Inhibition of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase Enhances Chemotherapeutic Effects on H460 Human Non-Small Cell Lung Cancer Cells through Activation of Apoptosis[1]

Yanping Hu, Marcel Bally, Wieslawa H. Dragowska, and Lawrence Mayer[2]

Department of Advanced Therapeutics, British Columbia Cancer Agency [Y. H., M. B., W. H. D., L. M.], and Celator Technologies Inc. [M. B., L. M.], Vancouver, British Columbia, V5Z 4E6 Canada

Abstract

The effects of Dox (Dox), paclitaxel (Taxol), and serum starvation on the regulation of XIAP (X-linked inhibitor of apoptosis), Bcl-2 phosphorylation, and apoptosis were evaluated in human H460 non-small cell lung cancer cells. Protein kinases that responded to these treatments as prosurvival elements in signal transduction were identified by simultaneously screening phosphorylation of protein kinases in H460 cells cultured in serum-free medium or treated with Dox. We demonstrated that Dox and Taxol induced apoptosis through down-regulation of XIAP and phosphorylation of Bcl-2 in a concentration-dependent manner without changing expression of Bcl-xL in H460 cells. These effects were paralleled by activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase protein. We identified that serum starvation and Dox reduced phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK), protein kinase C (PKC) α/β and c-Jun NH$_2$-terminal kinase. The MEK-specific inhibitor U0126 or PKC inhibitor staurosporine (STP) also down-regulated XIAP expression and induced apoptosis. Thus, our data suggest that apoptosis and down-regulation of XIAP induced by Dox exposure or serum starvation may be mediated through inactivation of the MEK/ERK and PKCα/β pathways. In support of this we demonstrated that the cytotoxic effects of Dox when combined with U0126 or STP were enhanced, *i.e.,* synergistic cytotoxic activities were demonstrated. The synergistic interaction of U0126 or STP with Dox was sequence- and concentration-dependent.

Introduction

It has become evident that anticancer drug-induced cell kill is, at least partially, mediated by programmed cell death or apoptosis (1). Progress has been made in the understanding of the events underlying drug-induced apoptosis, and several molecules have been implicated in this process. Among them, caspases are the central executioners of the apoptotic process, particularly caspase-3 (2). The Bcl-2 family of proteins plays a crucial role in regulation of apoptosis (3, 4). Overexpression of the antiapoptotic molecules Bcl-2 or Bcl-xL can cause resistance to anticancer drugs (5–8). In addition to Bcl-2 family members, other more direct effectors of caspase inhibition have been identified. These are XIAP,[3] cIAP1, cIAP2, and survivin (9–11). XIAP is the most potent member of the *IAP* gene family in terms of caspase inhibition and apoptosis suppression (9, 10, 12). Overexpression of XIAP has been shown to protect tumor cells from menadione, growth factor withdrawal, as well as anticancer drugs, mediated apoptosis (13–15). Down-regulation of XIAP using antisense oligonucleotides was shown to induce apoptosis and enhance chemotherapeutic activity against human NSCLCs H460 both *in vitro* and *in vivo* (16), as well as ovarian cancer cell lines (17). These reports suggest that XIAP plays a key role in the regulation of apoptosis in cancer cells. However, few studies have shown the effects of conventional anticancer drugs on XIAP expression during apoptosis.

Genotoxic stress has been shown to stimulate MAPKs including ERKs, JNKs/SAPKs, and p38 kinases. Usually, activation of the JNK and p38 pathways is involved in growth arrest and apoptosis (18–20), whereas the ERK cascade participates in many cellular programs such as proliferation, differentiation, and movement (21–23). High frequencies (30–50%) of constitutive activation of MEK and ERKs have been observed in many tumors derived from lung, pancreas, colon, ovary, and prostate (24–26). Such high frequencies of MEK/ERK activation in human tumors suggest that these

[3] The abbreviations used are: XIAP: X-linked inhibitor of apoptosis; IAP, inhibitor of apoptosis; NSCLC, non-small cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; Dox, doxorubicin; Taxol, paclitaxel; STP, staurosporine; CI, combination index; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH$_2$-terminal kinase; SAPK, stress-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FBS, fetal bovine serum.

proteins play an important role in supporting malignant progression and proliferation. Consequently, specific inhibitors could be developed against these protein kinases for cancer therapy. Indeed, this possibility is additionally supported by a study showing that a new MEK inhibitor, PD184352, inhibited the growth of human colon tumor xenografts *in vivo* (27).

In the context of apoptosis regulation, it has been reported that MAPKs are involved in the regulation of Bcl-2 phosphorylation and apoptosis induced by antimicrotubule agents such as *Vinca* alkaloids and taxanes in many tumors cells (28–30). A recent study showed that XIAP protection against apoptosis is achieved by two separate mechanisms, one requiring TAK1/JNK1 activation and a second involving caspase inhibition (31). It was suggested that apoptosis could be enhanced by the disruption of survival-associated MAPKs involved in signal transduction (32). This offers an attractive hypothesis that inhibition of specific survival protein kinase could enhance the activity of chemotherapeutic agents for the rational design of therapeutic agents and combination treatment regimens.

However, few studies have investigated the association of apoptosis induced by Dox with the activity of the MAPK pathway. In this report, we examined the effects of Dox- and Taxol-induced apoptosis on XIAP expression, as well as phosphorylation of Bcl-2 in human H460 NSCLC cells. In addition, we correlated these responses with changes in the specific protein kinases MEK/ERK, PKCα/β, and JNK. Because these protein kinases represent potential therapeutic targets for pharmacological intervention, we therefore studied the antitumor activities associated with combinations of cytotoxic drugs and protein kinase inhibitors

Materials and Methods

Tumor Cell Lines. The human NSCLC cell line (large cell type) NCI-H460 (H460) was obtained from the National Cancer Institute tumor repository and maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. Cells in exponential growth phase were used up to a maximum of 25 passages.

Western Blot Analysis. H460 cells were treated with specified agents for different times at various concentrations in complete medium or serum-free medium. The cells were lysed with ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, and 0.02% sodium azide) containing protease inhibitors (Complete-Mini protease inhibitor tablets; Boehringer Mannheim GmBH, Mannheim, Germany). After 30-min incubation on ice, samples were centrifuged at 10,000 rpm for 15 min and stored at −20°C. Protein content in the lysed extracts was determined using a detergent-compatible Bio-Rad assay (Bio-Rad Labs, Hercules, CA). Equal amounts of protein (40 μg/lane) were separated in, 12% SDS-polyacrylamide gels or 4–15% gradient SDS-polyacrylamide premade gels (Bio-Rad) and transferred to nitrocellulose membranes (Mandel, Guelph, Ontario, Canada). For bandshift assay of Bcl-2 phosphorylation, 12% SDS-polyacrylamide gel with 118:1 acrylamide:bis-acrylamide was used. The primary antibodies against XIAP (rabbit polyclonal; Aegera Oncology Inc., Ottawa, Ontario, Canada), Bcl-2 and Bcl-xL (mouse

monoclonal; DAKO, Glostrup, Denmark), caspase-3 and PARP (rabbit and mouse monoclonal, respectively; BD PharMingen, San Diego, CA) were obtained and used. The secondary antibody used was the appropriate horseradish-conjugated antimouse or antirabbit IgG (Promega, Madison, WI). Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England) and visualized after exposure to Kodak autoradiography film. Scanning densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify band intensities using volume/area integration. The amount of XIAP in cells was normalized to their respective β-actin lane levels.

MTT Assay. Growth inhibition of H460 cells was determined by the colorimetric MTT cell viability/proliferation assay. In brief, cells were incubated for 24, 48, or 72 h at 37°C in the presence of agents as indicated in the results. MTT (25 μg/well) was added to each well, and plates were incubated for 3.5 h at 37°C. The colored formazan product was then dissolved using 200 μl of DMSO. Plates were read using the microtiter plate reader (Dynex Technologies Inc., Chantilly, VA) at a wavelength of 570 nm. The percentage of viable cell in treatments with the different concentrations of agents was normalized to untreated controls. All of the assays were performed at least twice in triplicate. In some experiments as indicated in results, trypan blue was used to count viable cells. The concentration of agents required for IC_{50} was calculated as that giving a 50% decrease in absorbance as compared with controls incubated simultaneously without agents.

CalcuSyn software (Biosoft, Freguson, MO) was used to analyze data from MTT assay in which cells were exposed to agents alone or in combination with anticancer drugs and protein kinase inhibitors. The program provides a measure of the combined agents in an additive or synergistic manner. The CI equation in CalcuSyn is based on the multiple drug-effect equation of Chou and Talalay (33), and defines synergism as a more-than-expected activity effect and antagonism as a less-than-expected additive effect. Chou and Talalay (33) defined a parameter, CI, which assesses synergism (CI $<$ 1), additive (CI $=$ 1), or antagonism (CI $>$ 1).

Nuclear Morphology. Cells were treated with agents and incubated for 48 h at 37°C. Then cells were harvested and stained with 0.10 μg/ml 4′,6-diamidino-2-phenylindole for 30 min at room temperature. Cells were cytospun on a glass slide and viewed using a Leica microscope (Germany) at ×40 magnification under the UV fluorescent illumination. Digital images were captured using Imagedatabase V. 4.01 Software (Leica, Germany).

Screening Phosphorylation of Protein Kinases. H460 cells were treated with 1 μM Dox for 12 h or cultured in serum-free medium for 24 h. Cells were harvested and centrifuged at 4°C at 5000 rpm for 5 min. Cells pellets were sonicated 5 s in 0.5 ml of ice-cold lysis buffer pH 7.0 (20 mM 4-morpholinepropanesulfonic acid, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium PP_i, 2 mM sodium orthovanadate, and 0.5% NP40) and placed on ice for 30 min. The homogenate was centrifuged for 30 min at 100,000 $\times$ g in a Beckman Table Top TL-100. The resulting supernatant fraction was removed and

Table 1 Thirty-one phosphorylation sites in phosphoproteins of protein kinases tested

Abbreviation	Full name of protein	Epitope(s)[a]
Adducin	Adducin α	S662
CDK1	Cyclin-dependent kinase 1 (Cdc2)	Y15
CREB	cAMP responsive element binding protein	S133
ERK1/2	Extracellular regulated kinase 1	T202/Y204
GSK3α	Glycogen synthase kinase 3 α	S21/Y279
GSK3β	Glycogen synthase kinase 3 β	S9/Y216
JUN	Oncogene Jun	S73
MEK1	MAP kinase kinase 1	S217/S221
MEK3	MAP kinase kinase 3	S189
MEK6	MAP kinase kinase 6	S207
MSK1	Mitogen and stress activated protein kinase 1	S376
NR1	N-methyl-D-aspartate glutamate receptor subunit 1	S896
p38α MAPK	p38 alpha MAP kinase	T180/Y182
p70 S6K	S6 kinase p70	T389
PKBα (Akt1)	Protein kinase B α	T308/S473
PKCα	Protein kinase C α	S657
PKCα	Protein kinase C α	T638
PKCδ	Protein kinase C δ	T505
PKCϵ	Protein kinase c epsilon	S719
PKR	dsRNA dependent protein kinase	T451
RAF1	Oncogene Raf1	S259
RB1	Retinoblastoma	S780
RB1	Retinoblastoma	S807/S811
RSK1	Ribosomal S6 kinase 1	T360/S364
SAPK (JNK)	Stress-activated protein kinase	T183/Y185
SMAD1	SMA- and MAD-related protein 1	S463/S465
SRC	Oncogene Src	Y418
SRC	Oncogene Src	Y529
STAT1	Signal transducer and activator of transcription 1	Y701
STAT3	Signal transducer and activator of transcription 3	Y727
STAT5	Signal transducer and activator of transcription 5	Y694

[a] Y, tyrosine; S, serine; T, threonine.

immediately assayed for its protein concentration using a DC protein assay kit from Bio-Rad. BSA was used as the protein standard. The cell samples were boiled for 4 min at 100°C in the SDS-PAGE sample buffer including 31.25 mM Tris-HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), and 0.02% bromphenol blue (w/v). Thirty-one different phosphorylation sites of protein kinases were analyzed by Kinexus Bioinformatics Corporation (Vancouver, British Columbia, Canada), using antibodies that recognize phosphorylated epitopes (Table 1). A detailed assay method is described in Kinexus web site.[4] The experiment was repeated twice.

Results

Down-Regulation of XIAP in H460 Cells Treated with Dox and Paclitaxel. We examined the effect of Dox and Taxol on the expression of XIAP and Bcl-xL, as well as phosphorylation of Bcl-2 protein in H460 cells *in vitro*. Fig. 1*A* presents Western blot results of XIAP, Bcl-xL, and Bcl-2 protein levels in H460 cells treated for 24 h with different concentrations of
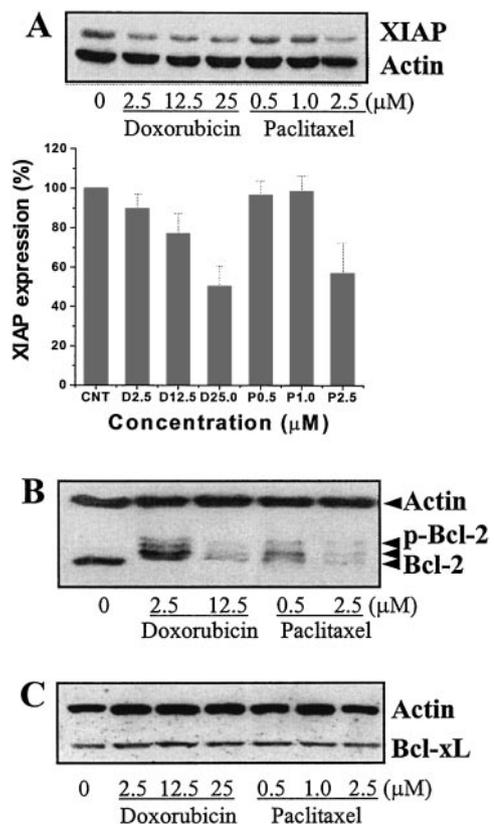
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[4] Internet address: http://www.kinexus.ca.

Fig. 1. Effects of Dox and Paclitaxel on apoptotic protein expression/phosphorylation in H460 cells treated for 48 h. *A,* XIAP levels were analyzed by Western blot (*top*) and the amount of protein was quantitated by densitometry (*bottom*). XIAP levels were normalized to cellular actin levels and compared with untreated control levels; *B,* Western blot analysis of phosphorylation of Bcl-2 protein (*p-Bcl-2*) and wild type Bcl-2 (*Bcl-2*); *C,* Western blot analysis of Bcl-xL protein levels; *bars,* ±SD.

Dox and Taxol. Dox down-regulated XIAP protein to 90.1, 77.3, and 50.4% of the control in a concentration-dependent manner at 2.5, 12.5, and 25.0 μM, respectively (Fig. 1*A*). When cells were treated with these concentrations of Dox, <40% of cytotoxicity was observed as judged by MTT assay. Similarly, Taxol decreased the XIAP expression down to 96.9, 98.4, and 57.3% of the control in a concentration-dependent manner at 0.5, 1.0, and 2.5 μM, respectively (Fig. 1*A*) that resulted in <30% cytotoxicity determined by MTT assay. Exposure of exponentially growing H460 cells to 2.5 and 12.5 μM of Dox, or to 0.5 and 2.5 μM of Taxol for 24 h resulted in Bcl-2 protein down-regulation as well as significant induction of Bcl-2 phosphorylation (Fig. 1*B*). The hyperphosphorylated Bcl-2 bands were detectable at lower drug concentrations than that required for significant reduction in XIAP expression. In contrast, the level of Bcl-xL expression remained unchanged when the H460 cells were treated with Dox at 2.5, 12.5, and 25 μM, or Taxol at 0.5, 1.0, and 2.5 μM for 24 h (Fig. 1*C*).

Induction of Apoptosis by Dox and Paclitaxel. To establish whether drug-induced down-regulation of XIAP protein expression as well as phosphorylation of Bcl-2 protein corresponded with the induction of the apoptotic cascade,
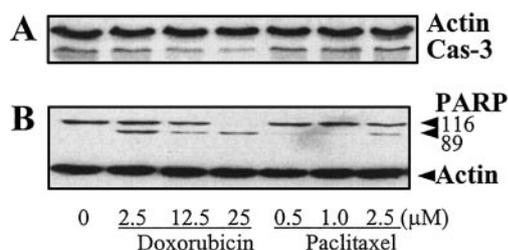
Fig. 2. Dox and Paclitaxel mediated caspase-3 and PARP activation in H460 cells treated for 48 h. *A,* effects of Dox and Taxol at different concentrations on the expression of pro-caspase-3 (*Cas-3*). *B,* the expression of PARP (full length, 116 kDa and processed 89 kDa). Protein expression was analyzed by Western blotting.

we analyzed levels of caspase-3 and PARP in treated H460 cells. As shown in Fig. 2*A,* H460 cells treated with Dox at 2.5, 12.5, and 25.0 μM activated and degraded procaspase-3 protein to 92.1, 63.5, and 39.8% of control procaspase-3 protein levels, respectively. After 24-h exposure to Taxol at 2.5 μM activated and degraded procaspase-3 protein to 74.6% of control procaspase-3 protein levels. Lower concentrations of Taxol (0.5 and 1 μM) did not affect expression levels of the procaspase-3 protein (Fig. 2*A*). Protein reduction was quantified by densitometry where the procaspase-3 level was normalized to cellular actin levels. PARP (M_r 116,000) was also cleaved to its predicted M_r 89,000 caspase-3-generated degraded fragment (Fig. 2*B*). These results suggested that the down-regulation of XIAP expression and phosphorylation of Bcl-2 protein induced by Dox and Taxol were associated with the apoptotic cell death.

Down-Regulation of XIAP in H460 Cells Cultured in Serum-free Medium. We also analyzed XIAP protein levels and growth inhibitory effects when H460 cells were cultured in serum-free medium. As shown in Fig. 3*A,* XIAP protein levels in H460 cells cultured in serum-free medium for 24 and 48 h were reduced to 74.0% and 45.9%, respectively, of expression levels observed in H460 cells cultured in 10% FBS medium. Fig. 3*B* demonstrates that the growth of H460 cells in serum-free medium for 24 and 48 h, respectively, was inhibited by 4.1% and 34.9% when compared with the growth rate of control cells in 10% FBS medium. This decreased cell growth was not associated with cell death, because no significant difference was observed in cell death of H460 cells cultured in serum-free medium up to 48 h compared with control cells grown in 10% FBS medium (<15% nonviable cells). These results suggest that down-regulation of XIAP was associated with cell growth inhibition before induction of cell death in H460 cells cultured in serum-free medium. Next, we determined the XIAP protein level in H460 cells cultured for 24 h in serum-free medium in the presence of Dox or Taxol. The results (Fig. 3*C*) showed that Dox, when added at concentrations of 2.5, 12.5, and 25 μM, caused substantial reduction in the XIAP protein levels, 12% of that measured in control cells, with cytotoxicity being <45% compared with untreated control cells. Paclitaxel decreased XIAP expression to 86, 62, and 25% of the control cells accompanied by 30–60% of cytotoxicity at 0.5, 1.0, and 2.5 μM, respectively (Fig. 3*C*). This down-regulation of
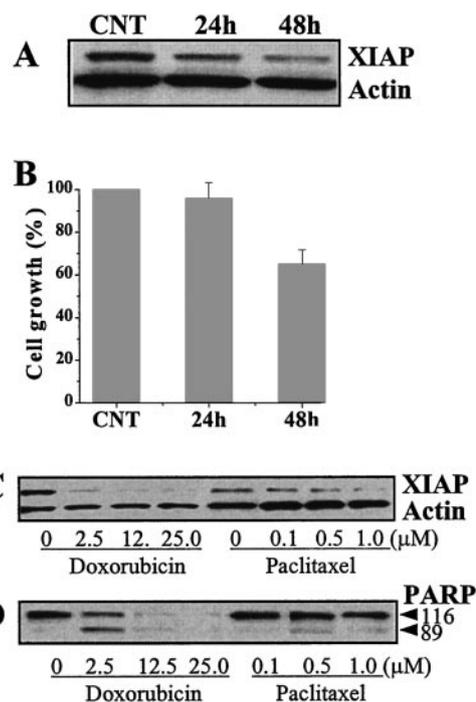

Fig. 3. Effects of serum starvation on the expression of XIAP protein and cell growth of H460 cells. *A,* reduction of XIAP protein expression and (*B*) reduction of growth rates of cells cultured in serum-free medium for 24 or 48 h compared with control cells (*CNT*) growing in 10% FBS medium. Enhanced reduction of XIAP protein (*C*) expression and PARP protein (*D*) expression (full length, M_r 116,000 and processed M_r 89,000) in H460 cells treated with Dox and Paclitaxel in serum-free medium for 24 h; *bars,* ±SD.

XIAP protein level in serum-free medium was more pronounced than down-regulation of XIAP in cells exposed to the same concentration of drugs cultured in 10% FBS medium (Fig. 1*A*). PARP was also cleaved to its predicted apoptosis-generated fragment (Fig. 3*D*) under these conditions. These results suggest that serum starvation enhanced down-regulation of XIAP, and induction of apoptosis by Dox and Taxol.

Changes in Phosphorylation State of Protein Kinases. To explore whether any specific cell signaling pathways were associated with the regulation of XIAP expression and apoptosis, we used a high throughput screening system to detect 31 phosphoprotein kinases (Table 1) in H460 cells. Cells were treated with 1 μM Dox for 12 h or cultured in serum-free medium for 24 h. The results shown in Fig. 4 and Table 2 clearly suggest the reduction in phosphorylation of protein kinases ERK1, MEK1/2, PKCα, PKCα/β, and JNK/SAPK. In H460 cells treated with 1 μM Dox for 12 h compared with control cells, phosphorylation of the protein kinases ERK1, MEK1/2, PKCα, PKCα/β, JNK/SAPK1, and JNK/SAPK2 were reduced as judged, by the relative trace quantities of 0.64, 0.73, 0.81, 0.71, 0.38, and 0.44, respectively. Also, in H460 cells cultured in serum-free medium for 24 h, the relative phosphorylation of the protein kinases ERK1, MEK1/2, PKCα, PKCα/β, JNK/SAPK1, and JNK/SAPK2 was 0.2, 0.15, 0.55, 0.31, 0.30, and 0.56, respectively, compared with the controls (Table 2). These results suggest that the
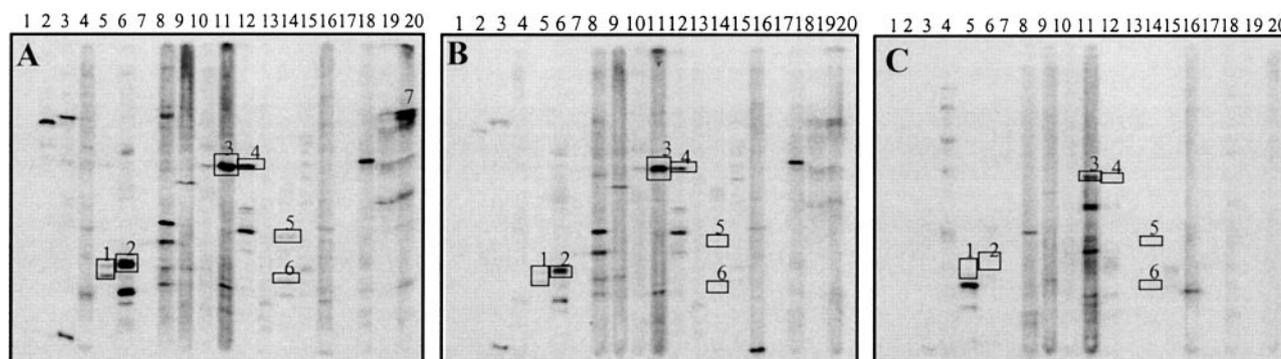
Fig. 4. Phosphorylation sites in phosphoproteins of protein kinases (listed in Table 1) in H460 cells; control (A), treated with 1 μM of Dox for 12 h (B), and cultured in serum-free medium for 24 h (C). Note: 1 = ERK1,2; 2 = MEK1,2; 3 = PKCα; 4 = PKCα/β; 5 = SAPK/JNK (M_r 46,100); 6 = SAPK/JNK (M_r 38,600). The assay method was described in "Materials and Methods."

Table 2 The reduced phosphorylation of protein kinases in H460 cells

No.	Protein kinases	Phos. epitope	Molecular weight (M_r)	Relative trace quantity[a]		
				Control	Dox (1 μM)	Serum free
1	ERK1/2	T202/T204	42,000	1.0	0.64	0.20
2	MEK1/2	S221	41,000	1.0	0.73	0.15
3	PKCα	S657	76,000	1.0	0.81	0.55
4	PKCα/β	T638/641	76,000	1.0	0.71	0.31
5	SAPK/JNK	T183/T185	46,000	1.0	0.38	0.30
6	SAPK/JNK	T183/T185	39,000	1.0	0.44	0.56

[a] Trace quantity: The trace quantity of a band is measured by the area under its intensity profile curve. Units are intensity × mm. The relative trace quantities were calculated based on the cells cultured in 10% FBS medium in which the level of phosphorylation of protein kinase was set as 1. The data is average of two separate experiments. The difference of data from the two separate experiments is <15% of the average values.

protein kinases ERK1, MEK1/2, PKCα, PKCα/β, JNK/SAPK1, and JNK/SAPK2 are the most affected members of the signal transduction pathways after treatment of H460 cells with Dox.

Down-Regulation of XIAP, and Induction of Apoptosis by U0126 and STP. To elucidate whether protein kinases MEK/ERK and PKC are functional regulators of XIAP expression and apoptosis in H460 cells, we treated cells (48 h) with a specific MEK/ERK inhibitor, U0126, and a broad protein kinase inhibitor, STP, an agent that is also a potent PKC inhibitor. U0126 down-regulated XIAP protein levels to 76% and 45% of control at concentration of 50 and 100 μM, respectively (Fig. 5A). Also, STP (0.5 and 1.0 μM) decreased XIAP expression by 19% and 44% relative to levels measured in control cells (Fig. 5A). However, U0126 and STP did not have any significant effect on Bcl-2 protein expression. Cells treated with U0126 and STP underwent morphological changes characteristic of apoptosis, including chromatin condensation and nuclear DNA fragmentation (Fig. 5B). These changes were rarely observed in the untreated cells.

Synergistic Activity of U0126 or STP in Combination with Dox or Paclitaxel. Given the apparent interrelation of MEK/ERK and PKC function with cytotoxicity induced by Dox and Taxol, we examined whether combined exposure of these agents could result in synergistic antitumor activity. The cytotoxicity of individual agents were first evaluated, and IC$_{50}$ values (72 h drug exposure) of U0126, STP, Dox, and Taxol were 25.12 μM, 0.02 μM, 0.02 μM, and 0.04 μM, re-

spectively. For the combination studies, U0126 was added to cells 1 h before Dox, such that a fixed molar ratio 625:1 or 312.5:1 was obtained. The cells were then incubated for 72 h. Dose effect and CI plots, generated by CalcuSyn software (Fig. 6, A and B), show that U0126 used in combination with Dox displays synergistic effects (CI values <1.0) with calculated CI values between 0.25 and 0.80 (Fig. 6B). However, when U0126 and Dox were added concurrently to cells at the ratios of 625:1 and 312.5:1, U0126 produced only additive effects with CI values between 0.9 and 1.1 (data not shown). Dose effect and CI plots in Fig. 6, C and D, indicate that STP used in combination with Dox has strong synergistic effects. STP and Dox were added at the same time at molar ratio of 10:1 (STP:Dox), and CI values between 0.1 and 0.5 were obtained (Fig. 6D). Similarly, STP and Dox added at molar ratio of 20:1 exhibited strong synergistic effects (data not shown). U0126 in combination with Taxol added at molar ratios of 375:1 and 750:1 exhibited strong synergistic effects when both drugs were added simultaneously (Fig. 6, E and F). Dose effect versus CI plots in Fig. 6, G and H, show that STP used in combination with Taxol at a molar ratio of 10:1 provided moderate to strong synergistic effects with CI values ranging between 0.2 and 0.7 (Fig. 6H).

Discussion

Lung cancer is one of the leading causes of cancer death despite recent advances made in early diagnosis and treat-
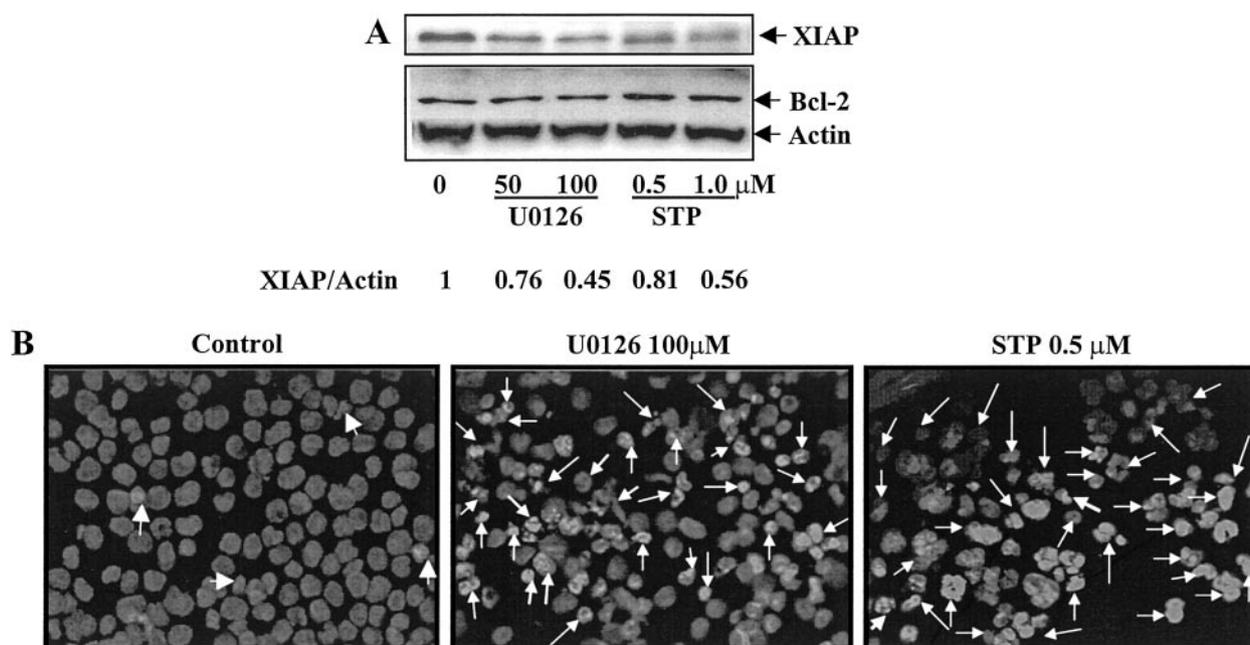
Fig. 5. Effects of the MEK inhibitor U0126 and the protein kinase inhibitor STP on the induction of apoptosis in H460 cells. *A,* expression of XIAP (*top*) and Bcl-2 protein (*bottom*) analyzed by Western blotting; *B,* morphological changes of H460 cells stained with 4′,6-diamidino-2-phenylindole. *Arrows* indicate cells showing DNA condensation and nuclear fragmentation.

ment. More than 75% of patients with NSCLC prove to be potential candidates for chemotherapy (34). However, chemotherapy produces rather poor response rates in NSCLC patients, with rare complete remissions. Clearly, new treatments must be developed to improve overall disease-free survival in NSCLC patients. A combination of conventional anticancer drugs with agents that activate additional apoptotic signals, or inhibit survival signals, may provide a rational molecular basis for novel chemotherapeutic strategies.

Activation of apoptosis in cancer cells can be induced by certain anticancer drugs through regulation of antiapoptotic proteins, such as Bcl-2 (1). XIAP is a relatively new antiapoptotic protein, and few studies have shown the effects of conventional anticancer drugs on its expression. It was reported recently that treatment with Dox and serum starvation strongly down-regulated XIAP mRNA in HL60 cells (35). In our study, we demonstrated that Dox and Taxol can downregulate XIAP protein expression in a concentration-dependent manner (Fig. 1*A*). The reduced XIAP protein is most likely because of down-regulated XIAP mRNA level (35), and because XIAP has ubiquitin protein E3 ligase activity, XIAP can be targeted for ubiquitin-proteasome-dependent degradation in response to apoptotic stimuli (36). Doxorubicin and Taxol can induce phosphorylation of Bcl-2 protein (Fig. 1*B*) at concentrations that are lower than that required for significant reduction of XIAP protein. These results suggest that Bcl-2 may be more susceptible than XIAP to the effects engendered after treatment of Dox or Taxol in H460 cells. The facts obtained here are consistent with both apoptosis regulators playing a functional role in the cytotoxic response of H460 cells to Dox and Taxol.

Chemotherapy-induced activation of cell survival pathways has been increasingly observed for conventional anticancer drugs. However, the signal transduction mechanisms that regulate apoptosis have yet to be clearly defined. Paclitaxel activates JNK/SAPK signaling cascades involved in apoptosis by phosphorylation and inactivation of Bcl-2 family member through Raf-1 in a variety of tumor cell lines (29, 30, 37). Paclitaxel can also cause the activation of the MEK/ERK pathway, which is considered a proliferation and cell survival pathway (38, 39). One study showed that activation of JNK, but not ERK, was associated with induction of apoptosis by Dox in the human T-cell leukemia line, H9 (40). However, our study has demonstrated that Dox treatment and serum starvation in H460 cells significantly reduced phosphorylation of MEK/ERK, JNK/SAPK, PKCα, and PKCα/β in comparison with cells cultured in 10% FBS medium. These data suggest that the MEK/ERK, JNK/SAPK, as well as PKCα and PKCα/β pathways are important mediators of survival signals in H460 cells.

Induction of apoptosis and down-regulation of XIAP were associated with reduction of phosphorylation and inactivation of MEK/ERK, JNK/SAPK, PKCα, and PKCα/β. Consequently, these protein kinases might represent potential therapeutic targets for pharmacological intervention. This is supported by our observation that the MEK/ERK-specific inhibitor U0126 and the protein kinase inhibitor STP, which is also a potential inhibitor of PKC, induced apoptosis through down-regulation of XIAP protein (Fig. 5*A*). These findings suggest that MEK/ERK and PKC play a key role in the regulation of XIAP expression in H460 cells. Similar findings were described in a study in which blockage of the ERK
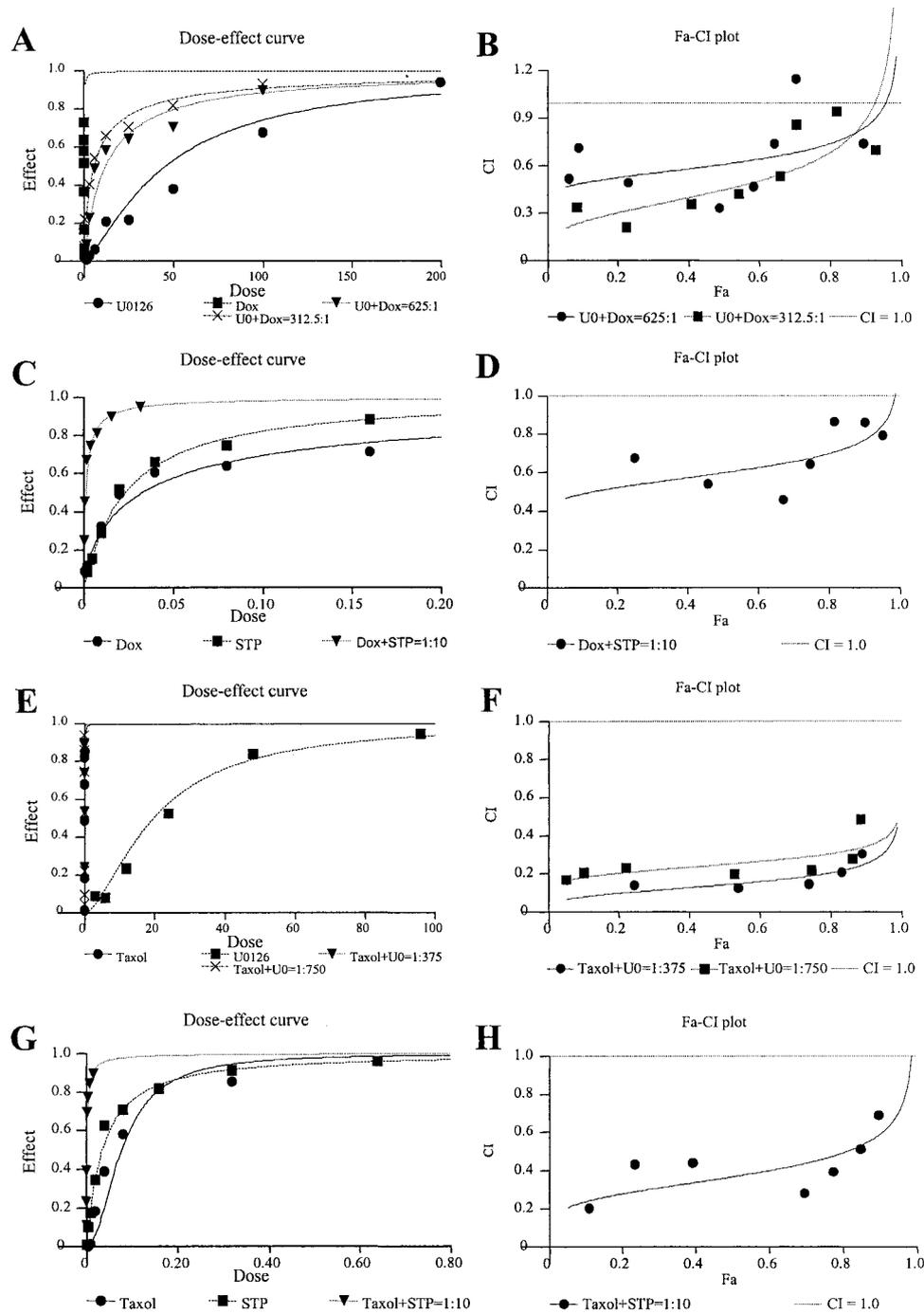
Fig. 6. MTT assay data plotted and analyzed by CalcuSyn software. *A, C, E,* and *G*, dose effect of Dox or Taxol alone or in combination with U0126 (*U0*) or STP. In plots *B, D, F,* and *H* data were analyzed for determination of CI. A CI index of <1 is synergistic, = 1 is additive, and >1 is antagonistic. Strong synergism is indicated by CIs of 0.1–0.3, and values of 0.3–0.7 or 0.7–0.85 are considered to indicate synergism or moderate synergism, respectively. All of the U0126 or STP combinations tested with Dox or Taxol exhibited moderate to strong synergism (*B, D, F,* and *H*).

pathway suppressed the growth of fibrosarcoma and renal cell carcinoma cells (41). Most importantly, our studies on combinations of protein kinase inhibitors U0126 or STP with Dox or Taxol demonstrated that U0126 or STP had significant synergistic effects when used to treat H460 cells. This synergism was dependent on several factors including schedule of drug exposure and drug concentration.

Consistent with the results of this study, others have shown that the combined treatment of Taxol plus a MEK1/2 inhibitor led to an enhanced cell death in human BT474 breast, OVCA194 ovarian, and H157 lung tumor lines (38, 39). Furthermore, pharmacological inhibition of MEK1 was able to increase the cytotoxic activity of ara-C by interruption of the protein kinase C signaling pathway (42). A new MEK inhibitor, PD184352, inhibited the growth of human colon tumor xenografts *in vivo*, and is being evaluated in ongoing trials as a single agent with the potential for combination therapy with Taxol (27). Our results as well as those from other groups suggest that the selective abrogation of survival signaling with the concomitant activation of proapoptotic

signaling pathways can markedly induce apoptosis and result in synergistic cytotoxicity. This may provide a promising approach for improving the response of tumors to conventional anticancer drugs.

Overall, our findings here illustrate that apoptosis induced by Dox and Taxol in H460 cells is associated with down-regulation of XIAP protein expression and induction of phosphorylation of Bcl-2. High-throughput screening assays of protein kinases may greatly improve our ability to identify molecular targets in tumor cells that will facilitate the development of the rationally designed therapeutic treatments that interrupt those targets. The implications of these findings are that combining conventional anticancer drugs such as Dox and Taxol with agents that inhibit specific survival signals may provide a molecular basis for novel chemotherapeutic strategies in the treatment of human lung cancer.

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Inhibition of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase Enhances Chemotherapeutic Effects on H460 Human Non-Small Cell Lung Cancer Cells through Activation of Apoptosis [1]

Yanping Hu, Marcel Bally, Wieslawa H. Dragowska, et al.

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