Treatment with MG-132 and TSA induced apoptosis in OS-RC-2 cells by increasing oxidative stress and decreasing the expression of NF-kappa B

MIQING XU[1], HUI XIE[1,2], YUN ZHONG[3], JUNJIAN MA[1], SHIMING LIU[3], HUI YANG[4,]

[1]Department of Geriatrics, Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China.
[2]Department of Kidney, Central Hospital, Kaiping, Guangdong, China.
[3]Guangzhou Institute of Cardiovascular Disease, Guangzhou, China.
[4]Department of Gastroenterology, Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China. Tel. +86-020-34153522. email: yanghui030454@gmail.com

Abstract. *Xu M, Xie H, Zhong Y, Ma J, Liu S, Yang H. 2015. Treatment with MG-132 and TSA induced apoptosis in OS-RC-2 cells by increasing oxidative stress and decreasing the expression of NF-kappa B. Nusantara Bioscience 7: 20-25.* Here we assessed the effect of the proteasome inhibitor MG-132 alone or in combination with the histone deacetylase inhibitor TSA on human renal cell on human renal cell carcinoma cells in vitro and we explored the underlying molecular mechanisms.OS-RC-2 cells were treated byMG-132 alone or in combination with TSA and/or NAc. The MTT assay and flow cytometry (FCM) were used to determine cell viability, ROS levels and apoptosis. Expression of Bax and NF-kappa B p65 proteins was quantified by Western blotting. MG-132significantly increased the level of ROS, inhibited cell growth and induced apoptosis in a dose- and time-dependent manner. Both ROS and apoptosis were further increased following the combined addition of MG132 and TSA. In all cases, expression of p65 was down-regulated. MG-132 alone or in combination with TSA induced apoptosis in OS-RC-2 cells through the generation of ROS and the down-regulation of p65 expression.

Keywords: Human renal cell carcinoma, MG-132, NF- B, p65, ROS, trichostatin A.

INTRODUCTION

Renal cell carcinoma (RCC) is responsible for 80%~90% of renal and 2%~3% of all adult malignancies. The incidence of RCC has been rising steadily. Currently, 62.7% of patients with renal cell carcinoma are asymptomatic, 15.2 % of these patients have metastatic lesions, and 11.9% of these are metastatic lesions that affect multiple viscera (Motzer et al. 2009). Advanced renal cell carcinoma is resistant to the current therapeutic options available: radiotherapy, chemotherapy and cytokine therapy. Therefore, there is a pressing need to develop novel therapeutic strategies for RCC.

Many tumors arise from epigenetic abnormalities or gene mutations (Virani et al. 2012). One of the identified defects is the level of histone acetylation. Histone acetylation is controlled by two enzymes with opposite roles, namely histone acetylation (HATs) and histone deacetylation (HDACs) enzymes. HAT spromote histone acetylation, stretch the nucleosome structure and activate transcription. In contrast, HDACs reduce histone acetylation and inhibit gene expression. Studies have shown that overexpression of HDACs can lead to tumor formation (Weichert et al. 2008a; Weichert et al. 2008b). Therefore, identifying regulators of HDAC expression may provide novel targets for therapeutic intervention. In fact, HDAC inhibitors (HDACi) are becoming a new class of promising anticancer drugs. Studies have also shown that HDACi suppress tumor growth in leukemia, mesothelioma

and cancers of the lung, breast, liver and prostate through their induction of cell cycle arrest, apoptosis and their inhibition of metastasis and tumor angiogenesis. Together, these characteristics enhance their efficacy in chemotherapy and radiotherapy (Kang et al. 2008; Qian et al. 2008; Rathkopf et al. 2010; Yang et al. 2011).

The proteasome system plays a role in cellular processes such as cell differentiation, cell cycle and apoptosis through the regulation of the turnover of proteins involved in these pathways (Sorolla et al. 2008; Sterz et al. 2008). Inhibiting the proteasome can lead to cell cycle arrest and apoptosis as cyclin synthesis is blocked and pro-apoptotic proteins accumulate (Sterz et al. 2008). Proteasome function could be targeted in cancer cells. These proteasome inhibitors trigger apoptosis by stimulating the formation of ROS in cancer cells and inhibiting the expression of NF-κB (Fan et al. 2011; Ko et al. 2010).

Several studies have shown that HDACi suppressed the progression of RCC through the induction of apoptosis and cell cycle arrest (Sato et al. 2012; Xu et al. 2013). Considerable attention has focused on the antitumor effect HDACi on RCC as well as proteasome inhibitors-induced apoptosis of cancer cells. However, very few studies have examined the combination antitumor effect between HDACi and proteasome inhibitors on RCC. Here, we treated human renal carcinoma OS-RC-2 cells with MG-132 alone or in combination with the HDACi trichostatin A (TSA) in vitro and explored the effects on apoptosis. We

found that MG-132 significantly increased the level of ROS in cells and consequently inhibited cell growth and induced apoptosis in a dose-and time-dependent manner. When combined with TSA, the rate of apoptosis was significantly increased. In addition, MG-132 alone or in combination with TSA suppressed the expression of p65. Together, these results suggest that MG-132 alone or in combination with TSA induced apoptosis through the generation of ROS and the suppression of NF- B expression.

MATERIALS AND METHODS

Reagents

TSA, MG-132 and the ROS inhibitor NAc were purchased from Sigma-Aldrich (St. Louis, MO).Fetal bovine serum (FBS), RPMI-1640 culture media, penicillin/streptomycin solution and trypsin were obtained from Invitrogen (Carlsbad, CA). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): Bax, p65 and GAPDH. MTT cell proliferation and cytotoxicity detection kits and the annexin V-FITC/PI apoptosis detection kit were purchased from Calbiochem (San Diego, CA). The Total ROS Detection Kit was obtained from Enzo Life Sciences (Farmingdale, NY).

Cell culture and treatment

OS-RC-2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 culture medium as specified by the cell bank. Cells were seeded at a density of 5×10^3 cells per well (96-well plates), 5×10^5 cells (T-60 culture dish), or 1×10^6 cells (T-100 culture dish) and cultured overnight in the presence of DMSO, NAc, TSA or MG-132 alone or in combination with TSA or NAc. The final concentration of DMSO in the culture medium was 0.1% in all experiments. Media was changed every 24 h and cells were pretreated with NAc for 2 hrs.

Cell viability assay

Cell viability was measured using an MTT cell proliferation kit and a cytotoxicity detection kit following the manufacturer's instructions. Briefly, OS-RC-2 cells were seeded in a 96-well culture plate and incubated with different concentrations of MG-132 (0.01, 0.05, 0.1, 0.5 and 1.0μM), 0.5μM TSA or MG-132 and TSA with or without 5 mM NAc for 24, 48, or 72 h. Absorbance at 550nm was measured on a microplate reader. Experiments were performed in triplicate and repeated four times.

Cell apoptosis assay

OS-RC-2 cells were treated with DMSO, MG-132 (0.05, 0.1, 0.5 or 1μM), 0.5μM TSA or MG-132 and TSA with or without 5 mM NAc for 48 h. At this point, cells were harvested and apoptosis was measured following the manufacturer's instructions using a NAVIOS™ flow cytometer. The apoptotic cells were quantitatively analyzed using Annexin V-FITC and propidium iodide (PI).

ROS Detection

OS-RC-2 cells (5×10^5 cells per T-60 culture dish) were treated with either DMSO, 0.1μM MG-132, 0.5μM TSA or MG-132 and TSA with or without 5 mM NAc pretreatment for 48h. At this point, cells were harvested and resuspended in 500μL ROS Detection Solution as specified by the manufacturer. Cells were stained for 30 min in the dark and ROS were measured by flow cytometry using a NAVIOS™ flow cytometer.

Western blotting

OS-RC-2 cells were collected and lysed in the CytoBuster™ protein extraction reagent containing protease inhibitors. Protein concentration was quantified using the BCA protein assay kit following the manufacturer's instructions. Samples were boiled, resolved on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to nitrocellulose membranes. Blocked membranes were incubated with antibodies for Bcl2, Bax, p65 or GAPDH as a loading control. The expression of the targeted proteins was measured using a chemiluminescence kit following the manufacturers' instruction. The intensity of each band was captured digitally and measured using image-pro-plus software.

Statistical analysis

The data are presented as mean±SD of at least 3 independent experiments and analyzed using a one- way analysis of variance (ANOVA) and SPSS 16.0 software. A P-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

MG-132 alone or combination with TSA decreased the viability of OS-RC-2 cells

To assess the growth inhibitory effect of MG-132 on OS-RC-2 cells, the MTT assay was used to measure the viability of OS-RC-2 cells exposed to various concentrations of MG-132 for 24, 48, and 72 h. As shown in Figure 1, MG-132 reduced the viability of OS-RC-2 cells in a concentration- and time-dependent manner (Figure 1A). To study the growth inhibitory effect of combination treatment on OS-RC-2 cells, OS-RC-2 cells were treated with a cocktail treatment of MG-132 (0.1μM) and TSA (0.5μM) and cell viability was measured by the MTT assay. The combination treatment was shown to have a synergistic time-dependent inhibitory effect on the survival of OS-RC-2 cells (Figure 1B). The Ros inhibitor NAc alone had no effect in regulating OS-RC-2 cell survival. However, MG-132 alone or combination with TSA-decreased OS-RC-2 cell survival was significantly increased by the Ros inhibitor NAc (Figure 1B).

MG-132 alone or combination with TSA induced apoptosis in OC-RC-2 cells

To investigate the effect of MG-132 on apoptosis, the Annexin V/PI assay was performed. OC-RC-2 cells were treated with various concentrations of MG-132 for 48h to induce apoptosis. MG-132significantly increased the
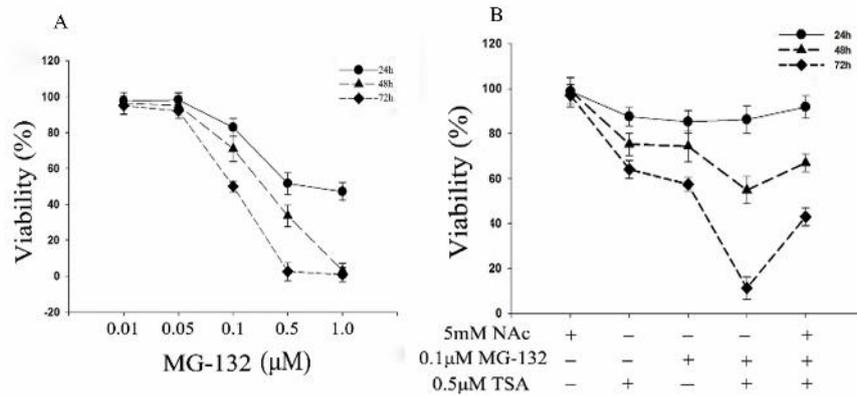
Figure 1. MG-132 alone or combination with TSA decreased the viability of OS-RC-2 cells. Cell viability was measured using the MTT assay. The survival rate of cells was calculated as a percentage of the control value. Data are expressed as mean±SD from three independent experiments.
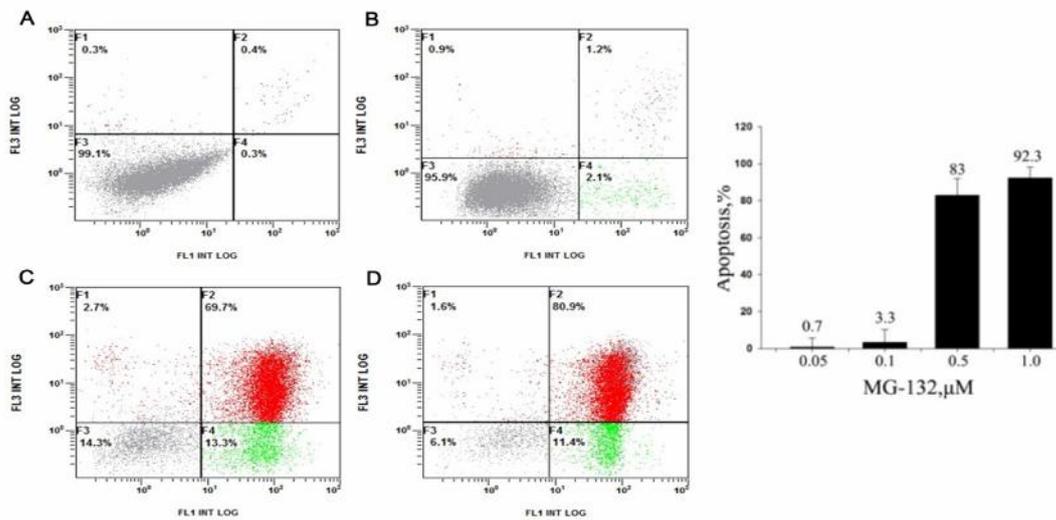

Figure 2. Effect of MG-132 on apoptosis of OS-RC-2 cells. Concentrations of MG-132: A. 0.05μM; B. 0.1μM; C. 0.5μM; D. 1 m. The percentage of apoptotic cells was determined by flow cytometry analysis of annexin V-/PI- stained cells. The experiment was repeated for 3 times.
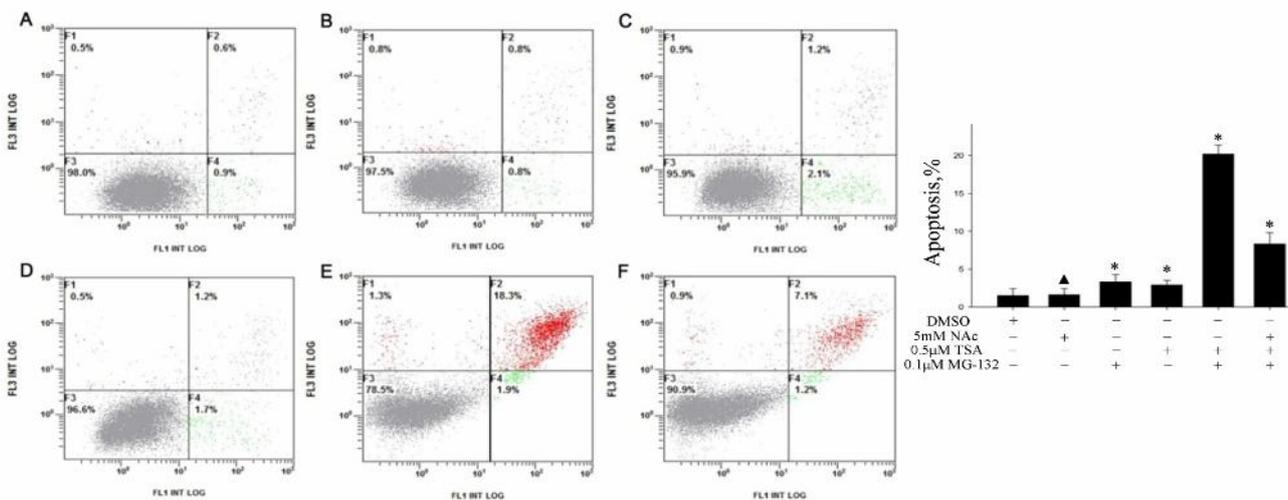

Figure 3. MG-132 in combination with TSA increased the rate of apoptosis in OS-RC-2 cells. Treatments: A. 0.1% DMDSO; B. 5mM NAc; C. 0.1μM MG-132; D. 0.5μM TSA; E. 0.1μM MG-132+0.5μM TSA; F. 0.1μM MG-132+0.5μM TSA G+5mM NAc. The percentage of apoptotic cells was determined by flow cytometric analysis of annexin V-/PI- stained cells. This experiment was repeated for 3 times. P>0.05, [*]P<0.05 vs DMSO-treated group, treatment time 48h.
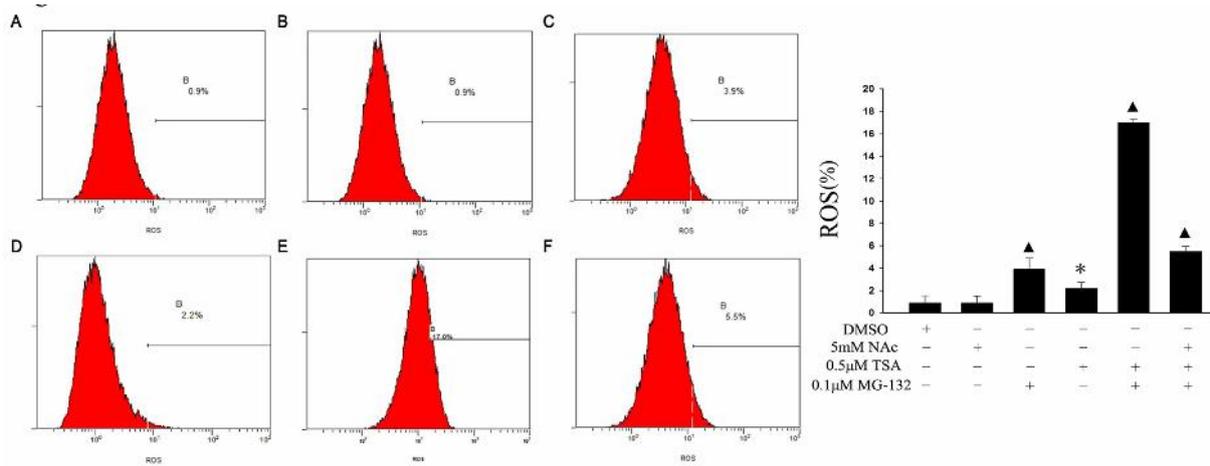
Figure 4. Effect of MG-132 alone or combined with TSA on the level of ROS. Treatments: A. 0.1% DMSO; B. 5mM NAc; C. 0.1μM MG-132; D. 0.5μM TSA; E. 0.1μM MG-132+0.5μM TSA; F. 0.1μM MG-132+0.5μM TSA +5mM NAc. The level of ROS was determined by flow cytometric analysis. This experiment was repeated for 3 times.　$P>0.05$, $*P<0.05$ vs DMSO-treated group, treatment time 48h..
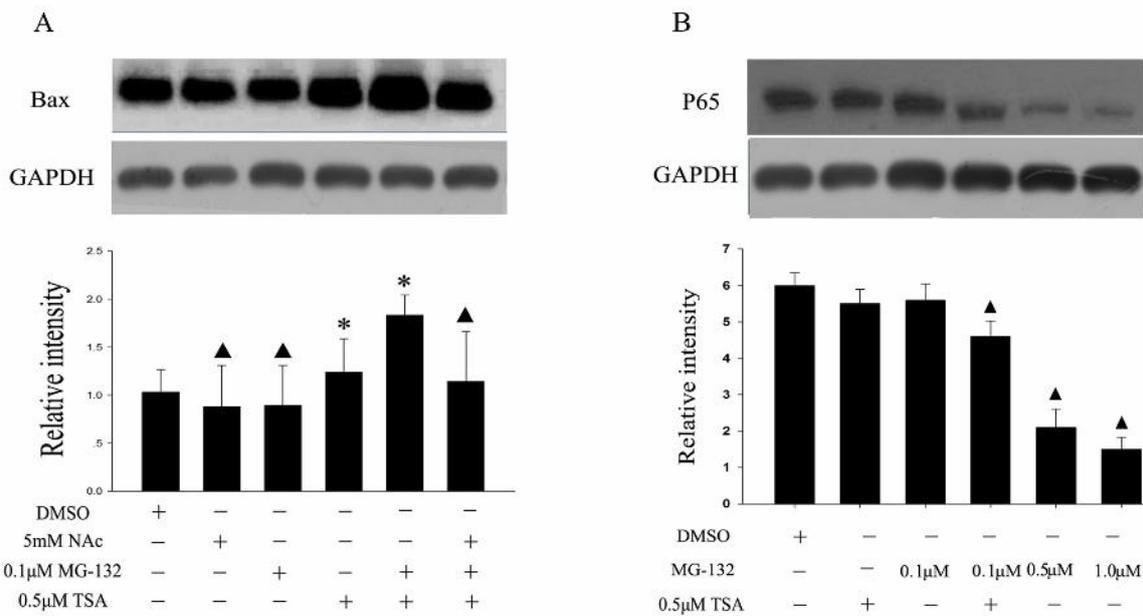

Figure 5. Effect of MG-132 alone or combined with TSA on the expression of Bax and P65 in OS-RC-2 cells. Protein expression was determined by Western blotting. GAPDH was used as the internal control. This experiment was repeated for 3 times. Bars represent mean±SD of 3 independent experiments. A: Bax, B:P65;　$P>0.05$,$*P<0.05$ vs DMSO-treated group.

percentage of apoptotic OS-RC-2 cells from 3.3% at 0.1μM ,and 83% at 0.5μM (p<0.01) (Figure 2). When OS-RC-2 cells were treated with 0.1μM MG-132 combined with 0.5μM TSA , the percentage of apoptotic OS-RC-2 cells (20.2%) was significantly increased than MG-132 or TSA treatment (p<0.01) (Figure 3). The combination treatment was shown to have a synergistically induced apoptosis in OC-RC-2 cells. When OS-RC-2 cells were pretreated 2 hr with NAc, the percentage of apoptotic OS-RC-2 cells decreased to 8.3% (Figure 3). The combination treatment-induced apoptosis of OS-RC-2 cells was significantly reduced by NAc.

The apoptotic effect of MG-132 alone or combined with TSA on OS-RC-2 cells was associated with oxidative stress

To investigate the ROS-generated effect of MG-132 alone or combined with TSA on OS-RC-2 cells, the ROS Detection Kit was performed to measure the ROS generation. After OS-RC-2 cells were treated with MG-132 (0.5μM), TSA or the combination treatment for 48h, the ROS generation was minor increased in the single treatment group, however, the ROS generation was significantly increased to 17.8% in the combination treatment group (p<0.01) (Figure 4).When OS-RC-2 cells were pretreated 2 hr with NAc, the percentage of ROS was

significantly decreased to 5.5% (Figure 4). It indicated that the apoptotic effect of MG-132 alone or combined with TSA on OS-RC-2 cells was associated with oxidative stress.

The expression levels of protein Bax and p65 were associated with the apoptotic effect induced by MG-132 and/or TSA treatment

Study was performed to investigate the effect of MG-132 and/or TSA treatment on the expression of Bax and p65 protein. Western blot analysis showed that MG-132 alone or combined with TSA increased the protein expression of Bax, and when pretreated 2 hr with NAc 5mM the protein expression of Bax decreased (Figure 5A). The protein expression of p65 was decreased in a concentration-dependent manner in OS-RC-2 cells in MG-132 treatment group. When OS-RC-2 cells were treated with MG-132 and TSA, a synergistic down-regulation of p65 was observed (Figure 5B).

Discussion

Several studies have reported that the proteasome inhibitor MG-132 induces apoptosis in multiple myeloma, squamous cell carcinoma and cutaneous melanoma cells through the activation of caspase 2, 3,8 and 9 (Chen et al. 2011; Li et al. 2007) . In addition, MG-132 has been reported to reduce cell proliferation and induce cell cycle arrest and apoptosis in glioma and gastric cancer cells through the activation of the c-*jun* and *erk* signaling pathways (Fan et al. 2011; Wu et al. 2010) . Here, the present data was shown that MG-132 exhibited a concentration- and time-dependent inhibitory effect on cell proliferation and promotes apoptosis of human renal cell carcinoma OS-RC-2 cells in vitro. Western blot analysis of cell lysates from treated cultures showed that the expression of the pro-apoptotic Bax protein was increased. Previous studies have reported that MG-132 can induce apoptosis in gliomas and endometrial tumor cells by increasing ROS (Fan et al. 2011; Llobet et al. 2008) . In our study, we found that the level of ROS increased in OS-RC-2 cells following treatment with MG-132 and this increase was inhibited by the ROS inhibitor NAc. In addition, cell proliferation and apoptosis were significantly reduced in the presence of NAc, suggesting MG-132 triggers apoptosis in OS-RC-2 cells by increasing the generation of ROS.

Previous studies have confirmed thatMG-132 and bortezomib, another proteasome inhibitor, can inhibit cancer cells from breast, lung, liver and prostate by inhibiting proliferation and inducing apoptosis through the down-regulation of the expression of the NF- B family subtype 65 (Ko et al. 2010; Sterz et al. 2008) . In our study, MG-132 was also observed to decrease the expression of p65 in OS-RC-2 cells in a concentration-dependent manner.MG-132has also been reported to inhibit cell proliferation and alter the phenotype of neuroendocrine tumors cells and to significantly prevent DNA repair in cancer cells without impacting normal cells (Sato et al. 2012; Takezawa et al. 2008) . In those studies, the concentrationofMG-132used ranged from 1 to 20μM. However, MG-132 was used at a concentration of 0.1μM

and efficiently induced apoptosis in OS-RC-2 cells. Therefore, MG-132is a promising therapeutic option for renal cell carcinoma.

Cancer therapy regimens involve a combination of different drugs. Studies have confirmed that HDACi (SAHA and TSA) inhibit cell proliferation, increase the generation of ROS, induce cell-cycle arrest and apoptosis (Miller et al. 2011; Sato et al. 2012) . In addition, several studies have reported that proteasome inhibitors can inhibit the expression of NF- B and increase ROS leading to apoptosis in cancer cells (Sterz et al. 2008) . Therefore, combining proteasome with HDAC inhibitors might result in a more effective anticancer regimen. In fact, the combination of MG-132 and an HDACi (SAHA, TSA, sodium butyrate) induces apoptosis in human breast cancer cells and retinoblastoma tumors through the inhibition of expression of the NF- B sub-types p65/p50 (Domingo-Domenech et al. 2008) . Bortezomib, another proteasome inhibitor, has also been reported to induce apoptosis synergistically in vivo and in renal cancer cell lines (Sato et al. 2012). In this study, MG-132 alone or in combination with TSA was used to treat renal cancer OS-RC-2 cells in vitro. Our results are similar to what has been reported for other proteasome and HDAC inhibitors, suggesting that the combination treatment has a stronger effect on the up-regulation of ROS, the inhibition of p65 and the induction of apoptosis in cancer cells.

Accumulation of ROS in cells can damage DNA and oxidize fat and protein which can lead to cardiovascular, metabolic or neurological disease, inflammation and cancer (Ziech et al. 2010) . Compared to normal cells, cancer cells have a higher basal level of ROS and are therefore more prone to go into apoptosis if ROS levels fluctuate slightly (Chen et al. 2011; Kim et al. 2012; Saito et al. 2006; Yang et al. 2012) . Several recent reports have highlighted the roles of several compounds such as jacaranone, antrodiacamphorata, gambogic acid, diosgenin and dihydroartemisinin on cancer cells where they serve to induce apoptosis by increasing ROS generation (Kim et al. 2012; Kong et al. 2012) . The present data was shown that a combination of MG-132 and TSA treatment increasing the generation of ROS and apoptosis in OS-RC-2 cells, it indicates that combination of MG-132 and TSA treatment has practical applications in therapy.

ROS can interact with NF- B through several signaling pathways. As ROS levels fluctuate, the expression of NF- B may be promoted or inhibited, and depending on the tumor type changes in the expression of NF- B may further influence ROS levels (Bubici et al. 2006; Morgan and Liu, 2011) . Here, we showed that 0.1 μM MG-132 alone or combination with0.5μM TSA increased ROS but did not upregulate the expression of p65 and did not induce apoptosis in OS-RC-2 cells. Szu-Ying (Wu et al. 2012) confirmed that Physalin F induced apoptosis in renal cancer cells by increasing the level of ROS and promoting the expression of NF- B. Therefore, increasing the generation of ROS and suppressing the expression of NF- B is an effective therapeutic option for renal cancer. MG-132 can also activate c-*jun* signaling pathways and induce apoptosis in prostate cancer cells and previous studies have shown

that TSA also induced apoptosis in OS-RC-2 cells through the activation of c-*jun* signaling pathways (Li et al. 2007; Xu et al. 2013) . Therefore, activation of c-*jun* is perhaps another mechanism through which TSA and MG-132induce apoptosis in renal cancer cells.

In summary, here we found that the basal level of ROS in OS-RC-2 cells is not high and TSA did not promote the expression of p65 in renal cancer cells. Addition of the proteasome inhibitor MG-132 increased ROS generation and induced apoptosis in renal cell carcinoma cells, an effect which was enhanced when MG-132 was used in combination with TSA. Finally, MG-132 alone or in combination with TSA inhibited the expression of p65 and increased the expression Bax, and this may be one of the mechanisms through which MG-132 promotes apoptosis in renal cell carcinoma.

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