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RESEARCH PAPER

Generation Dependent Safety and Efficacy of Folic Acid Conjugated Dendrimer Based Anticancer Drug Formulations

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ABSTRACT

Purpose Folate conjugated poly(propyleneimine) (PPI) dendrimer (FPPI) mediated anticancer therapy is being extensively discovered throughout the world. The present investigation was aimed at exploring the targeting potential of Melphalan loaded FPPI of different generations (MP-FPPI) for effective management of cancer.

Methods The MP-FPPI formulations were compared for drug entrapment efficiency, *in vitro* release profile, toxicology, folate receptor blockage assay, cell uptake assay, stability studies, and *in vivo* studies.

Results Upon increasing the dendrimer generation from fourth to fifth, the drug delivery parameters improved negligibly except the toxicological profile that improved exponentially. MTT assay in case of MCF-7 cells depicted the IC_{50} values of 8 ± 0.15, 0.9 ± 0.02, 0.2 ± 0.01 and 10 ± 0.17 μM, respectively in case of MP-FPPI3, MP-FPPI4, MP-FPPI5, and free Melphalan suggesting folate based targeting to be the efficacious approach to kill cancer cells. The median survival time for tumor bearing mice treated with MP-FPPI3, MP-FPPI4, MP-FPPI5 and free drug was found to be 23, 59, 62 and 26 days, respectively.

Conclusions The study concludes fourth generation PPI dendrimer to be superior carrier for folate based tumor targeting compared to third and fifth generation based formulations. This work is expected to provide a significant clue in the selection of "dendrimer generation" for folate mediated cancer targeting therapy.

KEY WORDS dendrimer generation · folate conjugation · toxicity · tumor targeting

INTRODUCTION

For over past one decade, our laboratory is continually involved in investigating the potential of dendrimer for targeting gene, proteins, peptides and bioactives (1,2). Moreover, the versatility of dendrimer has attracted researchers throughout the globe as is evinced from rising number of publications and patents in this field (3,4). Wide array of literatures are available that infers that drug loading efficacy as well as drug solubilization potential of dendrimer increases with increase in dendrimer generation. Higher generation dendrimers were observed to be eliciting high core-loading of drug and, consequently, slow and sustained drug release (5,6). At the same time with rise in dendrimer generation, there occurs a gradual rise in the number of peripheral amine groups that are accountable for its toxicity (7,8).

Jain and coworkers have examined and reported the influence of dendrimer generation on biocompatibility of dendrimer, wherein it was observed that dendrimers size is a

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predominant constraint related to its cytotoxicity (9,10). They recently examined the effect of necked dendrimer generation on drug delivery aptitude, and observed very high toxicities as evident by hemolysis, cytotoxicity and flow cytometry assays. This toxicity profile required their surface modification to neutralize their cationic charges. Conjugation with targeting ligands, especially folic acid, is widely explored concept (11–13). To best of our knowledge no efforts have been dedicated to investigating the drug targeting propensity of various generations of folate anchored PPI dendrimer, particularly in terms of drug delivery benefits and toxicity compromise. In this investigation single platform assessment of targeting potential of Melphalan loaded folate anchored PPI dendrimer of different generations (PPI3, PPI4 and PPI5) is proposed. This is a debut study exploring the effect of generation on the targeting potential of folate-conjugated PPI dendrimer and the outcome is expected to shed valuable information in selection of dendrimer generation.

In this research, we used folic acid as a model targeting ligand due to its promising acceptability in cancer targeting approach. The folate conjugation is also expected to enhance biocompatibility of cationic dendrimer due to locking of peripheral cationic groups. Another reason for use of folate based formulations is that, it provides an additional comparison of the effect of generation (which is our key object) on plain as well as folate based formulations.

MATERIALS & METHODS

Materials

Ethylenediamine and acrylonitrile (ACN) were purchased from CDH (India). Raney Nickel was purchased from Fluka (USA). Folic acid, EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride), DMSO (dimethylsulfoxide) and DCM (dichloromethane) were purchased from HiMedia (India). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma (Germany). Trypsin, tert-butoxycarbonyl (t-Boc), EDTA, and FITC (fluorocein isothiocynate) were purchased from Sigma Aldrich Pvt. Ltd. (St. Louis, Missouri, USA) All other chemicals were of reagent grade and used without further modification.

Synthesis of Folate-Conjugated PPI Dendrimer of Different Generations

EDA cored PPI dendrimers of different generations (PPI3, PPI4 and PPI5) were synthesized following the divergent approach as reported earlier (1,5,11). Briefly, t-Boc-protected folic acid was synthesized by the method reported earlier by us (1,5,11). Folic acid (10 mM) was dissolved in a mixture of DMSO: DCM (1:1 *v/v*); t-BOC (12 mM) was added to it and the resulting mixture was stirred continuously under dark for 2 days. This is followed by activation by adding EDC (5 mM) and stirred continuously for 4 h in dark (Fig. 1a).

Weighed quantities of PPI dendrimers (0.05 mM) of required generations (PPI3/PPI4/PPI5) were dissolved in 10 ml of DCM in different containers. The t-Boc-protected folic acid solution was added drop-wise to the container containing dendrimer solution (500 rpm; 37 ± 2°C). The reaction vessels were stirred continually in dark for 2 days, the content of reaction vessel was concentrated, piperidine (30% solution in DMF; two to three drops) was added, and the mixture was shaken for 3 min (Fig. 1b). Finally, the resultant product was collected by concentration under reduced pressure (washed twice with DMF) and the resultant product was characterized (UV, TLC, FT-IR, ^{1}H-NMR, SEC) for confirmation of synthesis.

Drug Loading in Formulations

Melphalan was loaded into folate conjugated PPI dendrimers (FPPI3/FPPI4/FPPI5) by equilibrium dialysis method as reported previously (1,5,11). Developed formulations were lyophilized [cryoprotectant (2% lactose)] and labeled as Melphalan loaded folate conjugated 3.0G PPI dendrimers (MP-FPPI3), Melphalan loaded folate conjugated 4.0G PPI dendrimers (MP-FPPI4) and Melphalan loaded folate conjugated 5.0G PPI dendrimers (MP-FPPI5).

In Vitro Drug Release Studies

The *in vitro* release of Melphalan from folate conjugated PPI (MP-FPPI3, MP-FPPI4 and MP-FPPI5) nanoformulations were studied in phosphate buffer saline (pH 4.0 and 7.4) containing 0.1 % *v/v* propylene glycol in a modified dissolution method at 37 ± 2°C reported earlier (5). The Melphalan concentration was determined in triplicate at different time points after appropriate dilutions by HPLC analysis (14).

Hemolytic Toxicity

The RBCs suspension (5%, 0.5 mL) was mixed with the 0.9 % *w/v* normal saline (4.5 mL), free Melphalan, and 0.5 mL formulations [folate conjugated dendrimers without drug (FPPI3, FPPI4, and FPPI5) and folate conjugated dendrimers with drug (MP-FPPI3, MP-FPPI4, and MP-FPPI5)] incubated for 60 min to allow interaction. After incubation, samples were centrifuged for 15 min at 1,500 rpm and supernatant was collected to quantify the hemoglobin content at λ_{max} 540 nm by UV spectrophotometrically considering 0.9 % *w/v* NaCl solution (normal saline) and deionized water

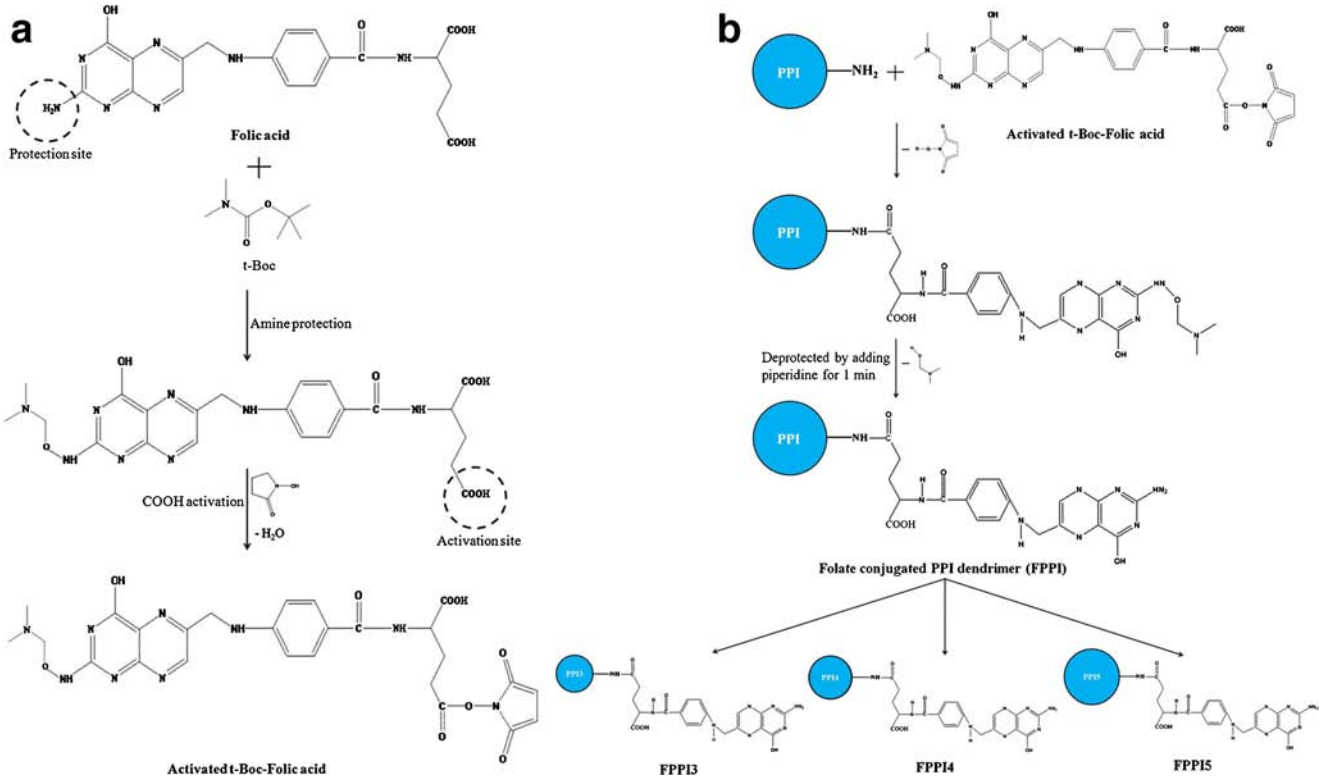

Fig. 1 Synthesis of (**a**) activated t-Boc protected folic acid; (**b**) FPPI dendritic formulations of different generations (PPI3, PPI4 and PPI5).

as 0 and 100% hemolysis, respectively (9,11). The percent hemolysis was calculated using the formula:

$$\text{Hemolysis}(\%) = \frac{Abs - \text{Abs}_0}{Abs_{100} - Abs_0} \times 100$$

where Abs, Abs_0 and Abs_{100} are the absorbance of samples, a solution of 0% hemolysis, and a solution of 100% hemolysis, respectively.

Cell Culture

We have selected MCF-7 and CASKI cells for cytotoxicity assay owing to existence of over-expressed folate receptor on their surface. We have used MCF-7 cell lines for flow cytometry studies on ground of its most robust nature compared to CASKI cells. MCF-7 (Human breast cancer) and CASKI (Leukemia) cells ($5X10^3$) were seeded evenly into 96-well flat-bottomed tissue culture plate (Iwaki; Japan) in folate deficient medium (HiMedia, India) supplemented with 10% Foetal Bovine Serum (FBS; Sigma, USA) and 1% Penicillin-Streptomycin (Sigma, USA) at 37 ± 0.5°C with 5% CO_2 in an incubator. The cells were sub-cultured every 48 h and harvested from sub-confluent cultures (60–70%) using 0.05% trypsin-EDTA.

In Vitro Cytotoxicity Assay

The *in vitro* MTT cytotoxicity assay of free Melphalan as well as developed formulations (MP-FPPI3, MP-FPPI4, and MP-FPPI5) were assessed on MCF-7 (human breast cancer) and CASKI (leukemia) cell lines (11,15). The 100 µL of each formulations to be tested was taken in cultured medium by adding into the 96 well plates (Iwaki, Japan) containing the MCF-7 and CASKI cells ($1x10^4$ cells/well) separately in 0.01 to 100 µM concentrations. MTT (20 µl, 5 mg/ml) in PBS (pH 7.4) was added after 48 h to each well followed by their incubation for 2 h at 37 ± 2°C, allowing viable cells to reduce the MTT into purple colored formazan crystals. These crystals were solubilized by adding 100 µl of Lysine-buffer (Tris HCl, 10 mM; NaCl, 75 mM; EDTA, 10 mM; Sodium dodecyl sulphate, 0.5% in water) containing Proteinase-K (0.15 mg/ml). The absorbance was measured at 570 nm in an ELISA plate reader at 37 ± 0.5°C (16).

Folate Receptor Blocking Assay

The assay is based on the principle of initial blockade of folate receptor by delivery of excess free folic acid (1 mM), followed by treatment with developed formulations (MP-FPPI3, MP-FPPI4, MP-FPPI5 and free drug) and analyzing its effect on cell viability (MTT assay), as well as on the IC_{50} shift (17,18). This study was performed according to the approved protocol

reported by our laboratory (7). Briefly, MCF-7 and CASKI cells were grown in culture flask for 2 days (37±2°C, 5% CO_2, 95% humidity), washed with PBS (pH 7.4) and, before trypsinization, cells treated with free folic acid (10 μM; 1 ml), followed by incubation for 2 h (37±0.5°C, 5% CO_2, 95% humidity). Cells were trypnised and were transferred to the 96 well plates (5×10^3 cells/ well) and incubated overnight. The samples were given appropriate treatment and the microplate was incubated for 2 days (37±0.5°C, 5% CO_2, 95% humidity). Laboratory protocol for MTT assay, as stated in previous section, was performed to determine the cell viability.

Flow Cytometry Studies

MCF-7 cells (1×10^5 cells /ml) were seeded on six well plates (Iwaki, Japan) using RPMI 1640 cell culture medium supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 mg/ml). When the cells were 60% confluent, they were treated with dendritic formulations and incubated for 24 h. After incubation, cells were detached using 0.05% trypsin–EDTA and washed with PBS (pH 7.4) at 4±0.5°C, followed by addition of annexin-propidium iodide dye (3 μl) and incubated for 15 min. The treated cells were centrifuged (1,500 rpm, 5 min) and obtained cell pellets were re-suspended with PBS and kept on ice till used. The number of cells in different phases of the cell cycle was determined in a flow cytometer (FACS caliber, Becton Dickinson, USA) by counting 10,000 events.

Fluorescence Microscopy Studies

FITC labeled formulation (5:1 mole: mole; 10 mg/ml in DMSO) was incubated for 8 h at room temperature (37± 0.5°C) with intermittent mixing. MCF-7 cells (5×10^4) were seeded in six-well plate (Iwaki, Japan) and incubated at 37°C under 5% CO_2 for 24 h. The medium was removed, and 2 ml of FITC conjugated formulations (FITC-MP-FPPI3, FITC-MP-FPPI4, and FITC-MP-FPPI5) were added and incubated for 1 h. Following this the formulation containing medium was removed, and the resulting cells were washed thrice with PBS and fixed with 2 % *v/v* formaldehyde in the PBS at room temperature for 10 min, and the samples were qualitatively analyzed under inverted fluorescent microscope (Leica, Germany) (19).

Tumor Induction in Mice

The tumor implantation was carried out by the method reported previously from our laboratory, with little modification (1,11). Eight- to-nine week-old female BALB/C mice (avg. body weight 25.0±2.0 g) were used for the *in vivo* studies. All the animal studies were conducted in accordance with the protocol approved by the Institutional Animal Ethical Committee of Dr. H.S. Gour University, Sagar, (M. P.) India.

Pharmacodynamics Study

The *in vivo* anti-tumor cancer targeting efficacy of the free drug and formulations [(MP-FPPI3, MP-FPPI4, and MP-FPPI5) (equivalent dose of Melphalan =1 mg/kg) suspended in PBS] was assessed in the tumor bearing BALB/C mice. The tumor bearing mice were randomly divided into five treatment groups (control, free drug, MP-FPPI3, MP-FPPI4 and MP-FPPI5) for treatment with formulations. At predetermined time intervals the tumor volume (cubic millimeters) was measured by electronic digital Vernier Caliper. Formula $V = 4/3\pi$ (1/2 length × 1/2 width × 1/2 depth) was used to compute tumor volume (1,20). Another group of animals was observed for survival life span using Kaplan-Meier survival curve.

Pharmacokinetic Study

For Pharmacokinetic study, tumors induced BALB/C mice were divided into five groups (control, free drug, MP-FPPI3, MP-FPPI4 and MP-FPPI5). Formulations and free drug solubilized in saline in the dosage of 1 mg/kg body weight were administered through intravenously in mice. Following formulation treatment, blood samples were collected from tail vein at specified time interval (0.16, 0.5, 1.0, 2.5, 5, 8, 12, and 24 h) and centrifuged at 3,000 rpm (Remi Elektrotech Ltd., Mumbai, India) for 10 min to separate RBCs and serum. The upper supernatant (serum) was collected separately with the help of micropipette and thoroughly mixed with 2 ml of ethyl acetate and subsequently centrifuged to remove the organic layer, evaporated to dryness and re-suspended in the mobile phase for analysis of the drug by HPLC (19).

Tissue Distribution Study

For bio-distribution studies, mice were divided into 5 groups (control, free drug, MP-FPPI3, MP-FPPI4 and MP-FPPI5) and each group was administered with same intravenous dose (1 mg/kg) of different formulations and Melphalan as in case of pharmacokinetic study. Two mice from each group were sacrificed at 2, 8 and 24 h, followed by dissection of the mice organs *viz.* spleen, kidney, liver and tumor. Weighed tissue samples were suspended in 2 ml ethyl acetate and homogenized for 5 min. The homogenate was transferred to an Appendroff tube and centrifuged at 3,000 rpm for 10 min at 4±0.5°C. The organic layer was subsequently transferred in to a second tube and evaporated to dryness under vacuum (11). The compound was re-suspended in 1 ml of the mobile phase, vortexed and then stored at deep freeze condition (-80±2°C) until analyzed for Melphalan content by HPLC method (19).

Stability Studies

The influence of accelerated conditions on the stability of developed formulations was studied by placing the samples (5 mL) separately in dark in amber colored and colorless (light conditions) glass vials at 0±2°C (T_1), ambient room temperature (RT) (27±2°C) (T_2) and 60±2°C (T_3) in controlled oven for a period of five weeks. Samples were analyzed every week for any visual changes like precipitation, turbidity, crystallization, color and drug leakage (however only terminal results were reported). The samples were analyzed after the specified time periods for turbidity, precipitation, crystallization and drug leakage (1,5).

Statistical Analysis

The statistical analysis was performed with Graph Pad Instat Software (Version 3.00, Graph Pad Software, San Diego, California, USA) using either unpaired *t* test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons. A probability $p < 0.05$ was considered statistically significant. The pharmacokinetic parameters were calculated using the Kinetica 5.0 PK/PD analysis software, Thermo Fischer Scientific, USA.

RESULTS

The accurately controlled architecture of dendrimers allows their surface modification as per the requirements, which makes them ideal carrier in the field of nano-medicine. Folate conjugation is sound concept in cancer chemotherapy. Since the initiation of dendrimers with the prominent resources to their credit, they have become the preferable option in folate mediated cancer therapy. Such a folate guided nano-carrier enters into cancer cells through receptor-mediated endocytosis (1,11–13). The purpose of present research was to compare the cancer targeting potential, hemolytic toxicity profile, *in vivo* pharmacokinetic and pharmacodynamic investigation and stability assay of folate-anchored PPI dendrimers of various generations under similar experimental condition and to find out the best dendrimer amongst them. For this, folate-conjugated PPI dendrimer of different generations (FPPI3, FPPI4 and FPPI5) were developed and characterized through FT-IR, ^{1}H-NMR spectroscopic and SEC analysis followed by loading of Melphalan. These conjugates were further compared for cancer targeting and drug delivery parameters through various *in vitro* and *in vivo* studies.

Synthesis and Characterization of Folate-Conjugated Dendrimer (FPPI3, FPPI4, and FPPI5)

EDA cored dendrimer was synthesized by divergent approach following established protocol reported previously by us (1,2,5). Subsequently folic acid was anchored on plain PPI dendrimers. The developed conjugates were characterized through FT-IR, ^{1}H-NMR, and SEC analysis. See the supporting data for detailed characterization.

Drug Loading

The percentage Melphalan loading was found significantly higher for FPPI dendrimers ($p < 0.001$) as compared to corresponding plain PPI formulations of respective generations (1,5). Folate conjugated PPI dendrimers displayed 17.82±1.16, 25.42±0.24 and 28.46±1.52% drug loading for MP-FPPI3, MP-FPPI4 and MP-FPPI5, respectively (Fig. 2a). The hydrophobic nature of dendrimer core may facilitate transport of hydrophobic Melphalan into the dendrimer core. Amount of drug loading was increased with each subsequent dendrimer generation. Presence of large cavity, more hydrophobic core and extra void spaces are the key reasons for higher drug loading inside the higher dendrimer generation, which provides additional space for drug retention as compared to lower dendrimer generation. Higher drug loading was observed in case of FPPI dendrimers compared to plain PPI based formulations, possibly because folate molecules that physically attached additional drug molecules along with drug molecules loaded inside the interior dendritic cavities. Higher drug loading inside the higher generation formulations (MP-FPPI4 and MP-FPPI5) compared to (MP-FPPI3) was possibly due to conjugation of extra folate molecules that physically attached additional drug molecules along with drug molecules loaded inside the interior dendritic cavities. The results are in agreement with existing reports wherein high level of drug loading was observed with folate modified dendrimers (1,21). However the extent of folate conjugation was directly related to generation of dendrimer. As the generation of dendrimers is increased, it may provide more scope for folate conjugation due to the availability of more amine groups. An interesting outcome is that shifting from MP-FPPI3 to MP-FPPI4 results in comparatively higher drug loading (1.42 times; $p < 0.005$) as compared to shifting from MP-FPPI4 to MP-FPPI5 (1.11 times; $p < 0.005$), possibly due to extra steric hindrance of fifth generation. This extra steric hindrance has also restricted the conjugation of more folic acid molecules. These results are in fine concurrence with the earlier report (1).

In Vitro Drug Release

In vitro drug release summary recommended sustained and controlled release of Melphalan from developed folate based formulations. Initially, a nonlinear release (faster release during first 3–4 h followed by sustained release) pattern has been exhibited by all the formulations at both the pH (4.0 and 7.4) due to leaching of drug from the surface. In contrast, release of encapsulated drug from the core is the prime reason for

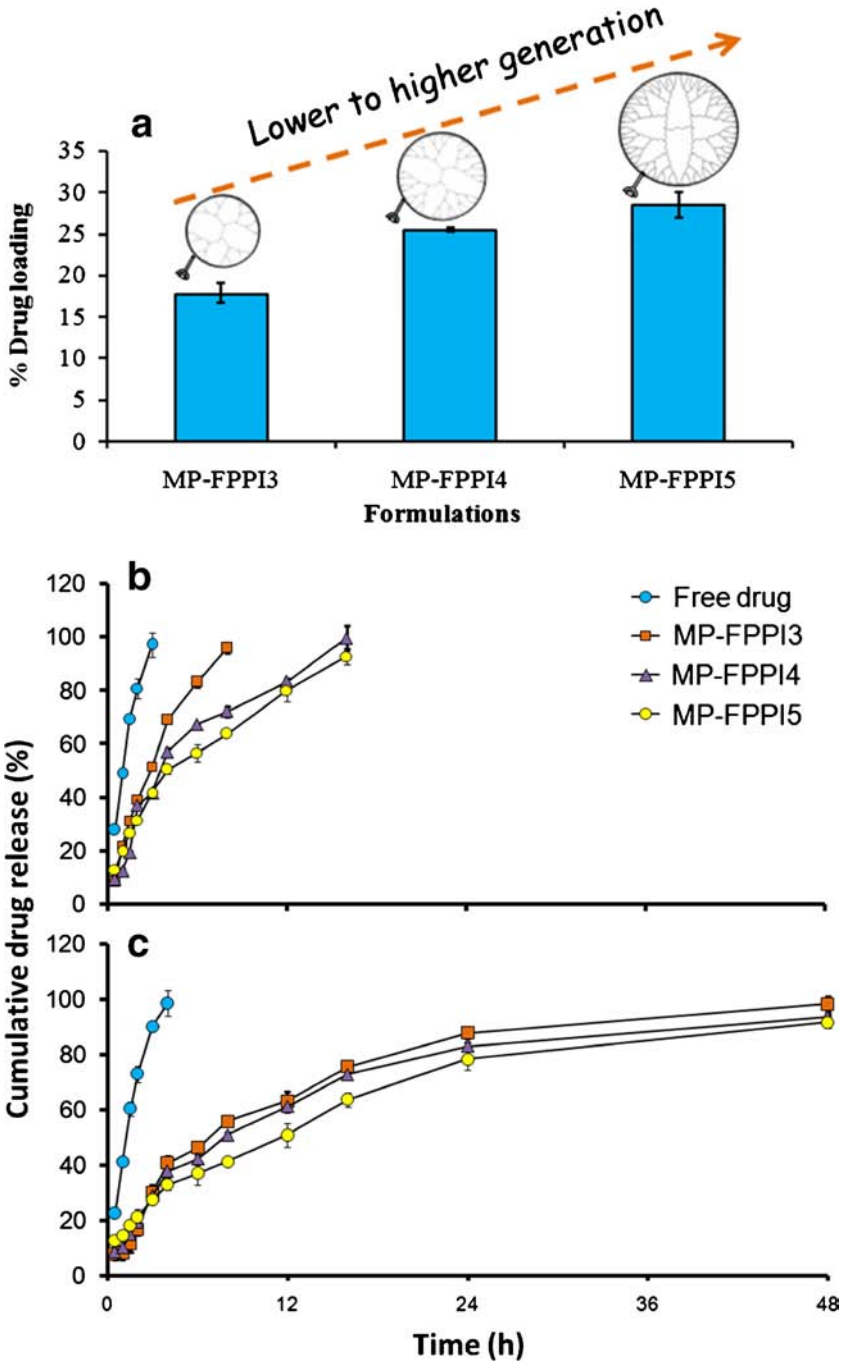

Fig. 2 (**a**) Comparative drug loading of dendritic formulations; Cumulative percentage Melphalan released from dendritic formulations in (**b**) PBS 4; and (**c**) PBS 7.4 as recipient medium ($n = 3$).

delayed and sustained drug release of the drug in the later phase.

Another finding of the drug release study is the pH-dependent drug release pattern. At acidic pH (pH 4.0), release pattern was found to be comparatively faster than at physiological pH (pH 7.4). This was due to repulsion of charges at acidic pH due to protonation of surface amine groups (Fig. 2 b&c). This charge repulsion results in an "extended conformation" of surface amine groups. However, alkaline pH causes collapse of dendrimer periphery on itself, known as 'back folding' (5,22). Thus, alkaline pH resulted in compact, globular dendritic structure leading to slower but controlled release of Melphalan from all the formulations.

After 12 h, the release pattern (pH 7.4) was found to be 63.18 ± 3.20, 61.14 ± 2.05, and $50.88 \pm 1.32\%$ drug release, and followed by 98.26 ± 2.67, 93.72 ± 0.73 and $91.74 \pm 2.25\%$ drug release after 24 h for MP-FPPI3, MP-FPPI4, and MP-FPPI5, respectively. The intensity of delayed release from folate based formulations has been found to be generation dependent. As the generation of dendrimer is increased,

delayed release behavior is also increased due to the additional resistance offered by extra layers present in higher generation dendrimers. Each additional layer has worked as barrier to drug release, which is increased with each generation number. The delayed and sustained release from higher generation based formulations is also attributed to the conjugation of more molecule of folic acid, as it led to more sealing at the dendritic periphery and hydrophobic interactions. These results are well in accordance with our recent reports (Fig. 2 b&c) (1,11,22). This comparative release profile clearly favor the importance of fourth generation based formulations as compared to fifth generation based formulations for their bioperformance on ground of their *in vitro* drug loading and cumulative drug release performance.

Hemolytic Toxicity

The hemolytic toxicity in this study was measured in terms of percent RBC hemolysis. Free Melphalan exhibited 13.18± 1.66% hemolysis, however folate based formulations FPPI3, FPPI4, FPPI5, MP-FPPI3, MP-FPPI4, and MP-FPPI5 displayed 3.04±0.14, 3.58±0.62, 5.86±0.24, 2.82±0.76, 3.06±0.94 and 5.22±0.66% hemolysis, respectively (Table I).

Our previous report depicted that as the generation of dendrimers increased the hemolytic toxicity was also increased (5). Interestingly, conjugation of folate reduced the hemolytic toxicity to greater extent but pattern was similar. These findings are similar to reported literature (5,9). The elevated toxicity of higher generation might be due to increment of terminal -NH_2 groups and multiple cationic charges. These cationic –NH_2 groups react with anionic cell components like plasma membrane, mitochondria, and nucleus caused cell lysis and toxicity. Another thrilling observation is that as we moved from fourth to fifth generation based formulation, a significant increment in hemolytic toxicity was observed as compared to moving from third to fourth generation based formulation. This might be due to rationally greater number of surface amine groups in 5.0G compared to 3.0G and 4.0G dendrimers.

In Vitro Cytotoxicity Assay

MTT cytotoxicity assay was performed on MCF-7 and CASKI cell lines. The observation conferred the IC_{50} value of MP-FPPI3, MP-FPPI4, MP-FPPI5, and free Melphalan to be 8±0.15, 0.9±0.02, 0.2±0.01 and 10±0.17 μM, respectively in case of MCF-7 cell lines (Fig. 3a), suggesting folate based targeting to be the efficacious approach to kill cancer cells. Results of our study are in agreement with available literature focused on folate based intra-tumoral delivery of anticancer drugs (5,21). Analogous observations were made in CASKI cell line wherein the IC_{50} values were observed to be 3.11±0.04, 0.93±0.06, 0.75±0.02 and 2.17±0.11 μM with MP-FPPI3, MP-FPPI4, MP-FPPI5, and free Melphalan, respectively (Fig. 3b).

Table I Percent Hemolysis of Different Dendritic Formulations ($n = 3$)

Formulations	3.0G	4.0G	5.0G
Melphalan	13.18±1.66		
FPPI	3.04±0.14	3.58±0.62	5.86±0.24
FPPIM	2.82±0.76	3.06±0.94	5.22±0.66

FPPI Folate conjugated PPI dendrimers, *MP-FPPI* Melphalan loaded folate conjugated PPI dendrimers

In vitro MTT cytotoxicity assay on MCF-7 and CASKI cell lines depicted the IC_{50} values of MP-FPPI4 and MP-FPPI5 to be significantly less ($p<0.005$) compared to MP-FPPI3, which clearly depict the competitive role of 4.0G based targeted dendrimer based formulations along with 5.0G dendrimers. All the folate conjugated nano-formulations depicted generation dependent targeting efficacy; as the generation was increased, the tumor targeting efficiency was also increased. The greater efficiency in case of higher generation might be ascribed to the presence of greater number of folate molecules attached with available peripheral amine groups. Another reason for higher efficacy may be the entrapment of more amount of drug due to the presence of larger cavity that improves the possibility for higher drug loading.

The numeric decline of IC_{50} values was comparatively much lower when we switched from third (MP-FPPI3) to fourth generation (MP-FPPI4) compared to switch from fourth (MP-FPPI4) to fifth (MP-FPPI5) generation, for both cell lines. This clearly delineates the utility of fourth generation based dendritic formulation compared to third and fifth generation based dendritic formulation in drug delivery and targeting propensity for cancer. After cumulative consideration of results of MTT assay along with the hemolytic toxicity, which is comparatively much higher for fifth generation; we can confidently favor the applicability of fourth generation dendrimers.

Folate Receptor Blocking Assay

Additionally, MTT assay was also performed in presence of excess free folic acid (1 mM) basically to understand the fate of drug delivery upon blockade of folate receptors. In case of MCF-7 cell lines, the IC_{50} values of MP-FPPI3, MP-FPPI4, MP-FPPI5, and free drug were found to be 76±0.12, 35± 0.28, 24±0.18 and 18.7±0.08 μM, respectively. Analogous pattern was also found in case of CASKI cell line, where IC_{50} values of MP-FPPI3, MP-FPPI4, MP-FPPI5, and free drug were found to be 95±0.16, 55±0.22, 50±0.32 and 2± 0.04 μM, respectively. In both cases, a clear upward shift of cytotoxicity curve was observed for all folate based formulations compared to free drug, inferring folate receptor to be a

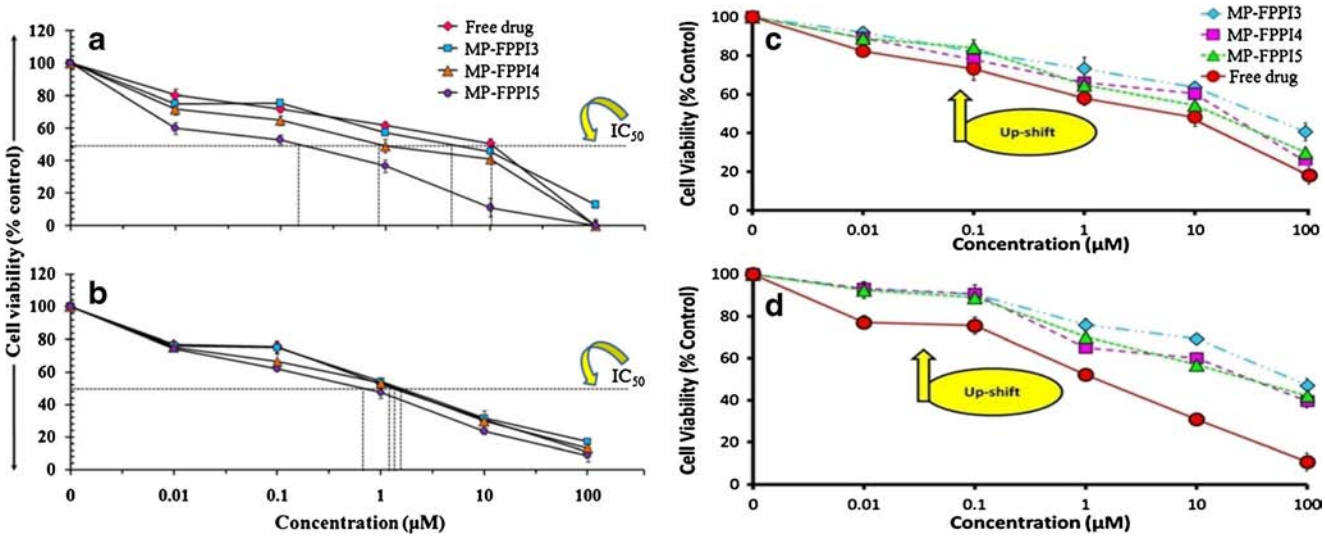

Fig. 3 Percent cell viability observed after 48 h post treatment of various systems by (A and B) MTT assay on (**a**) MCF-7 cells (**b**) CASKI cells; (**c** and **d**) folate receptor blocking assay (**c**) MCF-7 cells, and (**d**) CASKI cells ($n = 3$).

prime pathway for the uptake of these nano-formulations (Fig. 3 c&d).

Flow Cytometry Studies

Flow cytometry was carried out to evidently understand the cytotoxicity behavior of developed formulations. Maximum proportion of cells in live stage has been shown by control groups (>94.86%). The group treated with free drug demonstrated approximately 51.27±2.87% cells in apoptotic pathway, while the drug displayed approximately 42.88±1.95% cell death because of necrosis. It suggests the free drug to be enormously non-specific and toxic in nature.

Folate based formulations displayed 56.24±4.72, 61.78±1.82 and 73.94±5.38% cell death through apoptosis by MP-FPPI3, MP-FPPI4 and MP-FPPI5, respectively. However, in case of MP-FPPI5, 17.52±1.0% cells were observed to be in necrotic pathway as against mere 6.07±4.04 and 8.29±5.10% cell death by MP-FPPI3 and MP-FPPI4 formulations, respectively. So it can be concluded that in case of folate-conjugated formulations, the cells were killed by apoptosis mechanism, as evinced by higher proportions of cells in either early apoptosis or in apoptosis phase. It can be seen that all folate-conjugated formulations showed maximum apoptosis, which is due to the presence of folate receptors on the surface of cancer cells that escorted high tumor uptake and consequently high apoptosis (Fig. 4 a&b) (1,11). From the results of flow cytometry, PPI4 dendrimer based formulation emerges as better formulation compared to PPI3 and PPI5 based formulations on ground of its high cytotoxicity with low level of necrotic cell population. MP-FPPI3 also showed comparable cytotoxicity benefits however rapid drug release through this formulation makes it an inferior drug delivery nanocarrier.

Fluorescence Microscopy Studies

The cellular entry of dendritic conjugates was evaluated by fluorescence microscopy (Fig. 4 c-f). All the folate based dendritic formulations entered the cells, localizing mostly in the cytoplasm, however no fluorescence was detected in the nucleus. It is clearly evident from the figure that all the folate based dendritic formulations demonstrated punctuated distribution (higher uptake), which is a characteristic feature of endocytotic uptake. It can be concluded that all the folate based formulations showed an enhanced intracellular uptake due to folate receptor mediated endocytosis pathway. Similar results were obtained in our earlier studies (5). As compared to MP-FPPI3, much higher uptake was observed in case of MP-FPPI4 and MP-FPPI5. In fact, MP-FPPI4 showed greater fluorescence uptake compared to FPPI5M, justifying the selection of 4.0G based formulations for drug delivery.

Pharmacodynamic Study

Folate conjugated formulations showed a significant difference ($p<0.05$) in the tumor growth over time for all the generations. After 22 days tumor volumes for mice treated with MP-FPPI3, MP-FPPI4, and MP-FPPI5 were found to be 258.9±14.6, 215.6±15.2, and 196.4±13.1 mm^3, respectively (Fig. 5a). Based on principle of survival time, Kaplan-Meier survival curves were plotted for another group of animals. Result suggested that the median survival time for tumor bearing mice treated with free drug, MP-FPPI3, MP-FPPI4, and MP-FPPI5 was found to be 26, 23, 59 and 62 days, respectively (Fig. 5b). These results clearly project the higher tumor treatment potential possessed by the surface engineered dendritic formulations, which is well in accordance with

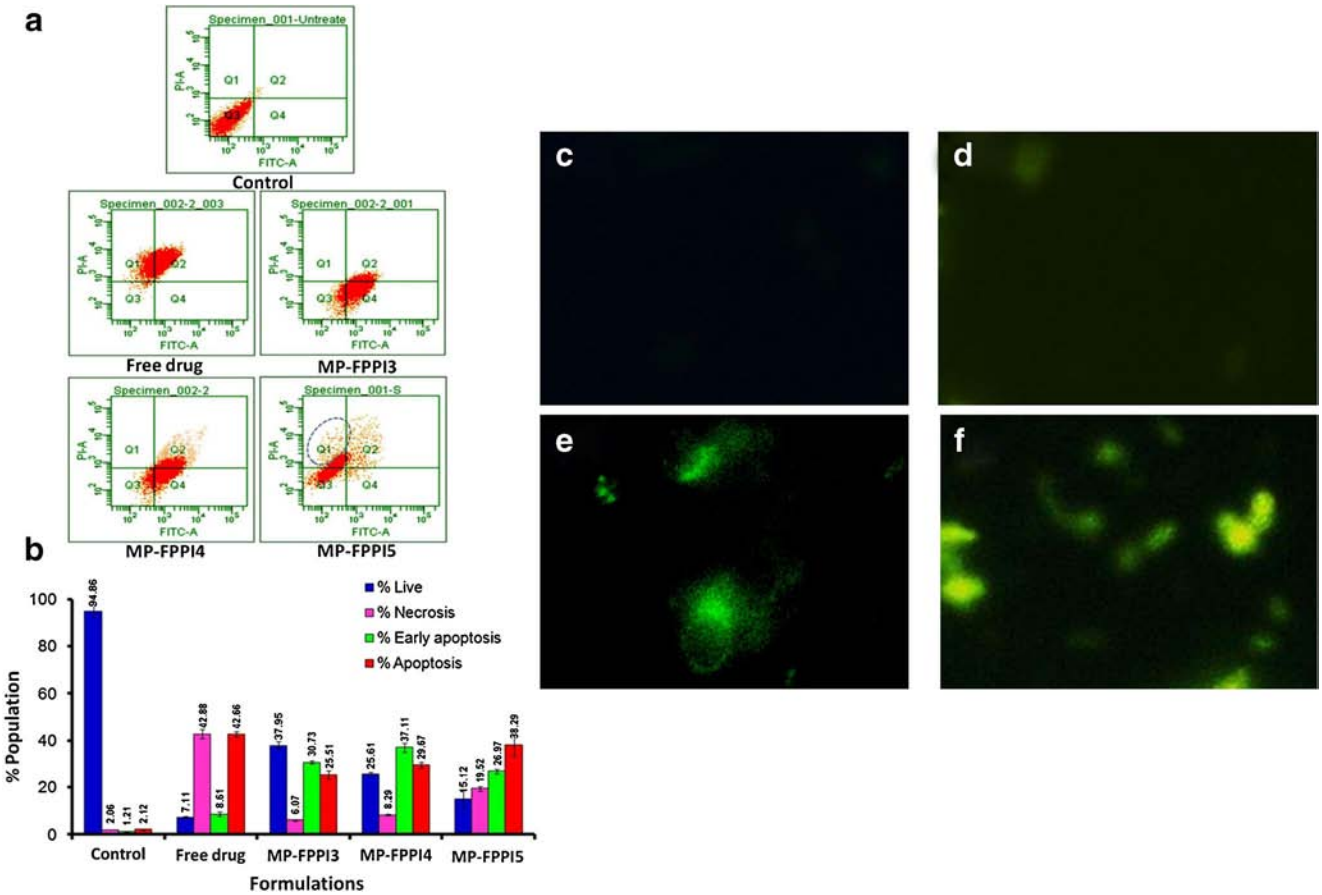

Fig. 4 (**a**) Flow cytometry assessment of MCF-7 cells treated with different dendritic formulations (**b**) Percent intracellular fluorescence activity calculated (considering the intensity of control samples as 100%) from flow cytometry; Fluorescence microscopy of MCF-7 cells treated with (**c**) control, (**d**) FITC tagged Melphalan loaded folate conjugated 3.0 G PPI dendrimer (FITC-MP-FPPI3), (**e**) FITC tagged Melphalan loaded folate conjugated 4.0 G PPI dendrimer (FITC-MP-FPPI4), and (**f**) FITC tagged Melphalan loaded folate conjugated 5.0 G PPI dendrimer (FITC-MP-FPPI5) *(n =3)*.

pharmacodynamic results. The median survival period for Melphalan loaded dendrimers formulations was found in the following order:

$$\text{MP}-\text{FPPI3} > \text{free drug} > \text{MP}-\text{FPPI4} > \text{MP}-\text{FPPI5}$$

Two major inferences can be drawn from the data. First, as the generation of dendrimers increased, its effectiveness in reduction of tumor volume was also increased. Secondly, MP-FPPI4 and MP-FPPI5 were found to be most effective among all the formulation in regards of inhibition of tumor volume efficiency.

Pharmacokinetic Study

Pharmacokinetic studies were carried out to investigate the release profile and retention of dendritic formulations in systemic circulation after intravenous administration. Plasma drug concentration with plain Melphalan solution was detected until fifth hours, following which no drug was detected in plasma. In contrast, all folate based formulations showed almost four times longer plasma drug concentration and it was proportional to dendrimer generations. As compared to free drug, folate conjugated formulations displayed higher drug concentration (C_{max}). Same pattern of drug level was also found with the *in vitro* drug release studies (Fig. 2 b&c). Observed pharmacokinetic parameters of free drug as well as formulations are shown in Table II. Pharmacokinetic parameters were found to be generation dependent; however MP-FPPI4 and MP-FPPI5 displayed comparable C_{max} suggesting MP-FPPI4 can be good alternative in drug delivery aspect due to toxicity concern.

Half life ($t_{1/2}$) of Melphalan was found to be lowest (1.23 ± 0.09 h) with elimination rate constant (K_{el}) of 0.562 ± 0.08 h^{-1} (Table II). Among folate based formulations, $t_{1/2}$ was found to be lowest for MP-FPPI3; however MP-FPPI4 and MP-FPPI5 displayed comparatively higher but almost similar $t_{1/2}$. Prolonged $t_{1/2}$ of folate based formulations may play a significant role in cancer targeting *via* the receptor-mediated endocytosis and promote site-specific delivery to tumor. As compared to free drug, folate based formulations increased the

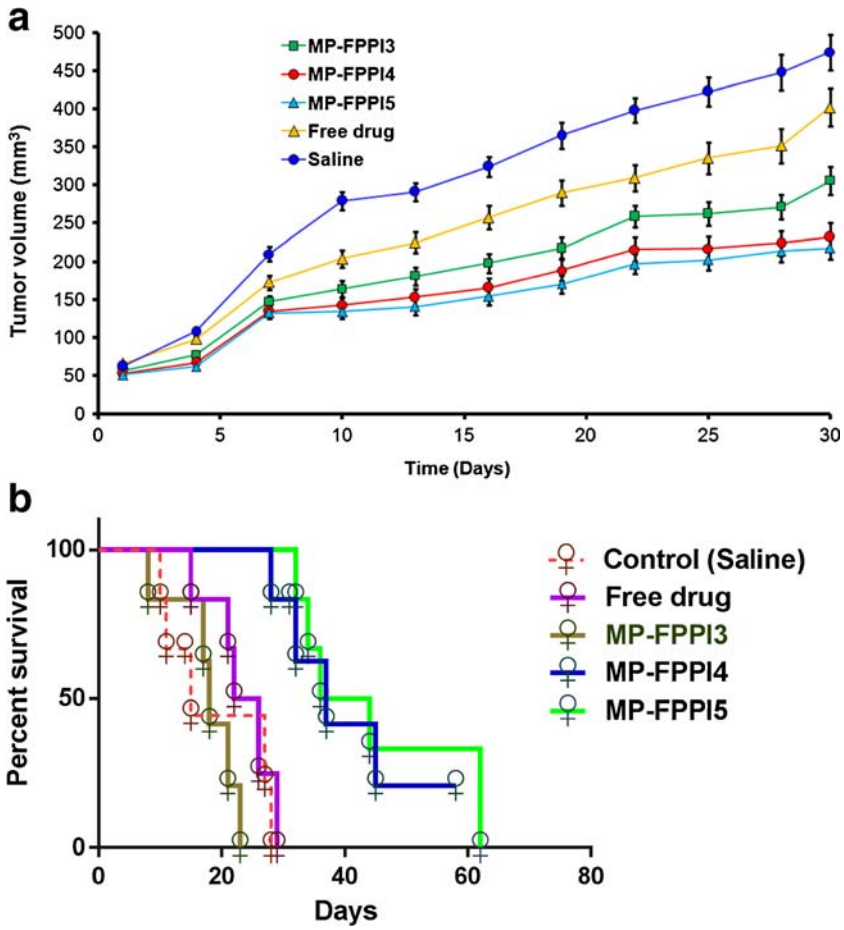

Fig. 5 (**a**) Screening graph for tumor growth following treatment of various dendrimer based nano-formulations; (**b**) Kaplan-Meier survival curves for various folate mediated dendritic formulations *(n =6)*.

half life by 1.32, 3.21, 3.56 fold, respectively for MP-FPPI3, MP-FPPI4 and MP-FPPI5. The formulations MP-FPPI4 and MP-FPPI5 demonstrated about comparable pharmacokinetic parameters similar to other *in vitro* and *ex vivo* results.

Tissue Distribution Study

The primary site of drug distribution was determined by investigating the bio-distribution studies. All the formulation displayed considerably higher concentration in kidney compared to other organs supporting that urinary excretion accounts for the major means of elimination of the drug as well as formulations from the body. Bio-distribution study revealed that the amount of drug available on tumor site was much higher in case of all folate based formulations compared to free drug. After 2 h, 4.24±0.02% of drug was recovered in tumor following intravenous injection of free drug solution as against to 2.24±2.26, 5.68±2.68 and 7.04±2.34% with MP-FPPI3, MP-FPPI4 and MP-FPPI5, respectively. However, very small fraction of drug *i.e.* 2.12±0.75 and 1.19±0.16% was recovered from tumor after 8 and 24 h, respectively following injection of free drug solution. On the other hand, MP-FPPI3, MP-FPPI4 and MP-FPPI5 showed 8.24±1.14, 9.54±0.98, 10.48±3.6 and 6.52±1.06, 11.64±2.11, 14.68±2.92% of injected dose in tumor after 8 and 24 h of injection (Fig. 6 a-c). Dendrimer size and interior hydrophobicity may be the key factors responsible for better bio-distribution performance of higher generation formulations. Our results are well in agreement with the available literature (23). This outcome is well supported by cell line based data along with *in vitro* assessment, hemolytic toxicity and cell line based assays, which clearly establishes comparable drug delivery potential of 4.0G PPI based targeted formulation (MP-FPPI4) along with 5.0G PPI based formulation (MP-FPPI5).

Table II Pharmacokinetic Parameters of Melphalan (Free Drug) and Its Formulations in Serum of BALB/C Mice

Formulation	C_{max} (μg/ml)	K_{el}	$t_{1/2}$ (h)
Free drug	4.54±0.04	0.562±0.08	1.23±0.09
MP-FPPI3	5.14±0.19	0.191±0.07	1.63±0.13
MP-FPPI4	5.22±0.07	0.175±0.03	3.95±0.27
MP-FPPI5	5.26±0.01	0.173±0.01	4.39±0.44

K_{el}, elimination rate constant; $t_{1/2}$, half-life; C_{max}, maximum drug concentration *(n =3)*; MP-FPPI3, Melphalan loaded folate conjugated 3.0G PPI dendrimers; MP-FPPI4, Melphalan loaded folate conjugated 4.0G PPI dendrimers; and MP-FPPI5, Melphalan loaded folate conjugated 5.0G PPI dendrimers

Stability Studies

Leakage of the drug from the dendrimeric carriers was used to assess the integrity of the system. The drug leakage was measured for the formulations (MP-FPPI3, MP-FPPI4 and MP-FPPI5) at various conditions of temperature (T_1, T_2, and T_3) after storage in dark (amber color glass vials) as well as light (colorless vials) for a period of 5 weeks. It was observed that the leakage was negligible at 0°C. Temperature-dependent drug leakage was observed in almost all the formulations. All the formulations, in general, were found to be most stable at room temperature in dark (Fig. 6d). In case of FPPI based formulations, PPI3 based formulation was found to be leakier compared to PPI4 and PPI5 based formulations. In general, drug leakage was higher in light than in dark, which may be attributed to structure cleavage due to temperature and light. Percent drug leakage was found to be least at RT compared to at 0±2°C and 60±2°C. At 0°C shrinking of dendrimeric architecture may be the possible reason leading to decreased cavity enclosing drug molecules and hence higher leakage. However, the greater leakage at 60±2°C may be due to increased solution kinetics. It can be inferred from the above results that folate conjugated dendrimer based formulation are more stable in dark at RT than at 0 and 60±2°C (24). The lesser drug leakage from these formulations might be ascribed to surface conjugation of folic acid, which imparts rigidity to the dendrimers. The dendritic box was a rigid structure with a tightly hydrogen bonded compact

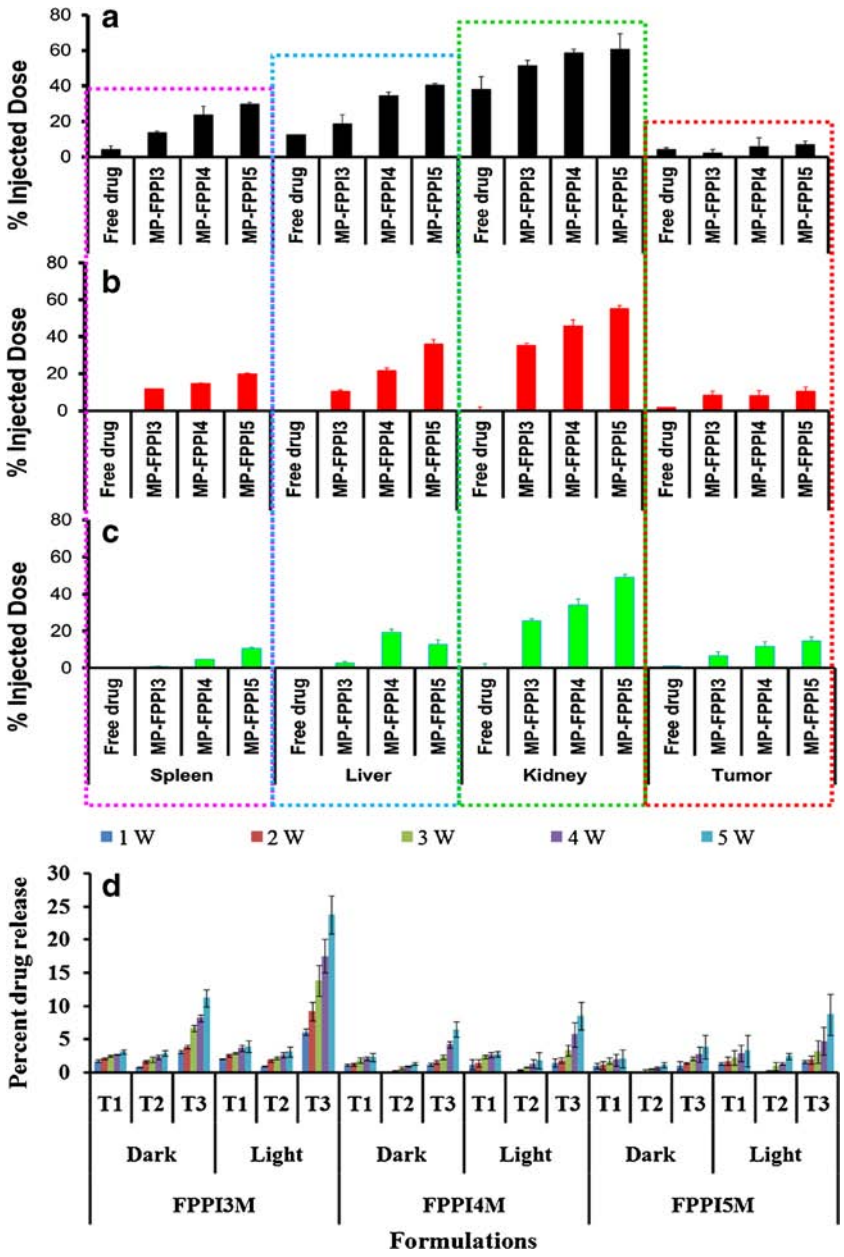

Fig. 6 Tissue distribution pattern of various dendritic formulations ($n = 6$) after (**a**) 2, (**b**) 8, and (**c**) 24 h following administration of different formulations; (**d**) Percentage drug release from folate mediated formulations under different storage conditions ($n = 3$). (T_1, T_2 and T_3 represent 0±2°C, 27±2°C and 60±2°C temperatures, respectively).

periphery. The folic acid conjugated dendrimers can be viewed as similar structures in which the periphery is almost sealed by bulky groups (25,26).

In conclusion, the developed formulations (MP-FPPI3, MP-FPPI4 and MP-FPPI5) were found to be safer compared to free drug in terms of hemolytic toxicity. Surface engineered dendrimers were able to reduce the cationic toxicity of the dendrimers due to reasons explained above and the results were similar to the previous reports (1,11,21). Toxicity of a formulation is a primary concern for its future applications and the developed formulations may prove their capabilities *in vivo* due to lesser toxicity. In addition, the formulation must be stable enough to withstand various environmental conditions to give its highest performance whenever used throughout its shelf-life. The prepared formulations were stable enough under normal storage conditions *e.g.* at room temperature, assuring easy storage and transportation, if so, in future.

DISCUSSION

The specially-made surface of dendrimers offers a prospect for their design and modification that are comparatively difficult with other types of nanocarriers. This will lead to their bright future in drug delivery. Our laboratory has suceesfully exlored delivery of a variety of drugs based on dendritic nanosystems. However, inspite of widespread applicability in pharmaceutical field, the use of dendrimers in biological systems is constrained because of their inherent toxicity. Folate conjugated dendrimers is a widely explored concept for the treatment of cancer, which also results into their toxicity reduction. Anticancer therapy based on third, fourth and fifth generation based PPI dendrimer is a time-honored concept. Till date, not a single report is available which compares the generation-dependent anticancer efficacy of PPI dendrimers on single platform. This paper desribes for the first time the comparative data pertaining to generation-dependent cancer targeting propensity of folate anchored PPI dendrimers. With respect to superior biocompatability, a clear projection for fourth generation has been observed compared to third and fifth generation. This work may focus the efforts of researchers towards PPI4 dendrimer based formulations as against widely explored PPI5 dendritic formulations. The present debut study report suggests that as compared to FPPI3 and FPPI5, FPPI4 is a promising and efficient targeted drug delivery system for cancer treatment. Thus FPPI4 could be considered as the optimized generation in case of PPI dendrimers.

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