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Design of 5-fluorouracil (5-FU) loaded, folate conjugated peptide linked nanoparticles, a potential new drug carrier for selective targeting of tumor cells†

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In the present investigation folate peptide (FA-Pep) conjugated 5-fluorouracil (5-FU) loaded nanoparticles were synthesized and their tumor targeting potentiality was monitored by different *in vitro* and *in vivo* techniques. FA-Pep-1 and FA-Pep-2 were synthesized and radiolabeled with $^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3$. $^{99m}\text{Tc}(\text{CO})_3$ -FA-Pep-1 exhibited promising tumor uptake in an *in vivo* model (nude mice bearing HeLa cell xenograft and Balb/c mice bearing B16F10 melanoma tumor) as compared to $^{99m}\text{Tc}(\text{CO})_3$ -FA-Pep-2. FA-Pep-1 was then conjugated with 5-FU-NPs (118 ± 4.3), as confirmed by the XPS study. These showed promising cytotoxic and apoptotic potential in B16F10 cell lines as compared to free 5-FU and unconjugated 5-FU-NPs. *In vivo* biodistribution and gamma-scintigraphy showed good accumulation of peptide conjugated NPs in the tumor region. Therapeutic efficacy studies in B16F10 tumor xenografts also exhibited substantial tumor growth inhibition. The above studies reveal that folate peptide conjugation may facilitate the tumor-targeting approach of 5-FU-NPs.

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Introduction

Recent years have witnessed the development of several targeting ligands, such as small receptor binding peptides, antibodies, folate receptor-specific peptides, and aptamers to deliver anticancer drugs selectively to the cancer cells sparing the normal healthy tissues.¹ Folate receptors (FR) overexpress on different types of cancer cells but are highly restricted in most normal cells and can be used as molecular tumor markers for selective delivery of anticancer drugs.^{2–6} Several folate conjugated bioactive molecules have been radiolabeled with technetium-99m for *in vitro* and *in vivo* evaluation to determine their FR binding ability.^{7,8} Folate-conjugated peptides were also studied to understand their ability to bind specifically to FR with high affinity comparable to that of folic acid.⁹

The nanoparticulate drug delivery system offers a versatile platform towards cancer research to which many modifications can be made to improve the target specificity, prolong systemic circulation, and promote extravasation favoring preferential accumulation in tumor tissue by enhanced permeability and retention physiology (EPR).¹⁰ Application of cell-

penetrating peptides (CPPs) for the development of site-specific drug targeting has received particular attention.¹¹ Among them, lysine¹² and arginine-rich CPPs¹³ are widely studied because of their simple structure. Moreover, literature reports have established that arginine and lysine based cationic peptides facilitate intracellular entry of macromolecular cargoes through lipid bilayers.¹⁴ This encouraged us to synthesize one lysine containing tripeptide (Lys-Gly-His; Pep-1) and one arginine containing octapeptide (Arg-Arg-Arg-Arg-Arg-Lys-Gly-His; Pep-2). These were conjugated with folic acid to achieve folate receptor-mediated endocytic delivery to cancer cells, while the terminal His-residue can be used for ^{99m}Tc -radiolabeling to monitor *in vivo* tumor targeting noninvasively.

The folate decorated peptides (FA-Pep-1 and FA-Pep-2) were radiolabeled with the $[\text{}^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ precursor and their *in vivo* tumor targeting ability was evaluated by conducting biodistribution and scintigraphic studies in B16F10 melanoma tumor-bearing Balb/c mice and HeLa tumor-bearing nude mice. Finally, the most promising targeting moiety (FA-Pep-1) was used to decorate PLGA nanoparticles (NPs) loaded with 5-fluorouracil (5-FU) to target the folate receptor overexpressed at the surface of FR-positive tumor cells.

Thus the aims of this study were to (1) construct folate conjugated to cell penetrating peptide with a simple linear structure (FA-Pep-1), (2) fabricate a 5-FU loaded PLGA based nanoparticle drug delivery system decorated with folate conjugated Pep-1, (3) evaluate *in vitro* at the cellular level by

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performing the cellular uptake and cell apoptosis and *in vivo* tumor accumulation through nuclear scintigraphy after ^{99m}Tc radiolabeling, and (4) monitor the therapeutic efficacy of the nanoformulation in a tumor-bearing animal model. It is expected that this potential drug delivery system can achieve better therapeutic efficacy of 5-FU, with reduced cytotoxicity to healthy normal tissue.

Materials and methods

General

Rink Amide MBHA resin (100–200 mesh), all side chain protected amino acids for syntheses, HOBT (*N*-hydroxybenzotriazole) and TBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate], HBTU [*N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uraniu hexafluorophosphate] and HOBT [*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluroniu hexafluorophosphate] were purchased from Novabiochem (Switzerland). Folic acid, DIPEA (*N,N*-diisopropylethylamine), picryl sulfonic acid, thioanisole, TFA (trifluoroacetic acid), FITC and DAPI (4',6-diamidino-2-phenylindole) were procured from Sigma (St. Louis, MD, USA). 5-Fluorouracil (5-FU) was procured from TCI Chemicals (Japan). PLGA 50:50 (PURASORB PDLG 5010 acid terminated) was purchased from PCAS (France). All other chemicals and solvents were of analytical grade and purchased from Merck India. $^{99}\text{MoO}_4^-$ was purchased from Bhabha Atomic Research Centre, Mumbai, India, and $^{99m}\text{TcO}_4^-$ was obtained by 2-butanone extraction from a 5 N NaOH solution of $^{99m}\text{MoO}_4^-$. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Waters Q-TOF Micromass instrument and MALDI was performed on a 4800 MALDI TOF-TOF instrument from Applied Biosystems, USA. The ^1H NMR spectra were recorded on a Bruker DRX 300 MHz spectrometer. All HPLC studies were done on a Waters HPLC system attached to a Waters 2489 UV/vis detector. Radioactivity in the eluate was monitored using a Berthold LB500 HERM radio HPLC monitor procured from Berthold Technologies GmbH (Bad Wildbad, Germany). Radioactivities of all samples were measured in a well type gamma counter (model LV4755) from Electronics Corporation of India Limited, India.

Synthesis of folate-conjugated peptides

Two linear peptides, one tripeptide (Lys-Gly-His; Pep-1) and one octa-peptide (Arg-Arg-Arg-Arg-Arg-Lys-Gly-His; Pep-2) were synthesized manually by using Fmoc protected amino acids on Rink Amide MBHA resin, following the Fmoc solid phase peptide synthesis strategy utilizing TBTU and DIPEA as a coupling reagent.^{15,16} The Dde group attached to the N^ϵ -group of Lys was removed by repetitive treatment with 2% hydrazine hydrate in DMF (3 × 5 min) and finally, the deprotected resin was washed with DMF (three times).¹⁷ A mixture containing five equivalents each of folic acid, HBTU, and HOBT and six equivalents of DIPEA dissolved in DMSO (4 mL) was added to the resin and stirred overnight under a nitrogen atmosphere. The resin was washed with DMF (4 times) and DCM (4 times) and

dried under vacuum for 24 h. The dried resin was then suspended in a cleavage cocktail containing TFA (95%), TIS (triisopropylsilane, 2.5%) and H_2O (2.5%) for two hours to get the folic acid conjugated peptides cleaved from the resin. Different side chain protected groups were also removed during the above treatment. The resin was separated by filtration; the crude peptides (FA-Pep-1 and FA-Pep-2) were precipitated from the filtrate by treatment with cold diethyl ether and purified by semi-preparative RP-HPLC using an XTerra C-18 column (7.8 × 300 mm ID, 10 μm particle size). The solvent system consisted of 0.1% TFA in H_2O (solvent A) and CH_3CN (solvent B). Elution was achieved by applying a linear gradient from 100% A to 85% A in 20 min at a flow rate of 2 mL min^{-1} . HPLC purification furnished pure FA-Pep-1 (0.332 g, 36.3%) and FA-Pep-2 (0.272 g, 25.3%); the purity was assessed by analytical HPLC (XTerra RP-18 column; 4.6 × 250 mm, particle size 5 μm) eluted as above (flow rate of 1 mL min^{-1}) and the molecular mass was confirmed by ESI and MALDI mass spectrometry. With FA-Pep-1 ESI-MS detected a peak for $[\text{M}]^+$ at m/z 763.69 (calcd for $\text{C}_{33}\text{H}_{41}\text{N}_{13}\text{O}_9$; 763.32), while for FA-Pep-2 MALDI-TOF MS found a peak for $[\text{M}]^+$ at m/z 1543.53, (calcd for $\text{C}_{63}\text{H}_{101}\text{N}_{33}\text{O}_{14}$; 1543.82). Both peptides were also characterized by the ^1H NMR results, which are given in the ESI[†] (Fig. S-5 and S-6).

To confirm the synthesis of Pep-1 and Pep-2, a portion of the resin was taken after coupling the last amino acid and treated with a cocktail mixture for 3–4 h (ref. 18) to cleave the peptide from the resin support. The crude peptide was precipitated (cold diethyl ether treatment) and checked by HPLC as stated above. In the case of Pep-1 ESI-MS detected a peak for $[\text{M}]^+$ at m/z 340, calcd for $\text{C}_{14}\text{H}_{24}\text{N}_6\text{O}_4$; 340.19. Similarly in the case of Pep-2 ESI-MS showed a peak for $[\text{M} + \text{H}]^{+1/2}$ at m/z 561.06 (calcd for $\text{C}_{44}\text{H}_{84}\text{N}_{26}\text{O}_9$; 1120.69).

Synthesis of $^{99m}\text{Tc}(\text{CO})_3$ -folate-peptides

Folate-peptide conjugates (FA-Pep-1, FA-Pep-2) were labeled with a freshly prepared $\text{fac-}[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ precursor pursuing the method reported earlier from this laboratory.¹⁹ The organometallic precursor $^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3$ was synthesized from freshly eluted $^{99m}\text{TcO}_4^-$ (185–370 MBq, in 0.5 mL saline). The peptide solution (4–5 mg dissolved in 0.2 mL of nitrogen purged water) was added to $^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3$ solution (0.3 mL, 74–185 MBq). The mixture was heated at 45 °C for 10–15 min maintaining the pH between 6.5 and 7.0, then cooled to room temperature, and analyzed by TLC (using silica gel strips as the stationary phase and acetone as the developing solvent); the chelation efficiency was determined by analytical HPLC following the same method as described under synthesis.

The radiochemical stabilities of $^{99m}\text{Tc}(\text{CO})_3$ -labeled folate-peptides in normal saline, serum, and histidine solution (10^{-2} M) at different time intervals (0, 1, 2, 3, 4 and 24 h) were ascertained by TLC as per the reported method.¹⁹

Octanol–water partition coefficients of the radiolabeled folate-peptides were determined and expressed as $\log P$ by a published procedure.¹⁹ Experiments were done on three samples from each labeled peptide conjugate and the mean ($\pm$ SD) was determined.

Scintigraphic imaging and biodistribution studies of $^{99m}\text{Tc}(\text{CO})_3$ -folate-peptides

The national regulations for the care and use of laboratory animals were observed in the study according to the guidelines approved by the Social Justice and Empowerment Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The experimental protocols were also approved by the Institutional Animal Ethics Committee.

In vivo biodistribution studies of the $^{99m}\text{Tc}(\text{CO})_3$ -folate-peptides were performed in B16F10 tumor-bearing Balb/c mice (body weight 25–30 g) as per a reported method,¹⁸ and in a female nude mice xenograft model of human cervical cancer. Freshly cultured HeLa cells (1×10^7 cells) suspended in a serum-free medium (0.1 mL) were inoculated subcutaneously into the right flank of Balb/c nude mice (body weight 18–20 g; procured from ACTREC, Mumbai). Tumor growth was monitored twice per week. A palpable tumor (around $1 \pm 0.1 \text{ cm}^3$) developed after four weeks.

B16F10 tumor-bearing animals in each group ($n = 5$) were injected separately either with $^{99m}\text{Tc}(\text{CO})_3$ -FA-Pep-1 or $^{99m}\text{Tc}(\text{CO})_3$ -FA-Pep-2 (0.03 mL, 8–12 MBq kg^{-1}) *via* the tail vein, whereas nude mice bearing subcutaneous FR-positive HeLa cells were similarly injected with $^{99m}\text{Tc}(\text{CO})_3$ -FA-Pep-1. Biodistribution studies at a 4 h post-injection time period were performed as per a reported method and the percent injected doses per g of tissue/organs were calculated.

Blocking studies ($n = 5$) were performed by co-administration of folic acid (100 μg per mouse in 0.1 mL PBS, pH 7.4) five min prior to the administration of radiolabeled-folate conjugated peptides to investigate folate receptor-mediated uptake.

Scintigraphic images of two of the HeLa tumor-bearing animals from the above sets were acquired at 4 h post injection under a GE Infinia Gamma camera equipped with the Xeleris Work Station.

Fabrication of 5-fluorouracil-loaded PLGA nanoparticles (5-FU-NPs)

5-FU-NPs were fabricated using the double emulsion (w/o/w) method, as reported earlier.²⁰ Briefly, an aqueous solution of 5-FU (5 mg in 1 mL distilled water) was added to PLGA (50 mg) dissolved in dichloromethane (2.5 mL) and emulsified by sonication. The emulsified mixture was then added to an aqueous solution (1% v/v) of Pluronic F-68 (30 mL) to form a secondary emulsion. The mixture was immediately sonicated (130 W and 20 kHz power supply) and subjected to overnight stirring to evaporate the DCM layer. The prepared 5-FU-NPs were recovered by ultracentrifugation (Sorvall Ultra Speed Centrifuge, Kendro, USA) at 40 000 rpm for 1 h at 4 °C and washed thrice with distilled water to remove the surfactant and free 5-FU. The pellet was finally lyophilized to obtain free-flowing powders.

Blank nanoparticles were prepared following the same procedure without adding 5-FU. FITC labeled nanoparticles were prepared as per the above method using FITC (5 mg) dissolved in acetone (1 mL) instead of 5-FU.

Characterization of 5-FU-NPs

5-FU-NPs (1 mg each) were dispersed in 1 mL of Milli-Q water by sonication, diluted and subjected to particle size distribu-

tion, mean diameter, polydispersity index (PDI) and zeta potential measurements using Malvern Nano ZS (Malvern Instruments, Worcestershire, United Kingdom), as reported earlier from this laboratory.²¹ Values reported are the mean $\pm$ SD of at least three different batches of formulations.

The shape and the surface morphology of 5-FU-NPs were ascertained by atomic force microscopy (5500 Agilent Technologies, Santa Clara, CA, USA).²¹

The internal structure of 5-FU-NPs was analyzed by TEM (FEI, TecnaiG²F-30 S-TWIN, The Netherlands).²¹

The yield, drug loading, and encapsulation efficiency were determined as reported by Jagadeeshan *et al.*²⁰ Briefly, the 5-FU loaded nanoparticle suspension as obtained after removal of DCM was centrifuged, the supernatant was separated and washings were done. All supernatants were pooled together, and the concentration of unencapsulated 5-FU in the supernatant was determined using a UV-vis spectrophotometer (SHIMADZU UV-1700) at 266 nm. The amount of 5-FU entrapped was obtained by subtracting the amount of unencapsulated 5-FU from the initial amount of 5-FU used. The yield, drug loading, and encapsulation efficiency were then calculated.

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of FA-Pep-1, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs were scanned in a FTIR spectrophotometer (Bruker, FTIR Tensor-27) as per a reported method²¹ to understand the possible chemical interaction occurring between the drug and the polymer matrix as well as to confirm the conjugation of the NH_2 function of FA-Pep-1 with the COOH function of PLGA.

Differential scanning calorimetric (DSC) study

To understand the physicochemical compatibility between 5-FU, FA-Pep-1, and the polymer matrix, DSC analysis was done using a differential scanning calorimeter (Pyris Diamond TG/DTA, PerkinElmer, Singapore) as per a method reported earlier from this laboratory.²¹

X-ray diffraction study

X-ray powder diffraction patterns of 5-FU, PLGA, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs were measured in an X-ray powder diffractometer (XRD-2000, Rigaku, Tokyo, Japan) as per an earlier reported method.²¹

Stability study

Lyophilized powdered 5-FU-NPs were stored at 4 ± 2 °C for 3 months and analyzed by DLS as stated above. Lyophilised NPs were also incubated in freshly collected rat serum (2 mg mL^{-1}), incubated for 1 h, 2 h and 4 h, and subjected to DLS analysis.

Conjugation of FA-Pep-1 with 5-FU-NPs

To enable efficient FR-targeting, 5-FU loaded PLGA-NPs were covalently linked to FA-Pep-1 through the COOH function of PLGA. Conjugation was done as per a reported method.^{20,22} Briefly to the suspension of 5-FU-NPs (20 mg) in PBS (10 mL)

of pH 7.4, 680 μL of EDC (from a stock of 1 mg mL^{-1}) and 250 μL of NHS (concentration 1 mg mL^{-1}) were added successively and placed under magnetic stirring for 3 h at room temperature and processed as per a reported method. The FA-Pep-1 conjugated 5-FU-NPs thus obtained were lyophilized and stored at 4 $^{\circ}\text{C}$ for further studies. The conjugation of FA-Pep-1 to 5-FU loaded NPs was confirmed by FTIR spectroscopy and XPS analysis.

Folate peptide conjugated FITC labeled NPs were prepared by replacing the 5-FU-NPs suspension with FITC labeled nanoparticle suspension and following the above-described procedure.

X-ray photoelectron spectrometric (XPS) study

X-ray photoelectron spectrometric (XPS) studies were undertaken to carry out the surface elemental analysis of FA-Pep-1 conjugated 5-FU-NPs for confirming peptide coupling. The diluted samples were placed on a clean silicon substrate, dried under vacuum, and analyzed in an Omicron Multiprobe Electron spectroscopy system (UK).²²

In vitro release of 5-FU from 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs

5-FU release was evaluated *in vitro* using the dialysis method as reported earlier.²⁷ Briefly dialysis bags (mol wt cut-off: 3.5 kDa, Thermo Fisher, USA), each containing accurately weighed samples of either 5-FU-NPs or FA-Pep-1 conjugated 5-FU-NPs (the 5-FU concentration in each was 0.5 mg mL^{-1}) suspended in PBS (2 mL), were placed in 200 mL PBS (pH 7.4) at 37 $^{\circ}\text{C}$ and stirred at 200 rpm throughout the experiment. At preselected time intervals, 1 mL of release medium was withdrawn and replaced with fresh PBS (1 mL) to maintain the sink condition. 5-FU release from the dialysis chamber into the release medium was determined by UV-spectrophotometric analysis at 370 nm.

In vitro cellular uptake study of 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs

Confocal microscopy. The cellular internalizations of FITC labeled NPs and FA-Pep-1 conjugated NPs were evaluated qualitatively in three FR-positive cell lines (KB, HeLa and B16F10) by confocal laser scanning microscopy (Leica DFC420C, Germany) as described earlier.²³ Cells were cultured in the respective medium (DMEM for HeLa and B16F10, MEM for KB) in coverslips placed inside a 35 mm dish and incubated (Heal Force HF-90, Japan) overnight at 37 $^{\circ}\text{C}$ in humidified air containing 5% CO_2 . Following 80% confluency in each dish, the medium was removed, replaced with fresh medium containing FITC loaded-NPs (50 $\mu\text{g mL}^{-1}$) and FITC loaded FA-Pep-1 conjugated -NPs (50 $\mu\text{g mL}^{-1}$), and incubated for 0.5 h. The rest of the procedure was the same as stated earlier.

Fluorescence activated cell sorting (FACS). Quantitative comparison of cellular uptake of FITC-labeled FA-Pep-1 conjugated and unconjugated NPs in both FR-positive (HeLa, KB, and B16F10) and FR-negative cell lines (A-549) was ascertained by flow cytometry as reported earlier (FACS Canto II™ cell sorter, BD Biosciences) and FACS Diva software (BD Biosciences).²³ An

increase in fluorescence intensity in the treated cells relative to that in the untreated control cells was reported.

Inhibition assays with free folic acid. The inhibition study was performed in FR-positive cell lines. FR-expressing cancer cells can be saturated with folic acid within one hour and the saturation plateau remains constant up to six hours.^{23,24} As described above, the medium containing 1 mM folic acid (FA) was added to the plated cells (1×10^6 per dish) in a 60 mm dish and incubated for 2 h to facilitate folate receptor (FR) saturation. In control experiments, cells were cultured only in the growth medium. Following incubation, the medium was replaced with fresh medium containing FITC-labeled-NPs and FA-Pep-1 conjugated NPs, and the rest of the procedure was the same as described above under the FACS study.

In vitro therapeutic efficacy studies of free 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs

Cytotoxicity assay. Cytotoxic potentials of free 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs were ascertained by MTT assay in the above three FR positive and one FR negative cell lines for 24 h. The cells were exposed to different concentrations of free 5-FU (1–700 $\mu\text{g mL}^{-1}$), 5-FU-NPs (0.2–400 μg of 5-FU mL^{-1}) and FA-Pep-1 conjugated 5-FU-NPs (0.2–400 μg of 5-FU mL^{-1}) and incubated as mentioned above; the rest of the procedure was the same as reported earlier.²⁵ The percentage of viable cells was determined from the absorbance ratio of treated to untreated cells.

Cell apoptosis assay. The cell apoptosis study was performed in B16F10 and HeLa cells using an Annexin-V-FITC/PI double staining assay kit supplied by BD Biosciences, USA, following a reported protocol.²¹ In brief, cells were cultured (60 mm dish at 37 $^{\circ}\text{C}$) and then treated separately with free 5-FU (2 $\mu\text{g mL}^{-1}$ for B16F10 and 30 $\mu\text{g mL}^{-1}$ for HeLa), 5-FU-NPs (0.8 $\mu\text{g mL}^{-1}$ for B16F10 and 20 $\mu\text{g mL}^{-1}$ for HeLa) and FA-Pep-1 conjugated 5-FU-NPs (0.4 $\mu\text{g mL}^{-1}$ for B16F10 and 10 $\mu\text{g mL}^{-1}$ for HeLa). The rest of the procedure was the same as done earlier. Analyses were performed in a flow cytometer (BD FACS) using FACSDIVA Version 6.2 software.

DAPI staining. The apoptotic effect of free 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs on the cell morphology (B16F10, HeLa) was further ascertained by DAPI staining following a method described earlier.²¹

In vivo evaluation

^{99m}Tc-labeling, biodistribution and scintigraphic imaging studies. ^{99m}Tc-radiolabeling of FA-Pep-1 conjugated 5-FU-NPs and 5-FU-NPs was performed by a direct radiolabeling method using stannous chloride dihydrate as a reducing agent, as reported earlier from this laboratory.^{21,25} The radiolabeling efficiency was evaluated by ascending instant thin layer chromatography (ITLC as per a reported protocol).

In vivo biodistribution studies of radiolabeled FA-Pep-1 conjugated 5-FU-NPs and 5-FU-NPs (at 4 h post injection) were performed in B16F10 tumor-bearing Balb/c mice (body wt 25–30 g) to ascertain organ uptake, expressed as a percentage of the injected dose per gram of tissue (% ID g^{-1}) or per organ as per a reported method.²⁵

Scintigraphic imaging studies (at 4 h post-injection) were also performed to ascertain tumor localization as per an earlier described method.

Evaluation of the antitumor activity of FA-Pep-1 conjugated 5-FU-NPs and 5-FU-NPs and free 5-FU. B16F10 tumor-bearing mice were divided into four groups ($n = 5$). Treatment (intraperitoneally) was started on the tenth day after tumor cell inoculation. Group 1 (control) animals were treated with saline, whereas each of the animals in groups 2–4 received 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs, respectively (dose 25 mg 5-FU per kg body weight) thrice weekly for three weeks.

Tumor growth was monitored regularly with a caliper to determine the tumor volume (mm^3) = (width² × length)/2.

Animals were sacrificed thirty-one days after cell implantation and the weight of the tumor was determined.^{27,28}

The tumor and a portion of the heart, liver, and lung was dissected out, fixed in 10% neutral buffered formalin, sectioned, and observed under a microscope following H&E staining.²⁶

Results

Synthesis and radiolabeling of folate-conjugated peptides

FA-conjugated peptides were purified by HPLC to achieve a purity higher than 90% (Fig. 1A). FA-Pep-1 was analyzed by ESI-MS and FA-Pep-2 by MALDI-TOF MS. ¹H NMR spectroscopic studies (Fig. S-5 and S-6†) of the products revealed the

Fig. 1 HPLC chromatograms of peptides, folate conjugated peptides, radiolabeled folate conjugated peptides, pertechnetate and tricarbonyl complex. (A) HPLC chromatograms (UV traces) of peptides and folate conjugated peptides; (B) radio-HPLC elution profile of radiolabeled folate conjugated peptides.

conjugation of the α γ -carboxyl group of folic acid with the side chain amino function of Lys in the peptide chain.

Folate-conjugated peptides were then radiolabeled with the $fac-[^{99m}Tc(CO)_3(H_2O)_3]^+$ core (yield > 96%, t_R = 6.2 min in radio HPLC). The donor groups of the His residue present at the N-terminal end of the peptide derivatives coordinated efficiently to the $fac-^{99m}Tc(CO)_3$ -core to produce $^{99m}Tc(CO)_3$ -labeled-FA-Pep-1 and FA-Pep-2 in substantially high yield (~95%), t_R = 13.3 and 10 min, respectively (Fig. 1B). $^{99m}TcO_4^-$ exhibited a retention time of 4.5 min under identical HPLC condition. The radiochemical purity (~96%) of $^{99m}Tc(CO)_3$ -labeled folate conjugated peptides was also ascertained by TLC in acetone.

Both $^{99m}Tc(CO)_3$ -FA-Pep-1 and $^{99m}Tc(CO)_3$ -FA-Pep-2 exhibited substantially high stability in normal saline and serum (around 95% after 1 h incubation and reduced to 92% after 24 h incubation). Histidine challenge assay also exhibited a 7% reduction in radiolabeling yield after 24 h incubation (Fig. S-7†). Negligible interactions of the complexes with the serum components and transchelator were observed. These results were consistent with the reported stability values of different peptide derivatives coordinated to the $^{99m}Tc(CO)_3$ -core.²⁶

The negative log *P* values of radiolabeled-FA-Pep-1 (−1.562) and FA-Pep-2 (−1.601) indicated the hydrophilic nature of the complexes.

Animal biodistribution and scintigraphic imaging studies of $^{99m}Tc(CO)_3$ -labeled folate conjugated peptides

$^{99m}Tc(CO)_3$ -FA-Pep-1 exhibited a substantially high tumour accumulation (2.0 ± 0.41 and $3.4 \pm 0.50\%$ ID g^{-1} at 2 and 4 h, respectively; Table 1) whereas these values were 1.9 ± 0.20 and $2.1 \pm 0.11\%$ ID g^{-1} at 2 h and 4 h, respectively, in the case of $^{99m}Tc(CO)_3$ -FA-Pep-2 in B16F10 tumor-bearing Balb/c mice. The log *P* values of both of the radiolabeled formulations were negative, supporting their hydrophilic nature as well as fast re-

nal elimination (50% of the injected dose eliminated within 2 h). Neither of the products exhibited any significant uptake in the stomach, indicating their *in vivo* stability.

Blocking studies (with cold folic acid) also showed about 75% and 58% reduction in the tumor muscle uptake of $^{99m}Tc(CO)_3$ -FA-Pep-1 and $^{99m}Tc(CO)_3$ -FA-Pep-2, respectively (Table 1), indicating the *in vivo* specificity of these peptide conjugates for FR-positive tumors.

A comparatively high tumor uptake, as well as favorably high tumor/muscle ratios, allowed us to conduct bio-distribution and scintigraphic imaging studies of $^{99m}Tc(CO)_3$ -FA-Pep-1 in a nude mice HeLa cell xenograft model. The bio-distribution results showed a promising tumor uptake ($4.8 \pm 0.3\%$ ID g^{-1}) and a significantly high tumor/muscle ratio (15.9) at a 4 h post injection time period (Table 2). Kidney uptake and renal elimination were also considerably high. Receptor blocking studies with free folate treatment demonstrated about 87% reduction in tumor uptake. Clear visualization of tumor mass (right flank area of the animal) was also observed during scintigraphic imaging studies under a gamma camera (Fig. 2A) which disappeared during the blocking study (Fig. 2B).

The promising tumor localization of $^{99m}Tc(CO)_3$ -FA-Pep-1 in an FR-positive animal tumor model allowed us to investigate the targeted delivery of 5-FU loaded PLGA-NPs decorated with FA-Pep-1.

Fabrication and characterization of 5-FU-NPs

5-FU was successfully encapsulated in biodegradable PLGA polymer by the double emulsification solvent evaporation method. 5-FU loaded PLGA nanoparticles were distributed in a narrow particle size range, and the average diameter was found to be 118 ± 4.3 nm (Fig. S8-A†), as determined by dynamic light scattering (DLS) with a PDI value of $0.440 \pm$

Table 1 Results of biodistribution studies of radiolabeled FA-Pep-1 and FA-Pep-2 in B16F10 melanoma tumor-bearing Balb/c mice at 2 and 4 h post injection. Results are expressed as the percentage injected dose per gram of tissue/organ (each value is the mean $\pm$ SEM of five animals per group)

Organ/tissue	B16F10 tumor-bearing mice					
	2 h		4 h		4 h	
	$^{99m}Tc(CO)_3$ -FA-Pep-1	$^{99m}Tc(CO)_3$ -FA-Pep-1	$^{99m}Tc(CO)_3$ -FA-Pep-1 + 100 μg free folate	$^{99m}Tc(CO)_3$ -FA-Pep-2	$^{99m}Tc(CO)_3$ -FA-Pep-2	$^{99m}Tc(CO)_3$ -FA-Pep-2 + 100 μg free folate
Heart	0.19 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.16 ± 0.02	0.15 ± 0.1
Blood ^a	1.8 ± 0.02	1.4 ± 0.02	1.3 ± 0.01	1.7 ± 0.1	1.5 ± 0.2	1.4 ± 0.1
Liver	11.2 ± 0.76	10.8 ± 0.15	11.0 ± 0.16	12.2 ± 0.26	10.5 ± 1	12.5 ± 0.17
Lungs	0.17 ± 0.02	0.2 ± 0.03	0.12 ± 0.02	0.16 ± 0.01	0.1 ± 0.04	0.11 ± 0.01
Spleen	0.1 ± 0	0.1 ± 0.06	0.23 ± 0.01	0.12 ± 0.07	0.13 ± 0.07	0.22 ± 0.11
Kidney	8.2 ± 0.10	7.2 ± 0.1	5.6 ± 0.1	9.2 ± 0.06	7.0 ± 0.5	5.4 ± 0.2
Intestine	12.2 ± 1.0	16.8 ± 1.7	17.7 ± 1.3	10.1 ± 0.3	16.4 ± 0.7	15.6 ± 0.62
Stomach	0.7 ± 0.21	0.6 ± 0.1	0.5 ± 0.11	0.7 ± 0.2	0.6 ± 0.0	0.7 ± 0.1
Urine	40.2 ± 0.02	42.8 ± 2.6	42.0 ± 1.2	41.1 ± 0.3	43.5 ± 3	42.1 ± 1
Tumor ^a	2.0 ± 0.41	3.4 ± 0.5	0.84 ± 0.11	1.9 ± 0.2	2.1 ± 0.11	1 ± 0.11
Muscle ^a	0.8 ± 0.05	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.05	0.6 ± 0.11	0.8 ± 0.06
Tumor/muscle	2.484	5.32	1.16	2.08	3.2	1.06
Tumor/blood	1.08	2.34	0.603	1.137	2.13	0.587

^a Percentage of injected dose per gram.

Table 2 Results of biodistribution studies of radiolabeled FA-Pep-1 in HeLa tumor-bearing nude mice at 4 h post injection. Results are expressed as the percentage injected dose per gram of tissue/organ (each value is the mean $\pm$ SEM of five animals per group)

Organ/tissue	HeLa tumor bearing nude mice	
	4 h	
	$^{99m}\text{Tc}(\text{CO})_3\text{-FA-Pep-1}$	$^{99m}\text{Tc}(\text{CO})_3\text{-FA-Pep-1} + 100 \mu\text{g free folate}$
Heart	0.13 ± 0.12	0.11 ± 0.13
Blood ^a	2.1 ± 0.11	2.2 ± 0.12
Liver	14 ± 0.13	11 ± 0.11
Lungs	0.16 ± 0.01	0.14 ± 0.02
Spleen	0.11 ± 0.01	0.12 ± 0.02
Kidney	12.8 ± 0.14	7.8 ± 0.14
Intestine	10.5 ± 0.11	9.5 ± 0.11
Stomach	0.1 ± 0.0	0.05 ± 0.0
Urine	41.2 ± 0.01	41.0 ± 0.1
Tumor ^a	4.8 ± 0.3	0.64 ± 0.01
Muscle	0.3 ± 0.06	0.37 ± 0.11
Tumor/muscle	15.9	1.71
Tumor/blood	2.27	0.289

^a Percentage of injected dose per gram.

0.050. The zeta potential value, an important characteristic of particles as it can influence particle stability, was found to be -28.2 ± 2.1 mV. AFM studies revealed a smooth spherical surface morphology along with the absence of aggregation or adhesion among the NPs (Fig. 3A and B), confirming the above-mentioned average diameter of NPs. TEM images also showed the monodispersive and spherical nature of the nanoparticles (Fig. 3C). The encapsulation efficiency and drug loading were found to be $56.0 \pm 2.2\%$ and 2.10 ± 0.01 , respectively (Table 3).

The 5-FU-NP formulation was stored at 4 ± 2 °C for 3 months, and the particle size and PDI were ascertained. These parameters were also evaluated after incubation with rat serum. No remarkable changes in particle size and PDI values were observed (Table 3).

FA-Pep-1 was attached to the surface of 5-FU loaded PLGA-NPs by EDC/NHS coupling reaction. The attachment of FA-Pep-1 onto 5-FU-NPs was confirmed by XPS studies. The spectra (Fig. 3H) showed peaks due to C, N, and O. The presence of an N-peak provided evidence for the conjugation of FA-Pep-1 on the NPs, whereas only C and O peaks appeared in the spectra of 5-FU-NPs.

The IR-spectra of FA-Pep-1 conjugated 5-FU-NPs (Fig. 3D) revealed a characteristic broad peak centering at 3451 cm^{-1} for the $-\text{NH}$ stretching vibrations of various amines and amides of peptide backbones, indicating the surface conjugation of FA-Pep-1 on 5-FU-loaded PLGA-NPs. Peaks at 1636 cm^{-1} and 1760 cm^{-1} accounted for the presence of C=O stretching bands of amide and carboxylic acid functions, respectively, of PLGA. The IR spectra of FA-Pep-1 also revealed

Fig. 2 Scintigraphic images of radiolabeled folate conjugated peptides. Scintigraphic images of (A) $^{99m}\text{Tc}(\text{CO})_3\text{-FA-Pep-1}$ and (B) $^{99m}\text{Tc}(\text{CO})_3\text{-FA-Pep-1} + 100 \mu\text{g folic acid}$ in HeLa tumor-bearing nude mice at 4 h post injection time periods.

Fig. 3 Characterization, physical parameters and drug release profile of nanoformulations. (A) AFM image; (B) three-dimensional view of the AFM image; (C) TEM image of 5-FU-NPs; (D) FTIR spectra of FA-Pep-1, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs; (E) DSC thermogram and (F) X-ray diffractograms of 5-FU, PLGA, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs; (G) *in vitro* release profile of 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs; (H) XPS study of 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs.

the characteristic broad band centering at 3420 cm⁻¹ for -NH stretching and a peak at 1677 cm⁻¹ accounting for the C=O stretching band of the amide function. Similarly, the IR spec-

tra of 5-FU-NPs revealed characteristics peaks of PLGA, at 3433 cm⁻¹ representing various -OH stretching bands and at 1760 cm⁻¹ due to C-O stretching bands of the -COOH function.

Table 3 Characterization and stability studies of 5-FU-NPs

Parameters	5-FU-NPs	5-FU-NP after 3 months storage at 4°C	Serum stability of 5-FU-NPs		
			1 h	2 h	4 h
Drug : polymer ratio	1 : 10	1 : 10	1 : 10	1 : 10	1 : 0
PLGA grade	50 : 50	50 : 50	50 : 50	50 : 50	50 : 50
Size (nm)	118 ± 4.3	124 ± 2.3	104 ± 2.0	109 ± 1.2	111 ± 2.1
PDI	0.44 ± 0.05	0.39 ± 0.07	0.23 ± 0.16	0.25 ± 0.17	0.29 ± 0.21
Zeta potential (mV)	-28.2 ± 2.1				
Drug loading (DL%)	2.1 ± 0.01				
Encapsulation efficiency (EE, %)	56.0 ± 2.2				

DSC analysis of free 5-FU revealed a sharp endothermic peak at 281.39 °C (its melting point), whereas the endothermic peak of PLGA appeared at 46 °C. The disappearance of the melting point curve of 5-FU in 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs revealed the uniform amorphous distribution of 5-FU inside the nanoparticles (Fig. 3E).

The information obtained from XR-diffractograms agrees with the results obtained from DSC analysis (Fig. 3F). Free 5-FU showed distinct peaks in its XRD, revealing its crystalline nature. The disappearance of such peaks in nanoparticulated formulations suggested that 5-FU on encapsulation was converted from a crystalline to an amorphous state.

The release profile of 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs exhibited a biphasic drug release pattern consisting of initial rapid release followed by a slower continuous release over six days (Fig. 3G). The release of drugs from folate-peptide conjugated NPs followed a release profile more or less similar to that of unconjugated NPs. Sustained release of the drug over a longer time period is desirable, as it reduces the dosing frequency and also facilitates the accumulation of the drug in the tumor tissue by the enhanced permeation retention effect of the nanoparticulated delivery systems.

Cellular uptake study of FA-Pep-1 conjugated NPs

Confocal microscopy studies were performed to understand the cellular uptake of FA-Pep-1 conjugated and unconjugated nanoparticles in FR-positive cell lines using FITC, a fluorescence marker. Fig. 4A–C depict the satisfactory cellular internalization of both conjugated and unconjugated formulations, as evidenced by the remarkable bright green fluorescence of FITC, mostly localized around the nuclei in the cytoplasm.

Quantitative analysis of cellular uptake (after 30 min incubation) by FACS (histograms and bar diagrams, Fig. 4D–I and III) revealed a 5.78 times higher uptake of FA-Pep-1 conjugated nanoparticles in KB cells as compared to the unconjugated one. The above internalization by the cells decreased significantly in the presence of excess FA (Fig. 4D–II and III). Similarly, the cellular uptake of FA-Pep-1 conjugated nanoparticles were 5.59 and 2.9 times higher than those of the unconjugated one in HeLa and B16F10 cells (Fig. 4E–I and III and F–I and III), respectively. In both cases, uptake as verified by MFI measurement was significantly decreased in the presence of excess FA (Fig. 4E–II and III and F–II and III). FA in the medium prevented the uptake of FA-Pep-1 conjugated NPs by the cells through competitive binding to folate receptors present on the surface of the FR-positive cell lines. However, no remarkable difference in MFI was observed between the conjugated and unconjugated formulations with an FR-negative cell line (Fig. 4G–I and II).

In vitro therapeutic efficacy studies of free 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs

Cytotoxicity assay. *In vitro* cytotoxicity assay of free 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs in different cell

lines revealed a concentration-dependent decrease in cell viability upon treatment for 24 h (Fig. 5A). The IC₅₀ value of FA-Pep-1 conjugated 5-FU-NPs in B16F10 cells was found to be 2.00 and 3.75 times lower compared to unconjugated 5-FU-NPs and free 5-FU, respectively. In HeLa and KB cells also the IC₅₀ values of FA-Pep-1 conjugated NPs were found to be comparatively less than those of the free drug and unconjugated NPs (Table 4), demonstrating that folate peptide conjugation can improve the drug efficiency.

The results also revealed the selectivity of the formulations in targeting the folate receptor. The free drug, as well as conjugated and unconjugated nanoparticles loaded with 5-FU, exhibited pronounced cytotoxic potential against B16F10 cell lines, which is likely due to the higher activity of 5-FU against B16F10 than HeLa and KB cells. This prompted us to perform thorough biological studies in B16F10 tumor-bearing Balb/c mice.

Cell apoptosis assay. As depicted in Fig. 5B, FA-Pep-1 conjugated 5-FU-NPs (IC₅₀ dose) induced a remarkably high (about 21% each in early and late phase) apoptosis of B16F10 cells whereas in HeLa cells at early and late phases these values were 6.1% and 24.0%, respectively (Fig. 5D). A considerable amount of necrosis of the above two cell lines was also observed. Unconjugated-NPs, as well as free 5-FU, also exhibited significant early and late apoptosis, as well as necrosis of the above two cell lines at a much higher dose, compared to FA-Pep-1 conjugated NPs. However, all these effects were much pronounced in B16F10 cells than in HeLa cells. The results clearly showed that FA-Pep-1 conjugation produced a superior anticancer effect.

However, the IC₅₀ values of the free drug as well as 5-FU loaded folate peptide conjugated and unconjugated formulations were much higher in KB cells as compared to the rest (Table 4). This could be attributed to drug resistance resulting in the efflux of drug molecules by P-glycoprotein (P-gp) pumps.²⁹ Because of the significantly low cytotoxic effect, the above apoptosis study was not conducted in the KB cell line.

Damage to the nuclear structure in HeLa and B16F10 cells as a result of apoptosis was investigated by confocal microscopy following DAPI staining. The majority of the cells in the treated group exhibited brightly stained nuclei with condensed chromatin exhibiting crescent-shaped profiles, which are distinctive of apoptotic cell death. In contrast, rounded intact nuclei appeared in untreated control groups (Fig. 5C and E).

In vivo studies in B16F10 tumor-bearing Balb/c mice

FA-Pep-1 conjugated 5-FU-NPs exhibited considerable cytotoxicity and apoptosis of B16F10 cells. This allowed us to undertake biodistribution and scintigraphic studies of technetium-99m labeled 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs in a B16F10 melanoma tumor-bearing mice model to ascertain tumor localization.

^{99m}Tc labeled FA-Pep-1 conjugated 5-FU-NPs exhibited a considerably high tumor accumulation ($4.8 \pm 0.12\%$ ID g⁻¹) at 4 h post-injection, the tumor to muscle ratio was also

significantly high (8.82). In contrast tumor uptake was considerably less in animals injected with radiolabeled 5-FU-NPs [Fig. S-9(A)†]. The relatively high tumor uptake of ^{99m}Tc labeled FA-Pep-1 conjugated 5-FU-NPs also permitted clear vi-

sualization of tumor mass during scintigraphic studies under a gamma camera [Fig. S-9(B)†].

As the tumor accumulation of FA-Pep-1 conjugated 5-FU-NPs was considerably high, we investigated the

Fig. 4 Cellular uptake of FITC labeled formulations observed by confocal microscopy and flow cytometric studies in three different cell lines at a 30 min post incubation time period. Confocal images of FA-Pep-1 conjugated FITC-NPs in KB (A.I), HeLa (B.I) and B16F10 (C.I) cell lines and unconjugated FITC-NPs in KB (A.II), HeLa (B.II) and B16F10 (C.II) cell lines; flow cytometric distribution of FA-Pep-1 conjugated FITC-NPs and unconjugated FITC-NPs in KB (D.I), HeLa (E.I), B16F10 (F.I) and A-549 (G.I) cell lines. The corresponding blocking studies (pre-incubation with folic acid) are shown in D.II (KB), E.II (HeLa) and F.II (B16F10). The above studies are represented by bar diagrams D.III (KB), E.III (HeLa), F.III (B16F10) and G.II (A549).

Fig. 5 Cytotoxicity and apoptosis study results. (A) Dose-dependent cytotoxicity study results of 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs against B16F10, HeLa, KB and A-549 cell lines at 24 h; flow cytometric analyses of cellular apoptosis of 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs in (B) B16F10 melanoma cells and (D) HeLa cells using Annexin-V-FITC and PI staining. DAPI staining of B16F10 melanoma (C) and HeLa cells (E) exhibits bright fluorescence due to condensed chromatin (apoptotic condition).

antitumor efficacy of free 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs in B16F10 tumor-bearing mice. Images of intact animals captured prior to sacrifice revealed the suppression of tumor growth upon treatment (Fig. 6C and D). Reduction in tumor volume was noticeably high in animals receiving FA-Pep-1 conjugated 5-FU-

NPs followed by 5-FU-NPs and free 5-FU as compared to the control group (Fig. 6A). Considerable reduction in tumor weight was also observed in animals treated with either FA-Pep-1 conjugated 5-FU-NPs (1.275 ± 0.64 g) or 5-FU-NPs (1.785 ± 0.16 g) as compared to saline-treated control (6.25 ± 0.89 g) and free 5-FU treated (4.28 ± 0.39 g) groups (Fig. 6B). Thus nanoencapsulation resulted in significant tumor suppression. There was no remarkable loss of body weight of animals suggesting that no significant toxicity was induced by the formulation.

Microscopic images of H&E stained tumor sections collected from treated animals revealed loss of cellular architecture and tissue compactness as well as the appearance of vacuolar structures in the necrotic region. In contrast, the control group revealed typical pathological features of closely

Table 4 The IC₅₀ value of 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NPs at 24 h

Cell line	5-FU ($\mu\text{g mL}^{-1}$)	5-FU-NPs ($\mu\text{g mL}^{-1}$)	FA-Pep-1 conjugated 5-FU-NPs ($\mu\text{g mL}^{-1}$)
B16F10	2	0.8	0.4
HeLa	30	20	10
KB	350	300	250
A549	10	8	8

Fig. 6 *In vivo* study results on a B16F10 tumor-bearing Balb/c mice model. (A) Changes in tumor volume monitored after treatment with 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NPs for 21 days, data are the mean $\pm$ SD (5 animals per group); (B) tumor weight as determined in the control, 5-FU, 5-FU-NPs, and FA-Pep-1 conjugated 5-FU-NP treated mice after their sacrifice on the 31st day from tumor implantation; (C) photographs of Balb/c mice bearing melanoma tumor and (D) stripped tumor tissue collected after 21 days treatment; (E) H&E staining of tumor tissue sections collected from the control and from 5-FU, 5-FU-NPs and FA-Pep-1 conjugated 5-FU-NP treated groups on the 21st day from treatment initiation.

arranged tumor cells with a clear nuclear structure. Microscopic images of lung and liver tissue sections did not show any noticeable sign of organ damage (Fig. 6E).

Discussion

Two linear peptides Lys-Gly-His (Pep-1) and Arg-Arg-Arg-Arg-Lys-Gly-His (Pep-2) were synthesized based on literature reports on the potential of arginine or lysine-containing peptides for increasing tumor accumulation.¹⁴ Each peptide was conjugated separately with folic acid through the side chain amino function of the lysine unit (Fig. 7). ^{99m}Tc radiolabeling through the terminal histidine residue facilitated non-invasive detec-

tion of tumor accumulation by SPECT and allowed monitoring of 5-FU loaded NP localization in the tumor area.

5-FU has been used as the first line therapeutic agent in different advanced cancer treatment protocols for the last 40 years.³⁰ Biological screening revealed the higher tumor accumulation of FA-Pep-1 in B16F10 tumor-bearing Balb/c mice and HeLa tumor-bearing nude mice. This allowed us to decorate 5-FU loaded NPs with FA-Pep-1 to achieve targeted delivery of 5-FU to FR positive tumor.

Confocal microscopy studies revealed an accumulation of FITC labeled NPs within the cytoplasm of the FR positive cell nucleus. This accumulation was also quantitated by the FACS study. Fluorescence imaging revealed that cellular uptake was

Fig. 7 Chemical structure of peptides and folate conjugated peptides. (A) Pep-1; (B) FA-Pep-1; (C) Pep-2; (D) FA-Pep-2.

related to the FR-expression level and was most likely due to FR-mediated endocytosis. Although the uptake was highest in the KB cell line, MTT assay revealed a significant decrease in the cell viability of treated B16F10 cells compared to HeLa and KB cells which possibly have relatively low sensitivity to 5-FU. Due to the potential cytotoxic effect of FA-Pep-1 conjugated 5-FU-NPs (IC_{50} $0.4 \mu\text{g mL}^{-1}$) against the B16F10 cell line, both *in vivo* scintigraphy studies (after ^{99m}Tc radiolabeling) and therapeutic efficacy studies of unlabeled

nanoformulations were conducted on Balb/c mice bearing B16F10 solid tumor. Scintigraphic studies revealed the significant accumulation of ^{99m}Tc -labeled FA-Pep-1 conjugated 5-FU-NPs in the tumor area. The conjugated NPs also showed promising B16F10 tumor growth inhibition as compared to unconjugated and free drug formulation. These findings indicate that FA-Pep-1 conjugation profoundly improves the targeting strategy as well as the therapeutic efficiency of 5-FU-NPs.

Conclusion

Several ligand targeted drug delivery strategies using folate and RGD peptides are in progress. Peptide conjugation may achieve specific binding to cancer cells for targeted drug delivery to enhance anticancer effects with the intracellular release of cytotoxic drugs.

Statistical analysis

All experiments were repeated at least three times, each time on a different day. All mean values of animal experiments were expressed as %ID per g of tissue or organs $\pm$ SD. Statistical analysis was performed by one-way ANOVA. Graphs were prepared using GraphPad Prism 5 and ORIGIN 8.0.

Conflicts of interest

The authors have no conflict of interest with any person or party.

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