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Methotrexate (MTX) Anticancer Conjugates with Biotin Using Pamam Dendrimers

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Abstract

Methotrexate (MTX) encapsulated in biotinelated dendrimer microparticles (MTX-BD) can be a useful tool for intra-tumor administration showing several benefits in minimizing systemic toxicities of MTX and improve its efficiency *in vivo*. To develop a biotinelated targeted drug delivery system, we employed dendrimer as a carrier. Biotin molecules were then conjugated on the surface of MTX-BD. The anticancer efficacy of biotin targeted MTX-BD was evaluated in mice bearing 4T1 breast carcinoma. A single dose of biotin targeted MTX-BD showed stronger in vivo antitumor activity than non-targeted MTX-BD and free MTX. By some days after treatment, average tumor volume in the biotin targeted MTX-BD treated group decreased to 17.6% of the initial tumor volume when the number of attached biotin molecules on MTX-BD was the highest. Mice treated with biotin targeted MTX-BD showed slight body weight loss (8%) 21 days after treatment, whereas non-targeted MTX-BD treatment at the same dose caused a body weight loss of 27.05% ± 3.1%.

Keywords: Pamam dendrimer**,** Biotin, Targeted drug delivery, *in-vivo* anticancer delivery, Biotinelated, Methotrexate, Conjugates.

INTRODUCTION

Methotrexate (MTX) is one of the most popular and safe anticancer drugs under the applied treatment dose (1,2). In order to obtain a better curative effect in clinical cases, MTX is also used in combination with other drugs for rheumatoid arthritis treatment (1,3,4). In addition, MTX is also used as an anticancer drug (5). Recently, MTX has been widely applied for the treatment of various cancers, such as hepatoma, osteosarcoma, leukaemia, lymphoma, gastric, breast, head and neck cancers (5–9). Many studies have demonstrated that MTX induces cancer cell death via apoptotic death pathways (10–14). Apoptotic death pathways can be divided into caspase-dependent and caspase-independent cascades (15,16). Concerning the MTX-induced apoptotic pathways, most studies have shown that MTX induces apoptosis via caspase-dependent cascades in many cancer cell lines (17–21). However, some studies have indicated that MTX can induce apoptosis via caspase-independent cascades in osteosarcoma cells (22,23). The present study found that MTX-induced apoptosis in Hep3B cells is via the caspase-dependent cascade, similar to most other studies (17–21). Considering that high-dose MTX treatments can cause renal and liver damage (35–37), combination treatments of low-dose MTX and other anticancer drugs are suggested and applied during clinical cancer therapy in order to enhance the anticancer effects and decrease MTX-induced side-effects (9,10,12,18,41). However, not all anticancer agents can enhance the anticancer effects of low-dose MTX. A recent study showed that aspirin can antagonize the MTX-induced cytotoxic effect on lung cancer cells (42). Alternatively, there have been many reports on the antioxidant activities of vitamin C (43–47). Moreover, some studies have demonstrated that vitamin C can exert anticancer activities in various cancer cells (48–52). The present study demonstrated that vitamin C can diminish MTX-induced increases in H_2O_2 levels. On the other hand, it is worth noting that vitamin C can help low-dose MTX exert a cytotoxic effect on Hep3B cells. Taken together, the study demonstrated that MTX activates the caspase-9/-3 cascade and induces increased H_2O_2 levels, causing cell cytotoxicity in

Hep3B cells, while more importantly, the present study is the first to demonstrate that vitamin C enhances the anticancer efficiency in MTX-treated Hep3 cells.

The side effects of anticancer drugs could limit the efficacy of cancer chemotherapy.1–2 Targeted drug delivery systems could diminish the adverse cytotoxic effects of anticancer drugs on normal tissues.3 Specific drug delivery to cancers could be achieved by adding a suitable targeting moiety on drug delivery systems.4 These targeting moieties could bind to certain types of receptors on the tumor cells and deliver cytotoxic drug to these cells specifically. Several targeting agents such as antibodies,5,6 peptides,7,8 vitamins,9 folic acid,10–14 and integrin15 could be used as suitable targeting moieties in targeted drug delivery systems. Among vitamins, biotin (vitamin H) has been used as a suitable targeting agent in several studies.16–18 Biotin receptors over expressed on the surface of different cancer cells could internalize sufficiently the biotin targeted drug delivery system into these cells.18

In this study biotin was used as a targeting moiety to design a tumor targeted drug delivery system based on a dendrimer conjugation strategy. The biotin targeted drug delivery system consisted of (i) Dendrimer as carrier; (ii) methotrexate (MTX) as anticancer drug; and (iii) biotin as a targeting moiety. MTX was conjugated to dendrimer and then MTX-BD (MTX-Biotinelated PAMAM Dendrimer were prepared by cross-linking the Dendrimer molecules using EDC cross-linker. The surface of MTX-HSA NPs was then functionalized by biotin molecules using a carbodiimde reaction. The targeting effect of biotin targeted MTX-HSA NPs to tumor cells were confirmed successfully in vitro.

Materials and Methods

Materials

Methotrexate USP was kindly donated by Sun Pharma Vadodara, India. Biotin was obtained from Sigma-Aldrich (St Louis, MO). 1-ethyl-3-(diaminopropyl) carbodiimde HCl (EDC), N-hydroxysuccinimide (NHS) and human serum albumin, HABA/Avidin and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Steinheim, Germany). RPMI-1640 modified medium and penicillin–streptomycin solution were obtained from Gibco Invitrogen (Calsbad, CA). Deionized water was used throughout the experiment. All other chemicals used were of reagent grade

Preparation of Biotinelated PAMAM Dendrimer Conjugate with Methotrexate MTX-BD)

Synthesis of G4–Biotinelated PAMAM Dendrimer

Vitamin Biotin was conjugated to the periphery of G4.0 PAMAM dendrimers *via* EDC coupling reaction as described in the literature.[30,31] Briefly, Biotin (0.335 g, 760 µmol) was dissolved in the mixed solvent of 18 mL DMF and 6 mL DMSO. To the above solution, EDC (2.06 g, 10.6 mmol) was added and stirred at room temperature for 1 h. The activated biotin was added dropwise to 90 mL aqueous solution of G4.0 PAMAM (0.337 g, 23.7 µmol) and the solution was stirred in dark under nitrogen atmosphere at room temperature for 3 days. The solution was concentrated by rotary evaporation, and then precipitated in acetone. The crude product was dissolved in water and dialyzed against deionized water for 2 days to remove the unreacted biotin. After lyophilization, the product was stored in dark. -

The number of biotin linked to the dendrimer surface was estimated by ^{1}H NMR (400 MHz). ^{1}H NMR (D_2O, 400 MHz, ppm): δ 8.66 (br, biotin, protons at 7 position of pterin ring), 7.61 (br, biotin, protons of phenyl next to amide group), 6.77 (br, biotin, protons of phenyl next to amino group), 3.50–3.05 (m, PAMAM, –CONHC***H***$_2$CH$_2$–), 2.98–2.76 (m, PAMAM, –CH$_2$C***H***$_2$NH– and –NC***H***$_2$CH$_2$CO–), 2.75–2.60 (m, PAMAM, –CH$_2$C***H***$_2$N<), 2.57–2.34 (m, PAMAM, 137-CH$_2$C***H***$_2$CONH–).

Cellular uptake and competition assay of Biotin

Before the experiment, the cells were cultured in FA-free RPMI 1640 medium for some days. Then the cells were harvested and seeded into 6-well culture plates at a density of 5×10^5 cells per well and incubated for 24 h. Free MTX and G4–FA–PEG/MTX were added at a MTX concentration of 10 μM and the cells were incubated for 2 h. Cells without drugs were used as a blank control. Thereafter, the cells were washed with cold PBS for three times and harvested. Since MTX has a quite strong fluorescence, it can be easily detected by FA Scan flow cytometer (Becton Dickinson FACS Calibur, Mountain View, CA, USA).[33]FL2-H filter (585 ± 21 nm) was used for the collection of fluorescence intensity and the events collected were ten thousands.

For the competition assay, FA in RPMI solution (1 mM) was added to the cells and incubated for 30 min in advance. Afterwards, free MTX and G4–FA–PEG/MTX were added to the cells for making the final MTX concentration of 10 μM and maintained for 2 h at 37 °C. The cells only with FA solution were utilized as a blank control.

The intracellular localization of MTX in C6 cells was detected with laser scanning confocal microscope (Leica SP2, Heidelberg, Germany). Cells were cultured in chambered cover slips for 24 h and treated with free MTX or G4–FA–PEG/MTX at a MTX concentration of 10 μM. After 2 h incubation, the cells were washed with cold PBS for three times, fixed with 4% (v/v) p-formaldehyde and finally stained with Hoechst 33258.

The competition assay was similar as the above.

Statistics analysis

Data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to determine significance among groups following the Bonferroni's post-test.

Charactrization

Identification of drug

Spectroscopy

The infrared spectrum of the drug was performed that is shown in figure no.3.2a. These infrared spectra of Methotrexate will compared with standard spectra of drug. Which confirm the identity of drug that is shown in figure no.3.2 b.

Figure: 3.2a I.R. Spectra of Methotrexate (Sample)

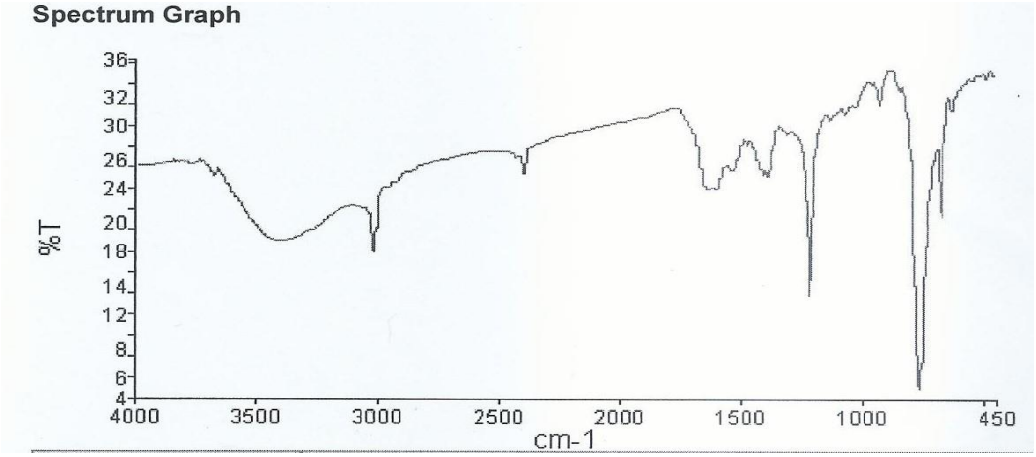
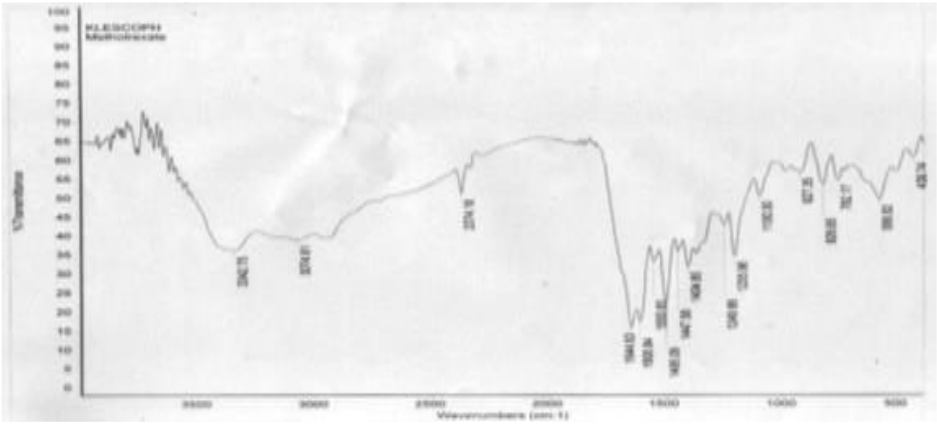
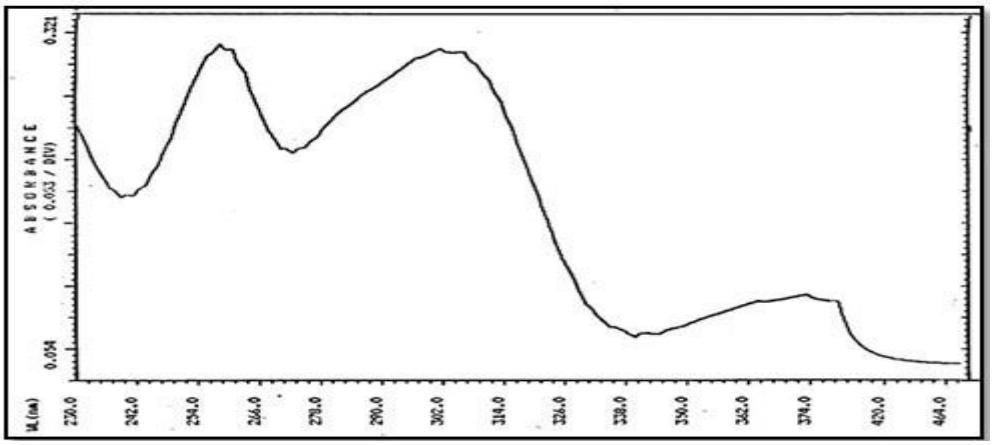


Figure: 3.2b I.R. Spectra of Methotrexate (Standard)



UV-SPECTROSCOPY



Particle size measurement

The particle size was measured with a zeta analyzer (Brookhaven Instruments Corporation, BIC) equipped with a 35 mW solid state laser (660 nm), using BIC particle sizing software (9kpsdw32, ver.2.3). All the samples including G4.0 PAMAM, G4–Biotinelated PAMAM dendrimer, G4–biotinelated–MTX and G4–Biotin–MTX were dissolved in 10 mM PBS (pH 7.4, 1 mg mL^{-1}) and filtered through a Minisart High-Flow, cellulose acetate 0.2 µm syringe filter (Sartorius Stedim Biotech GmbH, Germany) into the scattering cell. The measurement was carried out at 37 °C.

Particle Size Distribution

Size distribution was measured on Particle size analyzer (Malvern) in Central Drug Research Institute, Lucknow. The formulation of biotinylated G_4PAMAM dendrimer was filled in 15mL sample cell after initialization, of instrument, stirred and diluted to the concentration to obscuration >10 and < 20. The laser beam is directed through the sample. The light scattered by the particles moving through the leaser focus is recorded in angular region. The intensity distribution are collected by a multichannel analyzer and stored in PC.

Transmission Electron Microscopy (TEM)

TEM measurement was performed in Indian Institute of Toxicology and Research, Lucknow with the help of Tecnai G2 Twin spinet Netherland equipped with gratin odious TM CCD camera controller.

Procedure

Sample was taken and sonicate (bath sonicater) for 10 second. And we have taken the 2µl of the sample and 10 µl urinal acetate mixed properly. After five minutes one drop sample was tube and poured in TEM Gold Grid. Grid was dried in overnight (24hours) and further second coating was applied, again dried for 24 hours and examined the image under the TEM.

Drug loading

G4–biotinelated–PAMAM Dendrimer (153 mg, 5 µmol) was dissolved in 7 mL deionized water. MTX (58.1 mg, 100 µmol) in 7 mL deionized water was added dropwise to the dendrimer solution and stirred for 24 h in dark. The removal of free MTX was conducted by ultrafiltration and dialyzed twice against deionized water for 40 min. The absorbance at 482 nm from UV-vis spectra was used to calculate the percentage of MTX encapsulated.

The encapsulation of MTX was following the similar procedure except DMSO as the solvent.

Estimation of efficiency of loading of MTX BiotinylatedG_4 PAMAM dendrimers

Accurately measured quantity of 10 ml of MTX loaded solubilizate of biotinylated G_4PAMAM dendrimer vehicles (methanol) were dispersed in the HPLC mobile phase (Acetonitrile : water : 6:4), bath sonicated for 15 minutes. The sample was filtered using 0.45 µm membrane filter (Millipore), suitably diluted and analyzed by HPLC as reported.

***In vitro* MTX release**

The MTX release was carried on in PBS (pH 7.4) at 37 °C. 10.2 mg G4–MTX–PEG/MTX in 5 mL buffer solution was sealed in a dialysis bag (MWCO 8000) and immersed in 35 mL of the same buffer solution. A portion of 3 mL dialyzate was taken out at various time and replaced by 3 mL fresh buffer solution. The release of free MTX was performed in the same condition as a control. The released MTXs from the carrier were calculated with a standard curve draw by the ultraviolet spectrometry with the absorption wavelength of MTX at 302 nm.

In-vitro Release Rate Studies

A treated dialysis tube was used for in-vitro release studies. Formulation equivalent to 10 mg drug was introduced into prewashed dialysis tubing and placed in a beaker containing 200 ml freshly prepared PBS (pH 7.4). The sink condition was maintained by constantly stirring the buffer with the help of magnetic stirrer. Sample aliquots (5ml) were withdrawn periodically and replaced with equal volume of fresh PBS. Each sample was analyzed at 227 nm by U.V Spectrophotometer as reported method [18]. The data of release of MTX from biotinylated dendrimer formulation is calculated from standard curve [19].

Hemocompatibility studies

Hemocompatibility studies include haemolytic and haematological (blood count) evaluations for assessing in-vitro and in-vivo effects of administered dendrimer correspondingly on blood components. Haemolytic toxicity studies were performed following a slightly modified reported procedure [20].

Briefly, fresh whole blood from male Wister rats was collected using heparinised capillary in blood collecting vials (Himedia, India) and centrifuged at 2000 rpm for 15 min in an ultracentrifuge. RBC collected from bottoms were washed with physiological saline (0.9% w/v) until a clear colourless supernatant was obtained above the cell mass. Cells were resuspended in normal saline to obtain 2% erythrocyte concentration and this was further used for haemolytic toxicity studies. To 1.0 mL of RBC suspension 1.0 mL of physiological saline was added to serve as negative control, while replacing saline with deionised water was considered 100% haemolytic positive control. Dendrimer dispersions with and without MTX were mixed with equal volume of RBC suspension and incubated

at 37±0.2^0 C for 30 min with the gentle intermittent shaking. After incubation, haemoglobin content was measured spectrophotometrically at λ max 227 nm, of sample against control. The percent haemolysis was calculated for each sample by taking the absorbance of positive control ($Ab_{positive\ control}$) as 100% haemolytic sample, using following equation.

$$\text{Haemolysis} = \frac{\text{Absorbance sample } \lambda \text{ max228}}{\text{Absorbance positive control } \lambda \text{ max228}} \times 100$$

Haematological studies were performed in male Wister rats. Animals were divided in three treatment groups with three rats in each. MTX and formulation were administrated intravenously delivering 1.0 mg/kg of MTX to first, second and third group of animals, respectively. After lapse of 24 hour blood from each animal was collected and analyzed for RBC, WBC and differential counts in CDRI. All animal studies were performed in accordance with the guidelines of CPCSEA (Committee for the purpose of control and Supervision of Experiments on Animal, Ministry of culture, Government of India) and protocols were duly approved Institutional Animal Ethics Committee, RITM Lucknow.

Cytotoxicity assays

The cytotoxicity of free MTX and G4–biotin-/MTX was determined by sulforhodamine B (SRB) assay. C6 cells were seeded into 96-well plates at a density of 5 × 10^3 cells per well and cultured for 24 h before use. Then free MTX and G4–FA–PEG/MTX were added to the plates at a series of MTX concentrations from 0 to 10 μM. Blank G4–FA–PEG without MTX was used to test the cytotoxicity of carrier. After 48 h incubation, the cells were washed three times with PBS and treated by SRB staining assay. The absorbance at 540 nm was detected with a microplate reader and the cell viability was calculated using the following formula: survival% = ($A_{540\ nm}$ for the treated cells/$A_{540\ nm}$ for the control cells) × 100%, where the $A_{540\ nm}$ was the absorbance value. Each assay was repeated for at least three times. IC_{50} values were calculated from the dose–effect curve and expressed as concentration (μM) of MTX-equiv.[32]

The measurement of cytotoxicity of free MTX and G4–FA–PEG/MTX was following the same procedure as above.

Stability studies

Different formulation (5ml) and drug was kept in umber coloured vials. Properly sealed vials containing biotinylated G_4PAMAM dendrimer formulation were kept for stability studies over a month at room temperature and 40^{0}C. All vials were visually observed, and analyzed for drug content at the time intervals of 0, 1, 7, 15 and 30 days. The initial and final pH values were also measured [21].

In-vivo evaluation

The in-vivo performance of a drug delivery system is perhaps the most important criteria in its development as a clinically acceptable dosage form. In-vivo studies are carried out on a system promising in vitro performance on laboratory animals such as rats, mice, hamsters and monkeys etc. For a specific site drug delivery system in-vivo studies are conducted to ascertain the ability of the system in achieving compartmentalization of the drug in the targeted tissue or non targeted tissues. The blood levels as well as the urinary excretion of the drug may be monitored [22] have listed

various parameters which help in precise preclinical in-vivo evaluation of a target oriented drug delivery system.

Results

Synthesis of Biotinylated G_4PAMAM dendrimers

Biotinylated G_4PAMAM dendrimer was resulted in a high yield of fluffy white fibrous solid of melting point and yield 232^0C, 13.4 mg respectively).

Characterization of Biotinylated G_4PAMAM dendrimers

Product sample were scanned U.V data shows highest peak of λ_{max} 227nm.(Fig. 1)

Nuclear magnetic resonance spectroscopy

^{1}H NMR data revealed the presence of biotin ring juncture protons which were absent from the parent PAMAM dendrimer, however, the other characteristic peaks of the dendrimer (2.6-3.3 ppm) were observed in biotinylated G_4PAMAM dendrimer.(Fig..)

Mass Spectroscopy

The extent of biotinylation was quantified using mass spectroscopy. For PAMAMG_4 mass depicted 14 biotin molecules attached.(Fig.)

Infra red spectroscopy

The IR spectra of biotinylated G_4PAMAM dendrimer are shown in Fig.4. The interpretation confirms the formation of biotinylated G_4PAMAM dendrimer as peaks of all functional groups obtained in IR.

TABLE.1: Infra red spectroscopy

S.N.	Frequency of pure drug (in cm^{-1})	IR Assignments	Intensity
1	3681.17	Primary amine N-H stretching	25.27
2	3409.41	Secondary amine N-H stretching	18.93
3	3019.64	Stretching of sp2 C-H alkenes	17.97
4	1602.38	C=O ring stretch	23.9
5	1536.78	C-C stretch aromatic	25.77
6	1385.3	C=C stretch aromatic	25.24
7	1215.51	Medium sharp C-O stretching	13.83
8	757.13	C-H deformation (aromatic)	4.8
9	669.04	Out of plane bending =C-H	21.41

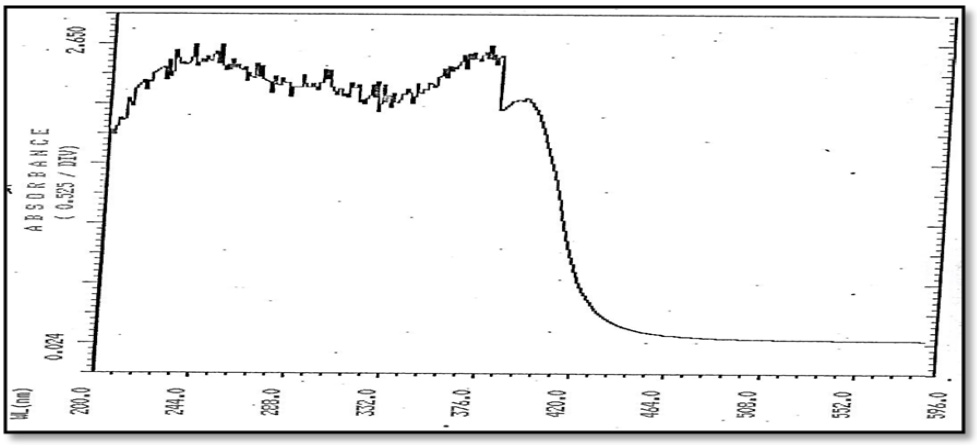


FIGURE:1. Infra red spectroscopy

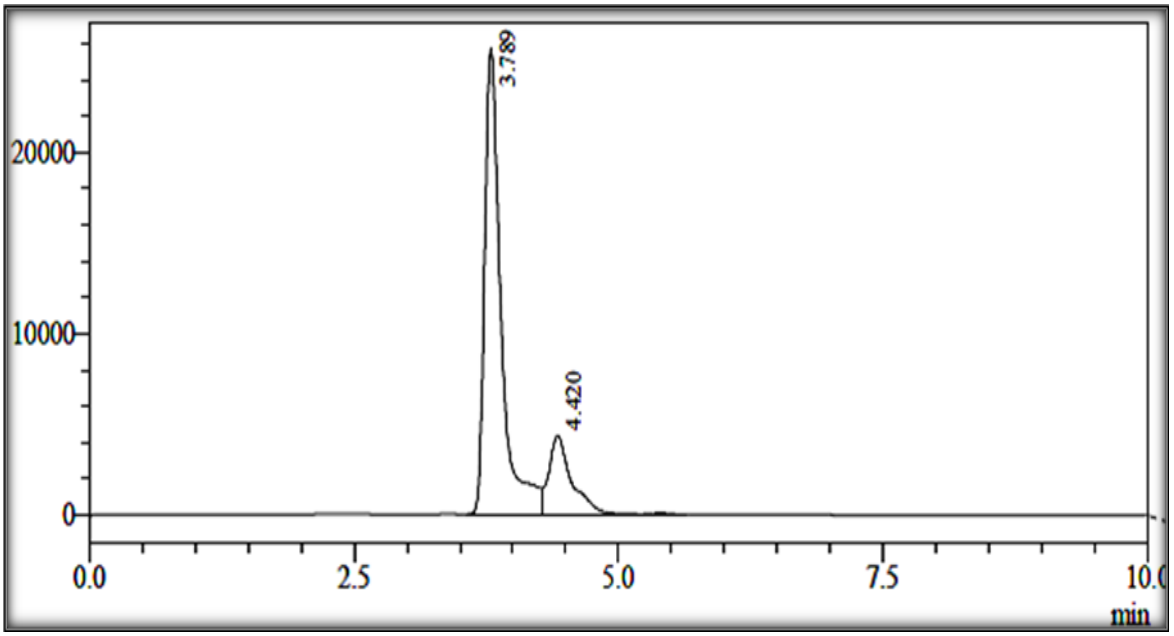


FIGURE:2. Infra red spectroscopy

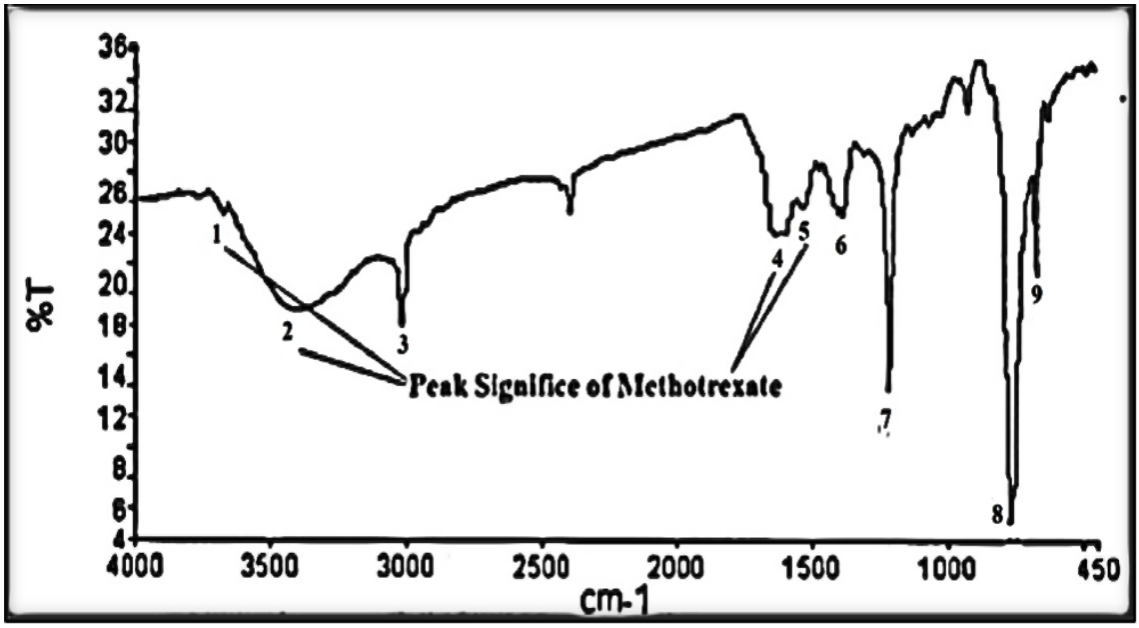


FIGURE:3. Infra red spectroscopy

3.6 Transmission Electron Microscopy (TEM)

The mean size of biotinylated G_4PAMAM dendrimer formulation was in the range of 22-30nm and was fairly uniform. The size of MTX containing formulation ranged from 22-35nm. The result are shown on the Fig.5

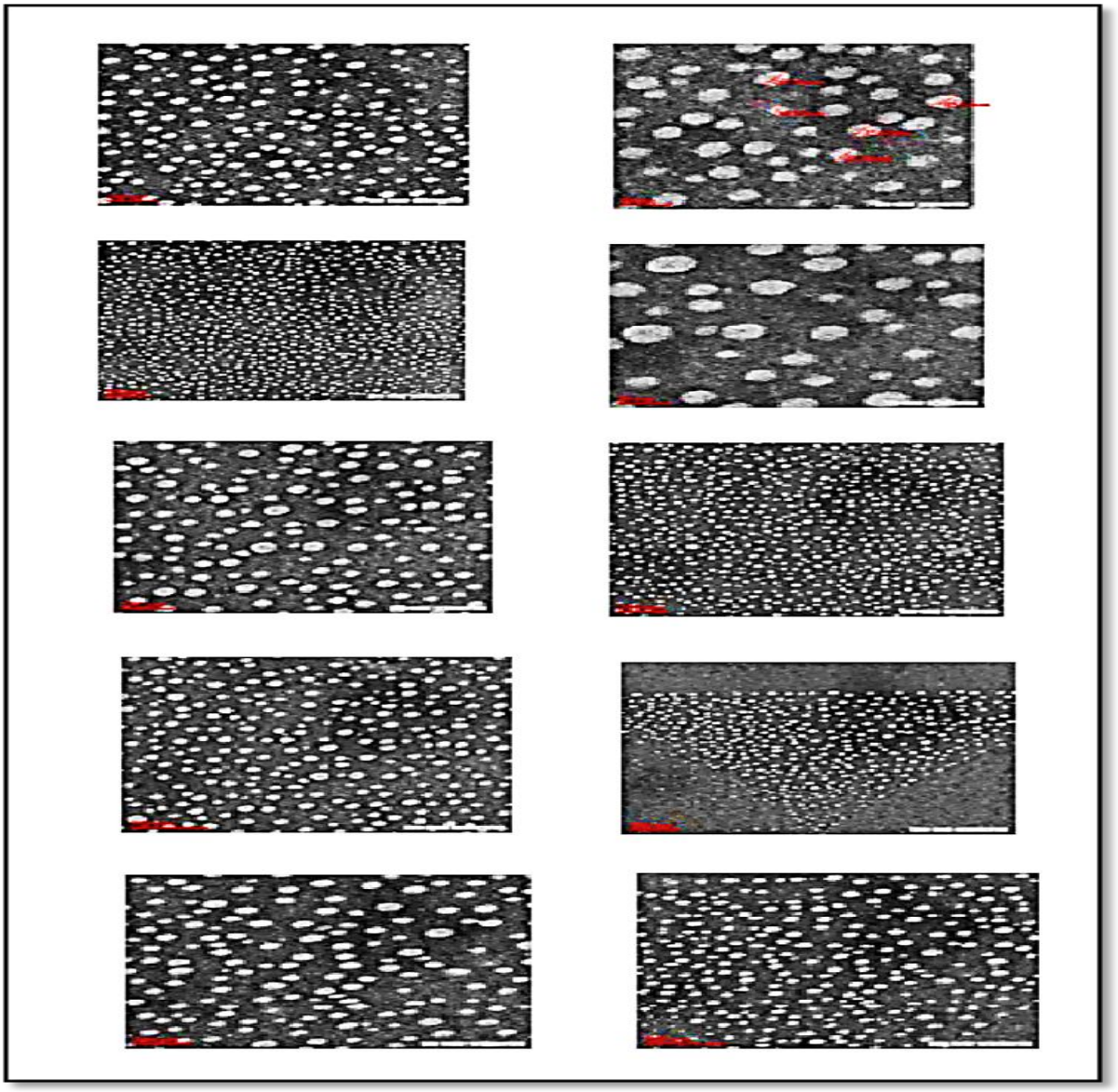


FIGURE:4. Transmission Electron Microscopy (TEM)

Size Distribution

The mean size of biotinylated G_4PAMAM dendrimer formulation was in the range of 0.2µm size. The particle sizes reported here were determined instrumentally using a Malvern's Mastersizer. The image is shown in **Fig.** No.6

S.N.	SYSTEM	SOLUBILITY AT DIFFERENT pH		
		pH-5	pH-7	pH-9
1.	**A-NH2**	0.02	0.09	0.16
		0.05	0.19	0.29
		0.07	0.38	0.46

Drug-loading efficiency of biotinylated dendrimers

Estimation of MTX loading efficiency of biotinylated G_4PAMAM dendrimer by HPLC revealed that the percentage of MTX encapsulated as calculated content 21.10%.

Release Rate

The release of MTX was about 12.5±0.17% in 24 hours. Release pattern is shown in the Table No.1 and Fig. 7.

Hemocompatibility studies

The Hemocompatibility studies shows reduction in hemotoxicity with biotinylated dendrimers.

4.0 Stability study

Biotinylated G_4PAMAM dendrimer formulation was stable in injection form. Degradation was less than 2% at room temperature and about 4%^{0}C in formulation from 30 days. Assay was more than 97.2% and 98% for formulation. The result are shown on the Table No. and Fig. .

Toxicity of dendrimer conjugates.

All mice were observed for the duration of the studies for signs of dehydration, inability to eat or drink, weakness, or change in activity level. No gross toxicity, either acutely or chronically up to 99 days, was observed regardless of whether the dendrimer conjugate contained MTX. The weight was monitored throughout the experiment and no loss of weight was observed; in fact, the animals gained weight. At each time point, a gross examination and histopathology of the liver, spleen, kidney, lung, and heart were done. No morphologic abnormalities were observed on the histopathology examination. No in vivo toxicity was noted in any animal group following the dendrimer injection.

Results and Discussion

Synthesis and characterization of G4–FA–PEG

To reduce the side effect of therapeutic agents, their targeting efficiency should be improved. One effective strategy is the modification of ligands onto the surface of the carrier. Here, we select FA as the functional ligand because of its comprehensive advantages of low immunogenicity, functional stability, over expression of receptors on cancer cells, and the nondestructive cellular internalization pathway. Furthermore, while multiple ligands are simultaneously bound on one carrier, the multivalent interactions with the receptors are much stronger than the corresponding monovalent interaction, which is called “cluster” effect.

In present study, FA was conjugated on the periphery of the fourth generation PAMAM through a carbodiimide mediated amide linkage by the reaction of exterior primary amino groups of PAMAM with the γ-carboxyl group of FA (Fig. 1). ^{1}H NMR measurement was used to analyze and calculate the number of FA conjugated on the periphery of PAMAM (Fig. 2a). From the integration ratio of the proton signals at 7.67 ppm (phenyl protons of FA) to those at 2.49 ppm corresponding to the protons of methylenes next to the carbonyl groups in PAMAM branches, the average number of FA attached on PAMAM exterior was 5.9 per PAMAM molecule. Similarly, the number of PEG conjugated on the periphery of PAMAM was evaluated to be 6.9 per PAMAM.

Conclusion

The hydrophobicity of the anticancer drug (MTX) creates major problem during the product development and presents a major hindrance in achievement of satisfactory bioavailability. Hence, solubility enhancement of these hydrophobic drugs (MTX) has always been a challenge to the Scientists. The use of PAMAM dendrimer as solubilizing agent has attracted the attention of many scientists due to its characteristic properties. Range of PAMAM dendrimer in their original or modified form has been tried successfully for enhancing solubility of hydrophobes. Studies comparing potential of PAMAM dendrimer in solubility enhancement to improve the delivery of

hydrophobic drugs. The toxicity of the amine-terminated dendrimers limits the clinical applications, yet due to its multifunctional nature. Studies are reported which show that masking the terminal amine groups by some means not only considerably improved the efficiency of PAMAM dendrimers in solubility enhancement but also made them more biocompatible. These studies suggest that pH of the medium, temperature, and solvents are the factors that influence the efficiency of dendrimers as solubilizing agent. The hydrophobic interactions are the possible mechanisms by which PAMAM dendrimers apply their solubilizing effect. The insufficiency of studies investigating the effect of temperature on dendrimer-mediated solubility enhancement is yet another aspect which needs increased concentration. As pharmaceutical products are manufactured and stored subject to various temperature conditions and they are exposed to 37 °C in the body, the necessity of relevant study design exploring these effects intensifies. The role of PAMAM dendrimer in solubility enhancement can become meaningful only if it results in subsequent enhancement in drug bioavailability. A detailed experimentation correlating *in vitro* and *in vivo* performance of dendrimers can yield substantial information, which could be useful in their development as drug delivery. Finally, it can be concluded that PAMAM dendrimer is highly effective for solubility enhancement to improve the drug delivery.

References

1. Svenson, S. Eur. J. Pharm. Biopharm. 2009,71, 445.
2. Palsson, L. O.; Tsiourvas, D.; Sideratou, Z.; Tziveleka, L. A. Curr. Top.Med. Chem. 2008,8, 1204.
3. Navath, R. S.; Kurtoglu, Y. E.; Wang, B.; Kannan, S.; Romero, R.; Kannan, R. M. Bioconjugate Chem, 2008,19, 2446.
4. Wang, C.; Wyn-Jones, E.; Sidhu, J.; Tam, K. C. Langmuir, 2007,23, 1635.
5. Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. J. ControlledRelease, 2000,65, 133.
6. Perumal, O. P.; Inapagolla, R.; Kannan, S.; Kannan, R. M. Biomaterials, 2008,29, 3469.
7. Chen, W.; Tomalia, D. A.; Thomas, J. L.Macromolecules,2000,33, 9169.
8. Milhem, O. M.; Myles, C.; McKeown, N. B.; Attwood, D'E. Int. J. Pharm. 2000, 197, 239-241.
9. Cheng YY and Xu TW.Eur J Med Chem2005; 40:1384–1389.
10. Amidon, G. L.; Lennernas, H.; Shah, V. P.; Crison, J. R. Pharm. Res. 1995, 12, 413.
11. Rawlinson, C. F.; Williams, A. C.; Timmins, P.; Grimsey, I., *International Journal of*Pharmaceutics.2007; 336, 42.
12. Garzo´n, L. C.; Martı´nez, F.J. Solution Chem. 2004, 33, 1379.
13. Chan, K. L. A.; Kazarian, S. G. Journal of Combinatorial*Chemistry*,2006,8, 26.
14. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. AdV. Drug DeliVeryReV, 46, 3-23.
15. Kubinyi, H. 1995, Pharmazie 2001, 50, 647-662.
16. Rangel-Yagui, C. O.; Pessoa, A., Jr.; Tavares, L. C.J. Pharm. Pharmaceut. Sci., 2005,8 (2), 147-163.
17. Brewster, M. E.; Esters, K. S.; Bodor, N. ,*International Journal of*Pharmaceutics. 1990, 59, 231-240.
18. Brewster, M. E.; Esters, K. S.; Loftsson, T.; Perchalski, R.; Derendorf, H.; Mullersman, G.; Bodor, N, Journal of*Pharmaceutical* Science. 1988,77, 981- 985.
19. Samaha, M. W.; Naggar, V. F. Int. J. Pharm. 1988, 421-429.
20. Barry, B. W.; El Eini, D. I. D. J. Pharm. Pharmacol. 1976, 28, 210- 218.

21. Devarakonda, B.; Hill, R. A.; De Villiers, M. M. Int. J. Pharm. 2004,284, 133-140.
22. Mahajeri et al; Quantitative LC Analysis of Methotrexate and its Application in Pharmacokinetic study of drug in Rat serum sample, Pharmaclogyonline 2:2011, 597-604.