

Induction of p27^{KIP1} as a Mechanism Underlying NS398-Induced Growth Inhibition in Human Lung Cancer Cells

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ABSTRACT

Increased expression of cyclooxygenase-2 (COX-2) causes enhanced production of prostaglandins, which are emerging as important mediators of growth stimulation of cancer cells. Overexpression of COX-2 has been found in human non-small cell lung cancer tissues and cell lines. In vitro and in vivo studies showed that nonselective cyclooxygenase inhibitors (like aspirin and indomethacin) may suppress growth of lung cancer cells and may prevent lung tumorigenesis induced by the tobacco-specific carcinogens. However, the molecular mechanisms that mediated the anticancer action of these inhibitors are not well defined. In this study, we examined the effect of a specific COX-2 inhibitor, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398), on high COX-2-expressing A549 lung cancer cells. Our results indicated that NS398 inhibited prostaglandin E₂ synthesis and induced G₁ growth arrest in these cells. NS398 specifically up-regulated cyclin-dependent

kinase inhibitor p27^{KIP1}, whereas the expressions of G₁-acting cyclins and cyclin-dependent kinases were not changed. Additionally, NS398 effectively suppressed cyclin E-associated kinase activity in A549 cells. The molecular mechanism responsible for the induction of p27^{KIP1} by NS398 was characterized. We found that NS398 did not induce p27^{KIP1} through transcriptional activation because this drug could not stimulate the p27^{KIP1} promoter. Metabolic labeling experiments showed that the synthesis rate of p27^{KIP1} protein was not altered by NS398. Conversely, pulse-chase assays demonstrated that degradation of p27^{KIP1} protein was obviously reduced in NS398-treated cells. We conclude that NS398 enhances p27^{KIP1} expression via post-translational regulation, and our results provide a new mechanism by which specific COX-2 inhibitors suppress proliferation of cancer cells.

Cyclooxygenases (COXs) are the rate-limiting enzymes involved in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. Two isoforms of COX, COX-1 and COX-2, have been identified (Smith et al., 1996). COX-1 is constitutively expressed in a wide variety of tissues, whereas COX-2 is a highly inducible gene that is activated by cytokines, growth factors, phorbol esters, and chemical carcinogens (Xie et al., 1991; O'Neill and Hutchinson, 1993). Study of the involvement of COX-2 in carcinogenesis was initiated by the observation that human colorectal tumors express high levels of COX-2, whereas the normal intestinal mucosa has low to undetectable COX-2 expression (Eberhart et al., 1994). Subsequent investigations indicated that overexpression of COX-2 could be detected in many human tumors (Subbaramaiah et al., 1996; Hida et al., 1998b; Sawaoka et al., 1998). These findings suggest that COX-2 may play an important role in tumorigenesis. However, the

mechanisms by which COX-2 and PGs support tumor growth are not clear. Enforced expression of COX-2 in rat intestinal cells results in an increase of Bcl-2 protein and resistance of the cells to apoptosis (Tsujii and DuBois, 1995). Thus, COX-2 expression may protect cancer cells from apoptosis induced by cytokines and reduce host defenses against tumor. Additionally, COX-2 expression may support tumor growth by promoting angiogenesis and enhancing metastatic abilities in cancer cells (Tsujii et al., 1998). Finally, the COX-2/PG-signaling pathway may directly enhance growth of cancer cells (Tjandrawinata and Hughes-Fulford, 1997).

Recent studies have demonstrated that increased expression of COX-2 was observed frequently in human non-small cell lung cancer (NSCLC), and elevated biosynthesis of PGs was found in NSCLC cell lines (Hubbard et al., 1989; Hida et al., 1998b). Additionally, recent works also indicated that aspirin, a nonsteroidal anti-inflammatory drug and a nonselective COX-2 inhibitor, may inhibit proliferation of NSCLC cell lines and may reduce the number of lung adenoma induced by the tobacco-specific nitrosamine 4-(methylnitro-

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; NSCLC, non-small cell lung cancer; NS398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; TBST, Tris-buffered saline/Tween-20.

samino)-1-(3-pyridyl)-1-butanone (Hida et al., 1998a). Moreover, activation of the ras or neu oncogene, which is frequently found in human NSCLC, also enhances the expression of COX-2 (Heasley et al., 1997; Vadlamudi et al., 1999). These results prompt us to speculate that COX-2 contributes to the development of lung cancer.

In this study, we investigated the effect of a specific COX-2 inhibitor, NS398, on COX-2-overexpressing A549 lung cancer cells. We found that NS398 suppressed synthesis of PGE₂ and induced G₁ growth arrest in A549 cells. Induction of p27^{KIP1} was observed in NS398-incubated A549 cells, whereas the expressions of G₁-acting cyclins and cyclin-dependent kinases (CDKs) were not changed. Furthermore, NS398 up-regulated p27^{KIP1} expression via post-translational regulation. Considered together, our results suggest that COX-2 inhibitors may modulate the expression of cell cycle regulatory proteins to suppress growth of cancer cells.

Experimental Procedures

Cell Culture and Reagents. A549 human NSCLC cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂ incubator at 37°C. NS398 and protein A/G-agarose and histone H1 were purchased from Calbiochem (La Jolla, CA). NS398 was dissolved in dimethyl sulfoxide to give a stock concentration of 20 mM. Antibodies against G₁ cyclins (D1, D2, D3, and E) and CDK 2, 4, and 6 and CDK inhibitors (CDKIs) p21^{WAF1}, p27^{KIP1}, and p57^{KIP2} were obtained from Santa Cruz (Santa Cruz, CA). Hoechst 33258 fluorescent dye was obtained from Sigma (St. Louis, MO). The human p27^{KIP1} promoter-luciferase fusion plasmid, p27PF, was kindly provided by Dr. T. Sakai (Department of Preventive Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan) (Minami et al., 1997).

Measurement of PGE₂. Cells were seeded at a density of 3×10^5 cells/well in 24-well plates and incubated with medium containing different concentrations of NS398 for 24 h. PGE₂ released into the medium was determined using an enzyme immunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ), following the manufacturer's protocol. The limit of sensitivity for detection of PGE₂ was 2.5 pg/ml.

Cell Viability and Apoptosis Assays. Cells were cultured in six-well plates in 10% FCS medium and were treated with different concentrations of NS398 for 48 h. After incubation, detached cells were collected by centrifugation and attached cells were harvested by trypsinization. Cells were pooled and viable cells were determined by trypan blue exclusion test. To evaluate the number of apoptotic cells, pooled cells were also fixed in 3% paraformaldehyde and stained with Hoechst 33258 dye, and apoptotic cells with condensed chromatin fragments were scored as described previously (Hung et al., 1999).

Analysis of Cell Cycle Distribution. Cells were cultured in the absence or presence of 100 µM NS398 for 48 h in 10% FCS medium. Control or NS398-treated A549 cells were fixed with 95% ethanol and stained with propidium iodide. Cell cycle distribution was analyzed by fluorescence-activated cell sorter flow cytometry (Becton Dickinson, Mountain View, CA) as previously described (Lee et al., 1999).

Immunoblotting. Cells were treated with different concentrations of NS398 for 24 h in 10% FCS medium. Cells were rinsed twice with ice-cold PBS and harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 µg/ml pepstatin A, and 2 µg/ml leupeptin) for 20 min on ice. Cell lysates were centrifuged at 12,000g for 10 min, and protein concentrations of the lysates were determined using a bicin-

chonic acid protein assay kit (Pierce, Rockford, IL). Proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat milk in Tris-buffered saline/Tween-20 (TBST; 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) overnight at 4°C. The blots were washed in TBST and incubated with various primary antibodies for 2 h at room temperature. After incubation, the blots were washed twice with TBST and incubated with peroxidase-conjugated secondary antibody for another 1 h and were developed by using the enhanced chemiluminescence system (Amersham Pharmacia Biotech). The blots were reprobbed with actin antibody to confirm the equal loading of the amount of protein in each lane.

Kinase Assay. Cells were cultured in the absence or presence of 100 µM NS398 for 24 h in 10% FCS medium, and cell lysates were prepared as described above. Cell lysates (200 µl) containing 300 µg of cellular proteins were immunoprecipitated with cyclin E antibody at 4°C for 4 h, and immunocomplexes were collected by adding protein A/G-agarose to the reaction mixtures at 4°C for another 1 h. In vitro kinase assays were performed as described previously (Lee et al., 1999).

Luciferase Assays. Cells were plated onto six-well plates at a density of 100,000 cells/well and grown overnight. Cells were transfected with 2 µg of p27PF plasmid with the LipofectAMINE method as described previously (Lee et al., 1999). Cells were then incubated in the absence or presence of NS398 (100 µM) for 24 h, and luciferase activity was measured. The relative light units per microgram of protein were calculated as an average of three independent experiments of duplicate samples.

Metabolic Labeling. Cells (1×10^6) were plated in 75-cm² flasks and grown overnight. Cells were cultured in the absence or presence of NS398 (100 µM) in 10% FCS medium for 24 h. After incubation, cells were washed with PBS and metabolically labeled with [³⁵S]methionine (100 µCi/ml) in methionine-free medium for 15 or 30 min. Cell extracts were prepared as described above, and equal amounts of proteins were incubated with anti-p27^{KIP1} antibody for 4 h at 4°C. Protein A/G-agarose was added to collect the immunocomplexes at 4°C for 1 h, and the immunoprecipitated proteins were resolved by SDS-PAGE. Radiolabeled p27^{KIP1} was visualized by autoradiography.

Protein Half-Life Determination. Cells (1×10^6) were plated in 75-cm² flasks and grown overnight. Cells were cultured in the absence or presence of NS398 (100 µM) in 10% FCS medium for 24 h. After incubation, cells were washed with PBS, metabolically labeled with [³⁵S]methionine (100 µCi/ml) in methionine-free medium for 1 h, and then chased with 10% FCS medium containing 100 µg/ml unlabeled methionine for 1, 3, or 6 h. Preparation of cell extracts, immunoprecipitation, and SDS-PAGE were performed as described above. Radiolabeled p27^{KIP1} was visualized by autoradiography, and the intensity of the signals was analyzed by a densitometer (Bio-Rad, Hercules, CA).

Results

Suppression of PGE₂ Synthesis and Induction of G₁ Growth Arrest by NS398 in COX-2-Overexpressing A549 Lung Cancer Cells. As shown in Fig. 1, analysis of PGE₂ in medium from COX-2-overexpressing A549 cells revealed significant synthesis and release of PGE₂ in these cells. NS398 inhibited PGE₂ synthesis and release in a dose-dependent manner. At the concentration of 100 µM, NS398 blocked PGE₂ synthesis by 70% in A549 cells. Additionally, NS398 suppressed proliferation of A549 cells. This drug at 100 µM inhibited cell growth by 35% after a 48-h treatment (Fig. 2). Recent studies have demonstrated that COX-2 inhibitors may exert their anticancer effect by inducing apoptosis; we tested whether reduced cell number caused by

NS398 was due to induction of apoptosis by this drug. We determined the number of apoptotic cells by fluorescent staining. As shown in Fig. 2A, NS398 at the concentration of 100 μ M did not significantly increase apoptosis in A549 cells after a 48-h incubation. These results suggest that NS398 may induce growth arrest rather than apoptosis in lung cancer cells. We next performed flow cytometric analysis to examine the alteration of cell cycle distribution in NS398-treated A549 cells. As shown in Table 1, 54, 26, and 20% of cells were presented in G₀/G₁, S, and G₂/M phase of the cell cycle, respectively, in proliferating A549 cells maintained in 10% FCS medium containing vehicle (0.5% dimethyl sulfoxide). On the contrary, the percentage of cells in the G₀/G₁ phase increased to 73%, and the percentage of cells in the S phase was reduced to 10% after treatment with NS398 (100 μ M) for 48 h. Collectively, these data indicate that NS398 blocks cell cycle progression of A549 cells in the G₁ phase.

NS398-Induced G₁ Growth Arrest Is Linked with Up-Regulation of p27^{KIP1} and Reduction of Cyclin E-Associated Kinase Activity. Recent studies indicated that progression of mammalian cell cycle is regulated by three distinct classes of proteins, including cyclins, CDKs, and CDKIs. Because NS398 blocked cell cycle progression in the G₁ phase, we tested whether the expression of G₁-acting cell cycle regulatory proteins was changed by NS398. Our results showed that NS398 at 100 μ M had a marginal effect on the expression of cyclin D1, D2, or E (Fig. 3A). Similarly, the expression of CDK 2, 4, or 6 was not regulated by NS398 (data not shown). On the contrary, NS398 significantly increased the level of p27^{KIP1} protein, but not p21^{WAF1} and p57^{KIP2}, in these cells (Fig. 3B). All of the immunoblots had been reprobed with actin antibody to confirm the equal loading of proteins in each lane. These data suggest that NS398-induced growth arrest in A549 cells is associated with induction of p27^{KIP1}. Previous studies have shown that p27^{KIP1} preferentially binds cyclin E-CDK complexes and inhibits their kinase activity. We tested whether up-regulation of p27^{KIP1} by NS398 may suppress cyclin E-associated kinase activity. In vitro kinase assays were performed by using histone H1 as a kinase substrate. Our results indicated that NS398 inhibited cyclin E-associated kinase activity in A549 cells in a dose-dependent manner (Fig. 4).

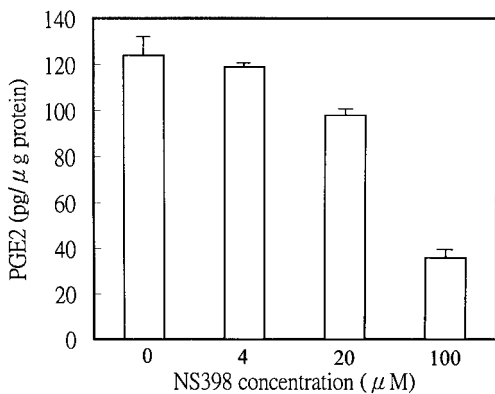


Fig. 1. NS398 inhibits biosynthesis of prostaglandin E₂ in A549 lung cancer cells. Cells were treated with different concentrations of NS398 for 24 h, and the amount of prostaglandin E₂ released into the medium was determined using an enzyme immunoassay kit. Data are the means of three experiments with the S.E. indicated.

NS398 Increases p27^{KIP1} Expression via Post-Translational Regulation. We next characterized the mechanism of induction of p27^{KIP1} by NS398. A549 cells were transfected with p27PF plasmid and incubated in the absence or presence of NS398 (100 μ M) for 24 h. Luciferase activity was

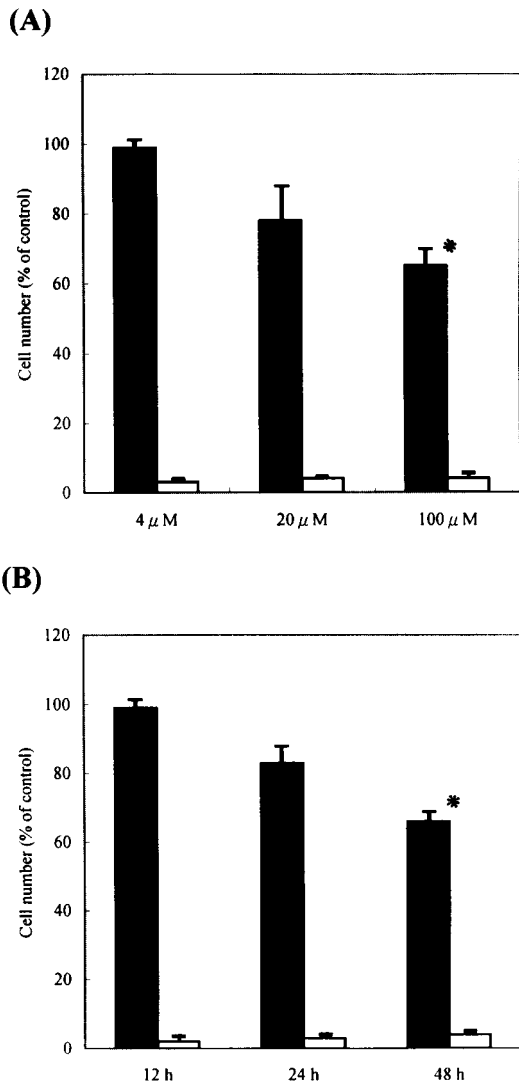


Fig. 2. NS398 induces growth arrest but not apoptosis in A549 cells. A, cells were treated with different concentrations of NS398 for 48 h. Detached cells were collected by centrifugation, and attached cells were harvested by trypsinization. Viable cells (■) were determined by trypan blue exclusion test and apoptotic cells (□) with condensed chromatin fragments were scored by fluorescent staining. B, cells were treated with 100 μ M NS398 for different time intervals. Viable and apoptotic cells were counted as described in A. Data are the means of three experiments with the S.E. indicated. **P* < .01 versus control.

TABLE 1
Effect of NS398 on cell cycle distribution of lung cancer cells
The percentage of cells in different phases represents the average of flow cytometric data in triplicates from three independent experiments. Results are expressed as means \pm S.E.

	Cell Cycle Distribution		
	G ₀ /G ₁	S	G ₂ /M
	% of total		
Control	53.8 \pm 1.0	26.2 \pm 0.7	20.0 \pm 0.4
NS398	73.4 \pm 1.2	9.5 \pm 0.6	17.1 \pm 0.8

assayed to determine the effect of NS398 on the p27^{KIP1} promoter. To our surprise, NS398 inhibited, but did not stimulate, the p27^{KIP1} promoter. NS398 at the concentration of 100 μ M suppressed the promoter activity by 60% (Fig. 5). These data suggest that NS398 does not induce p27^{KIP1} expression via transcriptional activation. We investigated whether NS398 induced p27^{KIP1} expression via translational or post-translational regulation. The rate of protein synthesis of p27^{KIP1} was examined by metabolic labeling assays. Cells were labeled with [³⁵S]methionine in methionine-free medium for a short time (15 or 30 min), and p27^{KIP1} was immunoprecipitated with specific antibody and resolved by SDS-PAGE. Radiolabeled p27^{KIP1} was visualized by autoradiography. As shown in Fig. 6A, the synthesis rate of p27^{KIP1} was not obviously changed by NS398. Thus, it seems likely that NS398 does not up-regulate the p27^{KIP1} level via an increase of translation. Conversely, compared with control cells, the degradation of p27^{KIP1} is dramatically reduced in NS398-incubated cells as determined by pulse-chase analysis. The half-life of p27^{KIP1} protein for control cells is esti-

mated to be 2.5 h, whereas the half-life of p27^{KIP1} protein for NS398-treated cells is >12 h (Fig. 6B). These results indicate that NS398 up-regulates p27^{KIP1} protein level via inhibition of degradation.

Discussion

Accumulating evidence indicates that COX-2 plays an important role in carcinogenesis. Either selective or nonselective COX-2 inhibitors have been found to inhibit growth of many types of cancer cells (Sheng et al., 1997; Duffy et al., 1998; Hida et al., 1998a; Molina et al., 1999). Moreover, in vivo investigations indeed demonstrated that COX-2 inhibi-

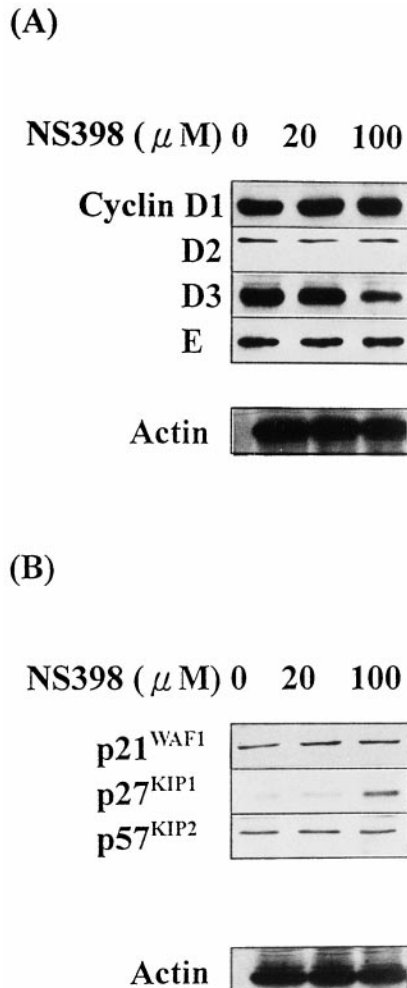


Fig. 3. Analysis of protein level of G₁ cyclins and CDKIs in A549 cells. Cells were incubated with different concentrations of NS398 for 24 h, and proteins were harvested for immunoblotting analysis. A, the blots were probed with antibodies against human cyclin D1, D2, D3, and E and then reprobed with anti-actin antibody to confirm equal loading of proteins in each lane. B, specific antibodies were used to detect the protein level of human p21^{WAF1}, p27^{KIP1}, and p57^{KIP2} in NS398-treated cells. The experiments were repeated three times, and similar results were observed.

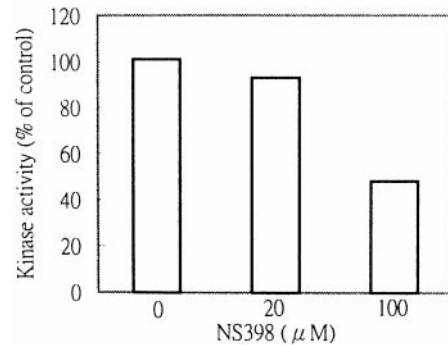
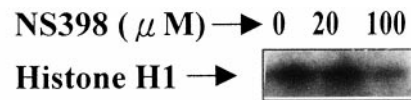


Fig. 4. Inhibition of cyclin E-associated kinase activity by NS398. Cells were incubated with different concentrations of NS398 for 24 h, and proteins were harvested. In vitro kinase assays were performed as described under *Experimental Procedures* by using histone H1 as the kinase substrate.

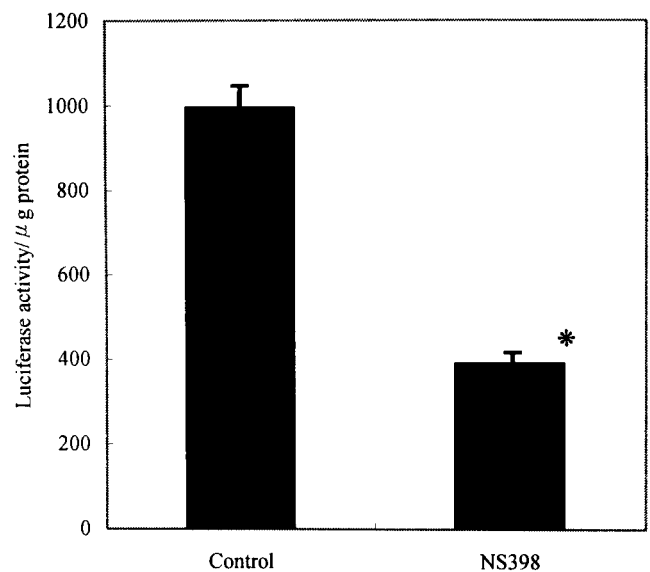


Fig. 5. NS398 inhibits, but does not stimulate, the human p27^{KIP1} promoter activity. Cells were transfected with the p27PF plasmid and incubated in the absence or presence of NS398 (100 μ M) for 24 h, and luciferase activity was determined. Results are the means of three independent experiments with the S.E. indicated. * P < .01 versus control.

tors may suppress growth of high COX-2-expressing colon and lung cancer cells grown in nude mice (Duperron and Castonguay, 1997; Sawaoka et al., 1998). However, the molecular basis for these anticancer effects is not well defined. Some COX-2 inhibitors potently induce apoptosis, whereas other inhibitors primarily induce cell cycle arrest. Additionally, different COX-2 inhibitors may exert different actions on various types of cancer cells. For example, a nonselective

COX-2 inhibitor, sulindac, was shown to induce apoptosis in HT-29 human colon cancer cells, but it induced growth arrest rather than apoptosis in human lung cancer cells (Duperron and Castonguay, 1997; Heasley et al., 1997). Therefore, the action of each COX-2 inhibitor on different types of cancer cells should be characterized separately.

Progression of the mammalian cell cycle is governed by cyclins, CDKs, and CDKIs (Hunter and Pines, 1994). Cyclins bind specific CDKs and activate their kinase activity to promote cell cycle progression. Conversely, two families of CDKIs negatively regulate the progression of cell cycle. The INK4 family members specifically bind CDK4 and CDK6 and block cyclin D association (Sherr and Roberts, 1995). On the other hand, the kinase inhibitor protein family members bind most of the cyclin-CDK complexes and inhibit their kinase activity (Massague and Polyak, 1995). Until now, only a few studies described the effect of COX inhibitors on the regulation of expression of cell cycle-related genes. Goldberg et al. (1996) demonstrated that sulindac increased the expression of p21^{WAF1} in human colon cancer cells. Other studies indicated that cell cycle parameters were unaffected by NS398 and indomethacin in colon and gastric cancer cells (Elder et al., 1997; Zhu et al., 1999). However, these studies did not investigate cyclins, CDKs, and CDKIs simultaneously and did not explore the molecular mechanism by which COX-2 inhibitors modulate the expression of these cell cycle regulators in their study. In this study, we provide the first evidence that a specific COX-2 inhibitor NS398 may up-regulate p27^{KIP1} expression via post-translational control and induce G₁ growth arrest in cancer cells.

Recent studies have indicated that the intracellular level of p27^{KIP1} protein is mainly regulated by translational or post-translational control. Proteolysis of p27^{KIP1} is mediated by ubiquitin-dependent and ubiquitin-independent mechanisms (Shireane et al., 1999). In the ubiquitin-dependent mechanism, p27^{KIP1} is phosphorylated by the cyclin E/CDK2 complex, which leads to ubiquitination. Ligation of ubiquitin and protein requires the sequential action of three enzymes. Activation of ubiquitin is achieved by a specific activating enzyme, E1, and activated ubiquitin is transferred to E2, a ubiquitin-carrier protein. Finally, ubiquitin is linked to a protein substrate by a ubiquitin-protein ligase (E3). Polyubiquitinated proteins are degraded by 26S proteasome. In the ubiquitin-independent mechanism, p27^{KIP1} is rapidly processed at its N terminus by 26S proteasome and then degraded to smaller peptides by a calpain-like protease. Whether NS398 affects the degradation of p27^{KIP1} through ubiquitin-dependent or -independent mechanism needs further investigation.

Most normal epithelial tissues, including breast, prostate, lung, and ovary, express high levels of p27^{KIP1} protein. However, a decrease of expression of this tumor suppressor gene is commonly found in many human cancers. Three recent studies have demonstrated that more than 70% of NSCLC tumors show reduced p27^{KIP1} immunoreactivity, and loss of this CDKI is linked with other predictors of poor prognosis and poor patient outcome (Esposito et al., 1997; Yatabe et al., 1998; Catzavelos et al., 1999). In addition, two of these studies clearly demonstrated that reduction of p27^{KIP1} in lung tumor tissues is due to enhanced degradation of this protein. Because mutations of the p27^{KIP1} gene are rarely found in human lung cancers, it is possible that an increase of p27^{KIP1}

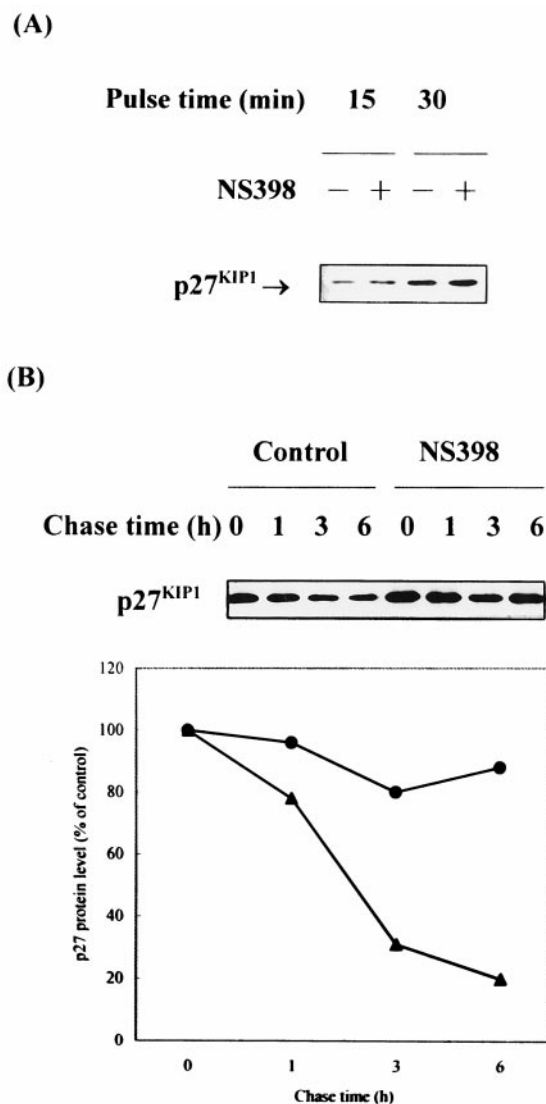


Fig. 6. NS398 increases p27^{KIP1} protein level via post-translational regulation. **A**, NS398 does not increase the protein synthesis rate of p27^{KIP1}. Cells were incubated in the absence or presence of NS398 (100 μ M) in 10% FCS medium for 24 h and metabolically labeled with [³⁵S]methionine in methionine-free medium for 15 or 30 min. Equal amounts of proteins were immunoprecipitated with anti-p27^{KIP1} antibody, and the immunoprecipitated p27^{KIP1} was resolved by SDS-PAGE. Radiolabeled p27^{KIP1} was visualized by autoradiography. **B**, degradation of p27^{KIP1} protein is inhibited by NS398. Cells were incubated in the absence or presence of NS398 (100 μ M) in 10% FCS medium for 24 h, metabolically labeled with [³⁵S]methionine in methionine-free medium for 1 h, and then chased with 10% FCS medium containing 100 μ g/ml methionine for 1, 3, or 6 h. Preparation of cellular proteins, immunoprecipitation, and SDS-PAGE were performed as described in **A**. Radiolabeled p27^{KIP1} was visualized by autoradiography, and the intensity of the signals was analyzed by a densitometer. A representative autoradiogram and the quantitative data for the labeled p27^{KIP1} in control (\blacktriangle) or NS398-treated (\bullet) cells are shown. Similar results were observed in another two independent experiments.

protein via inhibition of degradation by NS398 may be helpful for the treatment or prevention of lung cancer. Taken together, our results highlight a new mechanism by which COX-2 inhibitors suppress growth of human cancer cells.

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