

# Changes of NF- $\kappa$ B, p53, Bcl-2 and caspase in apoptosis induced by JTE-522 in human gastric adenocarcinoma cell line AGS cells: role of reactive oxygen species

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## Abstract

**AIM:** To identify whether JTE-522 can induce apoptosis in AGS cells and ROS also involved in the process, and to investigate the changes in NF- $\kappa$ B, p53, bcl-2 and caspase in the apoptosis process.

**METHODS:** Cell culture, MTT, Electromicroscopy, agarose gel electrophoresis, lucigenin, Western blot and electrophoretic mobility shift assay (EMSA) analysis were employed to investigate the effect of JTE-522 on cell proliferation and apoptosis in AGS cells and related molecular mechanisms.

**RESULTS:** JTE-522 inhibited the growth of AGS cells and induced the apoptosis. Lucigenin assay showed the generation of ROS in cells under incubation with JTE-522. The increased ROS generation might contribute to the induction of AGS cells to apoptosis. EMSA and Western blot revealed that NF- $\kappa$ B activity was almost completely inhibited by preventing the degradation of I $\kappa$ B $\alpha$ . Additionally, by using Western blot we confirmed that the level of bcl-2 was decreased, whereas p53 showed a great increase following JTE-522 treatment. Their changes were in a dose-dependent manner.

**CONCLUSION:** These findings suggest that reactive oxygen species, NF- $\kappa$ B, p53, bcl-2 and caspase-3 may play an important role in the induction of apoptosis in AGS cells after treatment with JTE-522.

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## INTRODUCTION

Apoptosis is an active cell death process, which requires specific gene regulation. A critical role for p53 in the execution of some forms of apoptosis has been suggested<sup>[1-6]</sup>. This protein is a sequence-specific DNA-binding protein, active as a transcription factor. It has been proposed that p53 may be involved in the cellular response to DNA

damage, producing arrest in the G<sub>1</sub> phase of the cell cycle to allow efficient repair of DNA before entry to S phase, or cell death if the damage is too large to be repaired<sup>[7,8]</sup>. Another gene implicated in apoptosis is bcl-2. The bcl-2 gene product functions as an anti-apoptotic signal, suppressing apoptosis induced by a wide variety of stimuli, including chemotherapeutic drugs and  $\gamma$  radiation<sup>[9-13]</sup>. The exact mechanism of bcl-2 in preventing apoptosis is still not clear. However, bcl-2 has been implicated in cellular control of their redox state<sup>[14]</sup>.

Previous studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) given *in vivo* to rodents and human can inhibit tumor growth<sup>[15,16]</sup>. JTE-522 is a novel NSAIDs, which is a specific inhibitor of cyclooxygenase-2 (COX-2) with significant anti-inflammatory and analgesic properties<sup>[17]</sup>. Some reported that JTE-522 possesses strong chemopreventive activity against colon carcinogenesis<sup>[18]</sup>, but the precise mechanism by which JTE-522 inhibits colon carcinogenesis is not clear. It is often attributed to specific inhibition of arachidonic acid metabolism via coenzymes. However, recent studies showed that the antitumor effect had little connection with NSAIDs inhibitory activity against cyclooxygenase, and was not prevented by exogenous supplementation of 16,16-dimethyl prostaglandin E<sub>2</sub>. Several groups have shown that certain NSAIDs induce apoptosis of tumor cell line, which is associated with the generation of reactive oxygen species (ROS)<sup>[19,20]</sup>. However, the signaling pathway leading to apoptotic cell death remains unclear.

ROS can play a central role in regulating cell proliferation and cell death. Evidence has been obtained that ROS such as superoxide and hydrogen peroxide can influence cell death triggered by internal cues (p53-mediated), external cues (TGF- $\beta$ -mediated) and immunogenic signals (TNF- $\alpha$ )<sup>[21,22]</sup>. In other instances, however, generation of ROS can inhibit apoptosis. Although the mechanism involved is still controversial, redox status and/or hydrogen peroxide have both been proposed as critical factors<sup>[19]</sup>. Therefore it is possible that ROS may play a role in regulating apoptosis in gastric epithelium.

The purpose of the present study is to identify whether JTE-522 can induce apoptosis in AGS cells and ROS are also involved in the process, and to investigate the changes in NF- $\kappa$ B, p53, bcl-2 and caspase in the apoptosis process.

## MATERIALS AND METHODS

### Cell line and reagents

Human gastric adenocarcinoma cell line AGS was provided by Cancer Institute, Zhongshan University. Cells were grown in RPMI-1640 medium and supplemented with 10% new bovine serum, penicillin G (100kU.L<sup>-1</sup>) and kanamycin (0.1g.L<sup>-1</sup>) at 37°C in a 5% CO<sub>2</sub>-95% air atmosphere. Antibodies used in this study included p53, bcl-2, I $\kappa$ B $\alpha$  and Beta actin were obtained from Santa Cruz. All other chemicals were purchased from Sigma Chemical Co (St. Louis,MO,USA).

### MTT assay

AGS cells growing on 96-well plates were treated with JTE-522 (0.1 mmol/L - 1mmol/L) for 72h, untreated cells served as a control. 10 $\mu$ L of the 2.5g.L<sup>-1</sup> stock solution of 3-[4, 5-dimethylthiaoly]-2,

5-diphenyl-tetrazolium bromide (MTT) was added to each well. After 1h of incubation at 37°C, the medium was removed, 50µl of the extraction buffer (10% Triton-X100; 0.1mol/L HCl) was added, and plates were gently shaken for 30min at room temperature. The optical densities were measured at 570nm.

### Morphological and biochemical analysis of apoptosis

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by electron microscopy (EM). Cells were pelleted and fixed with 30mL/L glutaraldehyde in PBS. EM analysis was performed as described previously<sup>[23]</sup>. Oligonucleosomal cleavage of genomic DNA was detected by agarose gel electrophoresis. In brief, genomic DNA isolated as previously described<sup>[24]</sup> was subjected to 1.5% agarose gel electrophoresis, followed by ethidium bromide staining.

### Assay for reactive oxygen species production

Generation of ROS was assessed using lucigenin. AGS cells grown in 75cm<sup>2</sup> culture flasks were incubated for 6h with JTE-522 (0.1-1mmol/L) in the presence or absence of 100µmol/L pyrrolidine dithiocarbamate (PDTc). The cells were then scraped off and washed in cold Hank's buffer. An aliquot containing 1×10<sup>6</sup> cells in 100µL of Hank's buffer was mixed in microtiter wells with 100µl of lucigenin prepared at a concentration of 40µmol/L. Light emission was detected using a Berthold LB96V luminometer for 3min.

### Assessment of caspase activity

Caspase-3 activity was measured using a caspase assay kit according to the supplier's instruction. In brief, caspase-3 fluorogenic substrates (Ac-DEVD-AMC or Ac-IETD-AMC) were incubated with JTE-522-treated with cell lysates for 1h at 37°C, then AMC liberated from Ac-DEVD-AMC or Ac-IETD-AMC was measured using a fluorometric plate reader with an excitation wavelength of 380nm and an emission wavelength of 420-460nm.

### Western blot analysis

The cells were lysed in lysis buffer (25mmol/L hepes, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5mol/L NaCl, 5mmol/L EDTA, 50mmol/L NaF, 0.1mmol/L sodium vanadate, 1mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.1g.L<sup>-1</sup> leupeptin (pH7.8) at 4°C with sonication. The lysates were centrifuged at 15000g for 15min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (42mmol/L Tris-HCl, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol and 0.002% bromophenol blue) was then added to each lysate, which was subsequently boiled for 3min and then electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated sequentially with antibodies against IκBα, p53 and bcl-2 and then with peroxidase-conjugated secondary antibodies in the second reaction. Detection was performed with enhanced chemiluminescence reagent.

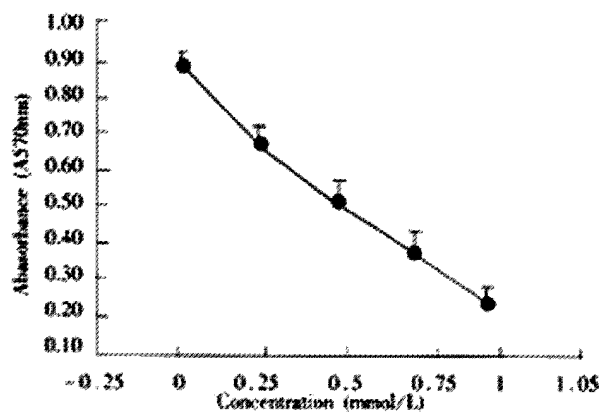
### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from AGS cells treated with JTE-522. Synthetic double-strand oligonucleotides of consensus NF-κB binding sequence, GATCCCAACGGCAGGGGA, were end-labeled with [<sup>32</sup>P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly (dI-dC) in a binding buffer containing 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at room temperature for 30min. For supershift assays, a total of 0.2µg of antibodies against p65 subunit of NF-κB were included in the reaction. DNA-protein complexes were resolved by electrophoresis in a 5% non-denaturing polyacrylamide gel, which was dried and visualized by autoradiography.

## RESULTS

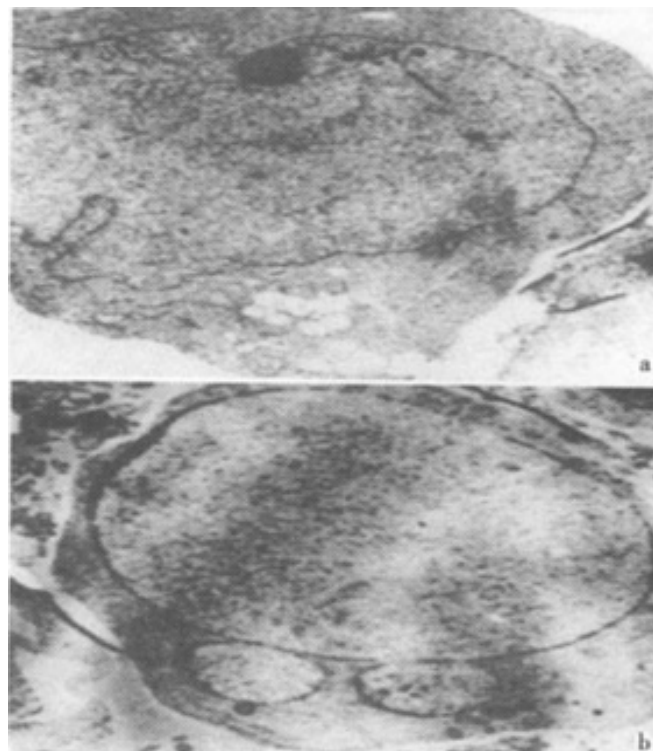
### Effect of JTE-522 on cell proliferation and apoptosis

AGS cells were incubated with various doses of JTE-522 for 72h. Analysis of cell viability using to MTT assay showed that JTE-522 significantly inhibited cell viability. The inhibition of cell viability was dependent on the dose of JTE-522 used (Figure 1).



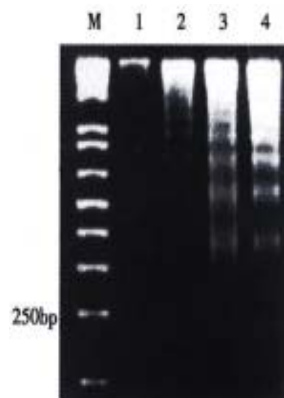
**Figure 1** Effect of JTE-522 on cell growth in AGS cells. The cells were treated with various concentrations of JTE-522 for 72h. The antiproliferative effect was measured by MTT assay. Results are the means±SD from three independent determinations.

The effect was due to apoptosis as demonstrated by EM and electrophoresis of genomic DNA. JTE-522-treated cells showed compacted nuclear chromatin with finely granular masses margined against the nuclear envelope and condensed cytoplasm, the nuclear outline was convoluted and the organelles were preserved (Figure 2) and led to oligonucleosomal cleavage of genomic DNA (Figure 3), which were hallmarks of apoptosis.

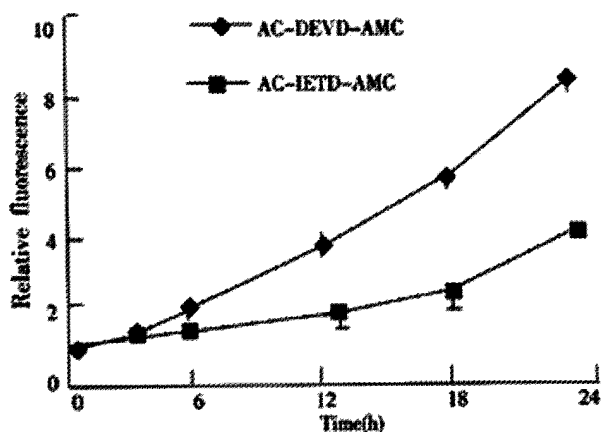


**Figure 2** Electro micrographs of JTE-522-treated AGS cells. Control AGS cells (A), or treated with 1mmol/L (B) JTE-522 for 72h, were examined by EM as in "Materials and Methods". Magnification: ×4000

We next investigated whether the activation of caspase was involved in JTE-522-induced apoptosis of AGS cells. JTE-522-induced apoptosis of AGS cells was accompanied by the induction of caspases activity as demonstrated by the cleavage of Ac-DEVD-AMC and Ac-IETD-AMC, respectively (Figure 4). These results indicated that JTE-522-induced cell death of AGS cells was a typical apoptosis associated with caspase activation.



**Figure 3** DNA ladder pattern formation of AGS cells. Cells treated with different concentrations of JTE-522 for 72h and the formation of oligonucleosomal fragments was determined by 1.5% agarose gel electrophoresis. M, DNA markers; lanes 1-4, AGS cells treated with 0, 0.25, 0.50, 1mmol/L of JTE-522



**Figure 4** Activation of caspase-3 activities by JTE-522 in AGS cells for indicated time period. JTE-522 treatment (0.75mmol/L) induced cleavage of Ac-DEVD-AMC and Ac-IETD-AMC, indicating activation of caspase-3 activity, respectively

#### Effect of JTE-522 on the production of ROS in AGS cells

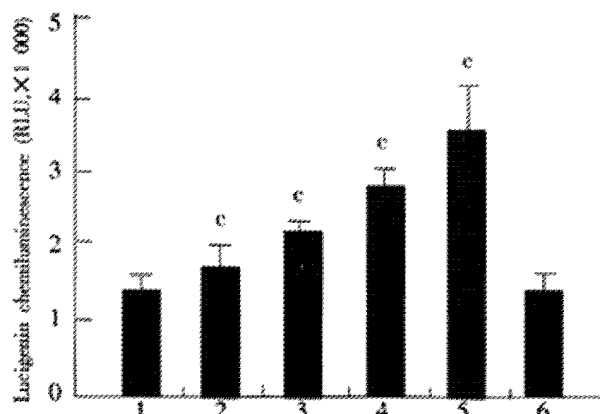
The effect of JTE-522 on the production of ROS in AGS cells, as assessed with lucigenin chemiluminescence, was shown in Figure 5. Chemiluminescence was significantly enhanced by incubation with various doses of JTE-522. This enhancement was prevented by co-incubation with PDTC at 100 $\mu$ mol/L. These results demonstrated the generation of reactive oxygen species in cells under incubation with JTE-522.

#### Effect of JTE-522 on the expression of p53, bcl-2, I $\kappa$ B $\alpha$ and the activation of NF- $\kappa$ B

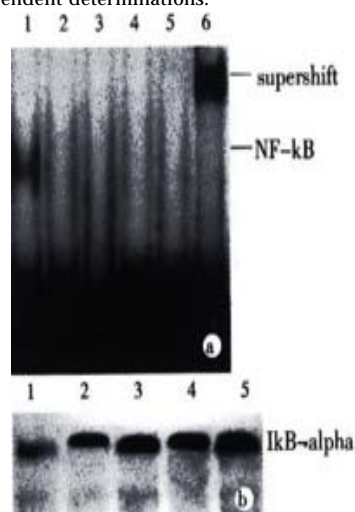
NF- $\kappa$ B plays a complex role in regulating programmed cell death. In many instances the inhibition of NF- $\kappa$ B activity can sensitize cells to death inducers<sup>[25]</sup>. In other instances, however, NF- $\kappa$ B activation has been found to play an important role in the induction of apoptosis. To determine whether the treatment with JTE-522 have any effect in the NF- $\kappa$ B transcriptional factors. We performed EMSA with nuclear extracts prepared from control or treated cells exposed to JTE-522 for various concentrations for 6h. The NF- $\kappa$ B specific complexes found in this cell line were almost complete inhibited in comparison with untreated

cells (Figure 6A). In accordance with result, an analysis of I $\kappa$ B $\alpha$  proteins level by Western blot demonstrated that the degradation of this protein was greatly inhibited during the apoptotic process (Figure 6B).

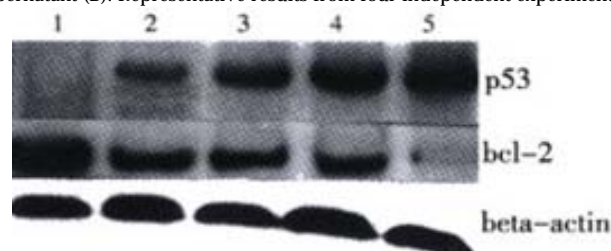
Additionally, by using Western blot we confirmed that the level of bcl-2 was decreased, whereas p53 showed a great increase following JTE-522 treatment. Their changes were in a dose-dependent manner (Figure 7).



**Figure 5** Effect of JTE-522 on the generation of ROS. AGS cells were incubated for 6h with JTE-522 (0.25-1mmol/L) in the presence or absence of PDTC at 100 $\mu$ mol/L. Lucigenin-associated chemiluminescence was measured for 3min with a lumiometer. (A). Lane 1: control; lane 2-5: AGS cells treated with 0.25, 0.5, 0.75, 1mmol/L of JTE-522; lane 6: JTE-522 (1mmol/L) +PDTC (100 $\mu$ mol/L) \* $P$ <0.01 vs control. Results are the means $\pm$ SD from three independent determinations.



**Figure 6** Effect of JTE-522 on NF- $\kappa$ B binding activity and I $\kappa$ B $\alpha$  degradation. Cells were treated with JTE-522 for 6h. Cells were harvested and EMSA was performed as described (A). Lane 1: control; lane 2-5: AGS cells treated with 0.25, 0.50, 0.75, 1mmol/L of JTE-522. The identity of DNA-complexed proteins was confirmed by supershift assays using antibodies against p65 subunit of NF- $\kappa$ B (lane 6). Immunoblot analysis of I $\kappa$ B $\alpha$  of corresponding cytosolic supernatant (B). Representative results from four independent experiments.



**Figure 7** P53, bcl-2 protein levels in AGS cells treated with JTE-522. Cell lysates were collected and processed at 6h. The whole cellular protein was electrophoresed in SDS-PAGE gel. Western blot was performed using antibodies against p53, bcl-2. Beta actin was used as a lane-loading control. (1) control; (2) 0.25mmol/L; (3) 0.5mmol/L; (4) 0.75mmol/L; (5) 1 mmol/L. Representative results from three independent experiments.

## DISCUSSION

Using cultured AGS human gastric adenocarcinoma cells, the observations described in this study demonstrate that JTE-522, a novel NSAIDs inhibits the growth of AGS cells and induces apoptosis in a concentration-dependent manner.

The onset of apoptosis is associated with the proteolytic activation of caspases. Caspases, a family of cysteine proteases, play a critical role in the execution of apoptosis<sup>[26-31]</sup>. They are synthesized as proenzymes that are processed by self-proteolysis and/or cleavage by another protease to their active forms in cells undergoing apoptosis. Caspase-3 is a major executioner of apoptosis. It is promoted during the early stage of apoptosis and the activated form is a marker for cells undergoing apoptosis<sup>[32]</sup>. After activation by initiators, the proform (p32) is cleaved to the active forms p20, p17, or p11, respectively<sup>[33]</sup>. Therefore, activation of caspase-3 in AGS cells in this study not only indicated the occurrence of apoptosis, but also implied the involvement of caspase-3 in JTE-522-induced apoptosis.

ROS have been found to play a central role in regulating apoptosis in numerous instances. Given that reduced rates of apoptosis may contribute to carcinogenesis, the regulation of cellular ROS production may be an important variable in the development of neoplasias. Other studies have also suggested a potential role for ROS in cancer suppression. For example, the p53 tumor suppressor protein activates the expression of ROS-generating proteins that increase cellular ROS production and eventually trigger apoptosis<sup>[34,35]</sup>. Increased ROS generation by chemopreventive agents may serve to compensate the lower levels of ROS generation in p53 null cells (AGS cells are p53 null)<sup>[36,37]</sup>. Animal studies have also implicated ROS in regulating carcinogenesis. Mice with elevated levels of glutathione peroxidase are more sensitive to skin carcinogenesis than their wild type counterpart<sup>[32]</sup>. A similar correlation has been made in the colon, where strains with higher levels of glutathione peroxidase activity have a higher cancer risk. The role of ROS in carcinogenesis is however likely to be complex given the potential mutagenicity of ROS. For example, the NSAIDs inhibition of cyclooxygenase has been proposed to suppress carcinogenesis by suppressing the production of peroxy radicals and the subsequent formation of mutagenic lipid peroxidation breakdown products<sup>[38]</sup>. The role of ROS in carcinogenesis may depend on the relative levels of different ROS generated, and where and when they are present. However, as demonstrated in this paper, we showed that JTE-522 increased ROS generation in AGS cells, and this increased ROS generation might contribute to the induction of AGS cells to apoptosis.

The bcl-2 protooncogene is unique among cellular genes for its ability in many contexts to block apoptotic cell death. A mechanism has been proposed in which bcl-2 regulates antioxidant pathways at sites of free radical generation<sup>[39]</sup>. The protein of bcl-2 also protects against apoptosis by blocking cytochrome C release hence this protein may have an antioxidant function<sup>[40]</sup>. In our experiment, the expression of bcl-2 decreased, and the p53 protein upregulated following JTE-522 treatment, these changes may implies that intracellular ROS may interfere with the expression of bcl-2 and p53, thereby contributing to inducing apoptosis in AGS cells.

In studying the mechanism by which the NSAIDs influenced cell death, common effects on the transcription factor NF- $\kappa$ B were noted. The NF- $\kappa$ B transcription factor is ubiquitous and can be detected under its inactive form in the cytoplasm of almost all cell types<sup>[41,42]</sup>. It suppresses the expression of cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in health and various pathological states<sup>[43,44]</sup>. Experimental data clearly indicate that NF- $\kappa$ B is a major regulator of the inflammatory reaction by controlling the expression of pro-inflammatory molecules in response to cytokines oxidative stress and infectious agents<sup>[45,46]</sup>. NF- $\kappa$ B is maintained under such an inactive cytoplasmic form by virtue of its association with an inhibitory molecule named I $\kappa$ B. I $\kappa$ B $\alpha$  is the best

characterized member of this family. In our current work, EMSA revealed that JTE-522 inhibited NF- $\kappa$ B activation. Western blot experiments demonstrated that this effect was mediated by inhibition of I $\kappa$ B $\alpha$  degradation. Determining the role of NF- $\kappa$ B in gastric carcinogenesis could help to guide the development of improved chemoprevention and treatment strategies.

In summary, JTE-522 inhibited cell growth and induced apoptosis in AGS cells. Increased ROS may play an important role in this caspase-3 mediated apoptotic process. The inhibition of NF- $\kappa$ B by JTE-522 may be mediated by preventing I $\kappa$ B $\alpha$  degradation. The precise relationship and importance of each of these factors in the apoptotic process should be established by more direct and profound analysis.

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