers showed no cytotoxic effect after 48 hours of treatment on the both PC3 and HCT cell lines as shown in figure 4.10 & figure 4.11 respectively. This effect was similar to the previous literature which states that negatively charged or neutral dendrimers reveal less toxicity as compared to positively charged dendrimers (figure 4.9); (Chen HT et al (2004), Ionov M et al (2011, El-Sayed M. et.al (2002)). Dendrimers having positively charged end groups may interact with negatively charged membrane and increase the permeability that facilitates the intracellular delivery of the bioactive molecules. However the higher generation of these dendrimers may result in disruption of membrane integrity followed by leakage of intracellular components which finally leads to cell death and toxicity. (Mecke A et al (2005), El-Sayed M et.al (2002)).
A slight increase in the cell number was observed in both PC3 and HCT cell lines as shown in fig 4.10 & fig 4.11, which may be due to the presence of chloride groups on the periphery of the dendrimers. Previous literature suggests that Cl\(^-\) affects cell growth and cell cycle progression in cell lines suggesting that a change in [Cl\(^-\)] play a critical role in the growth mechanism. (Xu B. et.al(2010), Hiraoka K. et al (2010)). These results suggest that there is no cytotoxic effect of dendrimers alone, thus suggesting that cytotoxic activity of drug-dendrimer conjugates would not have any effect due to the dendrimer.
Figure 4.10: Cytotoxic effect of mPEG(G2) and acetal PEG(G2) on PC3 cell lines after 48 hours of treatment.

Figure 4.11: Cytotoxic effect of mPEG(G2) and acetal PEG(G2) on HCT cell lines after 48 hours of treatment.
The validation of the acetal PEG(G2)-lupeol conjugate and mPEG(G2)-lupeol conjugate for the increased therapeutic efficacy of lupeol when compared to the free lupeol was done using MTT assay. The results showed a significant difference in the cytotoxicity of free lupeol to that of the conjugated lupeol.

Table 4.1: IC50 values of free lupeol, mPEG(G2)-lupeol conjugate, Acetal PEG(G2)-lupeol conjugate in different cell lines after 48 hr of treatment. The results are given in lupeol equivalent concentration.

<table>
<thead>
<tr>
<th>Cytotoxic molecule</th>
<th>IC50 (µg/ml)</th>
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<tr>
<td></td>
<td>PC3</td>
</tr>
<tr>
<td>Free lupeol</td>
<td>39.9</td>
</tr>
<tr>
<td>mPEG(G2)-lupeol conjugate</td>
<td>5.9</td>
</tr>
<tr>
<td>AcetalPEG(G2)-lupeol conjugate</td>
<td>7.9</td>
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Figure 4.12: Comparison of IC50 value of free lupeol, mPEG(G2)–lupeol conjugate, acetal PEG(G2)–lupeol conjugate on PC3 cell line after 48hr of treatment. The IC50 values are given in lupeol equivalent concentration.

Figure 4.13: Comparison of IC50 value of free lupeol, mPEG(G2)–lupeol conjugate, acetal PEG(G2)–lupeol conjugate on HCT-15 cell line after 48hr of treatment. The IC50 values are given in lupeol equivalent concentration.
We observed a significant difference in the cytotoxicity of the free lupeol when compared to the lupeol-dendrimer conjugates. The IC50 value of free lupeol in PC3 cell line after 48 hours of treatment was 39.19 µg/ml which was 6.6 folds more when compared to the mPEG(G2)-lupeol conjugate and 4.4 folds more when compared to acetal-PEG(G2)-lupeol conjugate (Fig. 4.12). Thus, these data demonstrated the cytotoxic activity of lupeol is increased upon conjugation to the dendrimers. Similar results were also observed in HCT-15 cell line, where in free lupeol showed a IC50 value of 42.28 µg/ml which was 3.9 folds and 3.7 folds more than mPEG(G2)-lupeol conjugate and acetal-PEG(G2)-lupeol conjugate respectively (Fig. 4.13).

The increase in activity could be attributed to the passive targeting effect of the dendrimers. Macromolecular prodrugs like dendrimer-drug conjugates can enter a cell in two ways i.e. either via diffusion route with free drug release into the cell or by an endocytosis route which involves the entire polymer-drug conjugate. The more ideal route for these macromolecular dendrimer-drug conjugates involves the endocytosis route (Ouchi T. and Ohya Y. (1995). The phenomena of enhanced permeability and retention (EPR) effect may help in better internalization of the macromolecular drug when compared to the free drug (Maeda H et.al( 2009), Greish K. (2010)).

To our knowledge this is the first report which shows increase in therapeutic effect of lupeol by conjugation to dendrimers.
In case of doxorubicin-dendrimer conjugates the conjugation is done via amide bond. The release of doxorubicin was measured under two pH conditions i.e. pH 7.4 similar to physiological pH and pH 5.5 similar to the mild acidic condition of tumour cells. The results showed (figure 4.14), the release of drug was more efficient in the acidic medium as compared to the physiological pH. 80.95% of doxorubicin was released from the conjugate at pH of 5.5 in comparison to 30.45% in pH 7.4 after incubation for 48 hours. This may be due to the following two reasons. First, the amide bonds between carboxylic groups of dendrimer and DOX, become more unstable in acid solution. Second, protonation of amino groups in DOX, under acid conditions, speeds its release.( Wence Ding and Lin Guo et al 2013, Sahu SK et al. 2012 ). Due to these release properties, doxorubicin-dendrimer conjugates releases more DOX in the mildly-acidic physiological environment of tumor than in the relatively neutral physiological environment of normal tissue. Taking advantage of its pH-sensitivity and release properties, the dendrimer conjugates can be delivered systemically, for targeted delivery and release of drug into tumor microenvironment.

![Graph showing drug release over time](image)

**Figure 4.14:** Doxorubicin release profile from Doxorubicin-dendrimer conjugates in Phospahte buffer saline pH 5.5 and pH 7.4.
The validation of the targeted and non targeted doxorubicin-dendrimer conjugates for the increased therapeutic efficacy of doxorubicin when compared to the free doxorubicin was done using MTT assay. The results showed a significant difference in the cytotoxic activity of the conjugated doxorubicin when compared to free doxorubicin. The study in this case was focussed on understanding the effect of active targeting of the system using folic acid, the effect of drug density and drug distribution on the cytotoxicity of the drug-dendrimer conjugates.

Table 4.2: IC₅₀ values of free doxorubicin and mPEG(G2.5)-doxorubicin conjugate, acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin conjugate in PC3 and HCT-15 cell line after 48hr of treatment.

<table>
<thead>
<tr>
<th>Cytotoxic molecule</th>
<th>IC₅₀ (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>PC3</td>
<td>HCT-15</td>
</tr>
<tr>
<td>Free doxorubicin</td>
<td>5.134</td>
<td>4.773</td>
</tr>
<tr>
<td>mPEG(G2.5)-doxorubicin conjugate</td>
<td>0.328</td>
<td>1.350</td>
</tr>
<tr>
<td>AcetalPEG(G1.5)-doxorubicin conjugate</td>
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<td>3.070</td>
</tr>
<tr>
<td>FA-PEG(G1.5)-doxorubicin conjugate</td>
<td>0.304</td>
<td>0.594</td>
</tr>
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</table>

The results show a significant difference in the cytotoxicity of the free doxorubicin to that of the conjugated doxorubicin. The cytotoxicity of free doxorubicin and the two non targeted dendrimer-doxorubicin conjugates i.e mPEG(G2.5)-doxorubicin conjugate and acetal PEG(G1.5)-doxorubicin conjugates were compared. Both the conjugates showed an increased efficiency of cytotoxicity of doxorubicin as compared to that of free doxorubicin. The IC₅₀ value of free doxorubicin in PC3 cell line after 48
hours of treatment was 5.134 µg/ml which was 15 folds more when compared to the mPEG(G2.5)-doxorubicin conjugate and 1.47 folds more when compared to acetal PEG(G1.5)-doxorubicin. Similar results were also observed in HCT-15 cell line, where in free doxorubicin showed an IC50 value of 4.773µg/ml which was 3.53 folds and 1.553 folds more than mPEG(G2.5)-doxorubicin conjugate and acetal PEG(G1.5)-doxorubicin conjugate respectively (fig. 4.15 & fig. 4.16).

On comparing the targeted FA-PEG(G1.5)-doxorubicin conjugate and the non targeted acetal PEG(G1.5)-doxorubicin conjugate with same number of doxorubicin molecules, a significant increase in cytotoxicity was observed in the targeted conjugate when compared to the free drug and the non targeted conjugate. The IC50 value of doxorubicin in the non- targeted conjugate was observed to be 11 folds and 5.16 folds more when compared to the targeted conjugate in PC3 and HCT-15 cell lines respectively (fig 4.15 & fig 4.16).

As mentioned in the case of lupeol-dendrimer conjugates the increase in cytotoxicity of non-targeted doxorubicin-dendrimer conjugates could be attributed to the passive targeting of the dendrimer conjugates which enables better accumulation of the drug inside the cells by the phenomena called enhanced retention and permeability effect(ERP effect).

The increase in cytotoxicity of targeted conjugates to the non targeted conjugates and the free drug can be attributed to the targeting effect of the conjugates. Active targeting requires the conjugation of receptor specific ligands that promote site specific targeting. The active targeting needs molecular recognition of the diseased cell (such as tumor cells) which over expresses unique receptors or antigens. Once these targets are recognized they can be targeted via the ligand-receptor, antigen-antibody interactions. Natural molecules such as folate or growth factors such as epidermal growth factors have advantages of lower molecular weights and lower immunogenicity than
antibodies when used as ligands for targeting cell surface receptors. Folic acid (FA) is a vital nutrient for the growth of cancer cells and can be used as a ligand due to high binding affinity and non-immunogenicity. Many human cancer cells over-express cell surface receptors of folic acid (about 100- to 300-times higher than in normal tissue), thus folate-conjugated dendrimers can be effective anti-cancer agents having higher affinity for cancer cells. Their higher receptor affinity and restricted interaction with normal tissues facilitate the delivery of therapeutic agents selectively to the target site (Sohail Akhter et.al (2013), (Yingjuan Lu et.al (2002)).

Figure 4.15: Comparison of IC50 value of free doxorubicin, mPEG(G2.5)-doxorubicin conjugate, acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin conjugate, PC3 cell line at 48hr of treatment. The IC50 values are given in doxorubicin equivalent concentration.
Figure 4.16: Comparison of IC50 value of free doxorubicin, mPEG(G2.5)-doxorubicin conjugate, acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin HCT-15 cell line at 48hr of treatment. The IC50 values are given in doxorubicin equivalent concentration.
To understand the effect of drug density and distribution of the cytotoxic molecule, we compared the effect of conjugation of the drug to two different generations of the acetal-PEG dendrimer i.e acetal PEG(G1.5)(OH)₄ with four functional groups present on the periphery for conjugation and acetal PEG(G2.5)(OH)₈ with eight functional groups present on the periphery for conjugation.

Table 4.3: IC₅₀ values in µg/ml of free doxorubicin, acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin acetal PEG(G2.5)-doxorubicin conjugate, FA-PEG(G2.5)-doxorubicin conjugate in PC3 cells and HCT-15 cells after 48 hr treatment.

<table>
<thead>
<tr>
<th>Cytotoxic molecule</th>
<th>IC₅₀ (µg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC3</td>
<td>HCT-15</td>
<td></td>
</tr>
<tr>
<td>Free doxorubicin</td>
<td>5.134</td>
<td>4.773</td>
<td></td>
</tr>
<tr>
<td>AcetalPEG(G1.5)-doxorubicin conjugate</td>
<td>3.480</td>
<td>3.070</td>
<td></td>
</tr>
<tr>
<td>FA-PEG(G1.5)-doxorubicin conjugate</td>
<td>0.304</td>
<td>0.504</td>
<td></td>
</tr>
<tr>
<td>AcetalPEG(G2.5)-doxorubicin conjugate</td>
<td>2.894</td>
<td>2.362</td>
<td></td>
</tr>
<tr>
<td>FA-PEG(G2.5)-doxorubicin conjugate</td>
<td>1.491</td>
<td>0.920</td>
<td></td>
</tr>
</tbody>
</table>
The comparison of IC₅₀ value of free doxorubicin and the two non targeted conjugates acetal PEG(G2.5)-doxorubicin conjugate (with eight functional groups), and acetal PEG(G1.5)-doxorubicin conjugate (with four functional groups) showed a significant increase in the cytotoxicity of the conjugated doxorubicin as compared to the free doxorubicin.

In case of non-targeted acetal PEG dendrimer-doxorubicin systems, acetal PEG(G2.5)-doxorubicin conjugate (with eight functional groups) the increase was more as compared to the acetal PEG(G1.5)-doxorubicin conjugate (with four functional groups) in both PC3 and HCT-15 cell line (fig 4.17 & fig 4.18). This difference may be attributed to the high drug density in case of acetal PEG(G2.5)-doxorubicin conjugate when compared to acetal PEG(G1.5)-doxorubicin conjugate.

Conjugation of folic acid as the targeting moiety to both the dendrimer-doxorubicin conjugates showed a further increase in cytotoxicity when compared to non targeted conjugates. However, in case of targeted conjugates we observed that the increase in cytotoxic activity was more in FA-PEG(G1.5)-doxorubicin conjugate when compared to FA-PEG(G2.5)-doxorubicin conjugate which was contrary to the results observed with non-targeted system (fig 4.17 & fig 4.18). This can be attributed to the high density of doxorubicin thereby, reducing the interaction between the target and the targeting moiety due to steric hindrance.
Figure 4.17: Comparison of IC50 value of free doxorubicin, acetal PEG(G2.5)-doxorubicin conjugate, acetal PEG(G2.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G2.5)-doxorubicin), acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin PC3 cell line at 48hr of treatment. The IC50 values are given in doxorubicin equivalent concentration.
Figure 4.18: Comparison of IC50 value of free doxorubicin, acetal PEG(G2.5)-doxorubicin conjugate, acetal PEG(G2.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G2.5)-doxorubicin), acetal PEG(1.5)-doxorubicin conjugate, acetal PEG(G1.5)-doxorubicin conjugate targeted using folic acid (FA-PEG(G1.5)-doxorubicin) HCT cell line at 48hr of treatment. The IC50 values are given in doxorubicin equivalent concentration.
According to literature, in both passive and active targeting approaches, dendrimers avoid their own uptake by the reticular endothelial system (RES) and consequently remain in the blood circulation for substantial amounts of time, thereby increasing their biological potency against specific tissues, such as tumors (Mignani S. et.al (2013)).

The results clearly showed that the conjugation of doxorubicin to the dendrimer was effective in increasing the therapeutic activity of the drug. In comparison to the non targeted conjugates the targeted conjugates exhibited high cytotoxicity, thereby inhibiting the proliferation of tumor cells efficiently. A lower half maximal inhibitory concentration (IC$_{50}$) value is found for targeted system than for non targeted system. These results demonstrate an increased drug delivery effect in receptor-targeted uptake of folic acid targeted conjugates by the cancer cells.