Induction of p53 expression and apoptosis by a recombinant dual-target MDM2/MDMX inhibitory protein in wild-type p53 breast cancer cells

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Abstract. The tumor suppressor gene p53 is often inactivated in breast cancer cells due to gene mutation or overexpression of its repressors (such as murine double minute 2 and murine double minute X). Inhibitors of murine double minute 2 (MDM2) and murine double minute X (MDMX) could lead to tumor suppression by restoration of p53 activity and such an approach is a promising strategy for future control of breast cancer. This study aimed to investigate the feasibility of the recombinant MDM2 and MDMX inhibitory protein in control of breast cancer *in vitro*. A cell-permeable dual-target MDM2/MDMX inhibitory protein was expressed in *E. coli* and incubated with p53 wild-type breast cancer cells. The data showed that this recombinant MDM2/MDMX inhibitory protein reduced the viability of MCF-7 and ZR-75-30 breast cancer cell lines and promoted cell cycle arrest and apoptosis by activation and stabilization of the p53 protein. Mechanistically, this MDM2/MDMX inhibitory protein increased the expression of p21, Bax and puma proteins, and inhibitory expression of MDM2 and MDMX proteins. This recombinant protein showed a better *in vitro* effect than that of nutlin-3α, a small molecule MDM2 inhibitor. The data further support the hypothesis that targeting of the p53 gene pathway could effectively control breast cancer.

Introduction

The tumor suppressor gene p53 plays a pivotal role in maintenance of genomic stability, possession of anticancer activity, protection against malignant transformation, induction of apoptosis when DNA damaged cells are beyond repair and inhibition of angiogenesis (1). However, more than 50% of human cancers contain mutated or a deleted p53 gene, while other tumors have had inactivation of the p53 gene (2). An early study showed restoration of endogenous p53 function holds considerable promise in the control of tumor progression (3). In normal homeostasis of cells, p53 levels are kept low through a continuous degradation of p53. In response to cell stress, such as DNA damage induced by ultraviolet irradiation, chemical agents or oxidative stress, the p53 gene becomes activated and the half-life of the p53 protein will be increased, leading to p53 accumulation in stressed cells. Subsequently, the p53 protein will change conformation to function as a transcription regulator to upregulate expression of the numerous downstream genes that are associated with cell cycle arrest, senescence, autophagy and apoptosis (4-7). In contrast, dysfunctional p53 protein loses its transcriptional activity and cannot suppress tumor growth, but rather accelerates tumorigenesis. Thus, investigation of p53 functions and its degradation could help in gaining knowledge how to effectively control cancer development and progression. The p53 protein is regulated by a complex network (8). Murine double minute 2 (MDM2), one of the important downstream genes of the p53 regulatory network, can regulate stability and activity of the p53 protein by binding to the N-terminal part of p53; thus, MDM2 is involved in cell growth inhibition, apoptosis induction and cell cycle control (9). MDM2 and p53 interact and regulate each other through an autoregulatory feedback loop (10). p53 can transcriptionally induce MDM2 expression, whereas MDM2 mediates ubiquitylation of p53, thereby marking p53 for nuclear export and proteasomal degradation. In contrast, murine double minute X (MDMX), homologous to MDM2, was indicated to not to be a p53 target gene and MDMX protein may participate in inhibiting p53 activity by interacting with p53 Box I, but that it lacks intrinsic ubiquitin-ligase activity (11); thus, it is also involved in the autoregulatory feedback loop of MDM2-p53 interaction (12). Therefore, the functions of the MDM2 and MDMX proteins are interdependent, but are not interchangeable in the regulation of p53 protein.

To date, studies on the p53 gene as a target of cancer therapy has made considerable progress, of which restoration of wild-type p53 function in tumor cells (13) has attracted the

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Table I. PCR primers used in this study.

p1:	5'-**GATATC**GGCCAGGTTAGCGT-3' ***Nco*I**
p2:	5'-**GGTACC**CGATGAGCGATAAAATTA-3' ***Eco*RV**
p3:	5'-**GATCC**GTATGGCCGCAAGAAGCGTCGCCAGCGCCGTCGCCG**GAGCT**-3' ***Bam*HI** ***Sac*I**
p4:	5'-**C**CGGCGACGGCGCTGGCGACGCTTCTTGCGGCCATAC**G**-3'
p5:	5'-**GTCCG**CCTCTGAGTTTGACGTTTGAGCATTATTGGGCGCAGTTGACGAGCGAAAA**CG**-3' ***Rsr*II**
p6:	5'-**GACCG**TTTTCGCTCGTCAACTGCGCCCAATAATGCTCAAACGTCAAACTCAGAGG**CG**-3' ***Rsr*II**

greatest attention. Thus, MDM2, a critical regulator of the p53 protein, is an attractive target for the development of novel antitumor agents (14). It has been shown that the function of the p53 protein can be activated by blocking the MDM2-p53 interaction, such as through the use of a small-molecule inhibitor nutlins to block the MDM2-p53 protein interaction (15). The surprising finding that decreasing MDM2 does not appear to increase p53 activity can be explained by recent studies indicating that DNA damage induces MDM2 self-degradation and an MDM2-denpendent degradation of MDMX, especially in MDMX overexpressed cancer cells (16), suggesting that it is essential for the p53 response that integrates the distinct and complementary roles of MDM2 and MDMX in p53 inhibition, and the role of MDM2-mediated degradation itself and MDMX for p53 activation. MDM2 and MDMX antagonists together lead to more potent activation of p53 in tumor cells for induction of tumor cell cycle arrest and apoptosis (17,18). Inactivation of p53 occurs in 15-50% of breast cancer cases (2). Restoration of p53 activity could lead to tumor regression and is therefore considered a useful strategy for treatment of breast cancer. In this study, we synthesized a cell-permeable dual-target MDM2/MDMX inhibitory protein that contains the transactivator (TAT) peptide for transduction across membranes and the scaffold protein (thioredoxin) displaying the MDM2/MDMX inhibitory peptide pDI (protein disulfide isomerase). This protein can bind to MDM2 and MDMX to disrupt their interaction with p53. We then investigated the antitumor activity of this protein in breast cancer cell lines.

Materials and methods

Cell lines and culture. Human breast adenocarcinoma cell line MCF-7 and human breast infiltrating duct carcinoma cell line ZR-75-30 (both with wild-type p53) were obtained from The Medical Research Center of Xi'an Jiaotong University (Xi'an, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% benzylpenicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2. Pancreatin/ethylene diamine tetraacetic acid (EDTA) (0.05%) was used to detach the cells for subculture.

Construction of expression vector and gene expression. We first amplified thioredoxin A (TrxA) to express the scaffold protein using polymerase chain reaction (PCR) with p1/p2 primers (Table I) and pET32a as a template. The 327 bp PCR product was purified and extracted using the Agarose-Gel extraction kit (Omegas, Norcross, GA). After DNA-sequence confirmation, the PCR product was inserted into the pMD18-T vector (Takara, Dalian, China). After amplification and DNA sequence confirmation, the pMD18-T-TrxA was digested with *Nco*I and *Eco*RV and the released fragment was further cloned into the prokaryotic expression vector pET40b (Novagen; Centurion, USA) to yield pET40b-TrxA. TAT sequences were amplified by using PCR with primers p3/p4 and cloned into pET40b-TrxA at *Bam*HI and *Sac*I sites to yield pET40b-TAT-TrxA. PDI was amplified by using PCR with primers p5/p6 and cloned into pET40b-TAT-TrxA at the *Rsr*II site to yield pET40b-TAT-TrxA-pDI.

The pET40b-TAT-TrxA-pDI and the control vector pET40b-TAT-TrxA were transduced into *E. coli* BL21 (DE3) (Novagen) for *in vitro* expression of the dual-target MDM2/MDMX inhibitory protein and the control protein, respectively, according to a previous study (19). Transformed *E. coli* was cultured for 8-12 h in Luria-Bertani (LB) medium containing 50 μg/ml kanamycin, seeded into the previous medium at a 1:50 (v/v) ratio, and then further cultured for 3-5 h at 37˚C. Protein expression was induced by the addition of different concentrations of Isopropyl β-D-1-Thiogalactopyranoside (IPTG) (0.1, 0.4, 0.7 and 1 mM). The time at which IPTG was added to the cultures was designated at 1, 3, 5, 7 and 9 h and the temperatures for induction of protein expression were set to 16, 25 and 37˚C, respectively. Based on the results, the optimal expression conditions were determined.

Protein purification. The *E. coli* culture was centrifuged at 4,000 x g for 20 min at 4˚C; the precipitates were resuspended in a bacterial lysate buffer containing lysozyme and sonicated on ice for 30 cycles (9 sec on, 9 sec off). The sonicated bacterial lysates were centrifuged at 4,000 x g for 10 min at 4˚C and then briefly washed with Buffer A (2 M urea, 500 mM NaCl, 50 mM PBS, pH 7.5). After centrifuging, the pellets were dissolved in Buffer B (8 M urea, 500 mM NaCl, 50 mM PBS, pH 7.5) containing 10 mM imidazole at 4˚C overnight. The next day, the mixture was loaded onto a pre-equilibrated Ni-NTA column for purification of newly synthesized protein. The column was washed three times with Buffer B containing 20 mM imidazole and eluted with Buffer B containing 100 mM imidazole.

Protein refolding. To remove the urea and refold the protein, the purified fractions of protein contents were pooled and gradually dialyzed in Buffer C (0.1 mM GSH, 0.01 mM GSSG, 500 mM NaCl, 10% glycerin, 400 mM arginine hydrochloride, 50 mM PBS, pH 7.5) with a decreasing concentration of urea followed by PBS buffer with 10% glycerin. Afterwards, the protein concentration was measured according to the Bradford method (20) using bovine serum albumin (BSA) as the standard. After filtration and sterilization, the proteins were stored in 50 mM PBS with 10% glycerin at -80˚C until use (within 6 months).

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (21) to measure whether the recombinant dual-target MDM2/MDMX inhibitory protein could inhibit interaction of MDM2 or MDMX with the p53 protein. Briefly, cell culture plates were coated with 2 μg/ml of p53 active motif peptide diluted in PBS overnight at 4˚C. Next, GST-MDM2-1-150 (GST-MDMX-1-200) (Abnova; Taipei, Taiwan) containing human MDM2 (MDMX), recombinant protein, control protein but did not contain pDI and dimethyl sulfoxide (DMSO) were added to the plates, respectively, and incubated for 1 h. Anti-GST monoclonal antibody (Abcam, Cambridge, MA) was added into the each well and incubated for 1 h at room temperature and subsequently washed with PBS thrice. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Abcam) was added to each well and incubated for 30 min. The optical density (OD) was measured at 450 nm using an ELISA reader.

Co-immunoprecipitation-western blot analysis. GST-MDM2-1-150 (GST-MDMX-1-200), anti-His antibody (Abcam) and recombinant protein or control protein were mixed together and incubated for 2 h at 4˚C. The binding proteins were precipitated by using a co-immunoprecipitation kit (Genmed, Shanghai, China) as described by Momand *et al* (22). The immunoprecipitated proteins were identified by using western blot analysis.

Protein extraction and western blot analysis. To determine the expression of p53, MDM2 and MDMX in breast cancer cells, the protein samples were fractionated by 10% sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA) for western blot analysis. The p53, MDM2 and MDMX proteins were detected using their corresponding primary antibodies at a concentration of 1 μg/ml followed by incubation with HRP-conjugated secondary antibodies (Pierce, Rockford, IL). The protein band was visualized using enhanced chemiluminescence (ECL, Pierce) and exposed to X-ray film.

Cell viability assay. Cell viability was assessed through a thizolyl blue (MTT) colorimetric assay. Briefly, breast cancer cells were seeded onto 96-well culture plates at a density of $2.5x10^3$ cells/well. The recombinant protein, control protein and PBS were added into each well with different gradient concentrations and the cells incubated for 48 h. At the end of experiments, 20 μl of MTT solution (5 mg/ml in 10 mM PBS; Sigma, St. Louis, MO) was added in each well and the cells incubated for 4 h at 37˚C. The culture medium was replaced with 200 μl of DMSO to dissolve the MTT metabolite and the OD measured at 490 nm using a micro-ELISA reader. Cell viability was calculated as the percentage absorbance relative to that of the control cultures.

Flow cytometry assay. Flow cytometry was performed to detect cell cycle distribution and apoptosis. For cell cycle distribution, the cells were stained with propidium iodide (PI). Breast cancer cells were treated with different proteins as indicated above. Cells were collected, washed twice with ice-cold PBS and then fixed in 75% ice-cold ethanol at 4˚C overnight. Cells were centrifuged at 600 x g for 5 min and the precipitate incubated with 2 mg/ml RNase A and then stained with 50 μg/ml PI containing 0.1% Triton X-100 and 0.02 mg/ml EDTA at 37˚C for 30 min. The percentage of cells in each stage of the cell cycle was determined by a flow cytometer and calculated by CellQuest software (BD Biosciences, Franklin Lakes, NJ).

To evaluate apoptosis, the cells were stained with Annexin V-FITC (fluoresecein isothiocyanate) and PI. Briefly, breast cancer cells treated with different proteins were collected and washed with PBS. The cells were resuspended with in binding buffer at a concentration of $1x10^6$ cells/ml and stained with 5 μl of Annexin V-FITC and 5 μl of PI and then subjected to flow cytometric analysis.

Statistical analysis. Statistical analysis was performed by using SPSS 16.0 software (SPSS, Chicago, IL). All data are presented as mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA and Student's t-test was used to evaluate the statistical significance of the mean. All statistical tests were two-sided and a p-value ≤ 0.05 was considered statistically significant. In each figure error bars represent standard error of the mean and statistical significance levels are noted as: $^{*}P<0.05$, $^{**}P<0.01$.

Results

Expression and quality control of the recombinant dual-target MDM2 and MDMX protein. After *in vitro* expression and purification this recombinant protein coupled with MDM2 and MDMX, co-immunoprecipitation-western blot analysis was performed to check the quality of the protein. The data showed that this protein was able to be immunoprecipitated by anti-MDM2 and anti-MDMX antibodies, indicating that this protein is functional (Fig. 1A).

Inhibition of MDM2-p53 and MDMX-p53 interaction using this recombinant protein. In order to determine whether the recombinant protein could inhibit MDM2-p53 and MDMX-p53 interaction, an ELISA was performed. The dual-target MDM2/MDMX inhibitory protein strongly disrupted both MDM2 and MDMX interaction with p53, compared to the controls. This protein inhibited interaction of MDM2/MDMX with p53 in a dose-dependent manner (Fig. 1B).

Inhibition of wild-type p53 breast cancer cell proliferation by this recombinant protein. Next, the effects of this recombinant protein on regulation of MCF-7 and ZR-75-30 cell viability were detected by treating them with different concentrations of the dual-target MDM2/MDMX inhibitory protein

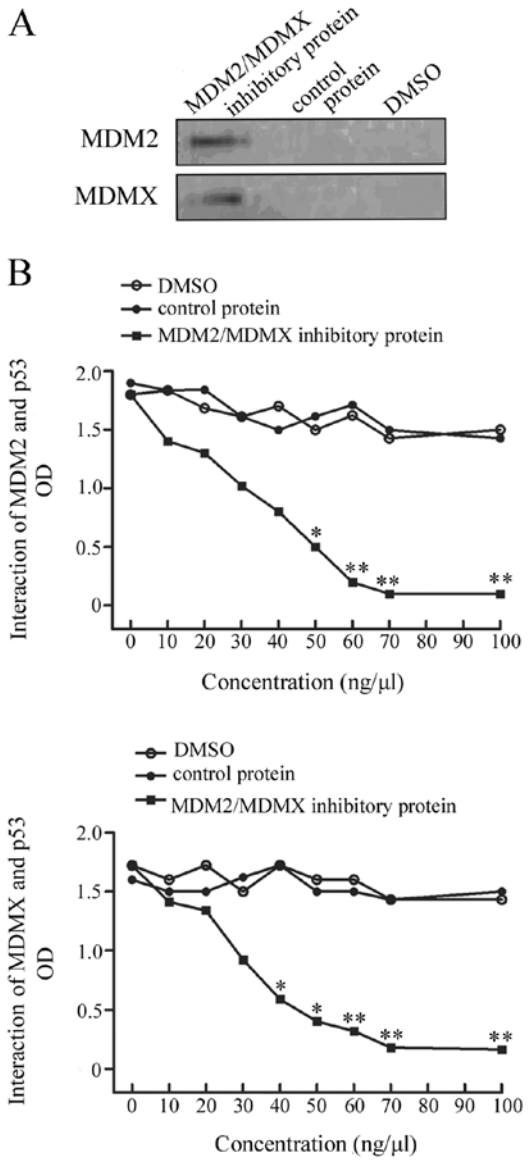

Figure 1. Quality of the recombinant dual-target MDM2/MDMX inhibitory protein. (A) The *in vitro* expressed dual-target MDM2/MDMX inhibitory protein binds to both MDM2 and MDMX protein, which was testified using co-immunoprecipitation-western blot analysis. (B) By the methods of ELISA, the recombinant dual-target MDM2/MDMX inhibitory protein inhibited interaction of both MDM2 and MDMX with p53 protein compared to the controls (DMSO and control protein without pDI). *P<0.05, **P<0.01.

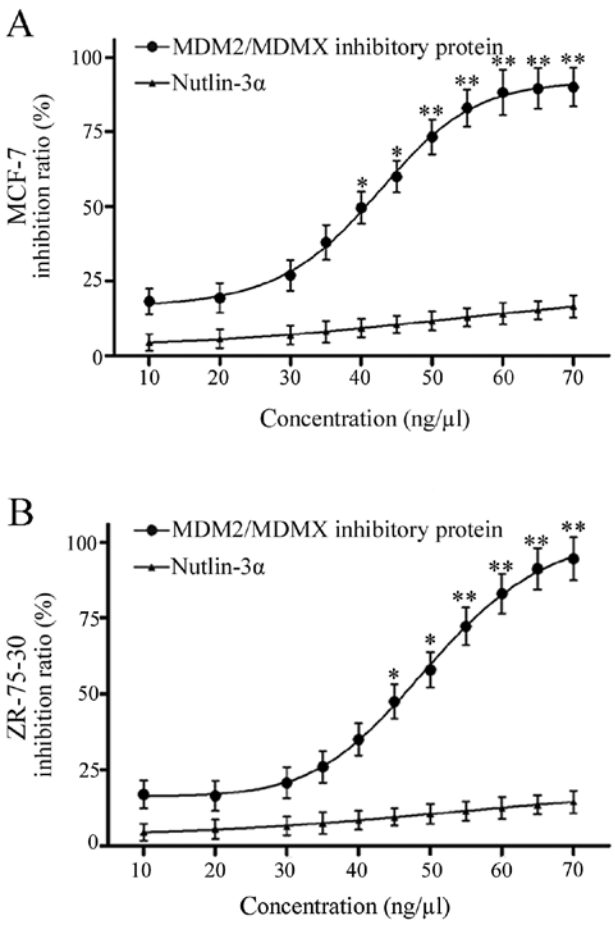

Figure 2. Effects of the recombinant dual-target MDM2/MDMX inhibitory protein on regulation of breast cancer cell viability as determined by an MTT assay. MCF-7 and ZR-7530 breast cancer cells were treated with the recombinant dual-target MDM2/MDMX inhibitory protein or nutlin-3α at the indicated concentration for 48 h prior to performing the MTT assay. *P<0.05, **P<0.01.

or nutlin-3α. MTT assay data showed that this recombinant protein inhibited the viability of MCF-7 cells and ZR-75-30 cells in a dose-dependent manner. The recombinant protein showed a stronger inhibition of cell proliferation than nutlin-3α, a well characterized MDM2 inhibitor (Fig. 2).

Induction of breast cancer cell cycle arrest by the recombinant protein. To assess the cause of the reduced cell viability by the recombinant protein, we analyzed cell cycle distribution using a flow cytometric assay. The percentage of the sub-G1 fraction of breast cancer cell lines MCF-7 and ZR-75-30 treated with this protein was obviously increased (72.49±0.90% and 76.43±2.07%, respectively). However, nutlin-3α treatment only induced a slight increase in the sub-G1 fraction in both MCF-7 cells (51.58±1.58%) and ZR-75-30 cells (60.53±0.64%) compared to the untreated MCF-7 and ZR-75-30 cells (49.64±1.42% and 48.23±0.64%, respectively) (P<0.05, Fig. 3A).

Induction of apoptosis in breast cancer cells by the recombinant protein. Increased sub-G1 phase of the cell cycle may indicate induced cell apoptosis; thus, we performed a flow cytometry/Annexin V-PI assay to detect the level of apoptosis. Treatment of MCF-7 cells with the dual-target MDM2/MDMX inhibitory protein, nutlin-3α or PBS had differential effects on apoptosis, resulting in 35.27±0.54, 10.34±1.13 and 7.41±0.83% of apoptotic cells, respectively. This recombinant protein caused a significant increase in the percentage of apoptotic cells compared to that of PBS-control or nutlin-3α-treated cells. The same treatment of ZR-75-30 cells resulted in 49.69±1.13, 18.92±0.64 and 9.65±0.73% of apoptotic cells, respectively. The recombinant protein was more effective than nutlin-3α treatment or PBS (P<0.05, Fig. 3B).

Inhibition of MDM2 and MDMX expression and activation and stabilization of p53 protein in breast cancer cells by the

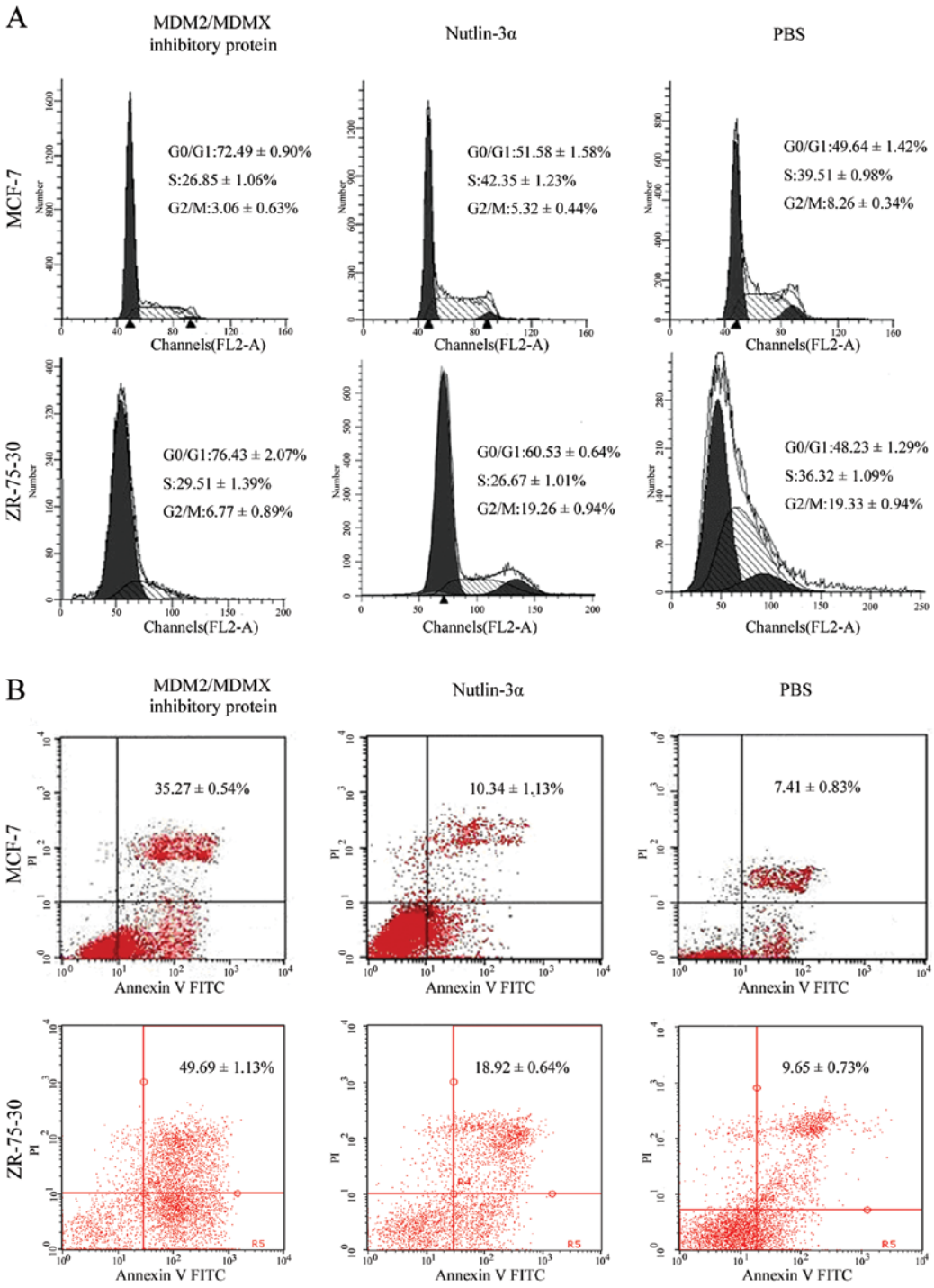

Figure 3. Effects of the recombinant dual-target MDM2/MDMX inhibitory protein on regulation of breast cancer cell cycle arrest and apoptosis. Both MCF-7 cells and ZR-7530 cells treated with the recombinant dual-target MDM2/MDMX inhibitory protein for 24 h induced more cell arrest at G0/G1 phase and apoptosis than the cells treated with nutlin-3α and PBS as a control.

recombinant protein. We demonstrated the usefulness of the dual target MDM2/MDMX inhibitory protein in regulation of breast cancer cell viability, cell cycle arrest and apoptosis. Next, we determined whether the dual target MDM2/MDMX inhibitory protein was able to inhibit MDM2 and MDMX expression and activate and stabilize p53 protein in breast cancer cells. Western blot analysis showed that the level of p53 expression was dramatically increased in MCF-7 cells treated with the recombinant protein compared to cells treated with nutlin-3α. ZR-75-30 cells also showed similar results. However, there was a sharp decrease in MDM2 expression and in MDMX level in MCF-7 and ZR-75-30 cells cultured with the recombinant protein compared to that of cells treated with nutlin-3α (Fig. 4A).

Induction of p21, Bax and puma expression in breast cancer cells by the recombinant protein. The molecular events after p53 gene activation were assessed by detection of cell

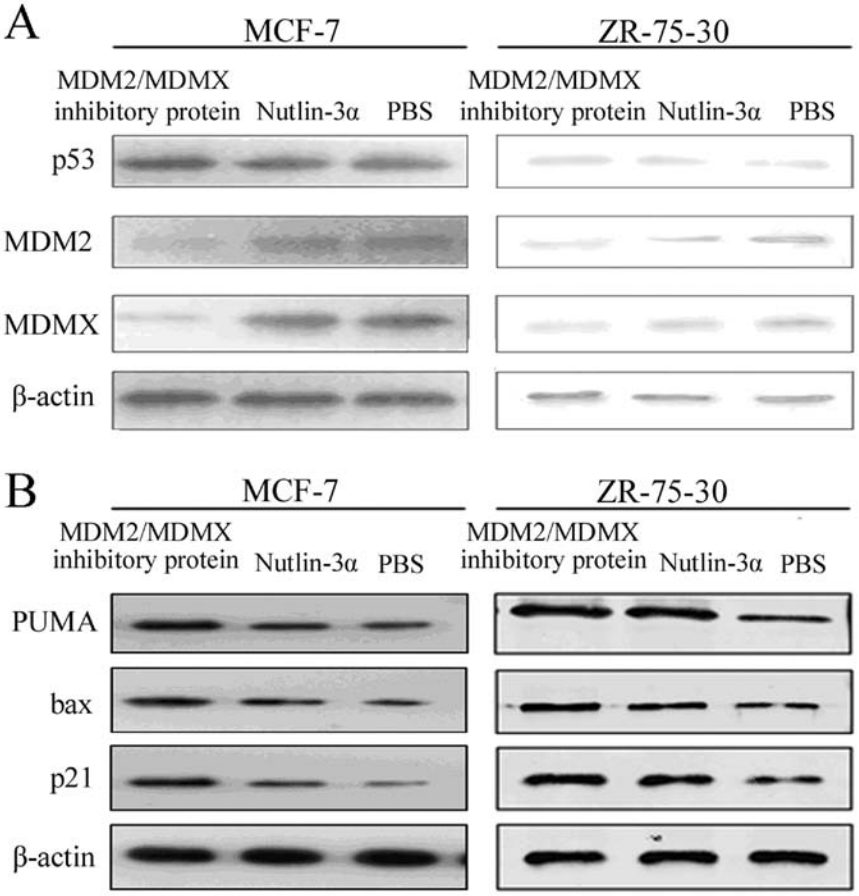

Figure 4. Effects of the recombinant dual-target MDM2/MDMX inhibitory protein on regulation of gene expression in breast cancer cells. MCF-7 and ZR-7530 cells were treated with the recombinant dual-target MDM2/MDMX inhibitory protein, nutlin-3α or PBS for 48 h and subjected to western blot analysis of gene expression.

apoptosis-regulatory proteins (Bax and puma) and the cell cycle inhibitory protein p21 using western blot analysis. In the wild-type p53 breast cancer cell lines, a marked increase in p21 expression was observed in the cells treated with the dual-target MDM2/MDMX inhibitory protein. In contrast to the inhibitor of MDM2 and MDMX, as control, nutlin-3α can also slightly change p21 expression (Fig. 4B). Breast cancer cells treated with the recombinant protein resulted in a significant increase in the levels of Bax and puma proteins, whereas such an effect was not observed in the nutlin-3α-treated tumor cells.

Discussion

In the current study, we determined the feasibility of a dual-target MDM2/MDMX inhibitory protein to control breast cancer by activation and stabilization of p53 protein *in vitro*. Our data demonstrated that i) the dual-target MDM2/MDMX inhibitory protein expressed in *E. coli* is functional, ii) treatment with the dual-target MDM2/MDMX inhibitory protein suppressed the viability of wild-type p53 breast cancer cells and induced cell cycle arrest and apoptosis of tumor cells, which was much more effective than that of the MDM2 inhibitor nutlin-3α, iii) the dual-target MDM2/MDMX inhibitory protein was able to inhibit MDM2 and MDMX expression and activate and stabilize the p53 protein, and iv) the dual-target MDM2/MDMX inhibitory protein increased expression of p21, Bax and puma proteins. Thus, inhibition of MDM2/MDMX proteins could be useful to activate and stabilize p53 protein for restoration of p53 tumor suppressor functions in breast cancer or other wild-type p53 silenced tumors.

Indeed, the ubiquitously expressed tumor suppressor p53 is a multi-functional protein that regulates cellular stress responses such as cell cycle arrest, apoptosis and cell senescence (23). Thus, the aim to restore p53 activity in human cancer has helped in developing a number of antitumor therapies in preclinical and clinical trials (24). Early studies utilized a gene therapy approach to deliver wild-type p53 cDNA into lung and head and neck cancer patients, which showed p53 antitumor activity (25-27). However, due to the toxicity and side-effects of the gene delivery vector or adenovirus, such an approach was disregarded. However, small molecules have been engineered to restore expression of wild-type p53 and the target-specific measure is more desirable (28). Numerous proteins have been described to regulate p53 pathway. Among these, MDM2 and MDMX stand out because of their importance. In the early stage, it was found that the MDM2 or MDMX-deficient mice died in the uterus, but these deficiencies were viable in a p53-deficient background, which indicating MDM2 and MDMX are not redundant p53 inhibitors.

In the current study, we regarded MDM2 and MDMX as the therapeutic targets in order to achieve full activation of p53. We designed and constructed a prokaryotic expression vector to carry a dual-target MDM2/MDMX inhibitory protein; the recombinant protein was expressed in *E. coli* and was found to be functional. Our *in vitro* data showed that this dual-target MDM2/MDMX inhibitory protein possessed a higher tumor inhibition rate, caused a larger increase in the sub-G1 fraction of cells, and promoted more p53 wild-type breast cancer cell apoptosis compared to nutlin-3α. These data indicate that the dual-target MDM2/MDMX inhibitory protein can re-activate p53 for its antitumor activity and is much more effective than the single MDM2 inhibitor nutlin-3α in antitumor activity. Our results confirmed data from previous studies that the dual-target MDM2/MDMX inhibitory protein also had antitumor activity in lung cancer, colon cancer and retinoblastoma cells (18,29). However, the reason for less effectiveness of MDM2-p53 inhibitors in nutlin-3α treated tumor cells may be because they failed to activate p53 especially in cells overexpressing MDMX (14,30,31), which further confirmed that MDMX plays a pivotal role inactivation and stabilization of p53 in cancer cells (32,33).

Furthermore, our current study further investigated the downstream gene of the p53 protein. Treatment of breast cancer cells with the dual-target MDM2/MDMX inhibitory protein significantly induced expression of the p53 protein, but downregulated MDM2 and MDMX expression compared to nutlin-3α treatment. Attributed to pDI, this recombinant protein was designed to downregulate MDM2 and MDMX proteins through binding to these proteins and disrupting their interaction with p53, which is in turn to upregulate p53 protein by increasing the half-life of p53 protein due to MDM2, the downstream gene of p53, can promote degradation of p53 via an autoregulatory feedback loop (34,35). MDMX as another important downstream gene of the p53 regulatory network, it shows little ubiquitylation activity towards p53 but enhanced activity of MDM2 (7) by an autoregulatory feedback loop of MDM2-p53 interaction (12).

Indeed, the dual-target MDM2/MDMX inhibitory protein was able to increase levels of p21, Bax and puma proteins, confirming the activity of p53 protein in the cells. P21 is the p53-regulated protein to control cell cycle progression and induce cell cycle arrest when overexpressed, while Bax and puma are the p53-targeted pro-apoptotic genes involved in cancer cell apoptosis. Normally, p53 regulates p21 expression to coordinate cell cycle G0/G1 phase checkpoint. High expression of both p21 and p53 is one of the reasons why the cell cycle arrests in G0/G1 phase in breast cancer cells treated with inhibitor of MDM2 and MDMX. Thus, expression of p21, puma and Bax protein was the effect of p53 activation and the latter was through suppression of MDM2 and MDMX by the dual-target MDM2/MDMX inhibitory protein in wild-type p53 breast cancer cells.

Further studies will evaluate the feasibility of this approach in a clinical trial. The recent studies estimated that the MDM2 and MDMX antagonists could be used in the treatment of 2-3 millions patients diagnosed with cancer per year, which could provide a strong incentive for the search for p53-based anticancer strategies, but the potential toxicity of such inhibitory proteins to normal cells remains to be determined. Moreover, potential addiction of tumor cells to overexpressed MDM2 or MDMX may make them uniquely vulnerable to p53 activating agents. In addition, combination with a low and non-toxic concentration of standard cytotoxic chemotherapeutic agents will produce favorable therapeutic indices. Clearly, the ability to obtain diverse p53-activating strategies opens up exciting opportunities for development and implementation of new therapeutic strategies for the large population of cancer patients. However, this study is a proof-of-principle for dual targeting of MDM2/MDMX to activate p53 protein antitumor activity. To overcome the bottleneck of this recombinant protein, a better strategy or approach is needed to generate a peptide with more stability, longer half-life and no immunogenicity to be used in patients. Thus, there is a long way ahead before the current knowledge can be applied for breast cancer therapeutics in the clinic.

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