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A Tumor-Specific Tissue-Penetrating Peptide Enhances the Efficacy of Chemotherapy Drugs in Gastric Cancer

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Purpose: C-end rule (CendR) peptides are found to enhance the penetration of chemotherapeutic agents into tumor cells, while GX1 is a peptide that homes to gastric cancer (GC) vasculature. This study aimed to synthesize a novel peptide GX1-RPAKPAR (GXC) and to explore the effect of GXC on sensitizing GC cells to chemotherapeutic agents.

Materials and Methods: Intracellular Adriamycin concentration analysis was applied to conform whether GXC peptide increases the penetration of chemotherapeutic agents into GC cells *in vitro*. The effect of GXC peptide on sensitizing GC cells to chemotherapeutics was validated by apoptosis assay and *in vitro*/*vivo* drug sensitivity assay. The specificity of GXC to GC tissue was validated by *ex vivo* fluorescence imaging.

Results: *In vitro*, administration of GXC significantly increased Adriamycin concentrations inside SGC-7901 cells, and enhanced the efficacy of chemotherapeutic agents by decreasing the IC_{50} value. *In vivo*, FITC-GXC specifically accumulated in GC tissue. Moreover, systemic co-injection with GXC peptide and Adriamycin statistically improved the therapeutic efficacy in SGC-7901 xenograft models, surprisingly, without obviously increasing side effects.

Conclusion: These results demonstrated that co-administration of the novel peptide GXC with chemotherapeutic agents may be a potential way to enhance the efficacy of anticancer drugs in GC treatment.

Key Words: GXC, GX1, C-end rule peptide, gastric cancer, penetration

INTRODUCTION

Gastric cancer (GC) is the fourth common cancer and the second leading cause of cancer-related death worldwide, with poor improvement in long-term survival over the last decade.[1] Chemotherapy remains one of the most important systemic treatments for advanced GC. Its therapeutic efficacy is limited by poor penetration of chemotherapeutic agents into tumor tissues and high levels of non-specific toxicity towards normal tissues or organs, which restricts the dose of the drug that can be safely administrated to patients.[2] Crossing the vascular wall and penetrating into the cytoplasm of tumor cells is a major challenge in GC chemotherapy.

Recently, peptides containing the R/K/XXR/K motif were found to exhibit a property of improving the poor penetration of tumor cells via binding to neuropilin-1 (NRP-1).[3] The R/K/XXR/K motif, which could partly regulate the cell transport system, is not active unless it occupies a C-terminal position in the peptide, and this effect is called C-end rule (CendR). Many kinds of CendR peptides have been developed by phage display technology, such as RPARPAR, iRGD, and so on.[3-5] However, the specificity of CendR peptides for GC is still limited.[4]

GX1 peptide (CGNSNPKSC), firstly identified by phage display technology, is a gastric tumor angiogenesis marker candidate, which could specifically bond to the endothelia of GC

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blood vessels.[6,7] The application of GX1 as a tumor targeting peptide for GC diagnosis and therapy has also been described.[6-12] In terms of this approach, the development of GC specific imaging probes, such as ^{64}Cu labeled GX1 and cy5.5 labeled GX1, has been undertaken for imaging of GC vasculature.[6,7] Furthermore, when GX1 is conjugated to rmhTNFα, the fusion protein is selectively delivered to targeted tumor sites, significantly improving the anti-tumor activity of rmhTNFα and decreasing adverse effects.[7] To this end, GX1 may be of use in strategies to improve the specificity of CendR peptides for GC.

In this study, we attempted to design and synthesize a novel GC-specific tissue-penetrating peptide GX1-RPAKPAR (GXC) and to investigate its efficacy of enhancing the sensitivity of GC to chemotherapeutics.

MATERIALS AND METHODS

Cell culture and peptide synthesis

The human gastric adenocarcinoma cell line SGC-7901 (maintained by the State Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases) was cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37ºC.

GXC peptide was conjugated with FITC, and the final products were purified by prep-high performance liquid chromatography (prep-HPLC) to remove unreacted ingredients and confirmed by mass spectroscopy and by HPLC with monitoring at 220 nm. RPAKPAR, GX1 peptide (CGNSNPKSC), GXC peptide (CGNSNPKSC-RPAKPAR), and FITC-GXC were all synthesized by GL Biochem Ltd, Shanghai, China.

Toxicity of peptides *in vitro*

For toxicity analysis of peptides (RPAKPAR, GX1, and GXC), 4.5×10^3 SGC-7901 cells were plated in 96-well plates for 24 h for adhesion, and then different concentrations of peptides were added. After 48-h incubation, cell viability was assessed using XTT {sodium 3′-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate} (Roche, Basel, Switzerland) assay. Firstly, XTT labeling reagent (final concentration of 0.3 mg/mL) and electron-coupling reagent (PMS, N-methyl dibenzopyrazine methyl sulfate) were mixed, and then 50 μL of the mixture and 150 μL of the cell culture medium were added to every well respectively. The cells were incubated with the reagents for 6 h. Finally, absorbance at 466 nm (A466) and 650 nm (a reference wavelength) of each well was read by a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher, Waltham, MA, USA).

In vitro drug sensitivity assay

SGC-7901 cells were plated in 96-well plates (4.5×10^3 cells/well). After 24 h of cellular adhesion, freshly prepared anticancer drugs, including vincristine (VCR, Sigma, St Louis, MO, USA), adriamycin (ADR, Sigma), 5-fluorouracil (5-FU, Sigma), and cisplatin (CDDP, Sigma), were added at final concentrations of 0.01, 0.1, 1 and 10 times the human peak plasma concentration for each drug: the peak serum concentrations are 0.4 μg/mL for ADR, 10 μg/mL for 5-FU, 2.0 μg/mL for CDDP, and 0.5 μg/mL for VCR. Also, three kinds of peptides, including GXC, GX1, and RPAKPAR (1×10^{-6} μmol/mL), were co-administrated with anticancer drugs respectively. After 48 h, cell viability was assessed using XTT assay. The concentration at which each drug produced 50% inhibition of growth (IC_{50}) was estimated by the relative survival curve. Independent experiments were performed at least three times.

Cell apoptosis assay

For cell apoptosis analysis, SGC-7901 cells were seeded in six-well plates (8×10^5/well). At 24 h thereafter, 5-FU and ADR was added into each well at the final concentration of 10 μg/mL and 0.4 μg/mL, respectively, and then three kinds of peptides of 1×10^{-6} μmol/mL were respectively added into each group. At 24 h after drug administration, flow cytometry was conducted to detect apoptosis by determining the relative amount of Annexin V-FITC-positive-PI-negative cells (FITC: fluorescein isothiocyanate PI: propidium iodide). The experiments were performed according to the manufacturer's protocol (BD Bioscience, Franklin Lakes, NJ, USA), and Modfit software (BD Biosciences) was used for the data analysis.

Intracellular ADR concentration analysis

For ADR fluorescence analysis, cells were stained with 10 μmol/mL of 3, 3′-dioctadecyloxacarbocyanine perchlorate (DIO) (Beyotime Biotechnology, Shanghai, China) for 15 min and then incubated in medium containing ADR (10 μg/mL) and GX1, RPAKPAR, and GXC (1×10^{-6} μmol/mL) for 2 h. The cells were then washed with fresh medium and placed into a Cellomics instrument (Thermo Fisher). ADR fluorescence was then read and imaged.

Specificity analysis of GXC to GC tissue *in vivo*

All animal studies were performed according to the internationally recognized guidelines approved by the Institutional Animal Care and Use Committee. About 1×10^7 of SGC-7901 cells were injected into the right flank of 4–6-week-old male nude mice (with a body weight of 20–25 g, n=3). Three weeks later, the mice were injected with 40 μmol/kg of FITC-GXC peptide, and 2 h later, tumor and organs were dissected and collected. FITC fluorescence was then imaged and quantified for specificity analysis (Thermo Fisher). *Ex vivo* fluorescence images were normalized and reported as photons per second per centimeter squared per steradian (p/s/cm^2/sr), and the mean fluorescence for each sample was reported.

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In vivo drug sensitivity assay

Approximately 1×10^7 of SGC-7901 cells stably transfected with lentiviral vector that expressed green fluorescent protein (LV-GFP) were injected into right flank of nude mice (about 4-week old, with a body weight of 20–25g, n=4 for every group). Two weeks later, the mice were intravenously injected with PBS, GXC (4 μmol/kg), ADR (1mg/kg), ADR (1mg/kg)+GX1 (4 μmol/kg), ADR (1mg/kg)+RPAKPAR (4 μmol/kg), and ADR (1mg/kg)+GXC (4 μmol/kg) every two days. Tumor weight was measured via optical imaging for GFP expressed by tumor cells *in vivo*. The mice were humanely killed on day 24, and the tumors were collected and weighed.

Statistics

All statistical analyses were performed using SPSS software (version 21.0, IBM Corp., Armonk, NY, USA). The data are presented as the mean±SEM of the mean. Student's t-test (two-tailed) or a one-way analysis of variance was employed to analyze the *in vitro* and *in vivo* data. $p<0.05$ was considered to be statistically significant different.

RESULTS

GXC peptide enhances the drug sensitivity of SGC-7901 cells *in vitro*

To explore enhancement of the anti-tumor effects of chemotherapeutics, it is necessary to investigate the cytotoxicity of RPAKPAR, GX1, and GXC to SGC-7901 cells. At the equivalent concentration of 10^{-3} μmol/mL, the relative cell viability (/control) of SGC-7901 cells was about 98.9% for RPAKPAR, 99.5% for GX1 and GXC. Moreover, at the physiologically required concentration of 10^{-6} μmol/mL, no significant difference in cell viability was found among three peptides (Fig. 1A). Hence, these three peptides did not influence the viability of the tumor cells.

Furthermore, XTT assays were conducted to investigate whether RPAKPAR, GX1, and GXC peptides could enhance the sensitivity of SGC-7901 cells to chemotherapeutic agents *in vitro*. The IC_{50} values of ADR, VCR, 5FU, and CDDP to SGC-7901 cells in GXC and RPAKPAR groups were significantly lower than those without peptides, respectively (Fig. 1B). No statistically difference in IC_{50} was observed between RPAKPAR and

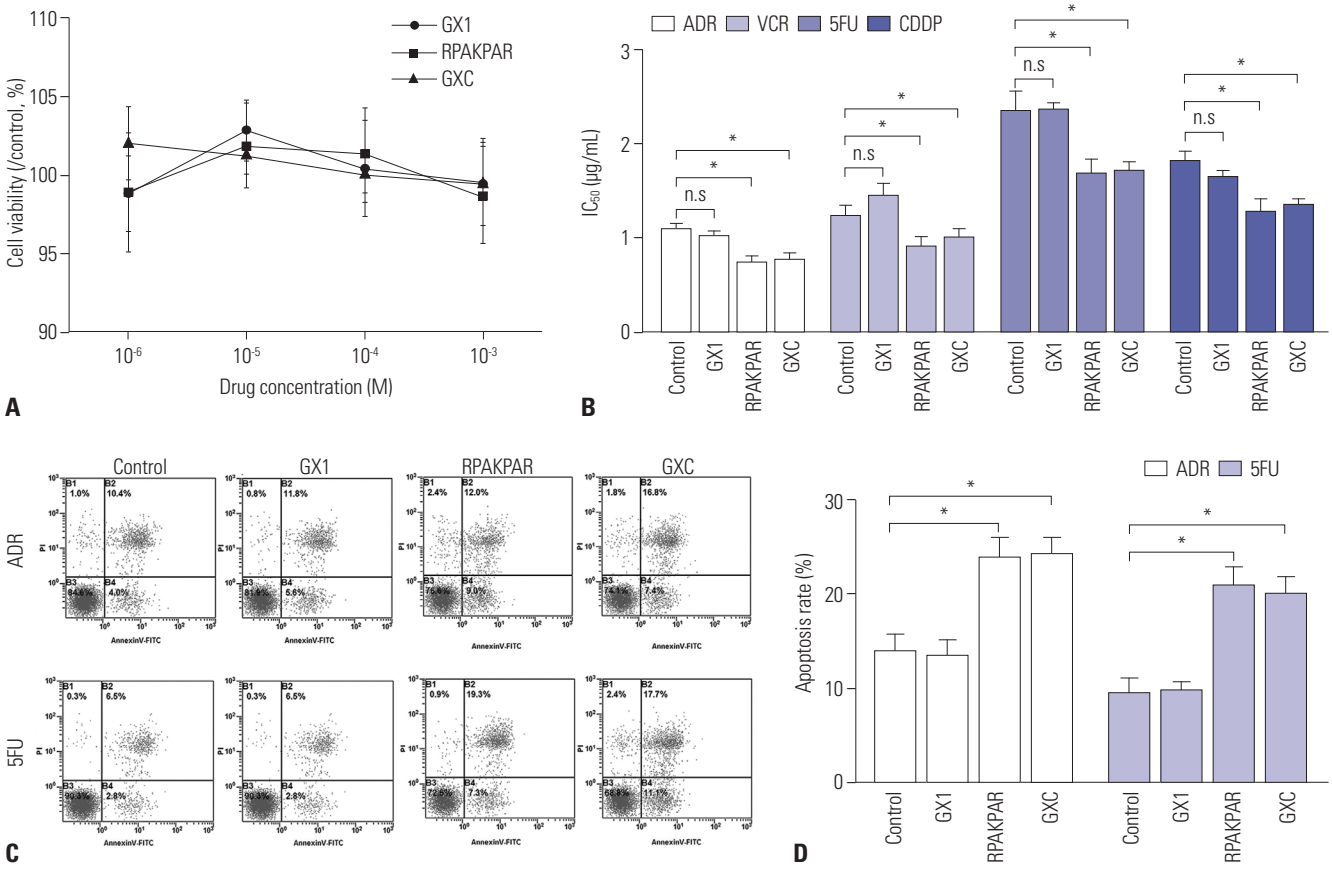

Fig. 1. GXC peptide enhanced the drug sensitivity of SGC-7901 cells *in vitro*. (A) Cytotoxicity of RPAKPAR, GX1, and GXC to SGC-7901 cells was acceptable. (B-D) RPAKPAR and GXC peptides sensitize gastric cancer cells to chemotherapeutics *in vitro*. (B) The effect of co-administration of three kinds of peptides on IC_{50} values of SGC-7901 cells to ADR, VCR, 5FU, and CDDP. (C) The apoptotic rates of SGC-7901 cells treated with ADR (0.4 μg/mL) or 5FU (10 μg/mL), co-administrated with GX1 (1×10^{-6} μmol/mL) or RPAKPAR (1×10^{-6} μmol/mL), respectively, for 24 h, were measured by flow cytometry. (D) The statistical analyses of cell apoptosis of SGC-7901 to ADR and 5FU. *$p<0.05$. GXC, GX1-RPAKPAR.

GXC groups. In addition, the same results were also obtained in MKN-45 and MGC-803 cell lines (Supplementary Fig. 1, only online), indicating that GXC could not only enhance the sensitivity of SGC-7901 cells, but also other gastric cell lines to chemotherapeutics. Compared with MKN-45 and MGC-803 cell lines, GXC seemed to exert greater effects in SGC-7901 cells. Hence, we chose the SGC-7901 cell line as the cell model for the following trials.

Meanwhile, cell apoptosis assay showed that the apoptosis rates for ADR and 5FU in RPAKPAR and GXC groups were respectively higher than that in ADR and 5FU alone, suggesting that both GXC and RPAKPAR could signally sensitized SGC-7901 cells to chemotherapeutic agents *in vitro*. Moreover, no significant differences in apoptosis rate for ADR and 5FU were found between RPAKPAR and GXC groups (Fig. 1C and D).

GXC peptide increases intracellular concentrations of ADR in SGC-7901 cells

To further prove that RPAKPAR and GXC could increase the penetration of ADR into SGC-7901 cells, intracellular concentration of ADR was measured using high throughput screening assay (Fig. 2A). The results indicated that co-administrated with peptides (GXC/RPAKPAR) leads to significantly higher intracellular ADR concentrations (red fluorescence) than that in GX1 group or ADR alone (Fig. 2B). These data showed that GXC and RPAKPAR could sensitize cells to anticancer drugs through increasing intracellular drug concentrations *in vitro*.

GXC peptide specifically binds to GC tissue

The specificity of GXC peptide to GC tissue was validated through *ex vivo* fluorescence imaging. To this end, FITC was chemically conjugated to GXC peptide. We further validated the specificity of FITC-GXC to solid tumor with orthotopic SGC-7901 xenograft models, and 40 μmol/kg of FITC-GXC was intravenously injected alone. Solid tumor and other normal organs, including liver, lung, spleen, kidney and heart, were dissected and subjected to *ex vivo* fluorescence imaging at 2 h after injection (Fig. 3A). As shown in Fig. 3B, the fluorescence intensity of FITC in solid tumor was significantly higher than that in other organs, demonstrating the high specificity of GXC to GC *in vivo*.

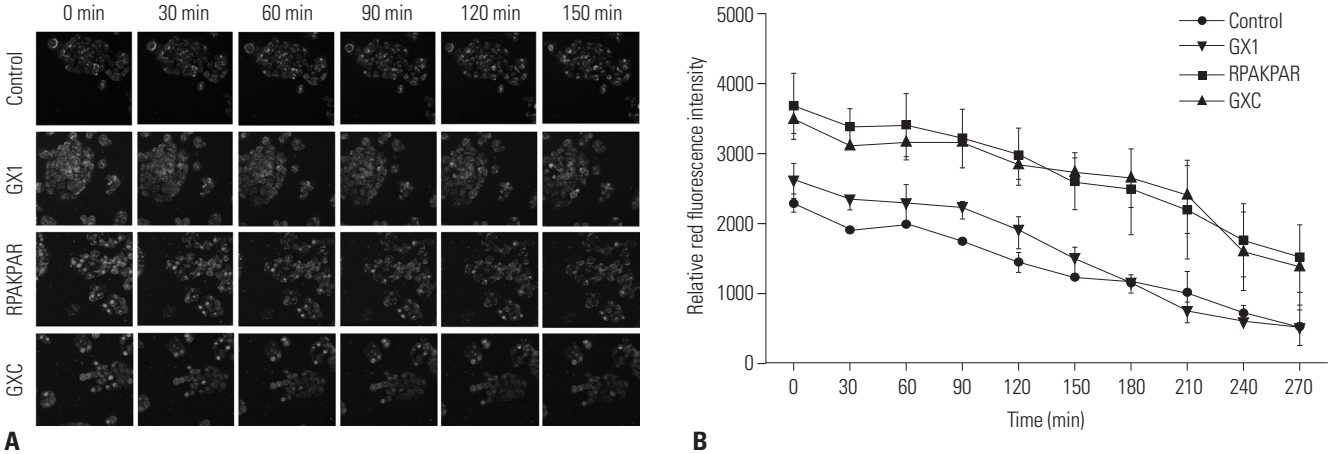

Fig. 2. GXC increased intracellular ADR concentrations *in vitro*. (A) SGC-7901 cells were treated with ADR (10 μg/mL) combined with GX1, RPAKPAR, and GXC (1×10^{-6} μmol/mL) respectively for 1 h. Green fluorescence indicates the cytoplasmic membrane stained with DIO, and red fluorescence indicates ADR. (B) The average red fluorescence intensity at different times. GXC, GX1-RPAKPAR.

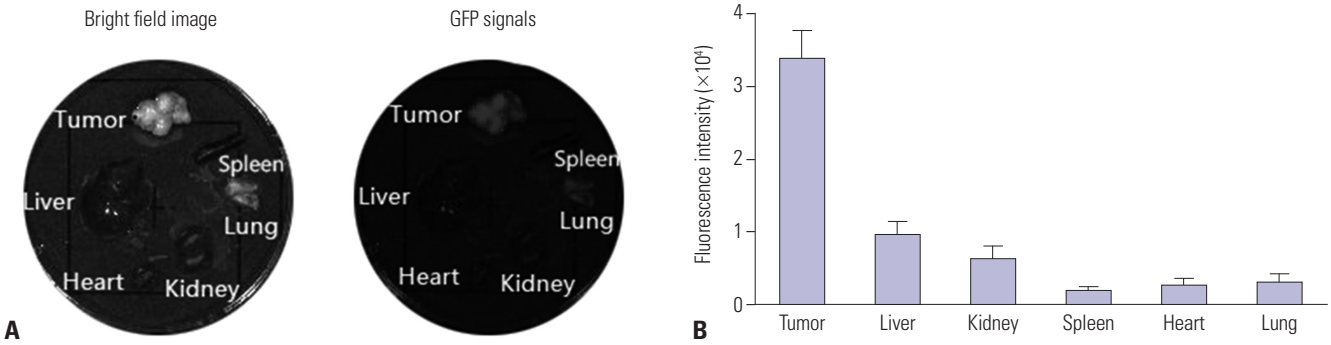

Fig. 3. GXC specifically accumulated in gastric cancer in nude mice bearing tumor xenografts derived from SGC-7901 cells. (A) *Ex vivo* imaging of tumor and normal tissues of FITC-GXC (40 μmol/kg) uptake at 2 h after euthanizing the mice. (B) Fluorescence intensity ratio of tumor and normal tissues were quantified using regions of interest analysis ($p<0.01$). GXC, GX1-RPAKPAR; GFP, green fluorescent protein.

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Co-administration with GXC enhances the efficacy of ADR *in vivo*

To prove the effect of GXC on drug delivery and efficacy, we administered the GXC peptide as a combination therapy with ADR to nude mice bearing tumor xenografts derived from SGC-7901 cells, which were transfected with GFP. Also, we monitored the mass of tumors by detecting the fluorescence intensity of GFP expressed by tumor cells (Fig. 4A). Hence, we detected the delivery efficiency of this combination therapy by comparing it to ADR alone. Mice were intravenously injected with different drugs every other day, and after treating for 24 days, tumors were dissected and collected for weighting. Combining ADR (1 mg/kg) with GXC (4 μmol/kg) resulted in lower fluorescence intensity of subcutaneous tumor than that in either the ADR+RPAKPAR or ADR group, which was in agreement with tumor weight analysis (Fig. 4B and C). Although both the fluorescence intensity and tumor weight of subcutaneous tumor in the ADR+RPAKPAR group was statistically lower than that in the ADR group; however, the body weight shift in the ADR+RPAKPAR group was also lower than that in the ADR group, suggesting that combining ADR with RPAKPAR could enhance both antitumor effects and side effects (Fig. 4D). On the other hand, no statistical significance in body weight shift was found between the GXC+ADR group and ADR alone, suggesting that combination therapy was significantly more effective than ADR+RPAKPAR and ADR alone in inhibiting tumor growth without increasing side effects.

DISCUSSION

There are many ways to penetrate cell membrane and vascular wall barriers. Several cellular and microbial proteins are capable of penetrating from outside the cell into cytoplasm, including homeodomain transcription factors (e.g., HIV-1 transactivator and Tat protein), the Antennapedia, and the herpes simplex virus-1 protein VP22.[9,10,13,14] Short cationic cell-penetrating peptides (CPPs) derived from these proteins retain their ability to internalize into cells and carry along cargo, including proteins, nucleic acids, and nanoparticles. However, these CPPs are not selective to almost all types of cells.[15] Recently, several tumor-specific CPPs have also been developed, such as Pep42 (CTVALPGGYVRVC) for melanomas, NRG (Asn-Gln-Arg) and cRGD [Arg-Gly-Asp-D-Phe-(NMeVal)] for angiogenic blood vessels, and iRGD (CRGDK/RGPD/EC) for various tumors (e.g., prostate cancer, breast cancer, and pan-

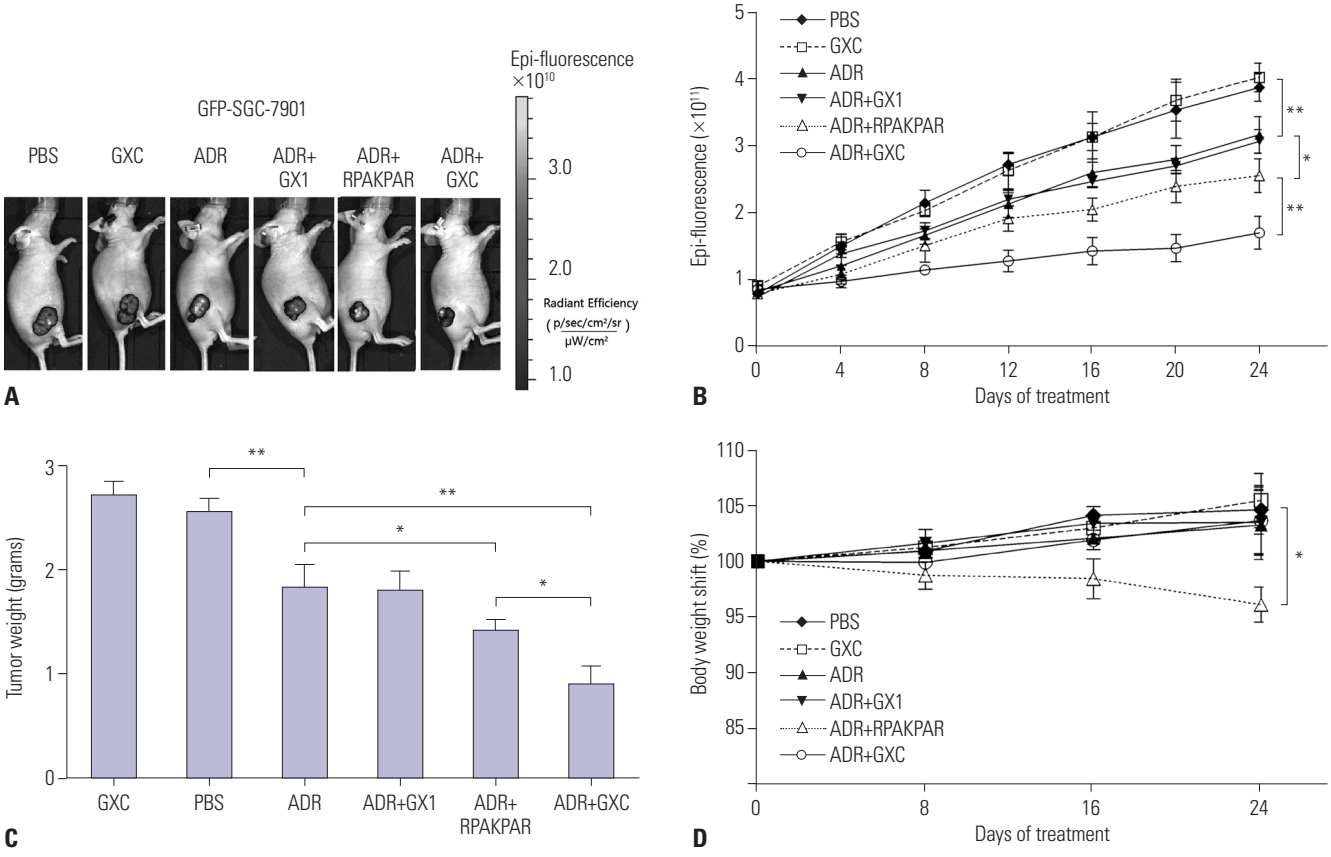

Fig. 4. Co-administration with GXC enhanced the anti-tumor effect of free ADR in nude mice bearing tumor xenografts. (A) Fluorescence imaging of GFP-SGC-7901 tumor models every 4 days. (B) Fluorescence intensity ratio of tumors was quantified by region of interest analysis. (C) The statistical analyses of tumor weight collected after treating for 24 days. (D) The statistical analyses of mice body weight shift. *p<0.05; **p<0.01. GXC, GX1-RPAKPAR; GFP, green fluorescent protein.

creatic adenocarcinomas).[4,16-21] Peptide iRGD could specifically bind to αv integrin in tumor tissue, and then cleaved by protease and exposed to a motif (R/K/XXR/K) for binding to NRP-1, which could mediate penetration into tissues and cells.[3] Conjugation to iRGD significantly improved the sensitivity of tumor-imaging agents and enhanced the activity of antitumor drugs.[20] Surprisingly, compared with conjugation to iRGD, the efficiency of chemotherapeutics co-injected with iRGD was deemed higher.[4] Recently, a study to reproduce those results failed to get iRGD to work.[22] However, a single failure to replicate results do not confirm that the initial trial of the 2010 paper[4] was wrong, which has also been validated by at least ten laboratories in the United States, Europe, China, South Korea, and Japan.[23] Above all, many molecules have been developed to enhance the permeability of tumor vasculature. However, none of them have been validated and used in GC treatment.

For GC treatment, the key point is to increase the specific targeting ability of an anticancer drug to tumor tissue so as to decrease unwanted side effects. Many researchers have attempted to conjugate anticancer drugs to some tumor-homing molecules [e.g., GX1-rmhTNFα, GX1-conjugated poly (lactic acid) nanoparticles], and these efforts have resulted in more effective antitumor activity than antitumor drugs alone in GC.[7,21]

In this study, we developed a novel strategy to deliver therapeutic agents into gastric carcinoma parenchyma using a unique tumor-specific tissue-penetrating peptide GXC, synthesized using GX1 and RPAKPAR peptides. GX1 could specifically bind to human GC vessels.[7] CendR peptides could regulate the cell membrane transport system via binding to cell-surface receptor NRP-1.[3] NRP-1 was found to play an important role in cell transport, angiogenesis, and development of the nervous and cardiovascular systems.[3,24] The C-terminal rule sequence of VEGF-A165, semaphorin 3A, and other ligands conjugate to the b1 domain of NRP-1, causing cellular internalization and vascular permeability.[25,26] NRP-1 was also reported to be overexpressed in human tumor vessels and tumor cells, including human GC cells, which might make GXC-induced enhancement in drug delivery more specific to GC.[26,27] Moreover, in some studies mouse/rabbit anti-human NRP-1 antibodies were used in immunofluorescence assay of tumor tissue harvested from nude mice models of human cancers, suggesting that the NRP1 structure in humans is similar to that in mice.[20,27]

In our study, several pieces of data supported that co-administration with GXC could enhance the chemotherapeutic effect of ADR in GC treatment *in vitro* and *in vivo*, which was in agreement with that of 5-FU-induced and ADR-induced cell apoptosis assay and intracellular ADR concentration assay. Our results also indicated that the fluorescence intensity of FITC-GXC accumulated in solid tumors was obviously higher than that in normal organs, conforming the high specificity of GXC to GC tissue *in vivo*. Finally, tumor treatment research demonstrated that co-administrating GXC with ADR as a combine therapy was more effective in inhibiting tumor growth than ADR alone.

We believe that GXC peptide has the following advantages: 1) combination therapy does not require anticancer drug modification, which may change the activity of anticancer drugs; 2) systemic administration of GXC results in a tissue penetration effect that appears to be selective for GC. Conceivably, the combination system can be further improved to enhance the penetration of probes for tumor imaging. Moreover, the pharmacokinetics, efficacy, and specificity of the GXC-mediated delivery need to be further validated.

In conclusion, systemic administration with GXC could sensitize GC cells to chemotherapeutics. Importantly, this therapeutic strategy did not require the drugs to be chemically conjugated to the peptide. The novel tumor-specific tissue-penetrating peptide GXC might provide a new potential therapeutic strategy for the clinical treatment of GC.

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