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Bcl-2/Bcl-xL inhibitor navitoclax increases the antitumor effect of Chk1 inhibitor prexasertib by inducing apoptosis in pancreatic cancer cells via inhibition of Bcl-xL but not Bcl-2

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Abstract

In our previous study, we showed that prexasertib, a checkpoint kinase 1 (Chk1) inhibitor, enhances the effects of standard drugs for pancreatic cancer, including gemcitabine (GEM), S-1, and the combination of GEM and S-1 (GS). The combination of prexasertib and GS has a strong antitumor effect and induces apoptosis in pancreatic cancer cells by downregulating anti-apoptotic protein Bcl-2. In the present study, we investigated the combined effect of GEM, S-1, and prexasertib with a selective Bcl-2 inhibitor (venetoclax) and a non-selective Bcl-2 inhibitor (navitoclax) in SUIT-2 pancreatic cancer cells. An MTT assay revealed that the combination of prexasertib with navitoclax showed a synergistic effect but the combination with venetoclax did not. Investigation of the pancreatic cancer cell lines SUIT-2, MIA PaCa-2, and BxPC-3 revealed that BxPC-3 also showed a high synergistic effect when combined with prexasertib and navitoclax but not venetoclax. Mechanistic analysis of the combined effect showed that apoptosis was induced. Bcl-2 knockdown with siRNA and prexasertib treatment did not induce apoptosis, whereas Bcl-xL knockdown with siRNA and prexasertib treatment resulted in strong induction of apoptosis. In addition, among the three cell lines, the combined effect of prexasertib and navitoclax resulted in increased apoptotic cell death because the protein expression levels of Bcl-xL and Chk1 were higher. Our results demonstrate that the combination of prexasertib and navitoclax has a strong antitumor effect and induces apoptosis in pancreatic cancer cells by downregulating Bcl-xL. Simultaneous inhibition of Chk1 and Bcl-xL could be a new strategy for treating pancreatic cancer.

Keywords Prexasertib · Venetoclax · Navitoclax · Checkpoint kinase 1 · Bcl-2 · Bcl-xL

Introduction

Pancreatic cancer is a highly malignant carcinoma with a dismal prognosis. The disease is often treated with drugs, so the development of more effective drug treatments is urgently required. The result of a randomized controlled trial comparing gemcitabine (GEM) with 5-fluorouracil (5-FU) in unresectable pancreatic cancer resulted in GEM becoming a standard drug worldwide [1]. The antitumor effect of S-1 (TS-1, Taiho Pharmaceutical Co. Ltd., Tokyo, Japan), an oral fluoropyrimidine developed in Japan, has already been demonstrated in single-agent or combination regimens for a variety of solid tumors [2–4]. A phase III trial conducted in Japan examining treatment of pancreatic cancer with S-1 showed that S-1 is non-inferior to GEM, and thus S-1 has become a key drug for pancreatic cancer in Japan, similar to GEM [5]. These key drugs (GEM and S-1, collectively called fluorinated pyrimidine anticancer drugs) are crucial for treating unresectable pancreatic cancer.

Our previous study using pancreatic cancer cells showed that the combination of S-1 and GEM is more effective than either drug alone, both in vitro and in vivo [6]. Mechanistic studies have shown that both drugs lead to increased phosphorylation of checkpoint kinase 1 (Chk1) [7]. Chk1 is activated when DNA is damaged, and it stops the cell cycle and catalyzes DNA repair [8]. Activation of Chk1 is a mechanism of anticancer drug resistance. We showed

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that prexasertib (LY2606368), which is a Chk1 inhibitor, enhances the effects of GEM, S-1, and the combination of GEM and S-1 (GS). The combination of prexasertib and GS has a strong antitumor effect and induces apoptosis in pancreatic cancer cells by downregulating anti-apoptotic protein Bcl-2 [9].

The Bcl-2 family regulates apoptosis and could be an attractive target for antitumor therapy [10]. Venetoclax (ABT-199) was developed as a selective Bcl-2 inhibitor, and it has been investigated in several clinical trials for treating chronic lymphocytic leukemia and approved by the US Food and Drug Administration [11–13]. Navitoclax (ABT-263) is a non-selective Bcl-2 inhibitor, inhibiting Bcl-2 and Bcl-xL, which is a member of the Bcl-2 family and has an anti-apoptotic effect [14]. Navitoclax is undergoing clinical trials in combination therapy for solid tumors [15].

In this study, we investigated the combined effect of GEM, S-1, and prexasertib with venetoclax or navitoclax in the pancreatic cancer cell line SUIT-2. In addition, we investigated several pancreatic cancer cell lines and conducted a mechanistic analysis of the combined effects.

Methods

Cell cultures

The human pancreatic cancer cell lines SUIT-2 and MIA PaCa-2, and the leukemia cell line HL-60 were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). The pancreatic cancer cell line BxPC-3 was purchased from ATCC (Rockville, MD). SUIT-2 cells, BxPC-3 cells, and HL-60 cells were grown in RPMI 1640 medium (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% low endotoxin fetal bovine serum (FBS) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), penicillin, and streptomycin. MIA PaCa-2 cells were grown in E-MEM medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% low endotoxin FBS (Sigma-Aldrich), penicillin, and streptomycin.

Antitumor agents

Venetoclax and navitoclax were purchased from MedChemExpress (Monmouth Junction, NJ). GEM, 5-FU, and 5-chloro-2,4-dihydroxypyridine (CDHP) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and prexasertib was purchased from Selleck Biotech (Osaka, Japan). S-1 consists of tegafur (FT), CDHP, and oteracil potassium [16]. Because FT is a prodrug of 5-FU [16], we used 5-FU instead of FT in this study. Furthermore, we omitted the oteracil potassium component because it is used to lower the incidence of gastrointestinal side effects, and instead used 5-FU combined with CDHP in a 1:2 ratio based on the blood concentration ratio of this combination in humans [17].

Antibodies

Monoclonal antibodies against caspase 3 (#9665, 1:1000 dilution), cleaved PARP (Asp214) (#5625, 1:1000 dilution), Mcl-1 (#94296, 1:1000 dilution), Bcl-2 (#2870, 1:1000 dilution), Bcl-xL (#2764, 1:1000 dilution), Bax (#2772, 1:1000 dilution), phosphorylated Chk1 (checkpoint kinase 1; Ser296) (#90178, 1:1000 dilution), phosphorylated Chk1 (Ser317) (#12302, 1:1000 dilution), phosphorylated Chk1 (Ser345) (#2348, 1:1000 dilution), Chk1 (#2360, 1:1000 dilution), and ɤH2AX (Ser139) (#9718, 1:1000 dilution) were purchased from Cell Signaling Technology (Danvers, MA). The monoclonal antibody against β-actin was purchased from Sigma-Aldrich (A5316, 1:5000 dilution). Purified mouse anti-cytochrome *c* (No.556433, 1:1000 dilution) was purchased from BD Biosciences (Franklin Lakes, NJ).

Cell proliferation assay

SUIT-2 cells were seeded in 96-well plates at a density of 1×10^3 cells/well and MIA PaCa-2 and BxPC-3 cells at a density of 2×10^3 cells/well. After culturing for 24 h at 37 °C, the culture medium was replaced with 0.2 mL of fresh medium containing each antitumor drug (venetoclax, navitoclax, GEM, S-1, and prexasertib) at each concentration. After a further 72 h at 37 °C, 20 µL of 0.5% MTT reagent (Sigma-Aldrich) in PBS was added to each well. The plate was incubated for 4 h at 37 °C, and 0.1 mL dimethyl sulfoxide (Kanto Chemical Co. Inc., Tokyo, Japan) was added to each well to dissolve the formazan crystals. The absorbance at 620 nm was measured in each well with a microplate reader. Half-maximal inhibitory concentration (IC_{50}) values of each drug were calculated using CalcuSyn (Biosoft, Cambridge, UK). The combination index (CI) was calculated using CalcuSyn and synergy level classifications of the combined effect for each drug were determined. A CI of less than 1 indicates synergy, a CI of 1 indicates additive effects, and a CI of more than 1 indicates antagonistic effects. All experiments were repeated at least three times.

Analysis of cell death

Cells were seeded on 6-well plates (1.0×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS. After 24 h at 37 °C, the culture medium was replaced with 2.0 mL of fresh medium containing prexasertib and/or navitoclax at each concentration. After a further 24 h at 37 °C, mono- and oligonucleosomes in the cytoplasmic fraction were measured using a Cell Death Detection ELISA kit

(Roche, Basel, Switzerland; cat. no. 1544675) according to the manufacturer's instructions. Floating and attached cells were collected and homogenized in 500 µL of incubation buffer. The wells of a 96-well plate were coated with anti-histone antibodies at room temperature for 1 h and incubated with the lysates, horseradish peroxidase-conjugated anti-DNA antibodies, and the substrate. The absorbance at 405 nm was measured in each well with a microplate reader.

Hoechst 33342 staining

To assess changes in nuclear morphology during apoptosis, cells were stained using fluorescent Hoechst 33342. Cells were seeded on 6-well plates (6.0×10^4 cells/well) in RPMI 1640 medium supplemented with 10% FBS. After 24 h at 37 °C, the culture medium was replaced with 2.0 mL of fresh medium containing, prexasertib (10 nM) and/or navitoclax (3 µM). After a further 24 h at 37 °C, the cells were stained with 1.2 mM Hoechst 33342 for 5 min at room temperature and examined under a fluorescence microscope.

Western blot analysis

Cells (3.0×10^5) were seeded in dishes and cultured for 24 h at 37 °C. The medium was replaced by fresh medium containing prexasertib (10 nM) or navitoclax (3 µM) and the cells were incubated for the indicated times (24, 48, and 72 h). Cells were rinsed with PBS and scraped or lysed (only HL-60) into cell lysis buffer M (FUJIFILM Wako Pure Chemical Corporation) dissolved in complete protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche). After incubation on ice for 20 min, cell lysates were obtained by centrifugation at 15,000×*g* for 15 min at 4 °C. Protein concentrations were determined and equal amounts (30 µg) of total protein were separated on 7.5–12% sodium dodecyl sulfate polyacrylamide gels at a constant current of 30 mA. Separated proteins were then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Burlington, MA) at 150 mA. The membrane was blocked with 1% Difco Skim Milk (BD Biosciences) and hybridized overnight at 4 °C with various primary antibodies. Membranes were probed with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Dako Denmark A/S, Glostrup, Denmark; cat. no. P0448 or P0260) and chemiluminescence was developed using a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA). The band intensities of Bcl-2, Bcl-xL, Chk1, and β-actin were analyzed using Image J version 1.41 (National Institutes of Health, Bethesda, MD).

Analysis of cytochrome c release

Cells (3.0×10^5) were seeded in dishes and cultured for 24 h at 37 °C. The medium was replaced with fresh medium containing prexasertib (10 nM) or navitoclax (3 µM) and the cells were incubated for the indicated time (24 and 48 h). The cells were washed with PBS and treated with 0.05% trypsin, and then resuspended in 100 µL of ice-cold digitonin lysis buffer (0.01% digitonin in PBS). After 5 min on ice, the cells were centrifuged at 14,000×*g* for 5 min, and the supernatant was subjected to western blotting analysis with a cytochrome *c*-specific antibody.

siRNAs and transfection of cells

Silencer Select Validated siRNA against Bcl-2 and Bcl-xL and Silencer Select Negative Control siRNA were purchased from Thermo Fisher Scientific. The day before siRNA transfection, cells (1×10^5) were seeded into 6-well plates and incubated overnight at 37 °C without antibiotics. The cells were treated with siRNA (final concentration of 5 nM) in RPMI 1640 medium in the presence of the DharmaFECT transfection reagent according to the manufacturer's instructions (GE Healthcare Japan, Tokyo, Japan). After incubation for 24 h at 37 °C, the medium containing the mixture of DharmaFECT and siRNA was replaced by RPMI 1640 medium containing 10% FBS and the cells were incubated for a further 24 h with or without prexasertib (0, 10, or 30 nM). Western blotting was performed to determine the expression level of each protein. Cell death was measured by a Cell Death Detection ELISA kit and Hoechst 33342 staining.

Statistical analysis

Data from the MTT assays and ELISA are expressed as the mean ± standard deviation. The IC_{50} values of venetoclax and navitoclax were compared using the unpaired *t*-test. Comparisons of more than two groups were made by one-way ANOVA with the post hoc Fisher's protected least significant difference test between each group. A p value of less than 0.05 was considered statistically significant. Statistical analysis was performed using BellCurve in Excel for Windows ver. 3.20 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Inhibitory effect of venetoclax or navitoclax on cell growth in pancreatic cancer cells

We performed an MTT assay to investigate the combined effect of venetoclax or navitoclax with the drugs GEM, S-1, or prexasertib against SUIT-2 cells. The combination of venetoclax with each of the drugs showed antagonistic effects (CI > 1) for many concentration combinations (Table 1). However, the combination of navitoclax and prexasertib showed a strong synergistic effect (CI < 1) for many concentration combinations (Table 2). An MTT assay was also performed to investigate whether venetoclax or navitoclax inhibits cell growth in SUIT-2, MIA PaCa-2, and BxPC-3 cells. These cells were more sensitive to navitoclax than venetoclax, as shown by its significantly lower IC_{50} value (Fig. 1a–c). In SUIT-2 and BxPC-3 cells, the combination of navitoclax and prexasertib showed a significantly higher cell growth inhibitory effect than the single agents, whereas this effect was not observed with venetoclax in all three cell lines (Fig. 1d–i).

Apoptotic effect of the combination of prexasertib and navitoclax

We determined the apoptotic effect of the combination of prexasertib and navitoclax to elucidate the mechanism by which the combination inhibited SUIT-2 and BxPC-3 cell growth. Apoptosis was quantified by using a Cell Death Detection ELISA kit, and the combination of prexasertib and navitoclax significantly increased the extent of apoptotic cell death compared with the single agents ($p < 0.05$) (Fig. 2a, b). Figure 2c shows the morphological changes in SUIT-2 cells treated with each drug for 24 h. Untreated control cells showed no change in nuclear morphology. The cells treated with prexasertib and navitoclax developed apoptotic features, including chromatin condensation and nuclear fragmentation.

Table 1 Combination indexes calculated for venetoclax + GEM, venetoclax + S-1, and venetoclax + prexasertib

	Venetoclax (μM)		
	1	2	3
GEM (ng/mL)			
0.1	–	2.016	2.226
0.3	0.828[a]	1.069	1.123
0.5	1.052	1.178	1.294
S-1 (μg/mL)			
0.1	2.682	1.979	1.549
0.2	1.128	1.072	1.145
0.3	0.778[a]	0.992[a]	1.041
Prexasertib (nM)			
10	1.249	1.345	1.376
20	0.882[a]	0.970[a]	1.026
30	0.970[a]	1.047	1.077

CI combination index, *GEM* gemcitabine

[a]Indicates synergy

Table 2 Combination indexes for navitoclax + GEM, navitoclax + S-1, and navitoclax + prexasertib

	Navitoclax (μM)		
	1	2	3
GEM (ng/mL)			
0.1	0.736[a]	0.871[a]	1.022
0.3	1.063	0.793[a]	0.974[a]
0.5	0.653[a]	0.808[a]	0.911[a]
S-1 (μg/mL)			
0.1	1.473	1.967	1.619
0.2	1.385	1.546	1.933
0.3	1.140	1.400	1.629
Prexasertib (nM)			
10	0.719[a]	0.890[a]	0.962[a]
20	0.529[a]	0.574[a]	0.698[a]
30	0.451[a]	0.559[a]	0.727[a]

CI combination indexes, *GEM* gemcitabine

[a]Indicates synergy

Effect of the combination of prexasertib and navitoclax on apoptotic pathways and Chk1 signaling

To analyze the detailed apoptotic pathway of prexasertib and navitoclax, molecules associated with apoptosis were investigated. The results for the detection of cleaved caspase 3 and cleaved PARP, which are expression markers for apoptosis, are shown in Fig. 3a. After treating SUIT-2 cells with prexasertib and navitoclax for 48 h, the expressions of cleaved caspase 3 and cleaved PARP increased in the navitoclax treatment group and the combination group. Subsequently, the Bcl-2 family that directly regulates apoptosis was examined (Fig. 3b). Expression of Mcl-1, an anti-apoptotic protein, was increased by navitoclax treatment. The expression of Bcl-2 decreased after 48 h of navitoclax treatment compared with the control. The expression of Bcl-xL, which is also an anti-apoptotic protein, and that of Bax, which is a pro-apoptotic protein, were unchanged. The release of cytochrome *c* into the cytoplasm 24 and 48 h after drug treatment increased strongly in the combination group (Fig. 3c). Figure 3d shows the effect of prexasertib and navitoclax on Chk1 signaling. From 24 h, prexasertib

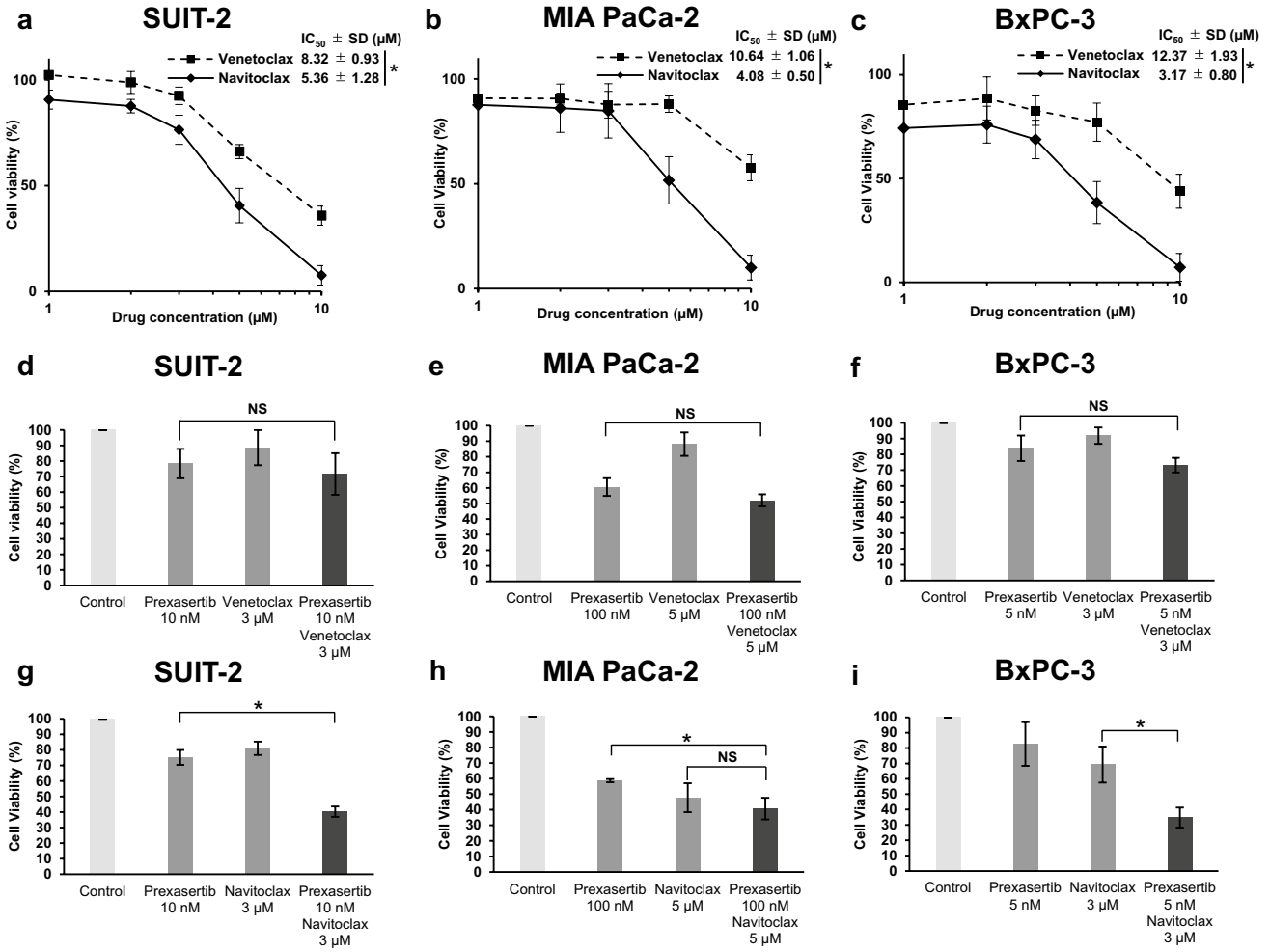

Fig. 1 Effects of venetoclax or navitoclax on SUIT-2, MIA PaCa-2, and BxPC-3. **a–c** Viability of cells treated with various concentrations of venetoclax or navitoclax for 72 h analyzed using an MTT assay. The IC_{50} values were determined at concentrations that showed 50% inhibition of cell growth. Effect of prexasertib combined with **d–f** venetoclax or **g–i** navitoclax, where cell growth was measured by MTT assay. Values shown are the mean ± SD from at least three independent experiments. $^*p < 0.05$ indicates a significant difference. NS: not significant

suppressed the phosphorylation of Chk1-S296, which is an autophosphorylation site that is the most important for the activation of Chk1. The combination of prexasertib and navitoclax increased the phosphorylation of Chk1-S317 and Chk1-S345, which are DNA damage markers, up to 48 h. The expression of ɣH2AX, which is also a DNA damage marker, was induced to a higher degree by navitoclax treatment and the combination treatment.

Induction of apoptosis by knockdown of Bcl-2 or Bcl-xL and treatment with prexasertib

We elucidated the mechanism of the synergistic effect of prexasertib and navitoclax further. Because navitoclax exhibits single-agent activity in tumors that are dependent on Bcl-2 or Bcl-xL for survival [14], we investigated Bcl-2 or Bcl-xL inhibition and its contribution to the effect of prexasertib combined treatment by using siRNA to selectively deplete Bcl-2 or Bcl-xL in the cells (Fig. 4a, b). Unexpectedly, Bcl-2 knockdown had no effect on apoptosis, and treatment with prexasertib did not result in apoptosis (Fig. 4c). However, knockdown of Bcl-xL increased the extent of apoptotic cell death, and knockdown of Bcl-xL with prexasertib treatment increased the extent of apoptotic cell death in a dose-dependent manner (Fig. 4d).

Nuclear morphological changes and molecular mechanism for knockdown of Bcl-xL and prexasertib treatment

Figure 5a shows the morphological changes of nuclei when Bcl-xL was knocked down and treated with prexasertib

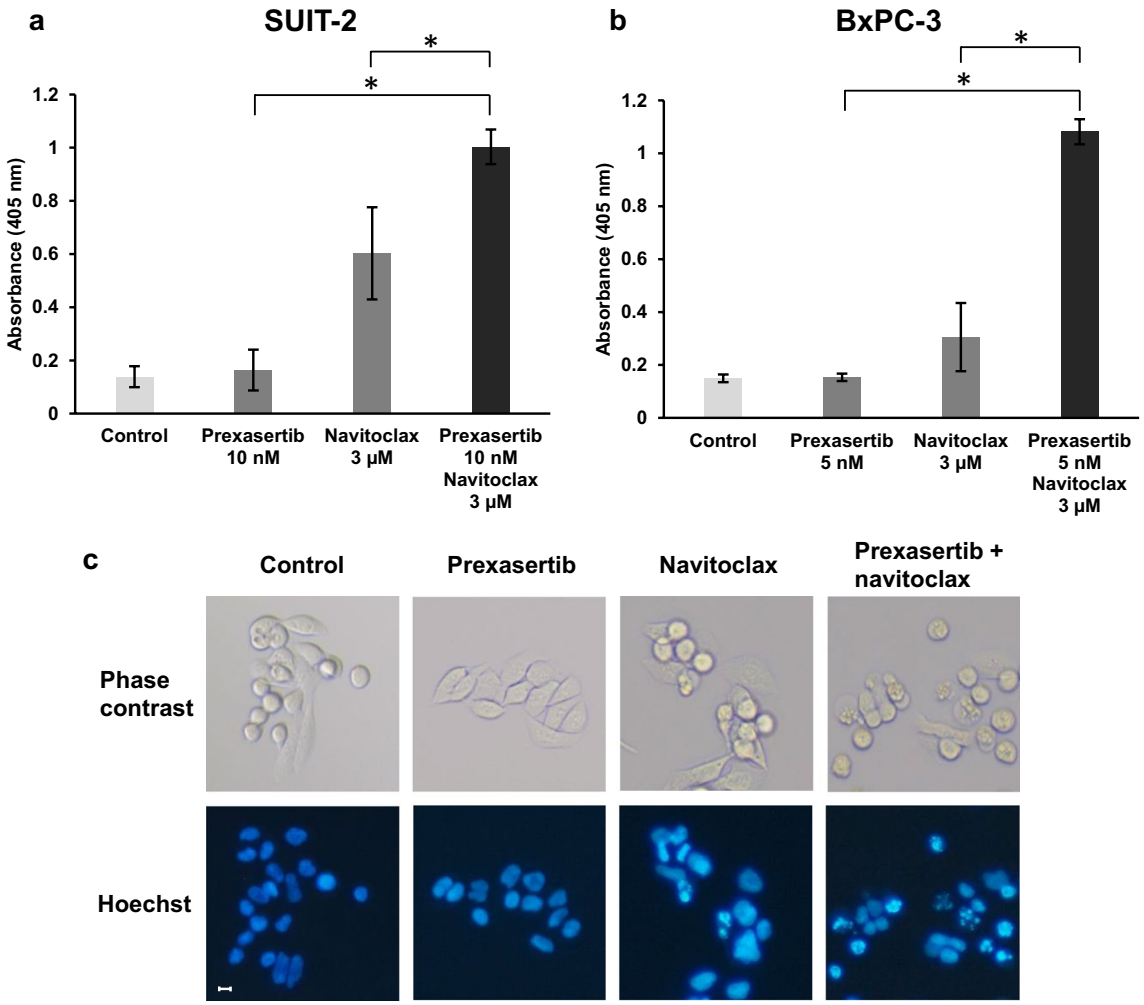

Fig. 2 Effect of the combination of prexasertib and navitoclax on apoptotic cell death. **a**, **b** SUIT-2 cells and BxPC-3 cells were treated with prexasertib and/or navitoclax for 24 h. Values shown are the mean ± SD. *$p < 0.05$ indicates a significant difference. **c** Analysis of morphological changes induced by prexasertib and navitoclax at 24 h in SUIT-2 cells. The cells were stained with Hoechst 33342 and the morphological changes were analyzed under a fluorescence microscope or assessed by phase contrast microscopy. The scale bar represents 10 μm

(30 nM) after 24 h. Untreated control cells showed no change in nuclear morphology. The cells treated with prexasertib and Bcl-xL knockdown developed apoptotic features including chromatin condensation and nuclear fragmentation. Figure 5b shows the detailed mechanism for prexasertib treatment and Bcl-xL knockdown. Knockdown of Bcl-xL using siRNA with prexasertib showed almost the same apoptotic signaling pathway to navitoclax combined with prexasertib. Expression of cleaved caspase 3 and cleaved PARP was increased in the cells treated with prexasertib and Bcl-xL knockdown. Cleaved Mcl-1 was increased, and this result was similar to the results for the combination of prexasertib, GEM, and S-1, which we previously reported [9]. Bcl-2 expression was most decreased in the prexasertib and Bcl-xL knockdown group. Prexasertib suppressed the phosphorylation of Chk1-S296. Expression of γH2AX was also induced to a higher degree by prexasertib and Bcl-xL knockdown.

Endogenous expression of Bcl-2, Bcl-xL, and Chk1 proteins in pancreatic cancer cell lines

We investigated whether differences in the effects of venetoclax, navitoclax, and prexasertib on each cell line were related to the endogenous expression levels of Bcl-2, Bcl-xL, and Chk1. HL-60 cells were detected as a control for Bcl-2 because venetoclax has been proven effective against this leukemia cell in clinical practice [12] and can be expected to have higher expression of Bcl-2 compared with other cancer cell lines. The results for the detection of Bcl-2, Bcl-xL, and Chk1 in each cell line are shown in Fig. 6a. Bcl-2 expression was significantly lower in the pancreatic cancer cell

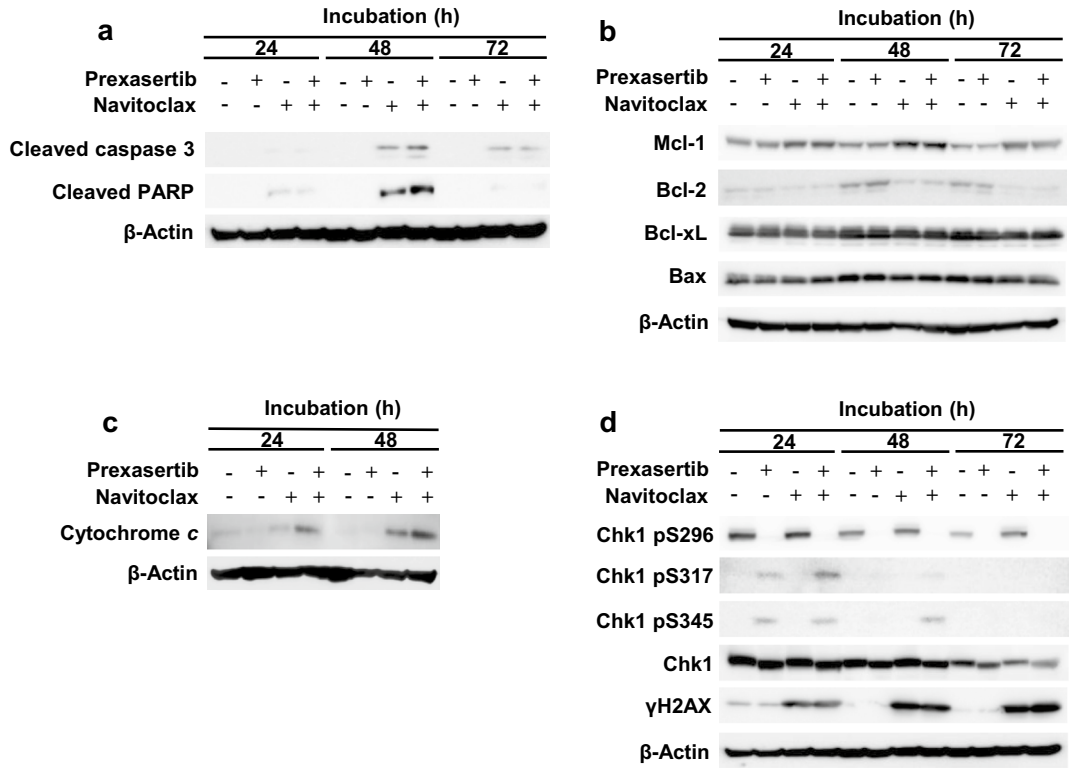

Fig. 3 Effect of prexasertib, navitoclax, and prexasertib + navitoclax on apoptosis and Chk1 in SUIT-2 cells. **a** Western blot analysis of the levels of cleaved caspase 3 and cleaved PARP. **b** Effect of prexasertib, navitoclax, and prexasertib and navitoclax on the Bcl-2 family. **c** Release of cytochrome *c* from mitochondria. SUIT-2 cells were treated for 24 and 48 h with prexasertib, navitoclax, or prexasertib and navitoclax. **d** Western blot analysis of the levels of Chk1 (pS296, pS317, pS345), Chk1, and γH2AX in SUIT-2 cells

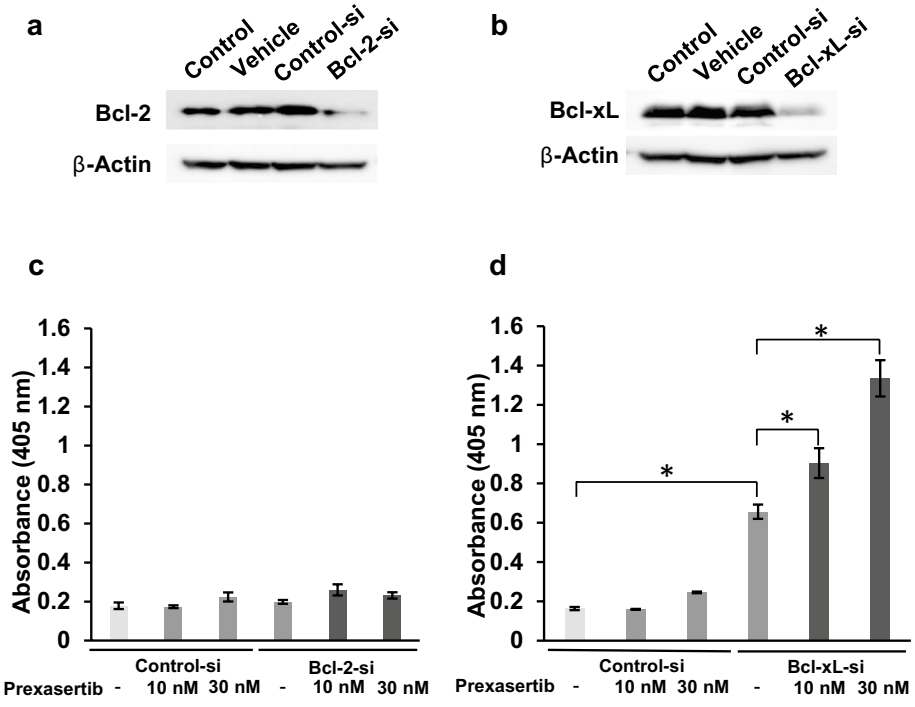

Fig. 4 Effect of prexasertib on apoptosis induction when Bcl-2/Bcl-xL is knocked down in SUIT-2 cells. Western blot analysis of the levels of expression of Bcl-2 or Bcl-xL and β-actin in SUIT-2 cells transfected with vehicle, non-silencing siRNA, and **a** Bcl-2 or **b** Bcl-xL specific siRNA. Apoptosis quantified by Cell Death ELISA in SUIT-2 cells treated with prexasertib for 24 h after transfection of non-silencing siRNA or **c** Bcl-2 or **d** Bcl-xL specific siRNA. Values shown are the mean ± SD. $^*p < 0.05$ indicates a significant difference

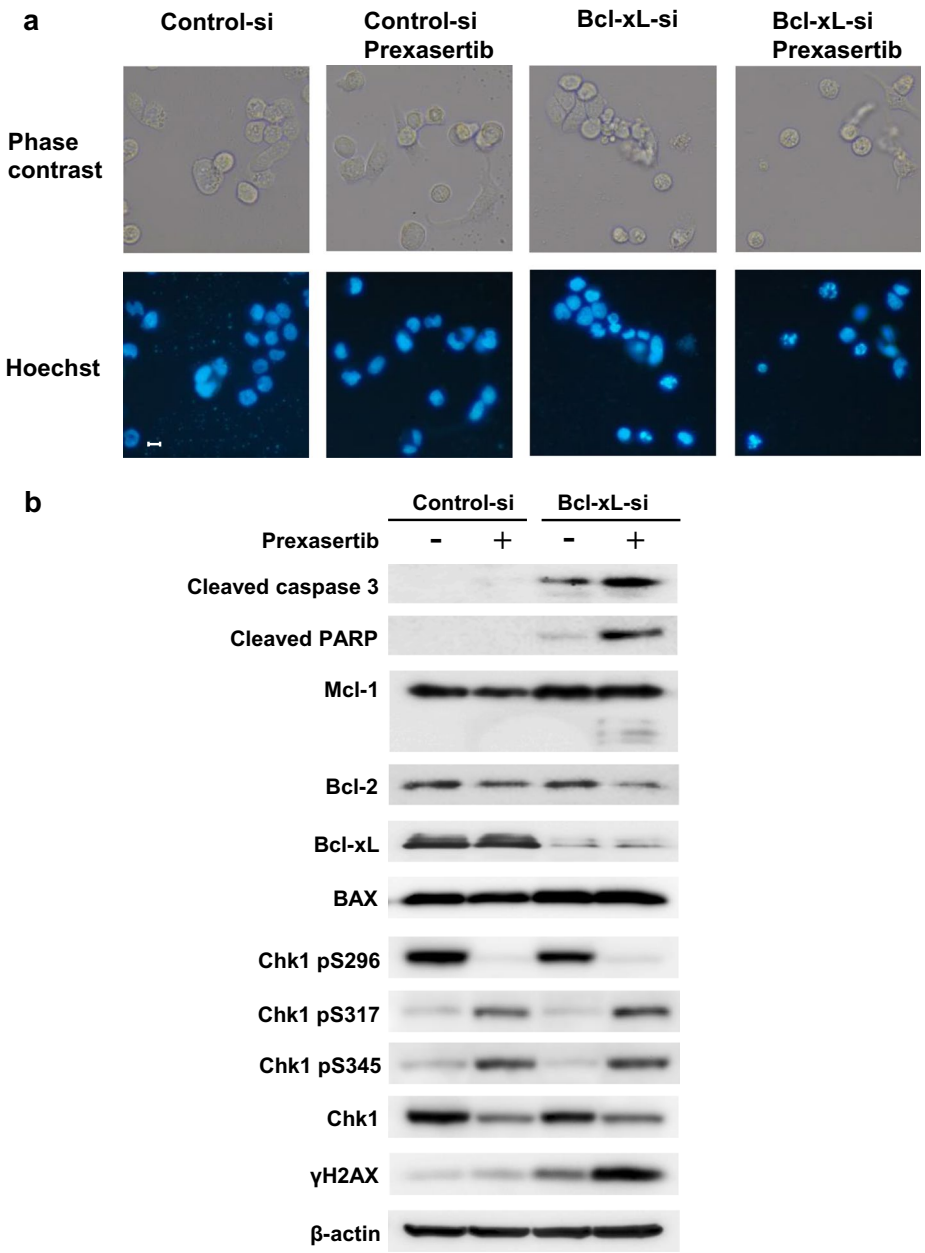

Fig. 5 Apoptosis induction by Bcl-xL knockdown using specific siRNA and prexasertib treatment in SUIT-2 cells. **a** Morphological changes analyzed by fluorescence microscopy or phase contrast microscopy in cells stained with Hoechst 33342. The scale bar represents 10 µm. **b** Western blot analysis of changes in the expression of apoptosis-related factors and Chk1 in cells treated with prexasertib for 24 h after transfection with non-silencing siRNA or Bcl-xL-specific siRNA

lines compared with HL-60 ($p < 0.05$) (Fig. 6b). However, the expression level of Bcl-xL was significantly higher in the pancreatic cancer cell lines than in HL-60 ($p < 0.05$) (Fig. 6c). MIA PaCa-2 showed lower expression of Chk1 compared with the other cell lines ($p < 0.05$) (Fig. 6d).

Discussion

In this study, we showed that combinations of prexasertib and navitoclax have a synergistic antiproliferative effect in pancreatic cancer cells. Furthermore, the synergistic antiproliferative effect was caused by inducing apoptotic cell death by inhibiting Bcl-xL but not Bcl-2.

Our previous studies showed that the combination of prexasertib, which is a Chk1 inhibitor, and GS had a strong antitumor effect and induced apoptosis in pancreatic cancer cells by downregulating Bcl-2 [9]. The Bcl-2 family regulates apoptosis and could be an attractive target for antitumor therapy [10]. We used Bcl-2 inhibitors venetoclax and navitoclax in combination with existing anticancer drugs to search for more effective treatments targeting Bcl-2. The effect of the single agent on the three pancreatic cancer cell lines was more sensitive for the non-selective Bcl-2 inhibitor navitoclax than for the selective Bcl-2 inhibitor venetoclax.

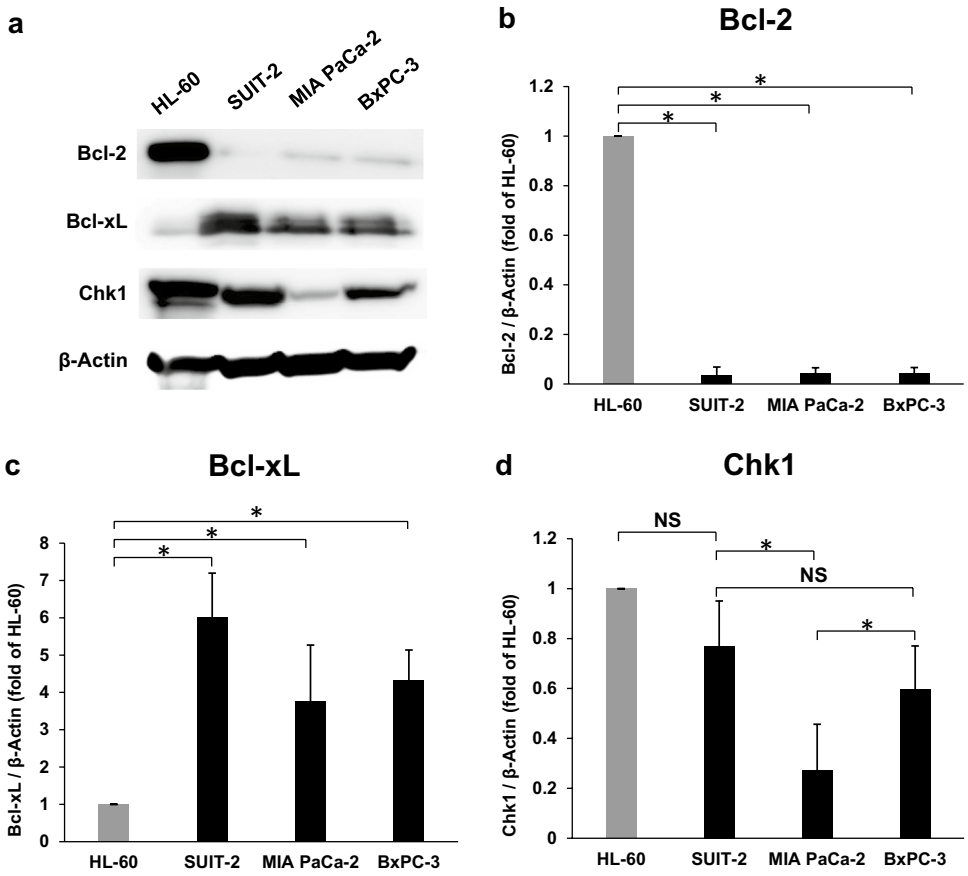

Fig. 6 Endogenous expression of Bcl-2, Bcl-xL, and Chk1 proteins. **a** Western blot analysis of the levels of Bcl-2, Bcl-xL, and Chk1 protein expression in HL-60, SUIT-2, MIA PaCa-2, and BxPC-3 cells. The relative band intensities of **b** Bcl-2, **c** Bcl-xL, **d** Chk1, and β-actin were quantified using densitometric analysis. Values are expressed as the mean ± SD of three independent experiments. Values shown are the mean ± SD. *$p < 0.05$ indicates a significant difference

Venetoclax has been reported to be more effective at higher Bcl-2 expression levels in vitro and in clinical trials [18, 19]. The intrinsic level of Bcl-2 is often not expressed in pancreatic cancer cell lines, and Bcl-xL and Mcl-1 are frequently expressed [20]. Our examination of the three pancreatic cancer cell lines and the leukemia cell line (HL-60) revealed that the pancreatic cancer cell lines also showed lower expression of Bcl-2 and higher expression of Bcl-xL compared with HL-60. Furthermore, analysis of data from the Cancer Genome Atlas and the Genotype Tissue Expression project showed that Bcl-2 levels of mRNA are lower in pancreatic cancer tissues than in normal pancreatic tissues, whereas Bcl-xL levels are higher in pancreatic cancer tissues than in normal pancreatic tissues [21]. In addition, the expression of Bcl-2 and Bcl-xL in human pancreatic cancer tissue is 23% and 90%, respectively [22]. Navitoclax is a non-selective Bcl-2 inhibitor, inhibiting Bcl-2, Bcl-xL, and Bcl-w [14]; therefore, navitoclax is effective against pancreatic cancer cell lines, and similar results have been reported [23].

DNA checkpoints are activated by DNA damage and they stop the cell cycle and catalyze DNA repair. Chk1 inhibitors are expected to selectively induce mitotic cell death in cancer cells [24]. Preclinical studies in solid tumor and blood cancer models have shown that prexasertib has antitumor activity as a single agent or in combination with other agents both in vitro and in vivo [25–29]. Prexasertib is undergoing multiple clinical trials and is being developed [30]. Results of phase I or phase II trials of prexasertib monotherapy or combination therapy have been reported, and their efficacy and tolerability have been verified in squamous cell carcinoma, ovarian cancer, and other advanced cancers [31–33]. However, there are no Chk1 inhibitors available clinically. Although the combination of prexasertib and navitoclax increased the phosphorylation of Chk1-S317 and Chk1-S345, which are DNA damage markers, prexasertib strongly suppressed the phosphorylation of Chk1-S296, which is the most important autophosphorylation site for the activation of Chk1 [24]. Our previous work also showed that prexasertib suppresses the autophosphorylation of Chk1-S296 [9].

The present study is the first to show that navitoclax enhances the antitumor effects of a Chk1 inhibitor. Furthermore, using specific siRNA also showed that the mechanism is due to Bcl-xL inhibition, not Bcl-2 inhibition. We also revealed that the increase in apoptosis was due to cytochrome *c* release into the cytoplasm and the caspase cascade. Simultaneous inhibition of Chk1 and Bcl-xL could be a new strategy for treating pancreatic cancer and other solid cancers. Venetoclax and the Chk1 inhibitor rabusertib (LY2603618) efficiently induce apoptosis in acute myeloid

leukemia cells [34]. Although the effects of drugs targeting the Bcl-2 family depend on the type of tumor, it is necessary to study new treatments targeting Chk1 inhibition and the Bcl-2 family, including Mcl-1.

Navitoclax has clinically significant side effects, which can lead to thrombocytopenia due to Bcl-xL inhibition [35, 36]. However, thrombocytopenia can be managed by decreasing the starting dose, and then confirming the platelet count and increasing the dose. Prexasertib also causes myelosuppression [32], and it is necessary to determine the dose and carefully manage the blood cell count when using navitoclax and prexasertib in combination. In addition, because the combination of the two drugs is expected to have a synergistic effect, it may be possible to decrease the dose of each drug.

The standard regimens currently used worldwide as chemotherapy for metastatic pancreatic cancer are FOLFIRINOX or GEM + nab-paclitaxel [37, 38]. However, these strong combination chemotherapy regimens are highly toxic and have limited efficacy; therefore, the development of more effective, safer new treatments is urgently required. It is also necessary to identify biomarkers to predict the effect of drug combinations; venetoclax is expected to be more effective when the expression of Bcl-2 is higher [19]. Similarly, gefitinib is more effective when there is a specific mutation in the EGFR gene [39]. Cancer chemotherapy requires personalization, and thus it will be necessary to be able to select and administer more effective treatments consisting of a Chk1 inhibitor and a Bcl-xL inhibitor by identifying biomarkers for predicting the effect. We have shown that the combined effect of Chk1 and Bcl-xL inhibitors is high in SUIT-2 and BxPC-3 cells, which have high expression levels of Bcl-xL and Chk1. In addition, the combined effect of Chk1 and Bcl-xL inhibitors was low in MIA PaCa-2, which has low expression levels of Chk1. These findings are expected to be useful in the field of personalized medicine. The expression levels of these biomarkers can be detected by immunohistological staining using biopsy or surgical specimens of tumors. Recently, companion diagnostics have been developed as in vitro diagnostic agents that clarify the genotype and expression level of target molecules and predict the treatment effect of chemotherapy. In the future, development of a minimally invasive method would be more useful for diagnosis based on these biomarkers, such as liquid biopsy.

Conclusions

The combination of prexasertib and navitoclax has a strong antitumor effect and induces apoptosis in pancreatic cancer cells by inhibiting the anti-apoptotic protein Bcl-xL. Simultaneous inhibition of Chk1 and Bcl-xL could be a new strategy for treating pancreatic cancer.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by YM. KT coordinated the experiments and the writing of the manuscript. OT supervised the research and experiments. KW, MH, and TH reviewed the data and coordinated the writing of the manuscript. YM coordinated the scientific work and the writing of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval No ethics approval/waivers were required.

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