Preparation and antitumor effect of a toxin-linked conjugate targeting vascular endothelial growth factor receptor and urokinase plasminogen activator

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Abstract

The aberrant signaling activation of vascular endothelial growth factor receptor (VEGFR) and urokinase plasminogen activator (uPA) is a common characteristic of many tumors, including lung cancer. Accordingly, VEGFR and uPA have emerged as attractive targets for tumor. KDR (Flk-1/VEGFR-2), a member of the VEGFR family, has been recognized as an important target for anti-angiogenesis in tumor. In this study, a recombinant immunotoxin was produced to specifically target KDR-expressing tumor vascular endothelial cells and uPA-expressing tumor cells and mediate antitumor angiogenesis and antitumor effect. Based on its potent inhibitory effect on protein synthesis, Luffin-beta (L β) ribosome-inactivating protein was selected as part of a recombinant fusion protein, a single-chain variable fragment against KDR (KDRscFv)-uPA cleavage site (uPAcs)-L β -KDEL (named as KPLK). The KDRscFv-uPAcs-L β -KDEL (KPLK) contained a single-chain variable fragment (scFv) against KDR, uPAcs, L β , and the retention signal for endoplasmic reticulum proteins KDEL (Lys-Asp-Glu-Leu). The KPLK-expressing vector was expressed in *Escherichia coli*, and the KPLK protein was isolated with nickel affinity chromatography and gel filtration chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis test demonstrated KPLK was effectively expressed. Result of *in vitro* cell viability assay on non-small cell lung cancer (NSCLC) H460 cell line (uPA-positive cell) revealed that KPLK significantly inhibited cell proliferation, induced apoptosis, and accumulated cells in S and G2/M phases, but the normal cell line (human submandibular gland cell) was unaffected. These effects were enhanced when uPA was added to digest KPLK to release L β . For *in vivo* assay of KPLK, subcutaneous xenograft tumor model of nude mice were established with H460 cells. Growth of solid tumors was significantly inhibited in animals treated with KPLK up to 21 days, tumor weights were decreased, and the expression of angiogenesis marker CD31 was downregulated; meanwhile, the apoptosis-related protein caspase-3 was upregulated. These results suggested that the recombinant KPLK may have therapeutic applications on tumors, especially uPA-overexpressing ones.

Keywords: A single-chain variable fragment against KDR, Luffin-beta, urokinase plasminogen activator, KDEL (Lys-Asp-Glu-Leu), KPLK protein, apoptosis, cytotoxicity, non-small cell lung cancer cell, CD31, caspase-3, solid tumor

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Introduction

Angiogenesis defines the process of endothelial cell (EC) budding from the preexisting vasculature, and it is essential for tumor cell surviving, growth, and metastasis.¹ Abundant blood supply brings oxygen and nutrients to all kinds of tissues, especially tumor tissue, and takes away waste products.² Binding with its two types of receptors, receptor 1 (FLT1) and receptor 2 (KDR), vascular endothelial growth factor (VEGF) acts as a positive regulator of angiogenesis^{3,4} and plays a critical role in the growth and

metastasis of solid tumors. Within this family of VEGF receptors, KDR (vascular endothelial growth factor receptor [VEGFR]-2/Flk-1) contributes significantly to tumor angiogenesis. The overexpression on the surface of tumor cells and tumor vascular ECs has made KDR a potent target in antiangiogenesis of tumors.^{5,6} Inhibition of the expression or activity of KDR could not only prevent tumor cells from amplification but also inhibit tumor angiogenesis and, thus, prevent tumor cells from growth and metastasis.^{7–9} The truncated KDR is able to inhibit the proliferation of

ECs¹⁰ and is an attractive candidate for target-tumor therapy.

Pro-urokinase plasminogen activator (pro-uPA) can bind to its membrane-anchored receptor, urokinase plasminogen activator receptor (uPAR), and produce the active form of uPA, which activates plasmin and matrix metalloproteases to promote the degradation of extracellular matrix. Also, these activated uPA-related proteins can stimulate growth factor receptors and their intracellular signaling pathways and lead to cell adhesion, migration, and proliferation.¹¹ Many reports have demonstrated that the increased levels of local uPA may indicate the poor prognosis in cancer patients.¹² Because uPA activity is preferentially upregulated in malignant tissues but not in normal ones,¹³ it is possible to establish a therapy by exploiting the high uPA activity in tumor tissue to selectively kill cancer cells while not damaging the surrounding and remote normal tissue cells. By integrating the uPA cleavage site (uPACs) into a recombinant cytotoxic protein, the active fragment of the protein can be released specifically in tumor tissue after the digestion by tumor-expressed uPA.

The direct interaction of ribosome-inactivating proteins (RIPs) with the large ribosomal RNA (rRNA) prevents protein synthesis by blocking the binding of ribosome to elongation factor 2 (EF-2) and displays a extensive inhibition on the proliferation of fungi, viruses, and tumor cells.¹⁴ Thus, the immunotoxin antitumor properties of RIPs, especially single-chain type I RIPs, have since been assayed extensively.^{15–18} Effective and safe immunotherapy has been achieved as treatment modality for tumors.¹⁹ Luffin is isolated from the seeds of the sponge gourd (*Luffa cylindrica*). It is a single-chain type I RIP and is known for possessing many biological properties, such as anti-HIV, antitumor, and abortifacient.^{20–23} Within the luffin family, we have previously shown that Luffin-beta (L β) fused with KDEL (Lys-Asp-Glu-Leu), a signal for protein retention in the endoplasmic reticulum, and uPA is cytotoxic to the non-small cell lung cancer (NSCLC) H460 cell line (uPA-positive cell^{24,25}) and the gastric carcinoma cell line.^{26,27}

In the present study, an immunotoxin fusion protein, a single-chain variable fragment against KDR (KDRscFv)-uPACs-L β -KDEL (named as KPLK) containing a single-chain variable fragment (scFv) was designed, expressed, and purified. Its tumor-specific recognition and cytotoxic effects was characterized. The fusion protein KPLK tandemly ligates scFv against KDR, uPACs SGRSA (uPACs, the minimized optimum substrate for uPA¹¹), L β , and KDEL. KPLK was purified through immobilized Ni²⁺ affinity chromatography. The cytotoxic and uPA-targeting activity of KPLK was determined through cell count kit-8 (CCK-8) and flow cytometry analysis *in vitro*, and its anti-angiogenesis and antitumor properties *in vivo* as well.

Materials and methods

Plasmids, bacterial strains, and cells

The pTA2 vector (Toyobo Co. Ltd., Osaka, Japan) was used to clone the L β cDNA. The pET-32a(+) vector (LabLife) was used to accommodate the fusion protein. *Escherichia coli*

strains BL21 (DE3) (Novagen, USA) and JM109 (Takara, Japan) were used for protein expression and preparation. The human NSCLC H460 cell line and the normal human submandibular gland cell (HSGC) line (Institute of Chemistry and Cell Biology, Shanghai, China) were grown in Iscove's Modified Dulbecco's Medium (IMDM) at 37°C and 5% CO₂. All media were supplemented with 10% fetal calf serum (Invitrogen, USA), 100 μ g/mL streptomycin, and 100 U/mL penicillin.

Construction of expression vector

To generate the recombinant vector expressing KPLK, the L β cDNA was first cloned into the pTA2 vector. KDRscFv cDNA was synthesized in Shanghai Generay Co. Ltd for Bioengineering, adding restriction enzyme sites and the G4SG4 linker²⁸ to link KDRscFv and L β and to assure proper space structure of the protein. The uPACs and KDEL peptides were fused at the C-terminal end of KDRscFv and L β cDNA, respectively. Subsequently, the fragment encoding target protein KPLK was subcloned into the pET-32a (+) vector at the downstream of thioredoxin (Trx) and enterokinase (EK) using the *HindIII* and *NcoI* restriction sites, resulting in pET/KPLK. The final fusion protein containing Trx, EK digestive site, KDRscFv, uPACs, L β , and KDEL was named as Trx-EK-KPLK (TEKPLK) (Figure 1(A)).

Expression and purification of the recombinant protein²⁹

pET/KPLK, which was identified by sequencing, was transfected into *E. coli* strain BL21 (DE3), and the transformants were grown in Luria-Bertani (LB) medium with shaking at 220 rpm for 15 h at 37°C. Then, the recombinant protein TEKPLK was induced by isopropyl- β -D-thiogalactopyranoside (IPTG, 1 mM) at 37°C for an additional 6 h. The culture was pelleted, resuspended in 30 mL lysis buffer (5 mM EDTA, 50 mM Tris-HCl, 0.15 M NaCl, 1 mg/mL lysozyme, 5 mM PMSF, 5 mM DTT, pH 8.0), and sonicated (400 W for 45 cycles, 5 s working, and 10 s free). The inclusion bodies were collected, dissolved in binding buffer (20 mM sodium phosphate, 8 M urea, 0.5 M NaCl, pH 7.4), and renatured in buffer (0.05 M Tris-HCl, 1 mM EDTA, 1 mM glutathione in reduced form, 0.1 mM glutathione in oxidized form, 0.5 M L-arginine, 0.15 M NaCl, pH 8.5). The recombinant protein TEKPLK was purified with an anion exchange column HiTrap SP.F.F (GE Healthcare, Piscataway, New Jersey, USA) and a Ni-NTA affinity column (GE Healthcare) and was eluted with increasing concentration of imidazole. The Trx-tag was removed to release protein KPLK by overnight EK digestion and loaded onto Ni-NTA affinity column for KPLK recovery and purification. The digestion and purification were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC), and the final product was frozen at -80°C until required.

Figure 1 Expression, purification, and digestion of TEKPLK. (A) The map of recombinant vector pET/KDRscFv-uPACs-L β -KDEL. (B) Bacterial cells were lysed and the recombinant protein was prepared as described in Section "Materials and methods". Samples from various steps were tested by SDS-PAGE. Lanes: 1: 100% eluent; 2: 50% eluent; 3: 30% eluent; 4: 10% eluent; 5: 3% eluent; 6: through liquid; 7: sample solution; M: molecular weight marker. The arrow shows the target product with the expected molecular weight of 75 kDa. (C) After EK digestion, two bands, a band at approximately 58 kDa (KPLK protein) and another band at approximately 17 kDa (Trx-tag protein), were found. Lanes: M: molecular weight marker; 1: TEKPLK without digestion; 2: TEKPLK digested with EK

Cell proliferation assessment

The effect of KPLK on cell proliferation was tested on the NCI-H460 cell line. The cells were serum-starved overnight, seeded into 96-well culture plates (4×10^3 in 150 μ L) and treated with KPLK (8 μ g/mL) alone, KPLK (8 μ g/mL) plus uPA (16 μ g/mL, HYPHEN BioMed, Neuville sur Oise, France), or KPLK (8 μ g/mL) plus PAI-1 (16 μ g/mL, plasminogen activator inhibitor-1,³⁰ Pepprotech, USA) 6 h later, respectively. As a control, PAI-1 (16 μ g/mL) or uPA (16 μ g/mL) alone was added to H460 cells, and KPLK (8 μ g/mL) plus uPA (16 μ g/mL) was added to HSGC in triplicate for each treatment. Regularly cultured H460 cells served as a blank control. Cell proliferation was determined by CCK-8 (Dojindo laboratory, Japan). At 1, 2, 3, and 4 days after the treatment, 10 μ L/well of the CCK-8 solution was added and incubated for 3 h. Then, values of optical density (OD) in different groups were automatically read at 450 nm.

Cell cycle analysis

NCI-H460 cells were cultured (1×10^6 cells/mL) in IMDM with 10% FCS in different flasks and were synchronized by 24 h serum starvation. Subsequently, the medium was changed with the one containing 8 μ g/mL KPLK only, 4 μ g/mL KPLK plus 8 μ g/mL uPA, or 8 μ g/mL KPLK plus 16 μ g/mL uPA. Regularly cultured cells also served as a control. After incubating for 48 h, the cells were harvested, fixed in 80% ethanol, stained with propidium iodide (PI, 4 μ g/mL; Sigma) in the presence of RNase (10 μ g/mL; Sigma), and analyzed by a flow cytometer.

Cell apoptosis test

To test the apoptosis-inducing effect of KPLK on tumor cells, NCI-H460 cells (8×10^4 cells/well) were seeded into six-well culture plates. Fresh medium containing KPLK, KPLK plus uPA, as above, was added into different wells after 24 h of culturing. The control cells were cultured in regular medium. After incubating for 48 h, the trypsinized cells were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and PI. Cell apoptosis was analyzed in triplicate for each treatment by a flow cytometer (BD FACS

Calibur, USA) and Cell Quest software (Modfit LT for Mac 3.0).

Experimental animals and tumor model

Nude mice (5–8 weeks of age, body mass 20–21 g) were obtained from the Center of Experimental Animals in the Third Military Medical University (TMMU) (qualified certification number: CQA 0101015# and 0103017#, Chongqing, China). The experimental procedures were approved by the Animal Care Committee of the TMMU and were in agreement with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. Animals were injected subcutaneously with H460 cells (1×10^7 cells in 200 μ L phosphate-buffered saline [PBS]) into the left axilla of each mouse. Nine days after the cell injection, animals were divided randomly into three groups after selecting animals with approximately 0.5 cm in diameter of tumor size. Intratumoral injections of KPLK (40, 80 mg/kg; $n=5$) every other day for a total of 21 days and up to five injection sites were applied according to the size of developed tumors. Control animals obtained the same injections of equal volume of PBS. On the 21st day, all animals were euthanized, weighed, and killed. After dissection, tumors were collected and weighed. Some tumors were sectioned for immunohistochemistry staining, while the others were frozen at -80°C until required.

Standard immunohistochemical staining with antimouse CD31 antibody (1:400, BD Pharmingen, Franklin Lakes, NJ, USA) was carried out to reveal tumor blood vessels. For the quantification of neoangiogenesis, the areas enclosed by CD31-positive blood vessels were selected using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA), and the area density of CD31 expression = CD31-positive area/the total area (%). Correspondingly, the number density of CD31 expression = the number of CD31-positive blood vessels per square mm.³¹

Total protein was extracted from tumors treated with KPLK or PBS and subjected to SDS-PAGE and immunological staining with goat antihuman caspase-3 antibody (sc-1225, Santa Cruz Biotechnology, Inc, USA) (1:500) was

Figure 2 Assessment of KPLK-specific inhibition effect on tumor cell. (A) Cytotoxic effects of KPLK on NCI-H460 cell line were assessed by CCK-8 assay. There was no noticeable difference among H460 cells alone, uPA, PAI-1, or HSGC groups through the observation period. KPLK and KPLK plus uPA or KPLK plus PAI-1 remarkably inhibited H460 cell proliferation from 24–96 h after the treatment, but the normal cell line (HSGC) was unaffected. The inhibitory effect ranked as KPLK + uPA > KPLK > KPLK + PAI. Data are presented as mean $\pm$ standard deviation (SD). (a) $P < 0.01$ versus H460 cells alone, uPA, PAI-1, or HSGC groups; (b) $P < 0.05$ versus KPLK; (c) $P < 0.01$ versus all other groups. (B) The effect of KPLK on the cycle distribution of H460 cell line. Cells were treated with KPLK, KPLK plus uPA for 48 h. The distribution of cell cycle was analyzed via propidium iodide staining and flow cytometry analysis. (a) Cells without treated as control; (b) cells treated with 8 μ g/mL KPLK; (c) cells treated with 4 μ g/mL KPLK plus 8 μ g/mL uPA; (d) cells treated with 8 μ g/mL KPLK plus 16 μ g/mL uPA. A significant increase of cell cycle in S and G2/M phases was revealed after the treatments, especially KPLK plus uPA treatments

conducted and visualized using SuperSignal® West Pico (Thermo).

Statistical analysis

Differences between treated samples and controls were calculated using the Student's *t*-test. Data were expressed as mean $\pm$ standard deviation. A value of $P < 0.05$ was required for statistical significance.

Results

Expression and purification of the target protein

A diagram of the pET/KDRscFv-uPAcs-L β -KDEL (pET/TEKPLK) expression vector is presented in Figure 1(A). After expressing TEKPLK in *E. coli* strain BL21 (DE3), the cells were collected and sonicated. TEKPLK was eluted by imidazole after passing the supernatant through an anion exchange column HiTrap SP.FF and an Ni-NTA affinity column. SDS-PAGE analysis confirmed that a molecular weight of approximately 75 kDa was detected in the inclusion bodies of transformed *E. coli*, which was consistent with the size of the expected full-length fusion protein of TEKPLK (Figure 1(B)). After EK digestion, two bands, a band at approximately 58 kDa (KPLK protein) and another band at approximately 17 kDa (Trx-tag protein), were found (Figure 1(C)). KPLK protein was separated from Trx-tag protein after another purification by the Ni-NTA affinity column. The yield of KPLK protein per liter of culture was about 5 mg. The purity of KPLK protein was about 99% validated by HPLC.

Recombinant protein inhibited cell proliferation

The treatment of KPLK plus uPA produced notable cell growth inhibition, but the normal cell line (HSGC) was unaffected. In contrast, the uPA or its inhibitor PAI-1 alone did not interfere with the H460 cell growth. KPLK alone could significantly inhibit the growth as early as at 24 h after treatment (Figure 2(A)).

The cell cycle disturbance effect of KPLK on H460 cells was assessed by flow cytometry after treatment with KPLK alone or in combination with uPA for 48 h. An accumulation of the KPLK-treated cells in the S phase and the G2/M phase was confirmed, and this was even more severe for the KPLK plus uPA-treated cells (Figure 2(B) and Table 1).

Recombinant protein induced cell apoptosis

Compared with normal cultured cells (Figure 3(A-a)), NCI-H460 cells incubated with 8 μ g/mL KPLK for 48 h showed detachment and death under observation through an inverted microscope (Figure 3(A-b)). When treated with 8 μ g/mL KPLK plus 16 μ g/mL uPA, most of the H460 cells detached and some cells had broken down (Figure 3(A-c)), although the normal cell line HSGC was unaffected (Figure 3(A-d)).

The apoptosis-inducing effect of KPLK on NCI-H460 cell line was tested by flow cytometry. The change of apoptosis was quantitatively shown as the percentage of cells positively stained by Annexin V/PI after the treatment of KPLK in combination with uPA for 48 h (Figure 3(B)). The percentage of apoptosis of the cells treated with 8 μ g/mL KPLK plus 16 μ g/mL uPA ($13.98\% \pm 2.89\%$, Figure 3(B-d)) was higher than that of the cells treated with 4 μ g/mL KPLK plus 8 μ g/mL uPA ($7.04\% \pm 1.14\%$, Figure 3(B-c)) or cells

Table 1 Cell cycle distribution after KPLK treatment (%) ($\bar{x} \pm \text{SD}$, $n = 3$)

Groups	G1	S	G2/M
H460 cell	69.16 $\pm$ 7.81	24.48 $\pm$ 2.95	6.36 $\pm$ 1.25
8 $\mu\text{g/mL}$ KPLK	65.97 $\pm$ 5.59	27.25 $\pm$ 3.12	6.78 $\pm$ 1.31
4 $\mu\text{g/mL}$ KPLK plus 8 $\mu\text{g/mL}$ uPA	57.20 $\pm$ 4.21*	31.65 $\pm$ 3.83*	11.16 $\pm$ 2.57*
8 $\mu\text{g/mL}$ KPLK plus 16 $\mu\text{g/mL}$ uPA	39.42 $\pm$ 3.32* [†]	41.63 $\pm$ 4.03* [†]	18.96 $\pm$ 2.86* [†]

* $P < 0.01$ versus control.[†] $P < 0.05$ versus 4 $\mu\text{g/mL}$ KPLK plus 8 $\mu\text{g/mL}$ uPA.

Figure 3 KPLK-induced cell death. (A) The harmful effect of KPLK on NCI-H460 cell line. H460 cells without treatment (a). After 48 h incubation, compared with the treatment of 8 $\mu\text{g/mL}$ KPLK alone (b), 8 $\mu\text{g/mL}$ KPLK plus 16 $\mu\text{g/mL}$ uPA treatment was able to kill more H460 cells (c), but the normal cell line (HSGC), which was treated with 8 $\mu\text{g/mL}$ KPLK plus 16 $\mu\text{g/mL}$ uPA, was unaffected (d). Scale bar = 100 μm . (B) The apoptosis-inducing effect of KPLK on NCI-H460 cells. Cells were treated for 48 h with different treatments stained with Annexin V and PI solution. Apoptosis was then detected via flow cytometry. The figure showed the percentage of apoptosis cells increased in different treatments. These figures are one representative experiment of three with similar results. (a) Cells without treatment as control; (b) cells treated with 8 $\mu\text{g/mL}$ KPLK alone; (c) cells treated with 4 $\mu\text{g/mL}$ KPLK plus 8 $\mu\text{g/mL}$ uPA; (d) cells treated with 8 $\mu\text{g/mL}$ KPLK plus 16 $\mu\text{g/mL}$ uPA

treated with 8 $\mu\text{g/mL}$ KPLK alone ($1.85\% \pm 0.79\%$, Figure 3(B-b)) or cells without treatment (Figure 3(B-a)) ($P < 0.01$). This result implied that L β is more potent in inducing cell apoptosis than KPLK.

Recombinant protein inhibited tumor growth

The antitumor effects of KPLK were evaluated using xenografted tumor models in nude mice. KPLK significantly inhibited tumor growth in H460 cell line xenografted nude mice (Figure 4(A), Table 2). At the end of the experiment, the tumors treated with KPLK (40, 80 mg/kg) were significantly inhibited compared with the tumors treated with PBS. The tumor growth inhibition was 25.23% and 41.83%, respectively, when compared with the PBS controls ($P < 0.01$). Furthermore, animals receiving KPLK had no apparent weight loss during the study, suggesting that KPLK in the range of treatment may be non-toxic to the mice.

Angiogenesis is one of the main components of granulation tissue formation and is crucial for tumor growth. The expression of CD31, a marker of neoangiogenesis, in tumors treated with KPLK was confirmed by immunohistochemical staining. Poor CD31 staining was seen in tumors treated

with KPLK (80 mg/kg) (Figure 4(B)). The area density of CD31 expression in tumors treated with KPLK (40 mg/kg, 0.254%; 80 mg/kg, 0.096%) was significantly lower than that in tumors treated with PBS (1.578%) 21 days after treatment. The number density of CD31 expression was also significantly lower in tumors treated with KPLK when compared with that of tumors treated with PBS at day 21 (Table 3).

To test the apoptosis-inducing effect of KPLK on tumors *in vivo*, Western blot was employed to detect the expression of apoptosis-related protein caspase-3. The result showed that the caspase-3 expression was increased in tumors treated with KPLK (40 mg/kg, 80 mg/kg) in contrast to tumors treated with PBS (Figure 4(C)), suggesting that KPLK could inhibit tumor growth in an apoptosis-induced manner, which was consistent with the *in vitro* results above.

Discussion

By replacing the receptor recognition domains of protein toxins, such as ricin from plant and diphtheria toxin and *Pseudomonas* exotoxin A from bacteria, with growth factors, cytokines, and antibodies, some tumor cell-selective cytotoxins have been established.³² These "immunotoxins"

Figure 4 Inhibitory effect of KPLK on tumor growth *in vivo*. (A) Pictures of tumors were taken at the end of the experiment. (B) Inhibitory effect of KPLK (40, 80 mg/kg) on angiogenesis. Shown are representative immunohistochemistry-stained tumor sections against CD31, a marker of neoangiogenesis. CD31 expression density within tumor was confirmed by the number density of CD31 expression in three regions of highest density in each section (assay was repeated in 3 sections per mouse and 3 mice were detected). Arrows, microvessels with positive CD31 staining. Scale bar = 100 μ m. $P < 0.01$ versus tumors treated with PBS. (C) Apoptosis-inducing effect of KPLK on tumor. Western blot analysis revealed that the caspase-3 expression was increased in tumors treated with KPLK (40, 80 mg/kg) in contrast to tumors treated with PBS. (A color version of this figure is available in the online journal.)

Table 2 Effect of KPLK on H460 cell line xenografted in nude mice

Groups	No. of mice		Body weight (g)		Tumor weight (g)	Inhibition rate (%)
	Start	End	Start	End		
PBS	3	3	20 $\pm$ 0.75	25 $\pm$ 1.36	1.53 $\pm$ 0.08	
KPLK (40 mg/kg)	3	3	20 $\pm$ 0.82	24 $\pm$ 1.02	1.14 $\pm$ 0.05	25.23
KPLK (80 mg/kg)	3	3	21 $\pm$ 0.69	24 $\pm$ 1.13	0.89 $\pm$ 0.03	41.83

Table 3 Quantification of CD31 expression in tumors

Groups	Area density of CD31 expression (%)	Number density of CD31 expression (number/mm ²)
	d21	d21
PBS	1.578 $\pm$ 0.251	0.042
KPLK (40 mg/kg)	0.254 $\pm$ 0.025*	0.015*
KPLK (80 mg/kg)	0.096 $\pm$ 0.013*	0.011*

* $P < 0.01$ versus tumors treated with PBS.

have been approved for clinical use based on their efficacy. Because these toxins are also uptook by normal tissues, the non-specific toxicity is an unsolved problem. Serious damage to normal tissue may be induced by a small amount of internalized toxin due to the high-efficient catalytic effect. To improve the tumor cell-targeting specificity of these cytotoxins is a key step for their clinical application.

The pharmacological properties and biological activities of RIPs have categorized them as potent immunotoxins³³⁻³⁵ and plant defense factors.^{36,37} The coupling of single-chain type I RIPs to cell-specific targeting molecules, such as cytokines and antibodies, could create an immunotoxin. L β , a type I RIP and one of the most toxic in the luffin family, exhibits antitumor activity as validated by our previous investigation and others.^{26,27}

In 1976, uPA was reported to be produced and released by cancer cells,³⁸ and subsequent investigations have revealed that overexpressed uPA are closely related with

malignant human tumors, including cancers of the colon,³⁹ ovaries,⁴⁰ breast,⁴¹ bladder,⁴² thyroid,⁴³ lung,⁴⁴ liver,⁴⁵ pleura,⁴⁶ pancreas,⁴⁷ and the head and neck¹¹ as well as monocytic⁴⁸ and myelogenous⁴⁹ leukemias. The uPA system is involved in the proliferation, invasion, and metastasis of tumor cells.^{50,51} Targeting cancer invasion-related factors has been tested as novel therapies to blockade tumor invasion and metastasis.⁵² Strategies to interfere the expression or the activity of uPA/uPAR include the use of inhibitors, antibodies, antisense oligonucleotides, and uPA and uPAR analogues.⁵³ However, these interventions lack a direct cytotoxic effect and can only slow down tumor progression. Several studies have targeted uPA and uPAR as an activator for exogenous protein toxins, such as the fusion of toxin catalytic domains to ATF.⁵⁴⁻⁵⁶ In this study, we exploited the tumor-derived plasminogen activators to specifically activate the cytotoxic effect of the recombinant L β fusion protein to kill tumor cells.

To reinforce the antitumor effect of L β while preventing possible cytotoxic effect on normal tissues and cells, the recombinant fusion protein KPLK with multiple functional components was constructed. A linker (G4SG4) connecting KDRscFv and L β proteins helped the proper folding of the KDRscFv and L β proteins and ensured the activity of the fusion protein. In addition, the uPACs was fused to the C-terminal end of KDRscFv. Once the uPACs was cleaved by tumor-expressed uPA, a fully active L β proteins could be released, which would increase the targeting specificity of the protein. KDEL, a signal for retention of proteins in the endoplasmic reticulum, was fused to the C-terminal end of L β , which enhanced the local concentration of the recombinant protein at endoplasmic reticulum, the site for protein

synthesis and L β targeting. The fusion protein was successfully expressed and purified with about 99% purity. Meanwhile, we investigated the antitumor effect of the fusion protein *in vitro* as follows.

As expected, KPLK plus uPA exhibited specific cytotoxic effects on tumor cells. KPLK-treated NCI-H460 cells showed cell damage, cell cycle arrest, apoptosis, and proliferation inhibition, while KPLK plus uPA treatment produced even more harmful impact. This result demonstrated that KPLK *per se* was harmful to tumor cell. This effect was further enhanced by addition of the exogenous uPA due to the release of fully activated L β and was partially abolished by the addition of PAI-1. Because it was confirmed that the NSCLC cells produce uPA,²⁴ this may partially contribute to the inhibitory effect of KPLK on cell proliferation. In contrast, the normal cell line (HSGC) with the same treatment was unaffected, suggesting that both KPLK and L β were not toxic to normal cells.

To evaluate the effect of the recombinant protein on tumor growth *in vivo*, we established NSCLC tumor models xenografted in nude mice, and the recombinant protein KPLK was used to treat the solid tumors. The tumor growth inhibition rate was about 25.23% and 41.83% when tumors were treated with 40 mg/kg KPLK and 80 mg/kg KPLK. Furthermore, animals receiving KPLK had no apparent weight loss during the study, suggesting KPLK in the range of treatment is non-toxic to nude mice.

Angiogenesis is crucial for tumor growth. VEGF as well as its receptor KDR is one of the most important factors that can promote tumor angiogenesis. Acting as the competing antibody, KDRscFv is able to prevent tumor angiogenesis by blocking the interaction between VEGF and endogenous KDR. In our solid tumor models of nude mice, immunohistochemistry analysis showed that the decreased expression of CD31 was seen in tumor treated with KPLK. The area density of CD31 expression in tumors treated with KPLK (40 mg/kg, 0.25%; 80 mg/kg, 0.11%) was significantly lower than that of tumors treated with PBS (1.58%) 21 days after treatment. The result suggests that KPLK could inhibit tumor growth by interfering tumor angiogenesis. In addition, the elevated caspase-3 in xenograft tumors treated with KPLK (40 mg/kg, 80 mg/kg) confirmed the induction of apoptosis of KPLK treatment *in vivo*.

As expected, the recombinant fusion protein KPLK presented a significant antitumor effect both *in vitro* and *in vivo* while not showing obvious toxicity to normal cells and tissues, and it may be a potential L β -based antitumor therapy.

Author contributions: JW conceived and designed the experiments and evaluated the statistical analysis and drafted the manuscript. YX did the main body of the study and performed statistical analysis. QL, DH, and XT carried out the animal, molecular biology studies and performed statistical analysis. LW, YS, and WZ carried out biochemical studies and performed statistical analysis of those studies. TY and CX were involved in drafting and revising the manuscript. All authors read and approved the final manuscript.

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