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Activation of p38 mitogen-activated protein kinase is necessary for gemcitabine-induced cytotoxicity in human pancreatic cancer cells

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Key Words: p38 MAPK, Akt, apoptosis, gemcitabine, pancreatic cancer.

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Abstract.

Background: Gemcitabine is a pyrimidine nucleoside analogue that is clinically active against pancreatic cancer. We have recently demonstrated that p38 MAPK is specifically activated by gemcitabine and pharmacological blockade of p38 MAPK signaling prevented gemcitabine-induced apoptosis in human pancreatic cancer cells. In this study, we further investigated to clarify that p38 MAPK is implicated in the cytotoxic action of gemcitabine. **Materials and Methods:** Cells expressing a dominant-negative mutant of p38 MAPK were generated. Clonogenic assay was used to assess the long-term effect on cancer cell viability in the human pancreatic cancer cells, PK1 and PCI43. The p38 MAPK activation level was assessed using an antibody specific to the phosphorylated form. **Results:** Gemcitabine increased the activation level of p38 MAPK in a dose-dependent manner and induced apoptosis in tested two human pancreatic cancer cells lines. The selective p38 MAPK inhibitors, SB203580 and SB202190, reduced gemcitabine-induced activation of p38 MAPK, prevented the gemcitabine-induced apoptosis and increased long-term clonogenic survival. Overexpression of a dominant-negative p38 mutant in cells resulted in the reduction of gemcitabine-induced p38 MAPK activation and apoptosis, and increases in clonogenic survival. **Conclusions:** These results strongly suggest that the activation of p38 MAPK signaling is necessary for gemcitabine-induced cell death in human pancreatic cancer cells. Based upon these results, we would suggest that molecules of p38 MAPK signaling pathways should be listed as novel targets for gemcitabine-based therapy.

Introduction

Gemcitabine (2',2'-difluorodeoxycytidine) is a pyrimidine nucleoside analogue whose activity has been shown in various solid tumors including pancreatic cancers (1-3). The major effect of gemcitabine is directed against DNA synthesis (4). The compound causes masked chain termination and ribonucleotide reductase inhibition (5, 6). However, how DNA damage induced by gemcitabine leads to apoptosis is still poorly understood. Elucidation of the mechanism by which gemcitabine kills cancer cells requires a deeper understanding of the signaling pathways activated by this drug in cancer cells.

Members of the p38 mitogen-activated protein kinase family (MAPKs) are strongly activated by genotoxic stress and inflammatory cytokines, leading to the regulation of cellular functions such as proliferation, differentiation, and survival (7-9). Several lines of evidence suggest that loss of p38 MAPK activation in mutant cells is associated with increased tumorigenesis (10), and that p38α-deficient cardiomyocytes and fibroblasts are more resistant to apoptosis induced by different stimuli (11). In addition, p38 MAPK activation has been detected in most healthy tissues but not in cell lines or cancer tissues including breast, lung, liver, bile duct, gastric, colorectal, renal cell, ovarian, and uterine cancers (12), suggesting that functional inactivation or silencing of the p38 pathway appears to be a defining feature of many types of cancer. Therefore, p38 MAPK signaling seems to be an attractive target in cancer therapy.

We previously described cellular changes associated with apoptosis and cell survival in human pancreatic cancer cell lines treated with gemcitabine, and demonstrated that p38 MAPK, but not JNK or ERK1/2, was activated in response to gemcitabine, suggesting that

p38 MAPK is specifically activated by gemcitabine (13). It was furthermore demonstrated that a selective p38 MAPK inhibitor, SB203580, significantly inhibited gemcitabine-induced apoptosis, indicating that p38 MAPK might be involved in the gemcitabine-induced cytotoxicity in human pancreatic cancer cells. In the present study, to further clarify the importance of p38 MAPK in the gemcitabine-induced cytotoxicity, we made a dominant-negative p38 MAPK mutant and used clonogenic assays to assess the long-term effect on cancer cell viability in the human pancreatic cancer cells.

Materials and Methods

Cell Culture. The human pancreatic adenocarcinoma cell lines PK1 and PCI43 were used in this study. The PK1 cell line was obtained from the Cell Resource Center for Biochemical Research (Tohoku University, Sendai, Japan) (14), and the PCI43 cell line was provided by Dr. H. Ishikura at Hokkaido University (Sapporo, Japan) (15). Cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37ºC. Experiments were performed using cells in the exponential phase of growth.

Drugs and Treatments. Gemcitabine was provided by Eli Lilly (Indianapolis, IN). The selective inhibitors of p38 MAPK, SB203580 and SB202190, were purchased from Calbiochem (San Diego, CA). Cells were incubated with the vehicle (DMSO) or gemcitabine in combination with or without pretreatment with p38 inhibitors. All experiments were performed in triplicate.

Expression Vector Construction and Transfection. The cDNA for human p38α was isolated and introduced into the expression vector as previously reported (16, 17). Briefly, the cDNA for full-length human p38α was obtained by RT-PCR using mRNA from PCI43 cells as a template. The product was cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Full activation of p38α requires phosphorylation of Thr180 and Tyr182 found within a Thr-Gly-Tyr tripeptide motif in the activation loop of the kinase (18). To generate a dominant-negative p38α mutant, site-directed mutagenesis was performed by PCR to replace Thr180 and Tyr182 with alanine and phenylalanine, respectively. An N-terminal hemagglutinin (HA) epitope-tag was introduced to distinguish between endogenous and transfected p38α. The p38α insert was sequenced on both strands to confirm the mutation.

PK1 and PCI43 cells were cultured in RPMI for 12 hours before transfection. Both cell lines were then transfected with empty vector or DN-p38α expression plasmids using GenePorter2 (Gene Therapy Systems, San Diego, CA) following the manufacturer's instructions. In all transfections, a constant amount of DNA was introduced by using an appropriate empty vector. Transfection efficiencies were assessed by checking for fluorescence of the enhanced green fluorescent protein (EGFP; BD Biosciences Clontech, Palo Alto, CA) under a fluorescence microscope. Cells were selected in 400 μg/ml G418 (GIBCO, Grand Island, NY) 2 days after transfection. DN-p38α-PCI43 cells were stably grown in the continuous presence of G418.

Western Blot Analysis and Antibodies. Polyclonal antibodies against phospho-p38 MAPK (Thr180/Tyr182) and phospho-Akt (Ser473) were obtained from Cell Signaling Technology (Beverly, MA). A monoclonal antibody recognizing the hemagglutinin (HA) epitope was

obtained from Babco (Richmond, CA). A monoclonal antibody against β-actin was obtained from Abcam (Cambridge, UK). Total protein extracts were prepared using lysis buffer [50 mM Tris-HCl (pH 7.5), 137 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μg/ml aprotinin]. The lysate was centrifuged, and the supernatant was collected. Samples were heated in SDS sample buffer for 5 min at 100°C. Protein concentrations were measured using the Protein Assay Kit (BioRad, Hercules, CA). Equal amounts of protein were loaded into each well and separated in 4-20% or 11-14% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in TBS-T [50 mM Tris (pH 8.0), 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20], and then exposed to primary rabbit antibodies against phospho-p38 or phospho-Akt, and primary mouse antibodies against HA or β-actin. Proteins were visualized with suitable horseradish peroxidase-conjugated secondary antibodies (Calbiochem) using the enhanced chemiluminescence detection system (NEN Life Science, Boston, MA).

Detection of Apoptosis. To assess morphological changes in chromatin structure of cells undergoing apoptosis, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). After transfection 24 h later, cells were incubated with gemcitabine for 4 h, then trypsinized gently. Trypsin-released adherent cells and cells that were floating in the medium before trypsin treatment were mixed, pelleted by centrifugation, washed with PBS and fixed with 3.5% paraformaldehyde for 20 minutes. Cells were then stained with DAPI (1 μg/ml),

mounted on glass slides, and analyzed using a fluorescence microscope to assess chromatin condensation and nuclei fragmentation. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. Cell staining experiments were performed in triplicate, and the percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. The incidence of apoptosis in each treatment was analyzed by counting 1,000 cells.

Clonogenic Assay. Clonogenic assays were carried out on growing cells in 100-mm dishes. Before the induction of apoptosis, cells were preincubated for 1 h with 20 μM SB203580 or SB202190. After treatment with 10 μM gemcitabine for 4 h, cells were washed twice with PBS and incubated in normal growth medium. For transfections, cells were plated in 100-mm dishes and transfected with an empty vector or the DN-p38α expression plasmid using GenePorter2 (Gene Therapy Systems) following the manufacturer's instructions. At 24 h after transfection, cells were incubated with 10 μM gemcitabine for 4 h. Cells were washed twice with PBS and incubated in growth medium in the presence of G418. Colonies formed within 2 weeks. These colonies were stained with 70% methanol and 1.5% crystal violet. Colonies were defined as >20 cells.

Results

Gemcitabine-induced Activation of p38 MAPK is Inhibited by the Selective p38 Inhibitors SB203580 and SB202190. We first confirmed that gemcitabine activates p38 MAPK in two human pancreatic cancer cell lines. As shown in Figure 1A, the activation level of p38 MAPK was low but detectable in the absence of gemcitabine in both cell lines. Activation

of p38 MAPK was markedly increased at a concentration of 10 μM gemcitabine at 24 h after the treatment. Next, we examined whether gemcitabine-induced activation of p38 MAPK is pharmacologically inhibited by selective p38 MAPK inhibitors. We used the pyridinylimidazole inhibitors SB203580 and SB202190 (16). Cells were preincubated with 20 μM SB203580 or SB202190 for 1 h, washed, and then incubated with 10 μM gemcitabine for 4 h. Cells were lysed, and the lysates were used for Western blotting. As shown in Figure 1B, both inhibitors strongly repressed activation of p38 MAPK by gemcitabine treatment, whereas the protein expression level of total p38 MAPK was unaffected.

Pharmacological Inhibition of p38 MAPK Activation Blocks Gemcitabine-induced Cell Death and Increases Clonogenic Survival. To test the biological role of p38 MAPK in response to gemcitabine, we used a specific inhibitor of p38 MAPK, SB203580. Cells were pretreated with 20 μM of SB203580 for 1 h, washed, and then incubated with 10 μM gemcitabine for 4 h. Cells were washed and cultured for an additional 72 h. As shown in Figure 2, there was an increase in the viability of both cell lines pretreated with a p38 MAPK inhibitor. The results obtained by these methods essentially matched those observed by flow cytometry analysis in our previous report (13). Next, to evaluate the clinical significance of our finding, we decided to assess the role of p38 MAPK using clonogenic assays, because the most important parameter in the efficacy of a chemotherapeutic drug is its long-term effect on cancer cell viability. Cells were pretreated with 20 μM of SB203580 and washed with PBS, then incubated with 10 μM gemcitabine for 4 h. Subsequently, the cells were seeded into normal growth medium and the growth of cell colonies was scored two weeks later. Figure 3 demonstrated that gemcitabine significantly reduced colony formation. Pretreatment with

SB203580 increased the number of colonies in both cell lines, suggesting that p38 MAPK signaling appears to mediate the gemcitabine-induced cytotoxicity in human pancreatic cancer cells.

Overexpression of the DN-p38α Mutant in Cells Inhibits Gemcitabine-induced Activation of p38 MAPK and Increases Clonogenic Survival. Although SB203580 and SB202190 are believed to be specific inhibitors of p38 MAPK, it is important to confirm experimental results obtained with these drugs through other strategies. Four different p38 MAPK members have been identified: p38α, β, γ, and δ. These proteins may have both overlapping and specific functions (19-21). Among them, p38α is broadly expressed and is the most abundant p38 MAPK family member, being present in most cell types. To further our studies, we expressed a DN-p38α mutant with the G418 resistance gene to examine whether overexpression of DN-p38α in cells affects gemcitabine-induced apoptosis and clonogenic survival. First, we tested the effect of a DN-p38α mutant on the activation level of p38 MAPK in response to gemcitabine. As illustrated in Figure 4, overexpression of a DN-p38α mutant resulted in the reduction of p38 MAPK activation levels in both cell lines. Wild-type p38α showed no inhibitory effect on p38 MAPK activation. Next, we found that gemcitabine-induced apoptosis was inhibited in cells expressing DN-p38α (Figure 5). Since results may differ depending on whether long-term survival or short-term apoptosis assays are performed, we transfected cells with pcDNA3-DN-p38α, and counted antibiotic-resistant colonies after 2 weeks in the presence of G418. As shown in Figure 6, relative to cells transfected with empty vector alone, less than 5% of the cells incubated with gemcitabine formed colonies. In contrast, incubation of DN-p38α-transfected cells with gemcitabine

blocked the inhibitory effect of gemcitabine on cell proliferation.

The serine/threonine kinase Akt is a well-characterized kinase that is known to play a critical role in anti-apoptotic signaling pathways. To address whether inactivation of Akt is involved in gemcitabine-induced apoptosis, we examined the effect of gemcitabine on Akt activation in the DN-p38 cells (Figure 7). The membranes probed with anti-phospho-p38 MAPK antibody in Figure 1B and 5A were reused for reprobing with anti-phospho-Akt (Ser473) antibody. No clear alteration of the Akt activation level was observed in gemcitabine-treated cells, indicating that Akt is not implicated in the gemcitabine-induced cytotoxicity mediated through p38 MAPK activation.

Discussion

Gemcitabine reportedly induces apoptosis in pancreatic cancer cells (22). However, little is known about the signaling pathway(s) leading to gemcitabine-induced apoptosis. We demonstrated in a previous report that the activation of the p38 MAPK signaling pathway is involved in the cellular response to gemcitabine in human pancreatic cancer cells (13). In the present study, we have further investigated the role of p38 MAPK signaling in gemcitabine-induced cytotoxicity using a molecular approach in human pancreatic cancer cell lines. We demonstrated that gemcitabine-induced apoptosis is significantly blocked by p38 MAPK inhibitors and by DN-p38α expression, suggesting clearly the proapoptotic role of p38 MAPK activation in gemcitabine-induced cell death. Furthermore, our results indicate, for the first time, that this event is a key component of gemcitabine-induced cell death, and that the biological outcome of p38 MAPK activation in response to gemcitabine is modulated by the inhibition of p38 MAPK signaling in human pancreatic cancer cells.

The present study confirmed the requirement of p38 MAPK activation in gemcitabine-induced cell death by using clonogenic assays. The clonogenic assay is the most reliable method for assessing cell killing after treatment with genotoxic agents (23). Short-term assays may lead to incorrect assessments of overall cell kill rates, largely because they ignore kinetic differences in the manifestation of cell death. Cells do not die immediately after treatment; they can take hours to many days before dying, and this is highly dependent upon the cell type and the toxic agent being investigated. It has been reported that the sensitivity of cells to toxic agents as assessed by their ability to form a colony is greater than that of the short-term assay, which measures the proportion of cells undergoing apoptosis (24). This supports our present data, which show differences in degree of apoptosis between the short-term assay (Figure 2 and Figure 5) and the clonogenic assay (Figure 3 and Figure 6).

The mechanisms underlying p38 MAPK-mediated apoptosis in response to gemcitabine remains to be clarified. With regard to this point, we tried to examine that Akt may be implicated in the mechanism. Akt is a key oncogenic survival factor, and activation of Akt has been shown to induce resistance to apoptosis induced by chemotherapeutic drugs or irradiation (25-27). Some reports demonstrated an association between the activation status of p38 MAPK and the Akt survival pathway in tumor cells. Liao *et al*. found that p38 phosphorylation is downregulated and Akt phosphorylation is upregulated in multiple human tumor tissues, whereas the staining intensity of phospho-p38 protein was relatively strong while that of phospho-Akt was very weak in normal organs and parallel healthy tissues (12). Tanaka *et al*. reported that stimulation of protein kinase C by phorbol 12-myristate 13-acetate promotes apoptosis in prostate cancer cells through activation of p38 MAPK and inhibition of

the Akt activity (28). These reports suggest that down-regulation of p38 MAPK activity is a common event in human cancer cells, particularly when the level of Akt activation is increased. On the other hand, p38 activation and Akt inactivation may contribute to increasing apoptosis induced by drugs. In the present study, we demonstrated that gemcitabine induced p38 MAPK activation but not Akt activation in cells transfected with empty vector or DN-p38α. Thus, our data failed to provide evidence that gemcitabine has an effect on phosphorylation of Akt in not only untrasfected but in DN-p38α-transfected cells.

In conclusion, our data indicate that gemcitabine induces phosphorylation of p38 MAPK, and that this event is a key component of pancreatic cancer cell death in response to gemcitabine. The proapoptotic functions of p38 suggest possible new approaches to targeted therapy and p38 MAPK modulators may have potential as chemotherapeutic drugs in gemcitabine-based therapy in human pancreatic cancer.

Acknowledgments

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Figure Legends

Figure 1. *(A)* Activation of p38 MAPK induced by gemcitabine in a dose-dependent manner. 24 h after treatment with gemcitabine at indicated concentrations, cells were lysed. Western blotting was performed using an antibody against phospho-p38 MAPK. As a loading control, an antibody against β-actin was used. The relative expression levels of phospho-p38 MAPK were calculated from densitometric data. The control (without gemcitabine treatment) was assigned a value of 1. *(B)* Gemcitabine-induced activation of p38 MAPK was inhibited by different specific p38 inhibitors, SB203580 or SB202190. After pretreatment with SB203580 or SB202190 for 1 h at a concentration of 20 μM, cells were washed, then treated with 10 μM gemcitabine for 4 h. Western blotting was performed using an antibody against phospho-p38 MAPK. As a loading control, an antibody against β-actin was used in each experiment.

Figure 2. Gemcitabine-induced nuclear fragmentation was detected by fluorescent microscopy. Cells were pretreated with 20 μM SB203580 for 1h and washed. After treatment with 10 μM gemcitabine for 4 h, cells were washed, and further cultured for 72 h. Both attached and detached cells were collected, fixed with 3.5% parafolmaldehyde and stained with DAPI. A total number of 1,000 cells was counted and the percentage of the cells showing nuclear morphological changes was calculated. *(A)* Representative photographs of the cells showing chromatin condensation and nuclei fragmentation analyzed with DAPI staining. *(B)* Results shown are the means of three experiments. *Bars,* SD

Figure 3. Clonogenic assay performed on cells treated with 10 μM gemcitabine. After treatment with gemcitabine for 4 h with or without pretreatment of SB203580, cells were washed, and medium was changed. Cells were cultured for 10 days, and fixed with 70%

ethanol. Next, cells were stained with crystal violet. Colonies consisting of more than 20 cells were counted. The relative number of colonies was calculated. *(A)* Photographs were taken at 2 weeks after gemcitabine treatment. *(B)* Colonies were counted 2 weeks later and normalized to control. *Bars*, SD.

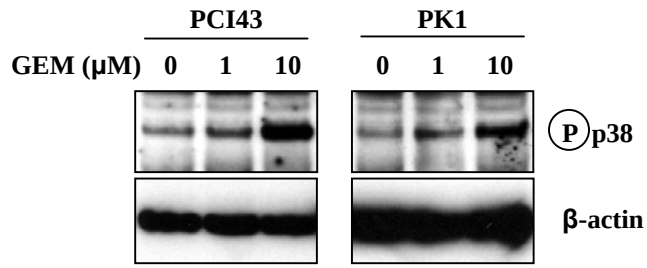
Figure 4. A dominant-negative p38 MAPK mutant (DN-p38α) inhibited gemcitabine-induced p38 activation in PCI43 and PK1 cells. At 24 h after transfection with an empty vector, DN-p38α or wild-type p38α, cells were treated with 10 μM gemcitabine for 4 h and lysed. Western blotting was performed to assess the expression levels of transfected HA-tagged DN-p38α and wild-type p38α, and the phosphorylation levels of p38 MAPK in PCI43 and PK1 cells.

Figure 5. *(A)* p38 MAPK phosphorylation in PCI43 cells stably expressing DN-p38α. Cells were transfected with pcDNA3 HA-tagged DN-p38α or empty vector, and selected in G418 (200 μg/ml). Western blotting was performed to confirm the stable expression of HA-tagged DN-p38α using an anti-HA antibody in PCI43 cells. Gemcitabine-induced phosphorylation of p38 was lower in PCI43 cells stably expressing DN-p38α than vector-transfected cells. *(B)* Gemcitabine-induced apoptosis in PCI43 cells stably expressing DN-p38α. Nuclear fragmentation was detected by DAPI staining. After treatment with gemcitabine for 4 h, cells were washed with PBS, and cultured for 72 h in the presence of G418 (200 μg/ml). Both attached and detached cells were collected, and fixed with 3.5% paraformaldehyde. A total number of 1,000 cells was counted under a fluorescent microscope, and the percentage of cells showing nuclear morphological changes was calculated. *Bars*, SD.

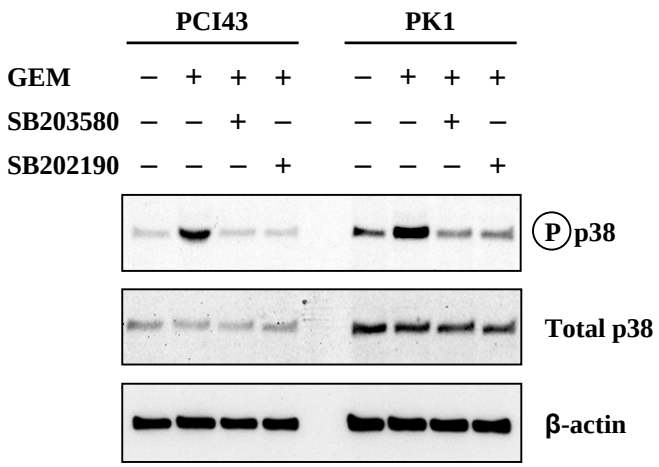
Figure 6. Clonogenic assay of PCI43 cells stably expressing DN-p38α treated with 10 μM gemcitabine. After treatment with gemcitabine for 4 h, cells were washed, and medium was changed. Cells were cultured for 10 days in the presence of G418 (200 μg/ml) and fixed with 70% ethanol. Cells were then stained with crystal violet. Colonies consisting of more than 20 cells were counted. *(A)* Representative photographs of colonies taken at 2 weeks after gemcitabine treatment. *(B)* Relative number of colonies was calculated. Results shown are the means of three experiments. *Bars,* SD

Figure 7. Phosphorylation levels of p38 MAPK and Akt in gemcitabine-treated cells. The membranes in Figure 1B and 5A were simultaneously probed with antibodies to both phospho-Akt (S473) and phospho-p38 MAPK.

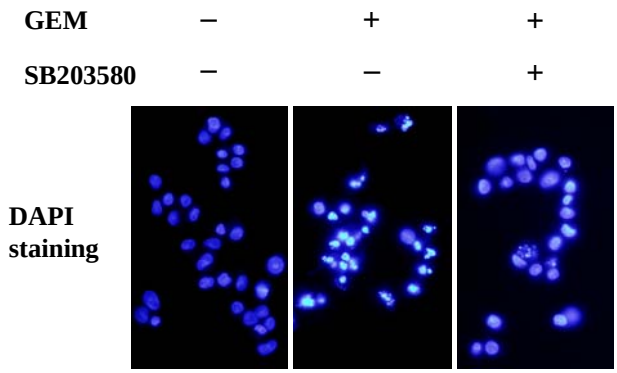
A.


PCI43
PK1
GEM (μM) 0 1 10 0 1 10
P p38
β-actin

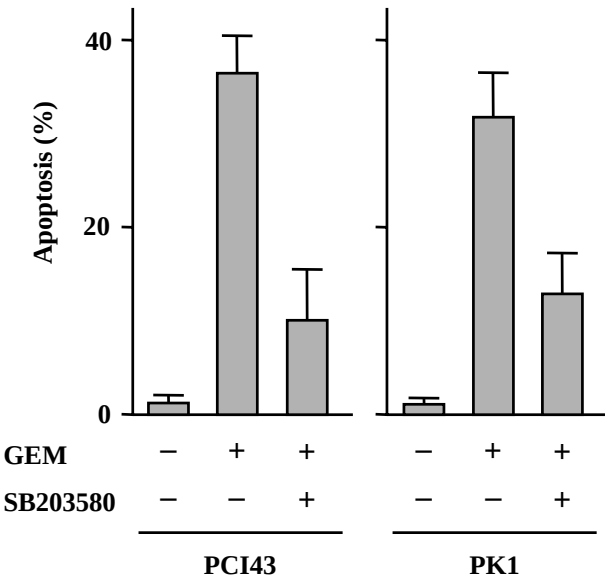
B.


PCI43
PK1
GEM – + + + – + + +
SB203580 – – + – – – + –
SB202190 – – – + – – – +
P p38
Total p38
β-actin

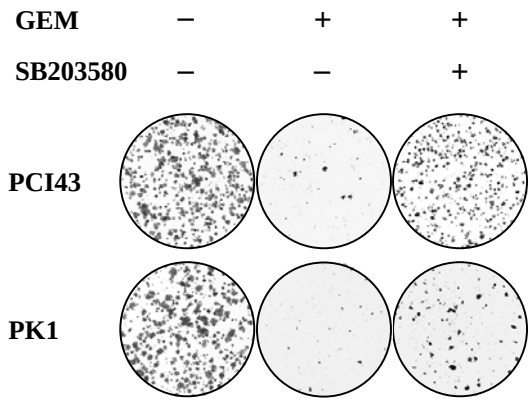
A.


GEM
–
+
+
SB203580
–
–
+
DAPI
staining

B.


Apoptosis (%)
40
20
0
GEM
–
+
+
–
+
+
SB203580
–
–
+
–
–
+
PCI43
PK1

A.


GEM
–
+
+
SB203580
–
–
+
PCI43
PK1

B.

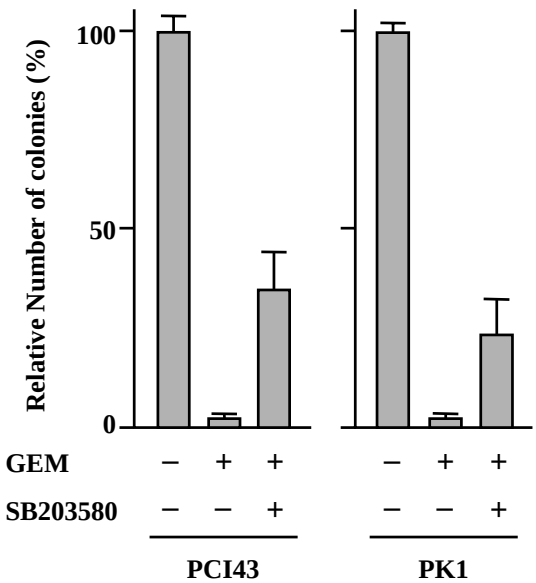
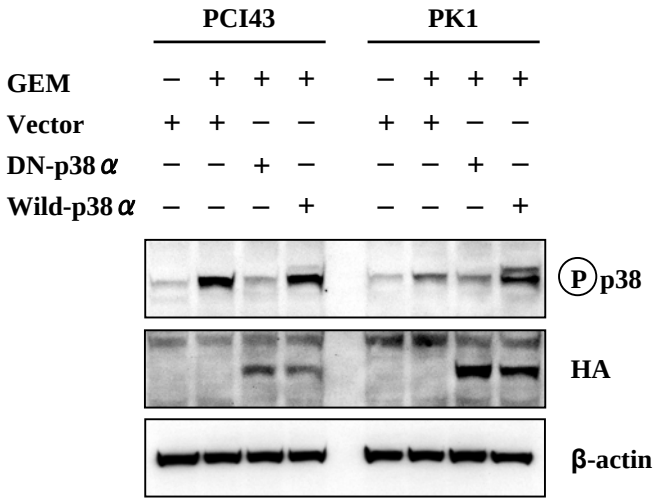
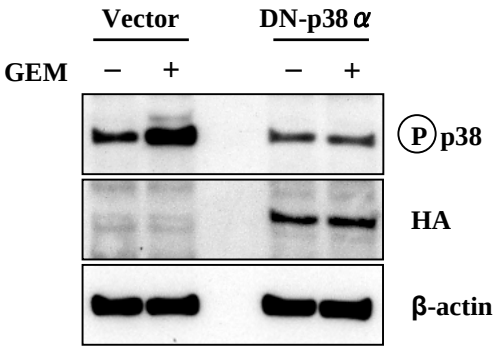

Relative Number of colonies (%)
100
50
0
GEM
–
+
+
–
+
+
SB203580
–
–
+
–
–
+
PCI43
PK1

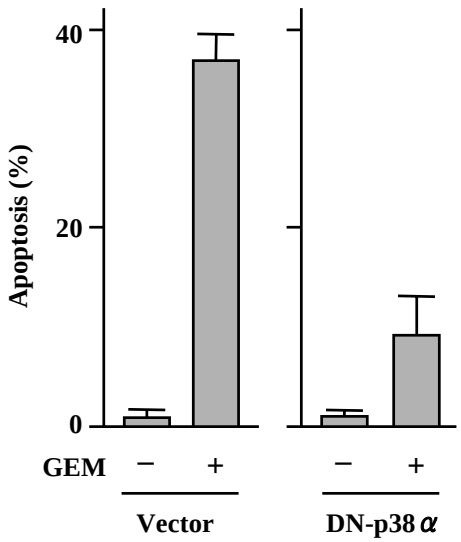
Fig. 4



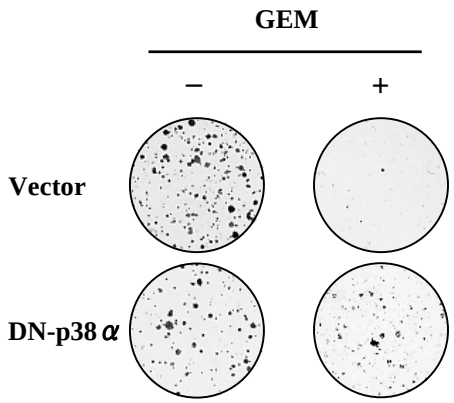
A.


Vector
DN-p38α
GEM
−
+
−
+
Ⓟp38
HA
β-actin

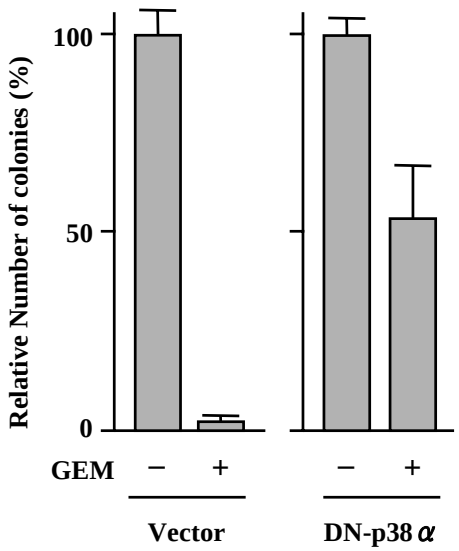
B.


40
20
0
Apoptosis (%)
GEM
−
+
−
+
Vector
DN-p38α

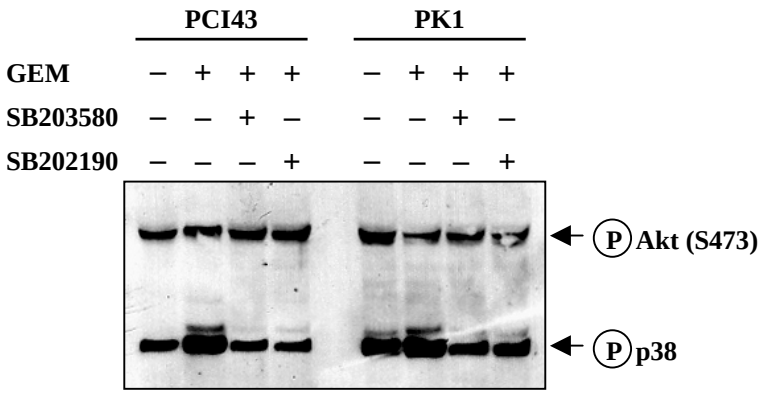
A.


GEM
−
+
Vector
DN-p38α

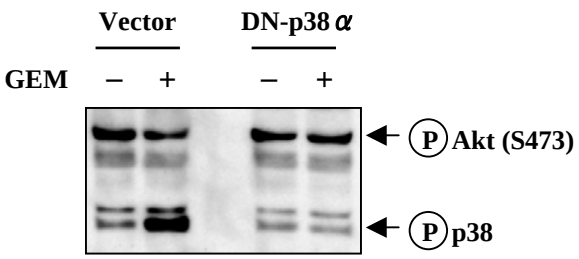
B.


Relative Number of colonies (%)
100
50
0
GEM
−
+
−
+
Vector
DN-p38α

A.


PCI43
PK1
GEM
SB203580
SB202190
P Akt (S473)
P p38

B.


Vector
DN-p38α
GEM
P Akt (S473)
P p38