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Development of a peptide-based bifunctional chelator conjugated to a cytotoxic drug for the treatment of melanotic melanoma†

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The cytotoxic drug gemcitabine (GEM) has been conjugated to receptor-binding peptides to target melanoma tumors. A hexapeptide having a Lys–Gly–His–Lys sequence (pep-1), an octapeptide with an Arg–Gly–Asp–Lys–Gly–His–Lys sequence (pep-2), a GEM-conjugated Lys–Gly–His–Lys peptide (GEM-pep-3) and a GEM-conjugated Asp–Gly–Arg peptide (GEM-pep-4) were synthesized and characterized. *In vitro* uptake of fluorescently labeled GEM-pep-3 and GEM-pep-4 on B16F10 cells was investigated. Fluorescence microscopy studies demonstrated significant uptake of GEM-pep-3 in the B16F10 mouse melanoma cell line. The peptides and GEM-coupled peptides were radiolabeled with [^{99m}Tc(CO)₃(H₂O)₃]⁺ and examined for *in vitro* cell binding in the B16F10 melanoma cell line and *in vivo* biodistribution and scintigraphic studies in a B16F10 melanoma tumor-bearing mice model. *In vitro* cellular uptake studies and biological evaluation confirmed significant deposition of GEM-pep-3 at the melanoma tumor site. The MTT assay depicted higher cytotoxic behaviour of GEM-pep-3 than free GEM. A considerable amount of cell apoptosis was also observed in B16F10 cells. Finally, the *in vivo* therapeutic efficacy study revealed a significant decrease in tumor growth in the GEM-pep-3-treated animal model. These studies reveal enough potentiality of GEM-pep-3 to treat melanoma and underline the need for further evaluation.

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Introduction

Recent contribution of research in different areas of modern biology includes finding novel ways to suppress tumor growth using low-toxicity therapies. An effective therapeutic approach is to eliminate cancerous cells while sparing the normal cells. Conventional chemotherapeutics (*i.e.* DNA alkylating agents) target proliferating cancer cells. These may damage healthy growing cells but fail to eliminate quiescent or non-proliferating cancer cells. As many primary and metastatic human tumors are associated with overexpression of different endogenous regulating peptides,^{1–3} conjugation of cytotoxic drugs to receptor-binding peptides is an attractive approach of various cancer research laboratories.^{4,5}

Advancement in the field of peptide synthesis over the last few decades has resulted in the development of new tumor-specific peptides exhibiting high affinity to receptors for research, diagnosis and therapy.^{6–8} Small, synthetic, receptor-binding peptides are preferable over proteins and antibodies for tumor targeting because of their easy chemical modification, favourable pharmacokinetics (high uptake in target tissues, rapid clearance from blood and non-target organs), and broad range of biological activities against many chronic diseases.⁴ In recent years, specifically designed radiolabeled small peptides have been increasingly being used for diagnostic imaging and radionuclide therapy in nuclear oncology.^{6,9} Highly sensitive molecular imaging techniques like single-photon emission computed tomography (SPECT) and positron emission tomography (PET) can be used to monitor tumor localization, progression as well as therapeutic intervention and control.^{10,11} Somatostatin, bombesin, neurotensin, vasoactive intestinal peptide and many others are currently under investigation for possible clinical applications in the diagnosis and treatment of human cancers.^{4,6} The overexpression of somatostatin receptors on various cancers led to the development of US FDA-approved ¹¹¹In-labeled DTPA-octreotide (¹¹¹In-octreoscan) as a diagnostic radiopeptide for scintigraphic imaging of neuroendocrine tumor in patients.¹² A wide variety of chelating agents and

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prosthetic groups have also been developed in recent years to radiolabel peptides with various radionuclides for scintigraphic imaging (^{99m}Tc , ^{123}I , ^{68}Ga , ^{64}Cu) and therapy (^{90}Y , ^{177}Lu).^{13,14}

Integrins are a class of cellular receptors known to bind extracellular matrix proteins. Overexpression of $\alpha\beta$ -integrins is associated with tumor angiogenesis. These integrins could serve as sites for targeting peptides with RGD (arginine-glycine-aspartic acid) or NGR (asparagine-glycine-arginine) motifs to home to tumor cells or tumor-associated blood vessels. Several studies by different groups have been conducted regarding the development of radiolabeled RGD-containing peptides to target integrin expression in various tumors.^{15,16}

Secreted protein acidic and rich in cysteine (SPARC) is an important matricellular protein that modulates cell-matrix interactions, cell adhesion to the extracellular matrix, cell cycle progression and the cell shape. It binds to several growth

factors, alters their biological activity, and influences apoptosis. In many cases, it augments the apoptotic cascade and enhances the effect of cytotoxic agents used in the treatment of malignancies. The Lys-Gly-His-Lys copper binding motif of the SPARC domain has been shown to induce angiogenesis *in vitro* and *in vivo*.^{17,18}

Cytotoxic drugs coupled to Asp-Gly-Arg or Lys-Gly-His-Lys peptides can exhibit great promise in targeting angiogenic markers on tumor vasculature. Gemcitabine is a nucleoside analog used to treat different types of cancers, *e.g.* breast, ovarian, non-small cell lung, pancreatic and bladder cancer. However, it undergoes rapid metabolism by cytidine deaminase in plasma and readily excretes through the kidney which limits its efficacy.

Chemical modification of GEM to improve its pharmacokinetics and conjugation of GEM with tumor-specific peptides¹⁹ have been undertaken with the goal of improving its

Scheme 1 Synthesis of GEM-pep-3.

cellular retention after delivery. For example, GEM conjugated to a gonadotropin-releasing hormone receptor ligand peptide offered a potential role in prostate cancer treatment when compared to free GEM.^{20,21}

In the present study, we synthesised a hexapeptide bearing a Lys-Gly-His-Lys motif (pep-1), an octapeptide bearing an Arg-Gly-Asp-Lys-Gly-His-Lys motif (pep-2), a gemcitabine (GEM)-conjugated Lys-Gly-His-Lys peptide (GEM-pep-3) and a GEM-conjugated Asp-Gly-Arg peptide (GEM-pep-4). The terminal histidine residue attached to each peptide acts as a chelating ligand for radiolabeling with technetium-99m (^{99m}Tc). A tyrosine residue was also inserted between the peptide ligand and the chelating site because aromatic amino acids (phenylalanine and tyrosine), which are primarily LAT 1 substrates, have attracted immense interest for developing useful tumor imaging agents;^{22,23} tyrosine being hydrophilic is preferred to support renal elimination. The peptide was coupled (*via* Asp) to the well-characterized anticancer drug gemcitabine through chemical conjugation following a solid phase peptide synthesis protocol (Schemes 1 and 2). The pep-

tide acts as a cargo as well as a bifunctional ligand. It provides an aspartic acid side arm at one end to conjugate GEM under mild conditions, whereas histidine at the other end can complex with the robust technetium tricarbonyl [Tc(CO)₃]⁺ core with high specific activity under mild, aqueous conditions reasonably close to physiological pH, with a minimal need for purification. The cytotoxic drug conjugated with peptides can exhibit great potential for tumor targeting and therapy.

Materials and methods

General

All chemicals and solvents were of either analytical or HPLC grade and used without further purification. Gemcitabine hydrochloride was purchased from TCI Chemicals (India) Pvt. Ltd. Rink Amide MBHA resin (100–200 mesh), all side chain-protected amino acids, and TBTU [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] were purchased from Novabiochem (Switzerland). DIPEA (*N,N*-diisopropyl-

Scheme 2 Synthesis of GEM-pep-4.

ethylamine), picrylsulfonic acid, thioanisole and TFA (trifluoroacetic acid) were procured from Sigma (St. Louis, MO, USA). $^{99m}\text{TcO}_4^-$ was obtained by 2-butanone extraction from a 5(N) NaOH solution of $^{99}\text{MoO}_4^-$ (purchased from BARC, Mumbai, India). Electrospray ionisation mass spectrometry (ESI-MS) was performed on a Waters Micromass Q-ToF micromass instrument and MALDI was performed on a 4800 MALDI TOF-TOF (Applied Biosystems, USA). HRMS (m/z) were measured using an LTQ Orbitrap XL instrument (Thermo Fischer Scientific, USA). ^1H NMR spectra were recorded on a Bruker DRX 300 MHz spectrometer. All HPLC studies were performed on a Waters HPLC system using an XTerra Prep C-18 column (7.8×300 mm, $10 \mu\text{m}$ particle size). Radioactivity in the eluate was monitored using a Berthold LB500 HERM radio HPLC monitor procured from Berthold Technologies GmbH (Bad Wildbad, Germany). Tissue and organ radioactivities were measured using a well-type gamma counter of Electronics Corporation of India Limited, India (Model LV4755). A B16F10 mouse melanoma cell line was used to understand the tumor targetability of the radiopharmaceuticals. The national regulations for the care and use of laboratory animals have been 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 have also been approved by the Institutional Animal Ethics Committee.

Synthesis of the peptides

The linear peptides were synthesized manually by a solid phase methodology on Rink Amide MBHA resin (100–200 mesh) using the traditional Fmoc SPPS method. The Rink Amide MBHA resin (0.1 mmol g^{-1}) was first treated with a 20% (v/v) solution of piperidine in DMF for 10 min under a nitrogen atmosphere to remove the Fmoc group. The resin was thoroughly washed with DMF. Coupling was performed using a DIPEA/TBTU methodology. Briefly, a five-fold excess (0.5 mmol) of Fmoc-protected amino acid [Fmoc-Lys(Boc)-OH in the case of pep-1, Fmoc-Arg(Pbf)-OH in the case of pep-2, and Fmoc-Asp(Oall)-OH in the case of GEM-pep-3 and GEM-pep-4], TBTU (5 equivalent), and DIPEA (10 equivalent) were mixed in DMF (3 mL), added to the resin, stirred at room temperature (under a nitrogen atmosphere) for 60 min, and then successively washed with DMF (3×25 mL) and DCM (3×25 mL). Progress of the amino acid coupling was monitored by colour change of picrylsulfonic acid/ninhydrin/chloranil in acetaldehyde. Deprotection of the Fmoc group was achieved by treatment with 20% piperidine in DMF as described above. The process was repeated with the next Fmoc-protected amino acid in the sequence as per the method described earlier from this laboratory^{24,25} to obtain Lys-Gly-His-Lys-Tyr-His (pep-1), Arg-Gly-Asp-Lys-Gly-His-Lys-Tyr-His (pep-2), Asp(Oall)-Lys-Gly-His-Lys-Tyr-His-Fmoc and Asp(Oall)-Gly-Arg-His-Fmoc. Pep-1 and pep-2 were cleaved by a standard procedure using a cocktail containing

TFA, phenol, 1,2-ethanedithiol, triisopropylsilane and water (85:5:5:2.5:2.5) as per the method reported earlier,^{21,22} whereas pep-3 and pep-4 were kept bound to the resin for coupling with GEM. The crude peptides (pep-1 and pep-2) were precipitated with cold diethyl ether, dissolved in water, and purified by semi-preparative HPLC on a Waters HPLC system (XTerra Prep C-18 column, 7.8×300 mm, $10 \mu\text{m}$ particle size). The solvent system consisted of 0.1% TFA in H_2O (solvent A) and MeOH (solvent B). Elution was achieved by applying linear gradients, from 100% A to 85% A for pep-1, pep-2 and GEM-pep-4 and 90% A to 60% A for GEM-pep-3, in 25 min at a flow rate of 2 mL min^{-1} . Purity was assessed by analytical HPLC using an XTerra RP-18 column (4.6×250 mm, $5 \mu\text{m}$ particle size) eluted with the above described solvent system at a flow rate of 1 mL min^{-1} . Detection of the peptide was achieved with a Waters 2489 UV/visible detector. HPLC purification furnished pure pep-1 (0.24 g, 80%) and pep-2 (0.23 g, 76%). Finally, the molecular mass was determined by ESI mass spectrometry and HRMS. In the case of pep-1, ESI-MS detected a peak for $[\text{M} + \text{H}]^+$ at m/z 768.49 (calcd for $\text{C}_{36}\text{H}_{54}\text{N}_{12}\text{O}_7$: 766.89); similarly, in the case of pep-2, ESI-MS showed a peak for $[\text{M} + \text{H}]^+$ at m/z 1096.56 (calcd for $\text{C}_{48}\text{H}_{74}\text{N}_{18}\text{O}_{12}$: 1095.21). Next, for pep-1, HRMS (E1) m/z calculated for $\text{C}_{36}\text{H}_{54}\text{N}_{12}\text{O}_7$ $[\text{M} + \text{H}]^+$: 768.4249, found: 768.4080; similarly, for pep-2, HRMS (E1) m/z calculated for $\text{C}_{48}\text{H}_{74}\text{N}_{18}\text{O}_{12}$ $[\text{M} + \text{H}]^+$: 1096.5720, found: 1096.5580. Both peptides were also characterized by ^1H NMR, the results of which are given in the ESI.†

Synthesis of gemcitabine-peptide conjugates

For the synthesis of GEM-pep-3 and GEM-pep-4, the all-group of the Asp acid derivative was selectively removed by treating the peptidyl resin ($\sim 0.1 \text{ mmol}$) with 3 equivalents of tetrakis(triphenylphosphine)palladium $[(\text{Ph}_3\text{P})_4\text{Pd}]$ in chloroform/acetic acid/*N*-methylmorpholine (37:2:1) for 4 h at room temperature (under a nitrogen atmosphere). The resin was washed with DIPEA (0.5%) and sodium ethyl dithiocarbamate (0.5%) in DMF. The deprotected free β -carboxyl group of Asp acid was activated by treatment with HBTU (0.5 mmol, 0.159 g) and DIPEA (1 mmol, 0.160 mL) in DMF for 30 min, then gemcitabine hydrochloride (0.5 mmol) was added, and the mixture was kept for 24 h under nitrogen purging. The Fmoc group of the terminal amino acid histidine was removed with 20% piperidine in DMF, and the product was washed with DMF and DCM. Finally, the peptide was cleaved from the resin by treatment with a cocktail and purified by HPLC as per the above described method to afford the desired GEM-pep-3 (0.21 g, 70%) and GEM-pep-4 (0.22 g, 73%).

GEM-pep-3 was characterized by ^1H NMR (Fig. S9†); MALDI-TOF MS found a peak for $[\text{M} + \text{H}]^+$ at m/z 1128.63 (calcd for $\text{C}_{49}\text{H}_{68}\text{F}_2\text{N}_{16}\text{O}_{13}$: 1127.16). Next, for GEM-pep-3, HRMS (E1) m/z calculated for $\text{C}_{49}\text{H}_{68}\text{F}_2\text{N}_{16}\text{O}_{13}$ $[\text{M} + \text{H}]^+$: 1128.5121, found: 1128.6300.

Similarly, GEM-pep-4 was characterized by ^1H NMR (Fig. S10†) and ESI-MS found a peak for $[\text{M} + \text{H}]^+$ at m/z 728.39

(calcd for $C_{27}H_{39}F_2N_{13}O_9$: 727.68). Next, for GEM-pep-4, HRMS (E1) m/z calculated for $C_{27}H_{39}F_2N_{13}O_9$ $[M + H]^+$: 728.3628, found: 728.4220.

Synthesis of 5,6-carboxyfluorescein-conjugated GEM-pep-3 and GEM-pep-4

The N-terminal Fmoc protection on His of GEM-pep-3 and GEM-pep-4 peptides bound to Rink amide was removed by treatment with 20% piperidine in DMF followed by washing with DMF. A solution of 5(6)-carboxyfluorescein (0.188 g, 0.5 mmol), HOBT (0.076 g, 0.5 mmol) and DIC (0.063 g, 0.5 mmol) in DMF was added separately to the abovementioned GEM-pep-CONH-resin (~0.1 mmol) and the mixture was purged under nitrogen for 24 h. The resin was filtered, washed successively with DMF (5 mL $\times$ 3) and dichloromethane (2.5 mL $\times$ 2), and cleaved with a cocktail mixture (6 mL, 4 h) containing TFA, phenol, 1,2-ethanedithiol, triisopropylsilane and water (85:5:5:2.5:2.5). The crude peptide was precipitated with cold and dry diethyl ether, filtered, and purified by semi-preparative HPLC on the abovementioned Waters HPLC system using the eluent 0.1% TFA in H_2O (solvent A) and MeOH (solvent B) to afford the desired GEM-pep-3-CF (0.193 g, 64%), where MALDI-TOF MS found a peak for $[M + H]^+$ at m/z 1486.38 (calcd for $C_{70}H_{78}F_2N_{16}O_{19}$: 1485.93), and GEM-pep-4-CF (0.198 g, 66%), where MALDI-TOF MS found a peak for $[M + H]^+$ at m/z 1086.51 (calcd for $C_{48}H_{49}F_2N_{13}O_{15}$: 1085.72).

Cell uptake study: confocal microscopy and fluorescence-activated cell sorting (FACS)

Internalization of the GEM-coupled peptides was confirmed qualitatively by confocal laser scanning microscopy on the B16F10 mouse melanoma cell line following the protocol reported earlier.²⁶ B16F10 cells (1×10^5) were plated on a glass cover slip placed inside a 35 mm cell culture dish, grown overnight to attain 80% confluence, and then incubated with GEM-pep-3-CF and GEM-pep-4-CF solutions in media (final concentration: $20 \mu\text{g mL}^{-1}$) for 2 h and 4 h, respectively, at 37 °C. Following incubation, the medium was removed; the cells were washed with ice-cold PBS (three times) and subjected to nuclear staining with DAPI for 5 min. The cells were again washed with PBS (two times); the cover slips were mounted with 60% glycerol mounting medium on a slide and observed under a fluorescence microscope (Leica DFC420C, Germany) using an FITC filter (λ_{ex} = 492, λ_{em} = 518 nm).

The uptake of GEM-pep-3-CF and GEM-pep-4-CF in B16F10 mouse melanoma cells was also evaluated quantitatively by flow cytometry as per the reported method.²³ Cells were cultured in DMEM-high glucose supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% GlutaMAX, 50 mM HEPES, and 0.125% BSA at 37 °C in a humidified incubator under a 5% CO_2 atmosphere. For flow cytometry experiments, cells were seeded at a density of 1×10^6 cells per well in 6-well plates and incubated at 37 °C for 24 h. After incubation, the medium was replaced with GEM-pep-3-

CF and GEM-pep-4-CF solutions in media (the final concentration was $20 \mu\text{g mL}^{-1}$) and incubated for 0.5, 1, 2 and 4 h. After further incubation for specified periods, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) to remove unbound compounds and subjected to trypsinization and centrifugation at 254g for 3 min at 4 °C. Finally, cell pellets were resuspended in PBS (pH 7.4), fixed by slow addition of cold ethanol, and subjected to flow cytometric analysis (FACS Canto IITM cell sorter, BD Biosciences). Flow cytometer channel voltage and gain were kept constant throughout the analysis. Approximately 10 000 cell detection events were evaluated to assess the uptake of GEM-coupled peptides by cells. The increase of fluorescence intensity in the cells treated with GEM-coupled peptides relative to that in the untreated control cells was reported.

Hemolysis study

The hemolytic potentials of different peptide analogs were ascertained following incubation with freshly collected rat RBCs suspended in phosphate-buffered saline. Blood samples (~3 mL) were collected from male rats (Sprague-Dawley), taken into heparinised tubes and centrifuged for 5 min (1000g) at 4 °C. The supernatant was discarded; erythrocytes were washed three times with PBS (pH 7.4) and suspended in the same. In order to examine the hemolytic effect, 190 μL of the RBC suspension was added to each well of a 96-well plate followed by the addition of 10 μL of different peptide derivatives (pep-1, pep-2, GEM-pep-3 and GEM-pep-4) dissolved in PBS in increasing concentration (e.g. 0.5, 1, 2.5, 10, 15, 30, 50, 75, 100 μM). In the negative control (blank), only 10 μL of PBS was added to 190 μL of erythrocyte suspension, whereas 10 μL of Triton X-100 (10%) was added to the erythrocyte suspension to obtain the positive control. Following incubation at 37 °C for 1 h with gentle stirring, the unlysed erythrocytes were separated by centrifugation at 10 000g for 5 min and the optical density (OD) of the supernatant was measured at 570 nm. The value was compared with the OD of the supernatant in positive control series where full lysis (by Triton X-100) occurred and the % lysis was calculated. The experiments were performed in triplicate to obtain the average value.

In vitro therapeutic efficacy studies

Cytotoxicity assay. The effect of GEM, GEM-pep-3 and GEM-pep-4 on the viability of the B16F10 cell line was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.²⁷ B16F10 cells were cultured as a monolayer in 75 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 using the aforementioned media. The cells were harvested by trypsinization, seeded (1×10^4 cells per well) in 96-well tissue culture plates containing growth medium, and incubated for 24 h at 37 °C in humidified air containing 5% CO_2 . The cells were then exposed to series of concentrations (0.01 to 1 μM) of GEM, GEM-pep-3 and GEM-pep-4. After 72 h of incubation, the medium containing the drug was removed. Then, 20 μL of

MTT solution (5 mg mL⁻¹ in PBS) and 80 µL of medium were added to each well, followed by incubation for another 4 h at 37 °C. The medium was carefully removed from each well before the addition of dimethyl sulfoxide (150 µL), and the optical density (OD) at 540 nm was measured using a microplate reader (GENios, Austria). The percentage of surviving cells was calculated from the absorbance ratio of treated to untreated cells. The drug concentration in which cell growth was inhibited by 50% (IC₅₀) was determined from the dose response curve (Fig. 4B). All experiments were repeated in triplicate.

Cell apoptosis analysis

Cell apoptosis was determined using an Annexin V-FITC/PI double staining assay kit supplied by BD Biosciences, USA. In brief, B16F10 cells (1 × 10⁶ per dish) were cultured in a 60 mm dish to attain 80% confluency, treated separately with GEM and GEM-pep-3 at a concentration of 0.12 µM, and allowed to incubate for 72 h at 37 °C under 5% CO₂. Then, the cells were washed with PBS, trypsinized, and centrifuged (1000 × g) for 5 min. The cell pellets were resuspended in PBS, redispersed after final washing in binding buffer (0.5 mL), stained with FITC-Annexin V and PI for 30 min in the dark, and analysed using BD FACSDIVA VERSION 6.2 software to quantitate apoptotic, necrotic and living cells.

DAPI staining was carried out to ascertain the apoptotic effect of GEM and GEM-pep-3 on cell morphology. Cells were treated for 72 h as per the method described above, washed twice with PBS, and fixed in 70% ethanol (-20 °C) for 15 min. After washing with PBS (thrice), the cells were treated with DAPI solution (4 µg mL⁻¹) for 5 min at room temperature and imaged under a confocal laser scanning microscope (Leica, DFC 420C, Germany). The images were processed using Leica application suite software.

Radiolabeling

The *fac*-[^{99m}Tc(CO)₃(H₂O)₃]⁺ precursor was prepared as per the reported method.^{28,29} Briefly, an aqueous solution (1.5 mL) containing Na/K tartrate (15 mg), Na₂CO₃ (4 mg) and NaBH₄ (5.5 mg) was purged with carbon monoxide for 6–8 min at room temperature, followed by the addition of freshly extracted aqueous ^{99m}TcO₄⁻ (0.5 mL, 185–370 MBq), and the mixture was heated at 80 °C for 20 min. The pH was adjusted between 6.5 and 7 with a mixture of 0.5 M phosphate buffer (pH 7.5): 1 N HCl (1:3) and the purity of the tricarbonyl precursor was analysed by HPLC. Labeling was carried out by addition of the freshly prepared tricarbonyl precursor (0.3 mL) to a 0.5 mL aqueous solution of peptide ligands (1 mg mL⁻¹) and heating at 50 °C for 10–15 min. The radiolabeling yield and radiochemical purity were determined by running TLC in pre-coated silica gel plates using acetone as the mobile phase and also by HPLC (methods described above).

Physicochemical evaluation

In vitro stability studies. The *in vitro* stability of ^{99m}Tc(CO)₃-pep-1, ^{99m}Tc(CO)₃-pep-2, ^{99m}Tc(CO)₃-GEM-pep-3 and ^{99m}Tc(CO)₃-GEM-pep-4 chelates was tested separately in normal saline and freshly collected rat serum. The labeled complex (0.1 mL) was mixed with either normal saline (0.9 mL) or rat serum (0.9 mL) and incubated at 37 °C for 24 h. Samples were withdrawn from the mixture at regular intervals (0, 1, 2, 4, 6 and 24 h) and analyzed by TLC. The stability of the radiolabeled peptide ligands was also studied by histidine (His) challenge. To 0.1 mL of each of the radiolabeled peptides, 0.9 mL of 10⁻² M His solution in saline was added and the mixture was incubated for 24 h at 37 °C and analysed by TLC at the abovementioned time intervals. All the results were expressed as mean ± SD of three experiments.

Partition coefficient. To determine the lipophilicity, radiolabeled peptide analogs (0.1 mL, 2–3 kBq) were added to a test tube containing 2 mL of 1-octanol and 2 mL of phosphate buffer (0.025 M, pH 7.4) and the mixture was vortexed for 1 min. The phase was separated by centrifugation (5000 × g) for 5 min, and 0.1 mL aliquots of each phase were taken in triplicate. Radioactivities of the samples were measured using a gamma scintillation counter. The partition coefficient (*P*) was calculated as the mean value of counts per minute per mL of the octanol layer divided by that of the buffer layer. Lipophilicity was expressed as log *P*.

In vitro cell binding and internalization. The cell binding study in cellular melanoma cell lines was performed according to the method described previously with a few modifications.³⁰ Cells were cultured in the aforementioned DMEM as per the method described above, distributed in 6 well plates (1 × 10⁶ cells per well), and cultured overnight. On the day of the experiment, the medium was removed and the cells were washed once with DMEM binding medium (described above). Subsequently, 1 mL of internalization medium (DMEM containing 1% FBS) and 0.15 mL PBS (pH 7.4) containing 0.5% BSA were added to each well, and ^{99m}Tc-labeled peptide analogs (0.02 mL, 15–20 MBq mL⁻¹) were added as mentioned above, followed by incubation at 37 °C in 5% CO₂ for different periods of time. The internalization was terminated at specified time intervals (0.5, 1, 2 and 4 h) by withdrawing the medium followed by washing twice with ice-cold phosphate-buffered saline (pH 7.2). Cell surface-bound (acid-releasable) and internalized (acid-resistant) radioligands were distinguished by washing with cold glycine buffer (0.05 M glycine solution, pH adjusted to 2.8 with 1 N HCl) for 5 min. Finally, the cells were treated with 1 N NaOH at 37 °C for 10 min to detach them from the plates and the supernatant was collected by centrifugation. The radioactivity of every fraction was measured using a gamma scintillation counter and the percentage of internalized radioactivity was determined as follows:

$$\frac{\text{The radioactivity in the supernatant}}{\text{The total radioactivity associated with the cells (surface-bound + internalized)}} \times 100$$

All experiments were repeated three times and the results were expressed as the mean $\pm$ standard deviation.

In vivo evaluation

Blood clearance study in rats. Blood clearance of ^{99m}Tc -labeled peptide analogs was studied in healthy Sprague Dawley rats weighing 275–300 g ($n = 3$) as per the reported method.²¹ Briefly, radiolabeled samples (2–4 MBq, 0.05 mL) were administered to the anaesthetized animals through a precannulated femoral vein. Blood samples (0.2 mL) were withdrawn from another precannulated femoral vein at specified time intervals (5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min). The samples were weighed and radioactivity was measured using a gamma scintillation counter. The percent injected dose per g (% ID per g) of blood sample at each time interval was calculated and plotted against the respective time interval to give the blood disappearance curve.

Biodistribution studies. The biodistribution of $^{99m}\text{Tc}(\text{CO})_3^-$ -labeled pep-1, pep-2, GEM-pep-3 and GEM-pep-4 was evaluated in B16F10 tumor-bearing Balb/c mice (body weight = 25–30 g). B16F10 cells were grown in cell culture as described earlier and a cell suspension (2×10^7 cells per animal) in saline was implanted subcutaneously into the thigh of the right hind leg of each mouse. A palpable tumor developed after 15–20 days of inoculation and the animals were used for biodistribution studies. The animals were well hydrated by intraperitoneal administration of saline (0.9%, 2 mL) for 1 h followed by injection of the $^{99m}\text{Tc}(\text{CO})_3^-$ -labeled peptide derivatives (0.03 mL, 10–12 MBq kg^{-1}) *via* the tail vein. The animals (five mice per group at each time point) were sacrificed at the appropriate time points (2 and 4 h). The organs or tissues of interest were excised, washed with normal saline to free from adhering tissues, and transferred into counting vials. The urinary bladder was clamped, dissected, and carefully transferred into counting vials. Blood samples were

Fig. 1 Chemical structures of pep-1 (A), pep-2 (B), GEM-pep-3 (C) and GEM-pep-4 (D). MM2 energy-minimized probable structures of pep-1 (E), pep-2 (F), GEM-pep-3 (G) and GEM-pep-4 (H).

obtained by puncture of the heart. The samples were counted against a suitable diluted aliquot of the injected solution as the standard and the results were expressed as either % ID per g of tissue or % ID per organ.

Scintigraphic imaging studies in tumor-bearing mice. Balb/c mice bearing B16F10 tumor were injected intravenously *via* the tail vein with 50 μ L (4–8 MBq) of each radiolabeled compound per animal. The animals were anaesthetised (ketamine injection intramuscularly), placed under a GE Infinia gamma camera equipped with a Xeleris workstation, and static images were acquired at 4 h post injection.

Antitumor efficacy studies. B16F10 melanomal cells (1×10^6) suspended in PBS (0.1 mL) were injected subcutaneously into the right hind leg of Balb/c mice ($n = 15$) and the progress of tumor development and change in body weight were monitored. The length and width of the tumor were measured using a caliper, and the tumor volume was calculated using the formula $(\text{length} \times \text{width}^2)/2$. When tumor dimensions reached about 100 mm³ in volume, the animals were randomly divided into three groups ($n = 5$ in each group) and injected through the tail vein with saline (control), GEM, and GEM-pep-3. Treatment started from day 10, free drug and drug-conjugated peptide solution in saline were administered

at a dose of 25 mg kg⁻¹ GEM thrice weekly for 21 days. The abovementioned dose was selected from a previously reported procedure.²⁷ Tumor growth was measured regularly in each animal to determine tumor volume. Finally, the animals were sacrificed on the 31st day after cell implantation. Tumors were removed, weighed and fixed in 10% neutral buffered formalin for histological studies.

Results and discussion

Receptor-binding peptides can be used as vehicles to deliver cytotoxic agents selectively to tumor lesions. The progress of targeting and treatment can be monitored efficiently by labeling with gamma-emitting radionuclides. This work was focused on the development, characterization and evaluation of peptide-conjugated GEM analogs radiolabeled with ^{99m}Tc using the tricarbonyl labeling technique. The amino acid sequence of the peptide was based on the KGHK domain of SPARC peptides and the RGD domain of integrin receptor-binding peptides.

Peptide synthesis and radiolabeling

The peptides and GEM-conjugated peptides (Fig. 1) investigated in this study were conveniently and successfully

Fig. 2 (A) HPLC chromatograms (UV traces) of peptides and GEM-peptides. (B) Radio-HPLC elution profiles of ^{99m}Tc(CO)₃-labeled peptides and GEM-peptides.

prepared in satisfactory yield ($\sim 80\%$) by following an Fmoc-based solid phase peptide synthesis. The well-known anticancer agent GEM was taken because of its small size and chemical stability. GEM was attached by manual synthesis to the deprotected free β -COOH group of Asp acid attached to the peptide sequence in the presence of HBTU and DIPEA to form stable amide linkage (Schemes 1 and 2). A His residue was introduced at the N-terminal end of all peptide derivatives because of the ease of radiolabeling with the *fac*- $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ core. Fluorescently labeled GEM-pep-3 and GEM-pep-4 were prepared in moderate yield ($\sim 65\%$) by attaching 5,6-carboxyfluorescein (CF) manually to the terminal His residue of the peptide in the presence of DIC/HOBt. The purity of all peptide derivatives was confirmed by HPLC analysis ($\sim 95\%$) and authenticated by either ESI or MALDI-TOF mass spectrometry (Fig. S1–S6[†]). Fig. 2A represents the RP-HPLC elution profiles of the peptide derivatives.

The radiosynthon generated by Alberto *et al.* has widely been used for the successful labeling of bioactive molecules achieving relatively high specific activity with full retention of biological properties.²⁸ In this study, radiolabeling of all peptide derivatives was carried out by using the *fac*- $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ precursor. The synthon was successfully prepared in high yield ($>97\%$) as verified by HPLC by reducing $^{99m}\text{TcO}_4^-$ with sodium borohydride in the presence of CO (g) at atmospheric pressure. Radio-HPLC analysis revealed the formation of the precursor exhibiting a single peak

($t_R = 7$ min), whereas $^{99m}\text{TcO}_4^-$ had a retention time of 4 min under identical HPLC conditions. Complexation was performed by the addition of an aqueous solution of peptide ligands to the freshly prepared *fac*- $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ precursor. The temperature and pH of the reaction were maintained at 50°C and 6.5–7.0, respectively. HPLC analysis of the labeled complexes revealed the formation of single radioactive components with substantially high radiochemical purity ($\sim 95\%$) which remained stable for at least 24 h after radiolabeling. The donor groups of the N-terminal His residue of the peptides coordinate efficiently to the *fac*- $^{99m}\text{Tc}(\text{CO})_3$ core ensuring excellent labeling efficiency. The radiochemical purity of the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides was also ascertained by TLC in acetone. The radiochromatograms of $^{99m}\text{Tc}(\text{CO})_3$ -pep-1, $^{99m}\text{Tc}(\text{CO})_3$ -pep-2, $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-3 and $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-4 showed single peaks at 12, 11, 14 and 11.5 min, respectively (Fig. 2B). The radiolabeled peptide derivatives exhibited higher retention profiles than the corresponding unlabeled entities. Complexation with the robust synthon precursor resulted in significant changes in chromatographic mobility during RP-HPLC.

In vitro evaluation of gemcitabine-conjugated peptides

Cellular uptake studies. The cellular uptakes of both GEM-pep-3-CF and GEM-pep-4-CF were assessed qualitatively by fluorescence microscopy in B16F10 melanoma cells.

Fig. 3 Fluorescence microscopy evaluation of the uptake of (A) GEM-pep-3-CF and (B) GEM-pep-4-CF at 2 and 4 h in the B16F10 melanoma cell line. Nuclei (blue) were stained with DAPI. Flow cytometric measurements of B16F10 cells incubated with GEM-pep-3-CF (C) and GEM-pep-4-CF (D) at different time points. Flow cytometric distribution profiles of GEM-pep-3-CF (E) and GEM-pep-4-CF (F) at different time points represented by bar diagrams.

Fluorescence microscopy is a powerful tool for determining the cellular localization of the pharmacophore. To monitor the cellular uptake, GEM-coupled peptides were labeled with 5,6-carboxyfluorescein (CF). To confirm the cellular internalization of the coupled peptides, B16F10 cells were incubated separately with GEM-pep-3-CF and GEM-pep-4-CF for 4 h. Cellular images are shown in Fig. 3A and B. The pharmacophore internalized into the cells giving bright green fluorescence and the nucleus appeared blue due to DAPI staining. For both compounds, pronounced internalization was observed at 4 h. However, cells incubated with the more cytotoxic GEM-pep-3 pharmacophore exhibited more intracellular cytoplasmic accumulation (Fig. 3A) when compared with those incubated with GEM-pep-4 (Fig. 3B).

Quantitative analysis of intracellular uptake behaviour of the GEM-coupled peptides was also performed in the B16F10 cell line using a flow cytometer. The relative extent of cellular uptake is presented in terms of mean fluorescence intensity (MFI) exhibited by the cells. Time-dependent cellular uptake

was evaluated and the maximum uptake was observed at 4 h. The MFI measured after 4 h of incubation with GEM-pep-3 was 4.4 times higher than that measured after 30 min of incubation, whereas it was only 1.8 times higher in the case of GEM-pep-4. The results clearly indicate substantially high cellular uptake of GEM-pep-3 (Fig. 3C and E) in comparison to GEM-pep-4 (Fig. 3D and F). The increased intracellular accumulation may lead to enhanced therapeutic efficacy.

Hemolytic activity. Cytotoxicity tests of the compounds towards rat erythrocytes were also performed. The hemolytic activity of the peptides and GEM-conjugated peptides at different concentrations, when incubated with rat erythrocytes, is depicted in Fig. 4A. The percentage hemolytic activity was calculated relative to 10% Triton X-100. No appreciable hemolysis (5–6% only) was observed with the compounds up to 50 μ M concentration, and this became around 7–9% when the concentration was doubled. Significantly low hemolytic activity indicates minimum chances of lysis of red blood cells and the formulations could be safe for intravenous administration.

Fig. 4 (A) Hemolytic activity profiles of pep-1 (—●—), pep-2 (—■—), GEM-pep-3 (—▲—) and GEM-pep-4 (—▼—). (B) MTT assay of GEM (—●—), GEM-pep-3 (—▲—) and GEM-pep-4 (—▼—) on B16F10 cells at different concentrations at 72 h. (C) Flow cytometric detection of apoptotic potential in control (saline), GEM- and GEM-pep-3-treated (IC_{50} dose) B16F10 cells using Annexin V-FITC and PI staining. (D) DAPI staining showing nuclear fragmentation (apoptotic conditions) in B16F10 melanoma cells treated with GEM and GEM-pep-3.

In vitro stability of $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptide derivatives

The *in vitro* stability of $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and gem-peptides incubated (37 °C) with normal saline or freshly collected rat serum was monitored by TLC up to 24 h at various intervals. The radiolabeling yields of the complexes were $\geq 95\%$, which became $\geq 92\%$ after 24 h of incubation in saline and serum (Table S2†). This indicates substantially high stability of the radiolabeled peptide analogs. Preferential high stability in serum also indicated minimum proteolytic degradation of the peptide backbone by plasma proteases. The $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptide complexes were also challenged with excess histidine to determine their stability against ligand exchange and decomposition. Known amounts of radiolabeled peptide derivatives were incubated with excess histidine and the samples were analysed by TLC at various time intervals up to 24 h. All the complexes exhibited about 5% decomposition after 24 h of incubation. The higher resistance ability of both free peptides and GEM-coupled peptides to histidine displacement indicates less possibility of transchelation of ^{99m}Tc to other high-affinity binding sites during *in vivo* studies. Chances of transfer of the radiolabel to other competing ligands *in vivo* are also minimal.

Lipophilicity and blood clearance

The lipophilic characteristic of the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and GEM-coupled peptides is important in determining the excretion pathway. Lipophilicity was determined by measuring the distribution of radiolabeled complexes between *n*-octanol and saline. $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-4, being the most hydrophilic, exhibited the lowest log *P* (−1.771) followed by $^{99m}\text{Tc}(\text{CO})_3$ -pep-2 (−1.339), $^{99m}\text{Tc}(\text{CO})_3$ -pep-1 (−0.978) and $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-3 (−0.629). Log *P* values as well as retention times of the radiolabeled complexes in RP-HPLC suggest low to moderate lipophilicity, predicting their excretion through the renal-urinary pathway.

The log *P* values in the negative side of the scale indicate the hydrophilic nature of the complexes. Because of this hydrophilic property, the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and GEM-coupled peptides cleared readily from the blood stream and showed biphasic blood disappearance profiles (Fig. 5A), an initial fast phase followed by a slow second phase.

Cell uptake and internalization analysis of radiolabeled peptide analogs

The uptake and internalisation of the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and GEM-coupled peptides at 37 °C were assessed in B16F10 melanoma cells for various time intervals. Fig. 5B and C depict the results of this study. After 30 min of incubation, the cellular uptakes observed for radiolabeled pep-1, pep-2, GEM-pep-3 and GEM-pep-4 were 4.76, 3.99, 4.99 and 2.9% of the total added activity, respectively. These values became 9.59, 6.11, 9.87 and 4.99%, respectively, after 240 min of incubation. However, in relation to the above cell associated activity after 30 min of incubation, 41.41% of $^{99m}\text{Tc}(\text{CO})_3$ -pep-1, 35.24% of $^{99m}\text{Tc}(\text{CO})_3$ -

pep-2, 42.34% of $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-3 and 30.45% of $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-4 were internalized, and this became maximum around 83, 76, 80 and 65%, respectively, after 240 min of incubation. The lysosomal proteases inside the cell probably degrade the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptide conjugate into peptide fragments. The internalized count could be due to the presence of metabolized ^{99m}Tc -peptide fragments within the lysosome.³¹

Animal biodistribution and scintigraphic studies

Tissue distribution studies of the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and GEM-coupled peptides in B16F10 melanoma tumor-bearing mice were conducted to ascertain localisation of the peptide analogs in tumor muscle. The uptakes (% injected dose per gram of tissue or organ) of the ^{99m}Tc -labeled peptide analogs in the urine, kidney, liver, intestine, blood, lung, spleen, stomach, normal and tumor muscle at 2 h and 4 h post injection (p.i.) time periods were determined (Table 2). The ratio of percentage injected dose per g of tumor developed in the right thigh muscle/control left thigh was then determined. The tumor uptakes of $^{99m}\text{Tc}(\text{CO})_3$ -labeled pep-1, pep-2, GEM-pep-3 and GEM-pep-4 were 1.83 ± 0.21 , 0.92 ± 0.29 , 1.717 ± 0.26 and $0.82 \pm 0.16\%$ ID per g of tumor muscle, respectively, at 2 h p.i., which at 4 h p.i. became 3.01 ± 0.38 , 1.09 ± 0.34 , 2.81 ± 0.32 and $0.83 \pm 0.16\%$ ID per g. The accumulation of radioactivity per g of tumor muscle was the highest in the case of $^{99m}\text{Tc}(\text{CO})_3$ -pep-1. Consequently, GEM coupled to the above peptide (GEM-pep-3) also exhibited substantial localisation in the tumor area, while $^{99m}\text{Tc}(\text{CO})_3$ -pep-2 exhibited moderate accumulation. The accumulation was lower in the case of $^{99m}\text{Tc}(\text{CO})_3$ -GEM-pep-4 (approximately 0.82–0.83% ID per g of tumor muscle throughout the period of study).

The biodistribution studies also revealed efficient clearance of the radiolabeled conjugates from the blood stream within 4 h post injection. $^{99m}\text{Tc}(\text{CO})_3$ -labeled pep-1 and GEM-pep-3 exhibited comparatively high tumor-to-muscle ratios (10.11 at 2 h and 18.81 at 4 h for pep-1; 10.64 at 2 h and 18.73 at 4 h for GEM-pep-3) as well as tumor-to-blood ratios (0.97 and 1.82, respectively, at 2 h and 4 h for pep-1; 0.95 and 1.77, respectively, at 2 h and 4 h for GEM-pep-3). Pep-2 is the RGD-peptide coupled to pep-1 and GEM-pep-4 is GEM conjugated to RGD. Both $^{99m}\text{Tc}(\text{CO})_3$ -labeled pep-2 and GEM-pep-4 exhibited comparatively lower tumor uptake as well as tumor-to-muscle and blood ratios than radiolabeled pep-1 and GEM-pep-3. All four $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptide derivatives excreted preferentially through the renal pathway (kidney + urine). Around 60–64% excretion was observed through this pathway during the 2–4 h study period. The compounds also exhibited some hepatic clearance (liver + intestine). About 23–28% of the injected dose cleared through the above pathway during the 2 to 4 h post injection time period. The uptake in other non-target organs was insignificant. Accumulation of a low amount of radioactivity in the stomach precludes the presence of free pertechnetate and indicates

Fig. 5 (A) Blood clearance profiles of $^{99m}\text{Tc}(\text{CO})_3$ -labeled pep and GEM-pep in mice after i.v. administration. Data shows mean $\pm$ SD ($n = 5$). (B) % cellular uptake and (C) % internalization of $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptides and GEM-peptides in B16F10 melanoma cells; normalized to 10^6 cells per well. (D) Scintigraphic images of $^{99m}\text{Tc}(\text{CO})_3$ -labeled pep-1 (I), pep-2 (II), GEM-pep-3 (III) and GEM-pep-4 (IV) in B16F10 tumor-bearing Balb/c mice at 4 h post injection time periods.

the *in vivo* stability of the radiotracers. Rapid and efficient clearance of radioactivity from most of the organs demonstrated the favourable biodistribution profile of the radiolabeled peptide analogs.

The tumor targeting efficacy of the $^{99m}\text{Tc}(\text{CO})_3$ -labeled peptide conjugates in a B16F10 melanoma tumor-bearing mice model was also evaluated by performing SPECT imaging under a gamma camera at 4 h post injection. Planar images of the tumor-bearing mice administered with ^{99m}Tc -labeled different peptide conjugates at 4 h post injection are shown in Fig. 5D. $^{99m}\text{Tc}(\text{CO})_3$ -pep-1 and GEM-pep-3 exhibited appreciable tumor uptake and noticeably high tumor-to-background contrast. But the uptake was comparatively lower in the case of technetium-labeled pep-2 and GEM-pep-4. However, in all cases, high levels of background activity were associated with the kidney, liver, gastrointestinal tract and urinary bladder. All these are in concurrence with the results obtained from the biodistribution studies.

Table 1 IC_{50} values of GEM and GEM-conjugated peptides in B16F10 cells

Compound	IC_{50} (μM)
GEM	0.260 ± 0.005
GEM-pep-3	0.120 ± 0.004
GEM-pep-4	0.210 ± 0.007

Therapeutic efficacy studies

Cytotoxicity study. The cytotoxicity of GEM, GEM-pep-3 and GEM-pep-4 was tested on B16F10 melanoma cells. Cells were treated with increasing concentration ($0.01 \mu\text{M}$ to $1 \mu\text{M}$) of the compounds and incubated for 72 h at 37°C . After incubation, cellular viability was evaluated by MTT assay (Fig. 4B). Inhibition of growth was measured in terms of IC_{50} , expressed in μM concentration. As shown in Table 1, the IC_{50} of GEM was $0.26 \mu\text{M}$, whereas those of GEM-pep-3 and GEM-pep-4 were $0.12 \mu\text{M}$ and $0.21 \mu\text{M}$, respectively. In general, both GEM-pep-3 and GEM-pep-4 exhibited higher cytotoxicity than the free drug; the IC_{50} value of GEM-pep-3 was more than two times lower than that of the free drug.

Apoptosis assay. From the cytotoxicity studies, it is evident that the IC_{50} dose ($0.12 \mu\text{M}$) of GEM-pep-3 is lower than that of GEM-pep-4 ($0.21 \mu\text{M}$). This encouraged us to determine the role of GEM-pep-3 in cellular apoptosis by Annexin V-FITC and PI staining in B16F10 melanoma cells. Apoptosis is a programmed cell death that plays an important role in tissue homeostasis. GEM-pep-3 induced significant apoptosis (61%) in B16F10 cells, whereas 26% apoptosis was observed when treatment was carried out with the free drug (Fig. 4C). Both compounds exhibited some necrotic effect (12–14%). The effect of GEM on cellular apoptosis was relatively lower than that of GEM-pep-3, which could induce better anticancer effects than GEM. Moreover, fluorescence microscopy

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

Organ	$^{99m}\text{Tc}(\text{CO})_3\text{-pep-1}$		$^{99m}\text{Tc}(\text{CO})_3\text{-pep-2}$		$^{99m}\text{Tc}(\text{CO})_3\text{-GEM-pep-3}$		$^{99m}\text{Tc}(\text{CO})_3\text{-GEM-pep-4}$	
	2 h	4 h	2 h	4 h	2 h	4 h	2 h	4 h
Heart	0.08 $\pm$ 0.001	0.06 $\pm$ 0.002	0.09 $\pm$ 0.002	0.07 $\pm$ 0.003	0.06 $\pm$ 0.003	0.04 $\pm$ 0.001	0.07 $\pm$ 0.004	0.05 $\pm$ 0.002
Blood ^a	1.88 $\pm$ 0.21	1.65 $\pm$ 0.12	1.50 $\pm$ 0.17	1.31 $\pm$ 0.08	1.79 $\pm$ 0.23	1.58 $\pm$ 0.16	1.13 $\pm$ 0.19	0.88 $\pm$ 0.12
Liver	18.15 $\pm$ 1.51	14.64 $\pm$ 1.62	21.17 $\pm$ 1.61	18.89 $\pm$ 1.49	17.65 $\pm$ 1.22	15.62 $\pm$ 1.37	16.89 $\pm$ 0.9	14.39 $\pm$ 0.99
Lungs	0.33 $\pm$ 0.07	0.24 $\pm$ 0.05	0.25 $\pm$ 0.06	0.19 $\pm$ 0.01	0.40 $\pm$ 0.09	0.25 $\pm$ 0.002	0.32 $\pm$ 0.07	0.20 $\pm$ 0.03
Spleen	0.63 $\pm$ 0.12	0.45 $\pm$ 0.07	0.76 $\pm$ 0.11	0.54 $\pm$ 0.03	0.69 $\pm$ 0.15	0.45 $\pm$ 0.18	0.44 $\pm$ 0.07	0.26 $\pm$ 0.13
Muscle ^a	0.18 $\pm$ 0.001	0.16 $\pm$ 0.007	0.16 $\pm$ 0.002	0.14 $\pm$ 0.002	0.17 $\pm$ 0.001	0.15 $\pm$ 0.001	0.17 $\pm$ 0.02	0.17 $\pm$ 0.01
Tumor muscle ^a	1.83 $\pm$ 0.21	3.01 $\pm$ 0.38	0.92 $\pm$ 0.29	1.09 $\pm$ 0.34	1.71 $\pm$ 0.26	2.81 $\pm$ 0.32	0.82 $\pm$ 0.16	0.83 $\pm$ 0.16
Kidney	17.15 $\pm$ 1.26	15.76 $\pm$ 1.57	16.12 $\pm$ 1.31	13.91 $\pm$ 1.29	18.96 $\pm$ 1.32	16.25 $\pm$ 1.31	4.44 $\pm$ 1.20	3.24 $\pm$ 0.26
Intestine	7.61 $\pm$ 0.52	8.32 $\pm$ 0.67	7.5 $\pm$ 0.19	6.30 $\pm$ 0.13	8.51 $\pm$ 0.54	6.28 $\pm$ 0.26	7.90 $\pm$ 0.50	9.06 $\pm$ 0.28
Stomach	0.23 $\pm$ 0.031	0.19 $\pm$ 0.022	0.29 $\pm$ 0.033	0.21 $\pm$ 0.026	0.25 $\pm$ 0.035	0.17 $\pm$ 0.023	0.36 $\pm$ 0.026	0.26 $\pm$ 0.24
Urine	42.15 $\pm$ 0.53	49.26 $\pm$ 0.51	41.15 $\pm$ 0.26	45.95 $\pm$ 0.71	41.21 $\pm$ 0.62	48.21 $\pm$ 0.81	53.11 $\pm$ 2.4	56.11 $\pm$ 1.18
Tumor/blood	0.97	1.82	0.61	0.83	0.95	1.77	0.72	0.94
Tumor/muscle	10.11	18.81	5.75	7.78	10.64	18.73	4.66	4.89

^a Percent injected dose per gram.

studies after DAPI staining of untreated as well as GEM- and GEM-pep-3-treated cells clearly exhibited nuclear fragmentation in treated B16F10 cells, which is a hallmark of cellular apoptosis (Fig. 4D). The highest number of fragmented structures and bubble-shaped nuclei was observed in the cells

treated with GEM-pep-3 as compared to cells treated with GEM. Thus, it could be concluded that GEM-pep-3 induced significant apoptosis as well as pronounced nuclear condensation in B16F10 cells. The biodistribution studies also revealed preferential accumulation of radiolabeled GEM-pep-3

Fig. 6 *In vivo* antitumor efficacy of GEM-pep-3 on a B16F10 melanoma tumor model. (A) Photograph of mice bearing melanoma tumor and (B) stripped tumor tissue. (C) Change of tumor volume upon treatment with GEM and GEM-pep-3 for 21 days; data shows mean $\pm$ SD ($n = 5$). (D) Tumor weight as determined in control, GEM- and GEM-pep-3-treated mice after their sacrifice on the 31st day from tumor implantation. (E) Body weight of animals on the day of tumor implantation (initial) and on the day of sacrifice (final) and % of tumor inhibition in the treated groups. (F) H & E staining of tumor tissue sections from control, GEM- and GEM-pep-3-treated groups at 21 days after treatment.

in the tumor area. This makes GEM-pep-3 an excellent candidate for targeted therapy. An antitumor efficacy study in an animal model was therefore conducted.

Antitumor efficacy studies. To understand whether the potent cytotoxic and apoptotic effect of GEM-pep-3 against B16F10 melanoma cells can be reproduced *in vivo*, Balb/c mice bearing melanoma tumor were treated with GEM and GEM-pep-3 at a dose of 25 mg kg⁻¹ GEM, thrice a week starting from day 10 after tumor implantation. Suppression of tumor growth was continuously monitored by measuring the tumor volume. It was observed that the tumor volume shrank in the animals receiving either GEM or GEM-pep-3 in comparison with the control group receiving saline (Fig. 6C). Substantial reduction in tumor volume was observed in mice treated with GEM-pep-3. It was 1.68 times more effective than GEM in inhibiting melanoma growth on day 31. Moreover, the dose of GEM in the formulation was low enough to produce any significant toxicity and no significant change in body weight of the animal was observed during the treatment schedule. The findings presented here also indicate that GEM-pep-3 exhibited potential melanoma suppression at a considerably low dose.

Tumor tissue was further investigated by HE staining (Fig. 6F). Histological sections of tumors (control group) showed appreciable tumor development with the formation of new blood vessels compared to those in the treated groups. All these observations reveal potential antitumor efficacy of GEM-pep-3 as compared to free GEM which was consistent with the *in vitro* experiments.

Conclusions

Cellular uptake and tissue biodistribution of peptide-conjugated gemcitabine were quantitatively measured after ^{99m}Tc-radiolabeling and intracellular localization was qualitatively confirmed by fluorescence microscopy following CF attachment. Confocal microscopic analysis visually confirmed substantial intracellular localization of GEM-pep-3 which correlated directly with scintigraphic and radiometric data. MTT assay and cellular apoptosis studies also revealed the antitumor potential of GEM-pep-3, which was successfully verified in a melanoma tumor-bearing animal model. The present data provides useful insight for designing and developing peptide-based bifunctional ligands to deliver cytotoxic cargos to tumor that overexpresses specific receptors for the peptides. Radiolabeling of this cytotoxic pharmacophore with a therapeutic radionuclide, *e.g.* Re-188, and evaluation of its potential as a therapeutic radiopharmaceutical are in progress. Synthesis of cytotoxic drugs attached to folic acid-conjugated peptides to target tumor cells by folate receptor-mediated endocytosis is also underway.

Conflicts of interest

The authors declare no competing interest.

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