Department of Orthopaedics, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Anti-ganglioside GD2 monoclonal antibody synergizes with cisplatin to induce endoplasmic reticulum-associated apoptosis in osteosarcoma cells

Weihong Zhu, Xinzhan Mao, Wanchun Wang*, You Chen, Ding Li, Hui Li, Pengcheng Dou

Received August 23, 2017, accepted September 28, 2017

**Corresponding author: Wanchun Wang, MD, Department of Orthopaedics, The Second Xiangya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011 China*
zuhweihong@csu.edu.cn

Pharmazie 73: 80–86 (2018) *doi: 10.1691/ph.2018.7836*

Cisplatin is an effective chemotherapeutic agent for osteosarcoma (OS) and has been shown to induce endoplasmic reticulum (ER) stress-associated apoptosis in human cancer cells. Ganglioside GD2-specific antibodies can inhibit tumor cell viability without involvement of the immune system. A recent study has shown that anti-GD2 monoclonal antibody (mAb) 14G2a effectively inhibits the viability and invasiveness of human OS cells. In this study, we explored the effect of anti-GD2 mAb and cisplatin alone and in combination on ER stress-associated apoptosis in osteosarcoma cells. MG-63 and Saos-2 human OS cells were treated with cisplatin and/or an-GD2 mAb 14G2a for 48 hours. Cisplatin and 14G2a dose-dependently induced apoptosis in MG-63 and Saos-2 cells. They in combination induced 70%-77% of apoptosis in MG-63 cells and 79%-85% of apoptosis in Saos-2 cells, exhibiting a synergistic effect stronger than addition of their individual effects over the control level. Showing no significant effect on the expression of protein kinase RNA-like ER kinase (PERK), cisplatin and 14G2a exhibited a marked synergistic effect on inducing phosphorylation/activation of PERK, phosphorylation/inactivation of eukaryotic translation initiation factor 2α (eIF2α), expression of CHOP, in parallel to inducing the caspase-3 activity and apoptosis in MG-63 and Saos-2 cells. The effects were abolished by lentivirus-mediated knockdown of PERK. Particularly, PERK knockdown abolished 63% and 65% of the combined apoptotic effect of cisplatin and 14G2a on MG-63 and Saos-2 cells, respectively. In conclusion, this study provides the first evidence supporting that cisplatin and 14G2a synergize to induce ER stress-associated apoptosis in human OS cells through activating the PERK ER stress pathway by synergistically inducing phosphorylation/activation of PERK. Our findings add new insights into the pharmacologic effects of anti-GD2 mAb in anticancer treatment and suggest that cisplatin plus anti-GD2 mAb could be a new effective therapeutic strategy for OS.

1. Introduction

As the most frequent primary bone malignancy, osteosarcoma (OS) comprises approximately 35% of all bone cancers and 2.4% of all malignancies in pediatric patients. Despite modern multidisciplinary treatments including chemotherapy and surgery, the 5-year survival rate of osteosarcoma patients remains 60%-70% (Ottaviani and Jaffe 2010). Standard chemotherapy for OS is based on a combination of different drugs: neoadjuvant therapy with methotrexate, cisplatin, and doxorubicin followed by surgery and post-operative chemotherapy (methotrexate, cisplatin, doxorubicin, cyclophosphamide, and vincristine) (Chou and Gorlick 2006). Cisplatin has been proven to be an effective chemotherapeutic agent for OS, while good responders to cisplatin chemotherapy show a better survival rate (Abe et al. 2002). In addition, it has been shown that chemotherapies with the inclusion of cisplatin have better outcomes for high grade OS (Anninga 2011).

Recent studies have revealed that cisplatin induces endoplasmic reticulum (ER) stress-associated apoptosis in human glioma cells, lung cancer cells, cervical cancer cells, and OS cells (Shi et al. 2016; Xu et al. 2015; Zhang et al. 2015; Zhao et al. 2014). The ER is an important cellular compartment for protein synthesis/folding. Various physiological and pathological conditions may lead to ER stress, which results in an accumulation of unfolded or misfolded proteins in the ER lumen (Berridge 2002; Jorgensen et al. 2003). This cellular stress subsequently causes an activation of the unfolded protein response (UPR) (Xu et al. 2015), which comprises three canonical signaling pathways: the protein kinase RNA-like ER kinase (PERK)/eukaryotic translation initiation factor 2α (eIF2α)/CHOP pathway, the inositol-requiring protein-1 (IRE1)/XBP1 pathway, and the activating transcription factor-6 (ATF6) pathway (Zhu et al. 2016). Moderate ER stress can be resolved by UPR to recover ER homeostasis, whereas severe and prolonged ER stress induces apoptosis (Xu et al. 2015). PERK is an ER transmembrane protein kinase that inhibits protein translation through phosphorylation/inactivation of eIF2α, which leads to increased expression of CHOP, a critical factor for triggering apoptosis in response to ER stress (Hotamisligil 2010; Hetz et al. 2011; Zinszner et al. 1998).

Intrinsic or acquired chemoresistance is the major reason for poor survival and disease relapse of OS patients (Huang et al. 2015). Novel molecular targeted drugs have recently emerged, but they have not been well established for the treatment of OS (Huang et al. 2015). Gangliosides are glycosilated lipid molecules belonging to the class of glycosphingolipids (Doronin et al. 2014). They are essentially located on the outer leaflet of the plasma membrane and can interact with transmembrane receptors or signal transducers involved in cell signaling (Liu et al. 2014). The tumor-associated ganglioside GD2 is an attractive target for immunotherapy. While its expression in normal tissue is restricted to the nervous system, it has been found in sarcoma, glioma, and small cell lung cancers, where it is associated with enhanced cell viability (Liu et al. 2014). GD2-specific antibodies can inhibit tumor cell viability without involvement of the immune system (Doronin et al. 2014; Horwacik et al. 2013). It has been shown that the anti-GD2 monoclonal antibody (mAb) is capable of decreasing viability of neuroblastoma cells in a dose-dependent manner (Cochonneau et al. 2013). Recent studies have shown that GD2 is highly expressed in OS tissues and cell lines (Liu et al. 2014; Roth et al. 2013). In addition, OS tissue

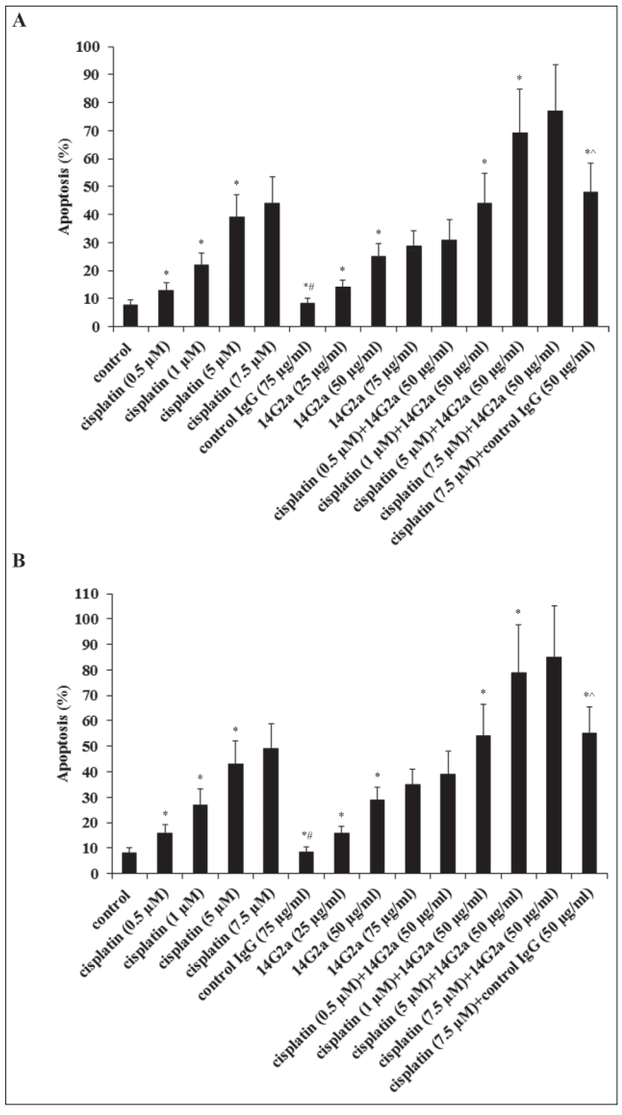

Fig. 1: Apoptosis in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a (*A*) MG-63 and (*B*) Saos-2 human OS cells were treated with cisplatin and/or 14G2a or control IgG at indicated concentrations for 48 h. Untreated cells were used as a control. The apoptosis rate (in percentage of total cells) was measured with a microplate reader-based apoptosis detection kit. *p<0.05 vs. immediate previous treatment; ^p<0.05 vs. cisplatin (7.5 μM); #p>0.05 vs. control.

obtained at the time of disease recurrence shows higher intensity of GD2 staining compared with samples obtained at initial biopsy and definitive surgery, suggesting that ganglioside GD2 may play an important role in OS cell survival. A recent study has shown that an anti-GD2 mAb effectively inhibits the viability and invasiveness of human OS cells (Liu et al. 2014).

In this study, we for the first time explored the effect of anti-GD2 mAb and cisplatin alone and in combination on ER stress-associated apoptosis in osteosarcoma cells.

2. Investigations and results

2.1. 14G2a promotes cisplatin-induced OS cell apoptosis by a synergistic effect

We used MG-63 and Saos-2 human OS cells as cell models in this study, for they express ganglioside GD2 on the cell membrane (Liu et al. 2014). We explored the individual and combined effect of cisplatin and 14G2a on apoptosis of MG-63 and Saos-2 cells, respectively. A mouse isotype-matched mAb purified from PK136 hybridoma culture supernatants was used as a control IgG for 14G2a. As shown in Fig. 1, both cisplatin and 14G2a dose-dependently induced apoptosis in MG-63 and Saos-2 cells. For 48 hours of treatment, cisplatin reached a dosage plateau at 5-7.5 μM in both cell lines, inducing 38%-43% of apoptosis in MG-63 cells and 42%-48% of apoptosis in Saos-2 cells (Fig. 1). 14G2a reached a dosage plateau at 50-75 μg/ml in both cell lines, inducing 25%-29% of apoptosis in MG-63 cells and 28%-34% of apoptosis in Saos-2 cells; in contrast, the control IgG showed no significant effect on the cells compared with the control (Fig. 1). At the plateau dosage, 14G2a but not the control IgG markedly promoted the apoptotic effect of cisplatin, inducing 70%-77% of apoptosis in MG-63 cells and 79%-85% of apoptosis in Saos-2 cells when the cisplatin dosage was at 5-7.5 μM (Fig. 1). The results indicated a synergistic effect between cisplatin and 14G2a, stronger than an addition of their individual apoptotic effect over the control level, suggesting that 14G2a could promote cisplatin-induced OS cell apoptosis by a synergistic effect.

2.2. Cisplatin and 14G2a synergize to activate the PERK/eIF2α/CHOP ER stress pathway in OS cells

PERK/eIF2α/CHOP signaling is a major ER stress pathway. Under ER stress conditions, PERK oligomerizes in ER membranes, inducing its autophosphorylation and activating the kinase domain (Xu et al. 2005). Then PERK phosphorylates and inactivates eIF2α, thereby globally shutting off mRNA translation and reducing the protein load on the ER, which, if persists, will lead to increased expression of CHOP, a critical factor for triggering apoptosis in response to ER stress (Hotamisligil 2010; Hetz et al. 2011; Zinszner et al. 1998). As shown in Fig. 2, the total PERK level was not significantly altered by cisplatin and/or 14G2a in MG-63 and Saos-2 cells; in contrast, both cisplatin and 14G2a dose-dependently induced phosphorylation/activation of PERK, while the control IgG showed no significant effect. 14G2a but not the control IgG markedly promoted cisplatin-induced phosphorylation of PERK, indicating a synergistic effect between cisplatin and 14G2a more than an addition of their individual effects over the control level (Fig. 2). As shown in Fig. 3, lentiviral transduction of shRNA knocked down the expression of PERK by over 80% in both MG-63 and Saos-2 cells, which readily abolished the individual and synergistic effects of cisplatin and 14G2a on phosphorylation/activation of PERK. As shown in Fig. 4, both cisplatin and 14G2a markedly induced phosphorylation/inactivation of eIF2α in MG-63 and Saos-2 cells; when used together, the two drugs exhibited a synergistic effect, which was more than an addition of their individual effects over the control level (Fig. 4). Knockdown of PERK abolished the individual and synergistic effects of cisplatin and 14G2a on inducing phosphorylation/inactivation of eIF2α (Fig. 4). In agreement with the findings above, knockdown of PERK abolished the individual and synergistic effects of cisplatin and 14G2a on inducing CHOP in MG-63 and Saos-2 cell (Fig. 5). We did not detect any significant effect, alone or in combination, of cisplatin and 14G2a on the IRE1 and ATF6 ER stress signaling pathways (data not shown). Taken together, the findings suggested a synergy between cisplatin and 14G2a in activating the PERK/eIF2α/CHOP ER stress pathway in OS cells.

2.3. Cisplatin and 14G2a synergize to induce ER stress-associated apoptosis in OS cells through the PERK signaling pathway

Caspase-3 is a major activated caspase present in ER stress-induced apoptosis (Loughlin and Artlett 2010; Syeda et al. 2013; Johnson et al. 2014). As shown in Fig. 6, both cisplatin and 14G2a markedly induced the caspase-3 activity in MG-63 and Saos-2 cells; when used together, the two drugs exhibited a synergistic effect, which was more than an addition of their individual effects over the control level. Knockdown of PERK did not show significant effect on the caspase-3 activity in untreated cells; however, it abolished approximately 43%/46% of cisplatin-induced caspase-3 activity and 32%/46% of 14G2a-induced caspase-3 activity in MG-63 and Saos-2 cells, respectively. In addi-

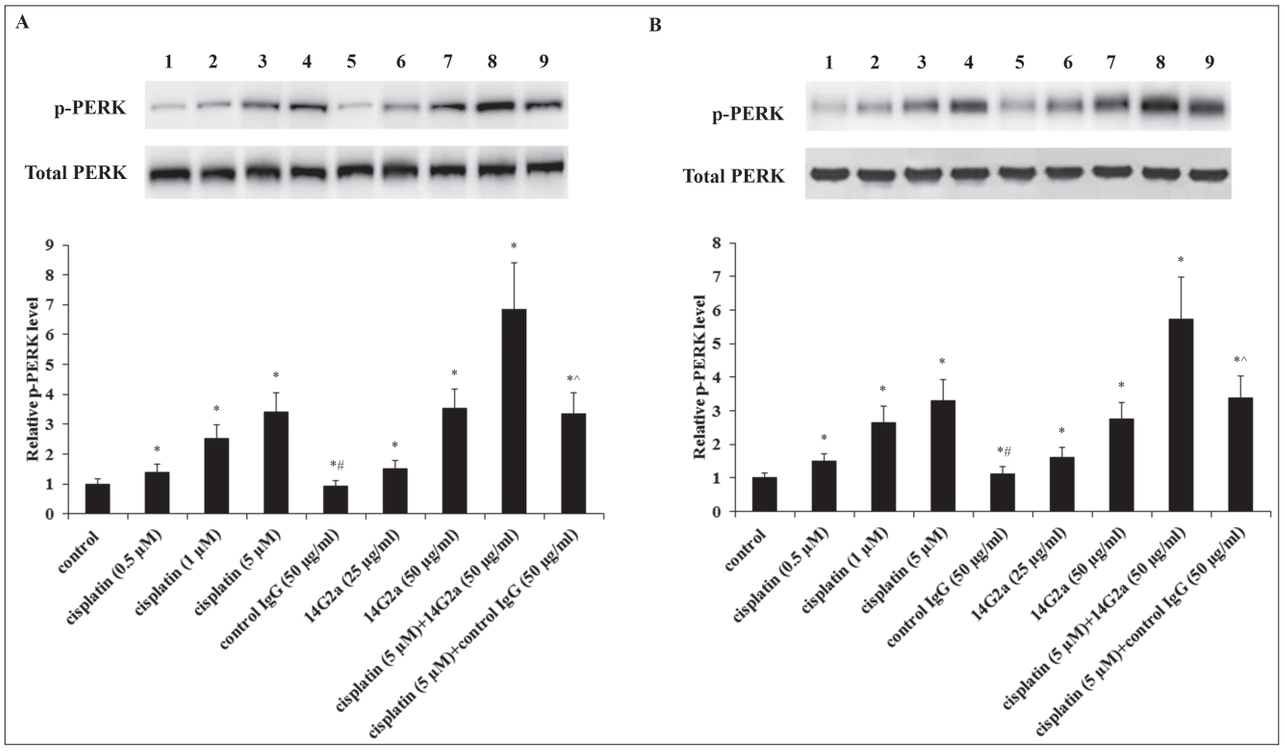

Fig. 2: Phosphorylated PERK (p-PERK) levels in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a
(*A*) MG-63 and (*B*) Saos-2 OS cells were treated with cisplatin and/or 14G2a or control IgG at indicated concentrations for 48 h. Then the cell lysates were subject to Western blot analyses to determine the protein levels of p-PERK and total PERK. Lysates from untreated cells were used as a control (*lane 1*). *Lane 2*, cisplatin (0.5 mM); *lane 3*, cisplatin (1 mM); *lane 4*, cisplatin (5 mM); *lane 5*, control IgG (50 mg/ml); *lane 6*, 14G2a (25 mg/ml); *lane 7*, 14G2a (50 mg/ml); *lane 8*, cisplatin (5 mM)+14G2a (50 g/ml); *lane 9*, cisplatin (5 mM)+ control IgG (50 mg/ml). There was no significant difference in the total PERK levels among the experimental groups. Density of the p-PERK blot was normalized against that of total PERK in each experimental group to obtain a relative density, which was expressed as fold changes to that of control (designated as 1). *p<0.05 vs. immediate previous treatment; ^p<0.05 vs. cisplatin (5 μM); #p>0.05 vs. control.

tion, knockdown of PERK abolished approximately 53%/55% of the combinatorial effect of cisplatin and 14G2a on MG-63 and Saos-2 cells, respectively (Fig. 6).

As shown in Fig. 7, knockdown of PERK did not show significant effect on apoptosis in untreated cells; however, it reduced approximately 15%/17% of cisplatin-induced apoptosis and 10%/13% of 14G2a-induced apoptosis in MG-63 and Saos-2 cells, respectively. In contrast, knockdown of PERK reduced approximately 39%/46% of apoptosis induced by combined treatment with cisplatin and 14G2a on MG-63 and Saos-2 cells, respectively, abolishing 63%-65% of the combined apoptotic effect of cisplatin and 14G2a [for MG-63 cells, (70-31)/(70-7.8$_{control}$)*100%=63%; for Saos-2, (79-33)/(79-8.2$_{control}$)*100%=65%]. Taken together, the findings suggested that cisplatin and 14G2a synergize to induce apoptosis in OS cells largely through the PERK ER stress pathway

3. Discussion

Cisplatin is an effective chemotherapeutic agent for OS and has been shown to induce ER stress-associated apoptosis in human cancer cells (Shi et al. 2016; Xu et al. 2015; Zhang et al. 2015; Zhao et al. 2014; Huang et al. 2015). Accumulating evidence indicates that GD2-specific antibodies can inhibit tumor cell viability without involvement of the immune system (Doronin et al. 2014; Horwacik et al. 2013). A recent study has shown that anti-GD2 mAb 14G2a effectively inhibits the viability and invasiveness of human OS cells (Liu et al. 2014). In the present study, we have shown that cisplatin and anti-GD2 mAb 14G2a synergize to induce apoptosis in OS cells largely by activating the PERK ER stress pathway.

Human OS cell lines MG-63 and Saos-2 reportedly express ganglioside GD2 on the cell membrane and thus were used cell models in this study (Liu et al. 2014). Our findings indicate an obvious synergistic apoptotic effect between cisplatin and 14G2a on the OS cells, for the two drugs used in combination exhibited a stronger apoptotic effect than an addition of their individual effects over the control level. Given the facts that intrinsic or acquired chemoresistance is the major reason for poor survival and disease relapse of OS patients (Liu et al. 2014) and that cisplatin is an important component of OS chemotherapy (Chou and Gorlick 2006; Liu et al. 2014), our findings suggest that cisplatin plus anti-GD2 mAb could be a new strategy to raise the therapeutic effect or to reduce chemoresistance for cisplatin-based OS chemotherapy.

We found that while cisplatin and 14G2a alone respectively induced relatively low ER stress-associated apoptosis, they in combination showed a marked synergistic effect on inducing ER stress-associated apoptosis, which made up 63%-65% of the combined apoptotic effect of cisplatin and 14G2a. The findings suggest that the synergistic apoptotic effect between cisplatin and 14G2a is largely attributable to a synergy between them in inducing ER-associated apoptosis.

Under cisplatin and/or 14G2a treatment, we only detected in OS cells significant changes in the PERK/eIF2α/CHOP pathway among the canonical ER stress signaling pathways. It has been reported that under ER stress conditions, PERK oligomerizes in ER membranes to induce its autophosphorylation and activate the kinase domain (Xu et al. 2005), which in turn leads to phosphorylation/inactivation of eIF2α and increased expression of CHOP, a critical factor triggering apoptosis in response to ER stress (Hotamisligil 2010; Hetz et al. 2011; Zinszner et al. 1998). We found that cisplatin and 14G2a synergize to activate PERK and its downstream signaling by inducing phosphorylation/activation of PERK, while showing no significant effect on the expression of PERK. The findings suggest cisplatin and 14G2a synergize to activate PERK signaling in OS cells at or before the oligomerization/autophosphorylation phase rather than altering the amount of PERK. The underlying mechanisms will be explored in our future studies.

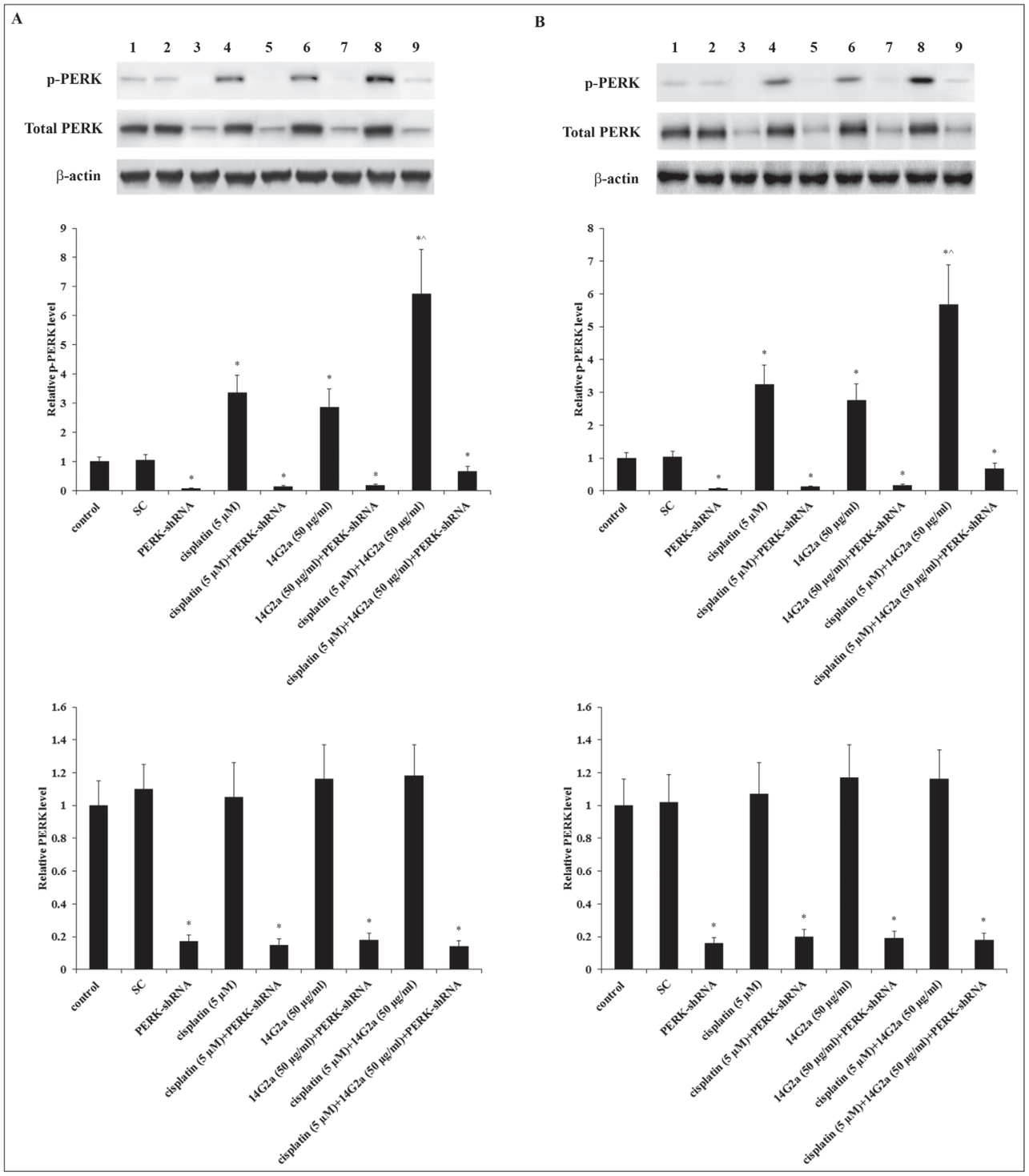

Fig. 3: Phosphorylated PERK (p-PERK) levels in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a with or without PERK knockdown
*(*A) MG-63 and (*B*) Saos-2 OS cells with or without transduction of human PERK-shRNA or scramble control shRNA lentiviral particles were treated with cisplatin and/or 14G2a at indicated concentrations for 48 h. Then the cell lysates were subject to Western blot analyses to determine the protein levels of p-PERK and total PERK. β-actin blotting was used as a loading control. Lysates from untreated cells were used as a control (*lane 1*). *Lane 2*, cells transduced with scramble control shRNA lentiviral particles (SC); *lane 3*, cells transduced with PERK-shRNA lentiviral particles (PERK-shRNA); *lane 4*, cisplatin (5 mM); *lane 5*, cisplatin (5 mM)+PERK-shRNA; *lane 6*, 14G2a (50 mg/ml); *lane 7*, 14G2a (50 mg/ml)+PERK-shRNA; *lane 8*, cisplatin (5 mM)+14G2a (50 mg/ml); *lane 9*, cisplatin (5 mM)+14G2a (50 mg/ml)+PERK-shRNA. Density of the p-PERK and the total PERK blot was respectively normalized against that of β-actin to obtain a relative density, which was expressed as fold changes to that of control (designated as 1). *$p<0.05$ vs. control and SC; ^$p<0.05$ vs. cisplatin (5 μM) and 14G2a (50 mg/ml).

Cisplatin is widely used against human solid tumors (Wang et al. 2014; Anninga et al. 2011) and has been shown to induce ER stress-associated apoptosis in human cancer cells (Hotamisligil 2010; Hetz et al. 2011; Zinszner et al. 1998). Anti-GD2 mAbs have been found effective against neuroblastoma and osteosarcoma (Hetz et al. 2011; Liu et al. 2014; Nishitoh 2012). Our study adds to the knowledge base that cisplatin and anti-GD2 mAb 14G2a synergize to induce ER stress-associated apoptosis in OS cells through synergistic activation of the PERK ER stress pathway. It puts forward interesting questions for future research: (1) Can

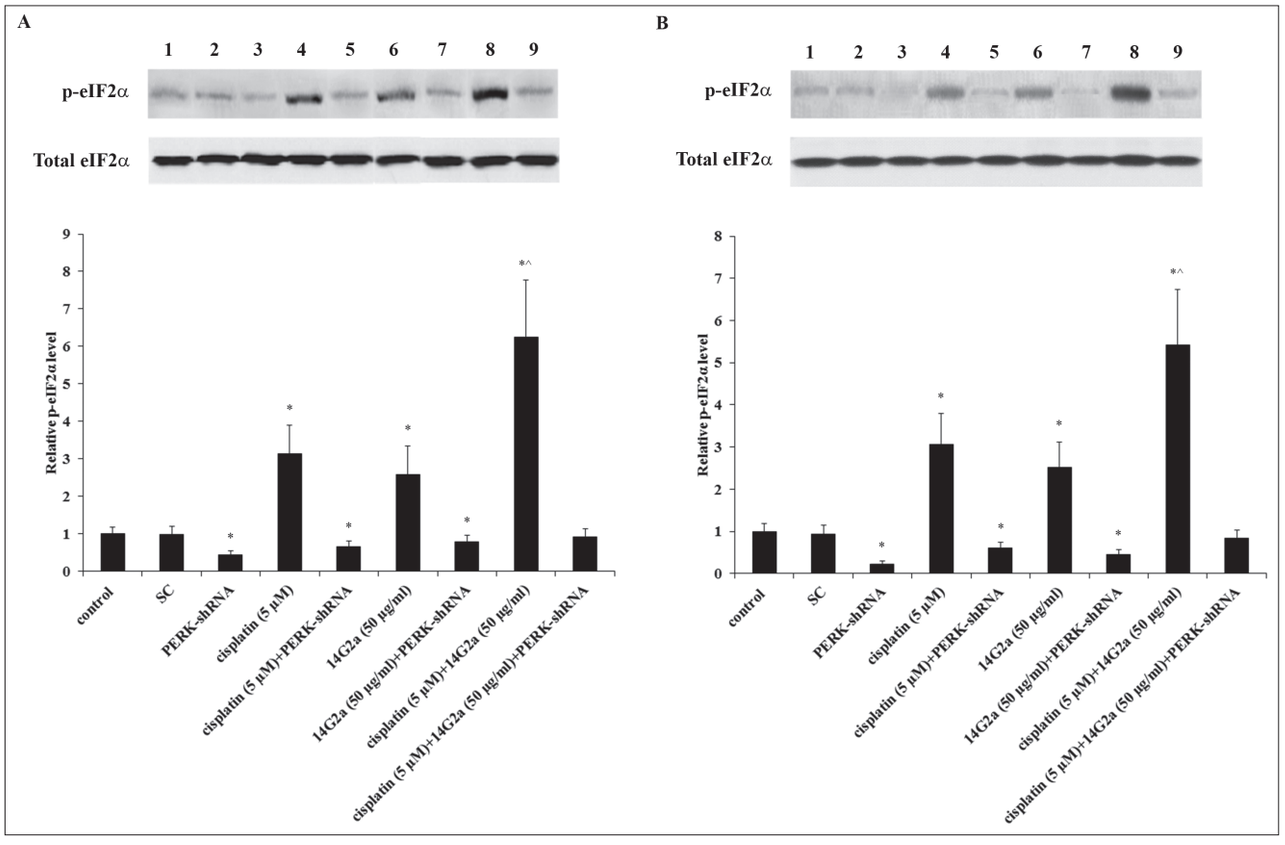

Fig. 4: Phosphorylated eIF2α (p-eIF2α) levels in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a with or without PERK knockdown
(A) MG-63 and (*B*) Saos-2 OS cells with or without transduction of human PERK-shRNA or scramble control shRNA lentiviral particles were treated with cisplatin and/or 14G2a at indicated concentrations for 48 h. Then the cell lysates were subject to Western blot analyses to determine the protein levels of p-eIF2α and total eIF2α. β-actin blotting was used as a loading control. Lysates from untreated cells were used as a control (*lane 1*). *Lane 2*, cells transduced with scramble control shRNA lentiviral particles (SC); *lane 3*, cells transduced with PERK-shRNA lentiviral particles (PERK-shRNA); *lane 4*, cisplatin (5 mM); *lane 5*, cisplatin (5 mM)+PERK-shRNA; *lane 6*, 14G2a (50 mg/ml); *lane 7*, 14G2a (50 mg/ml)+PERK-shRNA; *lane 8*, cisplatin (5 mM)+14G2a (50 mg/ml); *lane 9*, cisplatin (5 mM)+14G2a (50 mg/ml)+PERK-shRNA. There was no significant difference in the total eIF2α levels among the experimental groups. Density of the p-eIF2α blot was normalized against that of total eIF2α in each experimental group to obtain a relative density, which was expressed as fold changes to that of control (designated as 1). *p<0.05 vs. control and SC; ^p<0.05 vs. cisplatin (5 μM) and 14G2a (50 mg/ml).

anti-GD2 mAbs other than 14G2a also synergize with cisplatin to induce ER stress-associated apoptosis in OS? (2) Since cisplatin and anti-GD2 mAbs have been proved effective against various cancer cell types other than OS (Liu et al. 2014; Hotamisligil 2010; Hetz et al. 2011; Zinszner et al. 1998; Doronin et al. 2014; Horwacik et al. 2013; Cochonneau et al. 2013), can combined treatment with cisplatin and anti-GD2 mAbs result in synergized therapeutic effect in cancer cell types other than OS? If yes, by what mechanisms?

In conclusion, this study provides the first evidence supporting that cisplatin and 14G2a synergize to induce ER stress-associated apoptosis in human OS cells through activating the PERK ER stress pathway by synergistically inducing phosphorylation/activation of PERK. Our findings add new insights into the pharmacologic effects of anti-GD2 mAb in anticancer treatment and suggest that cisplatin plus anti-GD2 mAb could be a new effective therapeutic strategy for OS.

4. Experimental

4.1. Cell culture and treatments

MG-63 (Cat. No. CRL-1427) and Saos-2 (Cat. No. HTB-85) human OS cell lines was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 μM each of penicillin and streptomycin (Thermo Fisher Scientific, Beijing, China) in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were treated with cisplatin (Cat. No. Y0001018; Sigma-Aldrich, Beijing, China) at 0.5, 1, 5, and 7.5 μM and/or an-GD2 mAb 14G2a (Cat. No. sc-53831; Santa Cruz Biotechnology, Dallas, TX, USA) at 25, 50 and 75 μg/ml for 48 h; a mouse isotype-matched mAb purified from PK136 hybridoma culture supernatants was used as a control IgG for 14G2a. For PERK knockdown, the cells were transduced with human PERK lentiviral particles (Cat. No. sc-36213-V; Santa Cruz Biotechnology), which contain three expression constructs each encoding target-specific shRNA designed to knock down PERK; cells transduced with scramble control shRNA lentiviral particles (Cat. No. sc-108080; Santa Cruz Biotechnology) were used as a negative control for PERK knockdown. The cells were subject to subsequent experiments 24 h after transduction.

4.2. Cell apoptosis assay

MG-63 and Saos-2 cells with or without PERK knockdown were cultured at 7.5×10^4 cells per well in 96-well tissue culture plates in the presence of cisplatin and/or 14G2a treatment for 48 hours. Cell apoptosis was measured with a microplate reader-based TiterTACS in situ apoptosis detection kit (Cat. No. 4822-96-K; R&D systems, Minneapolis, MN, USA) as described by the manufacturer (Byun et al. 2012; Sen et al. 2012). Each experiment was repeated for three independent times in duplicates.

4.3. Real-time quantitative RT-PCR

RNA was prepared from MG-63 and Saos-2 cells using TRIzol reagent (Thermo Fisher Scientific) followed by purification with TURBO DNA-free System (Ambion, Austin, TX, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific). RT-qPCR was performed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific) and the fluorescent dye SYBR Green Master Mix (Thermo Fisher Scientific) as described by the manufacturer. The primers used were as follows: for CHOP, 5′-GCCTTTCTCCTTTGGGACACTGTCCAGC-3′ (forward) and 5′- CTCGGCGAGTCGCCTCTACTTCCC-3′ (reverse); for 18S rRNA, 5'-CCCTGTAATTGGAATGAGTCCAC-3' (forward) and 5'- GCTGGAATTACCG-CGGCT-3' (reverse). The PCR amplification condition was: 20 s at 95 °C followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Relative quantification of the CHOP mRNA level was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and normalized against that of 18S rRNA in the same sample. Each experiment was repeated for three independent times in duplicates.

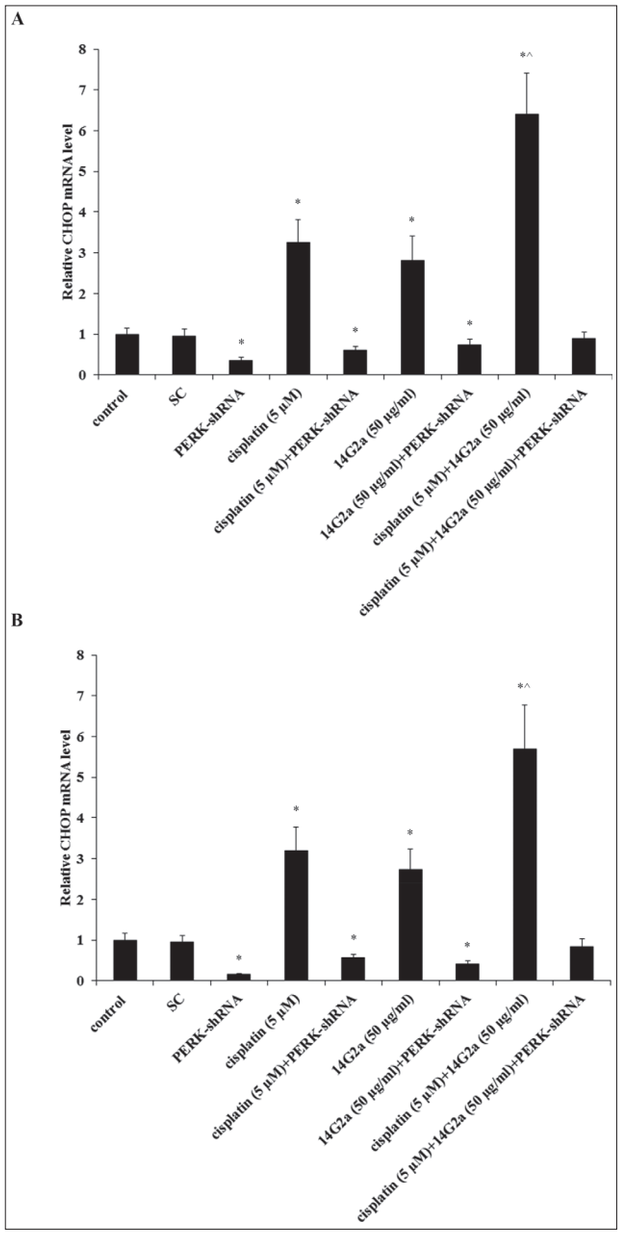

Fig. 5: Expression of CHOP in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a with or without PERK knockdown
*(*A) MG-63 and (*B*) Saos-2 OS cells with or without transduction of human PERK-shRNA or scramble control shRNA (SC) lentiviral particles were treated with cisplatin and/or 14G2a at indicated concentrations for 48 h. Then the CHOP mRNA levels were measured by real-time quantitative RT-PCR assays and expressed as folds changes to that of untreated control cells (designated as 1). *p<0.05 vs. control and SC; ^p<0.05 vs. cisplatin (5 μM) and 14G2a (50 mg/ml).

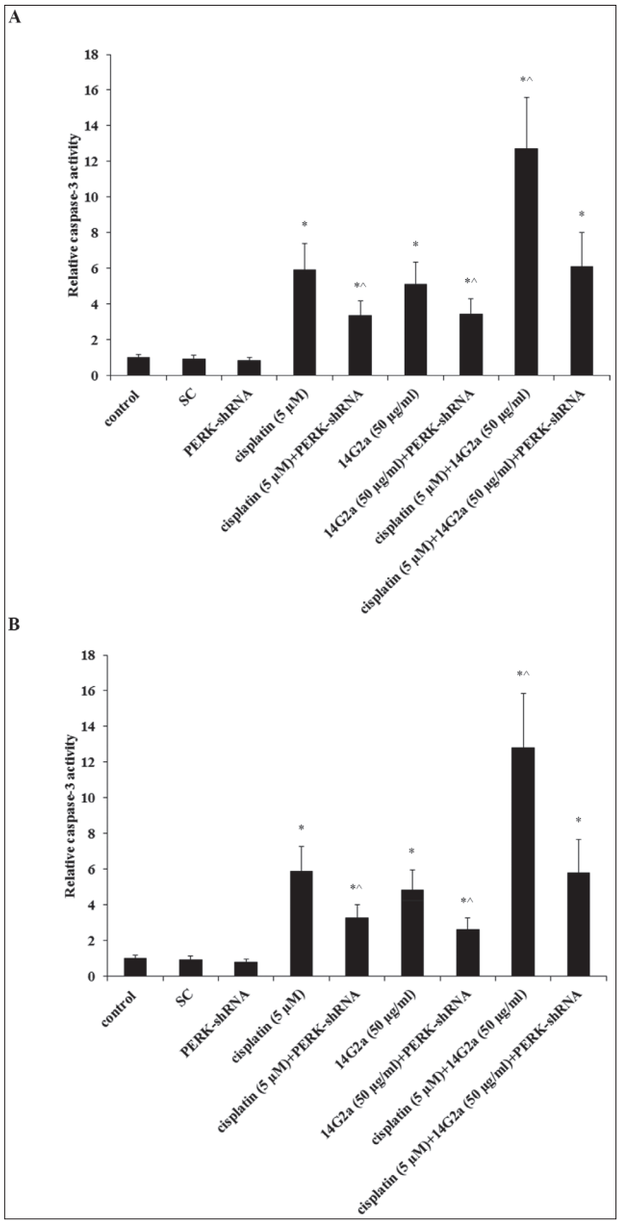

Fig. 6: Caspase 3 activities in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a with or without PERK knockdown
(A) MG-63 and (*B*) Saos-2 OS cells with or without transduction of human PERK-shRNA or scramble control shRNA (SC) lentiviral particles were treated with cisplatin and/or 14G2a at indicated concentrations for 48 h. The caspase-3 activity was measured with a colorimetric caspase 3 assay kit and expressed as fold changes to that of untreated control cells (designated as 1). *p<0.05 vs. control and SC; ^p<0.05 vs. cisplatin (5 μM) and 14G2a (50 mg/ml).

4.4. Western blot analysis

Whole cell lysates were extracted by incubating the cells with lysis buffer (50 mM Tris/HCl pH 7.2, 150 mM NaCl, l% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride; all purchased from Sigma-Aldrich) on ice for 30 min and removing cell debris by centrifugation at 2000 x g for 15 min at 4 °C. Equal amount of proteins for each sample were separated by 10% SDS-polyacrylamide gel and plotted onto a polyvinylidene difluoride microporous membrane (Millipore, Beijing, China). The membranes were blocked with 5% skim milk powder in TBS-T (Cat. No. SRE0031; Sigma-Aldrich) for 2 h and incubated for 1 h at room temperature with a 1:1000 dilution of rabbit anti-human phosphorylated PERK (p-PERK) (Thr 981) polyclonal antibody (Cat. No. sc-32577; Santa Cruz Biotechnology), rabbit anti-human PERK polyclonal antibody (H-300) (Cat. No. sc-13973; Santa Cruz Biotechnology), rabbit anti-human phosphorylated eIF2α (p-eIF2α) (Ser 52) polyclonal antibody (sc-101670; Santa Cruz Biotechnology), rabbit anti-human eIF2α polyclonal antibody (FL-315) (sc-11386; Santa Cruz Biotechnology), or mouse anti-human β-actin monoclonal antibody (Cat. No. sc-130301; Santa Cruz Biotechnology) and then washed in TBS-T and revealed using a bovine anti-rabbit (Cat. No. sc-2370; Santa Cruz Biotechnology) or bovine anti-mouse (Cat. No. sc-2371; Santa Cruz Biotechnology) secondary antibody (1:5000, 1 hour). Peroxidase was revealed with an ECL kit purchased from GE Healthcare (Shanghai, China). Three independent experiments were performed.

4.5. Caspase-3 activity assay

The activity of caspase-3 was determined using a colorimetric Caspase-3 Assay Kit (Cat. No. ab39401) from Abcam (Cambridge, MA, USA). Briefly, the assays were performed in 96-well plates by incubating 20 ml cell lysate protein per sample in 70 ml reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad, and 10% glycerol) containing 10 ml caspase-3 substrate (2 mM). The lysates were then incubated at 37 °C for 6 h, after which the samples were assayed using an iMark Microplate Absorbance Reader (Cat. No. 1681130; Bio-Rad Laboratories, Beijing, China) at 405 nm. Each experiment was repeated for three independent times in duplicates.

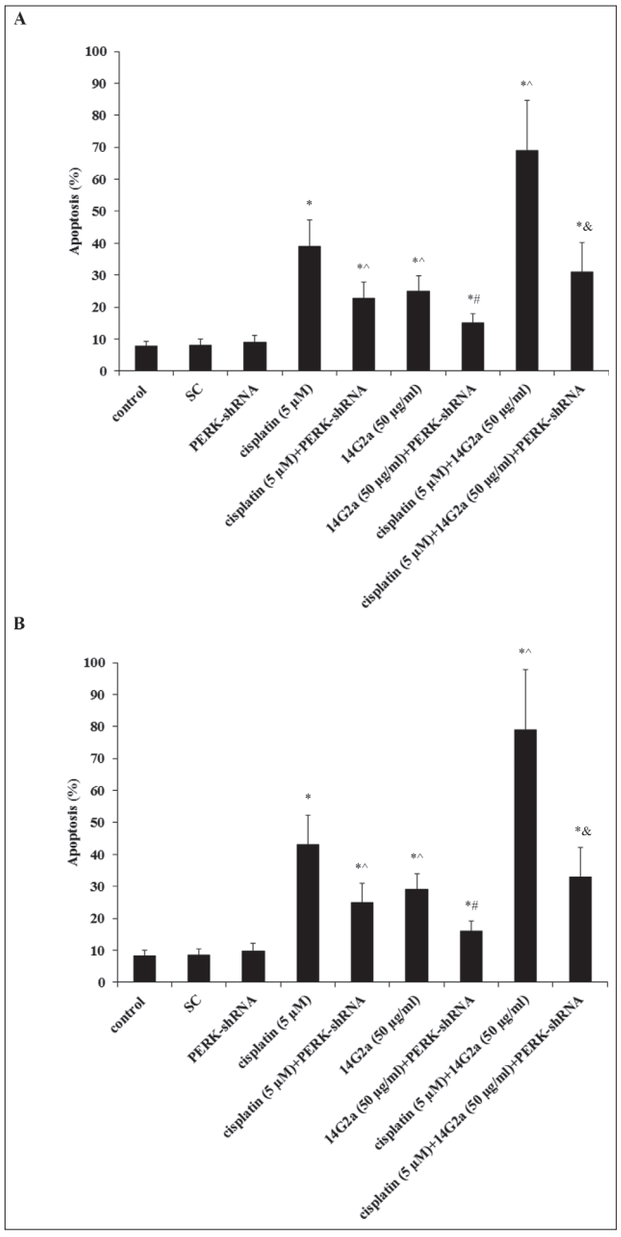

Fig. 7: Apoptosis in osteosarcoma (OS) cells treated with cisplatin and/or 14G2a with or without PERK knockdown
(A) MG-63 and (*B*) Saos-2 OS cells with or without transduction of human PERK-shRNA or scramble control shRNA (SC) lentiviral particles were treated with cisplatin and/or 14G2a at indicated concentrations for 48 h. Untreated cells were used as a control. The apoptosis rate (in percentage of total cells) was measured with a microplate reader-based apoptosis detection kit. *p<0.05 vs. control and SC; ^p<0.05 vs. cisplatin (5 μM); #p<0.05 vs. 14G2a (50 mg/ml); $^{\&}p$<0.05 vs. cisplatin (5 μM)+14G2a (50 mg/ml).

4.6. Statistical analysis

Statistical analyses were performed with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). All data values were expressed as means±SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by *post hoc* pairwise comparisons with Tukey's tests. p<0.05 was considered statistically significant in this study.

Acknowledgements: This work was supported by Hunan Provincial Natural Science Foundation (grants #14B4255 and #15A1624), Changsha, Hunan, China.

Conflicts of interest: None declared.

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