COX-2 contributes to P-glycoprotein-mediated multidrug resistance via phosphorylation of c-Jun at Ser63/73 in colorectal cancer

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Cross-drug resistance in multidrug-resistant (MDR) cells, which overexpress P-glycoprotein (P-gp) encoded by the MDR1 gene, is a major impediment to successful chemotherapy for colorectal cancer. In the present study, drug-sensitive HCT8 and multidrug-resistant (vincristine, VCR) HCT8/V colorectal cancer cell lines were used to examine the role of c-Jun NH2-Terminal Kinase- (JNK) signaling pathway in P-gp-mediated MDR associated with Cyclooxygenase-2 (COX-2). The results showed that SP600125, a JNK inhibitor, and NS-398, a COX-2 inhibitor, significantly reduced the degree of MDR in HCT8/V cells. This was accomplished by a significant decrease in gene level of MDR1 and protein level of P-gp in HCT8/V cells. Notably, addition of a JNK inhibitor had no significant effect on the expression of COX-2 in both HCT8 and HCT8/V cells. Interestingly, inhibition of COX-2 activity by a chemical inhibitor or its silence by small interfering RNA significantly decreased the level of phosphorylated c-Jun at Ser63/73 in HCT8/V cells. In contrast, upregulation of COX-2 significantly increased the levels of P-gp and c-Jun at Ser63/73 in HCT8 cells, but not in HCT8/V cells. Moreover, the intracellular vincristine accumulation in HCT8/V cells significantly increased after inhibiting COX-2 and JNK activity. Taken together, our study has provided the first direct evidence that COX-2 contributes to P-gp-mediated multidrug resistance via phosphorylation of c-Jun at Ser63/73 in colorectal cancer cells.

Introduction

Chemotherapy is one of the major treatment modalities for patients who suffer from colorectal cancer. The effectiveness of chemotherapy, however, is seriously limited by MDR, the phenomenon of simultaneous resistance to structurally unrelated drugs. Overexpression of P-glycoprotein (P-gp/MDR1), an integral membrane protein, represents one of the major mechanisms that contribute to the MDR phenotype. P-gp functions as a drug efflux pump that actively transports drugs from the inside to the outside of cells and causes a defect in the intracellular accumulation of drugs necessary for cancer cell killing. Multiple genes involved in MDR have been identified in well-characterized experimental systems, and their role in drug resistance has been confirmed by both in vitro and in vivo models (1). The multidrug resistance gene (MDR1), which encodes P-gp was often overexpressed in MDR cells. The most extensively studied form of MDR is the P-gp-associated MDR phenotype, and a number of studies have confirmed that P-gp overexpression in tumor cells correlates with poor prognosis for chemotherapy (2,3). Therefore, inhibition of P-gp activity and/or expression may reverse the MDR phenotype through enhancing intracellular accumulation of anticaner drugs. In the past two decades, there has been a worldwide effort investigating a large number of diverse chemical agents for their ability to overcome MDR through interacting with P-gp and inhibiting its function.

The c-Jun NH2-terminal kinase (JNK) is a member of the mitogen-activated protein kinase- signaling cascade that is typically activated in response to a cellular stress (4), including ultraviolet irradiation, ionizing radiation and exposure to hydrogen peroxide or tumor necrosis factor-α, which can trigger apoptosis (4–7). There are some reports showing that c-Jun phosphorylation is a hallmark of JNK activation. Osborn et al. (8) have found a link between JNK activity and MDR1 gene expression. Recent studies have shown that activation of JNK and c-Jun is associated with upregulation of the MDR1 gene (9,10).

Cyclo-oxygenase-2 (COX-2), an inducible form of the enzyme that catalyzes the first step in the synthesis of prostanoids, has been shown to be overexpressed in a wide range of tumors and possesses proangiogenic and antiapoptotic properties (11). A close association between MDR and COX-2 expression has been reported in human colon cancer (12,13). Experimental studies have shown that COX-2 modulates P-gp expression and is involved in the development of the MDR phenotype (14). However, the biochemical mechanism for this is not fully understood. In this study, we have explored the role of JNK-signaling pathway in COX-2-induced P-gp-mediated MDR using drug-sensitive human colorectal cancer HCT8 and multidrug-resistant HCT8/V cell lines.

Materials and methods

Cell culture and reagents

The human colorectal cancer HCT8 parental cell line and MDR HCT8/V cell line were obtained from Keygen Biotech Co., Ltd, Nanjing, China. The MDR HCT8/V cells displayed 20.24-, 40.86-, 3.16- and 3.59-fold resistance to vincristine (VCR), cisplatin, 5-fluorouracil and mitomycin C, respectively, compared with the parental cells (15). Cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO2 humidified atmosphere. HCT8/V cells were routinely maintained in a medium containing 2000 ng/ml VCR (Shenzhen Main Luck Pharmaceuticals Co., Ltd, Shenzhen, China) and incubated in a drug-free medium for at least 1 week before use. SP600125, a JNK-specific inhibitor, and NS-398, a COX-2-specific inhibitor, were obtained from Cell Signaling (Beverly, MA).

MDR1 promoter activity by vector transient transfection and dual luciferase assay

Cells (2 × 104) were seeded in each well of 96-well culture plates in 100 μl RPMI-1640 containing 10% fetal bovine serum and incubated at 37°C for 24 h in a 5% CO2 humidified atmosphere until cells reached 90–95% confluence at the time of transfection.

The MDR1 promoter recombinant vector pGL3-basic-MDR1 promoter (0.8 μg/well) was mixed with a control vector (10 ng/well) pRL-SV40 in 25 μl serum- and antibiotic-free RPMI-1640. The solution was mixed with 0.5 μl Lipofectamine 2000 reagent, diluted in 25 μl serum-free RPMI-1640 and incubated at room temperature for 20 min. Two vectors in 50 μl solutions were cotransfected into the cells after the cells were washed twice with serum- and antibiotic-free RPMI-1640. The cells were then incubated at 37°C for 12 h in a 5% CO2 humidified atmosphere after transfection with plasmids, the medium was replaced with 100 μl fresh serum-free RPMI-1640.

After incubation overnight, cells were washed with 100 μl phosphate-buffered saline and lysed by adding 20 μl lysis buffer (Shanghai Lai’an Biotech. Co., Ltd, Shanghai, China). After incubation for 15 min at room temperature on a rocking bed (200 r.p.m.), the lysate was centrifuged at 15 000g for 5 min at 4°C and the supernatant was harvested and analyzed using a commercial dual-luciferase assay kit (Shanghai Lai’an Biotech. Co., Ltd) according to the manufacturer’s instructions.

Abbreviations: COX-2, cyclooxygenase-2; mRNA, messenger RNA; MTT, 3-(4,4-dimethylthiazol)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; P-gp, P-glycoprotein; siRNA, small interfering RNA; TAMRA, 6-carboxytetramethylrhodamine; VCR, vincristine.

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Real-time quantitative reverse transcriptase–polymerase chain reaction assay

RNA isolation Total cellular RNAs were prepared using the RNAisol reagent (TaKaRa Biotechnology Co., Ltd, Dalian, China) according to the manufacturer’s instructions. An aliquot of RNAisol (1 ml) was added to each sample and incubated for 5 min at room temperature. Thereafter, 200 µl chloroform was added to each sample and shaken fiercely for 15 s. The samples were placed at room temperature for 2–3 min and centrifuged at 12 000g for 15 min at 4°C after a biphasic solution was formed. For the RNA precipitation, the aqueous phase (top) was transferred to a clean text tube and then 500 µl isopropanol was added. The samples were stored at room temperature for 5–10 min and centrifuged at 12 000g for 15 min at 4°C after a pellet was visible. To wash the RNA after removing the supernatant, 1000 µl 75% ethanol were added, vortexed and centrifuged at 8000g for 5 min at 4°C as the mol was carefully removed with pipetting, the RNA pellet was air-dried for 5–10 min and then dissolved in diethylpyrocarbonate (DEPC)-treated water with vortexing. RNA quality was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. The isolated RNA was quantified by spectrophotometry (optical density 260/280 nm). The specimens were then incubated for 10–15 min at 55–60°C and immediately frozen at −70°C.

Complementary DNA synthesis and real-time quantitative analysis

Reverse transcription was conducted using the PrimeScript™ RT–PCR Kit (TaKaRa Biotechnology Co., Ltd, One microgram of total RNA was used as templates to convert to complementary DNA. Briefly, reverse transcription was conducted using the PrimeScript™ RT–PCR Kit according to the procedures of specification. The following primers sequences were used for PCR amplification: human MDRI: 5'-CCACTCTCCATTTGACGCGC-3’ (forward), 5'-ACGTTTGCTGTAAGCCAA-3’ (reverse); and the TaqMan probe selected between the primers was fluorescent dye-labelled at the 5’-end and carboxyfluorescein (FAM) at the 3’-end, and the TaqMan probe was 5’-FAM-TGGCCTCAGGCAGCTTTGTC-3’-MARA-3’ (ShineGene, Shanghai, China; GenBank no. NM000927); GAPDH: 5’-gyceraldehyde-3-phosphate dehydrogenase: 5’-CCACTCTCCATTTGACGCGC-3’ (forward), 5’-ACGTTTGCTGTAAGCCAA-3’ (reverse), and the TaqMan probe was 5’-FAM-TGGCCTCAGGCAGCTTTGTC-3’ (ShineGene, Shanghai, China; GenBank no. AP261085). In brief, each PCR was performed in a total volume of 20 µl containing Premix EX TaqTM 10 µl, Rox reference dye 0.4 µl, upstream and downstream primers 0.4 µl, Taqman probe 0.8 µl, deionized H2O 6 µl and complementary DNA 2 µl. PCR cycling conditions were as follows: 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s. Each sample was measured in triplicate, and the data were analyzed by the delta method for comparing relative expression results [ratio, 2^((-CtSample-CtGAPDH)). GAPDH messenger RNA (mRNA) level was used to normalize RNA levels from various samples, and the mRNA expression level was expressed as a relative expression to the basal level.

Western blot analysis

Following treatment, cells were washed twice with ice-cold phosphate-buffered saline, and lysis buffer (50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate), containing the protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride), were added to each well dish. Cells were then scraped off the dish with a plastic policeman and transferred to labeled test tubes and lysed on ice for 15 min. The cell lysate was centrifuged at 14 000g for 15 min at 4°C. The protein content in the supernatant was determined by the bichroninic acid protein assay using a commercial kit (BCA Protein Assay Reagent; Merck & Co. Whitehouse Station, NJ). Protein samples were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to a polyvinylidine difluoride membrane. The membrane was incubated in the blocking buffer (10 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20), containing 5% non-fat powdered milk for 1 h. The membrane was incubated with a specific polyclonal antibody against total P-gp or c-Jun/ phosphor-c-Jun or COX-2 antibody (1:1000; Merck). Following incubation at 4°C overnight, the blot was washed thrice in tris-buffered saline-Tween (0.05% solution) and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Following three washes with tris-buffered saline-Tween solution, AB developer was added (1:1, vol/vol). Data obtained from the western blot experiments were analyzed by Bio-Rad Quantity One 1D Analysis software (Bio-Rad, Hercules, CA).

Transient transfection of COX-2

Cells were transfected with the plRES2-COX-2 or the control vector pRES2 using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Selection of cells stably overexpressing COX-2 and the control vector-transfected cells was achieved in the presence of 600 µg/ml geneticin (Invitrogen).

Small interfering RNA

Stealth small interfering RNA (siRNA) specific for human COX-2 was purchased from Invitrogen. The sequence of COX-2-specific siRNA was 5’-GCTGAAATTTACCCCTCTTAT-3’ and that in the control siRNA was 5’-TCTTCCAGGTCGTACG-3’. Cells stably overexpressing COX-2 were grown in antibiotic-free complete media to 80% confluence in 12-well plates and then transfected with 100 nM COX-2-specific or control siRNA using DharmaFECT Reagent 1 (Dharmacon, Inc, Lafayette, CO) according to the manufacturer’s protocol. Seventy-two and 24 h after transfection, cells were harvested for western blot analysis.

High-performance liquid chromatography analysis

High-performance liquid chromatography (Supelco Co., Ltd, St Louis, MO) analysis was performed on a 1200 system using a diamond C18 reversed-phase column (4.6 mm × 250 mm, 5 µm). The mobile phase consisted of methanol and water (55:45, vol/vol), potassium dihydrogen phosphate (0.06 mol/l) and adjusted to pH 5.0 at a flow rate of 0.7 µl/min. The sample volume injected was 20 µl. The detection wavelength was set at 297 nm.

Statistical analyses of data

Statistical analyses were conducted using the Statistical Package for the Social Sciences. Statistical significance was determined with one-way analysis of variance followed by Fisher’s least significant difference test. All results are reported as the mean ± standard deviation. A P value of <0.05 was considered to be a statistically significant difference.

Results

Blocking JNK and/or COX-2 downregulated the expression of the P-gp/MDRI in HCT8/V cells

To explore the mechanism of resistance to VCR in HCT8/V cells, MDRI promoter recombinant vector pGL3-basic-MDRI-promoter was constructed and its activity was determined in HCT8 and HCT8/V cells. Following co-transfection of the recombinant vector pGL3-basic-MDRI promoter and the control vector pRL-SV40 into cells, the MDRI promoter was activated significantly and the expression level dramatically decreased when treated with SP600125 or NS-398 at 20 μM for 2 h in HCT8/V cells. However, there was only a slight increase in the MDRI promoter activity after co-transfection and a minor change in the expression level when 20 μM SP600125 or NS-398 for 2 h was added to HCT8 cells and incubated for 2 h (Figure 1A).

To verify whether MDRI gene expression could be influenced by the JNK inhibitor SP600125 or COX-2 inhibitor NS-398, the treatment with the inhibitors was conducted in HCT8 and HCT8/V cells, respectively. Real-time quantitative PCR and western blot analyses were performed after cell harvest. The results showed that MDRI mRNA and protein levels significantly decreased in HCT8/V cells, however, only slight changes were observed in HCT8 cells (Figure 1B and C). The observations demonstrated that inhibition of JNK pathway or COX-2 activity led to significant suppression of the expression of MDRI and P-gp levels in HCT8/V cells, suggesting that both COX-2 and JNK were involved in the development of MDR in HCT8/V cells.
When HCT8/V cells were treated with SP600125 in combination with NS-398, a more remarkable decrease in the activity of MDR1 promoter was observed compared with MDR cells treated with the JNK or COX-2 inhibitor alone (Figure 1A). As shown in Figure 1B and C, lower levels of MDR1 mRNA and protein were observed in HCT8/V cells when treated with 20 μM SP600125 alone or the combination (+) for 2 h compared with cells treated with either inhibitor alone. However, the combined use of both SP600125 and NS-398 only gave rise to a slight effect on MDR1 promoter activity and MDR1/P-gp expression in HCT8 cells.

Blocking JNK did not alter the expression of COX-2 in HCT8 and HCT8/V cells

To determine the potential link between JNK and COX-2 and characterize the potential roles of JNK and COX-2 in MDR development, we compared the effects of JNK inhibition on COX-2 expression in HCT8 and HCT8/V cells. Western blot analysis showed that the expression of COX-2 in HCT8/V cells was nearly 4-fold higher than that in HCT8 cells (Figure 2). Addition of 20 μM SP600125 only slightly altered the level of COX-2 in both HCT8 cells and HCT8/V cells (Figure 2). The results showed that inhibition of JNK has no obvious effect on the expression of COX-2 in HCT8/V cells, which suggested that JNK could not regulate MDR1 expression via mediation COX-2 expression.

Blocking COX-2 by a chemical inhibitor or siRNA diminished the levels of p-c-Jun, but not c-Jun, in HCT8/V cells

Due to negligible effects of inhibition of JNK on the COX-2 expression, we further tested the role of COX-2 in the activation of JNK via examination of the contents of c-Jun and p-c-Jun in HCT8 and HCT8/V cells treated with 20 μM NS-398 for 2 h. Western blot analysis showed that the level of c-Jun was comparable in both cell lines and treatment of NS-398 only slightly change the levels of c-Jun in both cell lines (Figure 3A and B). However, the levels of Ser-63 and Ser-73 p-c-Jun were 2-fold higher in HCT8/V cells than those in its counterparts. Following the treatment of NS-398, there was a significant decline of the level of p-c-Jun in HCT8/V cells, whereas no significant change in the p-c-Jun level was observed in HCT8 cells (Figure 3C and D).

To corroborate above observations, we employed the siRNA technique to examine the importance of COX-2 in the activity of c-Jun-induced

Fig. 1. Effect of JNK and/or COX-2 inhibition on the expression of the MDR1 mRNA and P-gp levels in HCT8 and HCT8/V cells. Cells were cotransfected with recombinant vector pGL3-Basic-MDR1 promoter. Dual-reporter gene assays were performed in cotransfected cells with (+) or without (−) treatment of SP600125 (20 μM) or NS-398 (20 μM) for 2 h (A) and the data are expressed as firefly luciferase/renilla luciferase. Data are means ± standard deviations of values from triplicate experiments. †P < 0.01, inhibitor alone or combination versus control vehicle in HCT8/V cells; ‡P < 0.05, NS-398 + SP600125 versus NS-398 alone in HCT8/V cells; † ‡P < 0.05 NS-398 + SP600125 versus SP600125 alone in HCT8/V cells. Real-time quantitative PCR was performed to detect MDR1 mRNA in MDR HCT8/V cells and its parental HCT8 cells treated with 20 μM SP600125 or NS-398 alone or the combination (+) for 2 h (B) The data are representative of at least three experiments. #P < 0.05, † ‡P < 0.01 inhibitor alone or combination versus control vehicle in HCT8/V cells; † ‡P < 0.05 NS-398 + SP600125 versus NS-398 alone in HCT8/V cells. Western blotting assay was carried out to detect P-glycoprotein expression in MDR HCT8/V cells and its parental HCT8 cells treated with 20 μM SP600125 or NS-398 alone or the combination (+) for 2 h. (C) Western blotting with an antibody to β-actin was used to ensure equal loading of proteins in each lane. The bolts were photographed and quantitated for each sample; the data are the mean ± standard deviation of three independent experiments. *P < 0.05, † ‡P < 0.01 inhibitor alone or combination versus control vehicle in HCT8/V cells.

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MDR. HCT8 and HCT8/V cells were exposed to control or COX-2-specific siRNAs. Silencing of COX-2 resulted in a dramatic reduction in the level of p-c-Jun (63/73), but not c-Jun, compared with the cells transfected with the control siRNA (Figure 3C and D). However, knockdown of COX-2 by siRNA did not significantly alter the level of c-Jun and p-c-Jun (63/73) in HCT8 cells. The observations suggested that COX-2 exhibited a stimulative effect on the JNK activation and indicated that COX-2 played a role in the development of MDR in HCT8/V cells via the regulation of the activation of JNK.

Discussion

A significant challenge to the triumph of chemotherapy is drug resistance, by which cells resist to a number of structurally and functionally diverse therapeutic drugs including anticancer and antivirus agents, leading to poor prognosis. Drug resistance, either innate or acquired and especially in form (i.e. multidrug resistance, MDR) associated with P-gp/MDR1, multidrug resistance proteins and/or breast cancer resistance protein, presents a major impediment to achieve successful chemotherapy. One prototypical MDR to anticancer agents is mediated by overexpressed P-gp, a 170 kDa transmembrane glycoprotein encoded by the MDR1 gene. To date, P-gp, a member of the adenosine triphosphate-binding cassette transporter family, is a well-characterized drug efflux pump with substrate promiscuity and ability to export anticancer drugs and other cytotoxic agents from the tumor cells (16–19). This process has previously been interpreted on the basis of the ability of tumor cells to extrude or inactivate the cytotoxic agents or to modify their targets of action, leading to decrease in intracellular accumulation of anticancer drugs and lack of effectiveness in cancer cell killing.

In addition to the consequence of overexpression of P-gp, the development of MDR phenotype is probably associated with modifications of the activity of intracellular, which makes cancer cells more tolerated to cytotoxic agents. Recently, a strong correlation between expression of COX-2 and P-gp was revealed in tumor specimens derived from patients with breast cancer (20). Furthermore, it has been suggested that inhibition of COX-2 sensitized cancer cells to chemotherapeutic drugs via a functional blockade of P-gp and inhibition on its gene expression (21–27). All of these studies strongly suggest that COX-2 modulates P-gp expression and activity and is involved in the development of MDR phenotype.

Recently, it is convincible that JNK is one of the modulators to the development of the MDR phenotype (28–30). It has been reported that the activity of JNK has been implicated in the regulation of P-gp expression. JNK is a member of the mitogen-activated protein kinase family that binds the NH2-terminal activation domain of the transcription factor c-Jun and phosphorylated c-Jun (31). An anticipated consequence of JNK activation is an increased phosphorylation of sites in the NH2-terminal region of the nuclear substrate, c-Jun. It has been reported that JNK presented in a highly activated form in the P-gp-associated MDR variants of KB-3 cells, suggesting that JNK activation is an important component of the cellular response to several structurally and functionally distinct anticancer drugs and may also play a role in the development of MDR phenotype (32). Comerford et al. (33) reported that HeLa cells overexpressing MAPK kinase kinase-1 (MEKK-1) selectively activated JNK leading to increase in the levels of MDR1 mRNA and protein expression. Hypoxia-induced MDR promoter activity is inhibited in a dose-dependent manner by the JNK inhibitor SP600125 (34). Moreover, Takano et al. (35) reported that 5-aza-2'-deoxycytidine enhanced the cytotoxicity of vinblastine in

COX-2 overexpression stimulated JNK activation in HCT8 cells, but not HCT8/V cells

To further ascertain the effect of COX-2 on JNK phosphorylation and the role of COX-2 in the development of MDR, COX-2 was overexpressed in HCT8 and HCT8/V cells via transfection of recombinant vector pLRES2-COX-2 and the expression levels of P-gp, c-Jun and the β-actin were calculated and expressed relative to that in HCT8 and HCT8/V cells. Data are the mean ± standard deviation of three experiments. **P < 0.01, HCT8 versus HCT8/V cells.

COX-2 inhibition increased intracellular VCR accumulation and the sensitivity to VCR in HCT8/V cells

To further verify whether the activity of MDR1 was affected by incubation with the JNK inhibitor SP600125 or the COX-2 inhibitor NS-398, intracellular accumulation of VCR was determined in HCT8/V cells. As shown in Figure 5A, the results showed that the intracellular VCR accumulation in HCT8/V cells was significantly lower than that in HCT8 cells. Obviously, in the presence of NS-398 or SP600125, the intracellular levels of VCR were increased nearly 2-fold (Figure 5A). In agreement with these observations, the MTT assay revealed that HCT8/V cells exhibited a 10.49-fold higher resistance to VCR, compared with their parental chemosensitive counterparts (Table I). Following the treatment with SP600125 or NS-398, the IC50 values of VCR in HCT8/V cells decreased significantly from 191.08 ± 18.18 μg/ml to 50.34 ± 15.71 and 32.23 ± 7.48 μg/ml, respectively, compared with the control cells. These data further suggest that the reversion of MDR in HCT8/V cells was responsible for blocking JNK pathway or COX-2 expression.

We further examined the effects of incubation time and concentration of SP600125 and NS-398 on the cytotoxicity of VCR in HCT8/V cells. In HCT8/V cells, the IC50 values of VCR decreased in a time- and concentration-dependent manner (Figure 5B, C, D and E). Incubation of the cells with SP600125 or NS-398 for 1–4 h caused a significant decrease in the IC50 values of VCR in a time-dependent manner (Figure 5B and D). MTT results showed that both SP600125 and NS-398 at 5–40 μM significantly decreased the IC50 values of VCR in a concentration-dependent manner (Figure 5C and E). Thus, a concentration of 20 μM and an incubation time of 2 h for both inhibitors were used in all our inhibitory assays.

Fig. 2. Effect of JNK inhibition on COX-2 protein levels in HCT8 and HCT8/V cells. HCT8/V cells and its parental HCT8 cells were treated with 20 μM SP600125 for 2 h (+). Western blotting with an antibody to β-actin was used to ensure equal loading of proteins in each lane. The ratio of COX-2 to β-actin was calculated and expressed relative to that in HCT8 and HCT8/V cells. Data are the mean ± standard deviation of three experiments. **P < 0.01, HCT8 versus HCT8/V cells.
Caki-1 cells via JNK activation and subsequent suppression of the MDR1 gene.

Although a number of studies have showed that COX-2 and JNK-signaling pathway are associated with the P-gp-mediated drug resistance (27,36), the relationship between COX-2 and JNK in the development of MDR is elusive. In the present study, to characterize the role of COX-2 and JNK in the development of MDR, HCT8 and HCT8/V cell lines were treated with inhibitors of COX-2 and JNK. We observed that there was a high level of MDR1 mRNA and P-gp level in HCT8/V cells. Our data indicated that the expression of MDR1 mRNA and protein in HCT8/V cells was 5-fold higher than that in HCT8 cells. This observation is consistent with previous studies that the chemoresistance phenotype is associated with enhanced MDR1 gene expression (37,38).

To investigate the effect of COX-2 and JNK on MDR1 mRNA and protein expression in HCT8/V cells in vitro, we confirmed that blocking COX-2 or JNK downregulated the expression of the MDR1 gene (Figure 1A, B and C). Notably, combined using of inhibitors of JNK and COX-2, we found a dramatic downregulation in the activity of MDR1 promoter and expression of MDR1/P-gp. In sight of these data, it is possible that JNK and COX-2 can functionally superposed for each other as they have been shown to have the effect of decreasing MDR1 gene expression and P-gp level. In contrast to our study, Kang et al. (1) showed that FM3A/M cells became drug resistant due to overexpression of MDR1 associated with downregulation of JNK activity. In another study, JNK activity negatively correlated with the P-gp level in resistant gastric and pancreatic cancer cells (39). This reflects a complicated nature of MDR1/P-gp regulation by JNK with the involvement of other modulators.

In order to investigate the underlying mechanism of how COX-2 and JNK contribute to MDR development, this study characterized the role of COX-2 in regulating P-gp expression in human colorectal cancer MDR cells. In our study, reverse transcriptase–PCR and western blot assay showed that the level of MDR1 mRNA and protein in HCT8/V cells were significantly decreased after exposure to the COX-2 inhibitor NS-398. Our results are in agreement with previous studies, which showed that COX-2 inhibition reduced MDR1/P-gp expression and function in the MCF7 breast cancer cell line treated with increasing doses of doxorubicin (40). A previously study has demonstrated that REIC/Dkk-3 overexpression in MCF7/ADR cells caused c-Jun activation and P-gp downregulation in a JNK-dependent manner (41). Our study demonstrated the COX-2 overexpression in HCT8/V cells caused the c-Jun activation and P-gp upregulation. Although the link between c-Jun and COX-2 has been established
by Newton et al. (42) since 1997, it remains unclear how this link works and contributes to MDR development. Relevant to the present study are the findings that COX-2 expression depends on JNK-induced activator protein-1 (AP-1) transcriptional activity (43). Indeed, there is evidence that JNK was downstream of COX-2 (44). Our results provide a potentially direct link between JNK and COX-2: the phosphorylation of c-Jun by JNK activation seems to be stimulated by COX-2. Our data have shown that there was no significant change

![Diagram](https://example.com/diagram.png)

**Fig. 4.** Effect of COX-2 overexpression on JNK activation in HCT8 and HCT8/V cells. HCT8 and HCT8/V cells were cotransfected with vector pRES2 or COX-2 stably overexpressed vector pRES2-COX-2 and grown in complete media to 80% confluence. Cells were examined for the levels of P-gp, c-Jun and phosphorylated c-Jun by western blotting as described in Materials and Methods. Western blotting with an antibody to β-actin was used to ensure equal loading of proteins in each lane. (A) The ratio of P-gp (B), c-Jun (C), p-c-Jun (Ser63) (D) and p-c-Jun (Ser73) (E) to β-actin was calculated and expressed relative to that of control groups. Data are the mean ± standard deviation of three independent experiments. *P < 0.05, cells transfected with empty control vector pRES2 versus cells transfected with pRES2-COX-2 vector in HCT8 cells.
of the expression of COX-2, when JNK pathway was blocked by SP600125 (Figure 2); however, the level of p-c-Jun was significantly diminished when there was a reduced expression and activity of COX-2 in HCT8/V cells (Figure 3C and D). The reduced p-c-Jun protein levels might be ascribed to decreased COX-2 activity by a chemical inhibitor or siRNA approach in HCT8/V cells, resulting in decreased MDR1/P-gp expression. In contrast, overexpression of COX-2 in HCT8 cells enhanced the phosphorylation of c-Jun by JNK activation and stimulated the level of P-gp in HCT8 cells (Figure 4B, D and E). Transfection of the pLRES2-COX-2 vector into HCT8/V cells only resulted in a minor effect on P-gp and p-c-Jun expression, probably due to a high level expression of COX-2 and P-gp before transfection in the cells. These findings suggest that COX-2 stimulates the level of P-gp via enhanced JNK phosphorylation.

Since MDR cells often show a decrease in intracellular drug accumulation due to active efflux by P-gp (45), we checked on whether COX-2 and JNK inhibition affected intracellular VCR accumulation and the resistance in HCT8/V cells. Our study showed that the

Fig. 5. Effect of JNK or COX-2 inhibition on intracellular VCR accumulation and IC₅₀ values of VCR in HCT8/V cells. A validated high-performance liquid chromatography method was used to detect intracellular VCR accumulation in HCT8 and MDR HCT8/V cells, which were treated with 20 μM SP600125 or NS-398 for 2 h. (A) The data are representative of at least three experiments, which are presented as the mean ± standard deviation. ** P < 0.01 inhibitor alone versus control vehicle in HCT8/V cells. MTT assay was used to detect IC₅₀ values of VCR in MDR HCT8/V cells treated with SP600125 or NS-398 at 10 μM for 0.5, 1, 2 and 4 h (B and D, respectively). The inhibitor concentration effect on the IC₅₀ of VCR was also examined. HCT8/V cells were treated with SP600125 or NS-398 at 5, 10, 20 and 40 μM for 2 h (C and E, respectively), and the IC₅₀ values were determined by MTT assay. Data are the mean ± standard deviation of three experiments. * P < 0.05, ** P < 0.01, versus control.
Table I. Effect of JNK or COX-2 inhibition on VCR cytotoxicity in HCT8 and HCT8/V cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} to VCR (µg/ml)</th>
<th>Resistance fold compared with HCT8</th>
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<tbody>
<tr>
<td>HCT8/V</td>
<td>191.08 ± 18.18</td>
<td>10.49</td>
</tr>
<tr>
<td>HCT8</td>
<td>18.22 ± 7.12</td>
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</tr>
<tr>
<td>HCT8/V + SP600125</td>
<td>50.34 ± 15.71*</td>
<td>2.76</td>
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<tr>
<td>HCT8/V + NS-398</td>
<td>32.23 ± 7.48*</td>
<td>1.77</td>
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MTT assay was used to detect IC_{50} values of VCR in MDR HCT8/V cells and its parental HCT8, which were without or with 20 µM SP600125 or NS-398 for 2 h and refed with fresh medium at various concentrations of VCR for 48 h. The experiment was performed for thrice with similar results. Columns, mean of three independent experiments; bars, SD, RF, resistance folder.

*P < 0.01 versus HCT8/V.

intracellular accumulation of VCR was significantly increased in HCT8/V cells when treated with SP600125 or NS-398 (Figure 5A). The IC_{50} of VCR in HCT8/V cells also significantly decreased when JNK or COX-2 was inhibited (Figure 5 and Table I). These results are consistent with our findings that inhibition of JNK or COX-2 suppresses the expression and function of MDR1/P-gp and thus increase drug accumulation and sensitivity.

In this paper, our in vitro experiments presented have provided enough evidence that COX-2 contributes to P-gp-mediated MDR via regulation of JNK-signaling pathway. This include: (a) inhibition of JNK and COX-2 downregulates the expression of MDR1/P-gp in HCT8/V cells, (b) inhibition of JNK or COX-2 increases intracellular VCR accumulation and its cytotoxicity in HCT8/V cells, (c) inhibition of COX-2 by chemical inhibitor or its knockdown by siRNA downregulates p-c-Jun at Ser63 and 73 and (d) overexpression of COX-2 upregulates p-c-Jun (Ser63/73) in HCT8 cells.

In conclusion, we have shown that the first report of direct causal relationship between COX-2 expression and JNK activation in human colorectal cancer MDR cells. Our findings provided evidence that COX-2 and p-c-Jun overexpression were responsible for the development of MDR in HCT8/V cells. NS-398, a selective COX-2 inhibitor, could downregulate the expression of MDR1 mRNA and protein via JNK pathway. The observations, therefore, provided new insights into the regulation of P-gp expression in MDR cells and suggested new potential strategies for the reversal of P-gp-mediated anticancer drug resistance. However, other signaling molecules may also participate in the regulation of the activity of JNK in HCT8/V cells and thus contribute to MDR development. Further studies are needed to explore how COX-2, JNK and other signaling molecules interact in the development of P-gp-mediated MDR in cancer cells.

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References