Marine Alkaloid Polycarpine and Its Synthetic Derivative Dimethylpolycarpine Induce Apoptosis in JB6 Cells Through p53- and Caspase 3-Dependent Pathways

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Purpose. Polycarpine from ascidian *Polycarpa aurata* was previously found to be active against different human tumor cells. In this study, we investigated the antitumor mechanisms of polycarpine and its synthetic derivative, desmethoxyethoxy-polycarpine (dimethylpolycarpine), through the induction of apoptosis. This new knowledge regarding the proapoptotic action of polycarpine and dimethylpolycarpine should lead to a better understanding of their effects and development of a new class of anticancer drugs.

Methods. Apoptosis was clearly observed by flow cytometry and Western blotting using an antibody against cleaved caspase-3 as an apoptotic marker.

Results. Polycarpines differentially activated p38 kinase, JNKs, and ERKs in JB6 Cl 41 cells. The polycarpines-induced apoptosis was decreased in cells expressing a dominant-negative mutant of JNK. Both compounds stimulated p53-dependent transcriptional activity and phosphorylation. Induction of p53-phosphorylation at serine 15 was suppressed in JNK1 and JNK2 knockout cells. Furthermore, polycarpines were unable to induce apoptosis in p53-deficient MEFs in contrast to a strong induction of apoptosis in wild type MEFs, suggesting that p53 is involved in apoptosis induced by polycarpines. The p53 phosphorylation in turn was mediated by activated JNKs.

Conclusions. These results indicate that all three MAPK signaling pathways are involved in the response of JB6 cells to treatment with polycarpines. Evidence also supports a proapoptotic role of the JNKs signaling pathway *in vivo* and clearly indicates that JNKs are required for phosphorylation of c-Jun, activation of p53, and subsequent apoptosis induced by polycarpines.

KEY WORDS: anticancer action; apoptosis; marine alkaloid; p53.

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ABBREVIATIONS: EGF, epidermal growth factor; ERKs, extracellular signal-regulated protein kinases; FBS, fetal bovine serum; JNKs, c-Jun NH_2-terminal kinases; MAPKs, mitogen-activated protein kinases; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; polycarpine, bis[2-amino-4-(4-methoxyphenyl)-1-methyl-5-imidazolyl]disulfide dihydrochloride; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

INTRODUCTION

As a new source of anticancer compounds, marine organisms are receiving an increased level of interest. Several of these compounds (1–3) have been tested recently for their anticancer activity. A dozen of these compounds have recently been shown to demonstrate potent antitumor activities and are under different stages in clinical development (3). For example, a phase II clinical trial was completed for didemnin B as an antimelanoma drug but was not further investigated because of a serious subject anaphylactic reaction to the compound (4). However, the related compound, aplydin, is currently being tested in a similar clinical trial in Europe and Canada (4). One of the ecteinascidins, ET-743, showed promising results against soft tissue sarcomas in both phase I and II clinical trials run by PharmaMar SA (2).

The antitumor alkaloid polycarpine (Fig. 1A) was isolated from the ascidian *Polycarpa aurata* collected at Flinders Reef (Coral Sea) during the joint Soviet-Australian scientific expedition on board the research vessel *Akademik Oparin* in 1988 (5). Later, polycarpine and its derivative, dimethylpolycarpine (Fig. 1B), were obtained by total chemical synthesis with good yields (6). Compounds belonging to this group were previously studied at the National Cancer Institute using the panel of human tumor cell lines, and some of these compounds, including polycarpine, demonstrated potent antitumor properties *in vitro* (6). Polycarpine also showed significant anticancer activity *in vivo* against P388 murine leukemia, L1210 leukemia and carcinoma Ehrlich (6). In *in vitro* assays, polycarpine demonstrated strong inhibition of Raus sarcoma and avian myeloblastoma viruses reverse transcriptase activities, and also rat brain Na^+,K^+-ATPase activity (6).

Many chemopreventive substances may act through the induction of programmed cell death or apoptosis, which eliminates genetically damaged cells and cells induced improperly to divide. Therefore, apoptosis plays an essential role as a protective mechanism against tumorigenesis in the organism. (7,8). Many chemopreventive agents induce activation of p53, which normally exists in cells as a short-lived protein, and is very often implicated in the process of cell cycle arrest, DNA repair and apoptosis (9–11). Caspase activation also plays a central role in the execution of apoptosis and these pathways are initiated by cell surface death receptors and mitochondria (12).

Resveratrol, caffeic acid phenetyl ether (CAPE), and phenethyl isothiocyanate (PEITC) were reported to suppress epidermal growth factor (EGF)- or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced cell transformation and induce apoptosis in a JB6 P^+ Cl 41 mouse epidermal cell line (13–15). All of these cancer preventive substances and also the natural alkaloid, caffeine, induce apoptosis through a p53-dependent pathway, which depends on the activities of ERKs, p38 kinase and/or JNKs (13–16).

Virtually no information exists regarding the mechanisms that determine the anticancer properties of polycarpines. To study the mechanism, we investigated the ability of polycarpine and its derivative, dimethylpolycarpine, to induce apoptosis in mouse epidermal JB6 P^+ Cl 41 cells. Our results demonstrated that polycarpine and dimethylpolycarpine efficiently induce apoptosis and the apoptosis is mediated by the activation of p53-dependent and caspase 3-de-

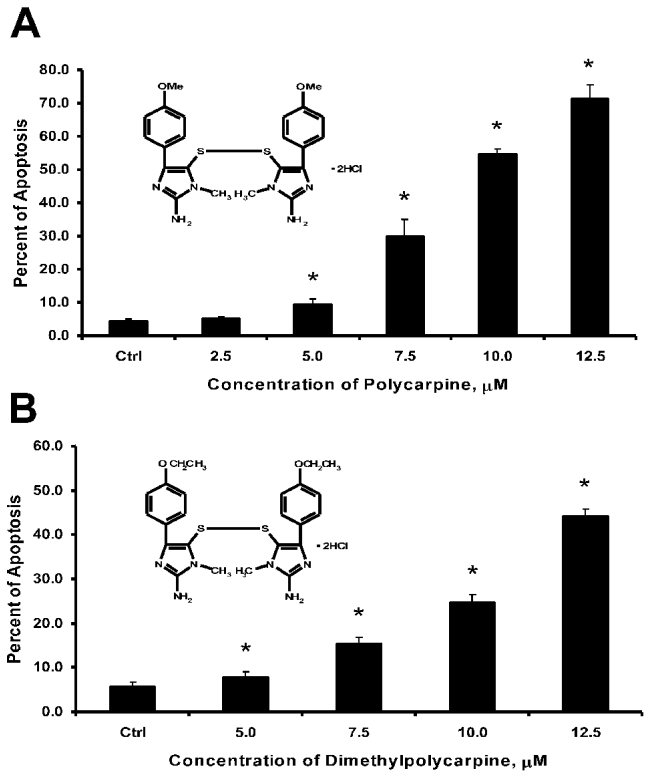

Fig. 1. The induction of apoptosis in JB6 Cl 41 cells by polycarpine and dimethylpolycarpine. JB6 Cl 41 cells were grown in 100-mm dishes, as described in "Materials and Methods," then treated with polycarpine (A) or dimethylpolycarpine (B) for 1 h in MEM without serum. Apoptosis was measured using annexin V and propidium iodide, with the Becton Dickinson FACSCalibur flow cytometer. Each bar indicates the mean ± SD of three independent experiments. *$p < 0.05$.

pendent pathways. The p53 activation occurs through its phosphorylation, which in turn is mediated by activated JNKs.

MATERIALS AND METHODS

Reagents

Polycarpine and dimethylpolycarpine (98% purity) were obtained by total chemical synthesis as previously described (6). Minimum essential medium (MEM) and Dulbecco's modified minimum essential medium (DMEM) were from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA). Penicillin/streptomycin and gentamycin were from Bio-Whittaker (Walkersville, MD, USA), L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). EGF was from Collaborative Research (Bedford, MA, USA). Luciferase assay substrate and Cell Titer 96 Aqueous One Solution Reagent (MTS) for the cell proliferation assay were from Promega (Madison, WI, USA). Mouse monoclonal IgG against p53, specific p38 kinase inhibitor, SB202190, and specific JNKs inhibitors, SP600125 and #420116 peptide-type inhibitor, were from Oncogene Research Products (Calbiochem, La Jolla, CA, USA). Protein A/G PLUS-Agarose immunoprecipitation reagent was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific mitogen-activated protein kinase antibodies against phosphorylated forms of p38 kinase, JNK1/2 and ERK1/2 were from New England Biolabs (Beverly, MA, USA). Caspase-3 and cleaved caspase-3 antibodies and the phospho-p53 antibody sampler kit were from Cell Signaling Technology Inc. (Beverley, MA, USA). The annexin V-FITC Apoptosis Detection Kit was from Medical & Biologic Laboratories (Watertown, MA, USA) and the chemiluminescent ECF substrate for Western blotting was from Amersham Pharmacia Biotech (Buckinghamshire, England).

Cell Culture

The JB6 P^+ Cl 41 mouse epidermal cell line and its stable transfectants Cl 41 DN-JNK1 $mass_1$, Cl 41 DN-p38 G7, Cl 41 DN-ERK2 B_3 $mass_1$, Cl 41 *p53* were cultured in monolayers at 37°C and 5% CO_2 in MEM containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (17). Wild-type ($Jnk^{+/+}$), $Jnk1^{-/-}$, and $Jnk2^{-/-}$, normal $p53^{+/+}$ or p53-deficient ($p53^{-/-}$) primary mouse embryonic fibroblasts (MEFs) were generated from embryonic day 14 mouse embryos and cultured for a maximum of 10 passages in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (18).

Western Blotting

Equal numbers of JB6 P^+ Cl 41 cells or subclones (6×10^5 to 7×10^5) were cultured for 12-24 h in 10 cm Petri dishes. After reaching 70–80% confluence, cells were starved for 24–48 h in 0.1% FBS-MEM or 0.5% FBS-DMEM. Cells were treated with the indicated concentrations of substances for 1 h or for the indicated time, then stimulated (or not stimulated) with EGF (20 ng/ml) for 30 min or UVB (4 kJ/m^2). After stimulation, the medium was removed and cells were washed once with ice-cold phosphate buffered saline (PBS) and lysed with 200 μl RIPA-buffer [1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The samples were sonicated and centrifuged at 15,000 × *g* for 15 min. The quantity of protein was measured by the Lowry method (19) and normalized against controls. The samples were heated to 95°C for 5 min, cooled on ice and centrifuged at 15000 × *g* for 5 min. Then the samples (20 μl) were resolved by 8% (15% for caspase-3 and cleaved caspase-3) SDS-PAGE. Immunoblot analysis of caspase-3, cleaved caspase-3, and phosphorylated ERK1/2, JNK1/2, and p38 kinases was carried out using specific antibodies against caspase-3, cleaved caspase-3, and phosphorylated sites of ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), p38 kinase (Thr180/Tyr182), respectively.

Immunoprecipitation Assay

Equal numbers of JB6 P^+ Cl 41 cells or wild-type (WT), $Jnk1^{-/-}$ or $Jnk2^{-/-}$ primary mouse embryonic fibroblasts (MEFs) (6×10^5 to 7×10^5) were cultured for 12-24 h in 10-cm Petri dishes in 5% FBS-MEM or 10% FBS-DMEM, then starved in 0.1% FBS-MEM or 0.5% FBS-DMEM for 48 h and treated with the indicated concentrations of polycarpine or dimethylpolycarpine for 1 h. Then, the cells were harvested with 0.3 ml of lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and transferred into

microcentrifuge tubes, kept on ice for 30 min, sonicated 3 times for 5 s, and centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant fraction was transferred to a new tube. The quantity of protein was measured and normalized to controls. Then 5 μg of p53 (Ab-1) monoclonal mouse IgG were added to each tube with lysate and incubated with gentle rocking overnight at 4°C. Then 50 μl of protein A/G PLUS-Agarose were added to each tube and incubated with gentle rocking for the next 3 h at 4°C. The protein-antibody-agarose complex was washed five times with 500 μl of lysis buffer. The supernatant fraction was carefully removed from the complex, and 50 μl of SDS sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% w/v bromophenol blue) were added. The samples were heated at 95°C for 5 min, cooled on ice and centrifuged at 15000 *g* for 5 min. The samples (20 μl) were resolved by 8% SDS-PAGE. After transferring to membranes, immunoblotting for phosphorylated p53 was carried out with phospho-p53 (Ser 15) antibody. Antibody-bound proteins were detected by chemiluminescence using ECF substrate for Western blotting and analyzed with the Storm 840 Image Analyzer (Molecular Dynamics, Sunnyvale, CA, USA).

Assay for p53-Dependent Transcriptional Activity

p53-Dependent transcriptional activity was assayed with a JB6 P^+ Cl 41 cell line stably expressing a luciferase reporter gene controlled by a p53 DNA binding sequence (PG-13). Viable PG-13 cells (8×10^3) suspended in 100 μl 5% FBS-MEM were added into each well of a 96-well plate. Plates were incubated overnight and then starved for 24 h in 0.1% FBS-MEM. Then, cells were treated with various concentrations of substances in 100 μl of 0.1% FBS-MEM. After incubation with substances for 24 h, the cells were extracted with lysis buffer (0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM DTT, 2 mM EDTA), and the luciferase activity was measured using the Luminoscan Ascent Type 392 microplate reader (Labsystems, Finland). Results are expressed as a p53-dependent transcriptional activity relative to untreated control cells.

Flow Cytometry

The onset of early and late apoptosis was analyzed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). JB6 P^+ Cl 41 cells or stable transfectants Cl 41 DN-JNK1 $mass_1$, Cl 41 DN-p38 G7, Cl 41 DN-ERK2 B_3 $mass_1$ were grown in 10 cm dishes to 50-60% confluence in 5% FBS-MEM. Wild-type (WT), *Jnk1*$^{-/-}$, or *Jnk2*$^{-/-}$ primary mouse embryonic fibroblasts, normal *p53*$^{+/+}$ MEFs or p53-deficient MEFs (*p53*$^{-/-}$) were grown in 10-cm dishes to 50–60% confluence in DMEM with 10% FBS. Then cells were treated with compounds in 0% FBS-MEM (for JB6 cells) or in 0% FBS-DMEM (for MEFs) for one hour. In the experiments with kinase inhibitors, cells first were pretreated with the inhibitor, and then with compounds. The concentration of the JNK inhibitor SP600125 was 10 μM and time of preincubation with cells was 15 min. In the case of peptide-type JNK inhibitor #420116, the concentration of the inhibitor used was 5 μM and preincubation time was 2 h. In the case of the p38 inhibitor SB202190, cells were pretreated with 10 μM SB202190 for 30 min. After treatment with compounds the medium was collected and attached cells were harvested with 0.025% trypsin in 0.1% EDTA in PBS. Trypsinization was stopped by adding 2 ml of 5% FBS in PBS. Cells were washed by centrifugation at 1000 rpm (170 rcf) for 5 min and processed for detection of early and late apoptosis using Annexin V- FITC and propidium iodide staining according to the manufacturer's protocol. In brief, 1-5 × 10^5 cells were collected after centrifugation, and resuspended in 500 μl of 1 × binding buffer (Annexin V-FITC Apoptosis Detection Kit, Medical & Biologic Laboratories, Watertown, MA, USA). Then 5 μl of Annexin V-FITC and 5 μl of propidium iodide were added, and the cells were incubated at room temperature for 5 min in the dark. Then Annexin V-FITC binding was analyzed by flow cytometry.

RESULTS

Polycarpine and Dimethylpolycarpine Induce Apoptosis in JB6 P^+ Cl 41 Cells

JB6 cells were treated with increasing concentrations of polycarpine (Fig. 1A) or dimethylpolycarpine (Fig. 1B) and harvested after 1 h. Apoptosis was assessed by flow cytometry using Annexin V-FITC and propidium iodide double staining. Apoptosis was clearly induced by these compounds in a dose-dependent manner (Fig. 1, A and B). Caspase-3 activation, as another marker of apoptosis induced by polycarpines, was then investigated.

Caspase-3 Activation Is Induced by Polycarpine or Dimethylpolycarpine

Caspase-3 is known to be important in the process of apoptosis. As indicated by Western blotting for cleaved caspase-3, either compound induced activation of caspase-3 in a time- and dose-dependent manner (Fig. 2, A and B). Although polycarpine is more effective apoptosis inducer on a concentration basis (Fig. 1, A and B), dimethylpolycarpine seems to be more effective in cleaving caspase-3 as it follows from Fig. 2, A and B. But more likely this result showed that the optimal time necessary to reach maximal concentration of cleaved caspase-3 is a little different for JB6 cells treated with polycarpine in comparison with the cells treated with dimethylpolycarpine. It may be explained by a higher hydrophobicity of dimethylpolycarpine that probably influences the binding of polycarpines to different proteins and as result has an effect on the velocity of the apoptotic process. These results clearly indicate that polycarpines may exert anticancer effects by inducing apoptosis. To study the molecular basis for apoptosis induced by polycarpine and dimethylpolycarpine, we then examined their effects on MAP kinase signal transduction pathways.

Phosphorylation of MAP Kinases Polycarpines Activity

Our investigations indicated that polycarpine or dimethylpolycarpine induces phosphorylation of p38 kinase (Fig. 3A), JNKs (Fig. 3B), and ERKs (Fig. 3C) in JB6 Cl 41 cells. However, MAP kinase phosphorylation as a result of treatment

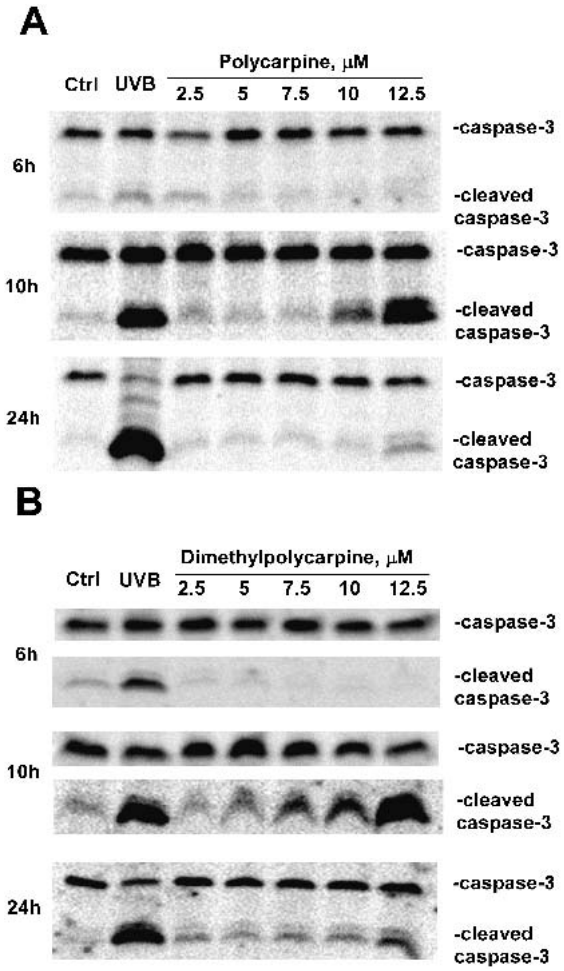

Fig. 2. Activation of caspase-3 in JB6 Cl 41 cells by treatment with polycarpine or dimethylpolycarpine. JB6 Cl 41 cells were grown in 100-mm dishes, as described in "Materials and Methods," then starved in 0.1% FBS/MEM for 48 h, and treated with the indicated concentrations of polycarpine (A) or dimethylpolycarpine (B) for the indicated times in 0.1% FBS/MEM. Cells were harvested at the indicated times and the cell lysates were subjected to Western blot analysis for detection of cleaved caspase-3 and total caspase-3. A representative experiment is shown.

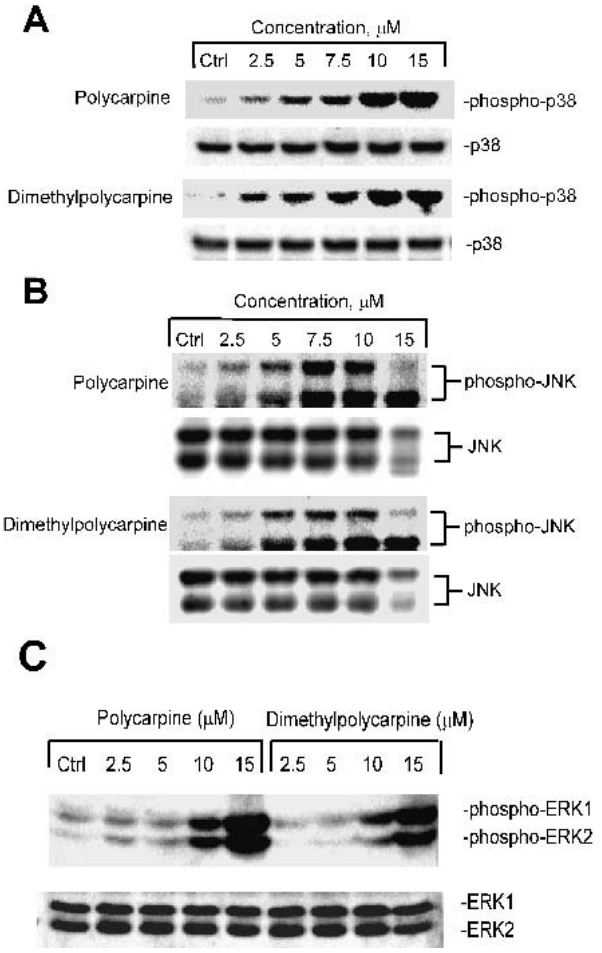

Fig. 3. Activation of p38 kinase, JNKs, and ERKs by polycarpine and dimethylpolycarpine. JB6 Cl 41 cells were cultured in 100-mm dishes as described in "Materials and Methods." Then, cells were treated with the indicated concentrations of polycarpine or dimethylpolycarpine, incubated for 1 h, and harvested. Then, the cell lysates were subjected to Western blot analysis using a specific antibody to detect phosphorylation of p38 kinase (A), JNKs (B), or ERKs (C). Untreated cells were used as a negative control. A representative experiment is shown.

with polycarpine or dimethylpolycarpine depended on the concentration of the compounds. Thus, polycarpine and dimethylpolycarpine already start to stimulate JNK1/2 and p38 kinase phosphorylation at a low concentration of 2.5 μM (Fig. 3, A and B), but ERK1/2 phosphorylation was induced only at 10 μM and higher concentrations of either compound (Fig. 3C). Then we studied the process of c-Jun phosphorylation in $Jnk^{+/+}$, $Jnk1^{-/-}$ and $Jnk2^{-/-}$ MEFs to show that polycarpine and dimethylpolycarpine induce JNKs activity.

Polycarpine and Dimethylpolycarpine Induced c-Jun Phosphorylation at Ser 73 in $Jnk^{+/+}$ MEFs, but Not in $Jnk1^{-/-}$ or $Jnk2^{-/-}$ MEFs

c-Jun is a well-known substrate for JNKs. Our results showed that both polycarpine and dimethylpolycarpine induced c-Jun phosphorylation at Ser 73 in $Jnk^{+/+}$ MEFs in a dose-dependent manner, while no activation was observed in $Jnk1^{-/-}$ or $Jnk2^{-/-}$ MEFs (Fig. 4, A and B). This result clearly indicated the induction of JNKs activity by polycarpines. To identify which signaling pathway is most critical for the proapoptotic action of polycarpine and dimethylpolycarpine, we then studied the effects of polycarpines on MTS reduction and induction of apoptosis in JB6 Cl 41 cells or cells expressing the dominant-negative forms of JNK1, p38 or ERK2 and in Jnk^{++}, $Jnk1^{-/-}$ or $Jnk2^{-/-}$ MEFs.

JNKs but Not p38 Kinase or ERKs Are Critical for the Proapoptotic Signal

First, we evaluated the role of MAPKs in the effect of polycarpine or dimethylpolycarpine on cell viability as indicated by MTS reduction. Time of incubation with the individual compound was 1 h. Expression of the dominant negative JNK1 dramatically reduced the inhibitory effect of polycarpine or dimethylpolycarpine on MTS reduction suggesting an increase in cell viability at higher concentrations of the

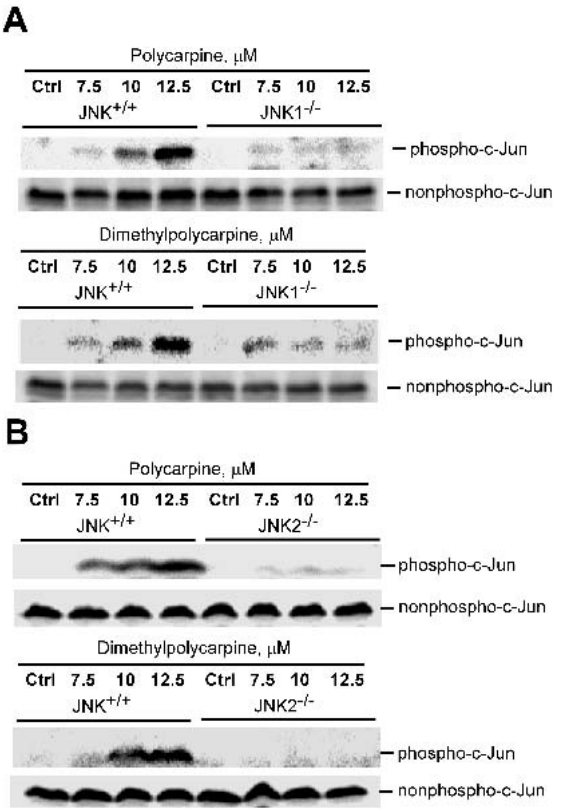

Fig. 4. c-Jun (Ser 73) phosphorylation induced by polycarpine and dimethylpolycarpine in MEFs. *Jnk*$^{+/+}$, *Jnk1*$^{-/-}$ (A) and *Jnk2*$^{-/-}$ (B) MEFs were cultured in 100-mm dishes as described in "Materials and Methods." Then, cells were treated with the indicated concentrations of polycarpine or dimethylpolycarpine, incubated for 1 h, and harvested. The cell lysates were then subjected to Western blot analysis using a specific antibody to detect phosphorylation of c-Jun at Ser73. Untreated cells were used as a negative control. A representative experiment is shown.

compounds in the absence of JNK1 (Fig. 5, A and B). On the other hand, the inhibitory effect on MTS reduction was actually increased for cells expressing the dominant negative p38 kinase (Fig. 5, C and D) or DNM-ERK2 (Fig. 5, E and F) in comparison with control JB6 Cl 41 cells. Furthermore and most importantly, we evaluated polycarpine and dimethylpolycarpine-induced apoptosis in cells overexpressing the dominant negative MAPK mutants or *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ MEFs. Results agree with those of the MTS assay and indicate that apoptosis was dramatically decreased, by about 20-70% in cells expressing DNM - JNK1 (Fig. 6, A and B) as well as by about 30-50% in *Jnk1*$^{-/-}$ or *Jnk2*$^{-/-}$ MEFs (Fig. 6, C, D, and E). In marked contrast, dominant negative mutants of p38 showed significantly increased, by about 20–50%, levels of apoptosis induced by polycarpines in comparison with control JB6 Cl 41 cells (Fig. 7, A and B). Expression of DNM-ERK2 had little effect on apoptosis induced by these compounds (Fig. 7, C and D). To further confirm the role of JNKs in polycarpine-induced signaling in JB6 P$^+$ Cl 41 cells, we used inhibitors of JNKs, including SP600125 and the peptide-type JNK inhibitor #420116. Results indicate that polycarpines-induced apoptosis was inhibited by about 20–50% by the JNKs inhibitors (Fig. 8, A and B). On the other hand, a 30-min incubation of cells with the specific p38 kinase inhibitor, SB202190, resulted in a significant increase in apoptotic cells (by about 20-30% at a 12.5 uM concentration of the compounds) compared with untreated cells (Fig. 8, C and D). These results support the significance of the JNK-mediated pathway in the induction of apoptosis by polycarpine or dimethylpolycarpine.

The p53 Tumor Suppressor Protein Is Involved in Apoptosis Induced by Polycarpine and Dimethylpolycarpine

To investigate the role of p53 in the induction of apoptosis by polycarpine and dimethylpolycarpine, we studied apoptosis in normal *p53*$^{+/+}$ MEFs and p53-deficient *p53*$^{-/-}$ MEFs. For either compound at concentrations of 10-12.5 μM, the number of apoptotic cells in normal *p53*$^{+/+}$ MEFs was about 2-4 times higher than in *p53*$^{-/-}$ MEFs (Fig. 9, A and B). This result strongly indicates that the p53 protein is involved in apoptosis induced by polycarpine or dimethylpolycarpine. To show that either compound stimulates p53 activity, we then studied the p53-dependent transcriptional activity and the p53 phosphorylation induced by polycarpines in JB6 cells.

Polycarpines Induce Phosphorylation of p53 at Ser15 and Activation of p53-Dependent Transcriptional Activity

When JB6 Cl 41 cells stably transfected with a p53-luciferase reporter gene (PG-13 cells) were treated with concentrations of polycarpine or dimethylpolycarpine from 2.5–12.5 μM for 24 h, we found a moderate (by 25–50%) induction of p53-dependent transcriptional activity (Fig. 9, C and D). This induction corresponds well with the stimulation of p53 protein expression and phosphorylation observed in JB6 Cl 41 cells treated with polycarpine or dimethylpolycarpine. We found that p53 phosphorylation occurs at Ser 15 as shown by immunoprecipitation and Western blotting (Fig. 10A). We showed that p53 phosphorylation is induced at a low concentration of 7.5 μM polycarpines. Also, increased levels of the non-phosphorylated p53 protein correlated well with the observed increase in p53 phosphorylation at Ser 15. Because our data showed that polycarpines induced apoptosis through a p53-dependent pathway and that JNKs play an important role in the induced apoptosis, we next determined whether JNKs mediate p53 phosphorylation.

JNKs Are the Major Mediators of Polycarpine- and Dimethylpolycarpine-Induced p53 Phosphorylation

To determine whether polycarpine-induced phosphorylation of p53 requires JNKs, we studied the phosphorylation of p53 at serine 15 induced by polycarpine or dimethylpolycarpine in *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, or *Jnk*$^{+/+}$ MEFs. The results showed that the polycarpine- and dimethylpolycarpine-induced p53 phosphorylation was suppressed in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ MEFs (Fig. 10, B and C) compared to *Jnk*$^{+/+}$ MEFs. These results indicated a requirement for JNKs in polycarpine- or dimethylpolycarpine-induced phosphorylation of p53 at Ser15 and subsequent apoptosis.

DISCUSSION

Many different types of marine alkaloids are active against human tumor cell lines. Among them are tetrahy-

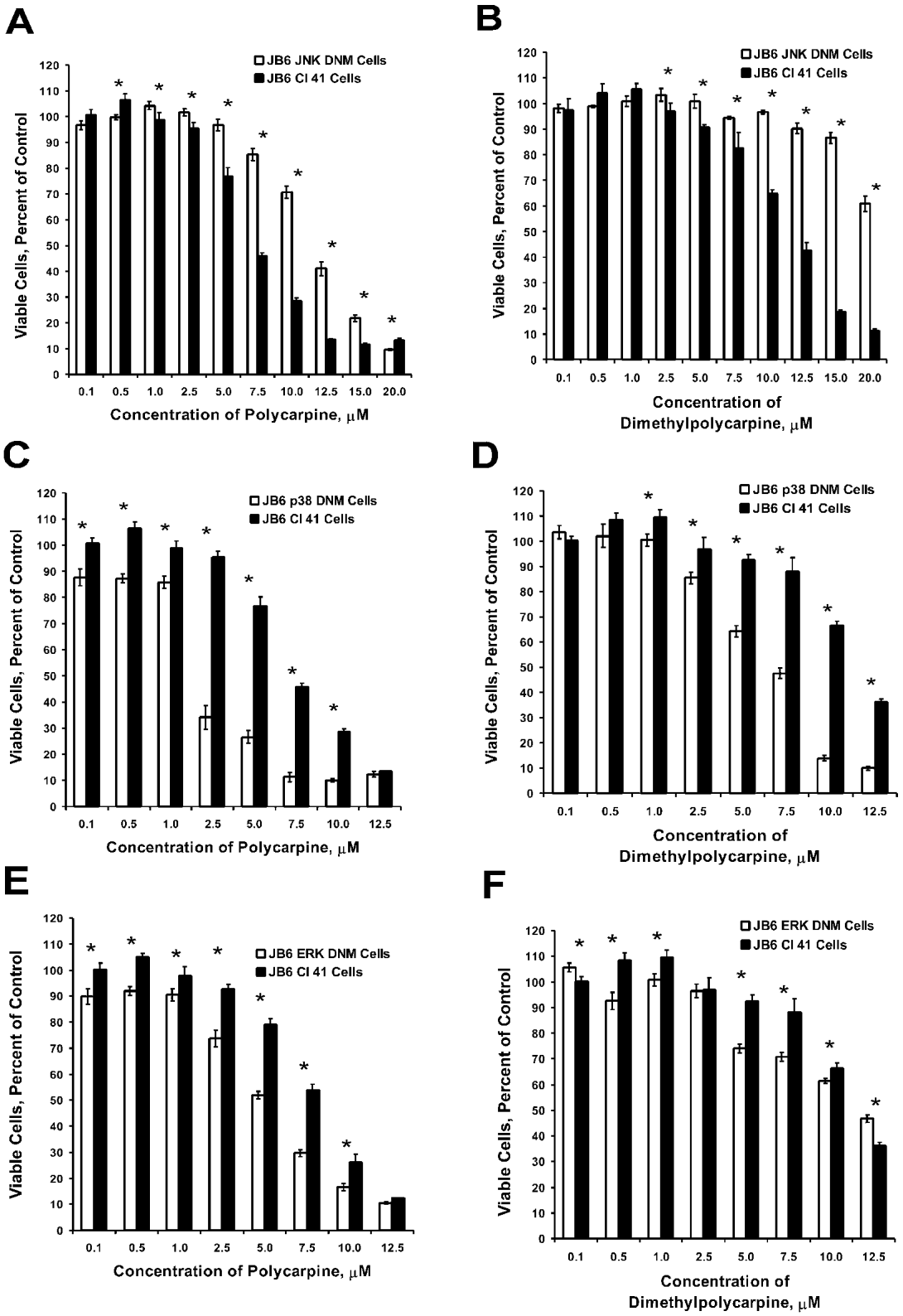

Fig. 5. The effect of polycarpine or dimethylpolycarpine on MTS reduction in JB6 Cl 41, JB6 Cl 41 DNM-JNK1, JB6 Cl 41 DNM-p38, and JB6 Cl 41 DNM-ERK2 cells. JB6 Cl 41 cells or JB6 Cl 41 cells with dominant negative forms of JNK1, p38, or ERK2 were cultured in 96-well plates as described in "Materials and Methods." Then, the medium was replaced with 0% FBS-MEM containing different concentrations of polycarpine or dimethylpolycarpine. The MTS reagent was added 1 h after the addition of these compounds. The MTS reduction was measured spectrophotometrically 2 h later. Data represent the percent of viable cells compared to percent of untreated control cells. Each *bar* indicates the mean ± SD from nine samples of three independent experiments. *$p < 0.05$.

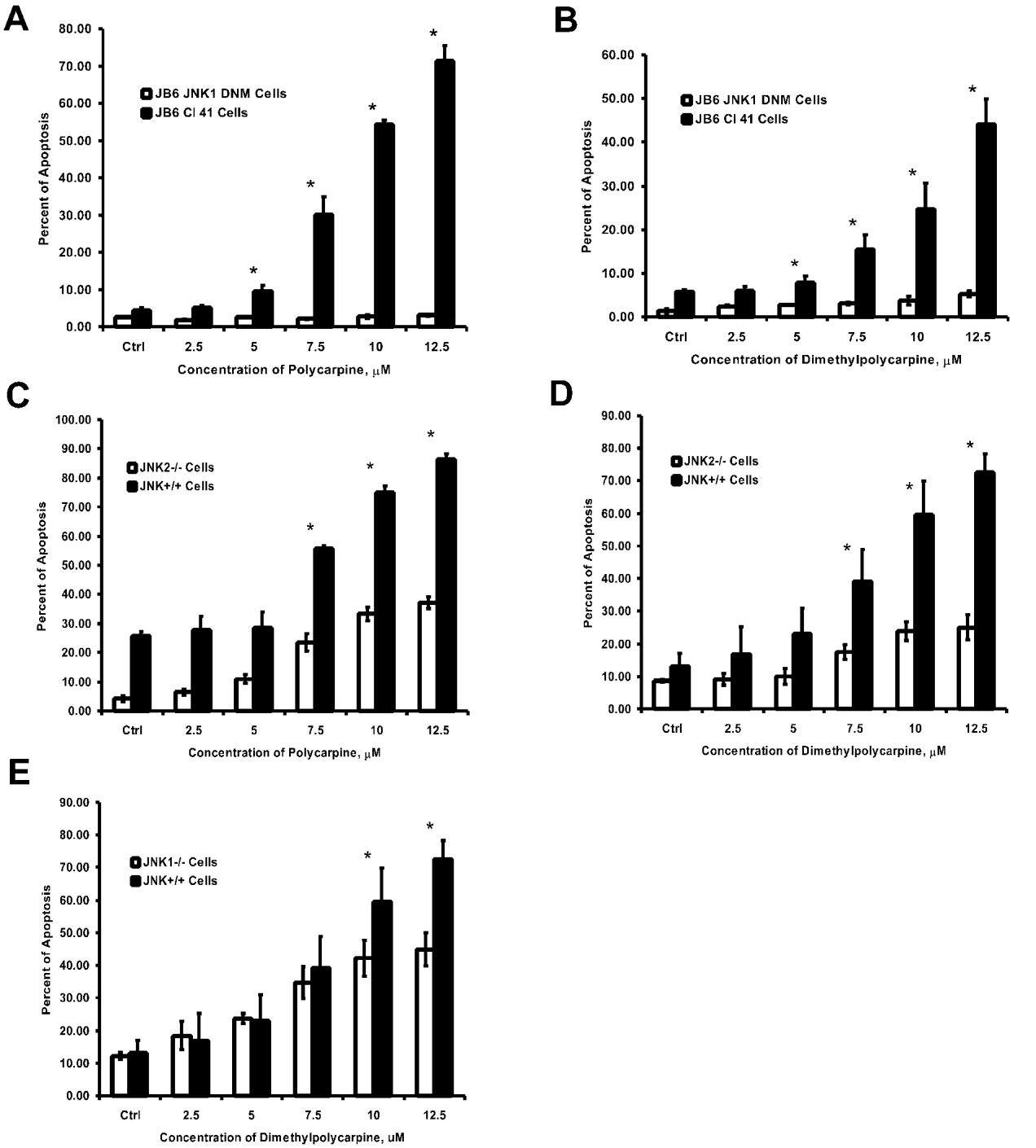

Fig. 6. The induction of apoptosis in JB6 Cl 41, DNM-JNK1 cells or in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ MEFs by polycarpine or dimethylpolycarpine. JB6 Cl 41 cells or MEFs were grown in 100-mm dishes, as described in "Materials and Methods." Then, cells were treated with polycarpine or dimethylpolycarpine for 1 h in MEM or DMEM without serum. Apoptosis was measured using annexin V and propidium iodide, with the Becton Dickinson FACSCalibur flow cytometer. Each bar indicates the mean ± SD of three independent experiments. *$p < 0.005$.

droisoquinoline (20), pyridoacridine (21), indole (22), indolocarbazole (23), guanidine (24), and various other types of alkaloids. At least one of them, ecteinascidin-743 (ET-743), which belongs to the tetrahydroisoquinoline type of alkaloids, is currently under phase II clinical investigation in Europe and the United States for treatment of soft tissue sarcoma and ovarian cancer (25,26). Marine imidazole-type alkaloid polycarpine isolated from ascidian *Polycarpa aurata* showed antitumor activity on a wide variety of human cancer cells including CNS cancer (SNB-75), colon cancer (HCT-116), melanoma (SK-MEL-5) and leukemia (MOLT-4), in concentrations from 0.02 to 9.00 μg/ml (6). However nothing was known until now regarding the mechanism, intracellular targets, or signaling pathways mediating the anticancer effect of

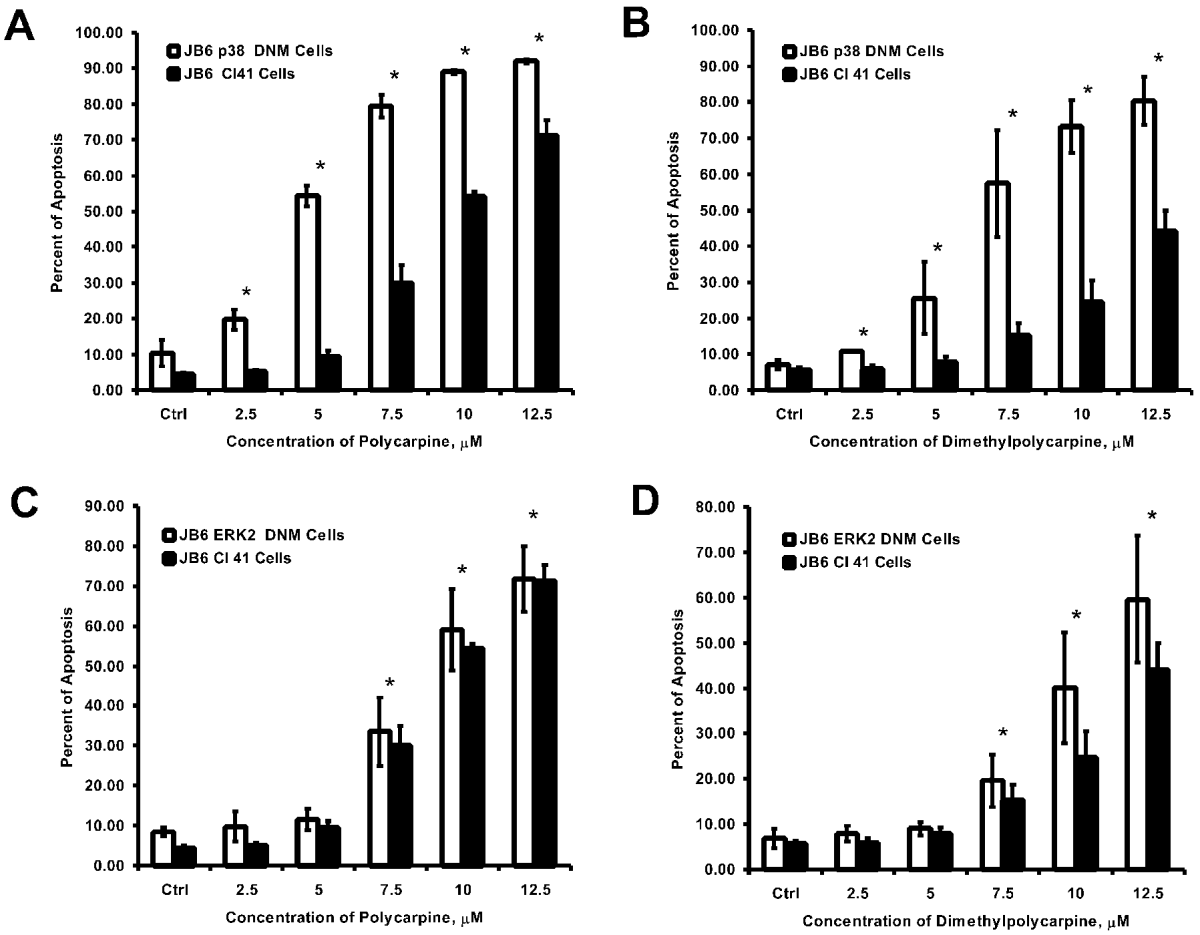

Fig. 7. The induction of apoptosis in JB6 Cl 41 DNM-p38 or DNM-ERK2 cells by polycarpine and dimethylpolycarpine. JB6 Cl 41, DNM-p38 or DNM-ERK2 cells were grown in 100-mm dishes, as described in "Materials and Methods," treated with polycarpine or dimethylpolycarpine for 1 h in MEM without serum. Apoptosis was measured using annexin V and propidium iodide, with the Becton Dickinson FACSCalibur flow cytometer. Each bar indicates the mean ± SD of three independent experiments. *$p < 0.05$.

this compound. We found by using flow cytometry that either polycarpine or its synthetic analog, dimethylpolycarpine, induce apoptosis in JB6 P^+ Cl 41 cells, suggesting that the antitumor activity of polycarpines may be due to induction of apoptosis in cancer cells. We also showed that the induction of apoptosis by polycarpines in JB6 cells is mediated by p53 and caspase 3 pathways, and that JNKs, but not p38 kinase or ERKs, are responsible for p53 activation. Three different MAPK signaling pathways have been identified in mammalian cells: ERKs, JNKs, and p38 kinases (27). This is the first report to show that polycarpines activate all three groups of MAPKs. In general, JNKs and p38 kinase are primarily activated by environmental stresses, whereas ERKs respond mainly to mitogenic and proliferative stimuli (27). Considerable progress has been made toward understanding the JNKs signaling pathway, but the biologic functions of JNKs *in vivo* are still uncertain. This is especially true as to whether the activation of the JNKs pathway is proapoptotic or antiapoptotic (28). Although the role of JNKs in apoptosis is not totally clear, activation of the JNKs signaling pathway has been implicated in apoptosis (29–32). Apoptosis is a general mechanism for removal of unwanted cells from organisms and plays a protective role against carcinogenesis (7–9). Evidence from both *in vivo* and *in vitro* experiments shows that apoptosis is involved in successful cancer treatment using many drugs and other chemical substances (8,13–16). In particular, the well-known marine alkaloids, ecteinascidine-743 or ascididemin, whose mechanisms of action are among the most well studied among all marine alkaloids, also promote apoptosis in tumor cells (33,34).

Our findings provide more evidence in favor of the proapoptotic action of the JNK signaling pathway in response to chemical stress. In our study, polycarpines (5 μM) induced dose-dependent activation and phosphorylation of JNKs in JB6 cells with subsequent apoptosis. The induction of JNKs activity by polycarpines was supported by Western-blotting experiments with phosphorylation of the c-Jun protein, which is the usual substrate of JNKs. Our experiments showed that polycarpines induced c-Jun phosphorylation in $Jnk^{+/+}$ MEFs but not in $Jnk1^{-/-}$ or $Jnk2^{-/-}$ MEFs in a dose-dependent manner. These data suggest that both JNK1 and JNK2 are required in c-Jun protein phosphorylation induced by polycarpines (Fig. 4, A and B). The crucial role of the JNKs signaling pathway in polycarpines-induced apoptosis in JB6 cells is clear from the cell proliferation and apoptosis experiments with DNM JNK cells (Fig. 5, A and B; Fig. 6, A and B), which exhibited a dramatic decrease in apoptosis induced by polycarpines. Apoptosis experiments using knockout $Jnk1^{-/-}$

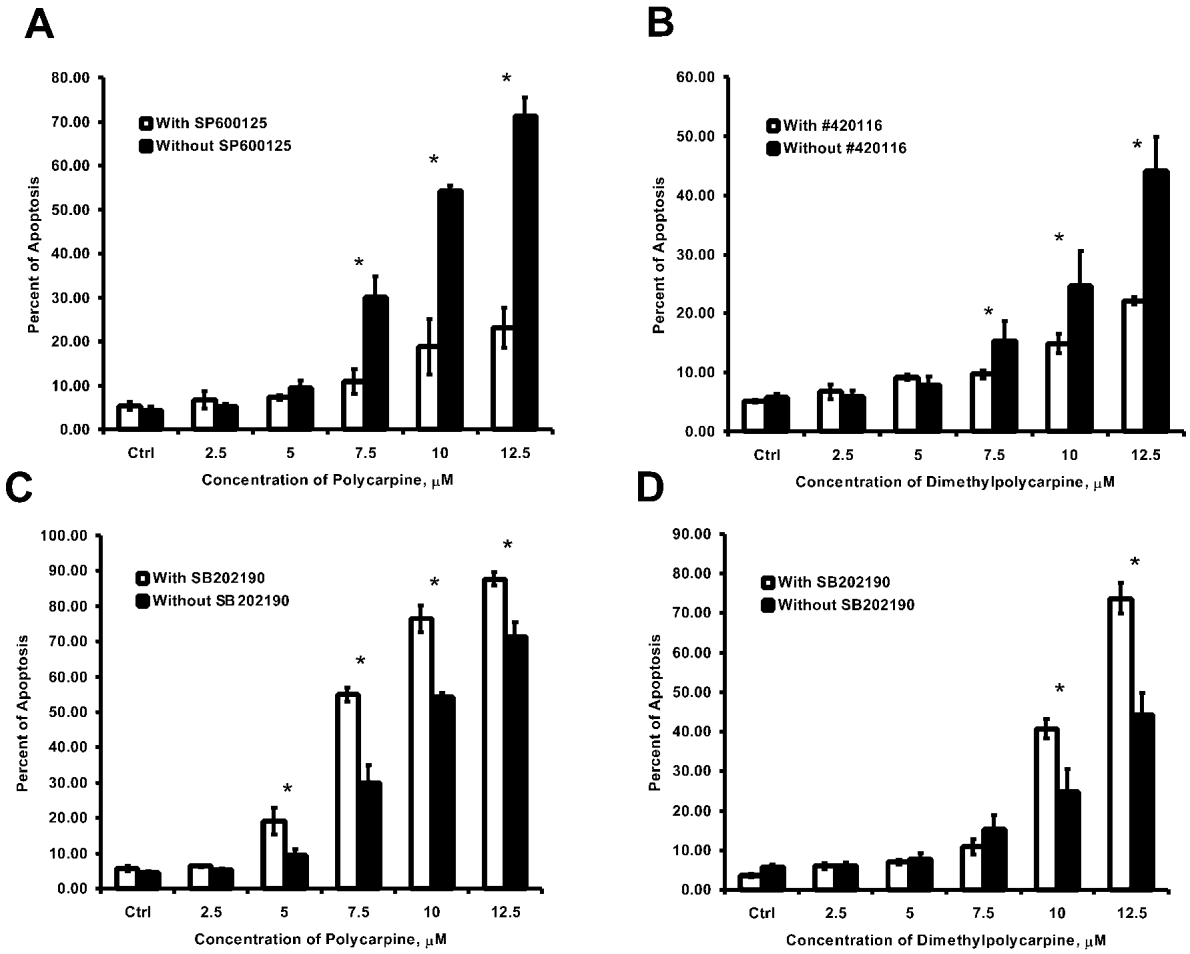

Fig. 8. The induction of apoptosis by polycarpine and dimethylpolycarpine in JB6 Cl 41 cells pretreated with JNKs or p38 inhibitors. JB6 Cl 41 cells were grown in 100-mm dishes, as described in "Materials and Methods." They were incubated with JNKs inhibitors: 10 μM SP600125 for 15 min or 5 μM concentration of the peptide-type inhibitor #420116 for 2 h, or with p38 inhibitor SB202190 for 30 min. Cells were then treated with the indicated concentrations of polycarpine or dimethylpolycarpine for 1 h in MEM without serum. JB6 Cl 41 cells not preincubated with the inhibitors were used as a control. Apoptosis was measured using annexin V and propidium iodide, with the Becton Dickinson FACSCalibur flow cytometer. Each *bar* indicates the mean ± SD of three independent experiments. *$p < 0.05$.

and *Jnk2*$^{-/-}$ cells (Fig. 6, C, D, and E) and also JB6 cells treated with JNKs inhibitors (Fig. 8, A and B) also support this conclusion. Evidence suggests that JNK1 and JNK2 protein kinases play a redundant but critical role in the regulation of apoptosis during early brain development in mice (35). We provide evidence here that deficiency in either JNK1 or JNK2 caused a reduced capacity of polycarpines to induce apoptosis and therefore JNK1 and JNK2 protein kinases are required in the polycarpines-induced apoptosis (Fig. 6, C, D, and E). Interestingly, at the same time and at the same doses as for JNKs, polycarpines start to induce p38 MAPK phosphorylation (Fig. 3A). The p38 MAPK pathway is crucial to inflammatory cytokine production and signaling (27,36,37). Several p38 MAPK inhibitors have been shown to block the production of IL-1, TNF, and other cytokines (37). Also, the p38 MAPK pathway is involved in the induction of several other inflammatory molecules, including COX-2 (38), which is known to play an antiapoptotic and tumor promotion role. Caco2 human colon cancer cells, transfected with COX-2, demonstrate increased metastatic potential (39) and rat intestinal epithelial cells overexpressing COX-2 demonstrate resistance to apoptosis (40). In our study, the p38 signaling pathway, contrary to the JNK signaling pathway, appears to be associated with cell survival and proliferation. The crucial role of p38 MAPK in this induction of cell survival pathway is clear from cell proliferation and apoptosis experiments with JB6 DNM p38 cells treated with polycarpines (Fig. 5, C and D; Fig. 7, A and B). We demonstrated a dramatic increase in apoptosis induced by polycarpines in the DNM p38 cells. Apoptosis experiments using JB6 cells treated with a p38 kinase inhibitor confirm this finding (Fig. 8, C and D). Antiapoptotic cell survival signaling pathways are considered to be promising targets for cancer chemotherapy (41). In comparison with JNKs, ERKs signaling also has been shown to have an opposing effect on apoptosis in rat PC-12 pheochromocytoma cells (30). In our experiments polycarpines induced ERKs phosphorylation in JB6 cells at high (10 μM and higher) concentrations of compounds (Fig. 3C). Cell proliferation and apoptosis experiments with JB6 DNM ERK cells (Fig. 5, E and F; Fig. 7, C and D) showed that the ERKs pathway didn't participate in apoptosis induced by polycarpines in JB6 cells. Future investigations are required to clarify a possible role for

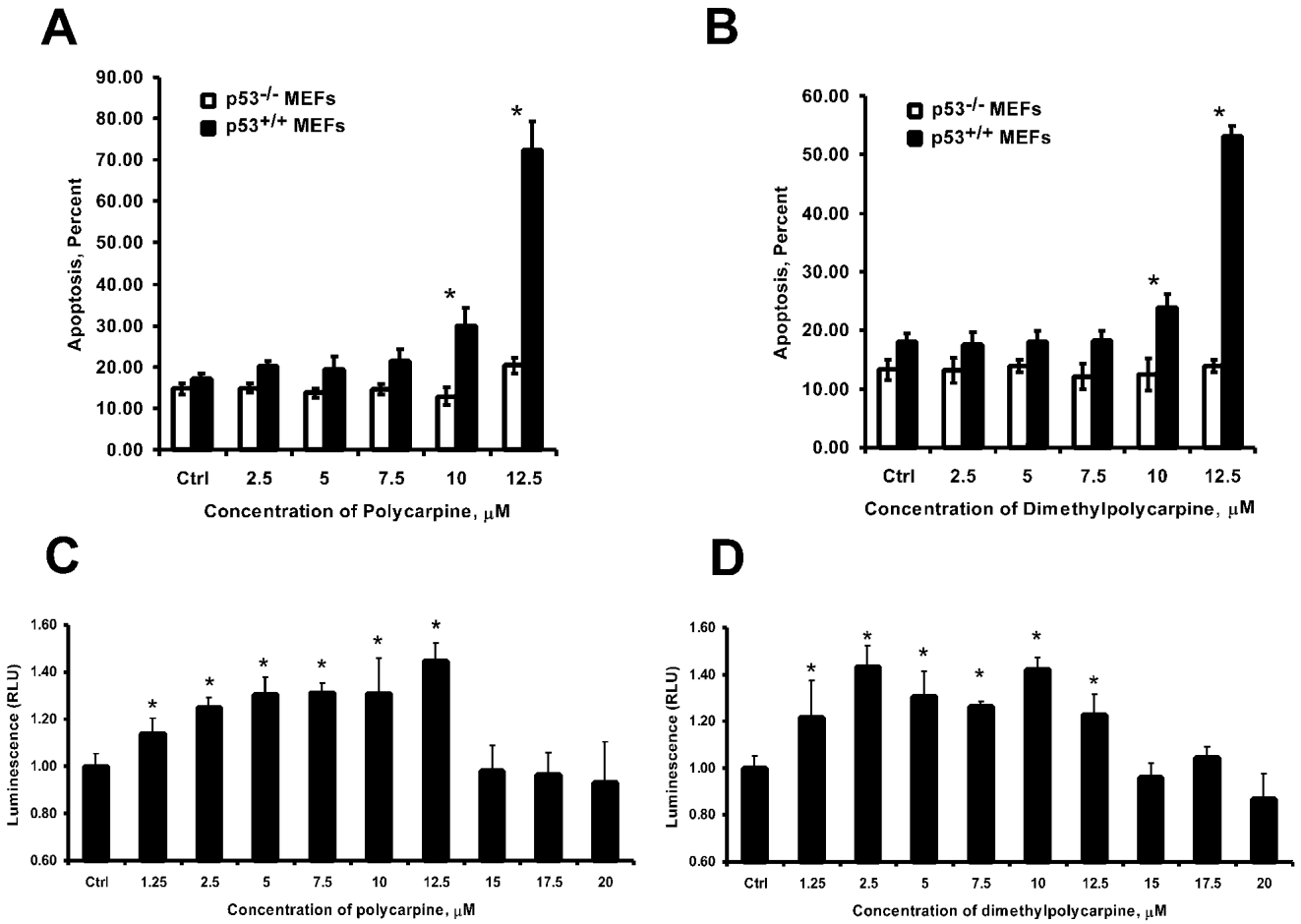

Fig. 9. The induction of apoptosis or p53-dependent transcriptional activity in $p53^{-/-}$ and $p53^{+/+}$ MEFs or in the JB6 Cl 41 p53 cell line (PG-13) by polycarpine and dimethylpolycarpine. $p53^{-/-}$ or $p53^{+/+}$ MEFs were cultured in 100-mm dishes as described in "Materials and Methods." Then, cells were treated with the indicated concentrations of polycarpine (A) or dimethylpolycarpine (B) and incubated for 1 h in DMEM without serum. Apoptosis was measured using annexin V and propidium iodide, with the Becton Dickinson FACSCalibur flow cytometer. JB6 Cl 41 p53-luciferase transfected cells (8×10^3) suspended in 100 μl of 5% FBS-MEM were added into each well of a 96-well plate. Cells were incubated overnight and then starved for 24 h in 0.1% FBS/MEM. Then, the medium was removed and the cells were treated with various concentrations of polycarpine (C) or dimethylpolycarpine (D) in 100 μl of 0.1% FBS/MEM. After incubation with substances for 24 h, the cells were disrupted with lysis buffer, and the luciferase activity was measured with the microplate reader Labsystems Multiscan MS (Finland). Results are presented as p53-dependent transcriptional activity relative to untreated control cells (1 unit). Each bar indicates the mean ± SD of three independent experiments. *$p < 0.05$.

ERKs in JB6 cells treated with polycarpines. A number of factors and signaling pathways in a cell can lead to apoptosis and one of the most important pathways extends through activation of the p53 tumor suppressor protein (9–11,42).

Phosphorylation of the p53 tumor suppressor protein plays an important role in regulating its activity (43). Mutations in Ser 15 and 20 impair p53 mediated apoptosis activity (44). Phosphorylation of p53 at Ser 15 is induced in response to several kinds of stress and leads to p53 activation (43,44). No one has reported data concerning p53 phosphorylation in response to the treatment with any marine alkaloid. Our data showed that in mouse JB6 Cl 41 epidermal cell line, polycarpine or dimethylpolycarpine induced p53 phosphorylation at Ser 15 (Fig. 10A). To determine whether activation of p53 is mediated by activated JNKs, we studied serine 15 phosphorylation of p53 in $Jnk1^{-/-}$, $Jnk2^{-/-}$, and $Jnk^{+/+}$ MEFs. The results showed that the polycarpines induced p53 phosphorylation at Ser15 in $Jnk^{+/+}$ MEFs, whereas in $Jnk1^{-/-}$ and $Jnk2^{-/-}$ MEFs, it was suppressed (Fig. 10, B and C). After its activation, p53 plays a coordinating role in the balance of gene expression, which leads to either cell growth arrest or apoptosis, thus preventing the proliferation of genetically damaged cells (9–11,42,43). p53 is the most frequently mutated gene in cancer and its deficiency is commonly associated with a sharply increased risk of cancer (45). The marine alkaloid, ET-743, induces a significant increase in p53 levels in cell lines expressing wild-type p53. Also, another marine alkaloid, ascididemin, induces apoptosis in human HL-60 leukemia cells, which are p53 deficient, indicating that in this case apoptosis occurs independently of p53 (34). In the present study, apoptosis occurred in $p53^{+/+}$ MEFs but not in $p53^{-/-}$ MEFs treated with polycarpines, (Fig. 9, A and B). These results strongly indicated that activated p53 is required for apoptosis induced by polycarpine or dimethylpolycarpine. Apoptosis is known to be mediated by a large family of cysteine aspartyl proteases known as caspases (46). Moreover, caspase activation plays a central role in the execution of apoptosis (12,47). Caspase 3 activation is involved in the pro-

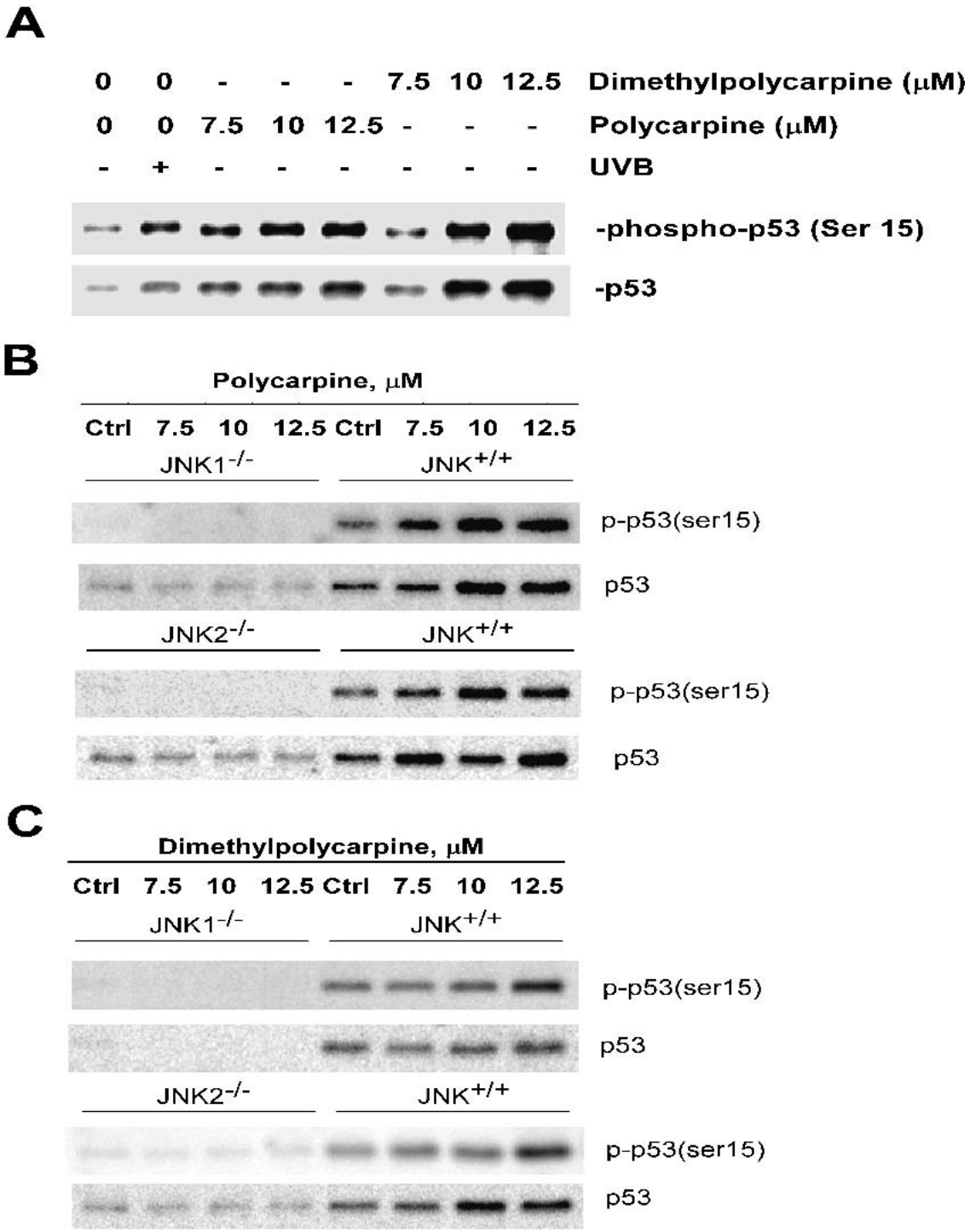

Fig. 10. p53 phosphorylation at Ser 15 induced by polycarpine and dimethylpolycarpine. Cells were grown in 100-mm dishes, as described in "Materials and Methods," starved in 0.1% FBS/MEM (A) or in 0.5% FBS/DMEM (B, C) for 48 h, and treated with the indicated concentrations of polycarpine or dimethylpolycarpine for 90 min (A) or for 1 h (B, C). Then the cells were lysed, and immunoprecipitation was performed as described in "Materials and Methods." The samples were resolved by 8% SDS-PAGE, and immunoblotting for phosphorylated p53 was carried out with antibodies against phosphorylation of p53 at Ser 15. A representative experiment is shown.

cess of apoptosis induced by the marine alkaloid, ET-743 (48) and evidence indicates that p53 can directly mediate caspase-3 activation with subsequent apoptosis (49,50). Our data showed that treatment of JB6 Cl 41 cells with polycarpines for 10 h induced the process of activation of caspase-3 through its cleavage (Fig. 2, A and B) resulting in subsequent apoptosis. The most active concentrations of polycarpines in the experiments with cleaved caspase-3 were in full agreement with the results of the apoptosis experiments using flow cytometry.

Thus, our study results indicate that all three main MAPK signaling pathways are involved in response to the treatment of JB6 cells with polycarpines. We provide supporting evidence for the proapoptotic role of the JNKs signaling pathway *in vivo* and show that JNKs are required for phosphorylation of c-Jun, activation of p53 nuclear factor, and subsequent apoptosis induced by polycarpines.

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