^{99m}Tc-labeled NGR-Chlorambucil Conjugate, ^{99m}Tc-HYNIC-CLB-c(NGR) for Targeted Chemotherapy and Molecular Imaging

Short title: ^{99m}Tc-labeled peptide drug conjugate, ^{99m}Tc-HYNIC-CLB-c(NGR)

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Abstract

Targeted delivery of chemotherapeutic drug at the tumor site enhances the efficacy with minimum systemic exposure. Towards this drugs conjugated with peptides having affinity towards a particular molecular target are recognized as affective agents for targeted chemotherapy. Thus in the present study tumor homing NGR peptide ligand was conjugated to DNA alkylating nitrogen mustard, chlorambucil (CLB). The peptide drug conjugate (PDC), CLB-c(NGR) was radiolabeled with ^{99m}Tc-HYNIC core to trace its pharmacokinetics and biodistribution pattern. *In vitro* cell binding studies of ^{99m}Tc-HYNIC-CLB-c(NGR) were carried out in murine melanoma B16F10 cells. The cytotoxicity studies carried out by incubation of the peptide/ drug/ peptide-drug conjugate with B16F10 cells demonstrated enhanced cytotoxic effect of PDC in comparison to either the peptide or the drug alone. *In vivo* biodistribution studies in C57BL6 mice bearing melanoma tumor showed maximum tumor uptake at 30 min p.i. (2.45 ± 0.28% ID/g) which reduced to 0.77 ± 0.1% ID /g at 3 h p.i. The radiotracer being hydrophilic cleared rapidly from heart, lungs, liver and muscle. The tumor-to-blood ratio and tumor-to-muscle ratios improved with time. This study opens avenues for conjugation of other targeting peptides with the drug chlorambucil for enhanced toxicity at the diseased site.

Keywords: Chlorambucil, NGR peptide, peptide-drug conjugate, ^{99m}Tc-HYNIC, cytotoxicity

1. Introduction

Chemotherapy is an important clinical tool for treatment of different types of cancers. However chemotherapeutic drugs do not spare normal cells during their attack at malignant sites posing limitation on the prolonged drug therapy needed in case of metastatic patients. The high dose administration of drugs during conventional chemotherapy is responsible for systemic toxicity and various other side-effects. Drugs conjugated with the targeting vectors like antibodies and peptides can serve as conduits for site-specific delivery and thus overcome the problem of non-specific accumulation of drugs in normal tissues.[1-3] The tumor vasculature (tumor homing) targeting arginine-glycine-aspartic acid (RGD) and asparagine-glycine-arginine (NGR) peptides have been explored and identified as effective drug carriers.[4-6] The RGD and NGR peptides conjugated with human tumor necrosis factor (hTNF) are reported to exhibit enhanced antitumor action at smaller doses.[7-9] The peptide–drug conjugates (PDCs) can thus precisely deliver the drug to the cancer cells and provide more efficacious results. The peptide-drug conjugate (PDC), NGR-hTNF is under clinical investigation for malignant pleural mesothelioma, colorectal, lung (small-cell and non-small-cell), liver and ovarian cancer, and soft tissue sarcomas.[10-12]

Chlorambucil (CLB) is a widely used chemotherapeutic drug in the treatment of chronic lymphocytic leukemia, lymphomas, breast and ovarian carcinoma and a variety of other solid tumors.[13,14] Chlorambucil, 4-{4-[Bis(2-chloroethyl)amino]phenyl}butyric acid with two reactive chloroethyl side chains belongs to the group of alkylating agents referred to as nitrogen mustards.[14,15] Chlorambucil exerts its cytotoxic action by forming inter-strand cross-links in DNA through alkylation at N7 of guanine base.[15,16] The goal of this study was to prepare NGR-chlorambucil conjugate for targeted chemotherapy enhancing clinical benefit of the drug. Radiolabeled PDCs can provide insight into the pharmacokinetics of PDC and the drug therapy response through non-invasive molecular imaging of tumor sites. ^{99m}Tc is an

ideal radioisotope for diagnostic purposes due to its favourable decay characteristics ($t_{1/2}$ = 6 h, 140 keV).[17,18] Amongst the various approaches available for ^{99m}Tc-labeling we focused on ^{99m}Tc-HYNIC core (HYNIC = 6-hydrazinonicotinic acid) as it offers high labeling efficiency and allows for preparation of molecular imaging probes with high specific activity and promising pharmacokinetic profile.[19,20] HYNIC also has an advantage of simple and easier conjugation with peptides either on-resin during solid phase peptide synthesis or in solution phase.[21-23]

In the present study cyclic NGR peptide, cKCNGRC was synthesized manually using standard Fmoc solid phase peptide synthesis. The peptide was then conjugated with the drug, chlorambucil and subsequently functionalized with HYNIC chelator. The peptide-drug conjugate, HYNIC-chlorambucil-cKCNGRC [HYNIC-CLB-c(NGR)] was radiolabeled with ^{99m}Tc. The radiotracer, ^{99m}Tc-HYNIC-chlorambucil-cKCNGRC [^{99m}Tc-HYNIC-CLB-c(NGR)] was investigated for its efficacy as tumor targeted molecular imaging probe. The *in vitro* cell binding study and cytotoxicity studies were carried out in human melanoma B16F10 cells. *In vivo* biodistribution studies were performed in C57BL6 mice bearing melanoma tumor.

2. Materials and Methods

2.1 General

The amino acid derivatives Nα-Fmoc-Nγ-trityl-L-asparagine, Nα-Fmoc-NΩ-(2,2,4,6,7-pentamethyl-2,3-dihydrobenzo[b]furan-5ylsufonyl)-L-arginine, Nε-Boc-Nα-Fmoc-L-lysine were purchased from Alfa Aesar, USA. Fmoc-Cys(Acm)-OH and Fmoc-Gly-OH were purchased from Sigma Aldrich. NovaSyn TGR resin was procured from Novabiochem, Germany. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) was procured from Alfa Aesar, USA. Chlorambucil, N,N-diisopropylethylamine (DIPEA), Piperidine, 6-chloronicotinic acid, di-tert-butyl dicarbonate,

sodium dihydrogen phosphate, disodium hydrogen phosphate, ethylenediamine diacetic acid (EDDA), N-[Tris(hydroxymethyl)methyl]glycine (Tricine) and hydrogen chloride solution 4.0 M in dioxane were procured from Sigma Aldrich, USA. Stannous chloride dihydrate was procured from Fluka. The peptide drug conjugate HYNIC- CLB-c(NGR) was purified using a semi-preparative HPLC system (JASCO, Japan) connected with JASCO-PU-2086 Plus, intelligent prep pump, JASCO UV-2075 Plus absorption detector and having a Megapak Sil C18-10 column (7.5 × 250 mm). The analytical HPLC measurements were performed on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and a Gina Star radiometric detector system, using a C_{18} reversed phase HiQ Sil column (5 μm, 4 x 250 mm). The eluting solvents (1 mL/min) used in HPLC were; H_2O (solvent A) and acetonitrile (solvent B) with 0.1% trifluoroacetic acid following the gradient: 0-3 min: 0% B; 3-5 min: 0-20% B; 5-10 min: 20% B; 10-20 min: 20-90% B; 20-22 min: 90-0% B; 22-30 min: 0% B. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis was carried out at the Mass spectrometry facility of Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC).

2.2 Synthesis

2.2.1 Synthesis of succinimidyl-6-hydrazinopyridine-3-carboxylate hydrochloride (NHS-HYNIC)

Synthesis of NHS-HYNIC was carried out following a reported procedure, using 6-chloronicotinic acid as the starting material.[23] Briefly, 6-chloronicotinic acid (1 g, 6.35 mmol) was added to 80% hydrazine hydrate (8 mL) and stirred at 100°C for 4 h. After cooling to room temperature the reaction mixture was concentrated under reduced pressure to give white solid. The solid was dissolved in water and adjustment of pH of the solution to 5.5 resulted in precipitate formation. The precipitate was filtered, washed with ethanol and dried in vacuum

to give 6-hydrazinopyridine-3-carboxylic acid (845 mg). Subsequently protection of hydrazine group was carried out by addition of triethylamine (365 µL, 2.62 mmol) and di-t-butyl-dicarbonate (285 mg, 1.31 mmol) to a solution of 6-hydrazinopyridine-3-carboxylic acid (200 mg, 1.31 mmol) in DMF and the reaction was stirred at room temperature for 16 h. The reaction mixture was then concentrated under reduced pressure to give a brown solid. The crude product was purified by silica gel column chromatography eluting with ethyl acetate to obtain 6-BOC-hydrazinopyridine-3-carboxylic acid. In the next step, a solution of 6-BOC-hydrazinopyridine-3-carboxylic acid (200 mg, 0.79 mmol) and N-hydroxysuccinimide (92 mg, 0.80 mmol) in DMF was added to a solution of dicyclohexylcarbodiimide (165 mg, 0.80 mmol) in DMF and stirred overnight at room temperature. Upon completion of the reaction, the reaction mixture was filtered and the filtrate was concentrated to dryness to obtain a brown residue. The crude product obtained was purified by silica gel column chromatography using ethyl acetate as the eluting solvent to obtain succinimidyl-6-BOC-hydrazinopyridine-3-carboxylic acid (105 mg).

To succinimidyl-6-BOC-hydrazinopyridine-3-carboxylic acid (100 mg) in a sealed flask under nitrogen environment, 4.0 M HCL in dioxane (2 mL) was added. The reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure and the residue was washed with THF and dried. A yellowish white solid (42 mg) was obtained. ESI-MS: *m/z* 251.1 observed (calcd. for $C_{10}H_{10}N_4O_4$: 250.1)

2.2.2 Synthesis of Chlorambucil-c(KCNGRC)-NH_2 [CLB-c(NGR)]

The fully protected peptide chain Lys(Boc)-Cys(Acm)-Asn(Trt)-Gly-Arg(Pbf)-Cys(Acm)-NH_2 was manually synthesized by standard Fmoc solid phase synthesis on NovaSyn TGR resin. Coupling of each amino acid was carried out using standard Fmoc strategy where 3-fold excess of amino acid as well as O-(7-azabenzotriazol-l-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU) was used along with 6-fold excess of N,N-

diisopropylethylamine (DIPEA) in dimethylformamide (DMF) for 90 min. The reaction was monitored by picrylsulphonic acid test and the Fmoc groups were removed by treatment with 20% piperidine in DMF for 30 min. The peptide at N-terminus was conjugated with chlorambucil, an anticancer DNA-alkylating drug, in presence of HATU and DIPEA. The peptide-drug conjugate was then cyclized by simultaneous deprotection and oxidation of acetamidomethyl thiol protected cysteines on solid support with thallium (III) trifluoroacetate and cleaved from the resin using a cocktail mixture of trifluoroacetic acid (TFA)/ethanedithiol/triisopropylsilane/water (94:2.5:2.5:1 v/v). The crude peptide-drug conjugate was precipitated, washed three times with diethyl ether, purified by semi-preparative HPLC and subsequently lyophilized to obtain white fluffy powder. MALDI-TOF MS: *m/z* 962.1 observed (calcd for $C_{38}H_{61}Cl_2N_{13}O_8S_2$: 963).

2.2.3 Synthesis of HYNIC-Chlorambucil-c(KCNGRC)- [HYNIC-CLB-c(NGR)]

The peptide-drug conjugate, CLB-c(NGR) was functionalized with the HYNIC chelator at ε-amino group of lysine and the reaction was carried out in solution phase. Briefly, NHS-HYNIC (2.2 mg, 8 µmol) was added to the purified CLB-c(NGR) (6 mg, 6 µmol) conjugate dissolved in DMF (500 µL) and stirred at room temperature overnight. The HYNIC-CLB-c(NGR) conjugate thus formed was purified by semi-preparative HPLC and characterized by mass spectroscopy. MALDI-TOF MS: *m/z* 1211.9 [M+CF_3COOH] observed (calcd for $C_{44}H_{66}Cl_2N_{16}O_9S_2$: 1098.1).

2.3 Radiolabeling

The purified HYNIC functionalized peptide-drug conjugate HYNIC-CLB-c(NGR) was radiolabeled with ^{99m}Tc using stannous chloride ($SnCl_2$) as reducing agent and EDDA and tricine as co-ligands. The conjugate HYNIC-CLB-c(NGR) (40 µg) was mixed with EDDA (200 µL, 50 mg/mL in 0.1N NaOH), tricine (100 µL, 200 mg/mL in distilled water), disodium hydrogen phosphate (6.4 mg), sodium dihydrogen phosphate (21 mg), $Na^{99m}TcO_4$

(500 μL, 370 MBq) and $SnCl_2$ (20 μg). The reaction mixture was subsequently incubated at 90˚C for 15 min. The radiochemical yield and purity (RCP) of the radiotracer was determined by TLC and HPLC respectively.

2.4 Determination of octanol-water partition coefficient (Log $P_{o/w}$)

Radiotracer (50 μL, ~3.7 MBq) was mixed with distilled water (950 μL) and *n*-octanol (1 mL) on a vortex mixer for about 1 min and then centrifuged (3500 g) for 5 min to get clear separation of the two layers. The radioactivity associated with 100 μL samples of both the aqueous and the organic phases was measured in well type NaI (Tl) counter. This was repeated thrice and the measurements were used to calculate the Log $P_{o/w}$ of the radiotracer.

2.5 Stability studies

The stability of the radiotracer, ^{99m}Tc-HYNIC-CLB-c(NGR) was determined in saline and human serum. The radiotracer was incubated in saline at 37˚C and the stability was assayed 6 h post-preparation by HPLC analyses. To determine serum stability, the radiotracer (50 μL) was incubated in human serum (250 μL) at 37˚C for 6 h. About 100 μL aliquot of this solution was withdrawn; the serum proteins were precipitated by the addition of 100 μL of acetonitrile. The mixture was centrifuged at 3500 g for 10 minutes and the supernatant was analyzed by TLC and HPLC to check the stability of the radiotracer in serum.

2.6 *In vitro* cell binding studies

The murine melanoma cells B16F10 were obtained from National Center for Cell Sciences (NCCS) Pune, India. The cells were cultured in Dulbecco's modified Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen Carlsbad, CA) and 1% antibiotic/antimycotic formulation. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. For cell binding studies, B16F10 cells (1×10^5) were seeded in 24-well tissue culture plates and incubated at 37˚C overnight. The cells were subsequently

incubated with ^{99m}Tc-HYNIC-CLB-c(NGR) (37 kBq/well) at 37˚C for 1 h. After incubation cells were washed twice with ice-cold phosphate buffer saline (PBS) and the cells were harvested by trypsinization. At the end of trypsinization, wells were examined under a light microscope to ensure complete detachment of cells. Cell suspensions were collected and radioactivity associated with cells was measured in a NaI (Tl) gamma counter. The activity in these cell suspensions as percentage of total input radioactivity was calculated to determine the cell uptake data. The experiment was carried out in triplicate. Specific uptake was determined by pre-incubation of cells with 100-fold excess of peptide [c(KCNGRC)]. Percentage inhibition values were calculated as [{difference between cell binding of radiotracer in absence and presence of cold peptide)/cell binding in absence of peptide}×100].

2.7 Cytotoxicity studies

To determine cytotoxicity of the peptide-drug conjugate the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out. All the samples were prepared with maximal final DMSO concentration <0.05%. The cells (3×10^3/well) were cultured in a 96-well plate overnight at 37˚C and subsequently exposed to varying concentrations of peptide (cNGR), drug (CLB) and PDC (CLB-cNGR) (1-50 µM) for 48 h. Cells treated with medium only served as control group. At the end of incubation, 20 µL of MTT solution (5 mg/mL in PBS) was added in each well and incubated for 3 h. After removing the supernatant, 150 µL of solubilizing solution (10% Triton X-100 in acidic iso-propanol) was added. The absorbance was measured at 570 nm with reference to 630 nm in BioTek Universal Microplate Reader (BioTek USA, Winooski, VT). The difference between these measurements was used for calculating the % of growth inhibition (% GI) in test wells compared to the control. The control cells were treated with medium containing only 0.05% DMSO solvent. All the tests were done in quadruplicates.

2.8 Biodistribution studies

Biodistribution studies were carried out in C57BL6 mice bearing murine melanoma (B16F10) tumor. The radiotracer (~3.7 MBq/animal, 100 µL) was injected intravenously into the tail vein of each mice (n = 4). The animals were sacrificed at different time intervals (30, 60, and 180 min), and the relevant organs excised for measurement of retained activity. For blocking studies the peptide, c(NGR) (15 mg/kg) was co-administered along with the radiotracer and mice were sacrificed at 60 min p.i. The excised organs were weighed and the activity associated with them was measured in a flat-bed type NaI (Tl) counter with suitable energy window for ^{99m}Tc. The activity retained in each organ/tissue was expressed as a percent value of the injected dose per gram (% ID/g ± S.D). All the animal experiments were carried out in compliance with the relevant national laws, as approved by the local committee on the conduct and ethics of animal experimentation.

2.9 Data analysis

Statistical data are reported as mean ± standard deviation (S.D.). Paired two-tailed Student's t tests were done to evaluate statistical significance, where $p<0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1 Synthesis and radiolabeling

The peptide-drug conjugate, HYNIC-CLB-c(NGR) (Scheme 1) could be prepared with >99% purity [as ascertained by UV-HPLC chromatogram (Figure 2)] by standard Fmoc solid phase synthesis method and was characterized by mass spectroscopy. Since HYNIC occupies a single co-ordination site EDDA and tricine were used as co-ligands to complete the coordination sphere and form a stable complex with ^{99m}Tc. The peptide-drug conjugate HYNIC-CLB-c(NGR) was radiolabeled with ^{99m}Tc with specific activity of 9.8 GBq/µmol.

Schematics of radiosynthesis of ^{99m}Tc-HYNIC-CLB-c(NGR) is presented in Figure 3. The radiochemical yield (RCY) and purity (RCP) of the radiotracer was determined by TLC and HPLC respectively. In TLC system using methanol: 1 M ammonium acetate (1:1, v/v) as the mobile phase ^{99m}Tc-HYNIC-CLB-c(NGR) and $^{99m}TcO_4^-$ had R_f = 1.0 whereas colloid, $^{99m}TcO_2$ remained at the point of spotting with R_f = 0. In case of 0.1 M sodium citrate mobile phase, R_f = 0 was observed for ^{99m}Tc-HYNIC-CLB-c(NGR) and $^{99m}TcO_2$ whereas $^{99m}TcO_4^-$ had R_f = 1.0. The RCY of ^{99m}Tc-HYNIC-CLB-c(NGR) was thus determined to be 98 ± 0.5%. The RCP as determined by HPLC radio-chromatograms for ^{99m}Tc-HYNIC-CLB-c(NGR) was 98 ± 1.5%. Retention time of ^{99m}Tc-HYNIC-CLB-c(NGR) was 14.1 min (Figure 3). The possibility of any interaction of chlorambucil moiety with the co-ligands, EDDA and tricine was investigated by incubation of the co-ligands with the peptide-drug conjugate, HYNIC-CLB-c(NGR) under similar conditions of radiolabeling. No change in the retention time/pattern of the peptide-drug conjugate, HYNIC-CLB-c(NGR) in UV-HPLC was observed thus ruling out the possibility of any adduct formation of chlorambucil with co-ligands. The radiotracer showed excellent *in vitro* stability in saline as no change in the HPLC radio-chromatogram pattern was observed after 6 h incubation period. The radiotracer was found to be 95% intact after 6 h incubation in human serum at 37˚C. Radio-TLC also confirmed the HPLC results and no increase in any colloidal species were observed on incubation of the radiotracer with human serum. These data suggest sufficient stability of the radiotracer for further carrying out *in vivo* studies.

The partition coefficient (Log $P_{o/w}$) was determined to be -2.19 ± 0.1 for ^{99m}Tc-HYNIC-CLB-c(NGR) suggesting that the radiotracer is highly hydrophilic.

3.2 *In vitro* cell binding studies

Cell binding studies were performed in murine melanoma B16F10 cells. The uptake value of 0.92 ± 0.09% was observed for ^{99m}Tc-HYNIC-CLB-c(NGR). During inhibition studies with excess of unlabeled c(NGR) peptide the uptake reduced to 0.59 ± 0.05%.

The cytotoxicity of the PDC was compared with that of the peptide, cNGR as well as with the drug, CLB alone by carrying out cell cytotoxicity assay in murine melanoma B16F10 cells. The cells cultured for a period of 24 h were incubated with the different concentrations of compounds for 48 h and the cytotoxicity of the compounds was determined by using MTT cell viability assay (Figure 4). The results are expressed as % growth inhibition compared with cells incubated with only the medium. Figure 4 demonstrates that the exposure of cells to PDC resulted in significant growth inhibition at all the concentrations as compared to the drug or the peptide alone. It was observed that nearly 25-fold excess concentration of the drug is required to achieve similar cytotoxicity as obtained on exposure to PDC. RGD peptide-chlorambucil conjugate has also been reported to show higher growth inhibition in B16F10 cells as compared to the drug or peptide alone.[24] The enhanced cytotoxic effect of PDC even at lower levels indicates clear advantage in reducing the systemic exposure and side effects of the drug. The conjugation of the drug with the targeting peptide would thus allow targeted delivery and killing of cancer cells increasing the chemotherapeutic efficacy of the drug.

3.3 Biodistribution studies

Results of biodistribution studies of ^{99m}Tc-HYNIC-CLB-c(NGR) in C57BL6 mice bearing melanoma tumor are summarized in Table 1. The maximum tumor uptake of 2.45 ± 0.28% ID/g was observed at 30 min p.i. The radiotracer cleared rapidly from non-target organs like heart, lungs, liver and muscle. The clearance of ^{99m}Tc-HYNIC-CLB-c(NGR) from the blood pool was reflected in the improved tumor/blood ratio with time. The tumor-to-

blood ratio and tumor-to-muscle ratios improved with time attaining a maximum of 2.5 ± 0.4 and 4.23 ± 0.57 at 180 min p.i. (Figure 5).

The blocking experiment carried out by co-injection of 15 mg/kg of cold c(NGR) peptide, resulted in 35% reduction in the uptake of ^{99m}Tc-HYNIC-CLB-c(NGR) in melanoma (B16F10) tumor.

The chemotherapeutic drug chlorambucil radiolabeled with ^{99m}Tc-tricarbonyl core using two different chelators containing N_2O and NNN as donor atoms has been reported.[25,26] The two ^{99m}Tc$(CO)_3$-labeled chlorambucil complexes had high log P values of 1.2 and 2.34 respectively and thus exhibited high liver and intestinal activities. ^{99m}Tc$(CO)_3$-chlorambucil complex with N_2O donors showed increase in the stomach activity indicating decomposition of the radiotracer. However the currently studied radiotracer tagged with the peptide had lower log P value -2.19±0.1 and the high hydrophilicity of the radiotracer was visible in its clearance from all the non-target organs. This suggests that off-target toxicity of drugs can be reduced by conjugation to the targeting vector, peptide. Radiolabeled peptide-drug conjugates offer an advantage of tracking the pathway of the drug, monitoring the reduction in tumor growth and thus ascertain the future application of PDC in targeted chemotherapy.

4. Conclusion:

This study reports the conjugation of chemotherapeutic drug, chlorambucil with the tumor targeting peptide, cNGR. The PDC showed enhanced inhibitory effect on the cell growth as compared to the peptide or the drug alone during cytotoxicity assays. The PDC was functionalized with HYNIC chelator for radiolabeling with ^{99m}Tc. The radiolabeled PDC, ^{99m}Tc-HYNIC-CLB-c(NGR) could be obtained in high radiochemical yield. The high hydrophilicity of the radiotracer resulted in rapid clearance from the non-target organs. This study thus paves the way for designing of different peptide-drug conjugates for targeted chemotherapy that can reduce the limitations while using the drug alone.

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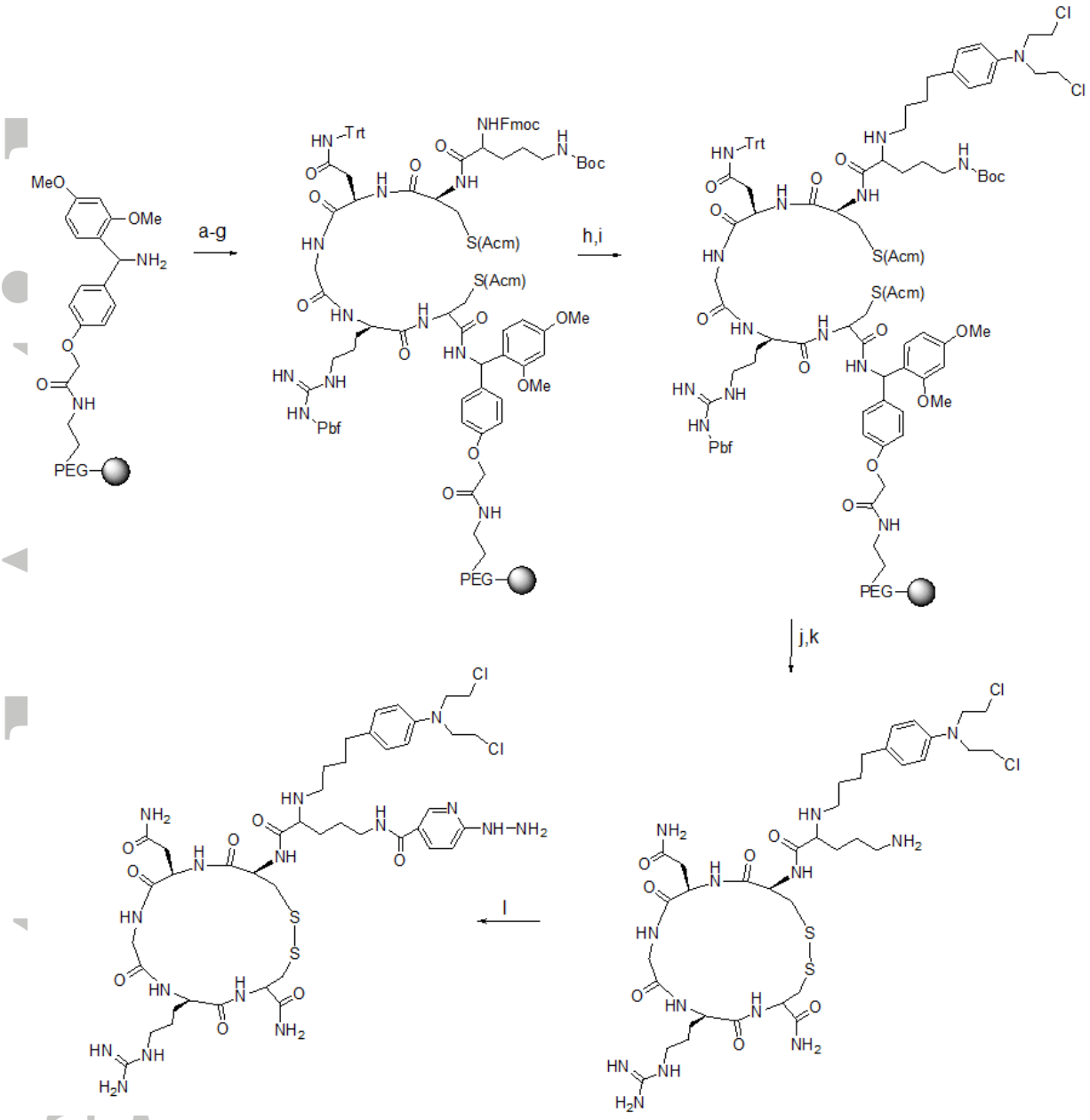
Scheme 1



Scheme 1: Synthesis of HYNIC-Chlorambucil-c(KCNGRC) [HYNIC-CLB-c(NGR)]

Reagents and conditions: a) N-Fmoc-Cys(Acm)-OH, HATU, DIPEA, DMF, rt, 2 h; b) 20% piperidine in DMF, rt, 30 min (x 2); c) N-Fmoc-Arg(Pbf)-OH, HATU, DIPEA, DMF, rt, 2 h; d) N-Fmoc-Gly-OH, HATU, DIPEA, DMF, rt, 2 h; e) N-Fmoc-Asn(Trt)-OH, HATU, DIPEA, DMF, rt, 2 h; f) N-Fmoc-Cys(Acm)-OH, HATU, DIPEA, DMF, rt, 2 h; g) N-Fmoc-Lys(Boc)-OH, HATU, DIPEA, DMF, rt, 2 h; h) 20% piperidine in DMF, rt, 30 min (x 2); i) Chlorambucil, HATU, DIPEA, DMF, rt, 2 h; j) Tl(III)trifluoroacetate, DMF, rt, 2 h; k) TFA/ethanedithiol/triisopropylsilane/water (94:2.5:2.5:1 v/v), rt, 2 h; l) Succinimidyl-6-hydrazinopyridine-3-carboxylate hydrochloride (NHS-HYNIC), DMF, rt, 12 h

Figure 2

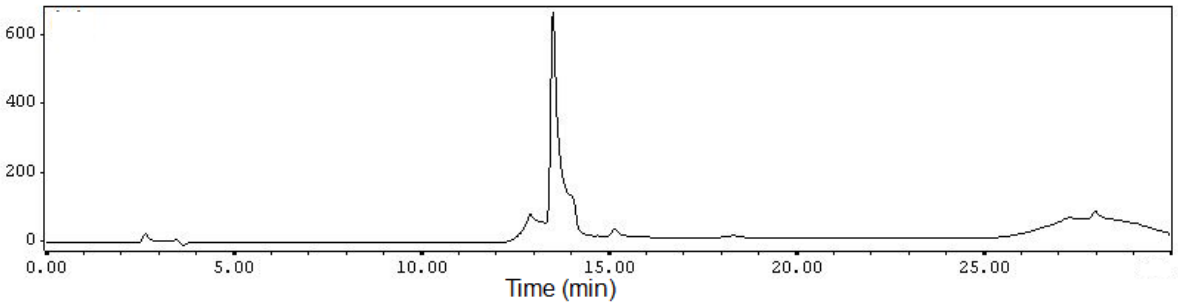


Figure 2: UV-HPLC (220 nm) chromatogram of HYNIC-CLB-c(NGR)

Figure 3

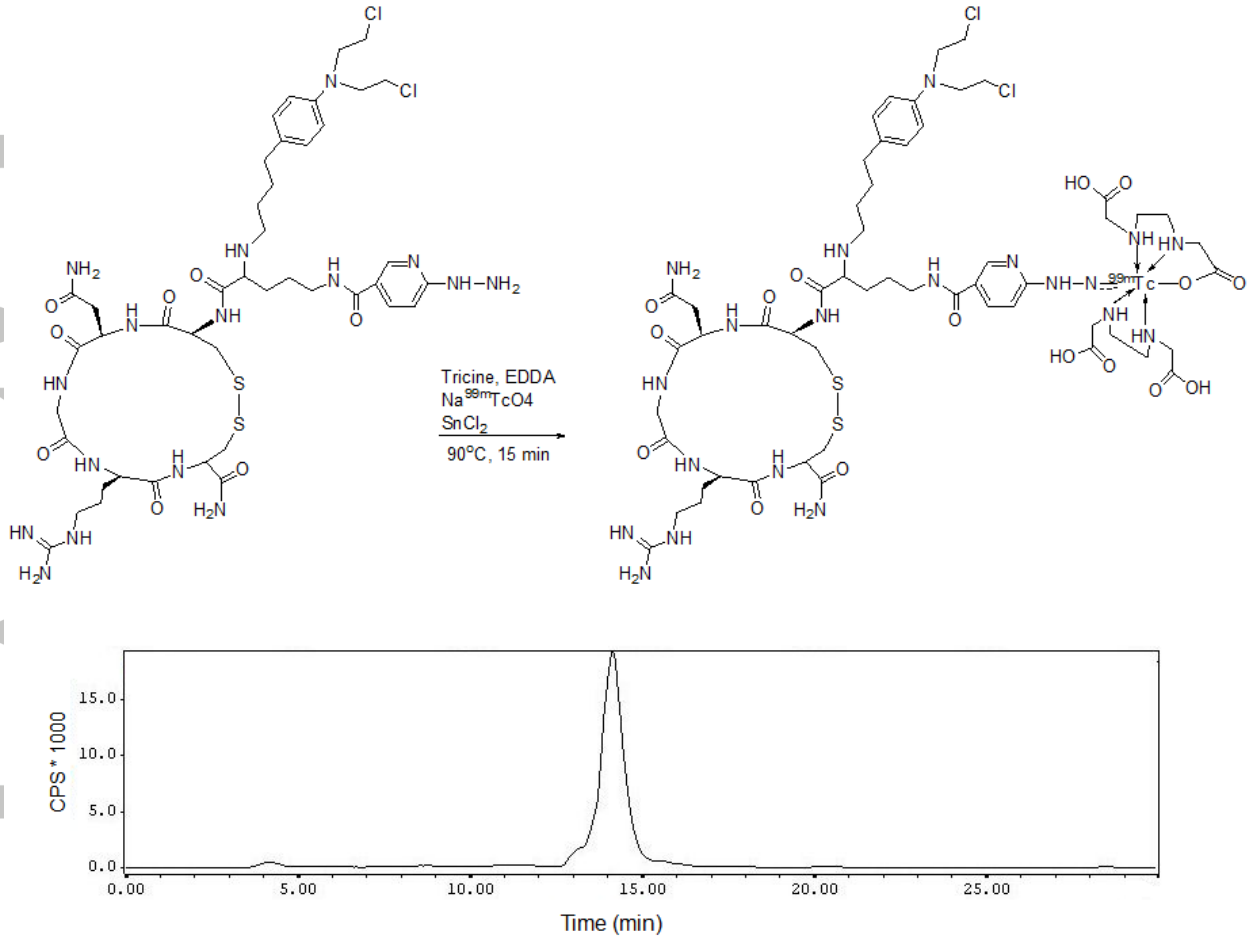


Figure 3: Radio-synthesis and HPLC radio-chromatogram of ^{99m}Tc-HYNIC-CLB-c(NGR)

Figure 4

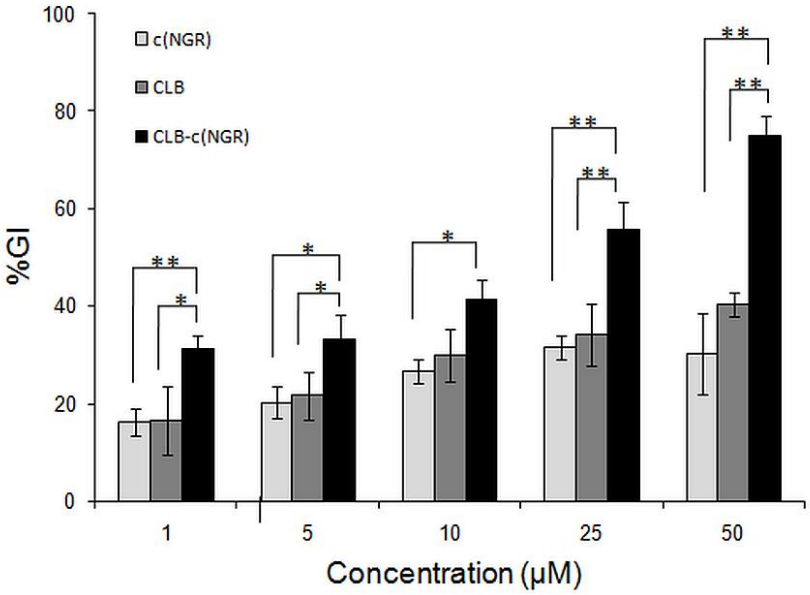


Figure 4: Growth inhibition effect of c(NGR), CLB and CLB-c(NGR) on B16F10 cells after 48h of exposure. The error bars represent standard deviation. *$p < 0.05$ and **$p < 0.005$.

Figure 5

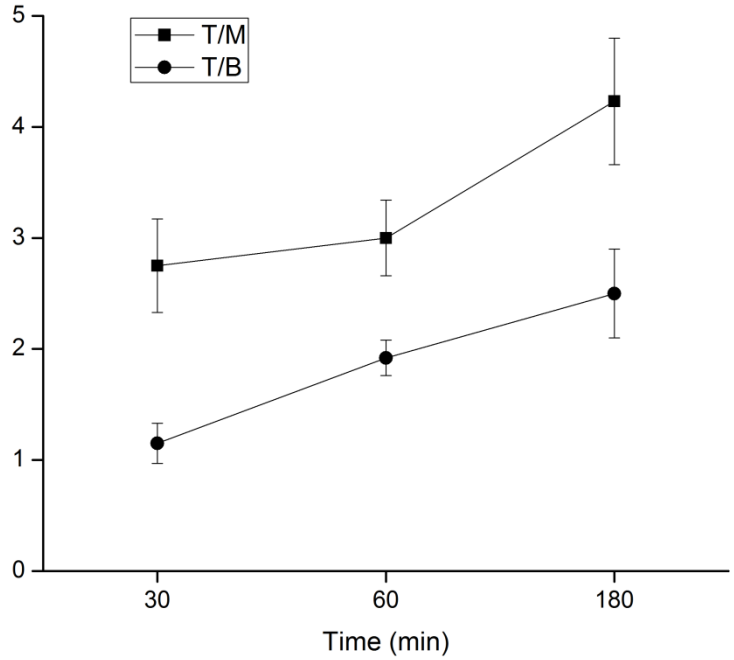


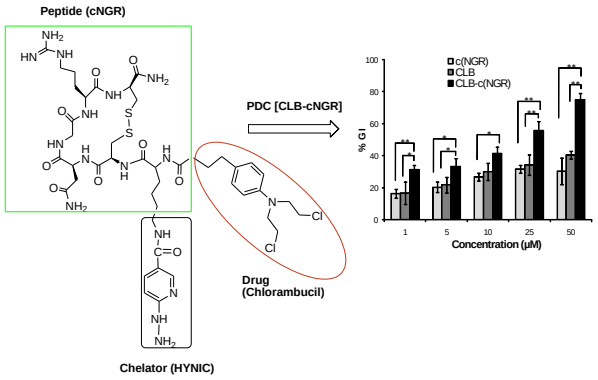
Figure 5: Variation in the uptake ratios of tumor/blood and tumor/muscle with time for ^{99m}Tc-HYNIC-c(NGR)-CLB

Table 1: Biodistribution pattern of ^{99m}Tc-HYNIC-CLB-c(NGR) in C57BL6 mice bearing melanoma (B16F10) tumor

Organs	**% ID/g (s.d)** (n = 4)			
	30 min	**60 min**	**60 min (Blocking)**	**180 min**
Blood	2.15 (0.11)	0.77 (0.15)	0.54 (0.04)	0.31 (0.24)
Liver	2.49 (0.76)	1.75 (0.03)	0.94 (0.12)	0.79 (0.32)
Intestine	3.32 (1.00)	4.03 (0.22)	2.73 (0.15)	3.25 (1.29)
Stomach	1.00 (0.35)	0.52 (0.22)	0.88 (0.08)	1.36 (0.77)
Kidney	3.82 (1.26)	1.78 (0.21)	2.73 (1.15)	1.44 (0.38)
Heart	0.33 (0.05)	0.67 (0.11)	0.35 (0.02)	0.28 (0.10)
Lungs	1.50 (0.55)	0.63 (0.38)	1.20 (0.4)	0.33 (0.19)
Spleen	0.47 (0.05)	0.68 (0.21)	0.41 (0.26)	0.50 (0.02)
Muscle	0.90 (0.25)	0.51 (0.10)	0.34 (0.12)	0.17 (0.06)
Tumor	2.45 (0.28)	1.48 (0.16)	0.93 (0.06)	0.77 (0.10)
T/B	1.15 (0.18)	1.92 (0.16)		2.50 (0.40)
T/M	2.75 (0.42)	3.00 (0.34)		4.23 (0.57)

^{99m}Tc-labeled NGR-Chlorambucil Conjugate, ^{99m}Tc-HYNIC-CLB-c(NGR) for Targeted Chemotherapy and Molecular Imaging

Kusum Vats[1], Drishty Satpati[*1], Rohit Sharma[1], Chandan Kumar[1], Haladhar D Sarma[2], Ashutosh Dash[1]



In this study the drug chlorambucil was conjugated with the peptide c(NGR) to prepare the peptide-drug conjugate (PDC), CLB-c(NGR) for targeted chemotherapy. The PDC exhibited enhanced cell cytotoxicity than peptide, cNGR or drug, chlorambucil alone. The radiolabeled PDC, ^{99m}Tc-HYNIC-CLB-c(NGR) being highly hydrophilic cleared rapidly from the non-target organs during biodistribution studies. The PDCs thus have a promising potential for future use in targeted chemotherapy.