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RESEARCH ARTICLE

A novel cell-permeable RDP-p53 fusion protein for specific inhibition on the growth of cancerous neural cells

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Abstract

Objective: There is 25–35% mutation rate of p53 in cancerous neural cells and this rate reaches 70–76% in glioma cell line. Complement of wild-type p53 has become a potential strategy for protein therapy of cancerous neural cells. Here we investigated the feasibility of a novel RDP-p53 fusion protein for anti-proliferation of cancerous neural cell and the possible mechanism, which would provide an effective approach for targeted delivery of p53 protein to treat cancerous neural cells.

Methods: The RDP-p53 fusion proteins are expressed in *Escherichia coli*, and they are labeled with FITC and rhodamine B by chemical modification. The fluorescence-labeled proteins are added to human hepatocellular carcinoma cells (HepG-2) and human neuroblastoma cells (SH-SY5Y) in order to investigate the possibility of RDP enhancing the cell uptake efficiency into neural cells as a cell-permeable carrier. The inhibitory effect of RDP-p53 on SH-SY5Y and human glioma cells (U251) was evaluated by MTT assay. Moreover, the anti-proliferation mechanism of RDP-p53 was determined by Apoptosis and Necrosis Assay Kit and flow cytometric analysis.

Results: The results showed that RDP-p53 could enter SH-SY5Y cells with high efficiency and selectively inhibit the growth of cancerous neural cells, including SH-SY5Y and U251. Also, cell apoptosis pathway and cell-cycle arrest at the G2/M phase were associated with the inhibition mechanism of RDP-p53 according to the data of flow cytometric analysis.

Conclusions: RDP-p53 could be a novel antitumor candidate for targeting treatment of cancerous neural cells.

Keywords

Cancerous neural cells, cell apoptosis, cell cycle, cell uptake, RDP-p53

History

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Introduction

Brain cancer remains a serious threat worldwide. The 5-year survival rate of patients with brain cancer is only 33.3% and even lower in malignant glioma (Anvari et al., 2014). Although surgical resection combined with radiotherapy and chemotherapy is effective temporarily, it is difficult to completely remove the tumor due to the infiltrate growth of cancers, as well as lost brain function that may result from injuries after resection (Pardridge, 2012; Singh & Subudhi, 2014). Non-invasive treatment to improve the survival of brain cancer-bearing organisms is a promising approach, but unfortunately, most chemotherapies fail due to the poor blood–brain barrier (BBB) penetration of drugs (Du et al., 2009). Therefore, it is clear that there is an urgent need for effective brain cancer delivery with highly cell penetration.

Although cell-penetrating peptides (CPPs) providing an effective approach to deliver cargoes into cells (Todorova, 2011; Li & Tsui, 2014) benefit from their transport ability because of their good properties of biodegradability, biocompatibility, low toxicity, and ease of synthesis (Heitz et al., 2009), the application of CPPs is limited due to their several drawbacks, including lack of cell specificity and low efficiency of BBB penetration. To address these problems, our group have recently developed a novel CPPs (named RDP), derived from rabies virus glycoprotein, to targeting-deliver proteins into brain cells following the systemic administration (Fu et al., 2012).

As a significant tumor suppressor, p53 has attracted numerous attentions for developing as an anticancer drug (Lu, 2005). Wild-type p53 plays a crucial role in the regulation of cell apoptosis, induction of cell-cycle arrest, recognition and repair of DNA damage, inhibition of cell malignant proliferation, and cell transformation in the cytoplasm and nucleus (Green & Kroemer, 2009; Yoshida & Miki, 2010; Goh et al., 2011). Deficiency of wild-type p53 could lead to the occurrence of tumors due to the genetic mutation or deletion (Boggs & Reisman, 2007), then significant disorder in cell growth, apoptosis, and DNA repair appears in the cells. Therefore, approaches that are able to recover p53 function in tumor cells are supposed to be developed as anticancer therapy. However, highly efficient carrier should be used in order to improve the low cell permeability of p53.

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In this study, we used a fusion protein RDP-p53 to investigate the feasibility of RDP as a carrier to specifically deliver p53 protein into the cancerous neural cells, and examine the biological function of RDP-p53. Additionally, we suggested the possible mechanism of anti-proliferation effect of the protein in cancer cells. The study will provide a simple and effective approach for the targeted delivery of p53 protein to treat cancerous neural cells.

Materials and methods

Expression of fusion protein

The plasmids pET28a/RDP-p53 and pET28a/p53 containing wild-type p53 gene were constructed by our laboratory (Zhao et al., 2014). To obtain the p53 and RDP-p53 protein, the bacteria containing the plasmids were cultured in the LB medium at 37 °C shaking incubator (180 rpm) for overnight. After incubation, the bacteria solutions were reactivated to an OD600 of 0.5–0.7, and then induced by IPTG with a final concentration of 1 mmol/l to express the fusion proteins at 25 °C. After incubated for 6 h, the cells were collected by centrifugation with 8000 rpm for 10 min at 4 °C and resuspended in binding buffer and then sonicated. The proteins were purified by Ni-NTA resin column (Amersham Pharmacia Co., Piscataway, NJ) and then analyzed by SDS-PAGE.

Preparation of fluorescence-labeled proteins

In order to prepare FITC and rhodamine B isothiocyanate (Rho B) labeled p53 and RDP-p53, the proteins and FITC/Rho B were dissolved in 0.5 mol/l carbonate buffered saline (CBS; pH 9.5) and mixed 30 min in a refrigerator. Subsequently, the solutions of FITC/Rho B were dropped in the protein solutions in a slow speed and reacted 5 h in the dark in a refrigerator. Meantime, 0.1 mol/l sodium carbonate acetate solutions were used to keep the pH of reaction mixtures in 9.0–9.5. After reaction, free fluorescein was dialysed by PBS at 4 °C. The final conjugates were stored in the dark at 4 °C for further use or freezed at −20 °C for storage purpose.

Cell culture

Human hepatocellular carcinoma cells (HepG-2) and human neuroblastoma cells (SH-SY5Y) were stored in our laboratory. Human glioblastoma cells (U251) were a gift from Miss Yu who is from the School of Pharmaceutical Sciences, Southwest University (Chongqing, China). HepG-2 and U251 were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. SH-SY5Y cells were grown in DMEM/F12 supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml. All media were obtained from HyClone (Logan, UT). Both cell lines were maintained in humidified 5% CO_2, and incubated at 37 °C in an incubator (ESCO, Timur, Indonesia).

Fluorescence analysis

HepG-2 and SH-SY5Y cells were cultured in a 35-mm cell culture plate, incubated for 24 h, and observed its morphology and cell confluence under the microscope. The fluorescence-labeled proteins were added to HepG-2 and SH-SY5Y cells in the presence of 2 ml DMEM or DMEM/F12 complete cell media for 3 h. After being washed three times with PBS, cells were incubated with DAPI (Beyotime, Jiangsu, China) for 30 min at a final concentration of 5 μg/ml, and then washed with PBS three times again. Cells were observed under the confocal microscope (ZEISS, Oberkochen, Germany), and the images were taken with an attached camera.

Cell apoptosis and necrosis assay

The cell apoptosis and the necrosis were detected by using Apoptosis and Necrosis Assay Kit (Beyotime, Jiangsu, China) according to the protocols of the manufacture. SH-SY5Y cells were cultured in 24-well plates at a certain concentration. Subsequently, proteins were added into the cells for a period of time, and then the detection reagents were mixed in the cells after observing the morphology under the microscope. Cells were washed three times with 500 μl PBS and photographed the fluorescence under the fluorescent microscope (Olympus, Tokyo, Japan).

Flow cytometric analysis of apoptosis and necrosis

SH-SY5Y cells and U251 cells were planted into 6-well culture plates and grown to 80% confluence. PBS, p53, and RDP-p53 were added to the cell culture plate at a final concentration of 100 μg/ml. After 24 h incubation, the cells were harvested and measured by flow cytometry (BD FACSVantage, San Jose, CA) in Chongqing Medical University (Chongqing, China).

Cell-cycle analysis

SH-SY5Y cells were treated with PBS, p53, and RDP-p53 for 24 h. After digestion and centrifugation, cells were fixed with 70% methanol at 4 °C and analyzed by flow cytometry.

Cell viability assay

U251 and SH-SY5Y cells were cultured at a density of 5×10^3 cells/well in 96-well plates, and the supernatant was discarded after overnight incubation. Different concentrations of RDP (Shanghai Ji'er Biotech. Co., Shanghai, China), p53, and RDP-p53 were prepared by DMEM and DMEM/F12, and added into the cell media. After incubated for 12 h, 24 h, 48 h, and 72 h, the cells were treated with 5 mg/ml MTT solution (20 μl) and incubated at 37 °C and 5% CO_2 for another 5 h. Subsequently, the media were removed from the cells and 150 μl DMSO was added into each cavity to dissolve the formazan crystals. The absorption at 490 nm was measured on a micro-plate reader (Bio-Rad, Hercules, CA). Each group was repeated three times.

Statistical analysis

All data were given as mean ± SEM. For comparison between three groups, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed. A p value of < 0.05 was considered to be statistically significant.

Results and discussion

RDP-p53 entered SH-SY5Y cells with high efficiency

To examine whether RDP-p53 could increase the cell uptake efficiency in comparison with p53, two different cell lines, HepG-2 and SH-SY5Y, were treated with fluorescence-labeled p53 or RDP-p53 for 3 h in a certain concentration (60 μg/ml). The results showed that a relatively weak signal was detected in the two cell lines following FITC and Rho B-labeled p53 protein treatment; however, the strong fluorescence intensity that located in the cytoplasm and nucleus was observed in SH-SY5Y cells, but not in HepG-2 cells, after treatment with fluorescence-labeled RDP-p53 fusion protein (Figure 1), suggesting that RDP could improve the transport efficiency of exogenous protein p53 into neural cells as a cell-permeable carrier, and last for at least 3 h. RDP is originated from Rabies virus glycoprotein (RVG), a main neuropathic pathogenic factor of rabies virus that could carry other virus into the central nervous system through the way of retrograde axonal transport (Mazarakis et al., 2001). According to the sequence analysis, the important nerve binding regions of RVG were 189–214 and 330–357 amino acid sequences (Tang et al., 2000; Li et al., 2010). RDP, whose partly sequences is derived from the 330–357 amino acid sequences of RVG, has been suggested to have the ability to transport protein and DNA into brain as a non-viral transfer

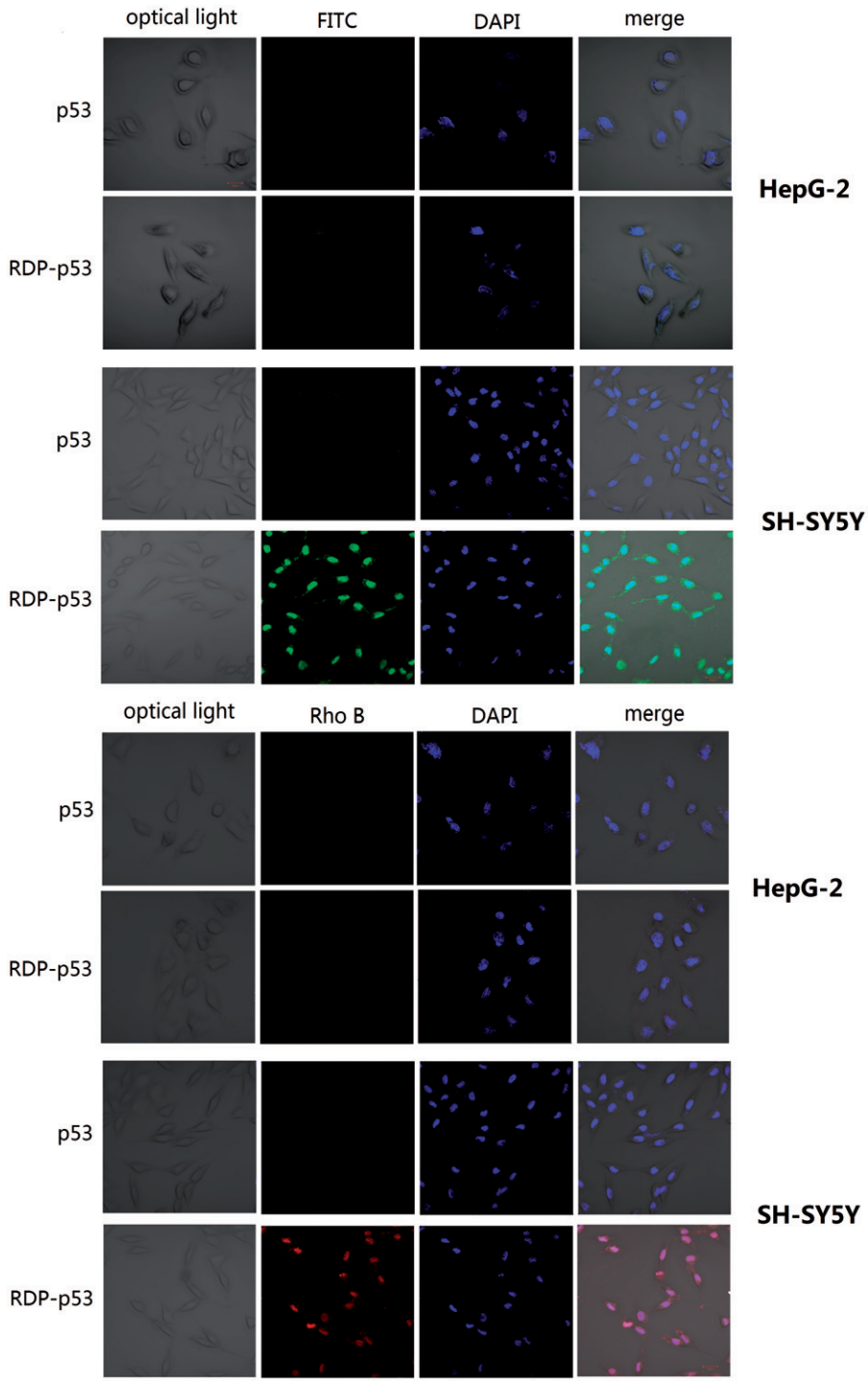

Figure 1. RDP-p53 selectively entered into the neuroblastoma cells observed under confocal microscope. HepG-2 and SH-SY5Y cells were first, respectively, incubated with FITC and rhodamine B isothiocyanate (Rho B) labeled p53 and RDP-p53 for 3 h. After washed with PBS three times, cells were stained by DAPI for 30 min and washed again. Magnification 400 × .

vector (Fu et al., 2013; Zhang et al., 2014), and the possible mechanism of specific cellular uptake may be associated with the clathrin-mediated endocytosis of $GABA_B$ receptor in neuron cells (Fu et al., 2013).

RDP-p53 obviously inhibited SH-SY5Y cell proliferation

The inhibition of RDP-p53 in cancerous neural cells is fundamental for further application. In order to examine the anti-proliferative features of the RDP-p53 in cancerous nerve cells, SH-SY5Y cells were first used. The cells were treated with different concentrations (10–100 μg/ml) of RDP-p53 for 12, 24, 48, and 72 h, and the cell viability was measured by MTT assay. The results showed that there were no obvious changes of cell viability after treated with RDP (100 μg/ml) for 12, 24, 48, or 72 h, suggesting that RDP alone does not affect cell growth (Figure 2A). Also, the survival rate of RDP-p53-treated cells was obviously lower than p53-treated cells that might be related to cell-permeability of this conjugates. Moreover, the cell viability of RDP-p53 treatment was significantly reduced following the increase of the incubation time and concentrations (Figure 2B), indicating that the proteins inhibited cell growth of SH-SY5Y in a time- and concentration-dependent manner. Thus, RDP-p53 might be a candidate for brain tumor treatment.

The mechanism of RDP-p53 induced cell death

p53 gene is regarded as the ''guardian of the genome'', and encoding product p53 protein can inhibit or kill the tumor cells by various ways (Hansen et al., 2007; Yu et al., 2009; Yamada, 2013; Yan et al., 2012) including up-regulation of the expression of pro-apoptotic gene, down-regulation of the expression of anti-apoptotic gene for induction apoptosis, and prevention of cell cycle entry into M phase from G2 phase (Choi & Kim, 2009). In this study, to determine the anticancer mechanism of RDP-p53, the proteins were added into the media of SH-SY5Y cells for a certain time. According to the protocol of the manufacture, there was weak red and weak blue fluorescence in normal cells, whereas apoptotic cells showed weak red and strong blue, and for necrotic cells, strong red and strong blue fluorescence were observed.

Following treatment with a certain dose RDP-p53 (40 μg/ml), a majority of cells appeared to be the weak red and strong blue fluorescence under the fluorescence microscope, whereas few showed strong red and strong blue fluorescence (Figure 3A), indicating RDP-p53 induced cell death mainly through apoptosis pathway, and RDP as a carrier does not influence the apoptosis function of p53. Besides, SH-SY5Y cells treated with RDP-p53 showed similar morphologic changes as the treatment with high concentration of p53 alone (100 μg/ml), including cell shrinkage, chromatin condensation, and the presence of ''apoptosis bodies'' under the optical microscope.

Additionally, the apoptosis rate and cell-cycle assay were detected by flow cytometry. The total percentage of apoptotic cells in PBS, p53, and RDP-p53 treatment groups were recorded as 2.34, 59.78, and 92.34%, respectively. The apoptosis rate of SH-SY5Y cells treated with RDP-p53 was relative higher than that of p53 (Figure 3B). As compared with the PBS group, the proportion of gated cells following p53 treatment in the G2 phase was 7.11%, while cells treated with RDP-p53 in the G2 phase was 9.84%, suggesting that RDP-p53 induced cell accumulation in the G2/M phase (Figure 3C). These data indicated that the inhibition mechanism on cell growth of RDP-p53 might be related to not only induction of cell apoptosis but also blocking of cell cycle in the G2/M phase.

Highly inhibitory effect of RDP-p53 on the growth of U251 cells

To test whether RDP-p53 showed an inhibitory effect on the other cancerous neural cells, U251 cells were treated with the protein at different concentrations and incubation times. As shown in Figure 4, the cell viability of RDP-p53 treatment

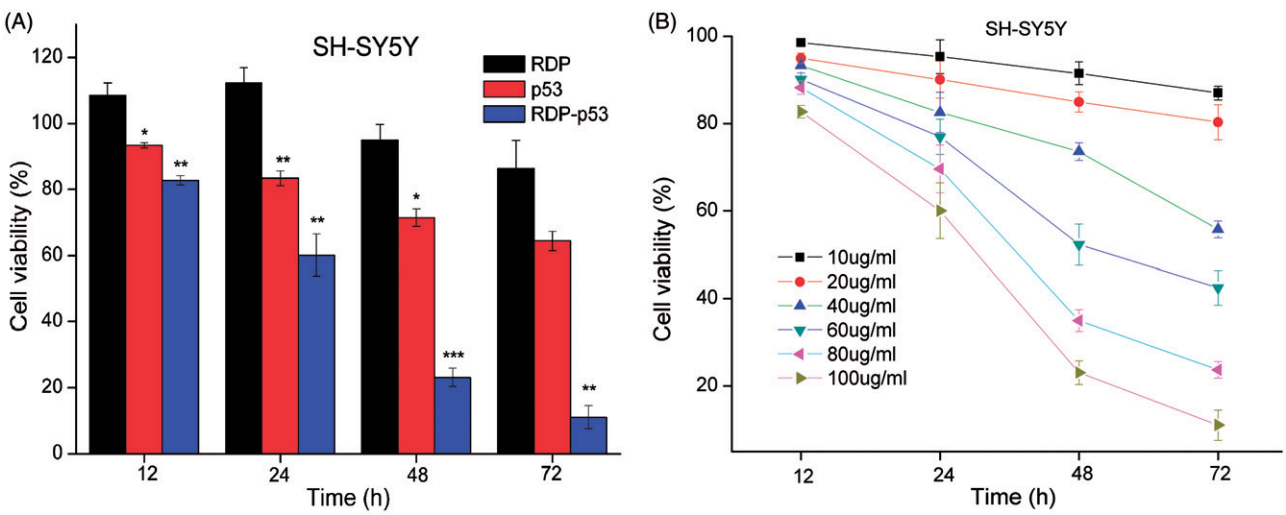

Figure 2. Cell viability of cell growth induced by p53 and RDP-53. (A) The survival rate of RDP, p53, and RDP-p53 on neuroblastoma cells at different reaction times. The final concentration of protein was 100 μg/ml and cells treated with RDP were considered as the control. Data are mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) The cell viability of RDP-p53 at different incubation times and different concentrations ($n = 3$). Cell viability was assessed with the MTT assay.

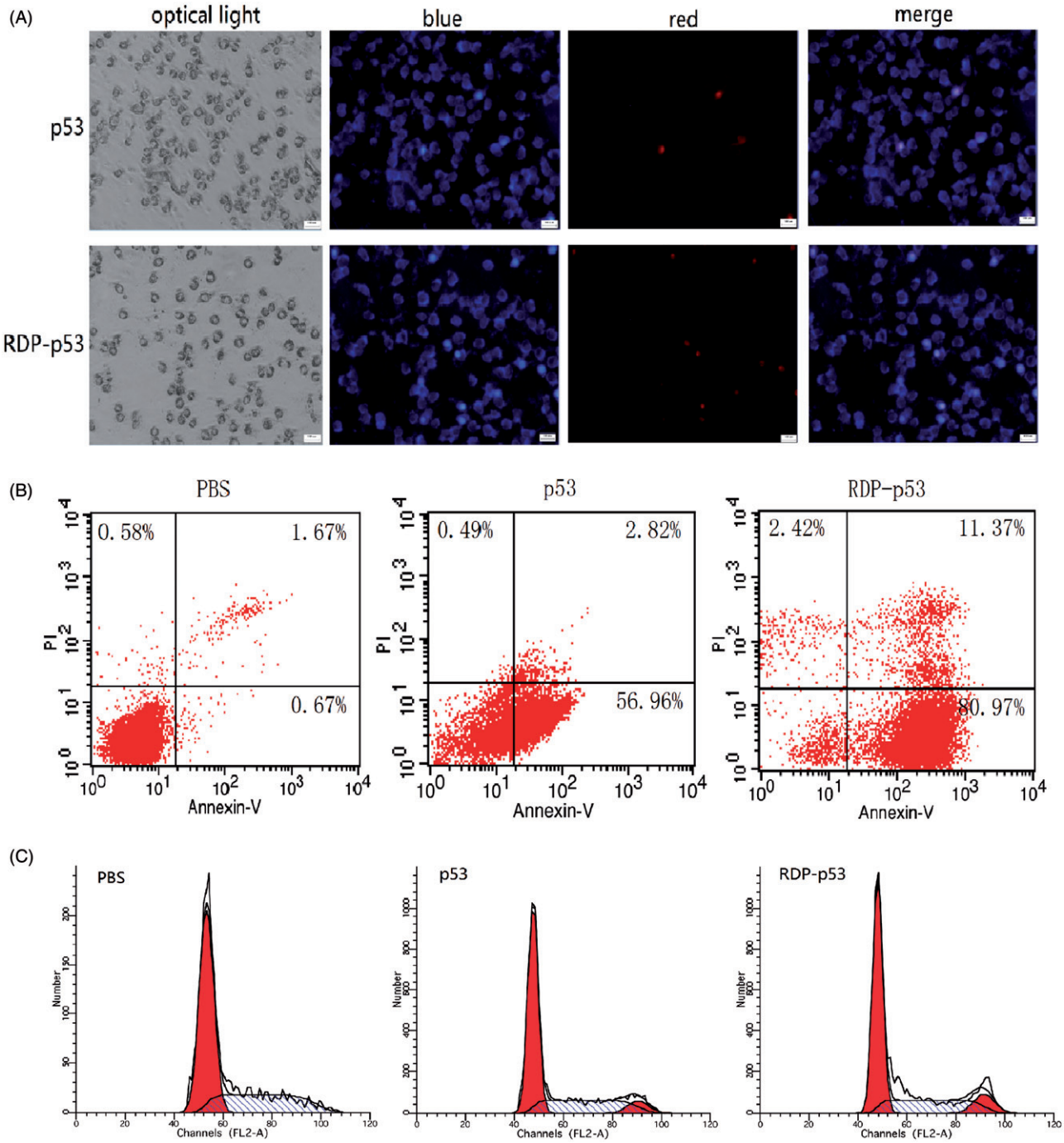

Figure 3. The mechanism of RDP-p53 in SH-SY5Y cells. (A) Strong blue with weak red fluorescence were observed in the apoptotic cells, clearly indicating that p53 showed the ability of inducing cells apoptosis. Original magnification is 100×. (B) Induction of apoptosis on SH-SY5Y cells following PBS, p53, and RDP-p53 treatment. SH-SY5Y cells were treated with protein for 24 h before detected by flow cytometric analysis. (C) Cell-cycle distributions in SH-SY5Y cells with PBS, p53, and RDP-p53 treatment.

was decreased with the increase of protein concentrations and treatment time. Moreover, the results of flow cytometry indicated that the total percentage of apoptotic cells of PBS, p53, and RDP-p53 treatment groups were, respectively, calculated as 8.67%, 13.06%, and 45.84%, suggesting that the antitumor mechanism of RDP-p53 on U251 was also associated with cell apoptosis (Figure 4C). Previous reports indicated that p53 plays a critical role in the initiation and the progression of cancerous neural cells, including gliomas (Nayak et al., 2004; Ohgaki & Kleihues, 2007). The mutation rate of p53 was 25–35% in astrocytoma tissues and this rate reached 70–76% in glioma cell line (Ishii et al., 1999). Activated-p53 protein as one nuclear transcription factor combined with the specific DNA binding element and improved the expression of downstream target genes could inhibit tumor growth. Therefore, delivery of wild-type p53 into the glioma has become an important strategy for glioma therapy. The results suggested RDP-p53, consisting of neuron-permeable peptide and p53, showed higher inhibition on U251 cell growth than p53 alone, which might be a novel antitumor candidate for protein therapy of malignant glioma.

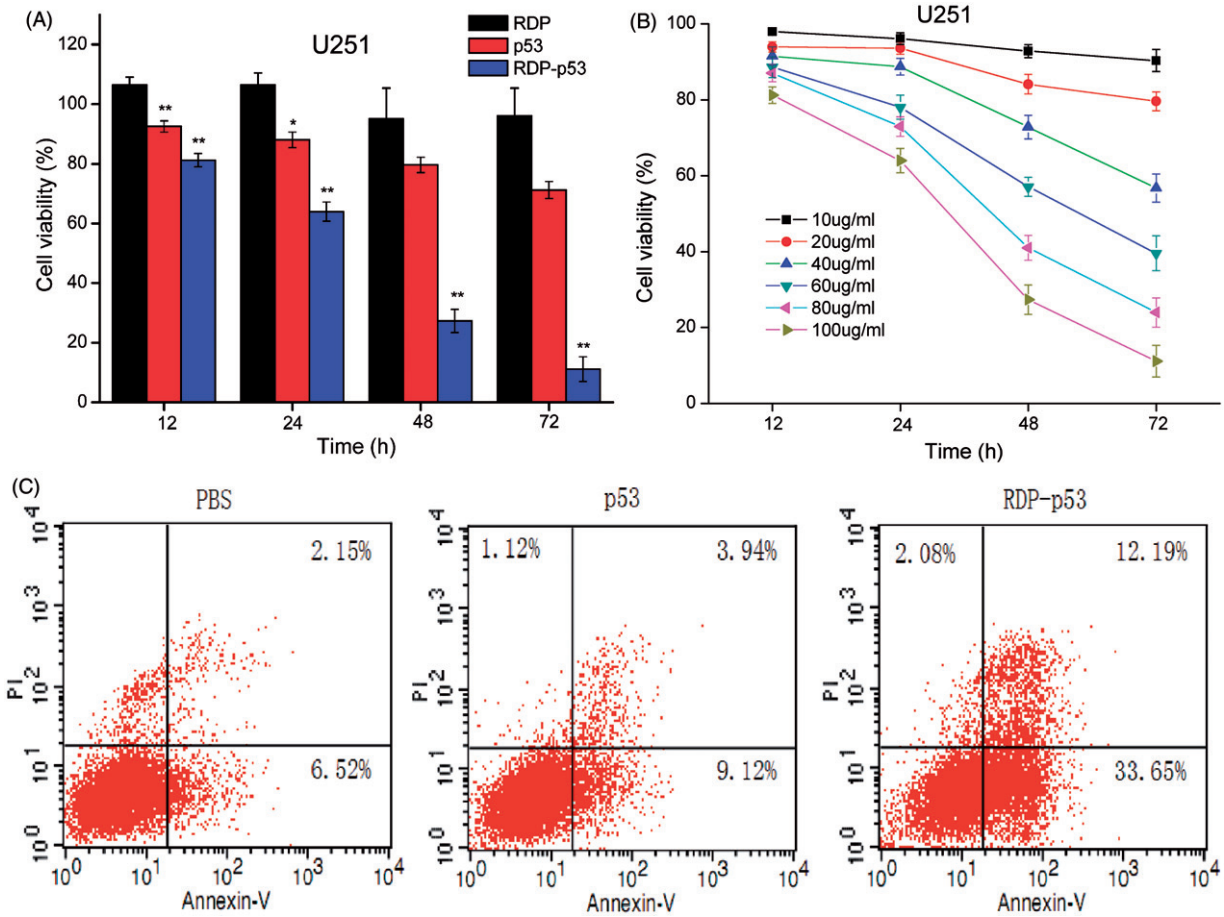

Figure 4. Cell viability of RDP-p53 in U251 cells. (A) The cell survival rate of RDP, p53, and RDP-p53 in U251 cells at different time points. The final concentration of protein is 100 μg/ml and cells treated with RDP were considered as the control. Data are mean ± SEM ($n = 3$). *$p < 0.05$, ** $p < 0.01$. (B) The cell viability of RDP-p53 with a series of concentration and different incubation times ($n = 3$). Cell viability was assessed with the MTT assay. (C) Induction of apoptosis in U251 cells with PBS, p53, and RDP-p53 treatment. U251 cells were treated with protein for 24 h before analysis by flow cytometry.

Conclusion

The study suggested that RDP, acted as a specific cell-permeable carrier, was able to improve the uptake efficiency of functional protein p53 into cancerous neural cells. The novel fusion protein RDP-p53 showed obvious inhibition on cell growth than p53 alone not only SH-SY5Y cells but also U251 cells, which might provide a potential and effective approach for protein therapy of brain tumors, including malignant glioma. The mechanism of RDP-p53 induced cell death mainly through apoptosis pathway and arrest cell cycle at the G2/M phase. RDP-p53 could be a novel antitumor candidate for treating cancerous neural cells.

Declaration of interest

The authors report that they have no conflicts of interest regarding the publication of this paper. This work is supported by the grants from the Natural Science Foundation of China (81273416).

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