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In vitro cytotoxicity, *in vivo* pharmacokinetic studies and tissue distribution studies of multifunctional citric acid dendrimers using the drug Cytarabine

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Abstract

Dendrimers are considered the emerging polymeric architectures, known for their well defined molecular-weight, polydispersity, uniformity and high-surface functionality. These nano-architectures are capable of encapsulating low-high molecular-weight drug moieties in their interior or exterior through covalent bonding and host-guest interactions. Further, large surface volume made researchers to implicate dendrimers in biomedical and therapeutic applications. Regardless of the massive applications, sometimes its use is limited because of the cytotoxicity produced. Considering this, the present research is focused on the synthesis and PEGylation of citric acid dendrimers. PEGylation is an act of conjugating polyethylene glycol to dendrimers that completely eliminates the toxicity issues associated with dendrimers and render them biocompatible. Cytarabine was loaded in the dendritic architecture to target specifically the tumor cells. Dendrimers are made tumor specific by incorporating certain agents that get cleaved in tumor environment. Synthesized dendrimers were studied for its effect on acute cytotoxicity, tissue-distributions and pharmacokinetic parameters.

Keywords: Citric acid, Cytarabine, Dendrimers, Leukemia, PEGylation, Tumor.

Introduction

Dendrimers represent a novel type of polymeric material, characterized by its repetitive branching, definite molecular weight, shape, size and uniform distribution. Its degree of control over molecular weight and shape gained interest of many researchers for encapsulation and drug delivery properties [1]. The nanosize of dendrimers facilitates the delivery of drug molecules to the target site, tumor cells through enhanced permeation and retention effect [2]. Drug molecules get conjugated to the dendritic system *via* physical attachment to the dendritic core or by covalent conjugation to the dendritic surface. Molecular moieties can be loaded both to the interior and exterior surface of the dendrimers [3]. A number of linker technologies have been investigated till date, but only dendrimers provide tumor specific drug release *via* incorporating acid labile hydrazones, esters and peptides that get cleaved by the enzymes present in the tumor environment [4]. When compared to other nano carriers, relatively small size of dendrimers enables them to get cleared off from the body *via* kidneys and escape phagocytosis by RES uptake [5]. Considering the use of dendrimers as carriers, it is essential that they should be bio-compatible and non-toxic [6].

Among different dendrimers available today, citric acid dendrimers are more biocompatible, water soluble and relatively less toxic [7]. Presence of multiple functional end groups at its terminal surface enables citric acid dendrimers to entrap a variety of molecules such as imaging agents, therapeutic agents, contrasting agents, targeting moieties etc [8]. Usually, biomolecules either get entrapped into the interior or get complexed to the exterior of dendrimers [9]. Drug-dendrimer interaction and water solubility are the two factors governing drug retention and its successive release from the host, dendrimers [10]. Being water-soluble, citric acid dendrimers prolong the half-life of encapsulated drugs. They take advantage of EPR effect which is commonly observed with tumor cells. Blood vessels of tumors are poorly aligned and possess proliferations in its structure [11]. Owing to this property of tumor cells, nano sized dendrimers easily pass into them without disturbing other healthy cells. Because of poor lymphatic drainage, dendrimers accumulate in tumor cells for a longer time to release the encapsulated drug into them, which simultaneously exhibit therapeutic activity and stand responsible for its destruction [12].

Cytarabine, an antineoplastic agent is found effective against cancers like acute myeloid leukemia, melanoma etc. In the present study, an attempt has been made to formulate PEGylated citric acid dendrimers and characterize them for different parameters like MTT assay, tissue distribution studies and Pharmacokinetic studies.

Materials and methods

Materials

Cytarabine was procured from Yarrow Chemicals, Mumbai; potassium permanganate, sodium carbonate, thionyl chloride, dimethyl form amide, pyridine, diethyl ether and N, N'- dicyclohexyl carbodiimide were purchased from Sd Fine Chemicals Ltd.,

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Mumbai; polyethylene glycol 400 and chloroform were purchased from Finar Chemicals Pvt. Ltd. Ahmadabad. All the chemicals used were of good analytical grade. *

Animals

Balb/c mice were purchased from National Institute of Nutrition (NIN), Hyderabad for their use in tissue distribution and cytotoxic studies. All the animals were inbred for experimental purposes in accordance with CPCSEA guidelines. Animals were housed in individually ventilaged cage system with a 12hr light/dark cycle. Noise, temperature and humidity were controlled as per the guidelines. They were supplied with standard rodent chow, water and bedding material before surgery.

Cell lines

B16F10 melanoma and K562 myeloid cell lines were procured from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS.

Method of Preparation

Citric acid dendrimers were prepared using Divergent technique and the steps involved in its synthesis are:
Synthesis of 5 generations of citric acid dendrimers
PEGylation of synthesized dendrimers
Drug loading of the PEGylated dendrimers

Synthesis of 5 generations of Citric acid dendrimers

Dendrimers synthesis can be conveniently studied in 3 steps
Step 1: 35.5gms of Potassium permanganate was dissolved in 694ml of water in a beaker, simultaneously; 3.75gms of Sodium carbonate was dissolved in 38ml of water in another beaker. The two solutions were mixed together with continuous stirring and 110ml of Polyethylene glycol was added and stirred on a magnetic stirrer for 3-4hrs to give out a homogenous mixture. The resultant mixture was filtered and the collected filtrate was heated on a mantle at 60-70 C with random cooling in between. This heating and cooling process was continued till a 100ml concentrated liquid retains. Resultant concentrate was covered with a layer of ether. Aqueous layer was separated out using a separating funnel and heated on a mantle at 70 C for the complete removal of ether. A white precipitate of Polyethylene glycol di acid formed is collected and is referred to as a 1st generation dendrimer.
Step 2: To synthesize a second generation dendrimer, dendrimer of 1st generation was made to react with Thionyl chloride in a round-bottomed flask provided with a condenser. This assembly was set on a magnetic stirrer for an hour and heated on a mantle at 70 C to yield chlorinated polyethylene glycol, a G1.5 dendrimer. Obtained dendrimer was dissolved in 20ml of Dimethyl Formamide and refrigerated for 24hrs on an ice-bath. On the other side, required amount of citric acid was dissolved in a beaker containing 10ml of DMF, followed by the addition of 110ml of pyridine. Both the reaction mixtures were placed on an ice-bath in a refrigerator overnight. After 24hrs, the solutions were taken out, mixed with each other and incubated for 6hrs at 55-60 C in an incubator to yield a second generation dendrimer. The resultant dendrimer was further purified by Column Chromatography technique [13].
2nd generation dendrimer was repeated with step 2 with increasing quantities of citric acid and thionyl chloride to produce a 4 generation dendrimer as shown in table-1.

Table-1: Formulation of Dendrimers

Formulation	$KMNO_4$	Na_2co_3	PEG400	Thionyl chloride	Citric acid	Pyridine	DMF	Drug
F1(G1)	35.5gm	3.75gm	110ml	-	-	-	-	-
F2(G2)	35.5gm	3.75gm	110ml	6ml	0.1g	100ml	30ml	100mg
F3(G3)	35.5gm	3.75gm	110ml	8ml	0.2gm	100ml	30ml	100mg
F4(G4)	35.5gm	3.75gm	110ml	10ml	0.4gm	100ml	30ml	100mg

PEGylation of dendrimers

Conjugation of polyethylene glycol to the dendrimers is called PEGylation. It is considered an important step in building of dendrimers as they impart stability to the drug incorporated within them with reduced side-effects and drug leakage from the carrier system [14].
For PEGylation, 1 mole of above synthesized dendrimers was dissolved in 10ml of DMF completely in a conical flask. N, N' Cyclohexyl carbodimide was added and the mixture was set undisturbed for 4 days at 40ºc. To this reaction mixture, 85ml and 150ml of polyethylene glycol and chloroform were added respectively to yield a clear solution and refluxed for 3-4 days. The resultant was filtered through a separating funnel by adding 5ml of water that completely removes the unreacted N, N'- dicyclo hexyl carbodimide [15].

Drug loading in the PEGylated dendrimers

Cytarabine was dissolved in distilled water, added to PEGylated dendritic solution and stirred on a magnetic stirrer for 24hrs for complete encapsulation of drug into dendrimers [13].

Characterization of synthesized PEGyalted dendrimers

Prepared dendrimers were characterized for the following parameters:

MTT cytotoxicity assay

MTT Assay was performed to determine the cytotoxicity of synthesized dendrimers. For this B16F10 melanoma cells and K562 myeloid cells were selected and seeded in 96-well plates at a density of $4.5x10^3$cells/well separately. On the other side, dendrimers were dissolved in some suitable solvent like DMSO and added to the well plates. Final concentration of dendrimers was adjusted to 0.001 to 10 µg/ml concentration range and incubated at 37^0c for 96hrs and the media was discarded. Each well was supplied with 100µl of MTT solution and incubated for 4hrs. Cells proliferate to produce blue colored formazon crystals, dissolved in DMSO under safety cabinet and placed in an orbital shaker for 10 minutes at room temperature. Finally absorbance of the above was recorded at 550nm by a micro plate reader [16].

Tissue distribution studies

Tissue distribution studies aim at studying the amount of drug being distributed to various body organs. Healthy balb/c mice were selected and B16F10 cell lines were injected subcutaneously. Later, the animals were sacrificed and the developed tumors were propagated in sufficient number of experimental animals.
Animals with $100mm^3$ tumor were selected, starved overnight and administered with 10mg/ml of synthesized drug-dendrimer complex through tail vein. Such animals were sacrificed at different time intervals like 10, 30, 60, 120 and 180 minutes after injection. Organs like spleen, liver, kidney and lungs along with tumor were isolated, washed with distilled water, weighed, and homogenized to 1 g/ml of tissue homogenate in PBS. Thus obtained homogenates were centrifuged at 1000rpm and the supernatants were analyzed using HPLC [17].

Pharmacokinetic studies

Pharmacokinetic studies were performed to know the drug's bioavailability and parameters such as T max, C max etc. B16F10 mice were chosen as model animals for pharmacokinetic studies. Animals under study were starved overnight. Control group was provided with saline and test was fed with dendrimer drug formulations orally. Throughout the course of experiment, water was supplied both for the control and test groups. Blood samples were collected at fixed time intervals like 0, 15, 30, 60, 120 and 180 minutes respectively and centrifuged for 10 minutes at 20000rpm. Supernatant plasma layer was separated and analyzed under HPLC. Data obtained was represented in terms of graphs against time (Hemmer, et al., 2013).

Results and discussion

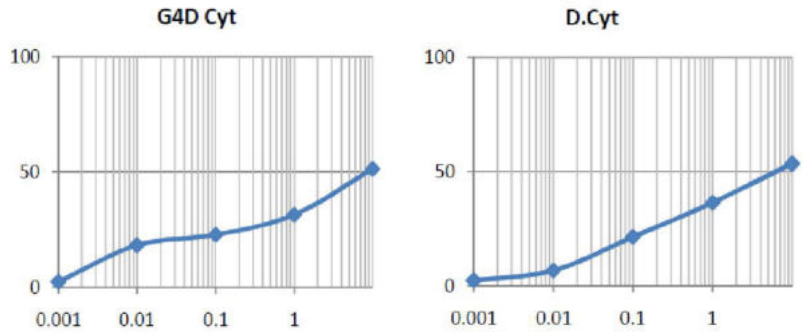
MTT assay

MTT assay was performed against B16F10 and K562 cell lines to determine the acute cytotoxicity. As per the results, drug, cytarabine concentration in drug-dendrimer complex was adjusted in the range 0.001 to 10µg/ml. At 0.001µg/ml, inhibition of cellular proliferation was found to be 2.47% and at 10 µg/ml, 51.39% (from figures 1 and 2).From the results, it is estimated that the cytotoxicity of formulated dendrimers is a result of interaction between dendrimers and cell surfaces. And as dendrimer generation increases, cytotoxic effect of dendrimers also increases significantly. A fifth/fourth generation dendrimer exhibits stronger cytotoxicity as compared to lower generations. As bulk of dendrimer increases, capability of a dendrimer to interact with its surrounding cells also increases gradually
.

Table-2: Tabular representation of MTT assay results against B16F10 cells lines induced balb/c albino mice

Concentration	DCYT	G4D.CYT	G5D
10	53.44	51.39	10.58
1	36.48	31.47	11.64
0.1	21.48	22.85	2.39
0.01	6.76	18.19	1.28
0.001	2.44	2.47	2.17
IC50value µg/ml	10	10	**>100**

DCYT= Drug cytarabine; G4D.CYT= 4th generation drug loaded dendrimer; G5D= 5th generation plain dendrimer.

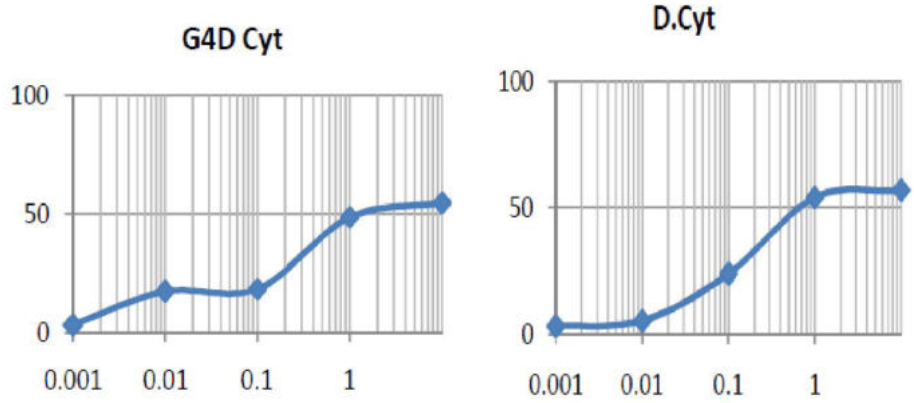


X Axis: Concentration ug/ml
Y Axis: Percentage Inhibition

Figure-1: Graphical representation of MTT assay results for the drug Cytarabine for B16F10 cell lines induced balb/c albino mice

Table-3: Tabular representation of MTT assay results against K562 cell lines induced albino balb/c mice

Concentration	DCYT	G4D.CYT	G5D
10	56.98	54.63	8.85
1	54.21	48.71	7.44
0.1	23.98	18.52	5.21
0.01	5.47	17.44	6.47
0.001	3.28	3.25	3.87
IC50value µg/ml	0.6	1	**>100**



X Axis: Concentration ug/ml
Y Axis: Percentage Inhibition

Figure-2: Graphical representation of MTT assay results for drug Cytarabine for K562 cell lines induced balb/c albino mice

Tissue distribution studies

Balb/c mice were used for studying tissue distributions of cytarabine. Soon after administering the drug, animals were sacrificed to study drug distribution pattern to various body organs. Tissue distributions of the drug, cytarabine by different organs is presented in figure 3 and as per the results of the study, tumor uptake of cytarabine increases significantly with time and was maximum at 180th minute i.e., 12.8µg/ml. The suggested mechanism for the uptake of cytarabine was supposed to be EPR effect. Enhanced permeability and retention due to poorly aligned blood vessels and poor lymphatic drainage respectively enables the drug cytarabine to interact with the tumor cells and exhibit its action.

Table-4: Tabular representation of tissue distribution study data in Tumor (µg/ml)

Distribution time (min)	10	30	60	120	180
DCYT	5.33	6.09	10.3	12.47	13.21
G4D.CYT	7.10	8.90	13.36	12.59	12.8
G5D	0	0	0	0	0

Table-5: Tabular representation of tissue distribution study data in Lungs (µg/ml)

Distribution time (min)	10	30	60	120	180
DCYT	5.33	4.14	7.75	12.90	12.47
G4D.CYT	6.85	9.95	12.17	12.50	13.58
G5D	0	0	0	0	0

Table-6: Tabular representation of tissue distribution study data in Liver (µg/ml)

Distribution time (min)	10	30	60	120	180
DCYT	4.02	5.23	10.06	11.7	12.50
G4D.CYT	4.93	8.84	14.3	12.48	14.4
G5D	0	0	0	0	0

Table-7: Tabular representation of tissue distribution study data in Kidney (µg/ml)

Distribution time (min)	10	30	60	120	180
DCYT	5.27	6.13	9.75	12.48	12.50
G4D.CYT	7.07	10.06	13.53	13.20	12.92
G5D	0	0	0	0	0

Table-8: Tabular representation of tissue distribution study data in Spleen (µg/ml)

Distribution time (min)	10	30	60	120	180
DCYT	4.65	5.37	9.95	13.36	13.7
G4D.CYT	5.15	8.52	13.20	13.72	13.12
G5D	0	0	0	0	0

Table-9: Tabular representation of tissue distribution study data for plasma

Distribution time (min)	10	30	60	120	180
DCYT	30.61	30.58	10.42	6.00	4.89
G4D.CYT	28.52	24.3	10.20	7.34	6.11
G5D	0	0	0	0	0

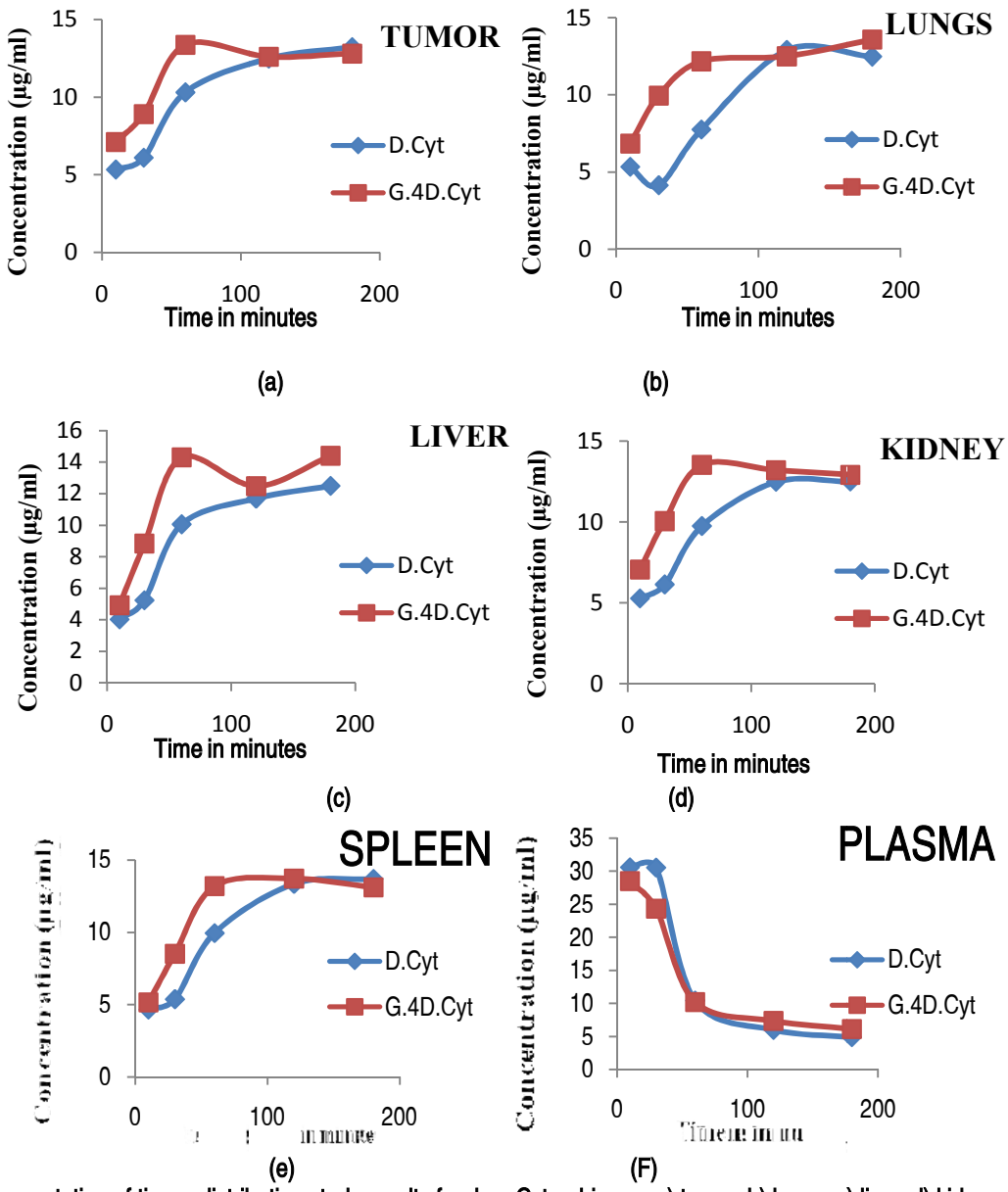


Figure-3: Graphical representation of tissue distribution study results for drug Cytarabine on a) tumor, b) lungs, c) liver, d) kidney, e) spleen, and f) plasma

Pharmacokinetic studies

Pharmacokinetic studies aim at quantifying the amount of drug released from the formulations after the IV administration of drug-dendrimer complex. From the results, C max of the drug-dendrimer complex and drug are 28.51 and 30.61 µg/ml respectively and figure 4 below shows the mean plasma concentration – time curve of cytarabine after its IV administration. AUC at the end of 30 minutes is 24.33 µg/ml.

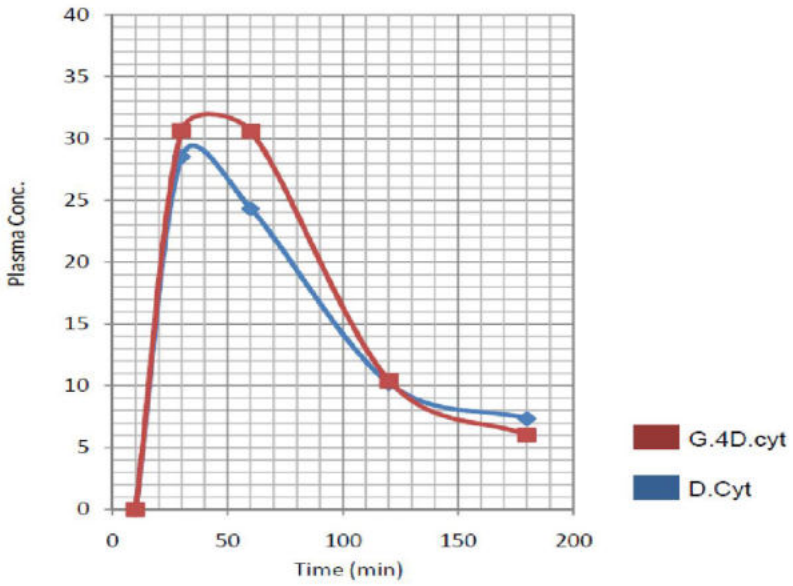


Figure-4: Mean plasma concentration – time curve of cytarabine

Conclusion

Dendrimers are an effective means for delivering a drug especially an antitumor drug to the tumor cells. In the present work, an attempt has been made to formulate PEGylated dendrimers. The synthesized dendrimers were characterized for MTT assay, tissue distributions and pharmacokinetic studies. Results from various studies proved that dendrimers are safe and strong enough for delivering the drug at the target site. During the study, it was found that dendritic generations play a major role. Higher generations have a greater impact on study parameters over lower generations.

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