MG-132 reverses multidrug resistance by activating the JNK signaling pathway in FaDu/T cells

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Abstract. Multidrug resistance (MDR) is a major impediment to cancer therapy. MG-132 has been identified to be effective against MDR in several types of cancer. However, the mechanism of MG-132 in head and neck squamous cell carcinomas remains unknown. Based on our previous study, the present detected *P-gp* and P-gp expression in hypopharyngeal carcinoma FaDu cells, revealing that their expression was lower than that observed in the MDR cell line FaDu/T. To reverse the MDR of FaDu/T cells, the present study introduced MG-132 and demonstrated that the high expression of *P-gp*/P-gp in FaDu/T cells was attenuated in a time-dependent manner. MG-132 also strengthened the sensitivity of FaDu/T cells to multidrugs. c-Jun N-terminal kinase (JNK) activation was further observed in FaDu/T cells. However, *P-gp*/P-gp did not decrease when FaDu/T cells were pretreated with SP600125. These results indicated that MG-132 reversed the MDR of hypopharyngeal carcinoma by downregulating *P-gp*/P-gp, and the underlying mechanism may be associated with the activation the of the JNK signaling pathway.

Introduction

A significant obstacle in the clinical treatment of cancer patients is multidrug resistance (MDR) (1). Many genes are reportedly related with MDR. Among them, the most significant are ATP-binding cassette (ABC) genes (2). Humans have 49 ABC genes, and the high expression of these genes in cancer patients results in decreased intracellular accumulation of chemotherapy drugs in spite of their different chemical structures (3). As the first identified and typical ABC transporter, *P-gp*/P-gp has the MDR characteristic of effluxing a number of commonly used chemotherapeutic agents, e.g., Taxol, doxorubicin, vincristine, vinblastine, colchicine, actinomycin D, and mitomycin C (4). Thus, the high expression of P-gp plays a critical role in many kinds of cancer-chemotherapy failure, and identifying approaches to overcoming P-gp-mediated drug resistance is urgent.

The development of MDR reversal has progressed for over 35 years (5). Classical chemosensitizers including verapamil, cyclosporine A, and PSC833 can reverse P-gp-mediated MDR, but their toxicity and other side effects *in vivo* limit their clinical application (6). To overcome their low efficiency and high toxicity in cancer treatment, the proteasome inhibitor MG132 has been found to be a potent P-gp-inhibitor (7,8). To elucidate the molecular basis underlying the reversal of *P-gp*/P-gp by MG-132 in head and neck squamous cell carcinomas (HNSCCs), we conducted an experiment on the hypopharyngeal carcinoma cell line FaDu and the multidrug resistance (MDR) cell line FaDu/T induced by Taxol that had been established in our previous study.

Materials and methods

Materials. The human hypopharyngeal carcinoma cell line FaDu and human bronchial epithelioid (HBE) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Media and serum were purchased from Gibco; Thermo Fisher Scientific, Inc.(Waltham, MA, USA). The primary antibodies anti-MDR1/P-gp, anti-actin, and anti-nuclear factor-κB (NF-κB) were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The primary antibodies anti-p-c-Jun N-terminal kinase (JNK) and anti-p-c-Jun were from Cell Signaling Technology. All other reagents were from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Cell culture and establishment of the resistant cell line FaDu/T. FaDu and HBE cells were cultured as a monolayer in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg streptomycin at 37˚C in humidified atmosphere composed of 95% air and 5% CO_2.

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The methods of establishing the resistant cell line (FaDu/T) has been described previously (9).

Cytotoxicity test. Cell viability was detected with cell counting kit-8 (CCK-8) assay kits. HBE cells were seeded in 24-well culture plates. The plates were placed in an incubator for 24 h, and the culture medium was changed to collect MG-132. CCK-8 assays were performed 48 h after treatment with different concentrations of MG-132. At the time of the CCK-8 assay, we added 10 μl of CCK-8 solution to each well of the plate, which was incubated for 2 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). Results were used to measure cell growth.

Reverse transcription (RT)- and semi-quantitative polymerase chain reaction (sqPCR). Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc). RT-PCR was performed using the ExScript RT reagent kit (Takara, Dalian, China) in a final volume of 20 μl containing 1 μg of total RNA, 4 μl of 5X ExScript buffer, 1 μl of dNTP mixture, 1 μl of oligo(dT) primer, 0.5 μl of ExScript RTase, 0.5 μl of RNase inhibitor, and RNase-free water to a volume of 20 μl. This reaction was performed at 42˚C for 15 min and terminated by heating at 95˚C for 2 min. PCR was performed following the manufacturer's instructions of Takara Taq™ under the following conditions: Pre-degeneration at 95˚C for 3 min, degeneration at 95˚C for 60 sec, renaturation at 58˚C for 45 sec, and elongation at 72˚C for 60 sec, for a total of 25 cycles. sqPCR was performed by running the products on a 1% agarose gel, and the bands were quantified using ImageJ v1.48 (National Institutes of Health, Bethesda, MD, USA). All experiments were conducted thrice. The P-gp primers were as follows: Forward, 5'-CTGCTCAAGTTAAAGGGGCTAT-3' and reverse, 5'-AACGGTTCGGAAGTTTTCTATT-3'. The actin primers were as follows: Forward, 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTT-3'.

Western blot analysis. Total protein was extracted using radioimmune precipitation assay buffer (Sigma-Aldrich; Merck KGaA) and protein lysis buffer following the manufacturer's protocols. Nuclear proteins were solubilized and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The Bradford method was used to determine the protein concentration of the supernatant. Samples (40 μg of total protein each) were used in western blot analysis with the first antibodies (*P-gp*/P-gp 1:400, mouse antihuman; actin, 1:2,000, mouse antihuman; P-JNK 1:1,000, rabbit antihuman; and p-c-Jun 1:200, goat antihuman). The bands of *P-gp*/P-gp, P-JNK, p-c-Jun, and actin were visualized at apparent molecular weights of 170, 46/54, 39 and 43 kDa, respectively. Relative OD ratio was calculated with NIH software Image J by comparing to actin from three experiments.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical calculations were performed using SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). One-way analysis of variance with a Bonferroni post hoc test were applied to analyze the variance, and P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA and protein levels of P-gp/P-gp in FaDu and FaDu/T cells. Compared with FaDu, *P-gp* (Fig. 1A) and P-gp (Fig. 1B) were upregulated in FaDu/T cells. ImageJ software was used to analyze the relative photodensity using actin as a loading control. Considering a value of 1 for FaDu groups, the relative photodensity of the FaDu/T-200 nM groups was as follows: *P-gp*/actin, 14.24±2.57 and 12.42±2.23; and P-gp/actin, 11.56±5.19 and 12.49±3.60, respectively. Statistical analysis showed significant differences between FaDu and FaDu/T cells (P<0.05).

Downregulation of P-gp/P-gp by MG-132 in FaDu/T cells. To assess the capacity of MG132 in the downregulation of P-gp, 1.5 μM MG-132 was applied in the present research. *P-gp*/P-gp expression in RNA (Fig. 2A) and protein (Fig. 2B) levels both decreased in a time-dependent manner. Considering a value of 1 for FaDu/T (0 h)/actin, the relative photodensity of *P-gp*/actin in FaDu/T groups at 12, 24, 48 and 72 h was as follows: 0.41±0.05, 0.17±0.06, 0.11±0.01 and 0.05±0.006, respectively, Statistical analysis showed significant differences between different time-points (F=252.47; P<0.05). Meanwhile, the relative photodensity of P-gp/actin in FaDu/T groups at 12, 24, 48 and 72 h was as follows: 0.73±0.12, 0.65±0.15, 0.42±0.05 and 0.20±0.02 respectively. Statistical analysis showed significant differences between different times (F=30.59; P<0.05).

Status of JNK signaling pathway in drug-sensitive FaDu cells and in FaDu/T cells treated with MG-132. To examine the activation status of JNK signaling pathway in Taxol-sensitive FaDu cells, these cells were treated with Taxol for 48 h, and then drug-sensitive FaDu cells were collected in a time-dependent manner. Western blot analysis showed that the JNK signaling pathway was activated in Taxol-sensitive FaDu cells. Furthermore, MG-132 functionally reversed the high expression of P-gp and promoted the relative protein level of the JNK signaling pathway phosphorylation in a time-dependent manner when FaDu/T cells were cultured in drug- and serum-free state for 24 h (Fig. 3).

SP600125, the inhibitor of the JNK signal pathway, inhibited the activation of this pathway. To ascertain the inhibitory effect of SP600125 on JNK signaling, we added MG-132 to FaDu/T cells for 24 h after adding SP600125. As shown in Fig. 4, the expression of p-JNK and p-c-Jun did not significantly change.

SP600125 inhibited the MG-132-induced downregulation of P-gp/P-gp in terms of RNA and protein levels. To ascertain the mechanism of MG-132 in downregulating P-gp, FaDu/T cells were pretreated with the JNK signal pathway inhibitor SP600125, followed by 1.5 μM MG-132 for 72 h. As shown in Fig. 5, in the absence of MG-132, FaDu/T cells with or without SP600125 pretreatment showed a similar expression of P-gp. By contrast, MG-132 treatment alone induced a significantly lower expression of P-gp, which can be reversed by pretreatment with SP600125. These results suggested that the JNK signaling pathway was involved in the MG-132-induced downregulation of P-gp in FaDu/T cells. Considering a value

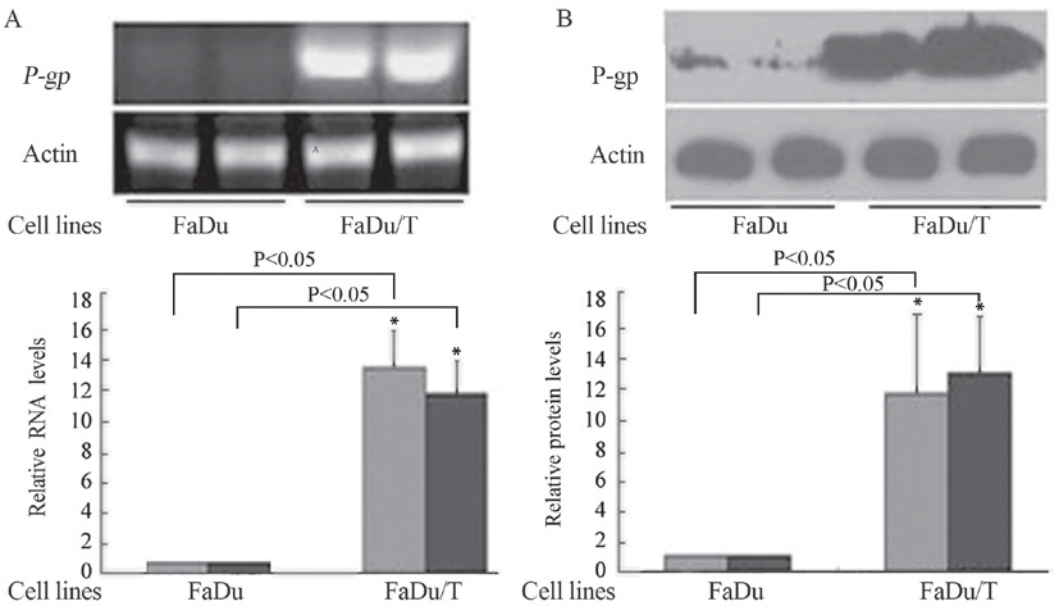

Figure 1. High P-gp expression in FaDu/T cell lines was detected at the mRNA and protein levels when compared with FaDu cells. (A) The multidrug resistance gene *P-gp* levels in FaDu and FaDu/T cells were detected by reverse transcription-quantitative polymerase chain reaction analysis, and (B) the relative protein P-gp levels were detected by western blot analysis. Densitometry revealed that the gene and protein expression of *P-gp*/P-gp increased in FaDu/T cells. Graphs show the quantification of RNA and protein bands by densitometric scanning. The relative RNA and protein levels were presented as the ratio of densities of *P-gp*/P-gp to actin bands; bars within the same group represent repeated bands. Results are expressed as the mean ± standard deviation. *P<0.05, as indicated. P-gp, P-glycoprotein.

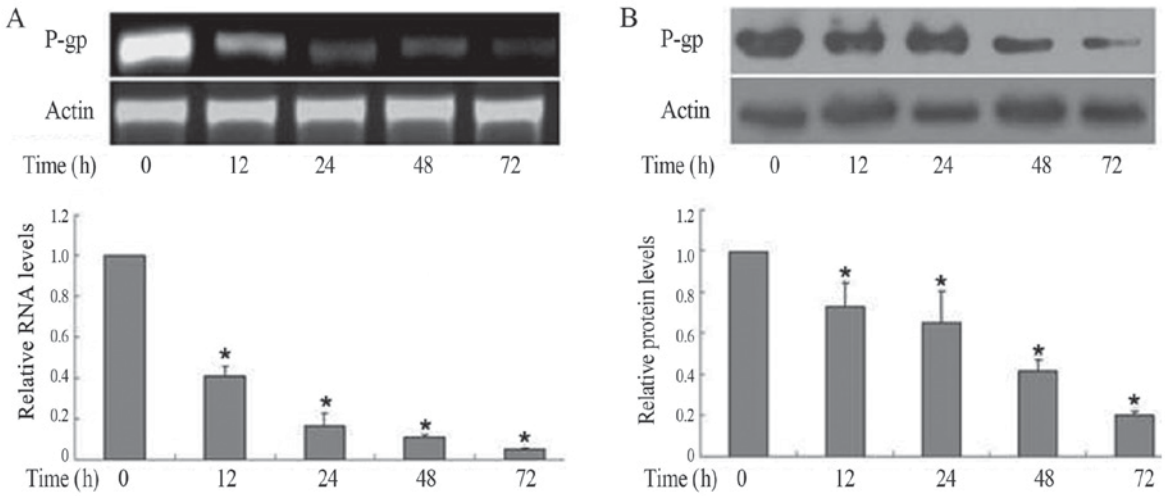

Figure 2. MG-132 downregulates the expression of P-gp at the mRNA and protein levels. The multidrug resistance (A) gene *P-gp* and (B) protein P-gp levels in FaDu/T cells were downregulated by MG-132 in a time-dependent manner. Quantification of RNA and protein bands by densitometric scanning is shown. The relative RNA and protein levels were presented as the ratio of densities of FaDu/T cells to actin bands. Results are expressed as the mean ± standard deviation. *P<0.05 vs. 0 h. P-gp, P-glycoprotein.

of 1 for FaDu/T/actin, the relative photodensity of *P-gp*/actin and P-gp/actin in FaDu/T cells, FaDu/T cells treated with SP600125 and MG-132, and FaDu/T cells singly treated single with SP600125 groups was as follows: 24.23±2.97, 24.65±3.77, 23.88±2.35; 3.24±0.36, 3.22±0.25, and 3.12±0.25, respectively. Statistical analysis showed a significant difference between different groups (P<0.05).

MG-132 inhibited the nuclear translocation of NF-κB in FaDu/T cells. Compared with FaDu cells, the nuclear protein levels of NF-κB in FaDu/T cells markedly increased when using lamin-A as a control. Considering a value of 1 for FaDu/T (0 h)/actin, the relative photodensity of FaDu/actin was 0.08±0.01. Statistical analysis showed a significant difference between FaDu and FaDu/T cells (P<0.05). However, the nuclear translocation of NF-κB was prohibited after short-time incubation of FaDu/T cells with MG-132. Considering a value of 1 for FaDu/T (0 h)/actin, the relative photodensity of FaDu/T/actin (24 and 48 h) was 0.69±0.27 and 0.16±0.01, respectively (Fig. 6). Statistical analysis showed that the difference between different time-points was significant (P<0.05).

Cytotoxicity of MG-132 to HBE cells. To evaluate the clinical value of MG-132, HBE cells were treated with MG-132 in a

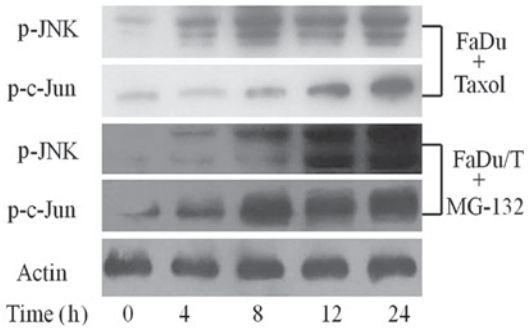

Figure 3. FaDu cells were treated with Taxol for 48 h. Western blot analysis showed that the JNK signaling pathway was activated in Taxol-sensitive FaDu cells. MG-132 activated the JNK signaling pathways in a time-dependent manner in FaDu/T cells. P-JNK and p-c-Jun represented the activation condition of the JNK signaling pathway. With the introduction of MG-132 in FaDu/T cells, the protein levels of p-JNK and p-c-Jun increased gradually. JNK, c-Jun N-terminal kinase; p-, phosphorylated.

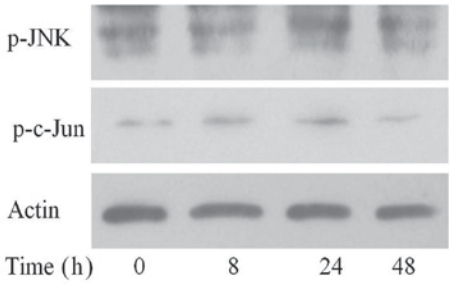

Figure 4. FaDu/T cells were pretreated with SP600125 for 24 h and then treated with MG-132. The protein levels of p-JNK and p-c-Jun did not markedly change. JNK, c-Jun N-terminal kinase; p-, phosphorylated.

concentration-dependent manner. Cell viability was detected with CCK-8 assay kits. As shown in Fig. 7, the viability of HBE cells were decreased significantly with increasing MG-132 concentrations. MG-132 exerted a cytotoxicity effect on HBE cells.

Discussion

To elucidate the molecular mechanism underlying the downregulation of membrane protein *P-gp*/P-gp by MG-132, we have previously established a multidrug-resistant cell line of FaDu to Taxol (FaDu/T) by stepwise exposure of normal FaDu cells to increased concentrations of Taxol for over 18 months. We find that *P-gp*/P-gp (P-glycoprotein) expression increases in FaDu/T cell lines and that the MDR of FaDu/T cells to DDP, 5-FU, Dox, and VCR is enhanced (9). However, when MG-132 was introduced into FaDu/T cells, in addition to decreased P-gp, the MDR to DDP, 5-FU, and VCR also decreased. Based on the above investigation (10), we speculated that P-gp overexpression may be mainly responsible for MDR in FaDu/T cells. Thus, the downregulation of P-gp by MG-132 was crucial to the reversal of MDR. Meanwhile, to clearly determine the mechanism of MG-132 in regulating P-gp in FaDu/T cells, we conducted further experiments.

As a proteasome inhibitor, MG-132 has anticancer effects through other cellular mechanisms, one of which is the activation of the JNK signal pathway (11). As a member of the MAPK family, the activation of the JNK signal pathway plays an important role in the growth, differentiation, and apoptosis of cancer cells (12). Several previous studies have suggested the existence of a negative binding site of AP-1 in the promoter region of the *P-gp* gene; thus, the activation of the JNK/c-Jun/AP-1 signal pathways can inhibit *P-gp* expression in human multidrug-resistant cells (13-15).

In the present study, with the application of MG-132 in FaDu or FaDu/T cells cultured in a drug- and serum-free state, we found decreased P-gp expression in FaDu/T cells a time-dependent manner. We also detected that the JNK signaling pathway was effectively reactivated in a time-dependent manner. These findings, together with theoretical studies, led us to the hypothesis that decreased P-gp expression in FaDu/T cell lines caused by MG-132 was regulated by the JNK signal pathway. However, our data were insufficient to show how AP-1 was involved in P-gp downregulation in FaDu/T cell lines.

To further address this question, FaDu/T cells were pretreated with SP600125 (16), a small molecule inhibitor of the JNK signaling pathway for 24 h. Results showed that the JNK signaling pathway was inactivated. Compared with FaDu/T cells treated with only MG-132, P-gp expression was not significantly decreased. All of the above results indicated that P-gp downregulation was attributed to the activation of the JNK signal pathway.

MG-132 is a potent inhibitor in the degradation of IκB proteins and thus, suppresses the nuclear translocation and activation of NF-κB. Given that the nuclear translocation of NF-κB was closely involved in P-gp expression (17,18), we wondered whether this pathway also existed in hypopharyngeal cancer cells. Fig. 5 shows that compared with FaDu cells, the nuclear translocation of NF-κB in FaDu/T cells increased, which was reversed when FaDu/T cells was in the presence of MG-132 for 48 h. Meanwhile, the expression of P-gp significantly decreased, thereby providing evidence that MG132 downregulated P-gp expression also probably by suppressing NF-κB nuclear translocation. However, FaDu/T cells were pretreated with SP600125, a small molecule inhibitor of the JNK signaling pathway for 24 h. Compared with FaDu/T cells treated with only MG-132, the expression of P-gp did not significantly decrease, although MG-132 can still suppress the activation of NF-κB under this condition. All of the above results indicated that NF-κB can upregulate P-gp when FaDu cells were initially exposed to Taxol. However, in FaDu/T cell lines, the downregulation of P-gp was attributed to the activation of the JNK signal pathway, and the inactivation of NF-κB affected only the termination of P-gp expression. These lines of evidence suggested that the downregulation of P-gp in FaDu/T cells was due to the activation of JNK signaling pathway. The interaction between the function of NF-κB and the JNK signaling pathway warrant further study.

In the present study, we presented evidence for the first time that MG-132 functionally downregulated P-gp expression by activating the JNK/c-Jun/AP-1 signal pathways, which promoted the negative regulation of AP-1 to P-gp. Therefore, the effects of MG-132 on P-gp downregulation may represent, at least in part, a novel strategy for overcoming the P-gp-related MDR of only FaDu cells to Taxol, and more cell lines of hypopharyngeal carcinoma to other chemotherapy agents such as DDP, 5-FU and Afatinib are needed to confirm the present findings in the future.

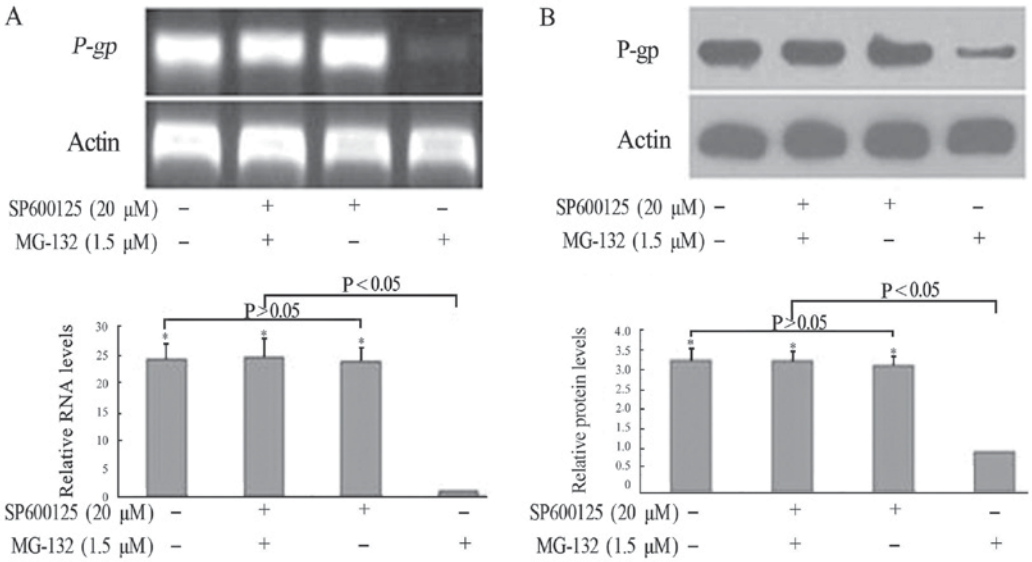

Figure 5. Effects of SP600125 on the MG-132-induced downregulation of P-gp expression at the gene and protein levels. The (A) mRNA and (B) protein levels of P-gp in FaDu/T cells pretreated with or without the JNK signal pathway inhibitor SP600125. In some groups, MG-132 was then introduced. FaDu/T cells underwent similar expression at the mRNA and protein levels (P>0.05). By contrast, MG-132 treatment alone induced a significantly low expression of *P-gp* and P-gp, which was reversed by pretreatment with SP600125. Graphs show the quantification of mRNA and protein bands by densitometric scanning. The relative mRNA and protein levels were presented as the ratio of densities of *P-gp*/P-gp to actin bands. Results are expressed as the mean ± standard deviation. *P<0.05, as indicated. P-gp, P-glycoprotein.

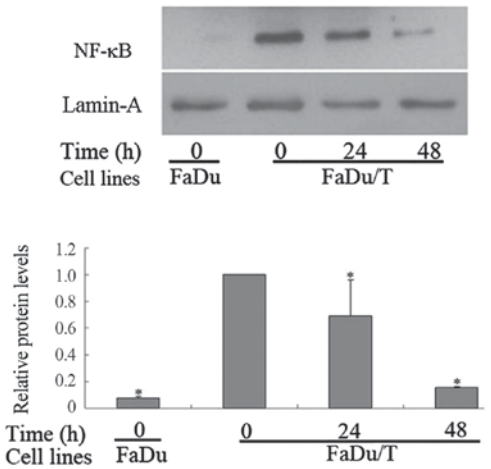

Figure 6. MG-132 inhibits the activation of NF-κB though nuclear translocation. Compared with the FaDu cell lines the, nuclear translocation of NF-κB in FaDu/T cells increased. Conversely, the nuclear translocation of NF-κB was prohibited following the incubation of FaDu/T cells with MG-132 for 48 h. Graphs show the quantification of nuclear NF-κB bands by densitometric scanning. Considering a value of 1 for FaDu/T (0 h)/lamin-A, the relative photodensity of FaDu/T/lamin-A (24 and 48 h) was 0.69±0.27 and 0.16±0.01, respectively. Statistical analysis showed that the difference between different time-points was significant. *P<0.05 vs. 0 h FaDu/T. NF-κB, nuclear factor-κB.

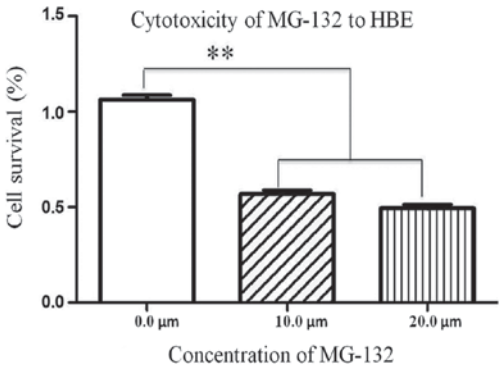

Figure 7. HBE cells were treated with MG-132 at various concentrations (0, 10 and 20 μmol/l) for 72 h, and then cells were subjected to cell counting kit-8 assay to measure cell viability. MG-132 induced HBE cell apoptosis in a concentration-dependent manner. **P<0.01, as indicated. HBE, human bronchial epithelioid.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JM and ZL conceived and designed the research and drafted the manuscript. XxL and XfL acquired, analyzed and interpreted the data, and performed statistical analysis. WX conceived and designed the study, and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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