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A novel delivery platform for therapeutic peptides

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ABSTRACT

Although many peptides have therapeutic effects against diverse disease, their short half-lives *in vivo* hurdle their application as drug candidates. To extend the short elimination half-lives of therapeutic peptides, we developed a novel delivery platform for therapeutic peptides using an anti-hapten antibody and its corresponding hapten. We selected cotinine because it is non-toxic, has a well-studied metabolism, and is physiologically absent. We conjugated WKYMVm-NH_2, an anti-sepsis therapeutic peptide, to cotinine and showed that the conjugated peptide in complex with an anti-cotinine antibody has a significantly improved *in vivo* half-life while retaining its therapeutic efficacy. We suggest that this novel delivery platform for therapeutic peptides will be very useful to develop effective peptide therapeutics.

1. Introduction

Many peptides have promising characteristics as drug candidates. However, due to their small size, in general they have extremely short elimination half-lives *in vivo* [1]. PEGylation can inhibit their renal clearance, but PEGylation usually requires time-consuming, peptide-specific optimization [2]. We hypothesized that an anti-hapten antibody could function as an alternative peptide carrier in complex with its corresponding hapten. For this purpose, the hapten must be non-toxic and physiologically absent and inert in animals and humans. Additionally, its metabolism must be well characterized. As a major metabolite of nicotine to which humans have long been exposed, cotinine is an appropriate hapten for this application. Cotinine is a relatively safe molecule with an LD_{50} of 2–4 g/kg in mice [3], and doses up to 1.8 g per day during a four-day period did not induce detectable negative effects and were well tolerated in humans [4]. Its metabolism in mammals is well known, with a serum half-life of approximately 16 h [5]. In human cotinine is absent physiologically and up to 160 mg cotinine intake for three consecutive days did not induce any physiological and behavioral effect [6].

The peptide Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$ (WKYMVm-NH_2) is a synthetic peptide that stimulates leukocytes, resulting in phospholipase C activity [7,8]. In terms of functional activity of WKYMVm-NH_2, the peptide stimulates phagocytic activity, superoxide anion production, increasing bactericidal activity in phagocytic cells such as monocytes and neutrophils [9–11]. The peptide also stimulates IFN-γ production in natural killer cells [12]. Like other chemokines, WKYMVm-NH_2 stimulates chemotactic migration of several different leukocytes, including monocytes, neutrophils, dendritic cells, and activated natural killer cells [12–14]. On the target receptors for the peptide, WKYMVm-NH_2 binds to the formyl peptide receptor (FPR) family; FPR1, FPR2, and FPR3 [14–17]. WKYMVm-NH_2 also binds to at least two different types of mouse FPR family; mFPR1 and mFPR2 [18,19]. Recently we demonstrated that administration of WKYMVm-NH_2 elicited effective therapeutic activity against polymicrobial sepsis [20]. However, injection of large amounts of the peptide (4 mg/kg twice per day for 2 days) was required, which might be due to its short half-life. In this study, we investigated anti-cotinine antibody/cotinine-conjugated therapeutic peptide as a new delivery platform.

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2. Materials and methods

2.1. Peptide synthesis and conjugation with cotinine

Peptides (WKYMVm-NH_2, wkymvm-NH_2) and cotinine-peptide conjugates (cotinine-WKYMVm-NH_2, cotinine-wkymvm-NH_2) used in this study were chemically synthesized by Peptron Inc. (Daejon, Korea).

2.2. Overexpression and purification of anti-cotinine rabbit/human chimeric antibody

The genes encoding variable regions of the heavy chain (VH) and light chain (VL) were amplified from anti-cotinine scFv–Fc expression vector [1] using (ATCCTGTTCCTGGTGGCCACCGCCACCG GCCAGTCGGTGAAGGAGTCC) and (ATCCTGTTCCTGGTGGCCACCGC CACCGGCGAGCTCGATCTGACCCAG) as 5′ primers and (TGAAGA GATGGTGACCAGGGTGCC) and (TAGGATCTCCAGCTCGGTCCCTCC) as 3′ primers. Human heavy chain constant region (CH1–CH3) and human light chain constant region (Cκ) were amplified from a human bone marrow cDNA library (Clontech Laboratories, Palo Alto, CA) using (GTCACCATCTCTTCAGCCTCCACCAAGGGC) and (GAGCTCGGATCCCTTGCCGGCCGT) as 5′ primers and (GAGCTG GAGATCCTACGGACCGTGGCCGCC) and (GCAAGCTCTAGACTAGCA CTCGCC) as 3′ primers containing annealing sites with VH and VL. Overlapping extension PCR was performed using (ACATC GGCTAGCCGCCACCATGGGCTGGTCCTGCATCATCCTGTTCCTG) and (ACTTAAGCTTGCGCCACCATGGGCTGGTCCTGCATCATCCTGTTCCTG) as 5′ primers and (GAGCTCGGATCCCTTGCCGGCCGT) and (GCAAGC TCTAGACTAGCACTCGCC) as 3′ primers to generate genes encoding the complete heavy chain and light chain fragments, respectively. The genes encoding heavy chain and light chain were digested with BamHI and NheI (New England Biolabs, Beverly, MA), and HindIII and XbaI (New England Biolabs), and inserted into an expression vector designed for expression of IgG in mammalian cells. The vector encoding IgG was transfected to CHO DG44 cells (Invitrogen, Carlsbad, CA) as described previously [21] and cultured in CD OptiCHO™ expression medium (GIBCO, Gaithersburg, MD) containing 100 U/ml penicillin and 100 g/ml streptomycin (GIBCO). Overexpressed IgG was purified by protein A gel affinity chromatography in accordance with the manufacturer's instructions (Repligen, Cambridge, MA).

2.3. Cell culture

Vector- or FPR2-transfected RBL-2H3 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 g/ml streptomycin. FPR2-transfected RBL-2H3 cells were also cultured with 250 μg/ml G418, as described previously [22].

2.4. Reactivity of the cotinine-WKYMVm-NH_2/antibody complex to FPR2-transfected RBL-2H3 cell in flow cytometric analysis

FPR2- or vector-transfected RBL-2H3 cells (1×10^5 cells per sample) were washed twice with PBS and once with flow cytometry assay buffer (1% FBS, 0.02% sodium azide in PBS). FPR2-transfected RBL-2H3 cells were incubated with 0, 1, 10, or 100 nM cotinine-WKYMVm-NH_2 and 100 nM anti-cotinine antibody for 30 min at 4 °C in 50 μl flow cytometric assay buffer. In parallel control experiments, the cells were incubated with 0, 1, 10, or 100 nM cotinine-WKYMVm-NH_2 and 100 nM control IgG (Palivizumab; Synagis®; Abbot Laboratories, Kent, UK), or 100 nM cotinine-wkymvm-NH_2 and 100 nM anti-cotinine antibody. In another control experiment, vector-transfected RBL-2H3 cells were incubated with 0, 1, 10, or 100 nM cotinine-WKYMVm-NH_2 and 100 nM anti-cotinine antibody. Cells were then washed with flow cytometric assay buffer twice and incubated with FITC-labeled anti-human Fc antibody (Thermo Fisher Scientific, Waltham, MA) diluted 1:100 in 50 μl flow cytometric assay buffer for 20 min at 4 °C. After washing twice with flow cytometric assay buffer, the cells were resuspended in 100 μl PBS, fixed with 100 μl 2% paraformaldehyde, and analyzed with a FACSCanto™ II flow cytometer (BD Bioscience, Heidelberg, Germany). Data were analyzed with FlowJo data analysis software (Treestar, Ashland, OR, USA).

2.5. Isolation of human neutrophils

The Institutional Review Board (IRB) of Ajou University Hospital specifically approved this study (approval ID: AJIRB-MED-SMP-10-074). We obtained written informed consent from all participants involved in this study. Peripheral blood was collected from healthy donors and human neutrophils were isolated by dextran sedimentation, hypotonic lysis of erythrocytes, and a lymphocyte separation medium gradient as described previously [23]. Isolated neutrophils were maintained in RPMI 1640 medium supplemented 5% FBS at 37 °C until used for experiments.

2.6. Calcium measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using Fura-2/AM as described previously [24]. Briefly, freshly isolated human neutrophils were incubated with 3 μM Fura-2/AM at 37 °C for 50 min in 4 ml fresh serum-free RPMI 1640 under continuous stirring. After washing three times with serum-free RPMI 1640, 2×10^6 cells were aliquoted for each assay in 1 ml Ca^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES [pH 7.3], 10 mM glucose, and 0.2 mM EGTA). The cells were stimulated with 1, 2.5, 5, 10, or 100 nM WKYMVm-NH_2 or cotinine-WKYMVm-NH_2, either alone or with anti-cotinine antibody (molar ratio of 2:1). As negative controls, 100 nM wkymvm-NH_2 or cotinine-wkymvm-NH_2 either alone or with 50 nM anti-cotinine antibody were used. Fluorescence changes were measured at 500 nm at excitation wavelengths of 340 nm and 380 nm using a RF-5301PC spectrofluorophotometer (Shimadzu Instruments Inc., Kyoto, Japan).

An increase in intracellular calcium concentration causes an increase in the fluorescence ratio of 340–380 nm excitation efficiency, the fluorescence ratios were meaured according to Grynkiewicz et al. [24].

2.7. Measurement of superoxide generation

Superoxide generation was measured by using the superoxide-dependent reduction of cytochrome c as described previously [25]. Human neutrophils (2×10^6 cells in RPMI 1640) were preincubated with 50 μM cytochrome c at 37 °C for 1 min and then incubated with 0, 10, 100, or 1000 nM WKYMVm-NH_2 or cotinine-WKYMVm-NH_2, either alone or with anti-cotinine antibody (molar ratio of 2:1). As negative controls, 0, 10, 100, and 1000 nM wkymvm-NH_2 or cotinine-wkymvm-NH_2, either alone or with anti-cotinine antibody (molar ratio of 2:1), were used. The absorption changes with cytochrome c reduction at 550 nm were recorded using a microplate reader (EL312e; Bio-Tek instruments, Winooski, VT) over 5 min at 1-min intervals. The initial absorbance value (0 min) was subtracted from each subsequent reading, and this value was converted to nanomoles of superoxide by dividing the absorbance by an extinction coefficient of 0.022 $\mu M^{-1}cm^{-1}$.

2.8. Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe, Gaithersburg, MD) as described previously [25]. Briefly, prepared human neutrophils were suspended in RPMI at a concentration of 1×10^6/ml, and 25 μl of this suspension was placed onto the upper wells of a chamber separated by a 3-μm polyhydrocarbon filter from the low well, which contained 0, 10 or 100 nM WKYMVm-NH_2 or cotinine-WKYMVm-NH_2, either alone or with anti-cotinine antibody (molar ratio of 2:1). As negative controls, 0, 10 or 100 nM of wkymvm-NH_2 or cotinine-wkymvm-NH_2, either alone or with anti-cotinine antibody (molar ratio of 2:1), were used. After incubation for 90 min at 37 °C, non-migrated cells were removed by scraping, and cells that migrated across the filter were fixed with 4% paraformaldehyde overnight. Fixed filters were dehydrated with 90%, 80%, and 70% ethanol and deionized water for 2 min, sequentially. After dehydration, filters were air-dried, and dried filters were stained with hematoxylin (Sigma–Aldrich, St. Louis, MO). Stained cells from each well were counted in five randomly chosen high-power fields (400×) [25].

2.9. Pharmacokinetic properties of cotinine-WKYMVm-NH_2 and cotinine-WKYMVm-NH_2/antibody complex

Pharmacokinetic studies were performed using aged (4–6 weeks old) male wild-type albino Institute of Cancer Research Center (ICR) mice (ORIENT BIO Inc. Seongnam, Korea). To determine the pharmacokinetic property of cotinine-WKYMVm-NH_2 and cotinine-WKYMVm-NH_2/antibody complex, mice were given a single slow-push bolus tail vein injection of cotinine-WKYMVm-NH_2 (0.5 mg/kg) with or without anti-cotinine antibody (10 mg/kg) in a final volume of 100 μl ($n = 9$ mice per bleeding time). Peripheral blood samples (~300 μl) were collected from the retro-orbital plexus at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, and 24 h from cotinine-WKYMVm-NH_2-injected mice and 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24, 48, 72, 96, 120, 144, and 168 h from cotinine-WKYMVm-NH_2/antibody complex-injected mice. After incubation at room temperature for 30 min, the samples were centrifuged at 800×g for 15 min. The sera were collected and stored at −80 °C for until analysis. The relative amount of cotinine-WKYMVm-NH_2 was determined by flow cytometric analysis. Serum samples were diluted (1:5) in 25 μl flow cytometric assay buffer and mixed with 25 μl 200 nM anti-cotinine antibody to allow cotinine-WKYMVm-NH_2 in the diluted sera to complex with the antibody. FPR2-transfected RBL-2H3 cells (1×10^5 cells per sample) were incubated with the mixture for 30 min at 4 °C, washed twice with flow cytometric assay buffer, and incubated with FITC-labeled anti-human antibody diluted 1:100 in 50 μl flow cytometric assay buffer for 20 min at 4 °C. The cells were washed twice with the flow cytometric assay buffer, resuspended in 100 μl PBS, fixed with 100 μl 2% paraformaldehyde, and subjected to flow cytometry using a FACSCanto™ II flow cytometer (BD Bioscience). Data were analyzed with FlowJo data analysis software. Statistical analysis was performed with GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, CA).

2.10. Pharmacokinetic properties of anti-cotinine antibody

Mice were given a single slow-push bolus tail vein injection of anti-cotinine antibody (10 mg/kg) dissolved in 100 μl PBS ($n = 3$ mice per bleeding time). Peripheral blood samples (~300 μl) were collected from retro-orbital plexus at after 0, 1, 3, 6, and 12 h, and 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, and 28 days. After incubation at room temperature for 30 min, the samples were centrifuged at 800×g for 15 min. The sera were collected and stored at −80 °C until analysis. Serum concentrations of anti-cotinine antibody were measured using an enzyme immunoassay. Twenty microliters bovine serum albumin (BSA)-conjugated cotinine (5 μg/ml) were added to each well of a microtiter plate and incubated overnight at 4 °C. After washing with PBS with 0.05% Tween 20, 150 μl blocking solution (3% BSA in PBS) was added to each well. After incubation for 1 h at room temperature, 50 μl of diluted serum samples (1:10–1:1000 dilution) or anti-cotinine antibody solution of known concentrations (50–1000 ng/ml) was added to each well. The plate was incubated for 1 h at room temperature and washed four times with PBS with 0.05% Tween 20. Then 50 μl/well horseradish peroxidase-conjugated goat anti-human antibody (Thermo Fisher Scientific) diluted 1:5000 in blocking solution was added. The plate was incubated for 30 min at room temperature and washed five times with PBS with 0.05% Tween 20. ABTS [2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] substrate solution (100 μl/well; Amresco, Solon, OH) was added, plates were incubated for 30 min at room temperature, and optical density was measured at 405 nm (Supplementary Fig. 1).

2.11. Cecal ligation and puncture

Cecal ligation and puncture (CLP) model was performed according to previous reports [20,26]. Briefly, ICR mice were anesthetized with intraperitoneal injections of Zoletil (50 mg/kg) and Rompun (10 mg/kg), and a small abdominal midline incision was made to expose the cecum. The cecum was ligated below the ileocecal valve, punctured once through both surfaces with a 22-gauge needle, and then returned to the central abdominal cavity. Sham-operated mice were subjected to the same procedure, but without ligation or puncture of the cecum. The abdominal incision was closed three layers with 6-0 nylon sutures. Each group was given a single slow-push bolus tail vein injection at 2, 14, 26, and 38 h after CLP. Survival was monitored once daily for 10 days. Survival data were analyzed using the log-rank test with Bonferroni test for post hoc comparisons, and statistical significance was set a priori at $p < 0.05$.

3. Results and discussion

3.1. Generation of cotinine conjugated WKYMVm-NH_2

In this study, we conjugated the WKYMVm-NH_2 peptide to cotinine and then tested whether the conjugated peptide within an anti-cotinine rabbit/human chimeric antibody [27] complex would retain its characteristics and exhibit therapeutic efficacy at lower doses. After WKYMVm-NH_2 was chemically synthesized and conjugated to carboxy-cotinine with a linker (Fig. 1A), the reactivity of the cotinine-WKYMVm-NH_2/antibody complex to FPR2 was monitored by flow cytometry using FPR2-transfected RBL-2H3 cells. The cotinine-WKYMVm-NH_2/antibody complex bound specifically to FPR2-transfected RBL-2H3 cells but not to FPR2-negative cells (Fig. 1B). Neither WKYMVm-NH_2/control IgG antibody nor negative control peptide wkymvm-NH_2 (D-Trp-D-Lys-D-Tyr-D-Met-D-Val-D-Met-$CONH_2$) conjugated to cotinine/antibody complex bounds to FPR2-positve cells (Fig. 1B).

3.2. Cotinine-WKYMVm-NH_2/antibody complex shows biological activity

We next asked if the cotinine-WKYMVm-NH_2/antibody complex retained the functional activity of the peptide in three *in vitro* assays. Both cotinine-WKYMVm-NH_2 and cotinine-WKYMVm-NH_2/antibody complex induced robust Ca^{2+} mobilization in human neutrophils, showing maximal activity at ~5 nM

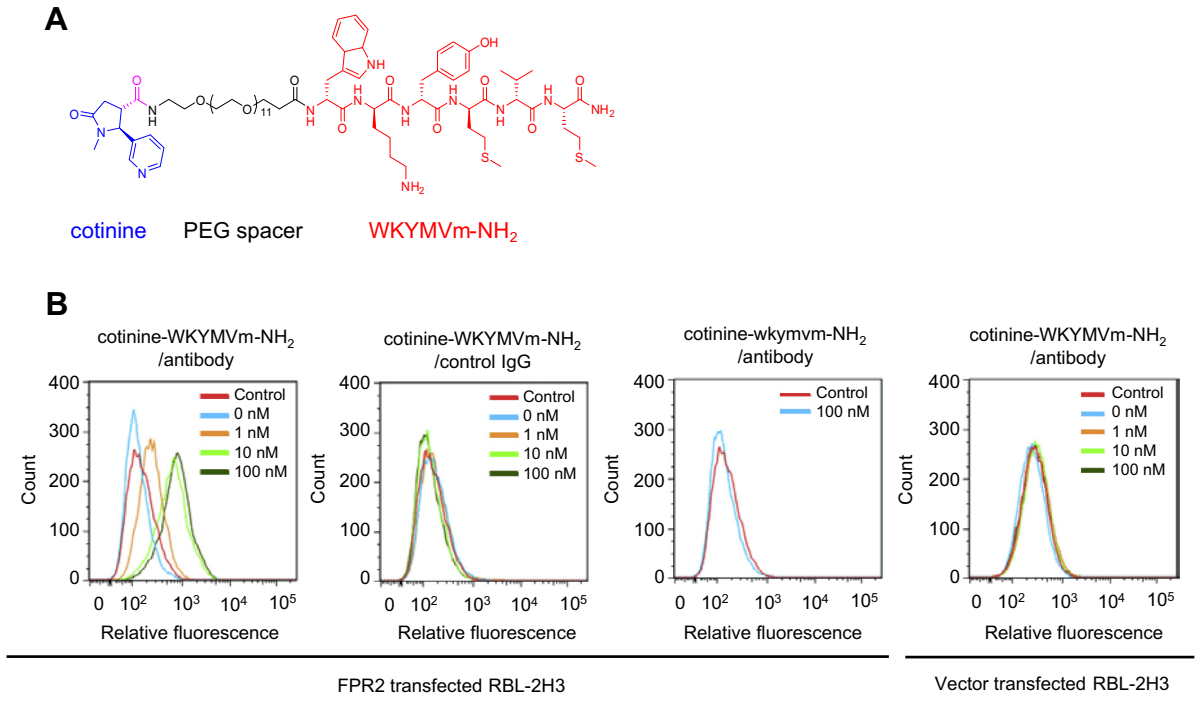

Fig. 1. *In vitro* characterization of the cotinine-WKYMVm-NH_2/anti-cotinine antibody complex. (A) WKYMVm-NH_2 conjugated to cotinine with a linker. (B) FPR2- or vector-transfected RBL-2H3 cells (1×10^5 cells/ml) were incubated with the indicated agents and FITC-labeled anti-human antibody at 4 °C for 20 min and subjected to flow cytometric analysis. Data are representative of three independent experiments (B).

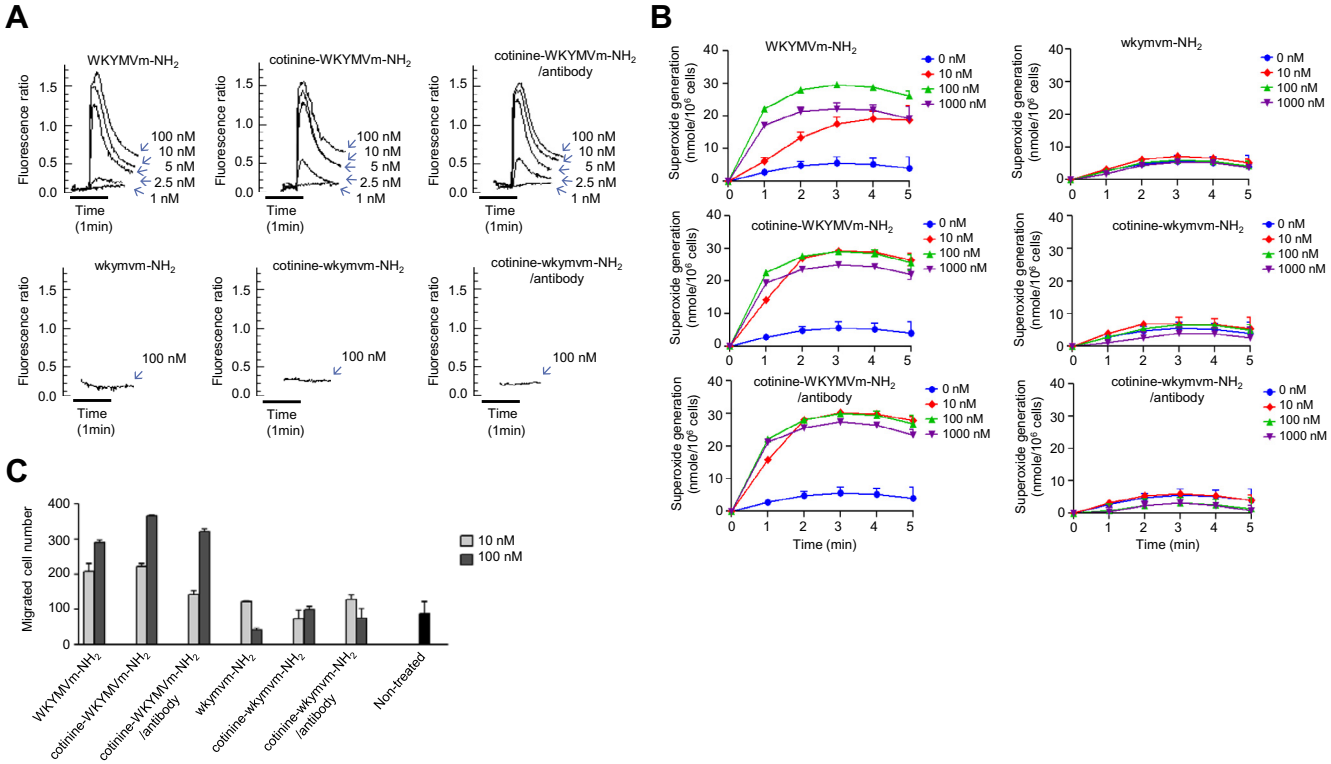

Fig. 2. Functional characterization of the cotinine-WKYMVm-NH_2/anti-cotinine antibody complex. (A) Fura-2-loaded human neutrophils (2×10^6 cells/assay) were stimulated with various concentrations of WKYMVm-NH_2 or other agents. The changes in fluorescence ratio (340/380 nm) were monitored. (B) Human neutrophils were stimulated with the different agents in the presence of cytochalasin B (5 μM). The amount of superoxide generated was measured using the cytochrome c reduction assay. (C) Isolated human neutrophils (1×10^6 cells/ml in serum-free RPMI) were added to the upper wells of a 96-well chemotaxis chamber and migration across the polycarbonate membrane (3-μm pore size) was assessed after 1.5 h at 37 °C. Migrated cells were counted in a high power field (400×). Data are representative of three independent experiments (A) or are mean ± S.E. of three independent experiments (B and C).

(Fig. 2A). The observed concentration-dependencies were similar to that of WKYMVm-NH_2. Both cotinine-WKYMVm-NH_2 and cotinine-WKYMVm-NH_2/antibody complex also stimulated robust neutrophil superoxide anion production, showing similar concentration-dependencies as WKYMVm-NH_2 (Fig. 2B). Cotinine-WKYMVm-NH_2, cotinine-WKYMVm-NH_2/antibody complex, and WKYMVm-NH_2 also triggered robust neutrophil migration with similar dose dependencies (Fig. 2C). In the three *in vitro* assays above, control peptide wkymvm-NH_2, cotinine-wkymvm-NH_2 conjugate, and cotinine-wkymvm-NH_2/antibody complex showed no significant activity. Taken together, these results suggest that neither the conjugation of WKYMVm-NH_2 to cotinine nor the formation of a complex with anti-cotinine antibody hindered the functional activity of WKYMVm-NH_2 as an agonist for FPR2.

3.3. Measurement of the in vivo stability of cotinine-WKYMVm-NH_2/antibody complex

We next measured the *in vivo* stability of cotinine-WKYMVm-NH_2/antibody complex in mice. Cotinine-WKYMVm-NH_2 conjugate (0.5 mg/kg) was injected intravenously with or without anti-cotinine antibody (10 mg/kg), and blood was drawn every 4 h for 24 h. Excess anti-cotinine antibody was then added to sera diluted in flow cytometric assay buffer, to allow cotinine-WKYMVm-NH_2 to form a complex with anti-cotinine antibody to facilitate quantification. The relative amount of WKYMVm-NH_2/antibody complex was monitored by flow cytometry using FPR2-transfected RBL-2H3 cells. In sera of mice injected with cotinine-WKYMVm-NH_2/antibody complex, total fluorescence was maintained for 8 h at greater than half its peak value and was above background for 16 h (Fig. 3). In contrast, the sera of mice injected with cotinine-WKYMVm-NH_2 showed a mean fluorescence intensity decrease to background by 1 h (Fig. 3). To determine the serum half-life, anti-cotinine antibody was injected intravenously at a dose of 10 mg/kg, and sera samples were collected every 96 h for 28 days (672 h). The serum half-life of anti-cotinine antibody was >6 days, as determined by enzyme immunoassay (Supplementary Fig. 1).

3.4. Therapeutic efficacy of cotinine-WKYMVm-NH_2/antibody complex against sepsis

The therapeutic efficacy of cotinine-WKYMVm-NH_2/antibody complex against sepsis was evaluated using the cecal ligation-puncture (CLP) animal model [20]. Mice ($n = 120$) were anesthetized, and a small abdominal midline incision was made to expose the cecum. The cecum was ligated below the ileocecal valve and punctured once through both surfaces with a 22-gauge needle, and the abdomen was closed. Immediately after the procedure, the mice were divided into six groups ($n = 20$ mice per group) and dosed every 12 h for 2 days with cotinine-WKYMVm-NH_2/antibody complex, cotinine-WKYMVm-NH_2, anti-cotinine antibody alone, or phosphate-buffered saline (PBS) vehicle control. The sham-operated mice were not treated. Treatment with cotinine-WKYMVm-NH_2/antibody complex (0.4 mg/kg) significantly improved the survival of CLP mice, with 80% of the test subjects surviving over 10 days, compared with 45% survival in the cotinine-WKYMVm-NH_2 (0.4 mg/kg) or WKYMVm-NH_2-treated group (0.2 mg/kg), or just 20% survival in the PBS-control treated group (Fig. 4).

Taken together, our results demonstrate that modification of a therapeutic peptide, in this case WKYMVm-NH_2, by conjugation

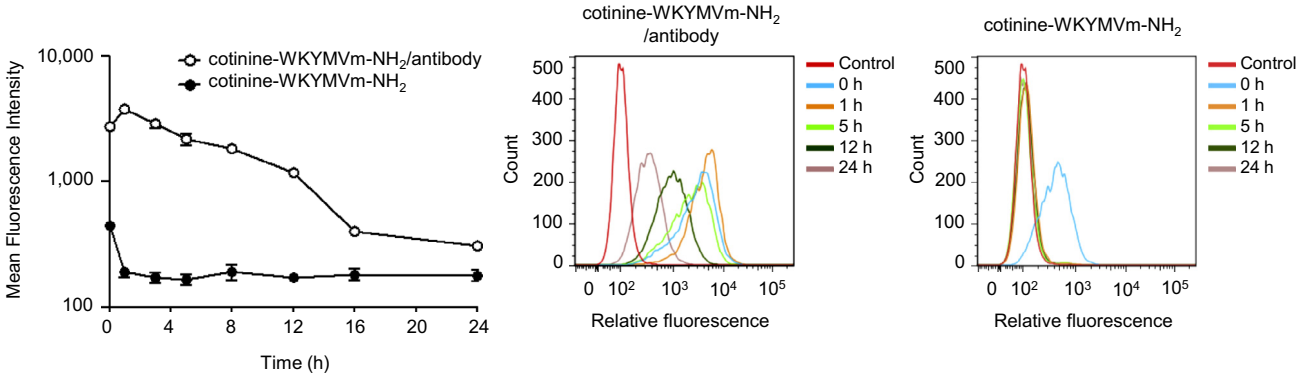

Fig. 3. *In vivo* stability of the cotinine-WKYMVm-NH_2/antibody complex. Cotinine-WKYMVm-NH_2 (0.5 mg/kg) was intravenously injected into mice alone ($n = 9$) or with anti-cotinine antibody (10 mg/kg; $n = 9$). The relative reactivity of sera to FPR2-transfected RBL-2H3 cells was monitored by flow cytometry after addition of excess anti-cotinine antibody. Data are mean ± S.E.

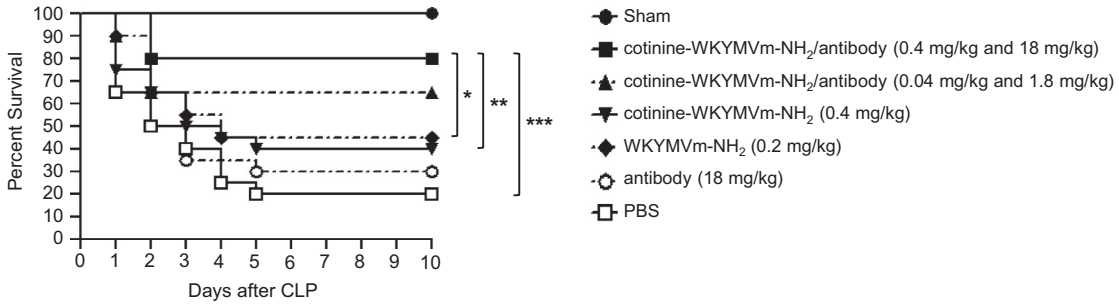

Fig. 4. *In vivo* efficacy of the cotinine-WKYMVm-NH_2/antibody complex against polymicrobial sepsis. Various doses of the indicated agents ($n = 20$ mice/group) were injected intravenously 2, 14, 26, and 38 h after CLP, and survival was monitored for 10 days. $^{*}p = 0.0241$, $^{**}p = 0.01$, $^{***}p = 0.0001$.

to cotinine hapten and binding to anti-cotinine antibody significantly enhanced the *in vivo* stability of the peptide while retaining its biological activity. Our results offer a novel delivery platform in which peptides are conjugated to cotinine and delivered via an anti-cotinine antibody in the development of bioactive peptides as therapeutic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.049.

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