Cell Death and Survival

Selective Protection of Normal Cells during Chemotherapy by RY4 Peptides

Xiao-Rong Wu, Lihua Liu, Zhi-Fu Zhang, Bing Zhang, Hongzhe Sun, Gerald L. Chan, and Na Li

Abstract

Mitochondrial targeted Szeto-Schiller (SS) peptides have recently gained attention for their antioxidative stress ability; however, the functional variations between normal and cancer cells have not been determined. Here, we report the results of such experiments conducted with a newly designed class of peptide called RY4, which is based on SS peptide sequence characteristics. The RY4 peptide exhibits distinct differences in antioxidative stress response between normal and cancer cells when challenged with chemotherapeutics like the glycolytic inhibitor dichloroacetate (DCA), the platinating agent carboplatin, and the DNA damage inducer doxorubicin. Interestingly, only normal human cells were protected by the RY4 peptide and catalase (CAT) activity was significantly enhanced in normal but not tumor cells when incubated with RY4. Pull-down, coimmunoprecipitation, and LC/MS-MS proteomic analysis demonstrated that RY4 and catalase are capable of forming protein complexes. Finally, in vivo efficacy was evaluated by intraperitoneal administration of RY4 into a lung cancer xenograft model, which revealed significant myocardiocyte protection from doxorubicin-induced cardiotoxicity without diminishing doxorubicin’s tumoricidal effects. Taken together, RY4 offers selective protection to normal cells from chemotherapy-induced toxicity by enhancing the activity of cellular antioxidant enzymes.

Implications: RY4 peptides selectively reduce chemotherapeutic-induced oxidative stress and represent a new class of chemoprotective agents with clinical potential. Mol Cancer Res; 12(10); 1–12. ©2014 AACR.

Introduction

Chemotherapy is still one of the most widely used and effective cancer treatment methods and is both used alone or in combination with surgery, radio-, immuno-, and/or epigenetic therapies to provide patients with cancer an extension in overall survival. However, a big limitation of chemotherapy is its nonspecific toxicity to normal cells during general administration against tumor cells. Many studies have demonstrated that multiple chemotherapy reagents damage normal cells through oxidative stress, for example, doxorubicin is well known for causing oxidative stress-induced cardio and neural toxicity (1); Carboplatin, another widely used chemotherapy compound, has been reported to induce oxidative stress-related ototoxicity in the inferior colliculus (2, 3). Therefore, it is critical to search for strategies to selectively reduce normal cell toxicity while preserving efficacy of chemotherapy regimens. Finding these strategies will require researchers to identify and exploit the subtle differences between normal and cancer cells (4).

Oxidative phosphorylation in mitochondria generates reactive oxygen species (ROS) as a byproduct of ATP production. After converting the superoxide anions into hydrogen peroxide (H2O2) through superoxide dismutase, H2O2 is further converted into H2O and O2 by enzymes such as catalase (CAT) to reduce H2O2 and hydroxyl radical caused damage on many cellular components and even cell death (5). Although antioxidant response enzymes possess a powerful ability to reduce ROS, their activity is regulated and they exhibit functional decline under pathologic conditions (6–8). According to Bowden and colleagues, catalase activity may play an important role during malignant progression and shows decline in established cancers (9). On the other hand, breast cancer cells (MCF-7) show impaired proliferation and migration ability when overexpressing catalase (10). These findings shine light on the possibility that differing selective protection through enzyme variations in normal and cancer cells can be exploited.

Szeto-Schiller (SS) peptides are a class of peptides characterized by their motif of alternating aromatic and basic amino acids (11). This class of peptide usually localizes to the mitochondrial membrane and demonstrates strong antioxidative ability (11, 12). Their antioxidant ability may be attributed to H2O2 scavenging and inhibition of lipid peroxidation. Tyrosine or dimethyltyrosine (Dmt)
residues in SS peptides may scavenge oxyradicals forming relatively unreactive tyrosyl radicals, further followed by radical–radical coupling to give dityrosin, or react with superoxide to form tyrosine hydroperoxide (13). Despite the wealth of information on this peptide class, the interaction of SS peptides with cellular antioxidative stress enzyme, such as catalase, has yet to be investigated. Moreover, to date no published report has compared the antioxidative effects of the SS peptides between normal and cancer cells.

In the current study, we have designed the RY4 peptides based on SS peptide sequence characteristics (H-D-Arg-Tyr-Lys-Tyr-OH) that show the ability to penetrate the cell membrane and localize to the mitochondria. RY4 peptides rescue normal cells but not cancer cells against some forms of chemotherapy [dichloroacetate (DCA), carboplatin, and doxorubicin]-induced cellular toxicity. The selective survival effects are mainly achieved by enhancing cellular catalase activity in normal cells. The interaction of RY4 peptide with catalase has been studied by pull-down, coimmunoprecipitation (Co-IP), and proteomics analysis. A H2122 xenograft lung cancer mouse model shows treatment with doxorubicin and RY4 results in less cardiomyocyte toxicity when compared with mice receiving doxorubicin alone. Addition of the RY4 peptide does not inhibit doxorubicin’s antitumor effects. Control peptide, SS20, does not exhibit normal cell rescue, catalase activity enhancement, and mouse cardiomyocyte protection effects. Taken together, RY4 peptide selectively reduces chemotherapy-induced oxidative stress impairments in normal but not tumor cells highlighting its clinical potential as a chemoprotective agent.

Materials and Methods

RY4 peptide and synthesis

RY4 peptide was designed by our group based on SS peptide structure characteristics. RY4, SS20, RY4-FITC and SS20-FITC, RH10, HY10, and His were synthesized by GL biochem Ltd with the sequences shown in Table 1.

Cell line and primary cell culture

WI38, MRC-5, HFL1 (human lung fibroblast cell lines), NCI-H2122 (human non–small cell lung carcinoma cell line), H460 (human lung carcinoma cell line), and Hela (human cervical cancer cell line) were purchased from the American Type Culture Collection and cultured as described in Supplementary Information.

Rat myocardiocytes and rat marrow stromal cells were primary cultures, prepared as the procedures described in Supplementary File.

RY4 treatment and cell viability tests in dichloroacetate, carboplatin, and doxorubicin-treated cells

For DCA and carboplatin experiments, a total of seven cell types, including three human normal lung fibroblasts (WI38, MRC-5, and HFL1), primary cultured rat marrow stromal cells, and three human tumor cell lines (H460, H2122, and Hela), were tested. For doxorubicin experiments, an additional primary cultured cell type, rat myocardial cells, was included. Excluding the groups of untreated cells (control), cells with RY4 0.1 mmol/L and SS20 0.1 mmol/L alone, the experimental designs are as follows.

For experiments using DCA: data were collected after 24 hours of toxin treatment: Cells with 75 mmol/L DCA treatment alone; Cells preincubated by RY4 (0.1, 1, 10, and 100 µmol/L) 4 hours + 75 mmol/L DCA; Cells preincubated by SS20 (0.1, 1, 10, and 100 µmol/L) 4 hours + 75 mmol/L DCA.

For experiments with carboplatin: data were collected after 24 hours of toxin treatment: Cells with 2 mmol/L carboplatin treatment alone; Cells preincubated by RY4 (0.1, 1, 10, and 100 µmol/L) 4 hours + 2 mmol/L carboplatin + RY4 (the same dose as last loading) 4 hours later; Cells preincubated by SS20 (0.1, 1, 10, and 100 µmol/L) 4 hours + 2 mmol/L carboplatin + SS20 (the same dose as last loading) 4 hours later.

For experiments with doxorubicin, data were collected after 48 hours of toxin treatment: Cells with 10 µmol/L doxorubicin treatment alone; Cells preincubated by RY4 (0.1, 1, 10, and 100 µmol/L) 4 hours + 10 µmol/L doxorubicin + RY4 (the same dose as last loading) 16 hours later; Cells preincubated by SS20 (0.1, 1, 10, and 100 µmol/L) 4 hours + 10 µmol/L doxorubicin + SS20 (the same dose as last loading) 16 hours later.

Cell viability was estimated using Trypan blue exclusion tests. Of note, 10 µL trypsinized cells were incubated with

Table 1. Peptide information

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
<th>Molecular weight</th>
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<tr>
<td>RY4</td>
<td>H-D-Arg-Tyr-Lys-Tyr-OH</td>
<td>629</td>
<td>Dissolve the peptides in sterile distilled water and store at 4°C</td>
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<td>SS20</td>
<td>H-Phe-D-Arg-Phe-Lys-NH2</td>
<td>640</td>
<td></td>
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<tr>
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<tr>
<td>FITC-SS20</td>
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<tr>
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</table>
10 μL Trypan blue (0.4%, Fisher Scientific Worldwide Co., Ltd.) for 2 minutes and unstained live cells were counted on a hemocytometer.

Primary cultures of myocardial cells exhibit spontaneous beating capability after 4 days in culture. Six independent fields for doxorubicin, RY4 + doxorubicin, and SS20 + doxorubicin groups were randomly picked to record the number of spontaneous beating cells via microscopy.

Three different passages of each cell line or three sets of independent primary cultures were tested with each toxin group. Experiments were performed in triplicate.

**Mitotracker and MitoSox staining in mitochondria**

To identify RY4 peptide localization, a final concentration of 0.1 mmol/L FITC-labeled RY4 or SS20 (FITC-RY4 or FITC-SS20; Table 1) was incubated with WI38 and Hela cells for 4 hours at 37°C. MitoTracker Red CMXRs (Life technologies) was added to the medium 20 minutes before the cells were washed with 0.1 mol/L PBS and fixed in 4% paraformaldehyde at 4°C for 1 hour. FITC and MitoTracker Red CMXRs signals were captured under Leica SP5 confocal system (Leica). Mitochondria from WI38 and Hela cells which had experienced 4-hour incubation of 0.1 mmol/L FITC-RY4 or FITC-SS20 were extracted following the instruction of Mitochondria Isolation Kit for Cultured Cell (Thermo scientific pierce). Images of fluorescence-labeled mitochondria and phase contrast of all mitochondria in the same field were captured by Olympus IX71 biologic microscope. Fluorescence stained and total mitochondria were counted (five images with ~1,000 mitochondria of each group), and their ratio was calculated.

MitoSox red mitochondrial superoxide indicator (Life Technologies) was able to penetrate and localize in mitochondria to be stimulated to fluoresce red when superoxide is present. Cellular superoxide levels were tested and compared by the indicator as described in Supplementary File.

**Catalase activity assay**

**Catalase activity assay** . CAT activity was measured using a catalase assay kit (Beyotime institute of Biotechnology) that works by detecting red color products generated by the reaction between peroxidase and N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine) arising from excess H2O2 not converted by catalase. In brief, cells were treated at sham, 0.1 mmol/L RY4 or 0.1 mmol/L SS20 for 4 hours. Cellular proteins were extracted as described in Supplementary Information. Proteins were mixed with kit-provided reagents following manufacturer’s instructions. The samples and the internal blanks were analyzed using a plate reader with a 520-nm filter. Enzyme activity was calculated according to standard curve generated by kit-provided reagents to further compare the fold changes among groups. Each cell line was tested from three independent cell passages and tested in triplicate.

**Catalase activity assay under SS peptide treatment.** To explore RY4 ability on catalase activity directly, active proteins of CAT were purchased from Sigma co. Ltd. Of note, 10 U/mL CAT were incubated with RY4 or SS20 peptides at (0, 1, 10, and 100 μmol/L) for 30 minutes. Enzyme activity was calculated using the same catalase activity kits mentioned above, all results were normalized by data of enzyme-free RY4 or SS20 groups.

**Semiquantification RT-PCR of catalase**

mRNA from cells treated with RY4 (0, 0.1, and 0.01 mmol/L) for 4 hours were extracted by TRIzol reagents (Life technologies) according to instructions provided by the manufacturer.

cDNA was generated from total RNA following the protocol of Transcript First-strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd). The primer sequences of catalase (327bp) and GAPDH (531bp) amplification were:

- catalase forward: 5′-CATTCCATCTCACAAGGTGTG-GCC-3′
- catalase reverse: 5′-AGCACGGTAGGGACAGTCAAG-GG-3′
- GAPDH forward: 5′-GGGCGCCTGGTCACCAGGGC-TG-3′
- GAPDH reverse: 5′-GGGGCCATCCACAGCTCTTCTG-3′

The gel band intensity was captured by Bio-Rad universal hood II (Bio-Rad Laboratories Headquarters) and analyzed using densitometry software of ImageJ 1.43u software. Catalase expression rates in different cell lines were normalized by GAPDH intensity (RT-PCR detailed parameters in Supplementary File). Three separate passages of each cell line (WI38, MRC5, HFL1, H460, H2122, and Hela) were used to extract RNA and run RT-PCR.

**Western blotting**

WI38, rat MSCs, rat myocardial cells, H460, H2122, and Hela cells were treated by RY4 peptides at sham, 0.1 and 0.01 mmol/L for 4 hours, washed by 0.1 mol/L PBS once, and scraped down from culture plates. Total protein was extracted as described in Supplementary File. After separation and membrane transfer, membranes were incubated with primary antibodies: rabbit polyclonal against catalase and mouse monoclonal against α-tubulin (Abcam), followed by anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody or anti-mouse HRP-conjugated secondary antibody (Zhongshan Goldenbridge Biotechnology Co., LTD). The results were imaged using Supersignal West Pico Chemiluminescent Substrate according to the manufacturer’s instructions (Pierce Biotechnology Inc.). Protein bands were quantified using ImageJ software, α-tubulin was used as a normalization control.

**Pull down, communoprecipitation, and LS/MS proteomic analysis**

In protein pull down experiments, 600 μg of WI38 cell lysates were incubated with 0.1 mmol/L RH10, HY10, and His (Table 1), respectively (details in Supplementary File).
For the first membrane, anti-mouse HRP-conjugated secondary antibody was used to detect mouse 6× His tag antibody in the system (positive control). For the second membrane, primary antibody of rabbit polyclonal to catalase IgG (Abcam) was incubated then followed by incubation with mouse anti-rabbit light chain (SouthernBiotech). The third one was incubated with rabbit polyclonal to p53 (Santa Cruz Biotechnology, Inc) followed by mouse anti-rabbit light chain (Southern Biotech; control). All primary and secondary antibodies were incubated with membranes at 4°C overnight. Results were developed by SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer’s instructions for the autoradiography.

Co-IP was conducted similar to the pull down experiments with the exception of the first step where 1 mmol/L RH10, HY10, or Histidine (His) was incubated with WI38 cells for 4 hours before protein extraction. Pull down and Co-IP experiments were repeated three times with three passages of each cell, respectively.

For proteomics analysis, WI38 COIP cell proteins by either Histidine (His), RH10, or HY10 were separated on gel. Silver-stained band areas were cut and submitted into high-performance liquid chromatography, then sent into LTQ-Orbitrap-XL (Thermo Scientific) for proteomics analysis. Mass spectrometry (MS) and tandem mass spectrometry (MS-MS) data were analyzed for protein identification using Eksigent software 2.09 (Eksigent Technologies). MS-MS data were searched against NCBI human protein database version of Oct 2010.

Animal experiments and heart impairment detections

All animal experiments were carried out with reference to the guidelines established by the Committee on Animal Care and Usage of Peking University (Beijing, China).

For the H2122 mouse xenograft model, 6 to 8-week-old male BALB/c nude mice were purchased from Beijing HFK Bioscience Co., LTD. A total of 5 x 10^6 NCI-H2122 cells were subcutaneously injected into right flank of each mouse. Tumor sizes were measured starting from day 5. Twelve days after injection, tumor-bearing mice were divided into six groups (9 mice/group) carrying a mean tumor volume of 0.09 cm^3 in each group. For group 1, mice were used as control and given saline injections; group 2 mice were injected intravenously with 6 mg/kg doxorubicin; group 3 and 5 mice were injected intraperitoneally with 25 mg/kg RY4 and 25 mg/kg SS20, respectively; group 4 and 6 mice were injected intraperitoneally with 25 mg/kg RY4 and 25 mg/kg SS20, respectively, four hours before receiving the doxorubicin injection. Injections of RY4/SS20 (groups 3, 4, 5, and 6) and doxorubicin (groups 2, 4, and 6) were given once a week for 4 weeks. The mouse body weights and tumor volumes were measured every 7 day until the completion of the experiments. By day 35, all mice were sacrificed, and tumors and hearts were dissected and weighed. Five hearts of each group were prepared in paraffin for tissue processing. Remaining hearts of each group were divided into half for protein extraction and half for hydroxyproline (Hyp). Tissue sections were cut at 10 μm by cryostat. Sections from each group of mice were stained with Masson’s Trichrome Staining (14).

Hearts for Western blotting tests were manually broken up and then, homogenized on ice, as described in the procedure for protein extraction above. Specific heart proteins were detected by mouse anti-cTnT antibody (Abcam) and labeled with anti-mouse HRP-conjugated secondary antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd). Mouse anti-β-actin (Abcam) HRP antibody was used as loading control. The results were analyzed as previously described. Heart Hyp level was detected according to instructions provided by the manufacturer (Nanjing Jian-Cheng Technology Co., LTD).

Statistical analysis

Both t test and ANOVA statistical analyses were performed by SPSS v. 13.0 for MitoSox staining, cell viability, RT-PCR, Western blotting, catalase activity assays, and animal studies. Histograms were generated by GraphPad prism 5 or Microsoft Excel.

Three passages of each cell line and three sets of independent primary cultures were used for data collection of all in vitro experiments. Nine mice of each group were used for in vivo data collection. P value less than 0.05 was considered statistically significant.

Results

RY4 peptide localization in cells and their antioxidative stress ability

After loading the FITC-conjugated RY4 peptides into culture medium, a FITC signal was detected as early as 0.5 hours, and lasted overnight. To specify FITC-RY4/FITC-SS20 localization, mitotracker, a red fluorescence dye that specifically localizes to mitochondria was added in combination to the culture medium. Leica SP5 confocal microscope captured identical mitochondrial staining patterns in WI38 and Hela cells when FITC-RY4/FITC-SS20 (green) and mitotracker (red) were coincubated (Fig. 1A–L). In RY4-treated cells, FITC signal distributed evenly and widely in WI38 cells (normal cell line; Fig. 1B), whereas in Hela cells (tumor cell line), they were clustered located at limited area of the cells (Fig. 1H). SS20-treated cells exhibited clustered FITC signal in both WI38 and Hela cells (Fig. 1E and K). After mitochondria isolation, the ratio of fluorescence stained mitochondria versus total mitochondria was 42.90% of RY4-WI38, 13.04% of RY4-Hela, 34.03% of SS20-WI38, and 19.39% of SS20-Hela (Supplementary Fig. S1; P < 0.05 or P < 0.01 vs. RY4-WI38).

In addition to peptide localization, RY4’s ability to protect against antioxidative stress was tested in WI38 cells. Three chemotherapy compounds that have been reported to induce cellular oxidative stress were selected to test the hypothesis here: DCA, carboplatin, and doxorubicin. MitoSox staining indicated a decreased amount of superoxide in WI38 cells exposed to RY4 peptide pretreatment.
(Fig. 1O, R, and U), the reduction rate varied from 32% (carboplatin), 40% (doxorubicin) to 45% (DCA), respectively ($P < 0.05$ or $P < 0.01$ vs. toxin alone, data not shown).

SS20 pretreatment did not exhibit superoxide reduction against the chemotherapy compound (Fig. 1P, S, and V). No fluorescence signal was detected in WI38 cells under MitoSox staining (Fig. 1M).

In addition to cellular decreases in superoxide concentration, RY4 peptides rendered protection to normal cells from DCA, carboplatin, and doxorubicin-induced cell death. Interestingly, the peptide-mediated survival function was not observed in tumor cells. Cell viability increased in three human fibroblast (WI38, MRC5, HFL1) cell lines and primary cultured rat marrow stromal cells when preincubated with the RY4 peptide from 0.1 μmol/L to 0.1 mmol/L for 4 hours compared with cells receiving chemotherapy alone (Fig. 2A, C, and E; $P < 0.05$ or $P < 0.01$), whereas no such protective effect was found in three human tumor cell lines (H460, H2122, and Hela cells; Fig. 2B, D, and F; $P < 0.05$ or 0.01). The normal cell selectively protection is not detected in SS20-incubated cells (Fig. 2A, C, and E). Doxorubicin induces observable cardiotoxicity on primary cultured myocardial cells, thus cell viability, MitoSox staining, and numbers of viable spontaneous beating cardiomyocytes were compared among groups.
of RY4/SS20 + doxorubicin and doxorubicin alone group. Of note, 0.01 and 0.1 mmol/L RY4 peptide concentrations increased myocardioyte viability by as much as 65% and 57%, respectively (Fig. 2G; \( P < 0.01 \)), reduced cellular superoxide concentration by 23% (Fig. 2H and I; \( P < 0.01 \)), and increased the number of spontaneously beating cardiomyocytes by 180% (Fig. 2J; \( P < 0.01 \)). SS20 did not exhibit protection effects on myocardioyte. No cellular viability reductions were detected in cells that experienced RY4 or SS20 treatment alone (Fig. 2).

The relation and working pattern of RY4 peptides and catalase
To further explore RY4’s relationship with antioxidative stress enzyme, catalase activity was examined (15, 16). Cell catalase activity was restored to maximal

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**Figure 2.** Cell viability of DCA, carboplatin, and doxorubicin-treated normal and tumor cell lines with or without RY4 and SS20 pretreatments. A and B, cell viability of WI38, MRC5, HFL1, rMSCs, H460, H2122, and Hela in control (complete medium, C), RY4 alone (R), SS20 alone (S), challenged by DCA at 75 mmol/L alone (toxin, T), 0.1 (R1), 1 (R2), 10 (R3), and 100 (R4) \( \mu \)mol/L RY4 peptides + DCA, 0.1 (S1), 1 (S2), 10 (S3), and 100 (S4) \( \mu \)mol/L SS20 peptides + DCA. C and D, cell viability of WI38, MRC5, HFL1, rMSCs, H460, H2122, and Hela in control (C), RY4 alone (R), SS20 alone (S), challenged by carboplatin at 2 mmol/L alone (toxin, T), 0.1 (R1), 1 (R2), 10 (R3), and 100 (R4) \( \mu \)mol/L RY4 peptides + carboplatin, and 0.1 (S1), 1 (S2), 10 (S3), and 100 (S4) \( \mu \)mol/L SS20 peptides + carboplatin. E and F, cell viability of WI38, MRC5, HFL1, rMSCs, H460, H2122, and Hela in control (C), RY4 alone (R), SS20 alone (S), challenged by doxorubicin at 10 \( \mu \)mol/L alone (toxin, T), 0.1 (R1), 1 (R2), 10 (R3), and 100 (R4) \( \mu \)mol/L RY4 peptides + doxorubicin, and 0.1 (S1), 1 (S2), 10 (S3), and 100 (S4) \( \mu \)mol/L SS20 peptides + doxorubicin. (Continued on the following page.)
function after incubating with 0.1 mmol/L RY4 for 4 hours in normal cells (WI38, MRC5, HFL1, rMSCs, and rMyocardial cells; \( P < 0.05 \)), whereas no significant improvement in enzyme activity was detected in RY4-incubated cancer cells (H2122 and Hela; Fig. 3A).

SS20 peptides did not exhibit cellular catalase activity enhancement in both normal and tumor cell lines (Fig. 3A).

To further explore any direct effects of RY4/SS20 on CAT, active CAT proteins extracted from bovine livers were purchased and prepared at concentrations of 10 U/mL of CAT, similar to the enzyme activity range detected in whole-cell extraction protein. Enzyme groups were incubated with final concentrations of 0, 1, 10, and 100 mmol/L RY4 or SS20 for 30 minutes, respectively. RY4/SS20 alone in solution (i.e., single group without enzymes in the system) was used to normalize. Experiments revealed as much as 1.98-fold increase of CAT activity with RY4 0.1 mmol/L (final concentration) compared with control (i.e., without any RY4), which suggests that RY4, but not SS20, has a direct effect on the activity of catalase (\( P < 0.01 \); Fig. 3B).

RY4 effects on the expressions of catalase were evaluated at the mRNA and protein level next. There was no detectable upregulation of catalase mRNA (in WI38, MRC5, HFL1, H460, H2122, and Hela; Fig. 3C and E), and protein (in WI38, rMSCs, rMyocardial cells, H460, H2122, and Hela; Fig. 3D and F) of catalase when cells were incubated with RY4 for 4 hours, which suggests that RY4 peptides may enhance oxidative defense by potentially increasing catalase activity without upregulating protein synthesis and thus, expression.

To better understand the interaction between RY4 peptides and catalase in cells, histidine (His) tag-conjugated RY4 peptides were used to conduct pull down experiments on WI38 lysates. As shown in Fig. 3G, catalase is pulled down by RH10 and HY10, when the His tag is conjugated at either RY4’s C or N terminus. Further tests were conducted by adding His, RH10, or HY10 peptides into WI38 cells before lysis and extraction.
of whole-cell proteins to detect existing His–protein complexes. Catalase was confirmed in RH10 Co-IP proteins (Fig. 3H). LS/MS proteomics analysis on His, RY4, HY10, and HY10 COIPed proteins confirmed catalase in RH10-binding protein profiles but not in His control (Table 2 and Supplementary Fig. S2).
RY4 peptides rescue H2122 xenograft tumor mouse hearts from doxorubicin-induced toxicity

To corroborate the observed protective effects, RY4 peptides appear to afford normal cells in culture, RY4 peptides were further examined in the H2122 xenograft mouse model. Mice carrying H2122 tumors were divided into six groups as described in Material and Methods. There was body weight reduction in doxorubicin alone, RY4 + doxorubicin, and SS20 + doxorubicin group (Fig. 4A; \(P < 0.05\) or 0.01). H2122 tumor volumes showed growth inhibition from day 21 in doxorubicin alone and RY4 + doxorubicin groups, and decreased to 48% (doxorubicin alone) and 52% (RY4 + doxorubicin) of tumor size compared with control’s by day 35 (Fig. 4B; \(P < 0.05\) or 0.01). Measurement of the dissected tumors from mice in doxorubicin alone group showed a 34% reduction of tumor volume and 27% reduction tumor weight, whereas RY4 + doxorubicin group showed a 36% and 25% reduction in tumor volume and weight, respectively, compared with the control mice (Fig. 4C and D; \(P < 0.05\)). SS20 + doxorubicin group showed both 21% reduction in tumor volume and weight compared with the controls’ (Fig. 4C and D; \(P = 0.05\) and 0.06, respectively). There were no significant changes in tumor size and tumor weight among control, RY4 injection alone, and SS20 injection alone groups (Fig. 4B–D).

Masson’s trichrome staining of mouse heart sections revealed well-arranged myocardiocyte myofibrils which connected each other tightly in control (saline) group, RY4 and SS20 injection alone group mouse hearts (Fig. 5A, C, and E). Mouse hearts of doxorubicin group and SS20 + doxorubicin group exhibited abnormal scar tissue structure with special blue staining compared with control’s (circulated area in Fig. 5B and F). Mouse hearts treated with RY4 + doxorubicin (Fig. 5D) showed well-arranged myofibrils as control group. Western blotting of cTnT, a protein specifically expressed in cardiac muscles (17), in six group mouse hearts yielded results (Fig. 5G) consistent with the Masson’s trichrome staining, that is, a strong cTnT signal in control, RY4 alone, and SS20 injection alone mouse hearts compared with doxorubicin group (Fig. 5H; \(P < 0.05\) or 0.01); RY4 + doxorubicin mouse hearts expressed higher cTnT levels versus doxorubicin group (\(P < 0.05\), which suggests that RY4 peptides may protect myocardiocytes from doxorubicin-induced damages, although RY4 + doxorubicin heart cTnT levels were still lower than the control’s (Fig. 5H; \(P = 0.00\)). Hyp, a predictor of tissue fibrosis, was measured in mouse hearts among six groups. Compared with doxorubicin group, low levels of Hyp were detected in control, RY4 alone, RY4 + doxorubicin and SS20 alone groups (Fig. 5I; \(P < 0.05\) or 0.01).

Discussion

Chemotherapy is one of the most widely used and effective cancer treatment strategies with multiple compounds developed and available for use today. Unfortunately, most of the compounds are also toxic to normal...
cells, which strongly restrict their therapeutic window, that is, the dose and frequency in vivo. Used in this study are carboplatin and doxorubicin, which are reported to cause oxidative stress resulting in ototoxicity in inferior colliculus (carboplatin; refs. 2, 3) or cardiotoxicity in hearts (doxorubicin; ref. 18). Dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor, has been suggested to hold antitumor properties as a function of its ability to inhibit the Warburg effect (19). However, strong neurotoxicity has been reported during DCA clinical trials where patients developed symptomatic peripheral neuropathy (20).

Figure 4. RY4 effect in H2122 xenograft tumor mouse models. A, H2122 tumor mouse body weights within 35 day experiments. B, comparisons of H2122 tumor volume among control (saline), doxorubicin (dox), RY4 25 mg/kg injection alone, RY4 25 mg/kg + doxorubicin, SS20 25 mg/kg injection alone, and SS20 25 mg/kg + doxorubicin groups. *, P < 0.05; **, P < 0.01, doxorubicin versus control group; #, P < 0.05; ##, P < 0.01, RY4 25 mg/kg + doxorubicin versus control group; &, P < 0.05; &&, P < 0.01, SS20 25 mg/kg + doxorubicin versus control group (A); data were reported as mean ± 1/2 SD. C, tumor volume comparisons among six groups after dissection. *, P < 0.05 versus control group, D, tumor weight comparisons among six groups after dissection. *, P < 0.05 versus control group. n = 9 of each group. Data were reported as mean ± 1/2 SD.

Figure 5. Mouse heart comparisons with different treatments. A–F, Masson’s trichrome staining of heart sections in groups of control (saline, A), doxorubicin (dox; B), RY4 25 mg/kg injection alone (C), RY4 25 mg/kg + doxorubicin (D), SS20 25 mg/kg injection alone (E), and SS20 25 mg/kg + doxorubicin (F) groups. The scale bar is 250 μm. G and H, Western blotting of cTnT in mouse hearts and quantification analysis normalized by β-actin. *, P < 0.05; **, P < 0.01 versus doxorubicin group; n = 4. Data were reported as mean ± 1/2 SD. I, Hyp level in mouse heart tissues. *, P < 0.05; **, P < 0.01 versus doxorubicin group. n = 4. Data were reported as mean ± 1/2 SD.
Two of the strategies currently being used to conquer chemotherapy side effects are (i) development of new, less toxic compounds and (ii) explore compounds that increase the therapeutic window by offering selective protection to wild-type cells. The first strategy is no doubt valuable and will continue to be explored as there should always be the endeavor of new and improved drug options; however, this option is time and capital intensive with many patients not able to benefit from the developments in time to be effective (21). The latter strategy is to selectively reduce existing chemotherapy reagent toxicity in normal cells. The current barrier to this approach is an inability to discover drugs that have a large therapeutic window in which normal cells are spared. Among other things, focus has recently been placed on plant extractions or using innocuous small chemicals to achieve the goal (22, 23).

In order to synthesize one SS peptide which exhibits high antioxidative stress ability however with low cost, we developed RY4 peptides. Interestingly, RY4 peptide exhibits selective survival effects on normal cells against chemotherapy-induced cell stress without interfering with its tumor killing effects in vitro and in vivo. Its selective protective effect on normal cells may be achieved by varied peptide distribution pattern between normal and tumor cells (Fig 1B and H); reducing cell oxidative stress challenge through catalase activity enhancements (Fig. 3A and B); by directly neutralizing superoxide via ROS scrubbing activity, or possibly by combinations of the above. RY4 peptides seem to amplify CAT activity when incubated with the purified enzymes in vitro for 30 minutes (Fig. 3B). Moreover, protein immunoprecipitation and proteomics analysis point toward a possible interaction with RY4 and CAT (Fig. 3G and H and Table 2). However, evaluation of CAT expression by RT-PCR and Western blotting suggests no detectable upregulations within 4 hours of RY4 peptide treatment (Fig. 3C–F). Interesting to note is that reductions of mRNA and protein have been noticed in some of the cell lines. This result could suggest that RY4-mediated reduction of superoxide concentration via enzyme activity improvement and/or ROS scrubbing may negatively regulate gene expression. SS20, one early reported SS peptide with less antioxidative stress capability, is used as control peptides (24), which does not exhibit selective survival effects on normal cells as RY4 peptides (Figs. 1 and 2). To better observe RY4 effects, all testing cells are subcultured for at least 48 hours to reach an exponential growth condition before any treatment from RY4/SS20 peptides and/or chemo, which requires a relatively high concentration of RY4/SS20 and chemo though. About 60% to 80% of cell viability reduction is detected by single chemotherapy treatment compared with control (Fig. 2), whereas RY4 could rescue 11% to 35% of cell viability for normal cells but not tumor cells.

Although it is still unclear the exact reasons of RY4 selective protection in normal cells, the results in Fig. 1 (Fig. 1B and H) exhibit varied RY4 distribution between WI38 and Hela cells, which could suggest a functional variation of the peptide. Catalase variation between normal and tumor cells may be another explanation. Previous studies have reported the abnormal catalase expression in cancer cells, for example, reduced catalase activity has been confirmed from 24 individual lung tumors compared with tumor-free lung tissues (25). By targeting tumor cell mitochondrial catalase, tumor progression and metastasis are able to be suppressed (26). On the other hand, it has been reported that certain compound is able to increase catalase activity without mRNA upregulation in cells, which indicates the possibility of activity up-and-down space of CAT in cells (27, 28). Such reports may help provide insight into why the protective effect of RY4 is minimized in cancer cells. Compared with RY4 peptides, SS20s exhibit neither normal cell protection effects, nor catalase activity enhancement, which suggests that catalase may be a crucial enzyme to fulfill RY4 unique function here.

In H2122 xenograft tumor mouse models, 25 mg/kg RY4 peptide pretreatment for 4 hours once a week is based on myocardioxenctomy experimental results (Fig. 2G); moreover, RY4 is stable and shows long clearance time detected by microwave stability tests (data not show here). Doxorubicin, RY4 + doxorubicin, and SS20 + doxorubicin group mice exhibit significant body weight reduction, which may be due to low carry-on of tumor weights. There is a slower tumor growth tendency in RY4 alone group compared with control ones in Fig. 4b (P > 0.05); however not shown in dissection results (Fig. 4C and D). It may due to tumor growth 3D structures and tumor measure limitation in live mice. By the end of animal experiments at day 35, no tumor inhibition blockage is detected by RY4 peptides application in RY4 + doxorubicin group (Fig. 4).

After tissue dissection, less damage is observed in RY4 peptide + doxorubicin treated mouse hearts than doxorubicin-treated ones (Fig. 5B and D). Western blotting results indicate reduced cTnT in doxorubicin treated mouse hearts, whereas cTnT recovery in RY4 + doxorubicin group (Fig. 5G and H), which confirms RY4s protect normal cells in vivo. Furthermore, there are significant hyp level decreases in RY4 + doxorubicin mouse hearts compared with doxorubicin group, which indicates RY4 functions on reducing heart damage and the following fibrosis (Fig. 5I; P < 0.01).

Further studies will focus on RY4 interaction to other antioxidative stress enzymes to better understand its mechanism of action in cells, which may help to expand RY4 selective targeting mechanisms. On the basis of FITC-RY4 localization image, RY4 other localization in cells needs to be explored as well. These studies should be repeated with other reported SS peptides as well. Moreover, it is still a question whether longer time of RY4 incubation may upregulate antioxidative stress enzyme expressions. Taken together, the RY4 peptide’s function in normal cells suggests its potential use in the clinic as a supplement of cancer chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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