Enhancement of Paclitaxel-induced Apoptosis by Inhibition of Mitogen-activated Protein Kinase Pathway in Colon Cancer Cells

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Abstract. *Resistance to chemotherapy represents a major obstacle to improving the survival of patients with colorectal cancer. In this study, the inhibition of the mitogen-activated protein kinase (MAPK) pathway was demonstrated to markedly enhance the apoptosis of colon cancer cells induced by paclitaxel, one of the key chemotherapeutic drugs widely used to treat various types of cancer. The treatment of the colon cancer cell lines SW480 and DLD-1 with paclitaxel resulted in increased activation of the MAPK pathway, which was blocked by PD98059, a MEK inhibitor. In both cell lines, MAPK inhibition by PD98059 led to a dramatic enhancement of the paclitaxel-induced apoptosis, as determined by cell cycle analysis and Hoechst 33342 staining, although the inhibitor alone did not affect apoptosis. This effect was restricted to paclitaxel since PD98059 did not alter the sensitivity to other drugs, including 5-fluorouracil (5-FU) and camptothecin (CPT). Importantly, selective blockage of the MAPK pathway by small interfering RNA (siRNA) also increased the apoptotic cell death induced by paclitaxel. These findings highlight the importance of the MAPK pathway in paclitaxel-induced apoptosis and suggest that a combined treatment with paclitaxel and MEK inhibitors could be an attractive therapeutic strategy against colon cancer.*

Colorectal cancer is one of the most common malignancies worldwide and its incidence has increased in Japan. Despite recent popularization of cancer screening programs, many patients with colon cancer are still diagnosed at an advanced stage. Surgical resection followed by adjuvant chemotherapy is a standard treatment option for patients with advanced colon cancer (1-3). However, a significant fraction of colon carcinomas show resistance to conventional chemotherapeutic agents, including paclitaxel (4, 5). To improve the prognosis of patients with colon cancer, the development of new therapeutic strategies based on our refined understanding of the molecular mechanisms responsible for drug resistance is needed.

Paclitaxel (taxol) is a member of the taxane class of agents that target microtubules (6). With its strong antineoplastic activity, paclitaxel is currently used for the treatment of a wide range of carcinomas including breast, ovarian and non-small cell lung cancer (6). However, the emergence of resistance remains a significant clinical problem and a major limitation to the application of this drug to other types of neoplasm (5). There have been several potential mechanisms reported to account for the paclitaxel resistance observed, such as the overexpression of P-glycoprotein, mutations in tubulin, and cellular total antioxidant capacity (7, 8).

Among the mechanisms related to paclitaxel resistance, altered cell signaling pathways have recently attracted considerable interest, because modulation of these pathways could be a novel anticancer strategy (9). Previous studies have described activation of the mitogen-activated protein kinase (MAPK) pathway in response to paclitaxel treatment (10-14). The MAPK pathway, implicated in the regulation of cell growth and survival, have been reported to be aberrantly activated in a broad spectrum of human tumors, especially in colorectal carcinomas (15, 16). Although the relevance of the MAPK pathway to the chemotherapeutic action of paclitaxel has not been fully understood, activation of this pathway could play a role in drug resistance. In fact, inhibition of the MAPK pathway has been shown to augment the sensitivity to paclitaxel in certain types of cancer (10-14). To our knowledge, however, the relationship between MAPK pathway activity and paclitaxel resistance has not been investigated in colon cancer.

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In the present study, the effect of MAPK pathway activation on the sensitivity to paclitaxel was determined in colon cancer cell lines.

Materials and Methods

Reagents and cell cultures. Paclitaxel, 5-fluorouracil (5-FU), and camptothecin (CPT) were purchased from Sigma-Aldrich (St. Louis, MA, USA). The MEK inhibitor, PD98059 was purchased from Calbiochem (San Diego, CA, USA). Two human colon carcinoma cell lines, SW480 and DLD-1, were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and penicillin (100 U/ml) at 37˚C in a humidified incubator containing 5% CO_2.

Immunoblot analysis. Whole cell lysates were extracted from the cultured cells using M-PER (Mammalian Protein Extraction) Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). The whole-cell extract (40-80 μg) was separated by electrophoresis on SDS-polyacrylamide gel, and transferred to nitrocellulose membranes (Protran, Dassel, Germany). The blots were then incubated with an anti-ERK (extracellular (signal)-regulated kinase) antibody (p44/42 MAP kinase (137F5) rabbit mAb; Cell Signaling Technology; Danvers, MA, USA; 1:500) or anti-phosphorylated ERK (phospho-p44/42 MAPK (Thr202/Tyr204) (197G2) Rabbit mAb, Cell Signaling Technology, 1:500) overnight at 4˚C. The blots were then incubated in horseradish peroxidase (HRP)-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 hour. Immunocomplexes were detected with electrochemiluminescence (ECL) plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad).

Flow cytometry. The cells (3.2×10^5 per well) were plated in 6-well plates and treated with appropriate combinations of drugs. Adherent and detached cells were harvested by trypsinization, and fixed in ice-cold 70% ethanol for at least 1 h. The cell pellets were washed twice with cold phosphate-buffered saline (PBS) and incubated for 30 minutes at room temperature in 1 ml PBS containing 50 μg propidium iodide (Sigma-Aldrich), 0.1% Triton X-100, 1 mM EDTA and 0.5 mg RNaseA. After staining, the samples were analyzed with a FACScan (BD Biosciences, San Jose, CA, USA) of 20,000 events per sample. The data from the flow cytometry were analyzed with ModFit LT software (Verity Software House, Topsham, MN, USA). Fragmented apoptotic nuclei were recognizable by their subdiploid (sub-G1) DNA content. The percentage of sub-G1 cells was recorded for each sample.

Hoechst 33342 staining. The cells were grown in 6-well plates in the presence or absence of appropriate drugs, washed in PBS and stained with Hoechst 33342. After staining, the cells were examined for nuclear changes (*i.e.*, chromatin condensation and nuclear fragmentation) characteristic of apoptosis under fluorescence microscopy (TE300; Nikon, Tokyo, Japan).

RNA interference. Small interfering RNA (siRNA) targeting p42 and p44 MAPK were purchased from Cell Signaling Technology. The cells were transfected with appropriate siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsberg, CA, USA) according to the manufacturer's instructions. Control transfections were performed with SignalSilence Control siRNA (fluorescein conjugate; Cell Signaling Technology).

Real-time RT-RCR. The total RNA was isolated with an RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA). The RNA was reverse transcribed into cDNA with a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Following reverse transcription, the cDNA was amplified with SYBR Premix Ex Tap (Takara Bio, Shiga, Japan) and the DNA Engine Option 2 System (Bio-Rad). A cDNA stock solution from SW480 colon cancer cells was serially diluted in 10-fold increments and simultaneously processed for PCR amplification for each primer pair to generate the standard curve. The forward primer for ERK1 was 5'-CAA CAT GAA GGC CCG AAA CTA CC-3' and the reverse primer for ERK1 was 5'-TAA CAT CCG GTC CAG CAG GTC AAG-3'. The forward primer for ERK2 was 5'-TAC ACC AAC CTC TCG TAC ATC G-3' and the reverse primer for ERK2 was 5'-CAT GTC TGA AGC GCA GTA AGA TT-3'. Each sample was run in triplicate.

Statistical analysis. The Student's *t*-test was used for statistical analysis. A *p*-value of less than 0.05 was considered significant.

Results

Effect of paclitaxel on MAPK pathway activity. Using Western blot analysis, both the SW480 and DLD-1 cell lines exhibited basal expression of phosphorylated ERK, indicating constitutive activation of the MAPK pathway and the expression increased in response to the treatment with paclitaxel (Figure 1). Treatment with the MEK inhibitor, PD98059, significantly inhibited both basal and paclitaxel-increased expression of phosphorylated ERK.

Effect of PD98059 on paclitaxel-induced apoptosis. Exposure to PD98059 alone at concentrations of 1-100 μM had almost no effect on the apoptosis of either the SW480 or DLD-1 cell lines, as measured by the sub-G1 fraction of cell cycle analysis (Figure 2a) or by nuclear fragmentation, a typical morphological feature of apoptosis (Figure 2b). Treatment of the SW480 and DLD-1 cells with paclitaxel at concentrations of 5-20 nM led to an increase in the G2/M population, followed by an increased fraction of the sub-G1 population ((17) and data not shown). While treatment with paclitaxel alone dose-dependently induced apoptosis in ~20-40% of the SW480 and DLD-1 cells, combined treatment with PD98059 markedly augmented the cytotoxic effect of paclitaxel, leading to the apoptotic cell death of approximately 40-70% (Figure 2c). PD98059 at concentrations 1-100 μM increased the paclitaxel-induced apoptosis in a dose-dependent manner (Figure 2d). Remarkably, the combined treatment with paclitaxel at 10 nM and PD98059 at 100 μM resulted in massive apoptotic cell death in over 70% of the cells, while treatment with each drug alone induced apoptosis in only a

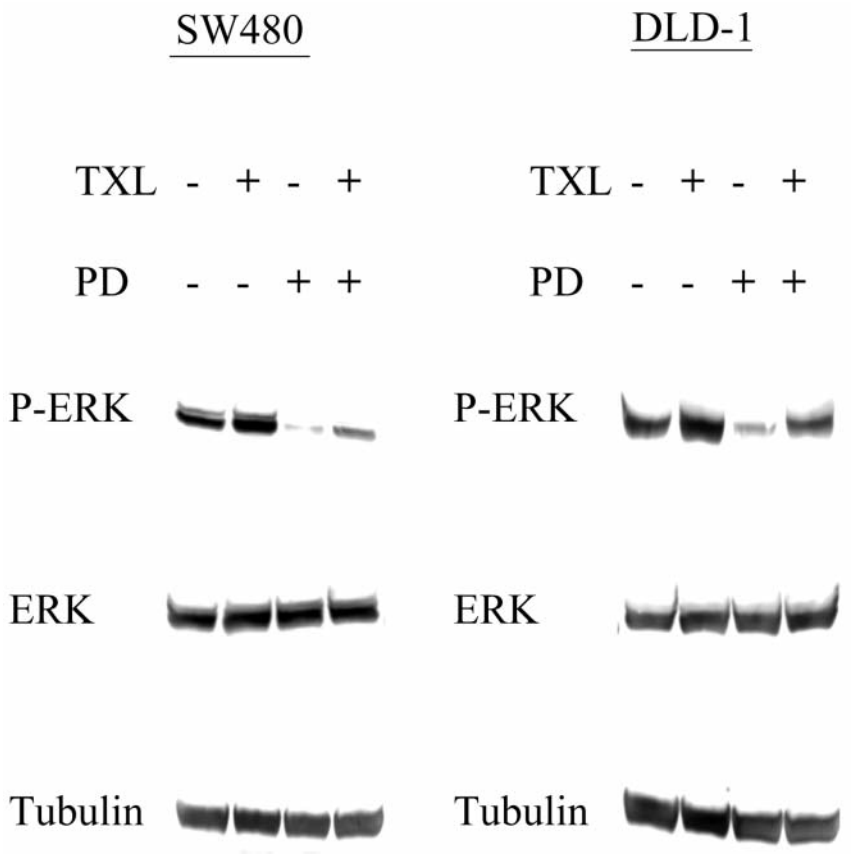

Figure 1. *MAPK pathway activity in colon cancer cell lines treated with paclitaxel in the presence or absence of the MEK inhibitor PD98059. SW480 and DLD-1 cells treated with or without paclitaxel (TXL) at 10 nM and PD98059 (PD) at 30 μM for 24 hours, and analyzed by immunoblot with antibody for phosphorylated ERK (P-ERK), total ERK (ERK) or tubulin.*

small fraction. The enhanced apoptosis by PD98059 was confirmed morphologically by nuclear staining with Hoechst 33342. The percentage of cells showing nuclear fragmentation increased by combined treatment with paclitaxel and PD98059 as compared to treatment with either drug alone (Figure 2e).

Effect of PD98059 on apoptosis induced by 5-fluorouracil and camptothecin. SW480 and DLD-1 cells were treated with 5-FU or CPT, drugs commonly used for the treatment of colon cancer, combined with or without PD98059. The treatment of each cell line with 5-FU (200 μg/ml) or CPT (20 μM) alone induced apoptosis in ~15-40% of cells (Figure 3). In both the cell lines, PD98059 at 30 μM, a concentration sufficient to augment the paclitaxel-induced apoptosis, did not alter the percentage of apoptosis induced by 5-FU or CPT (Figure 3), suggesting that the enhanced apoptosis by PD98059 was specific to paclitaxel.

Effect of MAPK inhibition by siRNA on paclitaxel-induced apoptosis. In order to confirm that the enhancement of paclitaxel-induced apoptosis by PD98059 was a result of the inhibition of MAPK activity and not of the possible non-specific effects of PD98059, another model was used to block the MAPK cascade. To specifically block the MAPK pathway activity, siRNA was used to knockdown p44 (ERK1) and p42 (ERK2) expression in the SW480 and DLD-1 cells. Transfection of siRNA targeting p44 and p42 resulted in ~70% and ~90% knockdown of p44 and p42 mRNA expression, respectively (Figure 4a). Western blot analysis confirmed a lower expression of both total and phosphorylated ERK by siRNA-mediated knockdown of p44 and p42, whether alone or in combination (Figure 4b). Although knockdown of p44 and p42 exerted almost no effect on apoptosis of the transfected cells, the resulting inhibition of MAPK activity drastically enhanced the apoptosis induced by paclitaxel (Figure 4c).

Discussion

In the present study, inhibition of the MAPK activity by PD98059 was demonstrated to markedly enhance the paclitaxel-induced apoptosis in two colon cancer cell lines in which constitutive activation of the MAPK pathway was

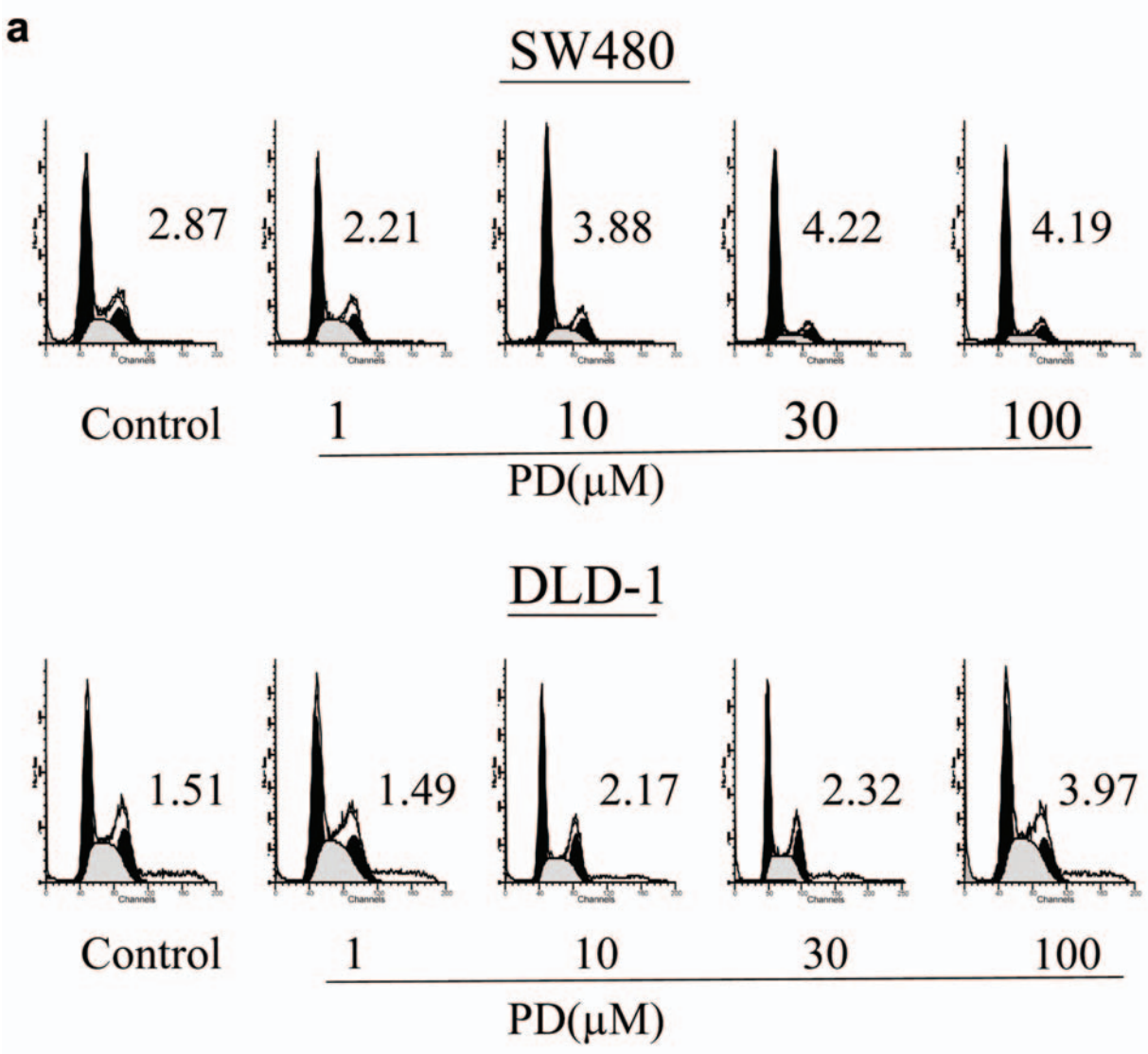

Figure 2. *Effects of PD98059 on paclitaxel-induced apoptosis in SW480 and DLD-1 colon cancer cells. (a) Cell cycle analysis after treatment with 0 (control)-100 μM of PD98059 alone. The value in each treatment group represents the percentage of the sub-G1 fraction. (b) Hoechst 33342 staining after treatment with 30 μM PD98059 alone. (c) Cell cycle analysis after treatment with paclitaxel (TXL) in the presence or absence of 30 μM PD98059. The value in each treatment group represents the percentage of the sub-G1 fraction. (d) Cell cycle analysis after treatment with 10 nM paclitaxel in the presence of 0-100 μM PD98059 at concentrations. The value in each treatment group represents the percentage of the sub-G1 fraction. (e) Hoechst 33342 staining after treatment with 10 nM paclitaxel with or without 30 μM PD98059. All treatments, 24 hours.*

detected. Furthermore, the siRNA-mediated knockdown of p44 (ERK1) and p42 (ERK2) expression similarly augmented the paclitaxel-induced apoptosis. These findings highlighted the importance of the MAPK pathway in paclitaxel-induced apoptosis and suggested that a combined treatment with paclitaxel and MEK inhibitors could be an attractive therapeutic strategy against colon cancer. Because a number of potent MAPK pathway inhibitors are being currently evaluated in clinical trials, our results are of great importance in the treatment of refractory colon cancer using paclitaxel or, possibly, similar microtubule-targeting drugs.

The MAPK pathway is known to be activated in a broad spectrum of human tumors through different mechanisms, including activating mutations in *RAS*, overexpression or activating mutations of epidermal growth factor (EGF) receptors and activating mutations of *RAF* (18, 19). Our present study is in agreement with other reports of constitutive activation of the MAPK pathway in a number of colon cancer cell lines and primary tumors (15, 16). Because activation of the MAPK signaling pathway is generally

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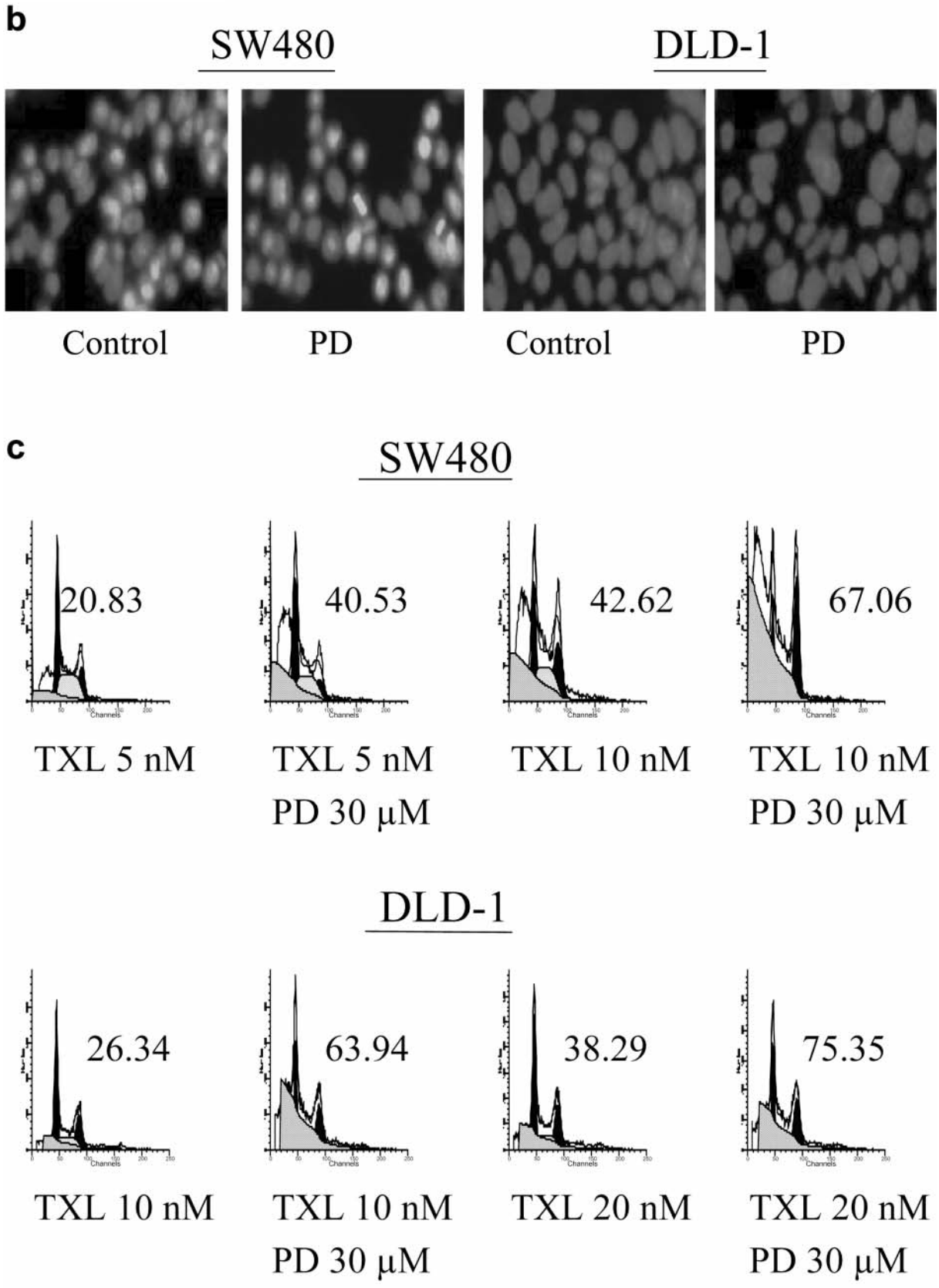

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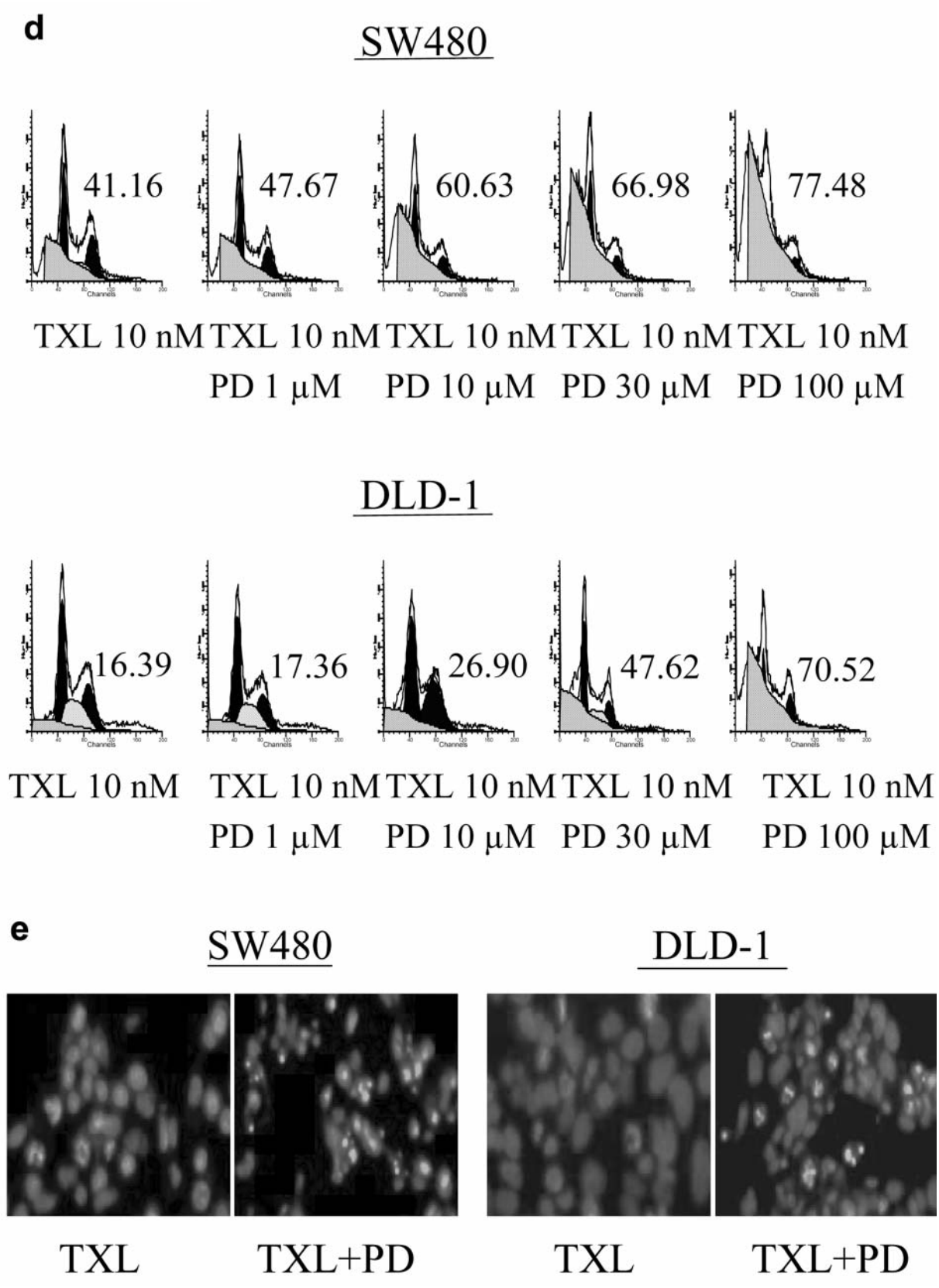


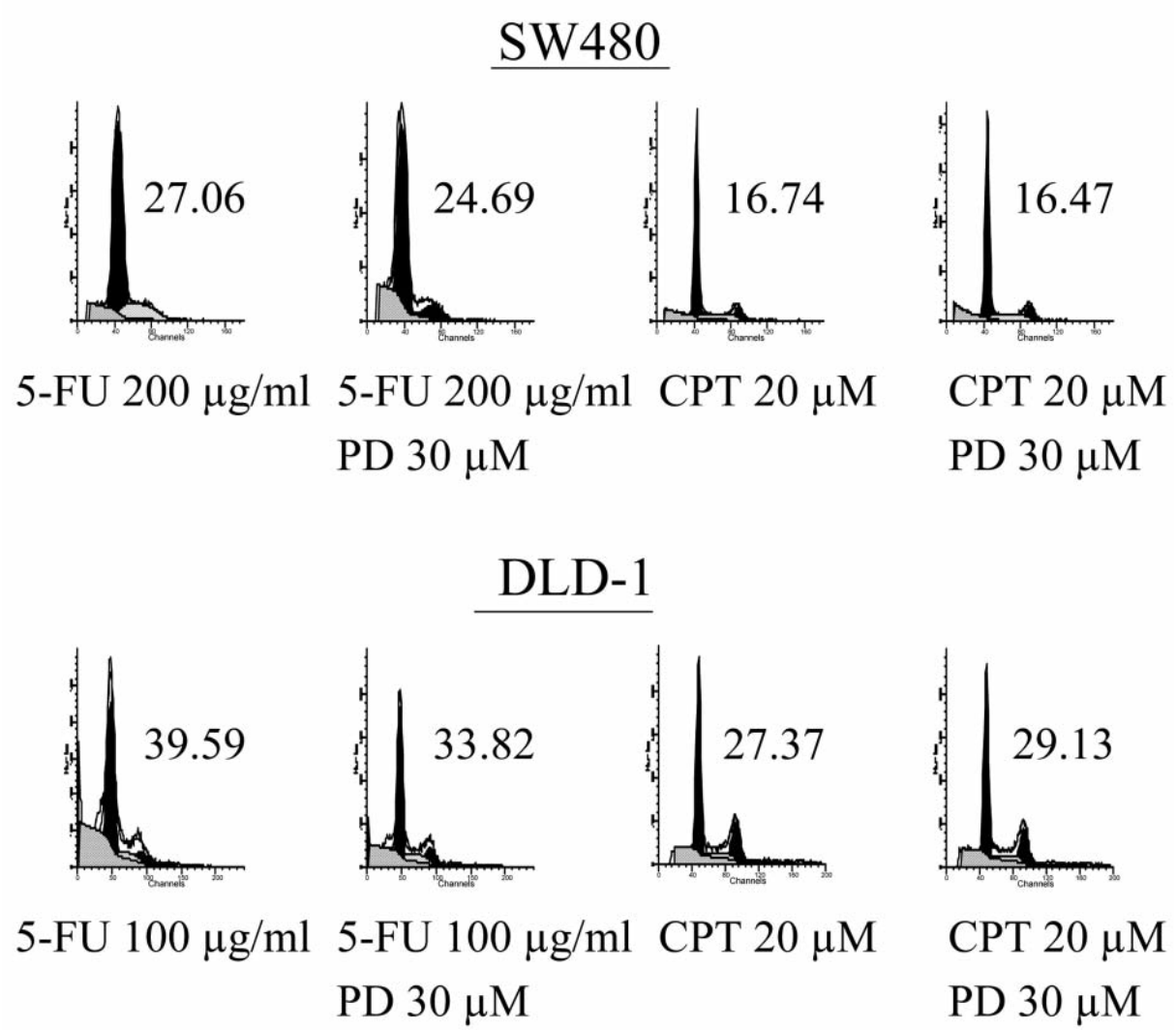
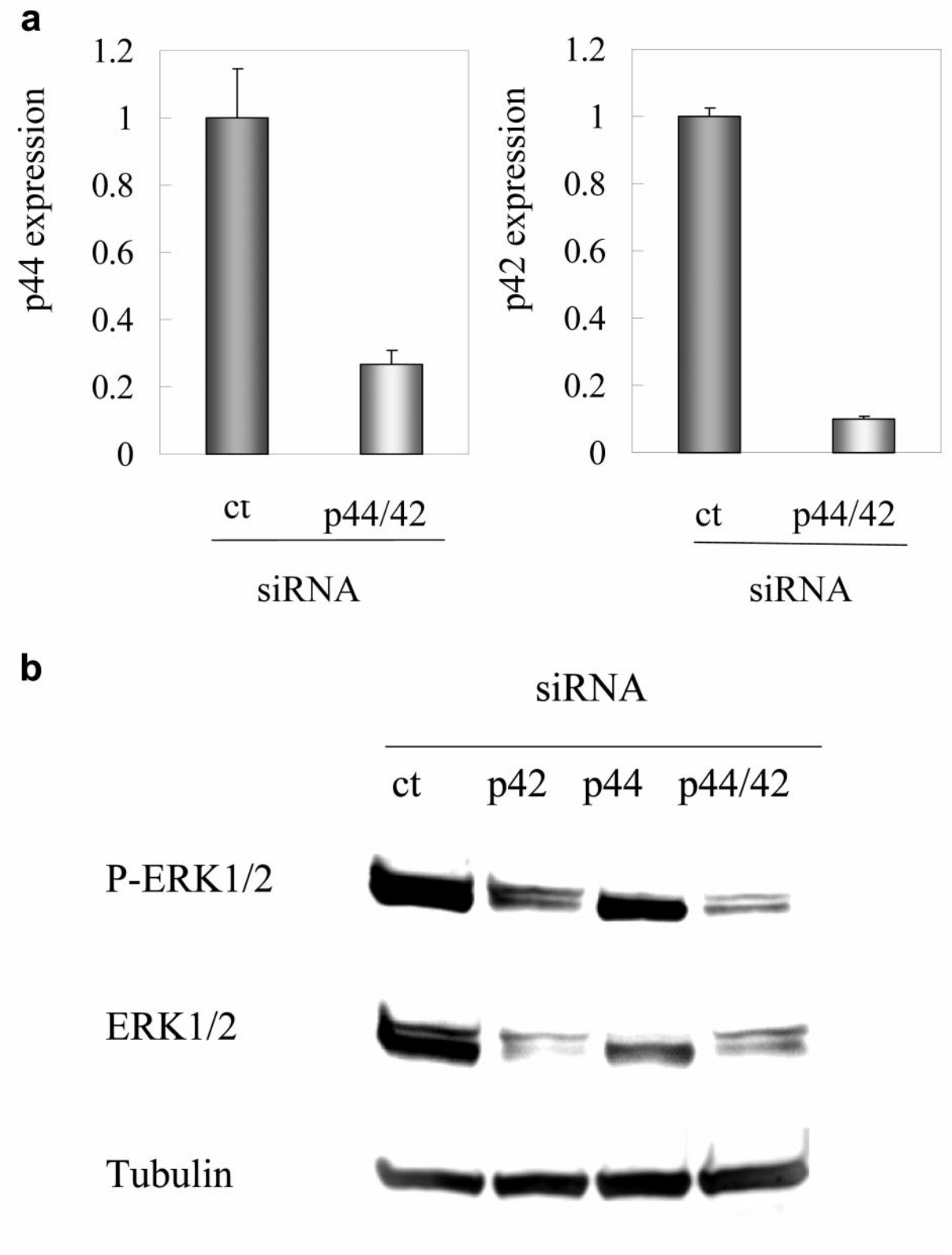


Figure 3. *Cell cycle analysis of SW480 and DLD-1 cells treated with indicated concentrations of 5-fluorouracil (5-FU) or camptothecin (CPT) in the presence or absence of 30 μM PD98059 for 48 hours. The value in each treatment group represents the percentage of the sub-G1 fraction.*

associated with enhanced cell proliferation and survival, this pathway has been considered an attractive target for anticancer therapies (18, 19). In fact, a variety of MAPK inhibitors have been developed and evaluated in clinical trials (18, 19). For example, a small molecule MEK inhibitor has been shown to inhibit the growth of colon carcinomas in animal models (20). These findings suggest that inhibitors of the MAPK pathway can be effective even as single agents.

Alterations in cell signaling pathways, particularly MAPK pathways, may play an important role in the complex mechanisms underlying the resistance to chemotherapeutic agents (9). The present results in colon cancer concur with previous reports showing that the MAPK pathway is activated by treatment with paclitaxel and that inhibiting MAPK activation can increase the sensitivity to paclitaxel in different types of cancer, including breast, ovarian and lung cancer (10-13). By contrast, it has been shown that inhibition of MAPK activation by PD98059 or SB203580 markedly reduced the ability of paclitaxel to induce apoptosis in MCF7 breast carcinoma cells and other cell lines (21). The different effects of MAPK inhibition on paclitaxel-induced apoptosis may be due to the different cell types with different genetic/epigenetic status and to the different concentrations of drugs used in these studies. Further studies (including *in vivo* experiments) are required, however, to validate our results and to establish the therapeutic role of MAPK inhibitors against colon cancer.

The exact mechanism by which inhibition of the MAPK pathway enhances paclitaxel-induced apoptosis remains largely unknown. It is generally suggested that activation of



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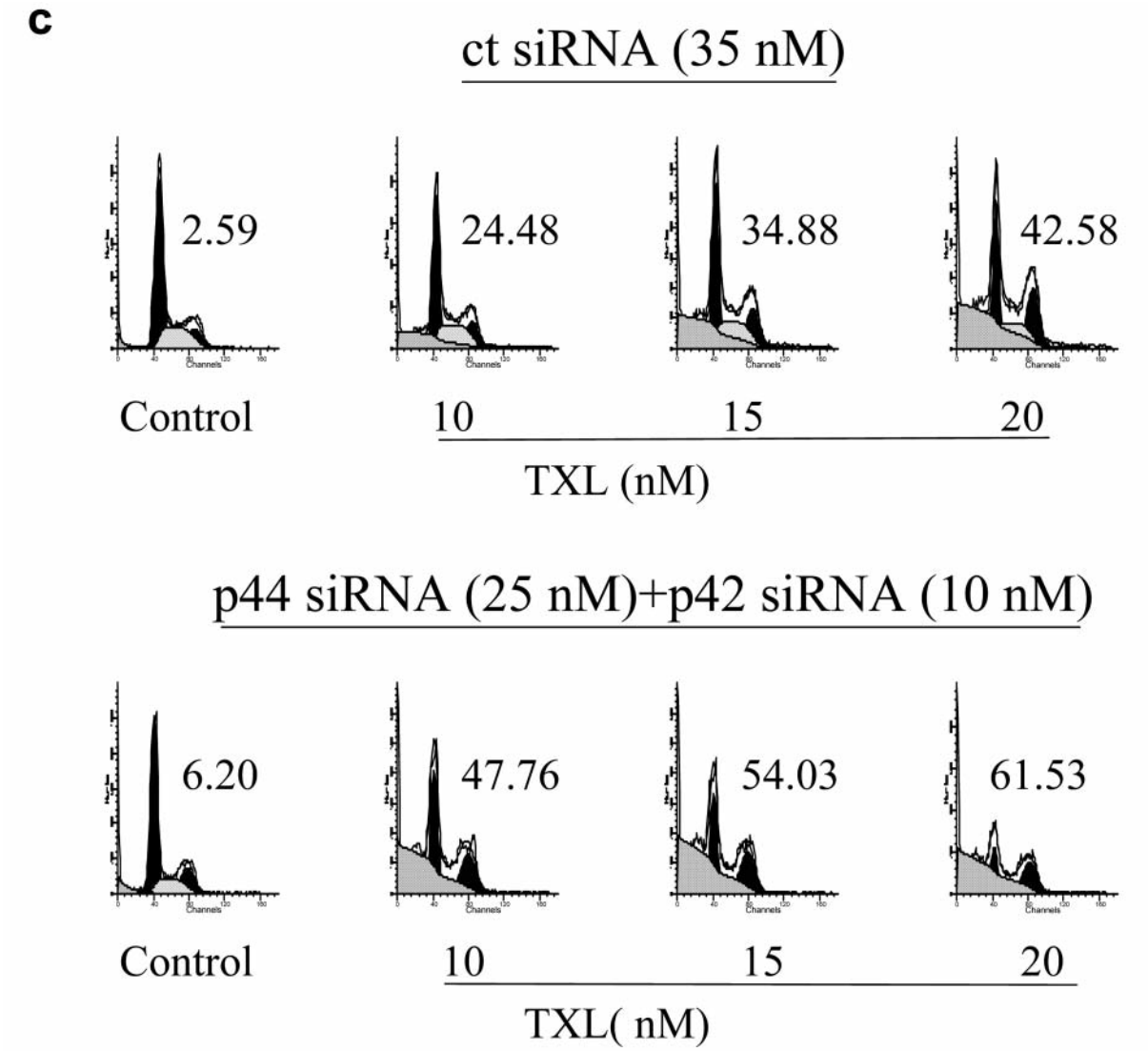

Figure 4. *Selective blockage of MAPK pathway by small interfering RNA (siRNA). (a) Real-time RT-PCR analysis of SW480 cells transfected with siRNA targeting p44 (ERK1) and p42 (ERK2), showing ~70% and ~90% knockdown of p44 and p42 mRNA expression, respectively. (b) Western blot analysis of SW480 cells transfected with siRNA targeting p44 (ERK1) and p42 (ERK2), either alone or in combination. (c) Cell cycle analysis of SW480 cells treated with paclitaxel after transfection with siRNA targeting p44 and p42 ERK. The value in each treatment group represents the percentage of the sub-G1 fraction.*

the MAPK pathway is associated with antiapoptotic processes. For example, activation of the MAPK pathway has been shown to lead to the phosphorylation of B-cell leukemia (Bcl)-2 protein (22), the suppression of the apoptotic effect of Bcl-2-associated death agonist (BAD) (23) and the accumulation of the p53 tumor suppressor (24). Interestingly, it was recently reported that inhibition of the MAPK pathway resulted in the down-regulation of P-glycoprotein and diminished cellular multidrug resistance (25), which could be another mechanism by which inhibition of the MAPK pathway enhances paclitaxel-induced apoptosis.

There is increasing interest in identifying inhibitors of cellular pathways that can synergistically increase the sensitivity to chemotherapeutic drugs. For example, cyclopamine, an inhibitor of the Sonic Hedgehog signaling pathway, has been shown to sensitize cancer cells to certain anticancer drugs, including paclitaxel (26). In most colon tumors, however, the Hedgehog pathway is not activated, suggesting that this pathway may not be an ideal therapeutic target (27, 28). Kawaguchi and coworkers have shown that simultaneous inhibition of the MAPK and phosphatidylinositol 3'-kinase pathways greatly enhanced sensitivity to paclitaxel in ovarian carcinoma (14). More recently, we demonstrated

that γ-secretase inhibitors dramatically enhanced taxane-induced mitotic arrest and apoptosis in colon cancer cells (17). These findings and the present study suggest that MAPK inhibitors, either alone or in combination with other pathway inhibitors, could be a new therapeutic strategy improving the effects of paclitaxel in patients with refractory colon cancer.

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