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ARTICLE *in* ONCOGENE · AUGUST 2005

Impact Factor: 8.46 · DOI: 10.1038/sj.onc.1208640 · Source: PubMed

Oncogene (2005) 24, 4813–4819

www.nature.com/onc

Oocyte-based screening of cytokinesis inhibitors and identification of pectenotoxin-2 that induces Bim/Bax-mediated apoptosis in p53-deficient tumors

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In this study, we demonstrate that a loss of p53 sensitizes tumor cells to actin damage. Using a novel oocyte-based screening system, we identified natural compounds that inhibit cytokinesis. Among these, pectenotoxin-2 (PTX-2), which was first identified as a cytotoxic entity in marine sponges, which depolymerizes actin filaments, was found to be highly effective and more potent to activate an intrinsic pathway of apoptosis in p53-deficient tumor cells compared to those with functional p53 both *in vitro* and *in vivo*. Other agents that depolymerize or knot actin filaments were also found to be toxic to p53-deficient tumors. In p53-deficient cells, PTX-2 triggers apoptosis through mitochondrial dysfunction, and this is followed by the release of proapoptotic factors and caspase activation. Furthermore, we observed Bax activation and Bim induction only in p53-deficient cells after PTX-2 treatment. RNA interference of either Bim or Bax resulted in the inhibition of caspases and apoptosis induced by PTX-2. However, the small interfering RNAs (SiRNA) of Bim blocked a conformational change of Bax, but Bax SiRNA did not affect Bim expression. Therefore, these results suggest that Bim triggers apoptosis by activating Bax in p53-deficient tumors upon actin damage, and that actin inhibitors may be potent chemotherapeutic agents against p53-deficient tumors.
Oncogene (2005) **24,** 4813–4819. doi:10.1038/sj.onc.1208640; published online 2 May 2005

Keywords: p53; apoptosis; actin inhibitor; chemosensitivity; Bim, Bax

Introduction

The majority of chemotherapeutic agents have been identified by their cytotoxic effects on panels of human tumor-derived cell lines. This approach has also revealed associations between cancer-specific mutations and drug sensitivity. Many studies have reported correlations between drug responsiveness and tumor genotypes. As the *p53* gene is inactivated in the majority of human cancers (Hollstein *et al*., 1991), much effort has therefore been spent to determine the effects of p53 inactivation on cancer cell response to therapeutic agents (Lowe *et al*., 1994; O'Connor *et al*., 1997).

Functional analyses of p53 protein have revealed that it is a transcription factor with sequence-specific DNA-binding activity (Farmer *et al*., 1992; Kern *et al*., 1992; Zambetti *et al*., 1992), and that it activates the transcription of several downstream target genes, including *p21*, an inhibitor of cyclin-dependent kinases (CDKs) (el-Deiry *et al*., 1993). p21 induction causes subsequent arrest in the G1 phase by binding to cyclin E- and A-associated cdk2 complexes (Gu *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993). Recently, we reported that p53 and p21 also inactivate cyclin B-associated cdc2 kinase by repressing the transcription of *cyclin B*, *cdc2*, and *cdc25C* genes, and that this repression is mediated by the cdk2-dependent phosphorylation of NF-Y (Park *et al*., 2000; Yun *et al*., 2003; Chae *et al*., 2004). Other recent studies have described additional *p53* target genes that are likely to activate cell cycle checkpoints and induce apoptosis (Vousden and Lu, 2002).

The loss of p53 function has been shown to determine cellular sensitivities to genotoxic agents (Lowe *et al*., 1993; Fan *et al*., 1994; McIlwrath *et al*., 1994) and mitotic spindle inhibitors (Trielli *et al*., 1996; Wahl *et al*., 1996; Debernardis *et al*., 1997; Fan *et al*., 1998). p53 was also found to modulate the cytotoxicity of anticancer agents. Experiments comparing transformed fibroblasts of normal and p53-deficient mice demonstrated that disruption of p53 function reduces the apoptosis induced by anticancer agents (Lowe *et al*., 1993). Subsequent *in vivo* studies provided further support for

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Received 6 December 2004; revised 5 February 2005; accepted 6 February 2005; published online 2 May 2005

this notion (Lowe *et al*., 1994; O'Connor *et al*., 1997), and thus, the inactivation of apoptotic response remains an attractive explanation for the poor responsiveness of p53 mutant tumors to these agents. Therefore, he identification of chemotherapeutic agents that activate p53-independent apoptosis is of fundamental importance for the development of treatments for p53-deficient tumors.

In this study, we report on novel cell-based screening system for identifying mitosis inhibitors. Through screening of natural compounds that inhibit the mitosis of activated oocytes, we identified 12 natural cytokinesis inhibitors. Among these, pectenotoxin-2 (PTX-2), which was first identified as a cytotoxic compound in marine sponges and later found to be an actin-depolymerizing agent (Draisci *et al*., 1996; Hori *et al*., 1999), was found to be highly toxic to p53-deficient cells. This finding led us to investigate the effects of PTX-2 and other actin inhibitors in tumors lacking a normal *p53* gene.

Results

Identification of mitosis inhibitors using ovulated oocytes

Most mammalian ovulated oocytes arrest in the metaphase of the second meiotic division unless artificially stimulated or fertilized (Choi *et al*., 1991; Masui, 1996). Using these synchronized cells, we developed a novel cell-based screening system to identify mitosis inhibitors. Ovulated oocytes are activated by treatment with ethanol, and then progress into the anaphase and complete mitosis. Thus, we applied 1100 natural compounds to screen for the mitosis inhibitors after treatment with ethanol. While normally activated oocytes completed mitosis and moved into the two-cell stage at 24 h and the four-cell stage at 48 h after ethanol treatment (Figure 1), some compound-treated oocytes failed to complete mitotic division, and remained in the one-cell stage, but with two nuclei (Figure 1). By using this approach, we identified 12 natural compounds that inhibit cytokinesis.

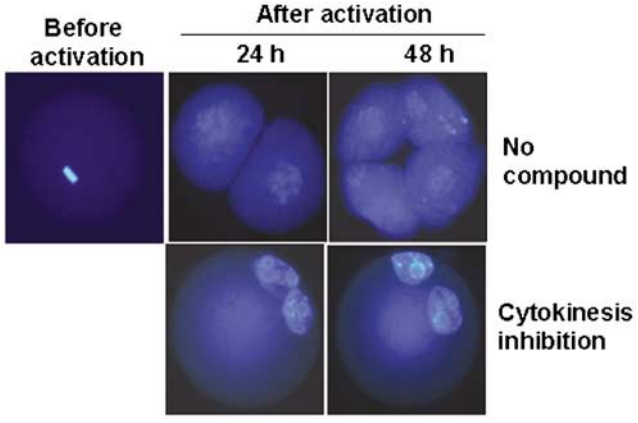


Figure 1 Identification of mitosis inhibitors using ovulated oocytes. Ovulated oocytes were treated with ethanol as described in 'Materials and methods'. After incubation with (lower panels) and without (upper panels), the natural compounds for 24 or 48, oocytes were fixed and stained with DAPI to visualize DNA

PTX-2 exhibits enhanced cytotoxic effect on p53-deficient tumors

We applied the cytokinesis inhibitors to human tumor cell lines and examined their cytotoxic effects. During this assay, we found that PTX-2, one of the inhibitors identified by screening, exhibited more enhanced cytotoxicity to p53-deficient tumor cells than p53-positive cells (data not shown). Thus, to compare the cytotoxic effects of PTX-2 to p53-positive and -deficient tumors, we employed HCT116 and its derivatives with both copies of p53 deleted and another expressing a dominant-negative mutant form of p53 ($p53^{-/-}$ and $p53^{281}$, respectively). Whereas the viability of HCT116 cells was largely unaffected by PTX-2 treatment at the concentrations tested, the HCT116 derivatives, $p53^{-/-}$ and $p53^{281}$, showed heightened chemosensitivity to PTX-2 (Figure 2a).

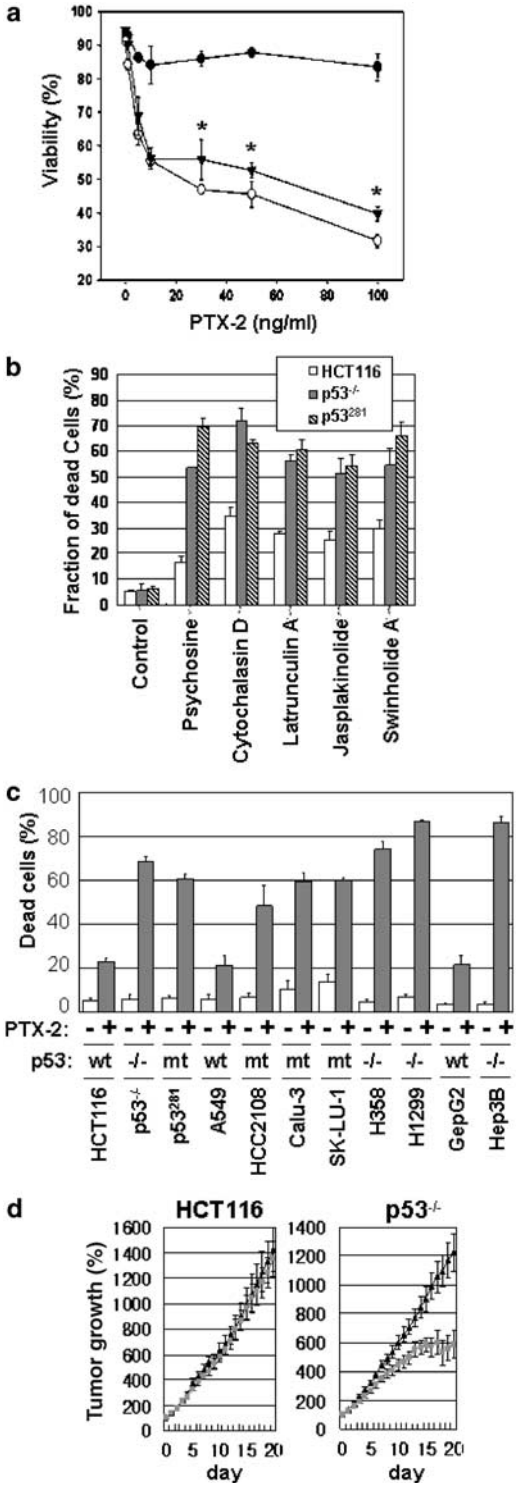
We also examined the cytotoxic effects of other actin inhibitors (Figure 2b). Psychosine causes filamentous actin to knot, whereas others, such as cytochalasin D (cyto D), latrunculin A, jasplakinolide, and swinholide A, are actin depolymerizers (Spector *et al*., 1999). All of the actin inhibitors tested in the present study showed more enhanced cytotoxicity to the HCT116 derivatives, $p53^{-/-}$ and $p53^{281}$, than parental cells (Figure 2b).

Loss of p53 sensitizes tumors to actin inhibitors

To test whether the status of p53 in tumor cells correlates with their chemosensitivities to PTX-2, we chose six independent human lung cancer cell lines (Figure 2c) and two well-known hepatocarcinoma cell lines (HepG2 and Hep3B), and investigated cell viabilities after PTX-2 treatment (Figure 2c). All of the tested cell lines without functional p53, such as HCT116 derivatives, Hep3B, and all the lung cancer cell lines except for A549, were much more sensitive to PTX-2 than p53-positive cells, such as HCT116, HepG2, and A549, although the degrees of sensitivity varied somewhat (Figure 2c).

Figure 2 Loss of p53 confers chemosensitivity to actin inhibitors. (**a**) Cells of HCT116 (•) and its derivatives, $p53^{-/-}$ (○) and $p53^{281}$ (▼), were treated with PTX-2 at the concentrations indicated and then cell viability was determined by trypan blue exclusion assay. Values represent means ± s.e.m. ($n = 3$). **(b)** HCT116 and its derivatives were treated with cyto D (10 μM), psychosine (50 μM), Latrunculin A (1 μM), Jasplakinolide (1 μM), and Swinholide A (100 nM) for 3 days and cell viability was determined as described above. **(c)** Human tumor cells carrying wild type (wt) or mutant (mt) p53 were treated with PTX-2 (30 ng/ml) for 3 days and viability was assessed by the trypan blue exclusion assay. Values represent means ± s.e.m. ($n = 3$). **(d)** Effect of PTX-2 on tumor growth in nude mice. After subcutaneous injections of parental HCT116 and $p53^{-/-}$ cells into nude mice, tumors were allowed to grow to a mean size of 200 mm^3 (0 day). For the following 20 days the mice received an intraperitoneal injection of PTX-2 (0.1 mg/kg, ■) or of the control vehicle (▲) daily. Tumor volumes were estimated daily ($n = 10$ for each day)

Applying these results therapeutically to tumors lacking p53, we compared the effects of PTX-2 on the tumor development of p53-positive and -negative tumor cells in nude mice (Figure 2d). We subcutaneously injected nude mice with HCT116 or its p53-null derivative ($p53^{-/-}$). After tumors grew to a mean size of 100 mm^3 (day 0), the mice were treated with a daily intraperitoneal injection of 0.1 mg/kg PTX-2 or its matching vehicle (PBS) for 20 days. PTX-2 did not affect tumor growth in the mice with HCT116 tumor cells, but the mean volume increase in tumors in those carrying p53-null cells receiving PTX-2 was reduced to 48% of that of the control group injected with PBS (Figure 2d). The body weights of mice were not significantly affected by PTX-2 (data not shown).



Actin damage triggers intrinsic death signals

After PTX-2 treatment, $p53^{-/-}$ cells, but not parental HCT116 cells, showed various apoptosis-specific phenomena, such as DNA laddering and morphological changes (data not shown). Cellular damage transduces intrinsic death signals to mitochondria, resulting in the disruption of mitochondrial membrane potential ($_m$) across the inner membrane, which in turn leads to the release of several proapoptotic molecules, including cytochrome *c* and Smac/Diablo, into the cytosol and to the activation of caspases (Vaux and Korsmeyer, 1999). Staurosporine (STS), an agent that potently destabilizes the mitochondrial membrane potential and triggers apoptosis (Green and Reed, 1998), was used as a control to determine the effects of actin inhibitors on the mitochondrial membrane potential. STS decreased $\Delta\psi_m$ before the appearance of apoptotic cells stained with propidium iodide (PI) regardless of p53, whereas actin inhibitors reduced $\Delta\psi_m$ in $p53^{-/-}$ cells, but not in parental cells (Figure 3a).

Consistent with mitochondrial dysfunction, cytoplasmic cytochrome *c* and Smac/DIABLO were increased in $p53^{-/-}$ cells (Figure 3b). Western blot analysis also showed that active forms of both caspase-9 and -3 were elevated in $p53^{-/-}$ cells under these conditions, but not in parental cells (Figure 3b). Consistently, the cleavage of poly(ADP-ribose) polymerase (PARP), a DNA repair protein that is cleaved by caspases during the execution phase of apoptotic cell death (Slee *et al*., 1999), was observed in p53-deficient cells (Figure 3b). An *in vitro* caspase activity also showed the significant activation of caspase-3 in $p53^{-/-}$ cells (Figure 3c). The caspase-dependent induction of apoptosis was supported by the observation that cell death was significantly inhibited by pretreating cells with the caspase-3 inhibitor z-DEVD-FMK (Figure 3d). Thus, apoptosis in cells treated with actin inhibitor is likely to be triggered by an intrinsic pathway, as was previously observed in DNA-damaged cells.

Apoptosis induced by PTX-2 is Bim/Bax-mediated

To identify the molecular mechanisms by which PTX-2 causes a loss of the mitochondrial membrane potential, we examined the protein levels of proapoptotic Bcl-2 family proteins. Owing to the absence of a change in the total protein level of Bax after PTX-2 treatment (Figure 4a), we investigated the possibility of Bax conformational changes. Immunoprecipitation analysis using anti-Bax 6A7 antibody and recognizing only the conformationally changed Bax protein showed that

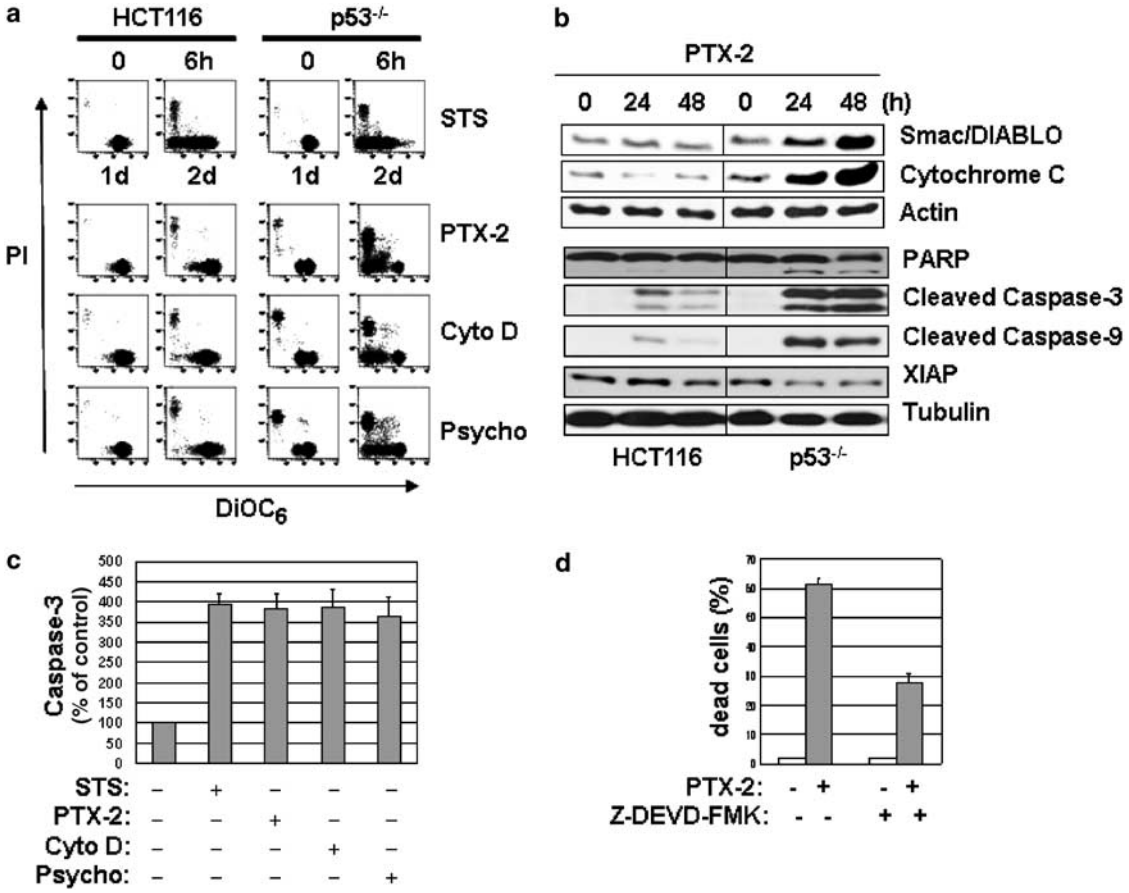


Figure 3 Apoptosis induction by actin inhibitor. (**a**) Mitochondrial dysfunction. Loss of $\Delta\Psi_m$ in cells treated with PTX-2 (100 ng/ml), cyto D (10 μM), psychosine (psycho, 50 μM), or STS (100 nM) for the times indicated. $\Delta\Psi_m$ was assessed by determining fluorescent dye ($DiOC_6$) uptake as described in 'Materials and methods'. **(b)** Cells were treated with PTX-2 (100 ng/ml) for the indicated times. Cytosolic fractions were then prepared and subjected to Western blot analysis (upper three panels). Whole cell extracts were prepared and assessed by Western blotting for PARP, cleaved caspase-3, cleaved caspase-9, and XIAP. **(c)** Enhancement of caspase-3 activity after the treatment with actin inhibitor. HCT116 $p53^{-/-}$ cells were treated with the three different actin inhibitors (100 ng/ml PTX-2, 10 μM cyto D, or 50 μM psychosine) for 2 days or with STS (0.1 μM) for 12 h. Caspase-3 activity was determined as described in 'Materials and methods'. **(d)** Prevention of cell death by a caspase-3 inhibitor. $p53^{-/-}$ cells were treated with PTX-2 (100 ng/ml) and a caspase-3 inhibitor (Z-DEVD-FMK, 25 μM) for 3 days. Cell viability was determined by trypan blue exclusion

active Bax was markedly elevated in p53-deficient cells, but not in parental cells (Figure 4a). Of the BH3-only proteins that activate multidomain proapoptotic members such as Bax (Cheng *et al*., 2001), we found that Bim was differentially regulated in $p53^{-/-}$ and parental cells, whereas $p53^{-/-}$ cells significantly induced Bim expression, parental cells did not (Figure 4a).

To determine whether either or both of Bax and Bim are critical for apoptosis induced by PTX-2, we used the SiRNA-mediated gene silencing technique. Transfection with SiRNA for either Bax or Bim inhibited the cell death induced by PTX-2 (Figure 4b). In addition, SiRNA for either Bax or Bim inhibited the activation of both caspase-3 and -9 (Figure 4c). Although SiRNA for Bim did not affect Bax expression, we addressed the possibility that conformational changes of Bax are dependent on Bim. Figure 4d shows that SiRNA for Bim blocked the activation of Bax proteins by PTX-2 (Figure 4d). These results imply that Bim is required for conformational activation of Bax after PTX-2 treatment.

Discussion

In this study, we demonstrate a novel cell-based screening system based on the use of mammalian ovulated oocytes to identify mitosis inhibitors (Figure 1). This system allowed us to screen for cytokinesis inhibitors, and, as a result, we found that PTX-2 is markedly toxic to p53-deficient tumors both *in vitro* and *in vivo* (Figure 2). Other agents that are known to cause actin damage via depolymerization or knotting of actin filament also showed enhanced cytotoxicity in p53-deficient tumor cell (Figure 2), implying that the loss of p53 sensitizes tumors to actin damage.

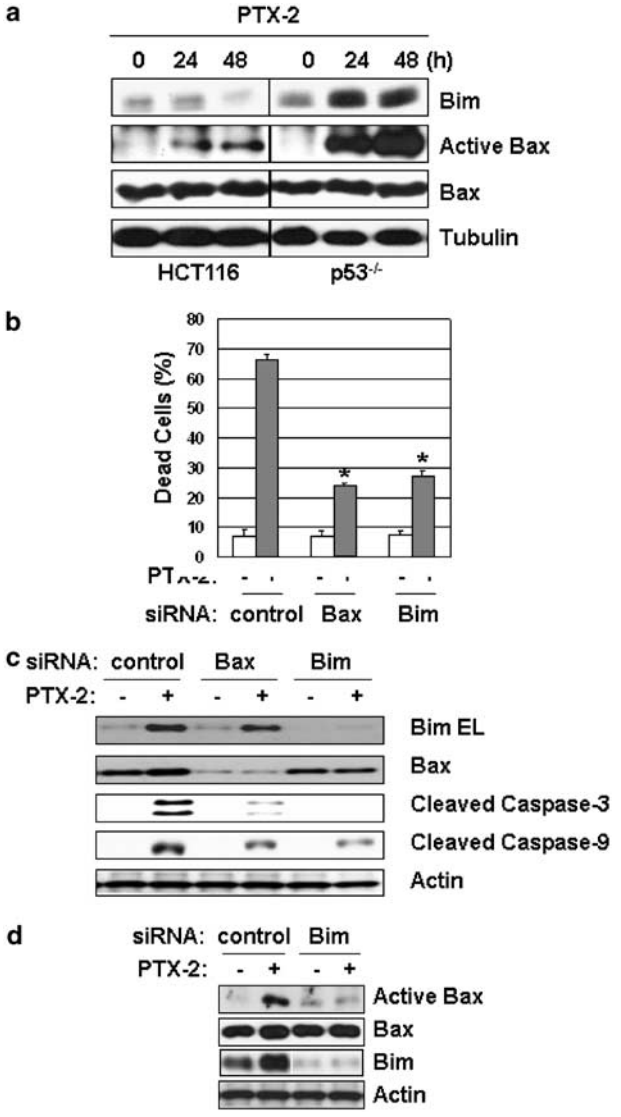


Figure 4 Role of Bax and Bim in the apoptosis induced by PTX-2. (**a**) PTX-2 induced a conformational change in Bax and the expression of Bim. Cells were treated with PTX-2 (100 ng/ml) for the indicated times. Active Bax was detected as described in 'Materials and methods'. **(b)** Prevention of apoptosis by knockdown of Bax and Bim. HCT116 and its derivative, $p53^{-/-}$ cells transfected with Bax or Bim SiRNA were treated with PTX-2 (100 ng/ml). After 2 days of treatment, cell viability was determined by trypan blue exclusion. **(c)** Knockdown of Bax and Bim blocked caspase activation. **(d)** Depletion of Bim protein by RNA interference resulted in the suppression of Bax activation in PTX-2-treated cells. HCT116 $p53^{-/-}$ cells transfected with Bax or Bim SiRNA were treated with PTX-2 (100 ng/ml) for 2 days

To examine why p53-deficient tumor cells are more chemosensitive, we analysed the cellular responses of $p53^{-/-}$ and of its parental cells after actin damage. It is known that mitochondria integrate death signals through Bcl-2 family members and coordinate caspase activation through the release of cytochrome *c* and Smac/DIABLO as a result of a loss of membrane potential ($\Delta\psi_m$) and increased permeability through the outer membrane. We showed that this mitochondrial apoptosis pathway is involved in the apoptosis induced by actin damage. Thus, our results suggest that some Bcl-2 family proteins are differentially regulated in $p53^{-/-}$ cells and parental cells.

The Bcl-2 family proteins consist of both anti- and proapoptotic members. They are critical death regulators that reside immediately upstream of mitochondria. Bax translocation to mitochondria is believed to play a key role in the induction of apoptosis by initiating the release of apoptotic factors such as Smac/DIABLO and cytochrome *c* when induced to do so by a variety of apoptotic stimuli (Gross *et al*., 1999). Bax translocation involves a conformational change that exposes the NH_2 terminus and the hydrophobic COOH terminus that targets mitochondria (Nechushtan *et al*., 1999). Bax conformation changes were induced by PTX-2 treatment (Figure 4a), and this was further supported by immunostaining with anti-Bax antibody (data not shown). Thus, the conformational change and translocation of Bax are likely to initiate PTX-2-induced apoptosis.

BH3-only proteins, including Bim, require for the activation of multidomain proapoptotic proteins, such as Bax, to exert their mitochondrial proapoptotic activity (Scorrano and Korsmeyer, 2003). Experiments with knockout mice have shown that Bim is essentially required for apoptosis (Bouillet *et al*., 1999). We found that Bim expression was induced in p53-deficient cells by PTX-2 treatment. Furthermore, the knockdown of either Bax or Bim by SiRNA-mediated gene silencing abolished PTX-2-induced apoptosis (Figure 4). This knocking down of the expression of Bax and Bim by RNA interference demonstrates that Bim-mediated Bax activation is essential for apoptosis induction in $p53^{-/-}$ cells after actin damage.

Although it is not clear which downstream effectors are involved in actin damage, and thus influence cell sensitivity to actin inhibitors, it can be speculated that the basis of the differential sensitivity occurs downstream of actin damage. Future studies will be directed to identify the regulator involved in the induction of Bim expression following actin damage, and also functions differentially according to the state of p53.

Materials and methods

Identification of chemical compounds that inhibit oocyte activation

The mice used in this study were ICR females, 3–4 weeks of age. To obtain ovulated oocytes, female mice were injected with 5 IU of pregnant mare's serum gonadotrophin (PMSG) and, 48 h later, with 5 IU of human chorionic gonadotrophin (hCG). Ovulated oocytes were collected from the ampulla of oviducts 14–16 h after the hCG injection. Cumulus-enclosed oocytes were isolated, and the cumulus cells were dispersed by incubation for 2–3 min in 0.1% hyaluronidase in culture medium (modified Whitten's medium containing 0.4% bovine serum albumin) at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air.

The cumulus-free oocytes were activated by treatment with 7% ethanol in culture for 7 min at room temperature (RT).

The activated oocytes were then rinsed three times in culture medium and incubated in the media containing various test compounds for 24 or 48 h. In all, 10–15 oocytes were used for each assay. The natural compounds library which consists of 950 methanol extracts from plants inhabiting in Korean peninsula and 150 single compounds from marine organisms was obtained from Biotech Institute of Gencross Corp. (Cheonan, Korea). Oocytes were then fixed with 1.8% paraformaldehyde in PBS (fixing solution) for 40 min at RT and stained with 4,6-diamidinophenylindole (DAPI) to visualize DNA (Sigma) under a Zeiss fluorescence microscope (Thornwood, NJ, USA).

Cell culture and transfection

HCT116, a human colorectal cancer cell line, and its p53-knockout derivative (denoted $p53^{-/-}$) were kindly provided by Dr Bert Vogelstein (John Hopkins Oncology Center, Baltimore, MD, USA). To select stable cell lines expressing $p53^{281}$, which is a dominant-negative mutant form of p53, we transfected HCT116 with pCMV-$p53^{281}$ plasmid (Yun *et al*., 1999). DNA transfections were performed using the $CaPO_4$ co-precipitation procedure (Graham and van der Eb, 1973). HCT116 and its derivatives, human hepatocarcinoma cell lines (HepG2, Hep3B), and lung cancer cell lines (Calu-3, SK-LU-1) were maintained in DMEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY, USA) and penicillin–streptomycin (50 U/ml). Other lung cancer cell lines (A549, HCC-2108, H358, H1299) were grown in RPMI1640 supplemented with 10% FBS and penicillin–streptomycin (50 U/ml). Unless otherwise indicated, drugs were added directly to cell media.

Gene silencing with small interfering RNAs

SiRNAs (sense and antisense strands) were purchased from Proligo (Paris, France). The sense strand sequences were as follows: Bim, 5′-CAAUUGUCUACCUUCUCGG(dTdT)-3′; Control, 5′-UUCUCCGAACGUGUCACGU(dTdT)-3′; and Bax, 5′-GGUGCCGGAACUGAUCAGA(dTdT)-3′ (Bidere *et al*., 2003; Reginato *et al*., 2003). Cells were transfected with SiRNAs by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. At 24 h after transfection, the cells were treated with PTX-2 (100 ng/ml) for 2 days, and then collected for either Western blot analysis, or trypan blue exclusion assay.

Cytotoxicity and apoptosis assays

Mitochondrial membrane potentials and viabilities were, respectively, assessed using 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (Molecular Probes, Eugene, OR, USA) and (PI) as described previously (Valavanis *et al*., 2001). Cells were seeded at 10^6 cells/100 mm dish 36 h before adding PTX-2 (100 ng/ml), cyto D (10 μM), psychosine (50 μM), or STS (0.1 μM). The cells were then incubated for the indicated times, trypsinized, washed, suspended in PBS, stained with $DiOC_6$ and PI, and analysed by flow cytometry (Coulter EPICS XL, Coulter Electronics, Hialeah, FL, USA). The activation of caspase-3 was detected using a Caspase-3 assay Kit (BD Pharmingen, San Diego, CA, USA), which uses *N*-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) as a substrate. Cell viability was determined by trypan blue exclusion using at least 300 cells in each group.

Western blot analyses

In all, 20 μg of protein was subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PolyScreen membranes (NEN, Boston, MA, USA). The membranes were then blocked with 5% nonfat dry milk in TBST buffer (Jung *et al*., 2001) and probed with antibodies. The following antibodies were used; antibodies against cytochrome *c*, poly(ADP-ribose) polymerase (PARP), and Smac/DIABLO (BD Pharmingen, San Diego, CA, USA); cleaved caspase-9, and cleaved capase-3 (Cell Signaling Technology, Beverly, MA, USA); actin, Bax, tubulin, and HSP60 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were detected using a horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat secondary antibody by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Subcellular fractionation

Cells were resuspended in an isotonic buffer (10 mM HEPES, pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A) and homogenized using a 26-gauge syringe needle. Cell homogenates were spun at 1000 *g* to separate out unbroken cells, nuclei, and heavy membranes. The supernatant was then spun again at 14 000 *g* for 30 min to yield the mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondrial fraction was then washed once with extraction buffer, and finally resuspended in RIPA buffer for Western blot analysis.

Detection of Bax conformational change

The detection of conformationally changed Bax was described previously (Yamaguchi *et al*., 2003). Briefly cells were lysed with Chaps lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Chaps) containing protease inhibitors. The cell lysates were then normalized for protein content and 200 μg of total protein was incubated with 1 μg of anti-Bax 6A7 monoclonal antibody (Upstate, Charlottesville, VA, USA) in 500 μl of Chaps lysis buffer for 6 h at 4°C. Then, 25 μl of protein A-agarose was added into the reactions and incubated at 4°C for an additional 2 h to precipitate conformationally changed Bax protein. After three washings in Chaps lysis buffer, the resulting immune complexes were subjected to SDS–PAGE immunoblot analysis with anti-Bax polyclonal antibody (Santa Cruz Biotechnology).

Nude mouse assay

To establish tumors in mice, 5×10^6 cells suspended in 100 μl of phosphate-buffered saline were injected subcutaneously into the right flanks of female nude mice (Balb/c, Charles River Laboratory, Hino, Japan). After tumors had grown to a mean size of 100 mm^3, the animals received a daily intraperitoneal injection of either of PTX-2 (0.1 mg/kg dissolved in 100 μl of PBS) or of the vehicle control (PBS) for 20 days. Tumor volume (V) was estimated from daily short- and long-axis data using the following formula: $V = 0.5 \times a^2 \times b$, where a and b stand for the short and long axes, respectively (Tsujii *et al*., 1998).

Acknowledgements
We thank Dr K Homma for his critical reading of the manuscript and the National Research Laboratory Program of the Korean Ministry of Science, the Molecular Aging Research Center, and National Cancer Control Program for financially supporting this work.

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