Title

Side Chain Protection as a Strategy to Improve Cell Penetrating Peptide Specificity

Author

Chen, Leo C

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Side Chain Protection as a Strategy to Improve Cell Penetrating Peptide Specificity

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Leo C Chen

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

Signed by:
Ian Bass Seiple
0F55EF21BB3D419...

Ian Bass Seiple
Chair

Signed by:
William DeGrado
DocuSigned by:
Michael Evans
40E92A530F764EE...

William DeGrado

Michael Evans

Committee Members

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Beyond his support, my co-mentors, Ian and Bill, have been instrumental in my growth. Like my dad, their constant positivity and support have driven me forward. Unexpectedly, their enthusiasm for even the smallest victories, whether completing a reaction or purifying a compound, was infectious and has been a great source of motivation. Even when I expressed my interest in teaching, they encouraged me and trusted me to teach a section of the reaction mechanisms course, as well as allowing me to teach at UC Berkeley, which provided invaluable teaching experience that will

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Contributions

The work presented in this dissertation was performed under the guidance and supervision of Ian B. Seiple, PhD, and William F. DeGrado, PhD. Collaboration with Ethel Tackie-Yarboi, PhD, Jesse Ling, PhD, and Michael J. Evans, PhD, was essential in carrying out the in vitro and in vivo portions of the experiments.

Side Chain Protection as a Strategy to Improve Cell Penetrating Peptide Specificity

Leo C Chen

Abstract

Cell-penetrating peptides (CPPs) offer immense potential for targeted delivery of therapeutic and diagnostic agents due to their ability to traverse cell membranes. However, their lack of cell specificity, driven by nonspecific electrostatic and hydrophobic interactions, limits their clinical applicability by causing off-target effects. To address this, we developed a side chain protection strategy that cages the positive charges of a cell penetrating peptide, modified temporin L, using granzyme B cleavable sequences. This approach inhibits membrane interactions until proteolytic activation, enhancing selectivity for tissues with elevated granzyme B activity, such as tumor microenvironments.

We synthesized branched constructs of mTL with lysine side chains masked by GZMB-cleavable sequences using solid-phase peptide synthesis. The constructs were characterized by circular dichroism, matrix-assisted laser desorption/ionization-time of flight mass spectrometry, and high-performance liquid chromatography to confirm sequence integrity and proteolytic cleavability. In vitro assays with HeLa cells demonstrated reduced cytotoxicity of the masked peptides, which was restored upon GZMB cleavage. Additionally, in vivo positron emission tomography imaging using a 64Cu-labeled construct validated enhanced activation in tumor tissues compared to healthy tissues.

These results demonstrate that side chain masking can effectively prevent nonspecific interactions and provide a platform for designing CPP-based delivery systems with improved specificity and safety profiles for therapeutic and diagnostic applications. This strategy holds promise for overcoming the limitations of conventional CPPs in clinical contexts.

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List of Abbreviations

A_{hx} - Aminocaproic acid

CD - circular dichroism

CPPs - Cell-penetrating peptides

DOTA - dodecane tetraacetic acid

DOTA-TGFDPEI - the DOTA-AhxFVQWFSK(Ac-IEPDFG)FLGK(Ac-IEPDFG)IEPD peptide construct

DOTA-TGF - the DOTA-A_{hx}FVQWFSK(FG)FLGK(FG)IEPD peptide construct

DOTA-mTL - the DOTA-A_{hx}FVQWFSKFLGKIEPD peptide construct

MALDI-TOF - matrix assisted laser desorption ionization-time of flight mass spectrometry

LC-MS - liquid chromatography–mass spectrometry

DPC - Dodecylphosphocholine

GRIP B - granzyme-targeting restricted interaction peptide specific to family member B

GZMB - granzyme B

mTL - modified Temporin L

PET - positron emission tomography

SPPS - solid-phase peptide synthesis

Introduction

Cell-penetrating peptides (CPPs) are short peptides capable of traversing biological membranes, enabling the transport of attached cargo into cells. This unique property has garnered significant interest for their potential in diagnostics and therapeutic applications, where CPPs can facilitate the selective delivery of diverse molecular cargoes to cell surfaces or intracellular targets. Examples of such cargoes include fluorophores for imaging, chemotherapeutic agents for cancer treatment, nucleic acids for gene regulation, and even full-length proteins as therapeutics. These versatile capabilities make CPPs a promising platform for advancing targeted delivery systems in medicine.[1,2]

Amphipathic cell-penetrating peptides are generally composed of hydrophobic and positively charged amino acid residues at physiological pH. Due to their small size, these peptides typically adopt a random coil structure in solution, as they lack a tertiary structure to shield the hydrophobic residues from bulk solvent. However, in the presence of biological membranes, amphipathic CPPs can transition to an alpha-helical secondary structure. In this conformation, the hydrophobic and hydrophilic residues align on opposite sides of the helix, forming a hydrophobic face that interacts with the lipid tails and a positively charged hydrophilic face that engages with the negatively charged phosphate heads of the lipid bilayer. This helical structure is generally essential for effective membrane binding.[3] The degree of interaction between CPPs and membranes can be modulated by tuning the peptide's hydrophobicity and charge, enabling outcomes ranging from membrane adherence to pore formation or complete

endocytosis into the cell. However, because these interactions are driven by nonspecific electrostatic and hydrophobic forces, they occur with all cell types possessing phospholipid membranes. Consequently, most CPPs lack the specificity needed for selective cargo delivery to particular cell types.

A common strategy to prevent nonspecific membrane binding involves linking a CPP to an anionic masking peptide, which effectively neutralizes the positive charges on the CPP.[2] With its positive charges shielded, the CPP cannot adopt the alpha-helical conformation required for interaction with negatively charged cell membranes. Furthermore, by incorporating a protease-cleavable sequence between the CPP and the masking peptide, CPP activity can be selectively activated in tissues where the target protease is upregulated. Given the prevalence of protease dysregulation in many diseases, protease-activatable masking strategies have become a widely used method for modulating CPP activity.

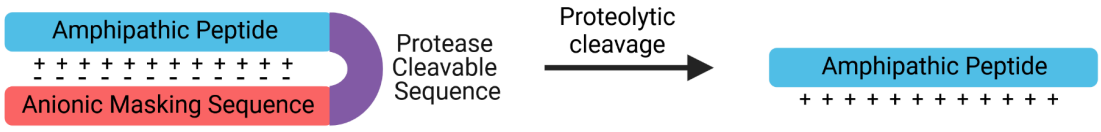

Figure 1.1 Linear protease-activatable masking strategy of cationic cell penetrating peptides.

Although many activatable CPP-based therapeutics have progressed to clinical trials, none have received FDA approval.[4] The primary challenges with these drugs are limited efficacy and toxicity, both stemming from a lack of cell and tissue specificity.[1,4] A key issue in their design is the non-negligible expression of target proteases in healthy

tissues, combined with functional redundancy among proteases. These factors lead to off-target activation and reduces the effective drug concentration in the intended tissues.[5]

Mizukami et al. investigated a protease-responsive strategy for modulating the activity of the cell-penetrating peptide temporin L (TL) by covalently caging its lysine side chain with a caspase-3 cleavable peptide sequence.[6] In a liposome-encased carboxyfluorescein release assay, the modified peptide STL1 showed significantly reduced membrane-damaging activity compared to TL, which was linked to its inability to form α-helical structures in the liposome environment, as confirmed by circular dichroism spectra. Upon cleavage by caspase-3, STL1 was converted to TL, restoring its activity and triggering carboxyfluorescein release. This work demonstrated that chemically caging lysine's positive charge offers an effective protease-responsive CPP protection strategy.

A study by Xu et al. similarly cages the positive charge on the lysine side chain of TL via conjugation to a cephalosporin antibiotic, which inhibits the membrane penetrating activity of TL.[7] When the construct was incubated with metallo-β-lactamases, the cephalosporin was hydrolyzed, releasing native TL and restoring its membrane-penetrating function. This protective approach was validated through multiple assays, including a cellular toxicity assay in HEK293 cells, a hemolysis assay with human red blood cells, and a minimum inhibitory concentration assay against susceptible *Escherichia coli*.

Inspired by these approaches, we present a side-chain protection strategy for the granzyme-targeting restricted interaction peptide specific to family member B (GRIP B) probe developed by Zhao et al.[8] GRIP B is a linear peptide-based imaging construct that incorporates a ^{64}Cu-labeled modified Temporin L (mTL), linked to a masking domain via a granzyme B (GZMB)-cleavable sequence. In tumor microenvironments with elevated GZMB levels, the enzyme cleaves the masking sequence, unmasking mTL and enhancing the localized accumulation of the probe. The radioactivity of ^{64}Cu is then interpreted using positron emission tomography (PET) to measure GZMB activity *in vivo*. This construct successfully detected GZMB activity from T cells activated with immune checkpoint inhibitors in three mouse cancer models.

In this work, we cage the two positive charges on mTL, as described by Zhao et al., using GZMB-cleavable sequences to prevent membrane interaction until proteolytic activation. This strategy not only frees the N-terminus for cargo conjugation but also allows for the incorporation of multiple protease-cleavable sequences. By leveraging this approach, we envision that incorporating multiple protease-cleavable sequences could exert a multiplicative effect on unmasking selectivity, thereby enhancing the contrast between tumor and healthy tissues and achieving selective delivery.

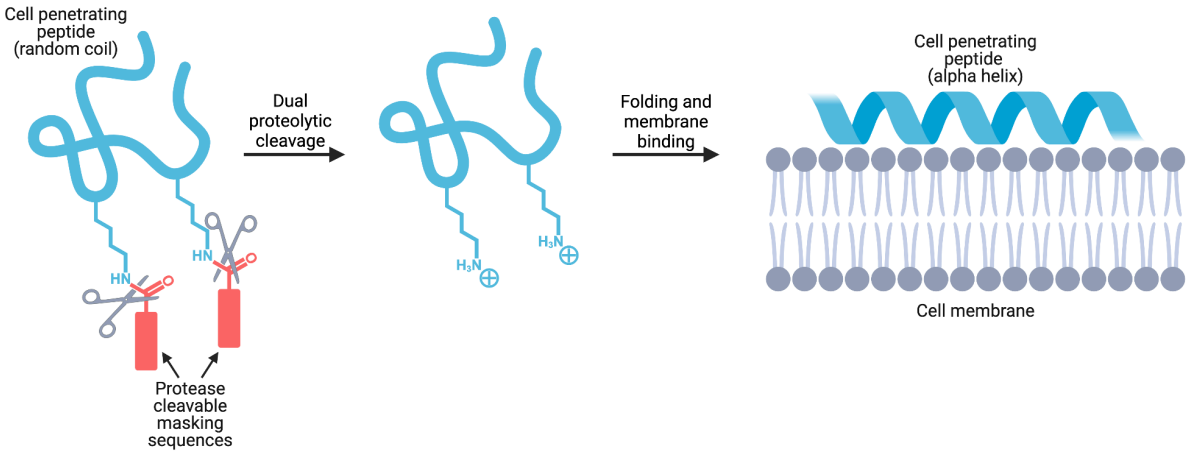

Figure 1.2 Lysine side chain masking strategy of cationic cell penetrating peptides that can be removed upon proteolytic cleavage

Methods

Peptide synthesis

All peptides were synthesized on a Biotage Initiator Alstra microwave synthesizer with 9-fluorenylmethoxycarbonyl-based solid-phase peptide synthesis (SPPS) using wang resin (Chem-Impex) with 0.3-0.8 mmol loading per gram of resin.

N-α-Fmoc-N-ε-4-methoxytrityl-L-lysine was incorporated into the mTL backbone via standard peptide coupling conditions. The methoxytrityl protecting group was later removed with TFA/TIPS/DCM (1:2:97). The resin was shaken with the solution for 15 minutes and washed with DCM. Incubation with the deprotection solution and wash was repeated until the solution no longer appeared orange/yellow (typically 4-5 rounds of incubation). The newly exposed amine of the lysine sidechain was further extended via 9-fluorenylmethoxycarbonyl-based SPPS to complete synthesis of branched constructs.

The peptides were globally deprotected and cleaved from the resin with a solution of TFA/TIPS/H2O (95:2.5:2.5), precipitated with cold diethyl ether, dissolved in DMSO, and purified using reversed-phase HPLC on a C18 preparative column (Axia) with a gradient of solvents A (water containing 0.1% TFA) and B (acetonitrile containing 0.1% TFA) at a flow rate of 10 ml/min. The peptides were then lyophilized until dry. Following successful purification, the peptides were confirmed to have the correct mass by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Shimadzu AXIMA Performance) using α-cyano-4-hydroxycinnamic acid (Sigma) as the matrix. The peptides were then dissolved in DMSO to the appropriate stock concentrations and subsequently used in all experiments as described.

Proteolytic time course

Lyophilized granzyme B human (Sigma-Aldrich) was first diluted to 18.7 uM in PBS, pH 7.4 and stored in -20C. Frozen stocks were thawed immediately prior to use. Lyophilized peptides were dissolved to a final concentration of 50 uM in PBS, pH 7.4 along with addition of the granzyme B human stock solution to a final concentration of 50 nM. The solution was incubated at 37 °C with shaking for 5 hours. 15 uL time points were collected at the beginning and at 1-hour intervals with addition of 15 uL of 1M HCl to quench GZMB activity. The time points were kept on ice until injection into the analytical HPLC.

Circular Dichroism

Dodecylphosphocholine (DPC) micelles were prepared by addition of DPC (Avanti Polar Lipids) into PBS, pH 7.4 to a concentration of 3mM. Lyophilized peptides were dissolved to 100uM in either PBS, pH 7.4 or in 3mM DPC/PBS, pH 7.4. Solutions were transferred to a 10-mm cuvette and circular dichroism data were collected on a Jasco J-810 (190-250 nm, 1 nm bandwidth, sextuplicate measurements).

Cell Culture

HeLa cells were cultured at 37 °C in a 5% CO2 atmosphere. The cells were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS and 0.1% penicillin-streptomycin. Media was replaced every other day until cells reached the desired confluency.

Viability Assay

HeLa cells (4 x 10^3) were seeded in a 96-well plate in cell culture media (100 µL) for 24 h at the atmosphere stated above. The media was then aspirated and treated with 100 µL of vehicle or drug at 100 µM (n=6) for 24 h at 37 °C. Cell viability was assessed using MTT. MTT media (20 µL, 5mg/mL) was added to each well for 2hrs and plate was centrifuged at 500 x g for 5 min after incubation, followed by media aspiration. The formazan crystals left were solubilized in DMSO and absorbance (540 nm) was measured using a microplate reader (SpectraMax M5). Data obtained was normalized to the vehicle. The graph shown is depicted as the mean ± SD vs. compound concentration.

Radiochemistry

[^{64}Cu]-$CuCl_2$ was purchased from University of Wisconsin Madison. C18 sep-pak cartridges (C18 Plus Short Cartridge, 360 mg Sorbent per Cartridge, 55–105 μm; or C18 Plus Light Cartridge, 130 mg Sorbent per Cartridge, 55–105 μm) were purchased from Waters. rad-HPLC was performed on a Hitachi Elite LaChrom HPLC System equipped with a gamma detector.

General procedure for labeling of peptides with ^{64}Cu

The peptide was mixed with 0.4 M NH_4OAc buffer (200 μL, pH adjusted to ~5.5). This solution was added to [^{64}Cu]-$CuCl_2$ and incubated at 37 °C for 30 min. After completion as determined by rad-HPLC, the crude mixture was diluted with 2 mL H_2O and passed through an C18 sep-pak cartridge (preactivated with 5 mL EtOH, followed by 10 mL H_2O). The cartridge was washed with 3 mL H_2O, followed by elution with 2 mL EtOH. The product was analyzed with rad-HPLC and dried under a stream of nitrogen.

For animal studies, the product was formulated in a final solution of saline with 10% DMSO and 10% TWEEN-80.

^{64}Cu-TempL-GFDPEI: According to general procedure, the reaction of TempL-GFDPEI (2 nmol) and 8.12 mCi [^{64}Cu]-$CuCl_2$ afforded ^{64}Cu-TempL-GFDPEI in 53% yield (decay corrected) and >98% radiochemical purity. Specific activity was 2140 mCi/μmol.

^{64}Cu-TempL: According to general procedure, the reaction of TempL (1 nmol) and 9.8 mCi [^{64}Cu]-$CuCl_2$ afforded ^{64}Cu-TempL in 35% yield (decay corrected) and >98% radiochemical purity. Specific activity was 2690 mCi/μmol.

Animal Studies

All animal procedures were approved by the UCSF Institutional Animal Care and Use Committee, and all studies were performed in accordance with UCSF guidelines regarding animal housing, pain management, and euthanasia. 6-8 weeks C57BL/6 mice were purchased from Jackson Laboratory and housed with free access to the water and food. An LPS model was established by intranasal (i.n.) instillation of 3 mg/Kg LPS (lipopolysaccharide) from *E.coli* 055:B5 (L2880, Sigma Aldrich) or saline. PET/CT was performed 4 days after LPS instillation.

For imaging studies, the mice were transferred to a Siemens Inveon small animal PET-CT system (Siemens, Erlangen, Germany) and imaged using a single static 20 min PET acquisition followed by a 10 min micro-CT scan for attenuation correction and anatomical co-registration. No adverse events were observed during or after injection of any compound. Anesthesia was maintained during imaging using isoflurane.

Immunofluorescence

Immunofluorescent staining (IF) was performed on frozen lung tissue samples. Samples were blocked using 0.5% bovine serum albumin (BSA) in 1X phosphate buffered saline (PBS). The primary antibody, Mouse Granzyme B Antibody (R and D Systems AF1865), was added at a 1:200 dilution and incubated at 4C overnight. This primary antibody was detected using Donkey anti-Goat IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific

A-11055) at a 1:2000 dilution. DAPI Nucleic Acid Stain (D1036, Invitrogen) was used to stain the nucleus, and samples were incubated at a 1:4000 dilution for 10 min at room temperature. Tissues were then sent to The J David Gladstone Institutes Histology and Light Microscopy Core Facility for imaging. IF slides were scanned in a whole slide scanner Leica Aperio Versa Slide Scanner with 20X; 0.80 Air, HC PL APO objective, and images were captured by Andor Zyla 5.5 sCMOS mono + RBG camera.

GZMB Homozygous Knockout Mice

Germline homozygous GZMB knockout cluster mice were created with a homozygous null mutation in the GZMB gene. GZMB knockout cluster mice were generously donated by Dr. T. Ley, originating from (Cell 1994 Mar 25;76(6):977-87, doi: 10.1016/0092-8674(94)90376-x).

PET Data collection and analysis

All *in vivo* PET data were viewed and analyzed by using open-source Amide software (amide.sourceforge.net). Its automated SUV calculation tool was used by entering decay-corrected injected activity and the animal weight. For each region of interest, a spherical region of interest (ROI, ~3 mm diameter) was drawn, and standardized uptake values (SUVs) were calculated by ROI statistics.

Results

Building upon the GRIP-B platform, a GZMB-cleavable branched construct with an amino acid sequence: DOTA-A_{hx}FVQWFSK(Ac-IEPDFG)FLGK(Ac-IEPDFG)IEPD

(DOTA-TGFDPEI) was synthesized. The mTL portion of the construct was synthesized with an aspartate loaded wang resin using 9-fluorenylmethoxycarbonyl-based SPPS. This was followed by deprotection of the methoxytrityl protecting groups on the lysine side chains, and the GZMB cleavable sequence was extended from the newly exposed amines via SPPS.

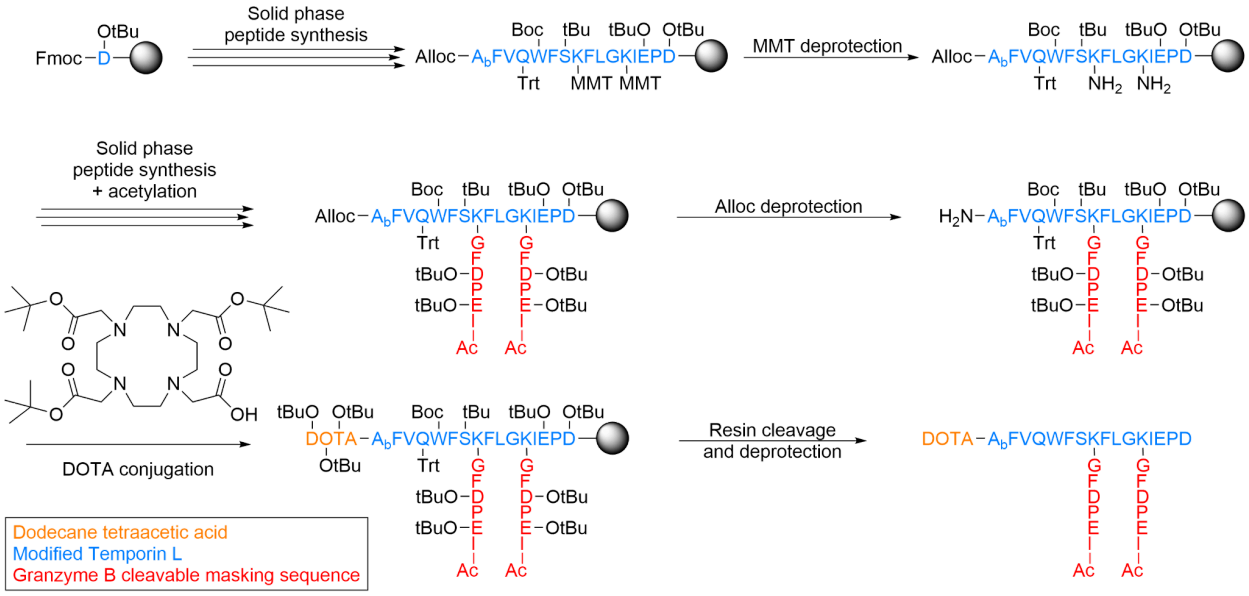

Figure 2 Synthetic scheme of protease-labile side chain protected mTL

After proteolytic cleavage with GZMB, the construct releases mTL containing a two amino acid phenylalanine-glycine scar on each lysine side chain with a post cleavage sequence DOTA-A_{hx}FVQWFSK(FG)FLGK(FG)IEPD (DOTA-TGF). With a proteolytic time course, the side chains of this construct were shown to be cleavable by GZMB and produced expected cleavage products through analysis with matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid

chromatography–mass spectrometry (LC–MS), and further matched the retention time of an authentic synthesized sample.

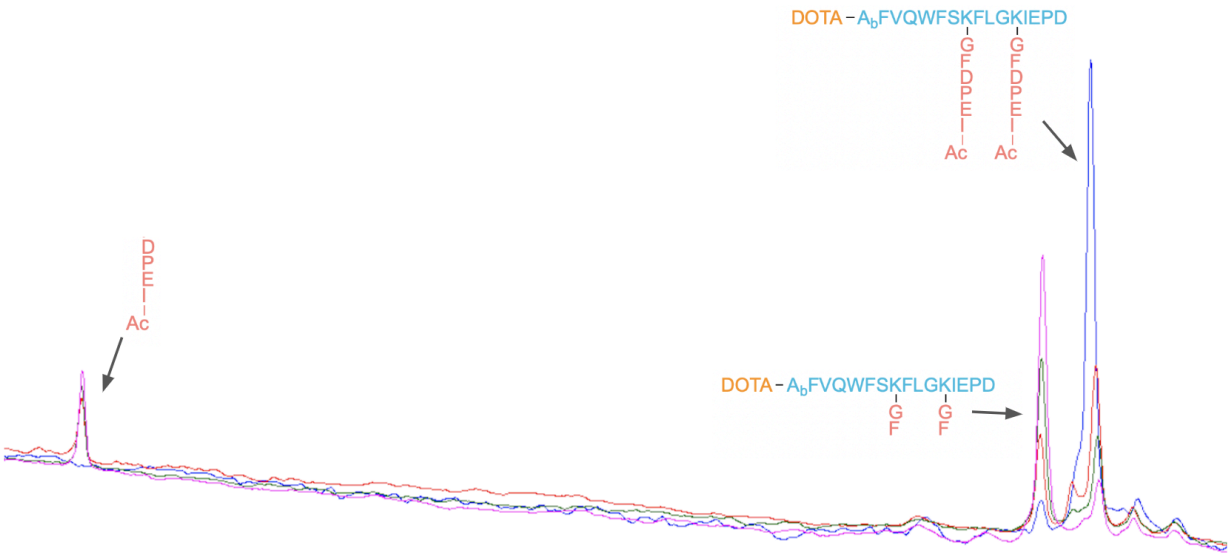

Figure 3.1 HPLC trace of Proteolytic cleavage time course of DOTA-TGFDPEI (50uM) with Granzyme B (50nM) over 5 hours.

Circular dichroism (CD) measurements demonstrate that DOTA-TGF assumes a randomly coiled structure in PBS alone, while adopting an alpha helical conformation in the presence of dodecylphosphocholine (DPC) micelles, indicating that the two amino acid scars do not prohibit membrane binding.

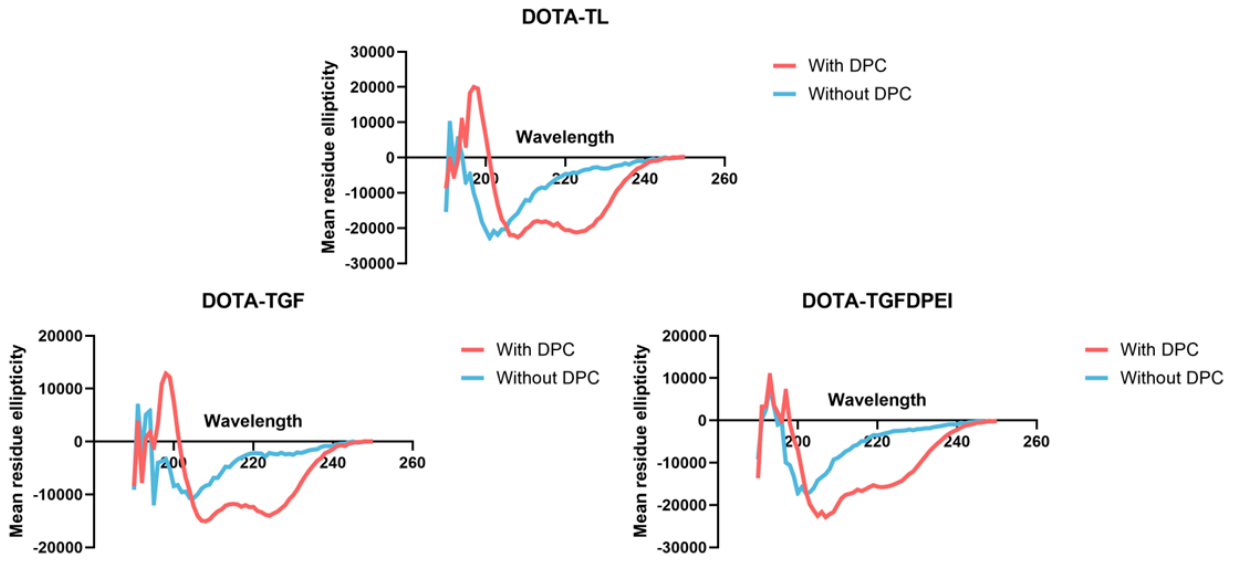

Figure 3.2 Circular dichroism spectra of DOTA-TL, DOTA-TGF, and DOTA-TGFDPEI in PBS or with DPC micelles in PBS.

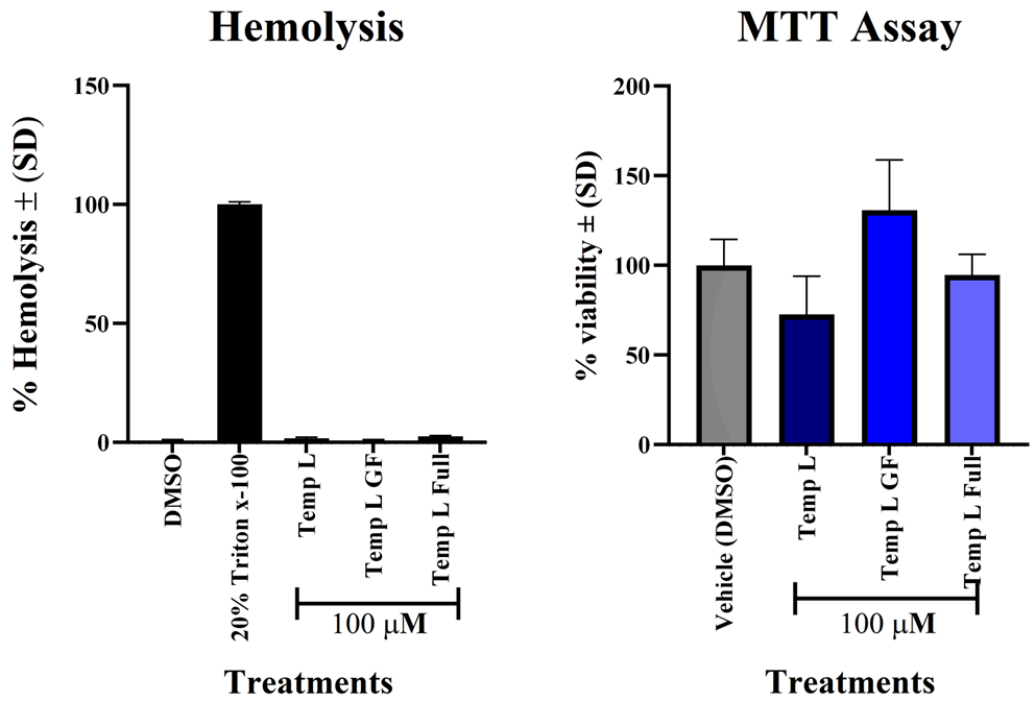

Figure 4.1 Hemolysis assay of DOTA-TL, DOTA-TGF, and DOTA-TGFDPEI with human erythrocytes. **4.2** Cell viability MTT assay of DOTA-TL, DOTA-TGF, and DOTA-TGFDPEI in HEK293 cells.

After confirming the cleavage activities with in vitro assays, we moved to test its action in vivo with PET/CT mouse imaging. Previous studies confirmed elevated granzyme B activities in the lungs of mice with infections, such as influenza. (ACS Infect. Dis. 2024, 10, 6, 2108–2117). In this study, we create a similar immune response model with lipopolysaccharides (LPS) from E. Coli based on our prior works (ACS Cent. Sci. 2021, 7, 10, 1638–1649).

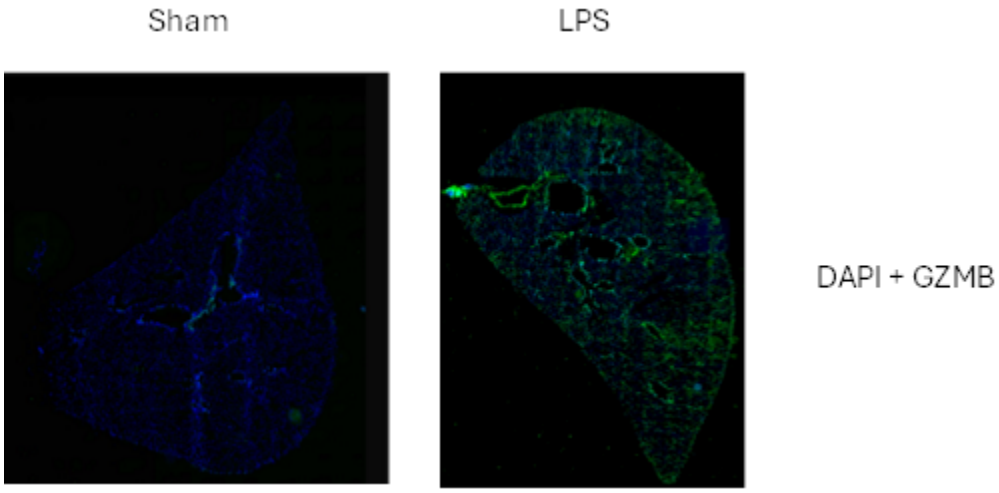

Figure 5.1 Immunofluorescence images showed higher expression of GZMB in LPS-instilled lung.

Hence, we instilled LPS into wild-type C57BL/6 mice (n = 4) to induce immune response, which was repeated for GZMB knockout mice (n = 3). Similarly, 2 sham cohorts (wild-type, n = 4; GZMB knockout, n = 3) were given PBS. After 4 days, the mice were injected with 64Cu-Temporin L-TGFDPEI for PET/CT scanning at 1 h, 3 h, 5 h, and 24 h post-injection (Figure 5.2).

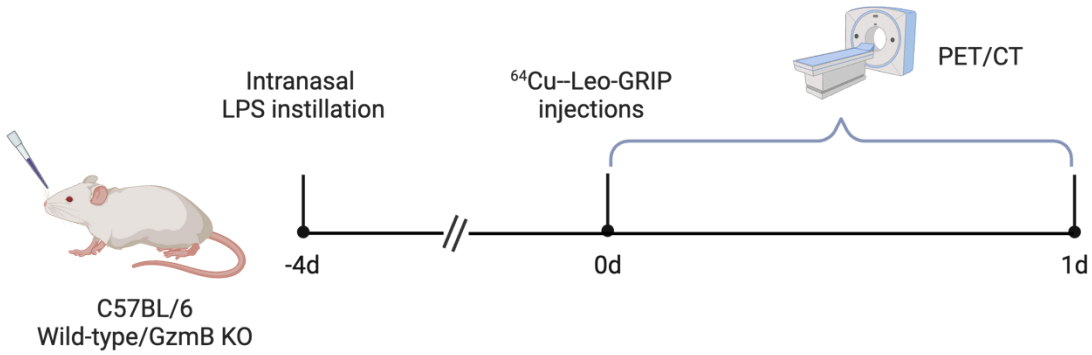

Figure 5.2 Experimental setup of mouse study with Temporin L-TGFDPEI.

The expression of GZMB was visualized with immunofluorescence (IF) of lung slides from C57BL/6 mice (Figure 5.1). The increased expression of GZMB is evident in LPS-instilled mice, while the expression level remains minimal for sham mice; this confirmed the effect of LPS in stimulating immune response. Representative PET/CT images of lungs are displayed in Figure 5.3. Region of Interest (ROI) analysis showed that the radiotracer uptake in lung of wild type C57BL/6 mice was the highest, reaching an SUVmean of ~2.2% ID/cc at 5 h and continue to 24 h post injection (Figure 5.3).

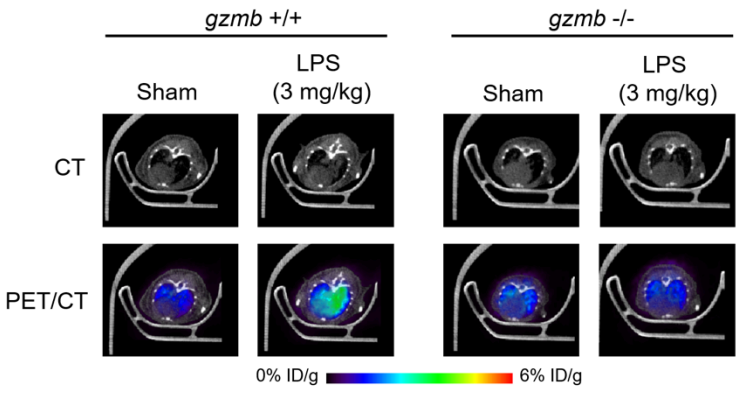

Figure 5.3 Representative transaxial [^{64}Cu]-TempL-GFDPEI mPET/CT images showing uptake in the lungs of C57BL/6 mice (wild type or Granzyme B knockout), which were treated with intranasal instillations of sham or LPS. Mice (n = 4) were injected with

[^{64}Cu]-TempL-GFDPEI on day 4 post-LPS instillation. Images are shown from one mouse per arm imaged at 5 h post injection of [^{64}Cu]-TempL-GFDPEI.

Radiotracer uptake in the lungs of wild type mice with PBS did not exceed 1.2% ID/cc at any time in our study. On the other hand, both LPS and PBS-instilled GZMB knockout mice had similar uptakes in lung, which peaked at around 3 to 5 h post injection. No significant differences were observed in the ROI analysis of other major organs (heart, kidney, liver, muscle; see supporting information). The uptake in lung of wildtype, LPS-instilled mice suggested the probe was cleaved in the presence of excess GZMB in the LPS-stimulated lungs, forming helix structures and retaining in the affected lung regions. The lack of GZMB expression in knockout mice precluded the cleavage of the probe, which results in a similar lung uptake as the sham, wild type mice.

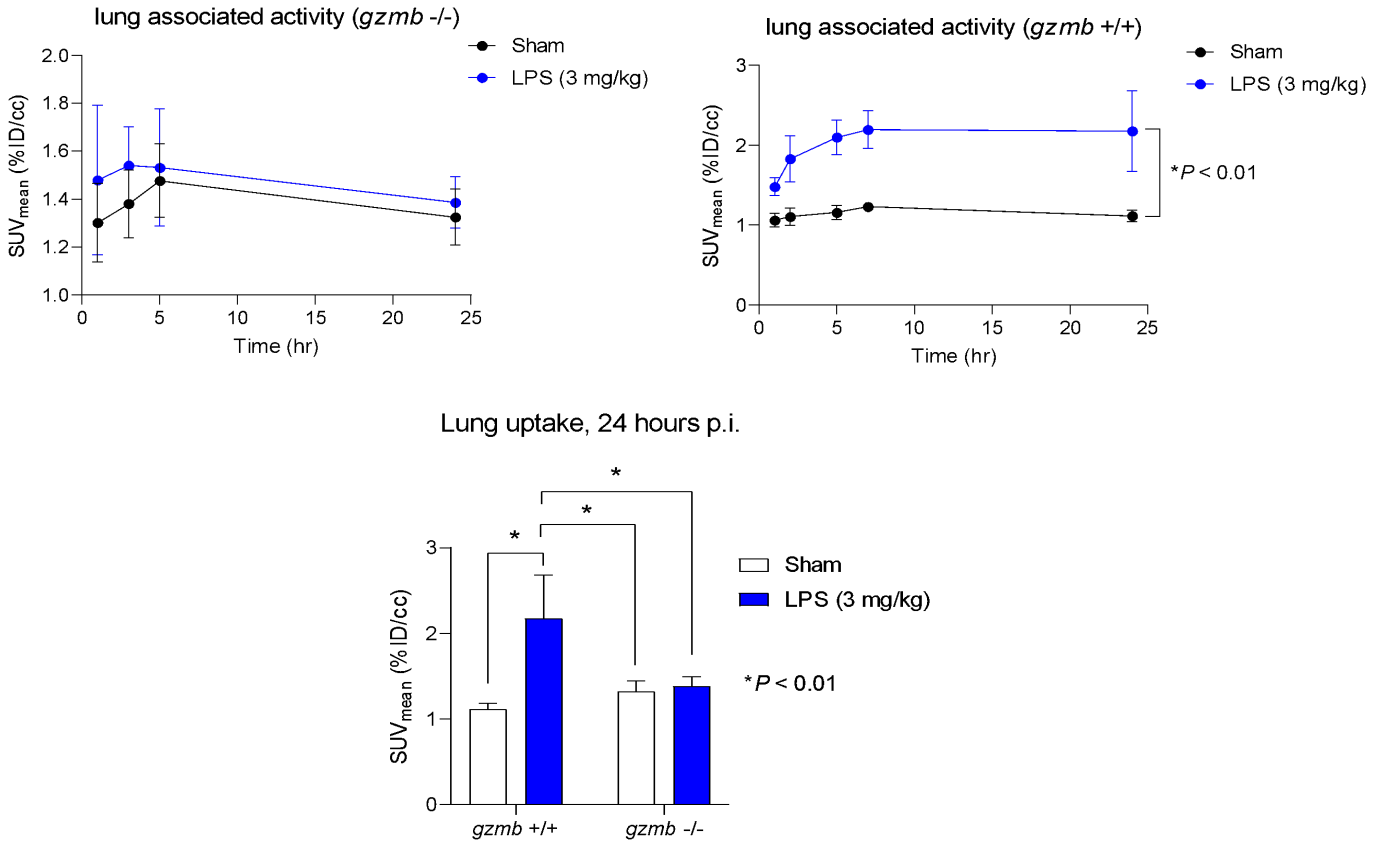

Figure 5.4 Region of interest (ROI) analysis of lung parenchyma shows higher radiotracer uptake in infected versus sham treated mice. * $p < 0.01$.

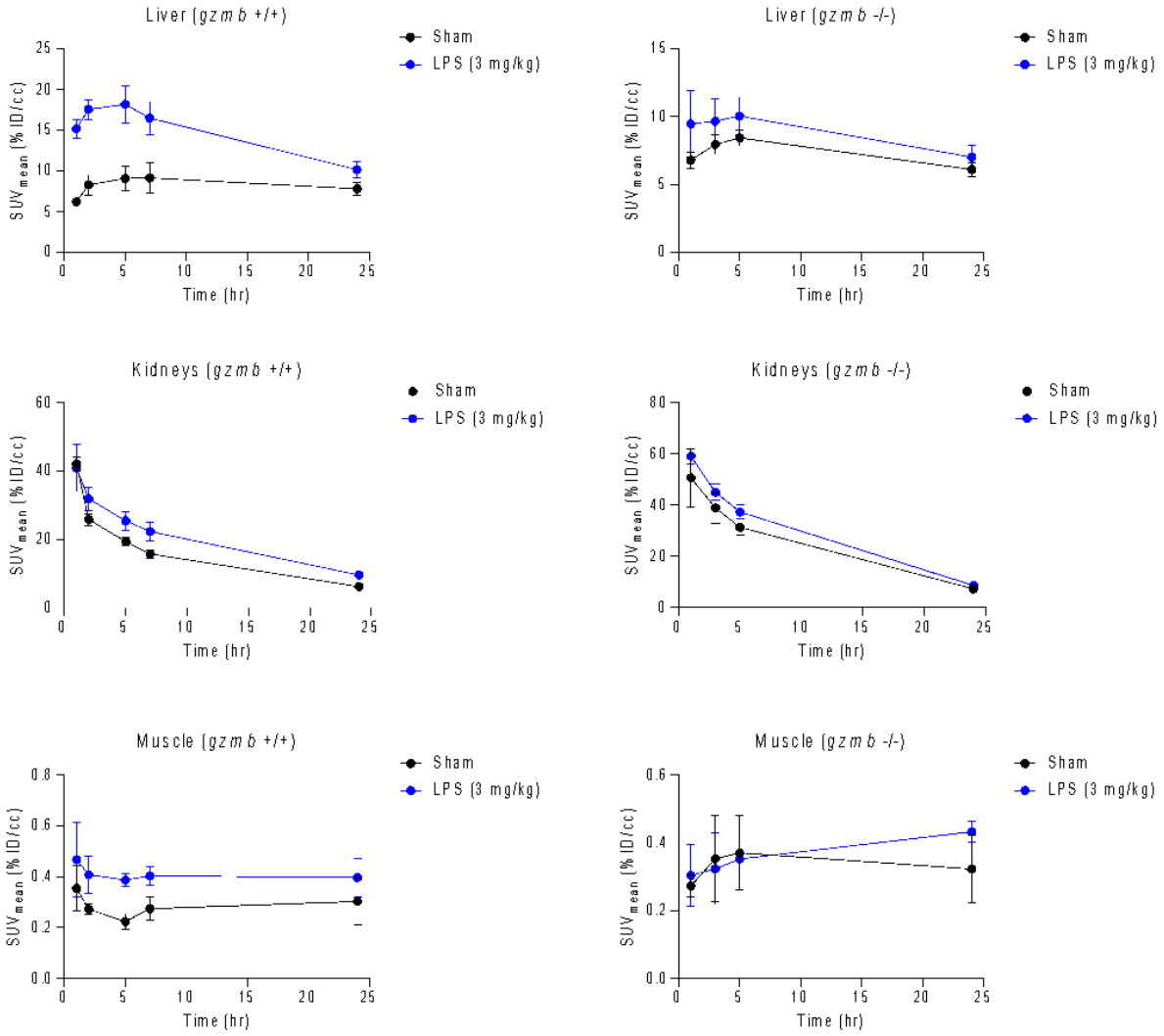

Figure 5.5 Region of interest (ROI) analysis of liver, kidney, and muscle over 25 hours.

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