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Development of TNKase specific cleavable peptide linked radioimmunoconjugates for radioimmunotherapy

Arutselvan Natarajan *, Pappanaicken R. Kumaresan, Sally J. DeNardo, Gerald L. DeNardo, Gary Mirick, Kit S. Lam

Division of Hematology and Oncology, Internal Medicine, University of California Davis, 1508 Alhambra Blvd., Room 3100, Sacramento, CA 95816, USA

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ABSTRACT

Radioimmunotherapy (RIT) is a method for selectively delivering radionuclides to cancer cells, while reducing the radiation dose to normal tissues. However, because of slow clearance of MAbs, normal tissues also received radiotoxicity. One of the promising strategies is linking on demand cleavable (ODC) peptides between radiometal chelates and the tumor targeting agents. We have tested this proof-of-concept by using ODC peptides designed to be cleaved only by TNKase and resistant to cleavage by enzymes present in the plasma and the tumor. TNKase-specific peptide linkers using L- and D-amino acids were screened by OBOC combinatorial peptide libraries. One of the best peptide was linked to radiometal chelate and ChL6-MAb to prepare radioimmunoconjugate (RIC). Optimization and characterization of the linker conjugation to MAb shows: (a) 1–2 peptides linked to each MAb; (b) immunoreativity >80%; (c) specific activity of the RIC 0.7–1 μCi/μg; (d) RIC stable over 7 days in human plasma and (e) radiometal chelated ODC peptide cleaved from the RIC in plasma by TNKase at clinical dose levels of 10 μg/ml. The percent release of radiochelate from RIC was 50% at 24 h and 85% over 72 h in vitro. This novel ODC-linked RIC could be a potential molecule for RIT.

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Monoclonal antibodies (MAb) linked to radiometal chelates used to deliver cytotoxicity in cancer therapy is termed radioimmunotherapy (RIT).[1–5] The delivery of an effective dose to tumor sites is challenging while sparing exposure to normal tissues and bone marrow. A number of approaches to improve the delivery of radiation dose specific to the tumor site and reduce the non-targeted radioactivity exposure to normal organs have been described.[6] The non-targeted radioactivity has been removed by various approaches, for example, increased renal excretion, immunoadsorption,[7] and pre-targeting procedures.[8–11] All of these methods demonstrated non-targeted radioimmunoconjugates clearance to some extent from normal organs.

Alternatively one can reduce radiotoxicity from the targeting molecules at the normal organs by cleaving a designed enzyme cleavable linker placed between radiochelates and immunoconjugates. For example, cleavable linkers were previously designed and utilized to cleave from hepatocytes by endogenous proteases such as liver-specific cathepsin[12–15] or by the exogenous hydrolytic enzyme-lactamase.[16] In a similar approach we have developed an on demand cleavable (ODC) peptide using human origin TNKase enzyme to cleave radiochelates from radioimmunoconjugate (RIC) to demonstrate the proof-of-concept.[17] In this approach the TNKase enzyme primarily restricted to the intravascular space, results in cleavage of only the circulating radiochelates from the RIC. This enzyme was derived from the recombinantly made tissue plasminogen activator (TPA), currently used as a thrombolytic agent in patients.[18]

In clinical studies for RIT treatment, first TNKase susceptible ODC–RIC will be administered to target the tumor and provide enough radioactivity followed by TNKase intravenously to cleave the ODC linker, thus DOTA radiochelate will be cleaved from the circulating RIC and excreted through kidneys.

The first ODC peptide linked RIC showed 50% immunoreactivity and cleaved 28% of the linkers from RIC at 10 μg TNKase dose in plasma incubated after 72 h. To increase the cleavable ability of the ODC peptide by TNKase at clinical dose level, we have screened a second one-bead-one-compound (OBOC) peptide library by established methods.[19,20] The OBOC method provided an ODC linker that is highly susceptible to TNKase but highly resistant to other proteases in plasma or tumor. From this library we have identified and synthesized a better peptide with efficient cleavage properties in plasma in order to reduce the TNKase dose and exposure time. Further the linkers' ligation method has been enhanced to preserve the tumor targeting of the RIC and to have minimum linkers for the effective enzyme susceptibility. Thus the present study was aimed at enhancement of novel ODC–RIC and has the following properties: high specificity, susceptibility to TNKase and increased immunoreactivity for better in vivo tumor targeting.

* Corresponding author. Tel.: +1 916 734 3787; fax: +1 916 703 5014.
E-mail address: arutselvan.natarajan@ucdmc.ucdavis.edu (A. Natarajan).

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ODC peptides were generated using fluorescent-quenched peptide libraries on PEGA beads through the OBOC combinatorial library method (MM1–3) by 'split-mix' approach.[17] Screening and sequencing of the peptides were carried out by established methods.[17] Table 1 shows a group of selected ODC peptides. The listed peptides were selected based on the following criteria: first they were stable in the presence of human plasma, normal tissues and tumor tissues; and second they were specifically susceptible to TNKase in plasma. Peptides were further characterized with mouse serum and we selected the best peptide (ayGRGrr) from the listed peptides in Table 1 for further study.

The peptide with the sequence of ayGRGrr was further modified at the terminals to create a bi-functional linker with one side DOTA to chelate radiometals and the other side with amino oxy to conjugate with chimeric L6 antibody (ChL6). Bi-functional modification of TNKase cleavable peptide (DOTA-spacer-ayGRGrr-spacer-K(Aoa)-NH_2) was carried out by a method similar to the synthesis of fluorescent-quenched peptides.[17] A short hydrophilic spacer was also introduced on both sides of the peptide linker using 2,2′(ethylenedioxy)-bis(ethylamine) and an anhydride component at the C-terminal end derived from succinic anhydride, respectively (Fig. 1A). The purity of the peptide was verified by both analytical HPLC and MALDI-TOF mass spectrometry. The bi-functionalized ODC peptide showed 95% purity and was further tested for the TNKase susceptibility and plasma stability.

Table 1
List of ODC peptides selected from the OBOC library and modified as bi-functional linker

SL#	ODC-peptide sequence[a]	Purity (%)		Radio chelatable DOTA (c) c/peptide	Specific activity (μCi/μg)
		TLC	HPLC		
1	ayGRGar	>90	>90	0.75	150
2	myGRGar	>90	>90	0.62	145
3	myGAGaa	>90	>90	0.52	135
4	ayGRGrr	>90	>90	0.53	135
5	myGRGrr	>90	>90	0.43	116

[a] Sequence contains L and D-amino acids each represents by a small and big fonts, respectively. ODC peptides were bi-functionalized with DOTA and aminooxy functional groups (Fig. 1A).

Cancer targeting immunoconjugate (IC) was prepared by placing a small cross-linker between ChL6 and the TNKase cleavable peptide (Fig. 1A). This strategy was adopted to control the number of linkers on each MAb. ChL6, an antibody targeted against a tumor antigen expressed on colon, lung, and breast cancer cells, was functionalized with keto groups at primary amines (mainly lysine) present in ChL6. The cross-linker levulinic succinimidyl ester was used to keto functionalize the ChL6. In a separate experiment we determined that 20 M excess of *N*-succinimidyl levulinic acetate to antibody yielded typically 3–5 keto groups per mole of ChL6 at the omega group of lysine and retained its immunoreativity equal to unmodified ChL6. This protocol allowed a reproducible number of keto functionality at the antibody without changing the biological property of the antibody. The number of cross-linkers conjugated to the ChL6 was estimated by analysis of unconjugated cross-linkers from the reaction mixture by HPLC.

DOTA-ODC-aminoxyamine was ligated to ChL6 by the ketone-oxime method[21] The ODC peptide conjugation to antibody was estimated by analytical HPLC, mass spectroscopy, and the DOTA estimation against $^{57}Co/CoCl_2$ method. To optimize the aminooxy ODC peptide ligation to keto functionalized ChL6, reactions were performed at various conditions. Table 2 shows the various molar ratios, time and pH utilized to optimize the formation of oxime bond. This ligation condition was further optimized at four different intervals of time (4, 8, 12, and 24 h) at RT for the best immunoreactive conjugates (Table 3). The best ODC–IC preparation was achieved at pH 5.5 with 1.5 M excess ODC peptide incubated 8 h at RT, yielded 1–2 linkers ligation per MAb and provided 0.8 metal chelatable DOTA. The metal chelatable DOTA was accounted 0.8 c/a

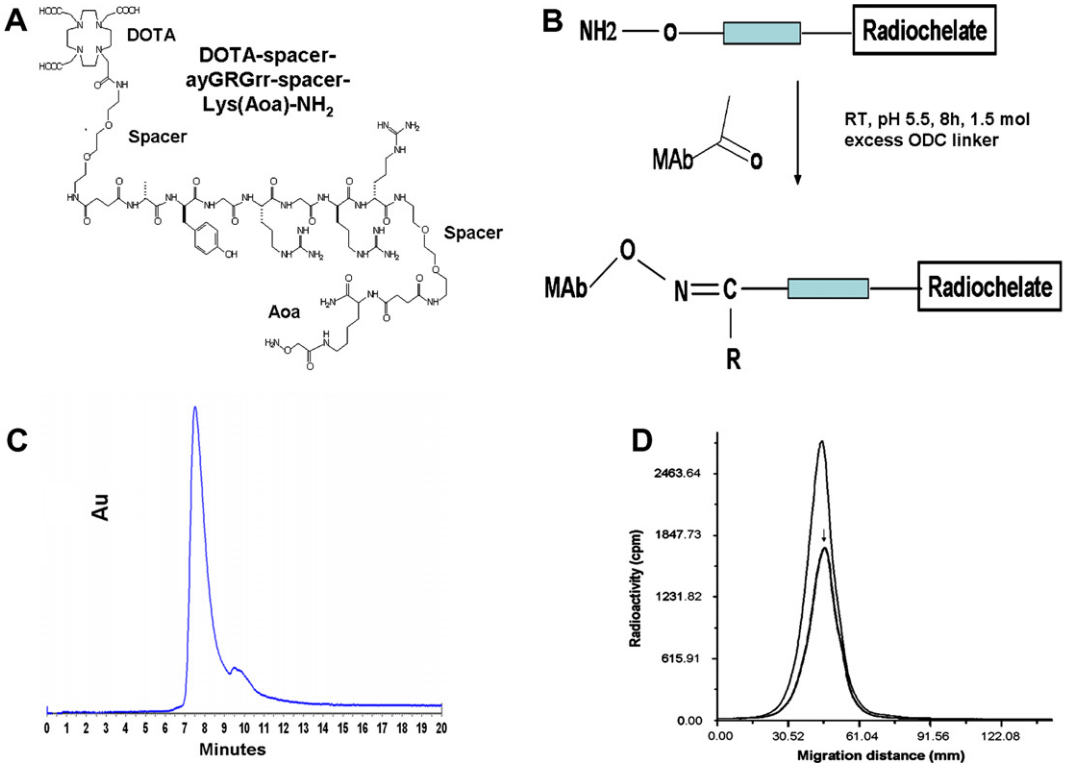

Figure 1. (A) Chemical structure of the TNKase cleavable ODC linker; (B) schematic diagram of ODC linker ligation to keto functionalized ChL6; (C) ODC-linked ChL6 analyzed by SEC-3000 HPLC, UV at 280 nm shows >90% pure; (D) ODC–RIC in plasma incubated over 7 days. Analysis of CAE at 45 min shows ODC–RIC stable in plasma 7 days (tall peak = day 1, and peak indicated by arrow = day 7).

Table 2
Optimization of ODC linker conjugation reaction with MAb at various molar ratio and pH

Expt #	Ratio of aminooxy peptide versus keto-MAb[a] (nmol/nmol)	Amount of aminooxy group ligated per MAb (nmol)	Metal chelatable DOTA availability per MAb	pH
1	0.5	0.4	0.3	
2	1	0.6	0.4	
3	1.5	1.2	0.8	5.5
4	2	1.5	1.1	
5	4	2.0	1.4	
6	0.5	0.3	0.2	
7	1	0.5	0.3	
8	1.5	0.8	0.5	6.0
9	2	1.2	0.8	
10	4	1.5	1.1	

[a] Each ChL6 molecules functionalized to an average five keto groups.

as against 1–2 c/a in comparison with linkers estimated by mass spectrometry, we are speculating this may be due to trace metals present in the reagents possibly chelated to DOTA during ODC–IC preparation.

The final immunoconjugate was greater than 95% pure by HPLC (Fig. 1C) and used to radiolabel with $^{111}InCl_3$ to prepare ODC–RIC. In a separate vial the control ^{111}In-2-iminothiolane (2-IT)-2-[p-(bromoacetamido) benzyl]-DOTA-ChL6 (2IT-RIC) was prepared as per the published procedure.[22] The ^{111}In-DOTA-ODC-ChL6 (on demand cleavable RIC) was purified by Sephadex-G50 column chromatography and the purity was 95% by HPLC and cellulose acetate electrophoresis (CAE) at 45 min (Fig. 1D, day 1). The specific activities of the ODC–RIC, and 2IT-RIC were 1 μCi/μg. The ODC–RIC was tested for immunoreactivity against ChL6 antigen positive breast cancer cells (HBT 3477) and ChL6 antigen negative lymphoma cancer cells (Raji). The ODC–RIC selectively bound to specific HBT 3477 cells but not to negative control Raji cells. Immunoreactivity of the ODC–RIC was greater than 80% to HBT 3477, but only 6% to Raji cells (Table 3). The RIC preparation with 1–2 ODC linkers showed the highest IR of about 80% (Table 3). Although, increased ODC peptides conjugation was observed at 4 M excess of ODC linkers per ChL6 at pH 5.5, the immunoreativity (IR) was reduced.

The ODC–RIC in plasma demonstrated greater than 95% stable in vitro determined by CAE at 45 min (Fig. 1D, day 7 trace) monitored for 7 days in human plasma. HPLC analysis indicated that $\geqslant$95% of the radiometal was associated with RIC, and $\geqslant$95% of RIC was in a monomeric form. This result indicated that chelated radiometals, linkers and protein were intact for 7 days.

To study ODC linker cleavage by TNKase enzyme, samples were analyzed at various times (4, 24, 48, and 72 h) by CAE at 45 min and HPLC. The degree of TNKase susceptibility was calculated by either decreased radioactivity at the peak corresponding to the ChL6 or increased radioactivity in the cleaved fractions corresponding to the peptide peak. Study after 4 h showed cleavage of the linker had started, evidenced by two different peaks corresponds to ODC–RIC and small molecular weight with peptide radiochelate. Incubation after 24 and 72 h showed the percent of linker cleaved from ODC–RIC was 50% and 85%, respectively (Fig. 2). The same experiment was performed using a control 2IT-RIC (^{111}In-2IT-ChL6) with TNKase enzyme the peaks corresponding to products were stable and no cleavage was observed at 72 h (see Supplement data Fig. 1).

Table 3
ODC linker conjugation with MAb at different reaction time versus immunoreactivity

ODC–RIC optimized at various reaction time (h)	Immunoreactivity	
	HBT %bound (+ control)	Raji % bound (− control)
4	80.8 ± 2.1	5.3 ± 1.4
8	80.5 ± 1.2	5.5 ± 2.0
12	67.9 ± 2.4	5.8 ± 1.2
24	46.3 ± 0.9	6.7 ± 0.8

Delivery of radionuclide for imaging and therapy of cancer using targeted antibodies, antibody fragments and peptides, directed towards tumor-associated antigens is an active area of research both by experimental and clinical trials.[9,14,23]However, in this modality radioactive uptake by various normal organs and tissues are very high and could induce toxicity, for example, liver, spleen and kidney. To reduce the radioactive uptake at normal organs, various approaches have been developed but the success towards the complete removal of RICs from the normal organs was not complete.

Keeping the RICs tumor uptake intact and clearing the radionuclides in normal tissues is challenging and many approaches have been developed. One of the approaches is placing an enzyme cleavable peptide or linker between MAb and radiochelates. The proof-of-the principle has been demonstrated by various groups; DeNardo et al.[14,22,24] extensively studied cathepsin B specific cleavable peptide, Beeson et al.,[16] used external B-lactamase enzyme cleavable peptide and Sutherland et al.[25] utilized protease enzyme cleavable peptide. In human clinical trials both prostate and breast cancers were compared to non-cleavable RIC, and the cleavable RIC showed a 30% mean dose decrease in liver while the tumor dose was similar with identical PK.[3] However, in this approach dose reduction was achieved only at the liver, but other normal organs were not effective.

Alternatively, we have developed another novel approach using TNKase enzyme cleavable peptide linked RIC for on demand cleavage. TNKase enzyme has been approved by the FDA as a thrombolytic reagent and the physiological characteristics are well established. In our previous report TNKase cleaved 28% of the radiochelate after 72 h using 10 μg/ml TNKase in human plasma in vitro and stable in plasma over 3 days. A published report[26] demonstrated that the differences in biodistribution of chelate were attributed to the different methods of conjugation of linker to the antibody, difference in the number of chelates and possibly different positions of attachment. Hence, here we have evaluated the new linkage and optimize the linkers ligation to MAb and established the conjugation method with various molar ratios of reactants, pH and incubation time.

In summary, in this study, we have screened focused OBOC library and panned a potential TNKase susceptible peptides with improved cleavage efficiency and stability in cancer cell supernatants,

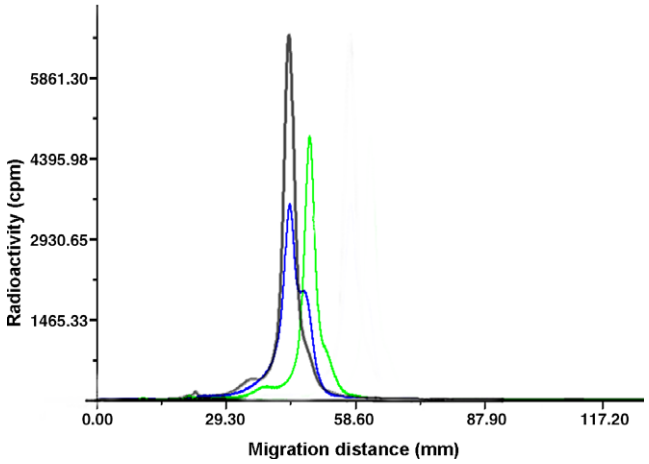

Figure 2. Study of TNKase susceptibility on ODC–RIC in human plasma at 0, 24, and 72 h. Analysis of CAE at 45 min shows TNKase (10 μg/ml) cleaved ODC over 24 h (blue trace, new peak appears at 52 mm) and 72 h (green peak, the trace completely shifted from 44 to 52 mm; >80% of the ODC peptide cleaved from the RIC).

mouse serum and human plasma. One potential ODC linker (ayGRGrr) was selected by in vitro and has evaluated its cleavable property to reduce radiation to normal tissues. This novel ODC peptide linked RIC showed increased immunoreactivity and specificity to TNKase with increased cleavable efficiency at clinical dosage level. These properties were achieved by controlling the number of linkers per molecule of MAb. Further in vivo study in animal model is ongoing and the study results may provide a novel molecule for imaging and therapy for RIT.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.097.

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