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D-SP5 Peptide-Modified Highly Branched Polyethylenimine for Gene Therapy of Gastric Adenocarcinoma

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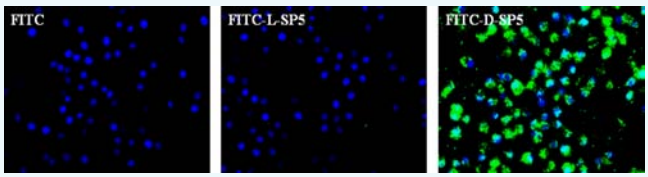
Supporting Information

ABSTRACT: Peptide-mediated targeting of tumors has become an effective strategy for cancer therapy. Retro-inverso peptides resist protease degradation and maintain their bioactivity. We used the retro-inverso peptide $_{D}$(PRPSPKMGVSVS) (D-SP5) as a targeting ligand to develop gene therapy for gastric adenocarcinoma. D-SP5 has a higher affinity for human gastric adenocarcinoma (SGC7901) cells compared with that of its parental peptide, $_{L}$(SVSVGMKPSPRP) (L-SP5). Polyethylenimine (PEI)/pDNA, polyethylene glycol (mPEG)-PEI/pDNA and D-SP5-PEG-PEI/pDNA were prepared for further study. Quantitative luciferase assays showed the transfection efficiency of D-SP5-PEG-PEI/pGL$_{4.2}$ was larger compared with that of mPEG-PEI/pGL$_{4.2}$. Flow cytometry assays revealed that the apoptosis rates of SGC7901 cells treated with D-SP5-PEG-PEI/pTRAIL were larger than mPEG-PEI/pTRAIL. Western blot assays indicated that the expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) protein in SGC7901 cells treated with D-SP5-PEG-PEI/pTRAIL was higher compared with that in cells treated with mPEG-PEI/pTRAIL. In vivo pharmacodynamics study revealed that D-SP5-PEG-PEI/pTRAIL could inhibit the growth of gastric adenocarcinoma SGC7901 xenografts in nude mice. Our results demonstrate that D-SP5-PEG-PEI is a safe and efficient gene delivery vector with potential applications in antitumor gene therapy.



INTRODUCTION

Gene therapy is a promising therapeutic strategy in serious acquired and inherited diseases including cancer.[1] Compared with regular chemotherapy, gene therapy has limited adverse side effects and drug resistance problems.[2] One essential challenge of gene therapy is the development of safe and effective delivery vectors.[3] Nonviral gene delivery systems play a significant role because of their lower immunogenicity, larger genetic payloads, and easier preparation.[4,5] Polyethylenimine (PEI) has been widely used in nonviral gene delivery systems because of its high transfection efficiency and high density of positive charge at low pH, which facilitates the mechanism of proton sponge-mediated endosomal escape.[6−8] The substantial cytotoxicity and instability of PEI polyplexes, however, has hindered its application. Many researchers have focused on PEI modification, including blocked copolymers of poly(ethylene glycol) (PEG) and PEI. PEGylation can shield the positive charge of the polyplex so as to reduce its cytotoxity. It also helps to increase the solubility and surface flexibility of the polyplex, which contributes to its colloidal stability. Also, the reduced interaction potential reduced interactions with erythrocytes, opsonization, and reticuloendothelial system phagocytosis.[9−16] Another challenge for gene delivery vectors is that they need to extravasate from the bloodstream to reach target tissues. This can limit their toxicity and aid transfection of the therapeutic gene into the intended tumor tissue.[3,17] Recently, targeting ligands, mostly peptides, have been used for drug delivery systems. Natural peptides are of the Lcon®guration (composed of L-amino acids), and when in blood, it is very easy for them to be degraded by the proteases in serum. Their short half-life and instability greatly influence their targeting effect. D-peptides can resist protease degradation and thus have higher stability in vivo.[18−20] However, simply replacing L-amino acids with D-amino acids can change the chiral structures of the parental peptides, leading to the loss of biological activity.[21] To solve this problem, Chorev and Goodman designed retro-inverso peptides, putting the sequence in a reverse order compared with the parental peptide, while changing the L-amino acids into D-amino acids. The retro-inverso peptides are very similar to the parental L-peptides with respect to side chain structure and thus retain the bioactivities of the L-peptides while gaining improved biological stability.[22,23] Some retro-inverso peptides have better binding affinity compared with the L-peptides. This may be explained by D-peptides binding to their targets with unique interface geometries not available to L-peptides.[24]

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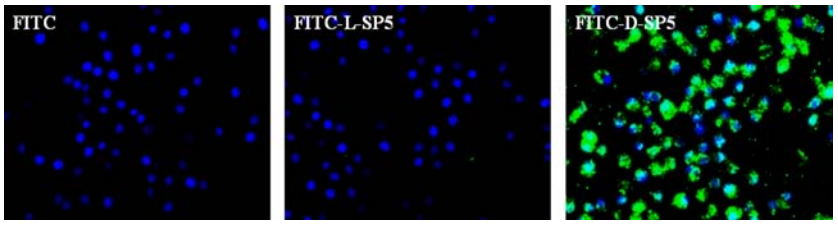
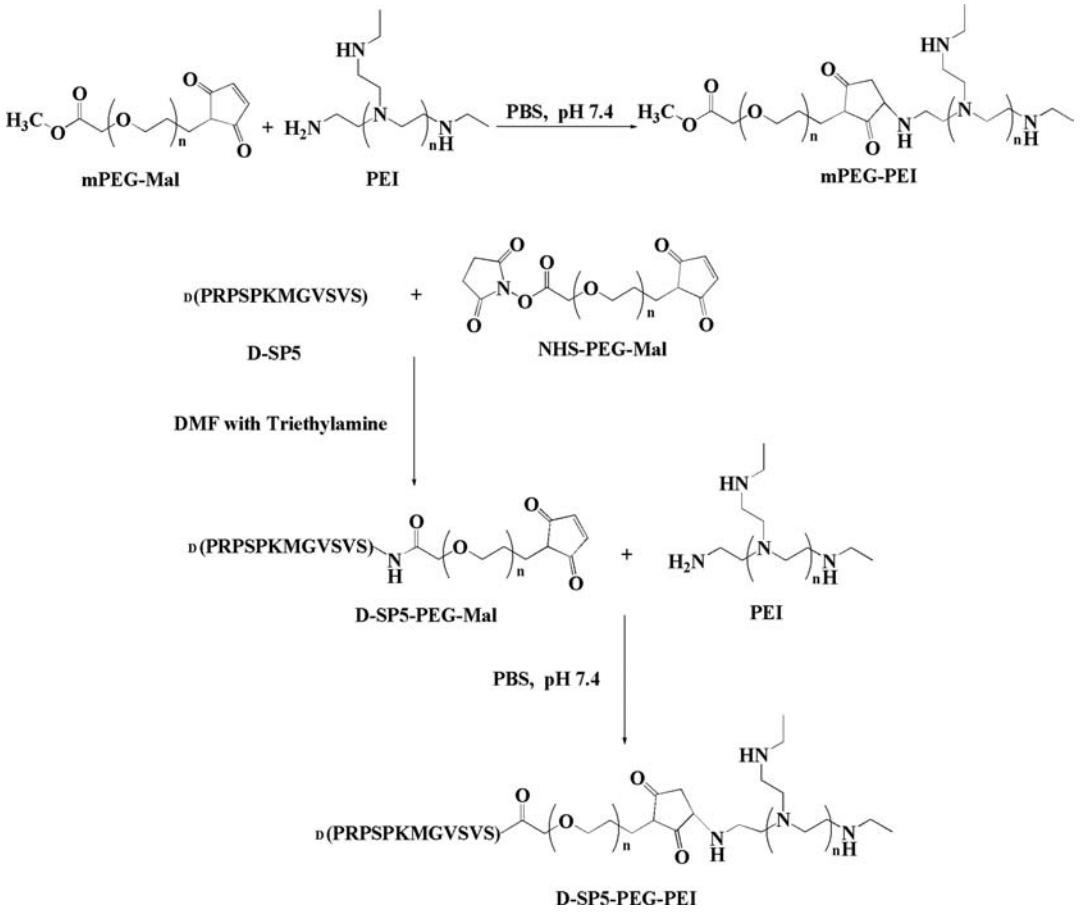


Figure 1. Uptake of FITC, FITC-L-SP5, and FITC-D-SP5 by SGC7901 cells.

Scheme 1. Synthesis of mPEG-PEI and D-SP5-PEG-PEI



L(SVSVGMKPSPRP) (L-SP5), which was previously reported as SP5-52, is a peptide that targets tumor vessels. It is a linear peptide consisting of 12 amino acids selected from a phage-displayed random peptide library.[25] Our earlier studies have transformed this peptide into a retro-inverso form, which we have named D(PRPSPKMGVSVS) (D-SP5). D-SP5 has a better binding affinity to its binding site and better biological stability than L-SP5, and we have used D-SP5 to build a chemotherapeutic drug delivery system: Dox-loaded D-SP5-PEG-DSPE micelles for the treatment of human mouth epidermal carcinoma KB xenograft tumor. This system achieved an enhanced therapeutic effect. We have also explored the in vitro binding property of D-SP5 in SGC7901 cells,[26] which demonstrated specific targeting. In this study, we pursued a further study of the characteristics of this peptide in targeting SGC7901 cells. We conjugated it with PEG-modified PEI to form a safe and efficient nonviral gene delivery carrier, with the purpose of achieving an effective treatment for gastric adenocarcinoma. To the best of our knowledge, the application of D-SP5 in a nonviral gene delivery system has not been previously reported.

RESULTS

Cell Uptake of the Peptides. The fluorescence images from the inverted fluorescence microscope (Figure 1) show that there was hardly any visible fluorescence in the FITC and FITC-L-SP5 groups while apparent fluorescence was seen in the FITC-D-SP5 group. This result indicated that SGC7901 cell uptake was remarkably increased for D-SP5, which demonstrated the targeting ability of the peptide. Therefore, we used D-SP5 to modify the gene delivery vector for subsequent studies.

Synthesis and Characterization of Polymers. mPEG-PEI and D-SP5-PEG-PEI were synthesized as indicated in Scheme 1, and ^{1}H NMR results are shown in Figure 2. The ^{1}H NMR showed signals at 2.4−2.8 ppm, which represent the H-carbon unit (−CH_2CH_2NH−), characteristic of PEI; signals at 3.4−3.7 ppm, which represent the H-carbon unit

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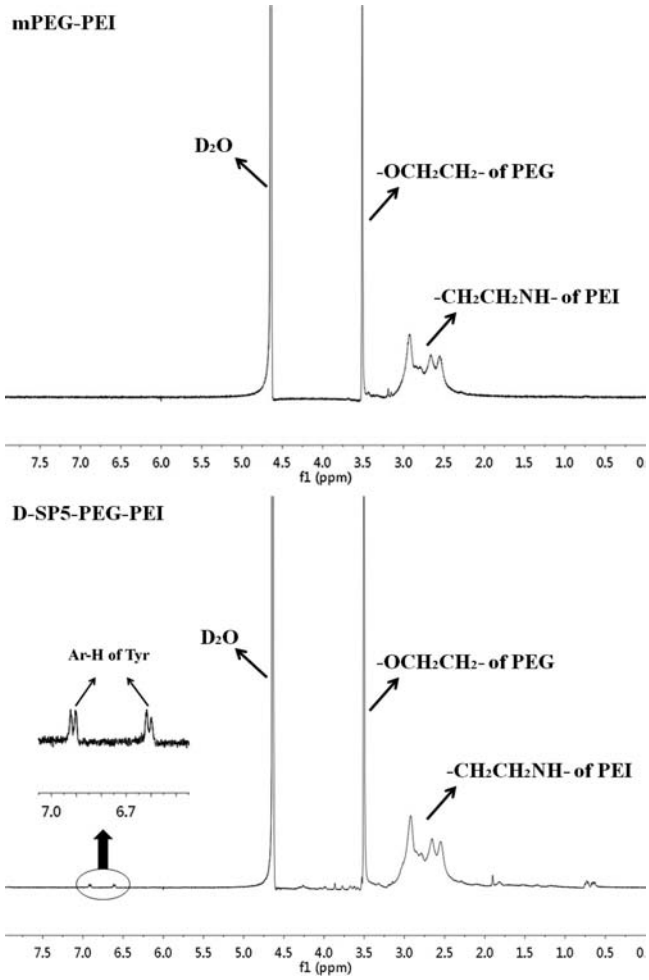

Figure 2. ^{1}H NMR spectra of mPEG-PEI and D-SP5-PEG-PEI in D_2O.

($-OCH_2CH_2$), characteristic of PEG, and signals at 6.5−7.0 ppm, which represent the phenyl group in D-SP5. These results indicate that D-SP5 was successfully linked to the polymer. The degree of PEG substitution in mPEG-PEI and D-SP5-PEG-PEI was calculated to be 5.80 and 6.25, which was closed to the theoretical value of 6.

Characterization of Complexes. The particle size and surface charge of complexes are important properties for gene delivery systems because they can affect transfection efficiency.[28,29] Table 1 shows the results of the mean size and zeta potential of polymer/$pGL_{4.2}$ complexes. The sizes of the complexes decreased as the N/P ratio of the complexes increased, while zeta potentials increased. The positive zeta potentials indicated that all polymers were capable of condensing $pGL_{4.2}$ effectively, which was also confirmed by agarose gel electrophoresis assays (Figure 3). At the same N/P ratio, PEI/$pGL_{4.2}$ complexes showed larger sizes and higher zeta potentials compared with mPEG-PEI/pDNA and D-SP5-PEG-PEI/pDNA complexes. Higher zeta potentials of PEI/pDNA complexes are caused by strong positive surface charges of PEI. In strong ionic solutions, including PBS, PEI derivatives are prone to aggregate, thereby forming large particles. PEGylation can shield part of the positive charge of PEI, enabling complexes with lower zeta potentials to form. Meanwhile, PEGylation can reduce aggregation and decrease particle sizes.[11,30]

Table 1. Size and Zeta Potential of Polymer/$pGL_{4.2}$ Complexes with Different N/P Ratios

sample	N/P	size (nm)	PDI value	zeta potential (mV)
PEI/$pGL_{4.2}$	8	1051.7 ± 31.56	0.301 ± 0.093	18.9 ± 0.38
	10	841.7 ± 28.92	0.212 ± 0.046	21.2 ± 0.81
	12	594.2 ± 57.52	0.284 ± 0.096	22.5 ± 0.57
mPEG-PEI/$pGL_{4.2}$	8	426.8 ± 13.92	0.240 ± 0.014	1.2 ± 0.27
	10	248.0 ± 12.34	0.221 ± 0.019	3.0 ± 0.34
	12	163.2 ± 6.32	0.146 ± 0.025	5.19 ± 1.06
D-SP5-PEG-PEI/$pGL_{4.2}$	8	466.6 ± 35.87	0.351 ± 0.055	1.27 ± 0.35
	10	316.2 ± 11.55	0.130 ± 0.028	3.0 ± 0.68
	12	247.7 ± 33.80	0.122 ± 0.062	5.9 ± 0.52

Figure 3. Agarose gel electrophoresis assay: Lane 1 is naked $pGL_{4.2}$. Lanes 2, 3, and 4 are D-SP5-PEG-PEI/$pGL_{4.2}$ at N/P ratios of 12, 10, and 8. Lanes 5, 6, and 7 are mPEG-PEI/$pGL_{4.2}$ at N/P ratios of 12, 10, and 8. Lanes 8, 9, and 10 are PEI/$pGL_{4.2}$ at N/P ratios of 12, 10, and 8.

Cytotoxicity Assay. An MTT assay was used to evaluate the cytotoxicity of PEI derivatives/$pGL_{4.2}$ complexes. Figure 4

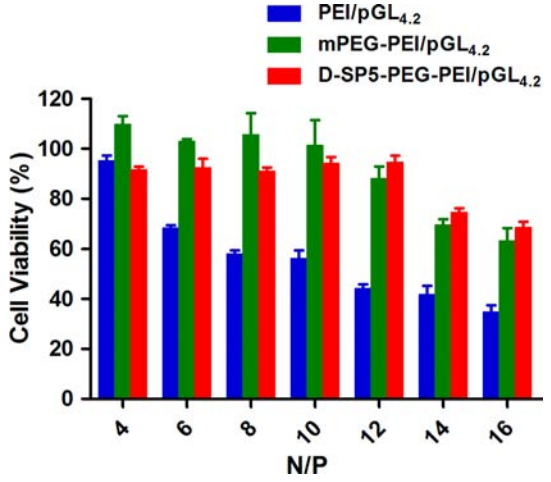

Figure 4. Viabilities of SGC7901 cells with mPEG-PEI/$pGL_{4.2}$ and D-SP5-PEG-PEI/$pGL_{4.2}$ compared with PEI/$pGL_{4.2}$.

shows that PEI/$pGL_{4.2}$ appears to be the most cytotoxic, with a cell viability of 44.1% at N/P = 12, while the cytotoxicity of mPEG-PEI/$pGL_{4.2}$ and D-SP5-PEG-PEI/$pGL_{4.2}$ was much lower, with a cell viability of 88.1% and 94.6%, respectively. The high toxicity of PEI is caused by its high density positive charge at all N/P ratios. It can be inferred that PEGylation of PEI reduces the positive charge of complexes, which diminishes the toxicity of the carriers.[11,31,32]

In Vitro Transfection Assay. We conducted two in vitro experiments to evaluate the transfection efficiency of the polymer/pDNA. The qualitative enhanced green fluorescent protein (EGFP) expression assay in Figure 5A showed that

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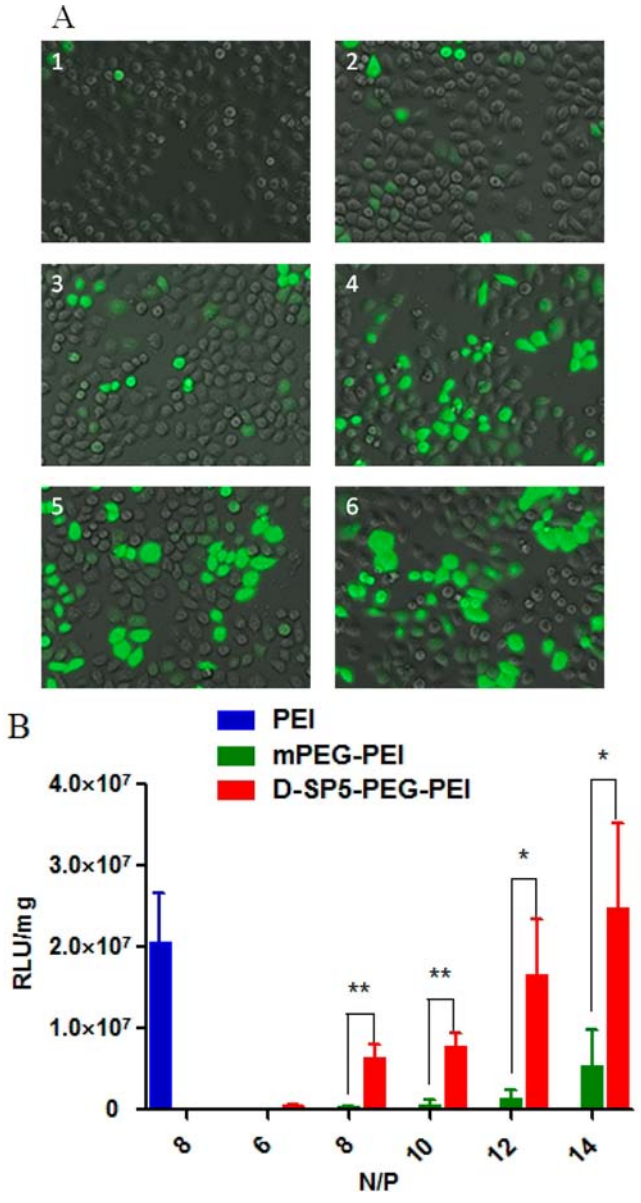

Figure 5. In vitro transfection efficiency of SGC7901 cells. A. Images 1, 3, and 5 are fluorescence images of mPEG-PEI/pEGFP-N2 at N/P ratios of 8, 10, and 12, respectively. Images 2, 4, and 6 are fluorescence images of D-SP5-PEG-PEI/pEGFP-N2 at N/P ratios of 8, 10, and 12, respectively. B. Luciferase expression levels of mPEG-PEI/pGL$_{4.2}$ and D-SP5-PEG-PEI/pGL$_{4.2}$ at different N/P ratios in transfected cells.

EGFP expression of the D-SP5-PEG-PEI/pEGFP-N2 complex was higher than that of mPEG-PEI/pEGFP-N2 at N/P ratios of 8, 10, and 12. The quantitative luciferase assay in Figure 5B showed that the transfection efficiency of D-SP5-PEG-PEI/pGL$_{4.2}$ was 19.1, 11.8, 11.3, and 4.6 times greater than that for mPEG-PEI/pGL$_{4.2}$ at N/P = 8 ($P < 0.01$), N/P = 10 ($P < 0.01$), N/P = 12 ($P < 0.05$), N/P = 14 ($P < 0.05$), respectively. Both the qualitative and quantitative experiments confirmed the improved transfection efficiency of D-SP5-PEG-PEI. It is reasonable to assume that the D-SP5 modification enhanced the targeting specificity of D-SP5-PEG-PEI/pDNA for SGC7901 cells.

In Vitro Pharmacodynamics Assay. *MTT Assay.* An MTT assay was used to evaluate the antitumor effect of polymers/pTRAIL complexes in vitro. Figure 6A shows that the rates of inhibition in SGC7901 cells treated with D-SP5-PEG-PEI/pTRAIL were 1.72, 1.64, and 1.96 times more than that of mPEG-PEI/pTRAIL at N/P = 8 ($P < 0.01$), N/P = 10 ($P < 0.01$), and N/P = 12 ($P < 0.001$), respectively, which indicated that D-SP5-PEG-PEI/pTRAIL has a better antitumor effect in vitro than mPEG-PEI/pTRAIL.

Flow Cytometry Assay. Apoptosis of SGC7901 cells was detected by annexin V-FITC/PI staining and analyzed by flow cytometry. The result is shown in Figure 6B. Under normal conditions, the level of apoptosis was very low (6.51%). The apoptosis rate increased to 13.09% at 8 h of drug administration (15.35% at 12 h of drug administration) after treating with mPEG-PEI/pTRAIL, and 82.48% at 8 h of drug administration (89.48% at 12 h of drug administration) after treating with D-SP5-PEG-PEI/pTRAIL. The apoptosis rate of D-SP5-PEG-PEI/pTRAIL has been slightly lower than PEI/pTRAIL (92.01%) at 8 h of drug administration, and almost equal with PEI/pTRAIL (92.77%) at 12 h of drug administration. This result confirmed the significantly higher apoptosis rate of SGC7901 cells induced by D-SP5-PEG-PEI/pTRAIL compared with that induced by mPEG-PEI/pTRAIL, and an equal rate of apoptosis induced by PEI/pTRAIL at 12 h of drug administration.

Western Blot Essay. The expression of TRAIL protein was determined by Western blot assay, and the result is shown in Figure 6C. It revealed a significantly higher level of TRAIL protein in D-SP5-PEG-PEI/pTRAIL treated cells than in mPEG-PEI/pTRAIL treated cells. This indicated that the increased inhibition of SGC7901 cells by D-SP5-PEG-PEI/pTRAIL was due to a higher expression level of TRAIL protein, and further confirmed the efficiency of D-SP5-PEG-PEI/pTRAIL.

In Vivo Pharmacodynamics Study. The tumor suppression effect of D-SP5-PEG-PEI/pTRAIL on SGC7901 cells is clearly shown in Figure 7A. We found the tumor volumes of the D-SP5-PEG-PEI/pTRAIL group were smaller than those of the mPEG-PEI/pTRAIL group with a tumor inhibition rate of 39.87%, which indicated a better therapeutic effect of D-SP5-PEG-PEI/pTRAIL. TUNEL assays were used to evaluate apoptosis in the tumor tissue of the mice. The result is shown in Figure 7B. The quantity of apoptotic bodies in the D-SP5-PEG-PEI/pTRAIL group was significantly more than in the other two groups, which demonstrated a higher apoptosis level in the tumor, and further confirmed the better tumor suppression effect of D-SP5-PEG-PEI/pTRAIL. H&E staining was used to examine the toxicity of mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL in the major organs: heart, liver, spleen, lung, and kidney. The results are shown in Figure 7C. Organ histopathology was evaluated in blind observations for the appearance of inflammatory cells, tissue inflammation, and structural damage. In the liver tissue of both mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL groups there was slight necrosis with inflammatory cell infiltration and hepatocyte swelling was also observed. The kidney tissue revealed a slight swelling of renal tubular epithelial cells and glomerulosclerosis in both mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL groups. The muscle fibers of the heart were partially damaged in both the mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL groups, but all the lesions were slight compared with that in a previous study of the toxicity of the gene vector PEI (MW 25000).[33] Other organs appeared to be normal compared with the control group.

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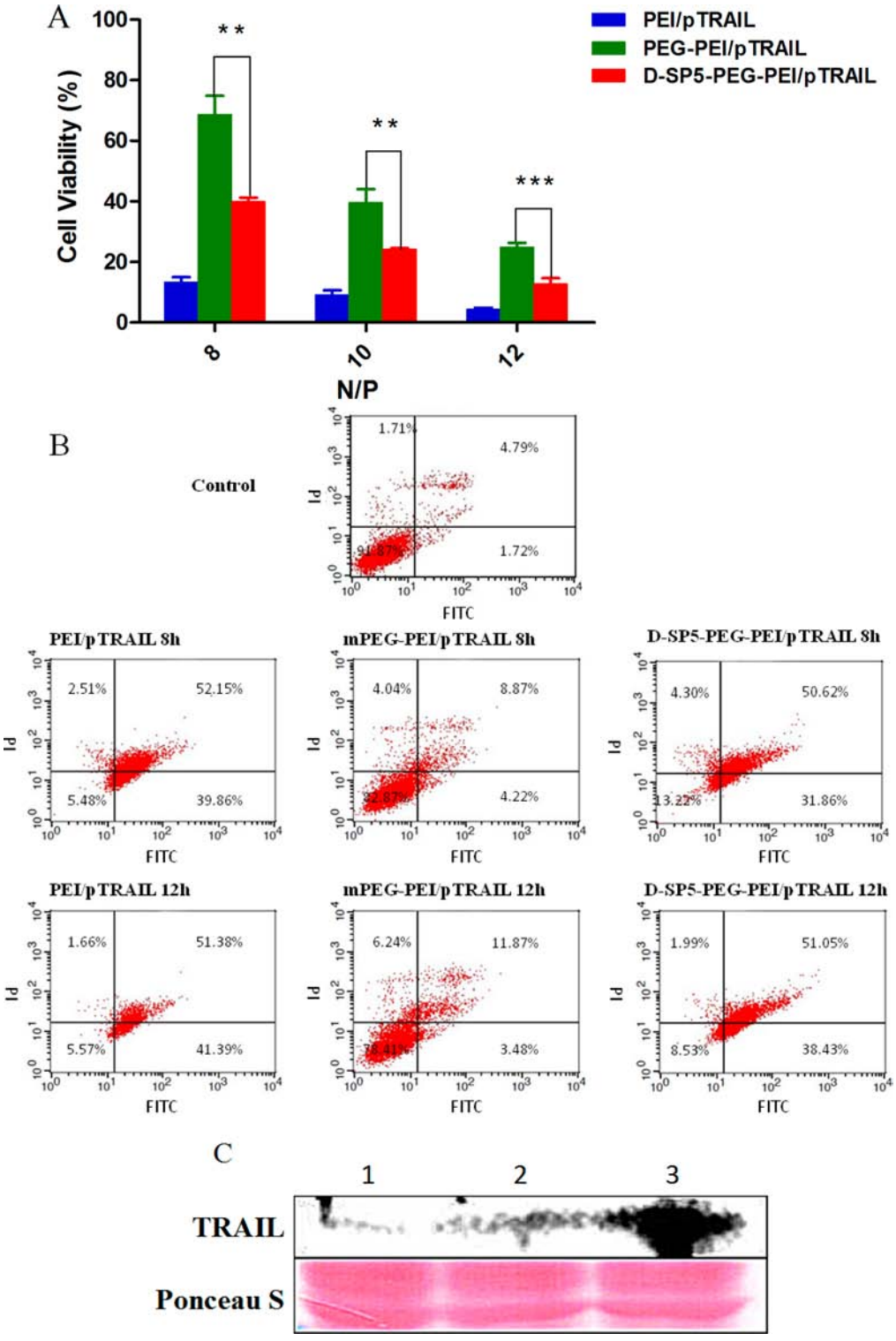

Figure 6. A. Viabilities of SGC7901 cells treated with mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL compared with that in cells treated with PEI/pTRAIL. B. Apoptosis of SGC7901 cells treated by mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL at N/P ratio of 12 at 8 h/12 h of drug administration. C. TRAIL protein expression of, 1, Control cells; 2, mPEG-PEI/pTRAIL treated cells; and 3, D-SP5-PEG-PEI/pTRAIL treated cells.

DISCUSSION

Gastric cancer is the fifth most common malignancy in the world, after cancers of the lung, breast, colorectum, and prostate. It is also the third leading cause of cancer death in both sexes worldwide according to World Health Organization (WHO). Surgery and chemotherapy are currently the major modalities for gastric cancer. Surgery is the primary treatment of gastric cancer, but it is not ideal in some cases, especially in advanced gastric carcinoma with metastasis. Multidrug resistance is the most frequent cause of failure in gastric cancer chemotherapy. Thus, new therapeutic strategies are urgently needed. Gene therapy is a promising molecular alternative in the treatment of gastric cancer.[34−36]

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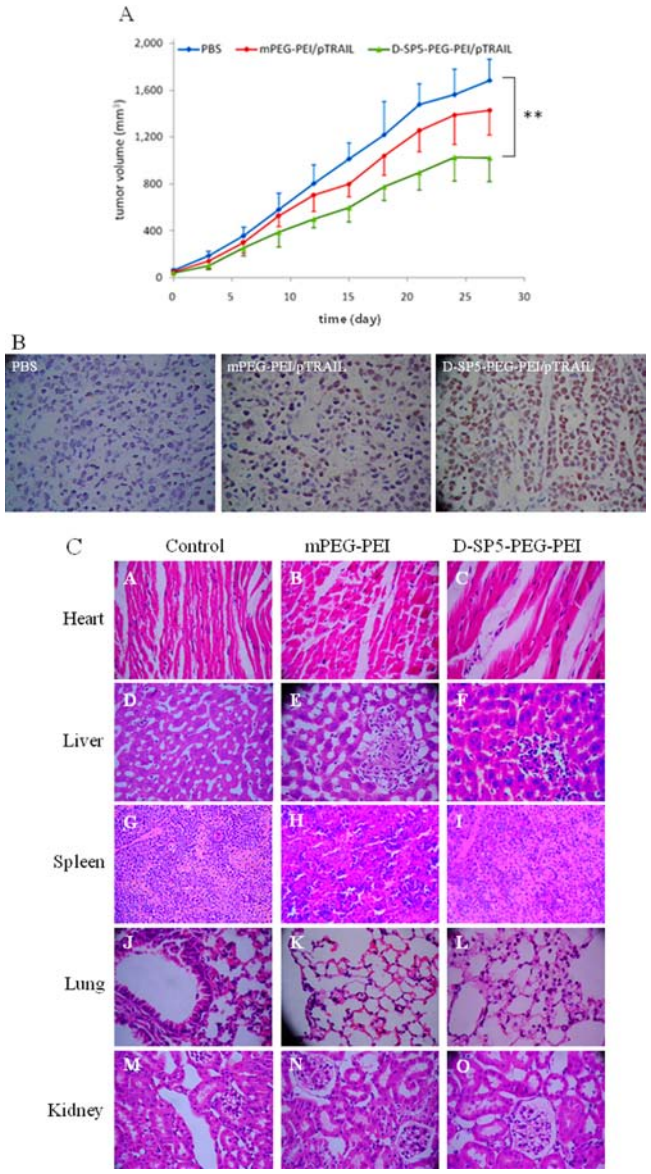

Figure 7. A. Tumor volumes in PBS, mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL groups (n = 8). B. Results of TUNEL assays. C. H staining of heart (A–C), liver (D–F), spleen (G–I), lung (J–L), and kidney (M–O) of mice treated with PBS, mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL, respectively.

The tumor necrosis factor-related apoptosis inducing ligand (TRAIL) gene was first identified and cloned by Wiley et al. in 1995 and its apoptotic pathway in tumors was demonstrated by Johnstone et al.[37,38] TRAIL protein can induce apoptosis in many types of malignancy after binding with the membrane-bound death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2), which are capable of signaling apoptosis without severe damage to normal cells. The bystander effect enhanced its antitumor activity, so it has been widely used in gene therapy of cancer.[39−45] In this study, we used the TRAIL gene (pTRAIL) as therapeutic gene to form a targeted gene delivery system D-SP5-PEG-PEI/pTRAIL for the treatment of gastric adenocarcinoma SGC7901 xenografts. D-SP5 has good affinity for SGC7901 cells as previously described, and the receptor-mediated endocytosis enhanced the capacity of D-SP5-PEG-PEI/pTRAIL to enter into cells, thus facilitating its antitumor effect.

Fluorescence images showed that the uptake of FITC-D-SP5 by SGC7901 cells was significantly greater than that of FITC-L-SP5, indicating a stronger affinity of D-SP5 for SGC7901 cells. This explains the targeting ability of D-SP5. D-SP5 was linked to difunctional NHS-PEG-Mal through a substitution reaction of the functional succinimide group with the amino group of D-SP5 to synthesis D-SP5-PEG-Mal. D-SP5-PEG-Mal was then introduced to PEI through an addition reaction of the functional maleimide group and the amino group of PEI to form the targeting gene delivery vector D-SP5-PEG-PEI. The MTT Assay showed a lower cytotoxicity of mPEG-PEI/$pGL_{4.2}$ and D-SP5-PEG-PEI/$pGL_{4.2}$ compared with PEI/$pGL_{4.2}$, which is consistent with our previous research.[30,46] The qualitative EGFP expression assay and the quantitative luciferase assay both showed an enhanced transfection efficiency of D-SP5-PEG-PEI/pDNA compared with the nontargeted mPEG-PEI/pDNA. This increase can be attributed to the high affinity of D-SP5 to SGC7901 cells, which was shown earlier.

We investigated the in vitro pharmacodynamics of the D-SP5-PEG-PEI/pTRAIL through MTT and flow cytometry assays. The MTT assay showed a better inhibition rate of D-SP5-PEG-PEI/pTRAIL at N/P ratios of 8, 10, and 12, and the flow cytometry assay revealed a significantly greater apoptosis rate of D-SP5-PEG-PEI/pTRAIL compared with the mPEG-PEI/pTRAIL treated group. Western blot assays demonstrated that the increased apoptosis rate was due to higher levels of TRAIL expression, which confirmed the antitumor effect of D-SP5-PEG-PEI/pTRAIL.

Although the in vitro transfection assay showed a better transfection efficiency at a higher N/P ratio (Figure 5B), the toxicity of the polymers demonstrated a significant cytotoxicity at N/P ratio of 14 and above (Figure 4). Together with the relatively better tumor inhibition at N/P ratio of 12 (Figure 6A), we finally chose N/P ratio of 12 to carry out our in vivo pharmacodynamics study. The in vivo pharmacodynamics study revealed that D-SP5-PEG-PEI/pTRAIL produced a better treatment effect, and TUNEL assays showed a higher apoptosis level in the tumor in the D-SP5-PEG-PEI/pTRAIL treated group. This demonstrated the in vivo targeting effect of D-SP5-PEG-PEI/pTRAIL could enhance suppression of the tumor. The H&E staining assay showed slight toxicity of both mPEG-PEI/pTRAIL and D-SP5-PEG-PEI/pTRAIL in the heart, liver, and kidney, and this demonstrated that PEGylation of PEI can reduce the in vivo toxicity to major organs. But on the other hand, it is not very satisfying of the in vivo antitumor effect of the D-SP5-PEG-PEI/pTRAIL considering the relatively good tumor suppression in vitro, we infer that this may be caused by the instability of the polymer/pDNA complex in vivo. We intend to engage in further study of this and wish to find a better solution in the future.

■ CONCLUSION

We conducted an intensive study on the retro-inverso peptide D-SP5 with respect to its ability to target SGC7901 cells and used it to build a targeted gene delivery vector, D-SP5-PEG-PEI. We found that D-SP5 possesses a high affinity for SGC7901 cells that contributed to the increased transfection efficiency of D-SP5-PEG-PEI with lower cytotoxicity. We chose the TRAIL gene (pTRAIL) as a therapeutic gene and prepared D-SP5-PEG-PEI/pTRAIL with which we obtained an enhanced level of apoptosis in SGC7901 cells in vitro. Furthermore, we used D-SP5-PEG-PEI/pTRAIL to treat nude mice with SGC7901 xenograft tumors and we achieved

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suppression of the tumor without obvious toxicity to major organs. We confirmed that the effect was due to a higher in vivo apoptosis rate in the tumor. In conclusion, D-SP5-PEG-PEI is a promising gene delivery system for antitumor therapy.

EXPERIMENTAL SECTION

Materials. $_{L}$(SVSVGMKPSPRP), $_{D}$(PRPSPKMGVSVS), $_{L}$(SVSVGMKPSPRP)-FITC (FITC-L-SP5), and FITC-$_{D}$(PRPSPKMGVSVS) (FITC-D-SP5) were synthesized by ChinaPeptides (Shanghai, China). High molecular weight PEI (branched, MW 25 000) was obtained from Sigma-Aldrich (Chemie GmbH, Munich, Germany). CH_3O-PEG-Mal (mPEG-Mal, MW 2000) and NHS-PEG-Mal (MW 2000) were purchased from Jenkem Technology (Beijing, China). Fluorescein-5-isothiocyanate (FITC) was bought from Fanbo Biochemicals (Beijing, China). 4′,6-Diamidino-2-phenylindole (DAPI) and the in situ cell death detection kit (POD) were obtained from Roche (Basel, Switzerland). Gelred was purchased from Bitoium (USA) and the Luciferase Assay Kit and the luciferase reporter gene plasmid ($pGL_{4.2}$) were products of Promega (Beijing, China). The BCA Protein Assay Kit and the apoptosis detection kit were purchased from Beyotime (Shanghai, China). The plasmid encoding EGFP (pEGFP-N2) was from Genechem Co. (Shanghai, China). The pORF-hTRAIL plasmid (pTRAIL) was a kind gift from Professor Chen Jiang (School of Pharmacy, Fudan University, China). A Plasmid Mega Kit was used to purify the plasmid DNA before application (Qiagen, Hilden, Germany). Anti-tumor necrosis factor-related apoptosis inducing ligand (TRAIL) antibody (ab2435) was purchased from Abcam (Cambridge, UK). The human gastric adenocarcinoma (SGC7901) cell line was obtained from the Shanghai Tumor Research Institute, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, USA).

Cell Uptake of Peptides. SGC7901 cells were seeded into 12-well plates at a density of 8×10^4 cells per well and incubated overnight to obtain approximately 70% cell confluence. FITC, FITC-L-SP5, and FITC-D-SP5 at a final concentration of 5×10^{-6} mol/L were added and incubation continued for 4 h. SGC7901 cells were washed with phosphate buffered saline (PBS; 0.01 M, pH 7.4) twice and fixed with 10% formaldehyde for 10 min. The cells were washed with PBS twice and the nucleus was dyed with DAPI for 15 min. The cells were washed with PBS twice and then overlaid with 10% buffered glycerol saline before being measured using an inverted fluorescence microscope (Leica, DMI4000B, Wetzlar, Germany).

Synthesis of mPEG-PEI. Based on our previous study and readjust accordingly,[27] mPEG-Mal (2.4 μmol) was dissolved in 0.5 mL PBS (0.2 M, pH 7.4). The solution was slowly added to PEI (0.4 μmol) dissolved in 1 mL PBS (0.2 M, pH 7.4) while stirring. The resulting mixture was stirred at room temperature for 6 h. The progress of the reaction was monitored by thin-layer chromatography (TLC) using a mixture of chloroform, methanol, *n*-butanol, and ammonia (v/v = 10/1.5/0.5/0.3) as the developing solvent. The reaction solution was then added to an Amicon Ultra filter tube (MWCO 10 000, Millipore, Billerica, MA, USA) to ultrafiltrate at 6000 × *g* and was subsequently washed five times with distilled water (dH_2O). The mPEG-PEI product was then collected and lyophilized.

Synthesis of D-SP5-PEG-PEI. According to our previous study with appropriate readjustment,[27] NHS-PEG-Mal (2.4 μmol) and D-SP5 (2.4 μmol) were dissolved in 1 mL dimethylformamide (DMF). 300 μL DMF with 33 μL triethylamine (TEA) was added to the above mixture in a dropwise manner and stirred at room temperature for 6 h. The reaction was monitored by HPLC. The final reaction product was purified via an AKTA explorer 100 system (GE, Fairfield, CT, USA) equipped with a Sephadex G-15 column. D-SP5-PEG-Mal was collected and lyophilized.

D-SP5-PEG-Mal (2.4 μmol) was added to PEI (0.4 μmol) dissolved in 1 mL PBS (0.2 M, pH 7.4), and the resulting mixture was stirred overnight. The final reaction was then placed in an Amicon Ultra filter tube and washed five times with dH_2O. The D-SP5-PEG-PEI product was collected and lyophilized.

Characterization of the Polymers. The mPEG-PEI and D-SP5-PEG-PEI polymers were characterized by ^{1}H NMR analysis using D_2O as the solvent on a 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA).

Preparation of Polymer/pDNA Complex. The polymer/pDNA was formed at desired N/P (nitrogen in cationic polymer per phosphate in nucleic acid) ratios with predetermined concentrations of pDNA and polymer solutions. The final pDNA concentration was 80 μg/mL. Equal volumes of pDNA solution were added to polymer solutions dissolved in PBS (pH 7.4), and then vortexed for 30 s followed by further incubation at room temperature for 30 min.

Characterization of the Polymer/pDNA Complex. *Size and Zeta Potential.* The mean particle size and zeta potential of the polymer/$pGL_{4.2}$ complexes were determined at 25 °C using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK).

Agarose Gel Electrophoresis Assay. Electrophoresis was performed to investigate the DNA condensation ability of the polymers. The polymer/$pGL_{4.2}$ complexes were prepared at the N/P ratios of 8, 10, and 12. These complexes were loaded on 1.0% (w/v) agarose gels with Gelred (3 μL 10 000× solution in 30 mL gel) and run in Tris acetate-EDTA buffer at 140 V for 40 min. DNA retardation was visualized and photographed by a KH-UVI ultraviolet light analyzer (Kanghe, Shanghai, China).

Cytotoxicity Assay. SGC7901 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated for 24 h at 37 °C. The medium was changed to fresh complete medium before adding the polymers/$pGL_{4.2}$ complexes. Freshly prepared PEI/$pGL_{4.2}$, mPEG-PEI/$pGL_{4.2}$, and D-SP5-PEG-PEI/$pGL_{4.2}$ with different N/P ratios (containing 2 μg of pDNA) of 4, 6, 8, 10, 12, 14, and 16 were added and coincubated for 12 h. The medium was changed to fresh complete medium and cells were incubated for a further 36 h. Twenty microliters of MTT (5 mg/mL) was added to each well and reacted for 4 h at 37 °C. The medium in each well was replaced with 150 μL dimethyl sulfoxide (DMSO) and incubated for additional 15 min. The absorbance at 490 nm was recorded by a microplate reader (PowerWave XS, Bio-TEK, Winooski, VT). Control cells received no treatment. The relative cell viability (%) of PEG-PEI/$pGL_{4.2}$ and D-SP5-PEG-PEI/$pGL_{4.2}$ treated cells was calculated by $A_{sample}/A_{control} \times 100\%$.

In Vitro Transfection. *Enhanced Green Fluorescent Protein Assay.* SGC7901 cells were seeded in 48-well plates at a density of 2×10^4 cells per well and incubated overnight at 37 °C. The medium was replaced with serum-free medium, and 50 μL of polymer/pEGFP-N2 complexes containing 2 μg

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pEGFP-N2 at N/P ratios of 8, 10, and 12 were added to each well. After incubation at 37 °C for 12 h, the medium was changed to fresh serum-free medium and cells were incubated for a further 36 h. Fluorescence images were visualized with an inverted fluorescence microscope (Leica, DMI4000B, Wetzlar, Germany).

Luciferase Assay. The plasmid $pGL_{4.2}$ encoding luciferase was used as the reporter gene in the luciferase assay. SGC7901 cells were seeded into 48-well plates at a density of 2×10^4 cells per well and incubated overnight prior to the transfection experiments. The medium was replaced with serum-free medium. 50 μL PEI/$pGL_{4.2}$, mPEG-PEI/$pGL_{4.2}$, and D-SP5-PEG-PEI/$pGL_{4.2}$ complexes containing 2 μg $pGL_{4.2}$ at various N/P ratios were added to each well and incubated for 12 h. Serum-free medium was replaced with fresh complete medium, and the cells were further incubated for 36 h. Luciferase activity was measured in accordance with relative light units (RLU) using an Ultra-Weak Luminescence Analyzer (Chuanghe, Beijing, China). The total protein concentration of transfected cell lysate was determined using a BCA protein assay kit following the manufacturer's instructions. The final transfection efficiency was calculated as relative lights units (RLU)/mg of total protein. All assays were conducted in triplicate and data are expressed in RLU/mg protein (±SD).

In Vitro Cell Apoptosis Assay. *MTT Assay.* SGC7901 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated for 24 h at 37 °C. The medium was changed to fresh serum-free medium before adding the polymers/pTRAIL complexes. Freshly prepared PEI/pTRAIL, mPEG-PEI/pTRAIL, and D-SP5-PEG-PEI/pTRAIL (50 μL) with different N/P ratios (containing 2 μg of pTRAIL) of 8, 10, and 12 were added and coincubated for 12 h. The medium was changed to fresh medium and cells were incubated for a further 36 h. Twenty microliters of MTT (5 mg/mL) was added to each well and reacted for 4 h at 37 °C. The relative cell viability (%) was determined as described in the cytotoxicity assay.

Flow Cytometry Assay. SGC7901 cells were seeded in 12-well plates (8×10^4 cells/well) and incubated for 24 h at 37 °C. The medium was changed to fresh serum-free medium before adding the polymers/pTRAIL complexes. Freshly prepared PEI/pTRAIL, mPEG-PEI/pTRAIL, and D-SP5-PEG-PEI/pTRAIL with N/P ratio (containing 2 μg of pTRAIL) of 12 was added and coincubated for 8/12 h. The medium was changed to fresh medium and cells were incubated for a further 40/36 h. The apoptosis rate was detected by an annexin V-FITC/PI apoptosis detection kit (Beyotime, Shanghai, China) with flow cytometry.

Western Blot Assays. SGC7901 cells were seeded in 12-well plates (8×10^4 cells/well) and incubated for 24 h at 37 °C. The medium was changed to serum-free medium before adding the polymers/pTRAIL complexes. Freshly prepared PEI/pTRAIL, mPEG-PEI/pTRAIL, and D-SP5-PEG-PEI/pTRAIL of N/P = 12 (containing 2 μg of pTRAIL) were added and coincubated for 6 h. The medium was changed to fresh medium and cells were incubated for a further 30 h. The cells in each well were collected and lysed in 40 μL cell lysate buffer. The total protein concentration of transfected cell lysate was determined using a BCA protein assay kit following the manufacturer's instructions. The total proteins were separated by electrophoresis and transferred to polyvinylidene fluoride membrane and TRAIL proteins were immunologically detected.

In Vivo Pharmacodynamics Study. All animal experiments were carried out in accordance with the guidelines evaluated and approved by the Ethics Committee of Fudan University. Male BALB/c nude mice of 4−6 weeks of age were obtained from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). The gastric adenocarcinoma model was established by subcutaneous injection of 3×10^6 SGC7901 tumor cells per mouse into the underarm region of the nude mice. The model nude mice were randomly divided into three groups ($n = 10$) when the tumor reached 50 mm^3 in size, and intravenously injected with freshly prepared mPEG-PEI/pTRAIL, D-SP5-PEG-PEI/pTRAIL (containing 40 μg pTRAIL in 200 μL PBS, N/P = 12), or PBS via the tail vein. This was repeated every 3 days, six times. The tumor volume was measured every 3 days until all the nude mice were sacrificed 11 days after the last drug administration.

TUNEL Assay. TUNEL assays were performed to evaluate the in vivo apoptosis of a tumor. Two mice from each group (PBS, mPEG-PEI/pTRAIL, and D-SP5-PEG-PEI/pTRAIL) were sacrificed separately on the 10th day after first administration of the drug. Tumor tissue was dissected for routine paraffin sectioning at 5 μm for TUNEL detection following the instructions of the in situ cell death detection kit. The DAB coloration method was used to reveal the level of apoptosis.

Histological Studies. Two mice from each group (PBS, mPEG-PEI/pTRAIL, and D-SP5-PEG-PEI/pTRAIL) were randomly selected from all the nude mice sacrificed, and the heart, liver, spleen, lung, and kidney were dissected and 5 μm tissue sections prepared at for H&E staining. Drug toxicity to these major organs was assessed by organ histopathology and blind observations for the appearance of inflammatory cells, tissue inflammation, and structural damage.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experiments. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00137.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Pack, D. W., Hoffman, A. S., Pun, S., and Stayton, P. S. (2005) Design and development of polymers for gene delivery. *Nat. Rev. Drug Discovery* 4, 581−93.

(2) Zhang, M., Garbuzenko, O. B., Reuhl, K. R., Rodriguez-Rodriguez, L., and Minko, T. (2012) Two-in-one: combined targeted chemo and gene therapy for tumor suppression and prevention of metastases. *Nanomedicine (London)* 7, 185−97.

DOI: 10.1021/acs.bioconjchem.5b00137
Bioconjugate Chem. 2015, 26, 1494−1503

(3) Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., and Anderson, D. G. (2014) Non-viral vectors for gene-based therapy. *Nat. Rev. Genetics 15*, 541−55.
(4) Pack, D. W., Hoffman, A. S., Pun, S., and Stayton, P. S. (2005) Design and development of polymers for gene delivery. *Nat. Rev. Drug Discovery 4*, 581−93.
(5) Mintzer, M. A., and Simanek, E. E. (2009) Nonviral vectors for gene delivery. *Chem. Rev. 109*, 259−302.
(6) Lungwitz, U., Breunig, M., Blunk, T., and Gopferich, A. (2005) Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm. 60*, 247−66.
(7) Varga, C. M., Tedford, N. C., Thomas, M., Klibanov, A. M., Griffith, L. G., and Lauffenburger, D. A. (2005) Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther. 12*, 1023−32.
(8) Benjaminsen, R. V., Mattebjerg, M. A., Henriksen, J. R., Moghimi, S. M., and Andresen, T. L. (2013) The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Mol. Ther. 21*, 149−57.
(9) Lv, H., Zhang, S., Wang, B., Cui, S., and Yan, J. (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Controlled Release 114*, 100−9.
(10) Florea, B. I., Meaney, C., Junginger, H. E., and Borchard, G. (2002) Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. *AAPS PharmSci. 4*, E12.
(11) Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., and Kissel, T. (2003) In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials 24*, 1121−31.
(12) Petersen, H., Fechner, P. M., Martin, A. L., Kunath, K., Stolnik, S., Roberts, C. J., Fischer, D., Davies, M. C., and Kissel, T. (2002) Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjugate Chem. 13*, 845−54.
(13) Owens, D. R., and Peppas, N. A. (2006) Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int. J. Pharm. 307*, 93−102.
(14) Tang, G. P., Zeng, J. M., Gao, S. J., Ma, Y. X., Shi, L., Li, Y., Too, H. P., and Wang, S. (2003) Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. *Biomaterials 24*, 2351−62.
(15) Sung, S. J., Min, S. H., Cho, K. Y., Lee, S., Min, Y. J., Yeom, Y. I., and Park, J. K. (2003) Effect of polyethylene glycol on gene delivery of polyethylenimine. *Biol. Pharm. Bull. 26*, 492−500.
(16) Park, M. R., Han, K. O., Han, I. K., Cho, M. H., Nah, J. W., Choi, Y. J., and Cho, C. S. (2005) Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. *J. Controlled Release 105*, 367−80.
(17) Galanis, E., Vile, R., and Russell, S. J. (2001) Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors. *Crit. Rev. Oncol. Hematol. 38*, 177−92.
(18) Hamamoto, K., Kida, Y., Zhang, Y., Shimizu, T., and Kuwano, K. (2002) Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiol. Immunol. 46*, 741−9.
(19) Nair, D. T., Kaur, K. J., Singh, K., Mukherjee, P., Rajagopal, D., George, A., Bal, V., Rath, S., Rao, K. V., and Salunke, D. M. (2003) Mimicry of native peptide antigens by the corresponding retro-inverso analogs is dependent on their intrinsic structure and interaction propensities. *J. Immunol. 170*, 1362−73.
(20) Hong, S. Y., Oh, J. E., and Lee, K. H. (1999) Effect of D-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide. *Biochem. Pharmacol. 58*, 1775−80.
(21) Verdoliva, A., Ruvo, M., Cassani, G., and Fassina, G. (1995) Topological mimicry of cross-reacting enantiomeric peptide antigens. *J. Biol. Chem. 270*, 30422−7.
(22) Chorev, M., and Goodman, M. (1993) A dozen years of retro-inverso peptidomimetics. *Acc. Chem. Res. 26*, 266−273.
(23) Chorev, M., and Goodman, M. (1995) Recent developments in retro peptides and proteins–an ongoing topochemical exploration. *Trends Biotechnol. 13*, 438−45.
(24) Welch, B. D., VanDemark, A. P., Heroux, A., Hill, C. P., and Kay, M. S. (2007) Potent D-peptide inhibitors of HIV-1 entry. *Proc. Natl. Acad. Sci. U. S. A. 104*, 16828−33.
(25) Lee, T. Y., Lin, C. T., Kuo, S. Y., Chang, D. K., and Wu, H. C. (2007) Peptide-mediated targeting to tumor blood vessels of lung cancer for drug delivery. *Cancer Res. 67*, 10958−65.
(26) Li, Y., Lei, Y., Wagner, E., Xie, C., Lu, W., Zhu, J., Shen, J., Wang, J., and Liu, M. (2013) Potent retro-inverso D-peptide for simultaneous targeting of angiogenic blood vasculature and tumor cells. *Bioconjugate Chem. 24*, 133−43.
(27) Wang, J., Lei, Y., Xie, C., Lu, W., Wagner, E., Xie, Z., Gao, J., Zhang, X., Yan, Z., and Liu, M. (2014) Retro-inverso CendR peptide-mediated polyethyleneimine for intracranial glioblastoma-targeting gene therapy. *Bioconjugate Chem. 25*, 414−23.
(28) Cherng, J. Y., van de Wetering, P., Talsma, H., Crommelin, D. J., and Hennink, W. E. (1996) Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharm. Res. 13*, 1038−42.
(29) Son, K. K., Tkach, D., and Patel, D. H. (2000) Zeta potential of transfection complexes formed in serum-free medium can predict in vitro gene transfer efficiency of transfection reagent. *Biochim. Biophys. Acta 1468*, 11−4.
(30) Ogris, M., Brunner, S., Schuller, S., Kircheis, R., and Wagner, E. (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther. 6*, 595−605.
(31) Moghimi, S. M., Symonds, P., Murray, J. C., Hunter, A. C., Debska, G., and Szewczyk, A. (2005) A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol. Ther. 11*, 990−5.
(32) Luo, X., Feng, M., Pan, S., Wen, Y., Zhang, W., and Wu, C. (2012) Charge shielding effects on gene delivery of polyethylenimine/DNA complexes: PEGylation and phospholipid coating. *J. Mater. Sci. Mater. Med. 23*, 1685−95.
(33) Xiong, M. P., Forrest, M. L., Ton, G., Zhao, A., Davies, N. M., and Kwon, G. S. (2007) Poly(aspartate-g-PEI800), a polyethylenimine analogue of low toxicity and high transfection efficiency for gene delivery. *Biomaterials 28*, 4889−900.
(34) Sutter, A. P., and Fechner, H. (2006) Gene therapy for gastric cancer: is it promising? *World J. Gastroenterol. 12*, 380−7.
(35) Zhang, D., and Fan, D. (2007) Multidrug resistance in gastric cancer: recent research advances and ongoing therapeutic challenges. *Expert Rev. Anticancer Ther. 7*, 1369−78.
(36) Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., et al. (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity 3*, 673−82.
(37) Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., et al. (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat. Med. 5*, 157−63.
(38) Johnstone, R. W., Frew, A. J., and Smyth, M. J. (2008) The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat. Rev. Cancer 8*, 782−98.
(39) LeBlanc, H. N., and Ashkenazi, A. (2003) Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ. 10*, 66−75.
(40) Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A. (2000) Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity 12*, 611−20.
(41) Qiu, S., Ruan, H., Pei, Z., Hu, B., Lan, P., Wang, J., Zhang, Z., Gu, J., Sun, L., Qian, C., et al. (2004) Combination of targeting gene-virotherapy with 5-FU enhances antitumor efficacy in malignant colorectal carcinoma. *J. Interferon Cytokine Res. 24*, 219−30.

DOI: 10.1021/acs.bioconjchem.5b00137
Bioconjugate Chem. 2015, 26, 1494−1503

(42) Kagawa, S., He, C., Gu, J., Koch, P., Rha, S. J., Roth, J. A., Curley, S. A., Stephens, L. C., and Fang, B. (2001) Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. *Cancer Res. 61*, 3330−8.
(43) Lee, J., Hampl, M., Albert, P., and Fine, H. A. (2002) Antitumor activity and prolonged expression from a TRAIL-expressing adenoviral vector. *Neoplasia 4*, 312−23.
(44) Seol, J. Y., Park, K. H., Hwang, C. I., Park, W. Y., Yoo, C. G., Kim, Y. W., Han, S. K., Shim, Y. S., and Lee, C. T. (2003) Adenovirus-TRAIL can overcome TRAIL resistance and induce a bystander effect. *Cancer Gene Ther. 10*, 540−8.
(45) Cao, X., Yang, M., Wei, R. C., Zeng, Y., Gu, J. F., Huang, W. D., Yang, D. Q., Li, H. L., Ding, M., Wei, N., et al. (2011) Cancer targeting gene-viro-therapy of liver carcinoma by dual-regulated oncolytic adenovirus armed with TRAIL gene. *Gene Ther. 18*, 765−77.
(46) Lei, Y., Wang, J., Xie, C., Wagner, E., Lu, W., Li, Y., Wei, X., Dong, J., and Liu, M. (2013) Glutathione-sensitive RGD-poly-(ethylene glycol)-SS-polyethylenimine for intracranial glioblastoma targeted gene delivery. *J. Gene Med. 15*, 291−305.

DOI: 10.1021/acs.bioconjchem.5b00137
Bioconjugate Chem. 2015, 26, 1494−1503