NS-398 enhances the efficacy of gemcitabine against lung adenocarcinoma through up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$ protein

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Gemcitabine is a chemotherapeutic drug widely used in the treatment of non-small cell lung carcinoma, especially in advanced lung adenocarcinoma. However, many patients with advanced lung adenocarcinoma show a resistance to gemcitabine. Overexpression of COX-2 has been found in human non-small cell lung cancer tissues and it´s cell lines. Evidences show that COX-2 is involved in drug resistance of tumor. However, It is unknown whether COX-2 inhibitor can augment the efficacy of gemcitabine against lung adenocarcinoma. In this study, A549 cells were treated with gemcitabine and/or NS-398. The cell viability was examined by MTT assay. The cell cycle distribution and apoptotic ratio were tested by flow cytometry. The levels of $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4a}$ and $p15^{INK4b}$ expression were detected by western blotting. After the cells were treated with gemcitabine along with NS-398, more cells were arrested in G_1 phase and went to apoptosis. The levels of $p21^{WAF1}$ and $p27^{KIP1}$ protein were elevated, while the levels of $p16^{INK4a}$ and $p15^{INK4b}$ protein were not changed. It can be concluded that NS-398 enhances the efficacy of gemcitabine against lung adenocarcinoma and the efficacy is associated with up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$protein.

Key words: NS-398, non-small cell lung carcinoma, $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4a}$, $p15^{INK4b}$.

Cyclooxygenases (COXs) are the rate-limiting enzymes in the conversion from arachidonic acid (AA) to prostaglandins (PG_S) and other eicosanoids. Two isoforms of COX, COX -1 and COX -2, have been identified to date [1]. COX-1 and COX-2 are various in expression and functions. COX-1 is constitutively expressed in almost all normal tissues and is important for maintaining homeostatic function [2]. COX-2 has been found highly expressed in pulmonary adenocarcinoma (ACA) [3], gastric cancer [4], prostate carcinoma [5], colorectal adenocarcinoma [6], and pancreatic adenocarcinoma [7]. Evidences have showed that COX-2 and the related downstream products exert effects on apoptosis resistance and tumor angiogenesis [8]. Moreover, COX-2 expression is associated with decreased host immunity [9] and tumor invasion and metastasis [10].

Disruption of the physiologic balance between cell proliferation and death is a general feature of all cancers**.** The inactivation or down-regulation of some negative regulators is involved in the formation of tumor cells characteristics of high aggressiveness and proliferation [11]. Cyclin-dependent kinase inhibitors ($CDKI_S$) are modulators which negatively regulate the activity of CDK-cyclin complexes. Two families of $CDKI_S$, INK^4 family and KIP family, have been identified. $p21^{WAF1}$ and $p27^{KIP1}$ belong to the latter, which can combined with the most of the CDK-cyclin complexes and favorably with $CDK_{4/6}$-cyclin D complexes [12]. $p16^{INK4a}$ and $p15^{INK4b}$ are members of INK family. This family mainly binds with $CDK_{4/6}$-cyclin D complexes [13, 14].

Gemcitabine(GEM) is one of the most effective chemotherapeutic drug administered to some patients with non-small cell lung carcinoma (NSCLC), but it is not effective on all the patients, especially on those with lung ACA. It is unknown whether NS-398 can enhance the efficacy of GEM against ACA and whether $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4a}$ or $p15^{INK4b}$ is involved in the process.

Materials and Methods

Cell line and chemicals. A-549 ACA cells (ATCC, USA) were maintained in 5% CO_2 incubator at 37°C with RPMI

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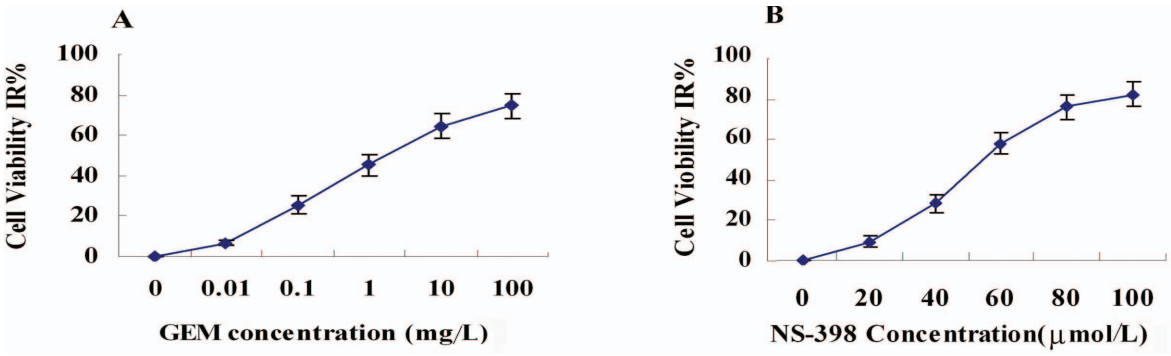

Figure 1. Effects of GEM or NS-398 of different concentrations on A549 cell viability. The cell viability of A549 cells at 12h, 24h, 36h and 48h were evaluated by MTT assay. Only the results of 24h were shown. The data were expressed with Mean±SD of inhibiting rate% (IR%=1-$A_{study\ group}$/$A_{untreated\ group}$×100%).

1640 medium(GIBICO,USA) which contains 10% fetal bovine serum(FBS), 2 mmol/L L-glutamine and 2.0g/L sodium bicarbonate. NS-398(Celecoxib, Cayman Chemical, USA) was dissolved in dimethyl sulfoxide(DMSO, Amresco, USA) and GEM(Gemzar, Lilly France S.A) in PBS. The stock concentration of NS-398 and GEM were 20 mmol/L and 1g/L respectively. Both NS-398 and GEM were diluted to the required concentrations with RPMI 1640 medium before each test. As for GEM solution, the ultimate concentration of DMSO was controlled no more than 0.1%. The stock solution of Dimethylthiazol-2-yl 2-5-diphenyltetrazolium bromide (MTT, Amresco, USA) was prepared by dissolving 5 mg of MTT in 1 mL of PBS and was filtered to omit particulates.

Cell viability assay. A549 cells (3-5×10^3) in 100μL were seeded into 38-mm^2 wells of flat-bottomed 96-well plates and incubated for 24h. The primary medium was then removed and the cultures were replaced with: new medium or medium containing different concentrations of NS-398 and/or GEM in triplicate. The cells were incubated for 6h, 12h, 24h or 36h. Four hours before each end of the incubation, 20μL of 5g/L MTT solution was added to each well. The formazan crystals were dissolved in 150μL DMSO. The absorbance at 570 nm was determined with a microculture plate reader.

Cell cycle distribution and cell apoptosis ratio analysis. A549 cells (1.25-1.5×10^5) in 1.5mL were plated in six-well plates and incubated for 24h. The following day, the primary medium was replaced with medium containing indicated concentration of NS-398 or/and GEM. At the end of the second 24h incubation, both the adherent cell layer and the cells floating in the medium were collected. 5×10^5-1×10^6 cells were washed. For cell cycle analysis, the cells were incubated with 2g/L RNase A in PBS (200μl) and 0.1g/L Propidium Iodide (PI, Sigma, USA) in 0.6% Nonidet P-40 on ice for 30 min. The DNA contents of samples were immediately measured by flow cytometry(Becton Dickinson). Cell cycle phase distribution was determined using CellQuest software (Becton Dickinson). Apoptosis was measured as recommended by the manufacturer of Annexin V-FITC Detection Kit (Keygen, China). Percentage of stained cells in each quadrant was quantified.

Western blotting analysis and density assessment. Untreated cells or cells incubated with NS-398 and/or GEM were harvested by trypsinization and lysed. Total cellular protein (20 μg) from each sample was subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween 20 for 2h at 4°C. The blots were probed overnight with mouse anti-human-p21^{WAF1}, anti-human-p27^{KIP1}, anti-human-p16^{INK4a} and anti-human-p15^{INK4b} monoclonal antibodies (Zymed, USA), then washed and probed with anti-mouse IgG -HRP (Zymed, USA). Immunoreactive material was detected by enhanced chemiluminescence. Equal protein loading was confirmed by reprobing blots with a mouse polyclonal antibody specific for β-actin (Zymed, USA). The density of all bands were assessed using the Kodak ID 3.6 computer software program.

Statistical analysis. Analysis was performed with One-way Analysis of Variance using SPSS 11.0 software. $p<0.05$ was considered as statistically significant.

Results

NS-398 and GEM decrease A549 cell viability. First, the efficacy of GEM and NS-398 to A549 cells was examined by MTT assay at different time points (6h, 12h, 24h and 36h) and with different concentrations. Our results demonstrated NS-398 inhibits the A549 cell viability in a dose-dependent manner (Fig. 1B), whereas, a time-dependent efficacy of NS-398 to this type cell did not appear (data not shown). Similar to NS-398, GEM inhibits the cell viability in a dose-dependent manner (Fig. 1A). After 24h treatment, NS-398 of 60 μmol/L or GEM of 1 mg/L inhibited about half of the cell viability. In order to avoid anti-proliferation effect of the drugs, NS-398 of 40μmol/L and GEM of 0.1 mg/L were employed in the following research.

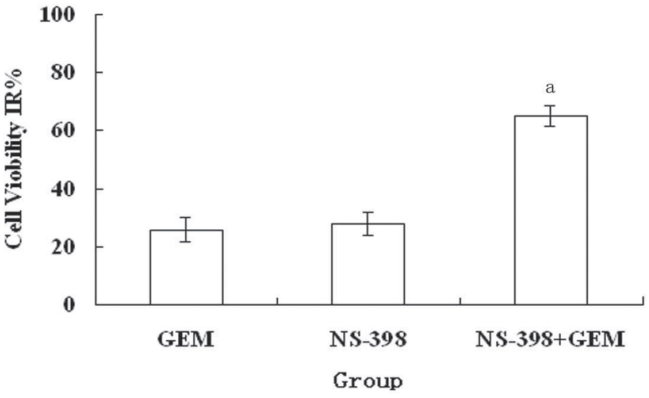

Figure 2. Combined effects of GEM plus NS-398 on A549 cell viability. The cell viability of A549 cells at 24h was assessed by MTT assay. The data were expressed with Mean±SD of inhibiting rate% (IR%=1-$A_{study\ group}/A_{untreated\ group}$×100%). [a]p<0.05 vs GEM or NS-398.

NS-398 enhances the cytotoxicity of GEM to A549 cells. Secondly, A549 cells were cultured with medium containing 0.1 mg/L of GEM and/or 40μmol/L of NS-398. As described in Fig. 2, GEM inhibits cell viability roughly by 25%. When the cells were treated with combined GEM and NS-398, it inhibits cell viability by 65% (p<0.05).

Combined effects of NS-398 and GEM on cell cycle progression. Thirdly, in order to elucidate the effects of NS-398 on cell cycle progression, the A549 cells treated with NS-398(40μmol/L) or/and GEM (0.1 mg/L) for 24h were analyzed by FCM. It appeared in Fig. 3 that cell fraction in $\mathbf{G_1}$ phase is 60.4±3.6% in GEM treated cells. The number reached 78.4±4.7% when the A549 cells were treated with GEM plus NS-398(p<0.05).

Synergistic effects of NS-398 and GEM on apoptosis. As shown in Fig. 4, the early apoptotic level is 11.7±3.4% in GEM treated cells. After A549 cells were treated with NS-398 and GEM simultaneously, early apoptotic cells increased to 28.4±4.9% (p<0.05). It can also be observed from our study neither NS-398 nor GEM treatments did not significantly increase the late apoptotic cells, even if the cells were treated with combined NS-398 and GEM (data not shown).

The levels of $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4a}$ and $p15^{INK4b}$ expression. To gain further insight into the mechanism by which NS-398 induces apoptosis and block cell cycle progression in G_1 phase, the levels of $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4a}$ and $p15^{INK4b}$ protein expression were examined. As depicted in Fig. 5, A549 cells express a moderate level of $p16^{INK4a}$, $p15^{INK4b}$, $p21^{WAF1}$ and $p27^{KIP1}$. $p21^{WAF1}$ and $p27^{KIP1}$ levels in cells treated with combined GEM and NS-398 are about 3-folds as compared with cells treated with GEM solely(p<0.01), whereas

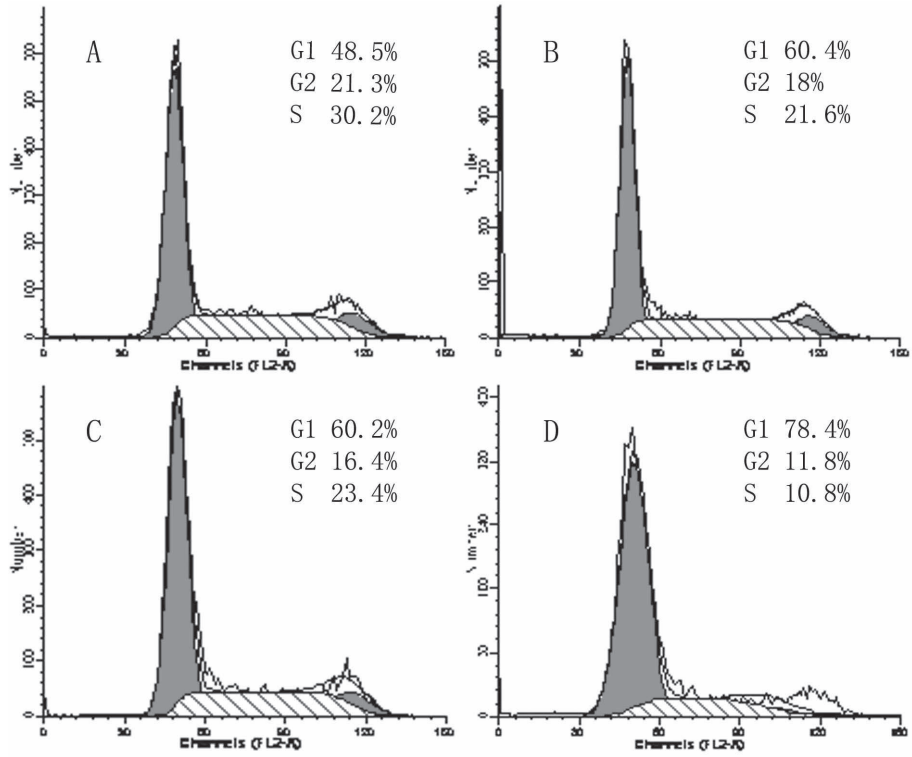

Figure 3. Effects of GEM or/and NS-398 on cell cycle distribution. A549 cells were cultured with: A. medium, B. medium containing 0.1 mg/L of GEM, C. medium containing 40μmol/L of NS-398, D. medium containing 0.1 mg/L of GEM and 40μmol/L of NS-398.

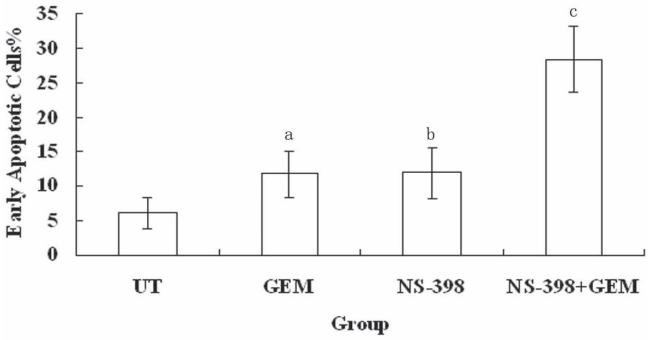

Figure 4. Effects of GEM or/and NS-398 on early apoptosis. A549 cells collected were stained with AnnexinV-FITC and PI. The data were expressed with Mean±SD. [a]p<0.05 vs UT, [b]p<0.05 vs UT, [c]p<0.01 vs UT, [c]p<0.05 vs GEM or NS-398.

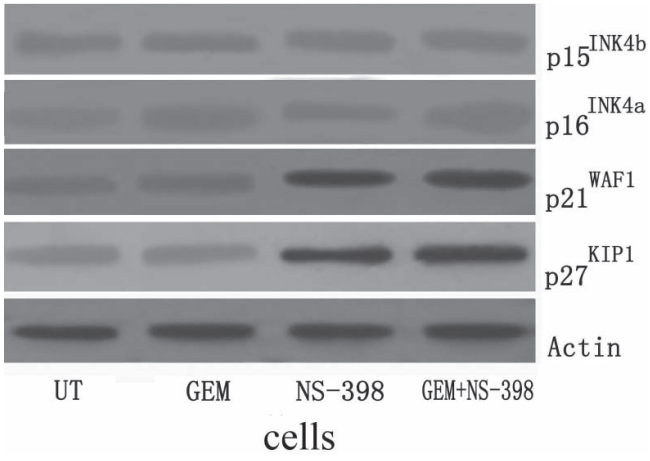

Figure 5. Effects of GEM and /or NS-398 on expression of p21^{WAF1}, p27^{KIP1}, p16^{INK4a} and p15^{INK4b} protein. Cellular protein of A549 cells were isolated and tested with western blotting. The densities of bands in each group were calculated and were compared with the untreated cells.

the levels of p16^{INK4a} and p15^{INK4b} expression remain unaltered(p>0.05).

Discussion

It was convincingly showed in our study that NS-398, a selective COX-2 inhibitor, can augment the efficacy of GEM against human lung ACA in vitro. These effects were proved by decreased cell viability, increased cell number in G_1 phase and enhanced apoptotic cell number in cells treated with NS-398 and GEM than those treated with simple GEM. Furthermore, p21^{WAF1}and p27^{KIP1}, but not p16^{INK4a} and p15^{INK4b} were found to be involved in the regulation of NS-398-mediated anti-proliferation and growth inhibitory procedure.

p21^{WAF1} and p27^{KIP1} are regulators of cell cycle progression. NS-398 mainly arrests cells in G_1 phase but not in G_2/M phase according to our study. The results implied G_1-associated regulators of cell cycle are involved in the process. The levels of p16^{INK4a}, p15^{INK4b}, p21^{WAF1} and p27^{KIP1}protein expression proved our hypothesis.

p21^{WAF1} is a tumor suppressor gene which locates in the downstream of p53 and is regulated by p53 through transactivation-dependent or independent mechanisms. p21^{WAF1} activated by p53 down-regulates the activity of CDK-cyclin complexes, maintains the Rb-E_2F complexes and represses the transcription of E_2F-regulated genes. As a result, the cell cycle transition from G_1 to S phase is prevented [15, 16, 17]. p27^{KIP1} is regarded as a tumor suppressor gene too. It plays an important role in conduction of extracellular signals and has influence on cell differentiation. Similarly, over-expression of p27^{KIP1} can slower cell cycle progression [18].

p21^{WAF1} and p27^{KIP1} are also regulators of apoptosis. It is still controversial about the effects of enhanced p21^{WAF1} expression on cells' apoptosis status. Previous data demonstrated that targeted over-expression of p21^{WAF1} increases apoptosis rate, or disruption of p21^{WAF1} function leads to lowered apoptosis rate [19, 20]. It is not quite clear about the potential mechanisms through which p21^{WAF1} promotes apoptosis. These mechanisms could be related to its ability to interact with or possibly regulate components of the DNA repair machinery [21]. However, there are contrary results which showed an anti-apoptosis role of p21^{WAF1} [22, 23]. As for the effects of p27^{KIP1}, the results are similar with p21^{WAF1}. Most of the data showed p27^{KIP1} is a positive regulator of apoptosis [24, 25].

The results above indicate that NS-398 plays an anticancer role not only through cell cycle arrest in G_1 phase but also via apoptotic mechanisms.

According to previous data, the altered level of p21^{WAF1} protein expression occurred at two levels. One is at transcriptional level at which p21^{WAF} is regulated through p53-dependent or p53-independent mechanisms [26]. As p21^{WAF1} promoter contains two conserved p53-binding sites, the alteration of p21^{WAF1} may occur in response to p53 after DNA damage [27]. In the p53-independent pathway, p21^{WAF1} transcription may be activated by a variety of transcriptional factors which are induced by a number of different signaling pathways [26]. The other is at post-transcriptional level. At this level, p21^{WAF1} can be degraded by proteasome in an ubiquitin-dependent or -independent manner [28, 29]. In regard to p27^{KIP1,} a large number of studies showed that p27^{KIP1} gene rarely mutate and its mRNA level remains stable in cell cycle, therefore the enhanced expression of p27^{KIP1} protein caused by NS-398 is chiefly through post-translational pathway. Some results have proved the hypothesis [30].

In summary, the study proved our previous hypothesis that NS-398 can enhance the efficacy of gemcitabine against lung ACA. Our results also showed this effect of NS-398 is performed not only through arrest of cell cycle in G_1 phase but also via apoptotic mechanisms. Furthermore, it was found

in our study that some of the CDKIs were involved in the process.

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