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Differential Roles of PDK1 and Akt1 Expression and Phosphorylation in Breast Cancer Cell Resistance to Paclitaxel, Doxorubicin, and Gemcitabine

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Running title: Role of PDK1 Versus Akt1 in Breast Cancer Chemosensitivity

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ABBREVIATIONS: PI3K, phosphoinositide 3-kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; IGF-1, insulin-like growth factor-1; S241, serine 241 of PDK1; T308, threonine 308 of Akt1; S473, serine 473 of Akt1; siRNA, small interfering RNA oligonucleotides; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; GSK, glycogen synthase kinase 3; ELISA, enzyme-linked immunosorbent assay

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ABSTRACT

3-Phosphoinositide-dependent protein kinase-1 (PDK1) and Akt1 are two closely related components of the phosphatidylinositol-3 kinase (PI3K) pathway, which is aberrantly regulated in breast cancer. Despite the importance of PDK1, few studies have evaluated it as a potential target for cancer therapy compared to studies of Akt1. We hypothesized that PDK1 is a superior target in the PI3K pathway. To test this, we first used a mouse mammary cell line retrovirally infected to express human PDK1 or Akt1 for comparative studies of treatment with paclitaxel, doxorubicin, and gemcitabine. Overexpression of PDK1 or Akt1 conferred similar resistance to treatment with paclitaxel or doxorubicin when compared with control cells. However, the PDK1-overexpressing cells were more resistant to gemcitabine than were the Akt1-overexpressing cells. We next correlated the expression and activation-specific phosphorylation of PDK1 and Akt1 with the cytotoxic effects of the same agents in several human breast cancer cell lines. Cells with high levels of phosphorylated PDK1 were more resistant to gemcitabine-induced apoptosis than were cells expressing high levels of phosphorylated Akt1. To further validate this observation, we used small interfering RNA oligonucleotides to selectively knockdown PDK1 or Akt1 expression in MCF7 human breast cancer cells. We found that knockdown of PDK1 expression sensitized MCF7 cells to gemcitabine-induced apoptosis more effectively than did knockdown of Akt1 expression in the same cells. Our findings show that PDK1 may be a superior alternative to Akt1 as a target for sensitizing breast cancer cells to chemotherapeutic agents, particularly gemcitabine.

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Introduction

Phosphatidylinositol-3 kinase (PI3K) generates specific inositol lipids that play important roles in the regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal changes (Vivanco and Sawyers, 2002; Cantley, 2002). The aberrant PI3K pathway plays an important role in breast cancer tumorigenesis and breast cancer resistance to conventional chemotherapy (Fry, 2001; West *et al.*, 2002), and its abnormality may occur at several levels during its signal transduction in the tumors of breast cancer patients. For example, overexpression of human epidermal growth factor receptor-2 (HER2), which activates PI3K through heterodimerization with other members of the family, is found in 25-30% of breast cancers (Slamon *et al.*, 1987; Slamon *et al.*, 1989). The phosphatase and tensin homologue deleted on the chromosome ten (PTEN) tumor-suppressor gene, which counteracts the activity of PI3K, is frequently mutated or decreased at the protein level in advanced breast cancers (Bose *et al.*, 2002; Nagata *et al.*, 2004). The activity of proto-oncogene Akt, particularly Akt2, which is one of the major downstream substrates of PI3K-mediated cell signaling, is increased in approximately 40% of breast cancer tumors (Sun *et al.*, 2001). The current paradigm for targeting the PI3K pathway for breast cancer treatment includes agents directed to HER2, such as trastuzumab and pertuzumab, and to FRAP/mTOR (FKBP and rapamycin-associated protein/mammalian target of rapamycin kinase), such as temsirolimus/CCI-779. Several novel inhibitors for PI3K and Akt are in preclinical and early clinical phases of study.

3-Phosphoinositide-dependent protein kinase 1 (PDK1) is a 63-kDa serine/threonine kinase that serves as a master regulator of a group of protein kinases known as the AGC family (cAMP-dependent or cGMP-dependent protein kinases and protein kinase C) (Alessi *et al.*, 1997b; Alessi *et al.*, 1997a; Stokoe *et al.*, 1997; Stephens *et al.*, 1998; Mora *et al.*, 2004). PDK1

is highly expressed in the majority of established human cancer cell lines (Fry, 2001) and in the tissue specimens of breast cancer patients (Lin *et al.*, 2005). Phosphatidylinositol 3,4,5-triphosphate generated by PI3K at the plasma membrane directs membrane localization of PDK1 through its pleckstrin homology domain, resulting in the autophosphorylation of PDK1 at S241 within its activation loop, which is essential for PDK1 kinase activity (Casamayor *et al.*, 1999). PDK1 phosphorylates the activation loop of a number of protein serine/threonine kinases of the AGC kinase superfamily, including Akt, serum- and glucocorticoid-induced kinase, protein kinase C isoforms, and the p70 ribosomal S6 kinase (Stokoe *et al.*, 1997; Stephens *et al.*, 1998; Pullen *et al.*, 1998; Le Good *et al.*, 1998; Williams *et al.*, 2000). Experimental expression of PDK1 alone was sufficient to transform mouse mammary epithelial cells, a process believed to depend on PDK1-mediated activation of protein kinase Cα rather than activation of Akt1 (Zeng *et al.*, 2002; Xie *et al.*, 2003).

Despite its important role, few studies have evaluated PDK1 as a potential target for cancer therapy. One earlier study showed that inhibition of PDK1 with antisense oligonucleotides led to the inhibition of cell proliferation and induction of apoptosis in a glioblastoma cell line (U-87) (Flynn *et al.*, 2000). 7-Hydroxystaurosporine (UCN-01) has also been reported to inhibit PDK1 (Sato *et al.*, 2002), but at higher doses it also inhibits other kinases, such as Chk1 and Chk2 (Graves *et al.*, 2000; Yu *et al.*, 2002), making its activity less specific (Davies *et al.*, 2000). Recently, some progress has been made toward developing PDK1-specific inhibitors (Feldman *et al.*, 2005; Zhu *et al.*, 2004).

The purpose of this study was to evaluate a potential role of PDK1 and to compare it with that of Akt1 in conferring resistance to chemotherapy to breast cancer cells. We used mouse mammary cells retrovirally infected to express human PDK1 or Akt1 and compared them for the

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cytotoxic effects of treatment with paclitaxel, doxorubicin, and gemcitabine, which are used in clinics for treating breast cancer patients. We also correlated the expression and activation-specific phosphorylation levels of PDK1 and Akt1 with the cytotoxic responses of several human breast cancer cell lines treated with the same agents. Finally, we compared the effects of selective expression knockdown of PDK1 and Akt1 with small interfering RNA oligonucleotides (siRNA) on the sensitivity of breast cancer cells to treatment with these agents. Our results show that PDK1 could be an alternate or even superior target in the PI3K pathway for sensitizing breast cancer cells to chemotherapy.

Materials and Methods

Cell Lines and Culture. The COMMA-1D mouse mammary cells expressing PDK1 and Akt1 have been described previously (Zeng *et al.*, 2002). The human breast cancer cell lines (MDA453, BT474, SKBR3, MCF7, MDA157, MDA468, T47D, ZR75B, MDA435, and MDA231) were purchased from American Type Culture Collection (Manassas, VA). All cell lines were grown and maintained in Dulbecco's modified Eagle's medium or F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C.

Western Blot Analysis and Blotting Antibodies. Following various treatments described in the figure legends, the cells were harvested with a rubber scraper and washed twice with cold phosphate-buffered saline (PBS). Cell pellets were lysed and set on ice for at least 10 min with a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin. The lysates were then cleared by centrifugation, and the supernatants were collected. Equal

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amounts of lysate protein were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then Western blot analyses were performed with various specific primary antibodies. The primary antibodies directed against total Akt, S473-phosphorylated Akt1, T308-phosphorylated Akt1, total PDK1, S241-phosphorylated PDK1 and T202/Y204-phosphorylated p44/42 mitogen-activated protein kinase (MAPK) antibody were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-total MAPK antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Specific signals were visualized using the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

Colorimetric Assay for Cell Survival and Proliferation. The assays were conducted in 24-well culture plates containing 0.5 ml of medium/well. Following attachment of cells to the plates overnight, they were pulse treated for 6 h with various concentrations of paclitaxel, doxorubicin, and gemcitabine and then cultured in drug-free medium for an additional 72 h. Cell growth and survival were assessed by incubation of the cells for 2 h at 37°C in a CO_2 incubator with 50 μl/well of 10 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)] (Sigma-Aldrich, St. Louis, MO). The cells were then lysed with a lysis buffer (500 μl/well) containing 20% sodium dodecyl sulfate in dimethyl formamide/H_2O (1:1, v/v), pH 4.7, at 37°C for at least 6 h. The cell lysates were measured for optical absorbance at a wavelength of 595 nm. The viable cells (viability) were expressed as a percentage of the optical absorbance relative to the optical absorbance of the corresponding control or untreated cells.

Expression Knockdown of PDK1 and Akt1 with siRNA. The "Smart-pool" siRNA of PDK1 or Akt1 was purchased from Dharmacon, Inc. (Lafayette, CO.) and was transiently transfected into MCF7 breast cancer cells with the FuGENE-6 transfection reagent, following the instructions provided by the manufacturer (Roche Diagnostics Corp., Indianapolis, IN). A

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random siRNA sequence was used as a nonspecific control. Forty-eight hours after the transfection, the cells were harvested by scraping the cells from the plates and lysed with a buffer described. The efficacy of gene expression knockdown by the siRNA was determined by Western blot analysis with respective specific antibodies described in figure legends. The protein level of β-actin was used as a loading control.

Quantification of Apoptosis. An enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Corp.) that quantitatively measures the cytoplasmic levels of histone-associated DNA fragments (mononucleosomes and oligonucleosomes) was used as we previously reported (Liu *et al.*, 2000). This colorimetric enzyme immunoassay was performed exactly according to the manufacturer's instructions.

Nuclear Staining with Hoechst 33258. Untreated or treated cells were washed twice in PBS containing 0.1% bovine serum albumin and then resuspended to a concentration of 1×10^6 cells/ml in the PBS buffer. The cell suspension was then mixed with an equal volume of a solution containing 10 mM Hoechst dye, which can bind to AT-rich regions of the DNA and is excited by ultraviolet fluorescence light with a 465-nm wavelength. The mixture was incubated for 15 min at 37°C. The cell suspension was then fixed in 1% paraformaldehyde (dissolved in PBS) and analyzed under a fluorescence microscope. The percentage of apoptotic cells was determined by counting the nuclei with condensed and fragmented chromatin. Three different experiments were performed; at least 300 cells in different areas were analyzed per condition. Counting was performed in a blinded manner by two investigators.

Statistical Analysis. Values are provided as mean ± standard error of mean. Statistical analysis was performed using the unpaired Student's *t* test. Significant differences are indicated for p values < 0.01 (*).

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Results

Differential Cell Signaling and Chemosensitivity of Mouse Mammary Epithelial Cells (COMMA-1D) Expressing High Levels of PDK1 or Akt1. To evaluate whether PDK1, in comparison with Akt1, may be a novel target for sensitizing breast cancer cells to conventional chemotherapy, we used COMMA-1D mouse mammary cells retrovirally infected with either human PDK1 cDNA (C8000) or Akt1 cDNA (C8014) as an experimental model (Zeng *et al.*, 2002). We compared these cells with control vector-infected cells (CVCC) for cytotoxic effects of paclitaxel, doxorubicin, and gemcitabine.

We first explored the difference in cell signaling between these isogenic cells at basal levels when cultured in medium containing 0.5% fetal bovine serum and after stimulation with IGF-1 (Fig. 1). T308 and S241 are the respective phosphorylation sites located on the activation loops of Akt1 and PDK1 and are necessary for their kinase activities (Casamayor *et al.*, 1999). Overexpression of PDK1 alone in C8000 cells led to a constitutively higher level of PDK1 phosphorylation on S241 (lane 3 vs lane 1 of the PDK1-S241 blot), as the phosphorylation level was not further increased following IGF-1 treatment (lane 3 vs lane 4 of the same blot). In contrast, overexpression of Akt1 alone in serum-starved C8014 cells did not lead to a higher level of phosphorylation of Akt1 on T308 when compared with the control cells (lanes 5 vs 1 of the Akt1-T308 blot), but they did show a moderately higher level of T308 phosphorylation after IGF-1 stimulation (lanes 6 vs 2 of the same blot). The increase in PDK1 expression and phosphorylation alone in C8000 cells was not accompanied by a corresponding increase in the level of Akt1 phosphorylation on T308 (lane 3 of the Akt1-T308 blot), but stimulation of these cells with IGF-1 induced a much higher level of Akt1 phosphorylation on T308 than that in C8014 cells after the same stimulation (lanes 4 vs 6 of the same blot), despite that C8014 cells

express a higher level of Akt1. This result is consistent with previous findings that Akt1 phosphorylation on T308 by PDK1 not only depends on the expression level and activity of PDK1 but may also require adequate levels of phosphoinositols to bring Akt near PDK1 for phosphorylation and that the level of T308 phosphorylation is dependent more on the expression level of PDK1 than on that of the Akt1 protein itself (Nicholson and Anderson, 2002).

In contrast to the changes in the levels of Akt1 phosphorylation on T308 and PDK1 phosphorylation on S241 in the serum-starved control cells (CVCC), the level of Akt1 phosphorylation on S473 increased substantially after IGF-1 stimulation despite the low levels of Akt1 and PDK1 in these cells. Increased levels of S473 phosphorylation of Akt1 were also found in PDK1-overexpressing C8000 cells and Akt1-overexpressing C8014 cells. This observation suggests that the level of S473 phosphorylation of Akt1, compared to that of T308, was more similar to S241 of PDK1 and was more dependent on the expression level of the protein itself. A notable difference between the levels of S241 phosphorylation of PDK1 and S473 phosphorylation of Akt1 is, however, that only the latter was further increased by stimulation with IGF-1. The results indicated that increased levels of Akt1 phosphorylation on S473 may be found with either increased expression of PDK1 protein or Akt1 itself or cellular stimulation by IGF-1. Also, the results imply that phosphorylation of Akt1 on T308, which may occur without a concurrent increase in the level of phosphorylation of Akt1 on S473, is a better indicator of PDK1 activity, whereas phosphorylation of Akt1 on S473 may be elevated by multiple factors.

Stimulation of the cells with IGF-1 also increased the levels of phosphorylated MAPK and GSK3. The increase in the level of MAPK phosphorylation was most evident in the PDK1-overexpressing C8000 cells, whereas a moderately higher level of GSK3 phosphorylation was found in the PDK1- and Akt1-overexpressing C8014 cells after treatment with IGF-1. Of note,

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despite high level of phosphorylated Akt in C8000 cells upon IGF-1 stimulation, the MAPK (ERK1/2) was activated to a greater extent than the vector-transfected cells, suggesting that a potential inhibitory effect on Raf-1/B-Raf signaling by Akt was not operative in the setting of our experiments (Zimmermann and Moelling, 1999; Rommel *et al.*, 1999).

The results from these studies highlight the existence of differences in cell signal transduction among these isogenic cells following expression of transduced PDK1 and Akt1 cDNA, which is essential for comparing their respective effects on drug sensitivity. While the activity of Akt1 can be regulated independently of PDK1, any change in the activity of PDK1 may result in a change in overall Akt1 activity. Because the level of Akt1-S473 was minimally affected by expression of PDK1 compared with that of Akt1-T308 (Fig. 1), we used the levels of PDK1-Ser241 and Akt1-S473 to represent the respective activity levels of PDK1 and Akt1 in our studies.

We next assessed the cytotoxic responses of these cells to treatment with paclitaxel, doxorubicin, or gemcitabine (Fig. 2A). When measured by MTT assays, overexpression of PDK1 in C8000 cells and Akt1 in C8014 cells rendered an overall moderate resistance to treatment with paclitaxel (dose ranged from 0.001 to 1 μM), doxorubicin (from 0.001 to 1 μM), or gemcitabine (from 0.005 to 50 μM) when compared with treated control cells. A notable difference between the PDK1-overexpressing C8000 and Akt1-overexpressing C8014 cells was their response to treatment with gemcitabine. The C8000 cells were consistently more resistant to gemcitabine than were the C8014 cells. The increase in resistance to the treatments with paclitaxel, doxorubicin, and gemcitabine shown by the MTT assays was verified by two additional independent assays measuring the cytoplasmic levels of histone-associated DNA fragments (Fig. 2B) and the levels of nuclear fragmentation or condensation in the cells stained

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with Hoechst 33258 (H33258), a DNA-binding fluorescence dye (Fig. 2C). Compared with the results of control cells treated with paclitaxel (0.01 μM), doxorubicin (0.01 μM), or gemcitabine (5 μM), the levels of apoptosis induced in C8000 and C8014 cells were lower. The level of apoptosis of gemcitabine-treated C8000 cells was statistically significantly lower than that seen in CVCC or C8014 cells, suggesting that PDK1 expression conferred a higher resistance, particularly to gemcitabine treatment.

Expression and Phosphorylation of PDK1 and Akt1 in a Panel of Human Breast Cancer Cell Lines and Their Role in Chemosensitivity. We then attempted to correlate the levels of PDK1 and Akt1 expression and activation-specific phosphorylation in established human breast cancer cell lines with their cytotoxic responses to the same treatments. The expression and activation-specific phosphorylation levels of PDK1 and Akt1 varied greatly among the cell lines we used (Fig. 3). Some cell lines (MDA453, SKBR3, MCF7, T47D, MDA435, MDA231) expressed higher levels of PDK1 than others (BT474, MDA157, MDA468, ZR75B). In all cells except MDA231 cells, total PDK1 levels generally correlated with S241-phosphorylated PDK1 levels. In contrast, the levels of T308 phosphorylation of Akt1 did not seem to directly correlate with the protein levels themselves; furthermore, its levels were not always associated with PDK1 or S241-phosphorylated PDK1 levels. These variations were not surprising considering the heterogeneity in genetic background and differences in context of biochemical signaling activity in the individual cell lines. Compared with the other cell lines in the panel, MDA157 and MDA468 cells contained intermediate levels of S241-phosphorylated PDK1, but the levels of T308-phosphorylated Akt1 in the two cell lines were very high. On the other hand, MCF7 and T47D cells had high levels of S241-phosphorylated PDK1 but very low levels of T308-phosphorylated Akt1. Furthermore, the levels of Akt1 phosphorylation on T308

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and S473 did not seem to be coupled always. For example, the baseline levels of Akt1 phosphorylation on S473 in MCF7 and T47D cells were comparable to the levels in other cells despite the low baseline levels of Akt1 phosphorylation on T308. In contrast, BT474 and ZR75B cells had relatively high levels of Akt1 phosphorylation on T308, but the levels of Akt1 phosphorylation on S473 were lower than those in other cells having similar levels of T308-phosphorylated Akt1.

We examined the cytotoxic responses to treatment with doxorubicin, paclitaxel, and gemcitabine of the ten breast cancer cell lines in Figure 3. The dose dependent responses of the ten cell lines to treatment with paclitaxel or doxorubicin were largely the same, but a clear bifurcation occurred in cell lines with differential levels of PDK1 and Akt1 when they were treated with gemcitabine. Figure 4 shows the results for two breast cancer cell lines (MDA453 and SKBR3) that contain high levels of S241-phosphorylated PDK1 (and T308-phosphorylated Akt1) but low levels of S473-phosphorylated Akt1 (Fig. 3) and another two breast cancer cell lines (MDA231 and MDA468) that have high levels of S473-phosphorylated Akt1 but low levels of S241-phosphorylated PDK1. MDA453 and SKBR3 cells, which express relatively high levels of S241-phosphorylated PDK1, were more resistant to gemcitabine than were MDA468 and MDA231 cells, which express relatively low levels of S241-phosphorylated PDK1 but high levels of S473-phosphorylated Akt1. The median inhibitory concentration of gemcitabine was at least two log scales higher in the MDA453 and SKBR3 cells than in the MDA468 and MDA231 cells.

In some other cell lines with high levels of PDK1, such as T47D, MDA435 and MCF7 cells, we did not see a preferential resistance to gemcitabine treatment (data not shown). Thus, the observed relevance of high level of PDK1 expression with differential cell sensitivity to

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treatment with gemcitabine appears be cell type- and context-specific. To determine a definitive role of PDK1 expression level in differential resistance of cells to gemcitabine, we used siRNA to selectively knock down the expression of PDK1 and Akt1 and evaluated the sensitivity of the targeted cells to treatment with paclitaxel, doxorubicin, or gemcitabine (Fig. 5). We chose MCF7 cells as a model because they express high levels of both PDK1-S241 and Akt1-S473 (Fig. 4), which allowed us to compare the effects of PDK1 and Akt1 on drug sensitivity in cells with same genetic context. MCF7 cells are also technically better than other cell lines for transfection and undergo a high percentage of siRNA uptake. Reduction in the expression of siRNA was maximized after treatment of cells for 72 to 96 hours in MCF7 cells (Fig. 5A). We therefore used this experimental condition in the subsequent experiments. Reduction in the expression level of PDK1 and Akt1 by the siRNA was successful in either untreated cells or those treated with paclitaxel (0.01 μM), doxorubicin (0.01 μM), or gemcitabine (5 μM) (Fig. 5B). The expression knockdown of PDK1 was specific as evidenced by the unaffected level of Akt, and likewise, the expression knockdown of Akt1 was achieved without noticeably affecting the level of PDK1. The expression knockdown of PDK1 or Akt1 was accompanied by a reduced level of GSK3 phosphorylation, particularly of GSK3α, in the cells without affecting the total level of GSK3. To ascertain that the expression knockdown of PDK1 had no significant effect on either the total Akt level or the activated form of Akt, and *vice versa*, we also measured activation-specific phosphorylation of PDK1 and Akt1 following siRNA treatment. As expected, we found no such effect (Fig. 5C). This finding indicated that the knockdown of the targeted molecules was specific and successful.

We then examined to what extent the selective expression knockdown of PDK1 and Akt1 had on MCF7 cell sensitivity to treatment with paclitaxel, doxorubicin, and gemcitabine (Fig. 6).

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Compared with paclitaxel or doxorubicin, gemcitabine induced the least apoptosis in MCF7 cells when measured by an ELISA (Fig. 6A). Expression knockdown of PDK1 and Akt each slightly induced apoptosis in MCF7 cells as shown by higher basal levels in the cells without exposure to the chemotherapeutic agents. The expression knockdown of Akt and PDK1 moderately enhanced the induction of apoptosis by paclitaxel and doxorubicin (Fig. 6B and C). Notably, expression knockdown of PDK1 greatly enhanced the induction of apoptosis by gemcitabine, measured by both the apoptosis ELISA and nuclear staining of the cells with Hoechst 33258. Expression knockdown of Akt also enhanced the induction of apoptosis by the same dose of gemcitabine, but to a much lesser extent.

Discussion

In this study, we demonstrated the differential roles of PDK1 and Akt1 in conferring cellular resistance to paclitaxel, doxorubicin, and gemcitabine. Notably, compared with Akt1 expression, PDK1 expression, which is known to activate several AGC family members in addition to Akt, had a much greater effect on cellular resistance to gemcitabine. Our findings suggest that PDK1 might be a better target than Akt1 when used as a target for sensitizing cancer cells to gemcitabine treatment.

Because PDK1 acts upstream of Akt1 and activates a broader spectrum of signal molecules than Akt1, it is likely that the greater resistance to gemcitabine conferred by PDK1 is caused by signaling molecules within the network in addition to Akt1. One such candidate molecule is protein kinase Cα, which we have shown has elevated activity in PDK1-overexpressing cells but not in the Akt1-overexpressing cells (Zeng *et al.*, 2002). This speculation is also supported by a recent study showing that MCF7 and MDA231 breast cancer cell sensitivity to gemcitabine was markedly enhanced by treatment of the cells with protein

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kinase C modulator bryostatin 1 (Ali *et al.*, 2003).

Despite that PDK1 acts upstream of Akt1, increased PDK1 kinase activity does not necessarily equate to full activation of Akt1. PDK1 consists of an N-terminal kinase domain and a C-terminal pleckstrin homology domain, appears to exist in an active, autophosphorylated configuration, and is refractive to additional activation upon cell stimulation with agonists that activate PI3K, such as insulin (Alessi *et al.*, 1997a; Pullen *et al.*, 1998; Casamayor *et al.*, 1999). Among the five identified sites that are phosphorylated, S241 within its activation loop is essential for PDK1 activity (Casamayor *et al.*, 1999). Because of its constitutively active status, PDK1 regulates its substrates in a manner dependent on whether the substrates, rather than PDK1 itself, are converted to a form that can be phosphorylated (Alessi, 2001). In contrast, Akt1 contains an N-terminal pleckstrin homology domain, followed by a central kinase catalytic domain and a C-terminal tail. Activation of Akt1 is a multistep process involving both membrane binding via its pleckstrin homology domain and phosphorylation. At least two residues of Akt1 are rapidly phosphorylated, including T308 and S473 (Alessi *et al.*, 1996). Full activation of Akt requires phosphorylation of the molecule on both sites (Scheid *et al.*, 2002) and a few additional sites including two autophosphorylation sites (T72/S246) of the molecule (Li *et al.*, 2006). T308 lies in the kinase activation loop and is phosphorylated by PDK1 (Alessi *et al.*, 1997b; Stephens *et al.*, 1998), whereas a *bona fide* S473 kinase remains unknown. Some candidate kinases have been suggested, including MAPK-activated protein kinase 2 (Alessi *et al.*, 1996; Rane *et al.*, 2001), integrin-linked kinase (Delcommenne *et al.*, 1998), DNA-dependent protein kinase (Feng *et al.*, 2004), and rictor-mammalian target of rapamycin complex (Sarbassov *et al.*, 2005). Depending on the circumstances, the phosphorylation on these two sites is sometimes separable. For example, IGF-1 failed to induce T308 phosphorylation of Akt1 in PDK1-/- embryonic stem

cells but still phosphorylated Akt1 on S473 in a manner sensitive to the inhibition of PI3K by LY292004 (Williams *et al.*, 2000). In contrast, staurosporine inhibits Akt1 phosphorylation at T308 but not at S473 (Andjelkovic *et al.*, 1999; Hill *et al.*, 2001).

As mentioned, a potential role of PDK1 in protecting cells from chemotherapy-induced apoptosis is much less well documented in the literature than is Akt1 (Knuefermann *et al.*, 2003). Our data showed that overexpression of PDK1 conferred resistance to chemotherapy similar to that conferred by overexpression of Akt1. In particular, the PDK1-overexpressing cells appeared to be more resistant to gemcitabine than the Akt1-overexpressing cells. The mechanism of this difference when compared with the results for paclitaxel or doxorubicin is still not known. Paclitaxel, doxorubicin, and gemcitabine are three distinct chemotherapeutic agents with distinct mechanisms of action for cytotoxicity. Both paclitaxel and doxorubicin are important components of the current breast cancer chemotherapy regimen, whereas gemcitabine is still being evaluated for treatment of breast cancer patients (Blackstein *et al.*, 2002). Our findings may have important implications for the clinical application of gemcitabine for treating patients with breast cancer. Compared with paclitaxel or doxorubicin, gemcitabine is generally less effective for inducing apoptosis in breast cancer cells. However, our data suggest that its relatively weak apoptosis-inducing activity may be remarkably enhanced by concurrent inhibition of PDK1 in breast cancer cells.

Based on our findings, we conclude that PDK1 may be a preferred molecular target for sensitizing breast cancer cells to chemotherapeutic agents, particularly to gemcitabine. Together with our earlier result that overexpression of PDK1 enhances tumorigenesis (Zeng *et al.*, 2002; Xie *et al.*, 2003), our current findings strongly justify the development of PDK1-specific inhibitors to treat human cancers.

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Footnotes

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Figure legends

Fig. 1. Comparison of phosphorylated protein levels in COMMA-1D mouse mammary epithelial cells retrovirally infected for expressing high levels of PDK1 or Akt1. Control vector-infected cells (CVCC), PDK1 expression vector cells (C8000), and Akt1 expression vector cells (C8014) grown to subconfluence were incubated in serum-free medium overnight. The next day, cells were treated with either vehicle or 10 nM IGF-1 for 30 min at 37ºC. The cells were then scraped off the culture plates with a rubber policeman, lysed, and prepared for Western blot analysis using specific antibodies as indicated. Equal loading of protein samples in each lane was confirmed by incubating the blots with anti-β-action antibody. Protein levels were detected by enhanced chemiluminescence detection.

Fig. 2. Differential responses of PDK1- and Akt1-overexpressing COMMA-1D cells to paclitaxel-, doxorubicin-, and gemcitabine-induced cytotoxicity. A, The control vector-infected cells (CVCC), PDK1 expression vector cells (C8000), and Akt1 expression vector cells (C8014) were subjected to a 6-h pulse treatment with the indicated concentrations of paclitaxel, doxorubicin, or gemcitabine in culture medium containing 0.5% fetal bovine serum. After treatment, the cells were cultured for an additional 3 days with drug-free medium containing 10% fetal bovine serum. Cell viability was then assessed by an MTT assay as described in Materials and Methods. Cell viability was calculated by normalizing the optical absorbance of cell lysates at a wavelength of 595 nm with the value of the corresponding untreated cells plus or minus standard deviation (SD). Some SDs are smaller than the symbol size. B and C, Cells were treated for 6 h in culture medium containing 0.5% fetal bovine serum and vehicle only, 0.01 μM paclitaxel, 0.01 μM doxorubicin, or 5 μM gemcitabine. The untreated or treated cells were then

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cultured for an additional 24 h with drug-free medium containing 10% fetal bovine serum. B, The cells were harvested and lysed for quantitative measurement of apoptosis by ELISA as described in Materials and Methods. C, The cells were harvested and stained with the Hoechst 33258 dye, and cells with fragmented or condensed nuclei were recorded under a fluorescent microscope and expressed as a percentage of the number of total nuclei. * $p < 0.01$ when compared with same treatment in control cells (CVCC) or as indicated.

Fig. 3. Levels of expression and phosphorylation of PDK1 and Akt1 in a panel of established human breast cancer cell lines. A total of 10 breast cancer cell lines maintained in culture medium supplemented with 10% fetal bovine serum were harvested by scraping, and then lysed. Equal amounts of cell lysates were subjected to Western blot analysis using specific antibodies as indicated, and protein levels were detected by enhanced chemiluminescence detection.

Fig. 4. Differential responses to paclitaxel-, doxorubicin-, and gemcitabine-induced cytotoxicity of breast cancer cells. MDA453, SKBR3, MDA468, and MDA231 cells were treated for 6 h with the indicated concentrations of paclitaxel, doxorubicin, or gemcitabine in culture medium containing 0.5% fetal bovine serum. After treatments, the cells were cultured for an additional 3 days in drug-free medium supplemented with 10% fetal bovine serum. Cell viability was then assessed by an MTT assay as described in the Materials and Methods. Cell viability was calculated by normalizing the optical absorbance of cell lysates at a wavelength of 595 nm with the value of the corresponding untreated cells plus or minus standard deviation (SD). Some SDs are smaller than the symbol size.

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Fig. 5. Expression knockdown of PDK1 or Akt1 by siRNA. MCF7 cells were transiently transfected with the "Smart-pool" siRNA of PDK1 or Akt1 with a protocol provided by the vendor (Dharmacon). A random siRNA sequence was used for nonspecific control of the experiments. A, Cells were exposed to a transfection medium containing a Smart-pool siRNA of PDK1 for a 24-h period followed by additional incubation as indicated. Cells were harvested every 24 h after the siRNA transfection period. Samples at each time point were collected and prepared for Western blot analysis with a PDK1-specific antibody. B, MCF7 cells were exposed for 24 h in transfection medium containing a Smart-pool siRNA of PDK1 or Akt1 or control siRNA. Cells were then incubated for 6 h in fresh medium containing vehicle, 0.01 μM paclitaxel, 0.01 μM doxorubicin, or 5 μM gemcitabine. After the treatment, cells were kept in drug-free medium for an additional 24 h before harvest and preparation for Western blot analysis with antibodies directed against PDK1, Akt1, total GSK3, and phosphorylated GSK3. Equal protein loading of samples was verified by the level of β-actin. C, MCF7 cells were treated with PDK1 and Akt1 siRNA as described for panel B. Cell lysates were assayed by Western blot analysis for the levels of activation-specific phosphorylation of PDK1 after Akt siRNA treatment and *vice versa*.

Fig. 6 Effects of expression knockdown of PDK1 or Akt1 by siRNA on the cellular responses to treatment with paclitaxel, doxorubicin, or gemcitabine. A, MCF7 cells were pulse treated with the indicated concentrations of paclitaxel, doxorubicin, or gemcitabine for 6 h in culture medium containing 0.5% fetal bovine serum. After the treatment, cells were kept in a drug-free medium for an additional 24 h before harvesting in preparation for measurement of apoptosis by ELISA. B and C, MCF7 cells were subjected to transfection with the "Smart-pool" siRNA of PDK1 or

Akt1 or a control siRNA and subsequent treatment with the same agents as described in Fig 5. B, Cells were then harvested and lysed, and lysate samples with equal amounts of protein for each cell line were analyzed by quantitative ELISA for apoptosis as described in Materials and Methods. C. Separate dishes of the cells were harvested and stained with the Hoechst 33258 dye. Cells with fragmented or condensed nuclei were recorded under a fluorescent microscope and expressed as a percentage of the number of total nuclei. $*p < 0.01$ when compared with treatment of the cells with siRNA or as indicated.

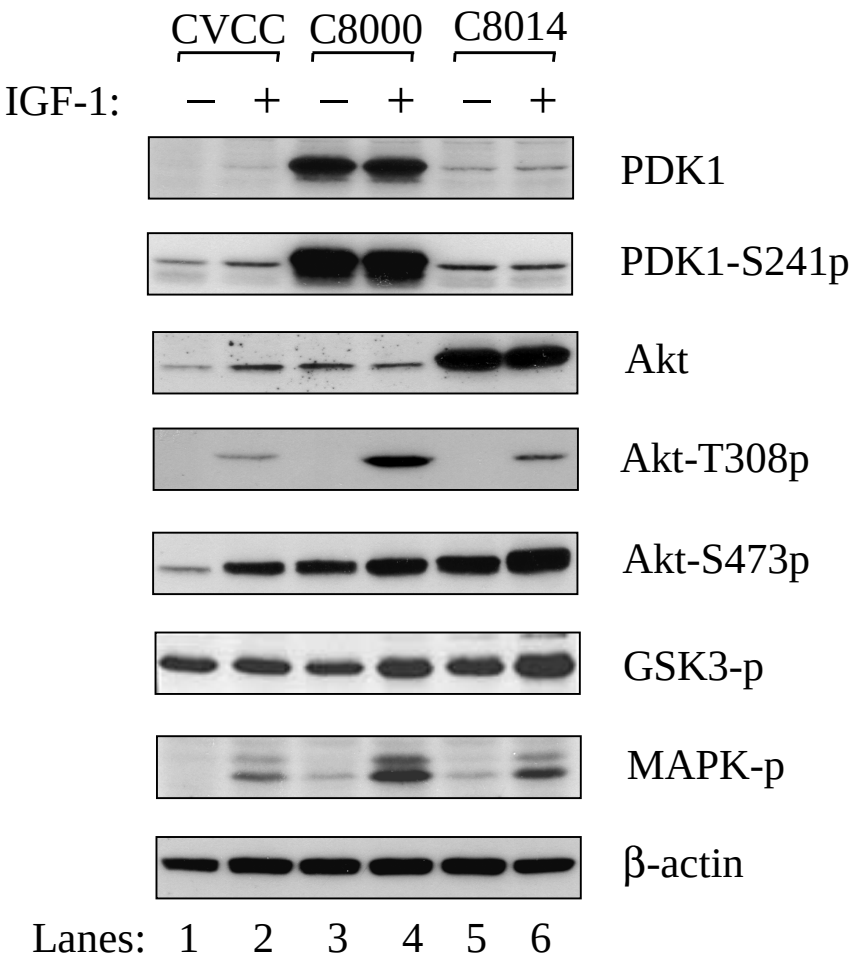

Figure 1

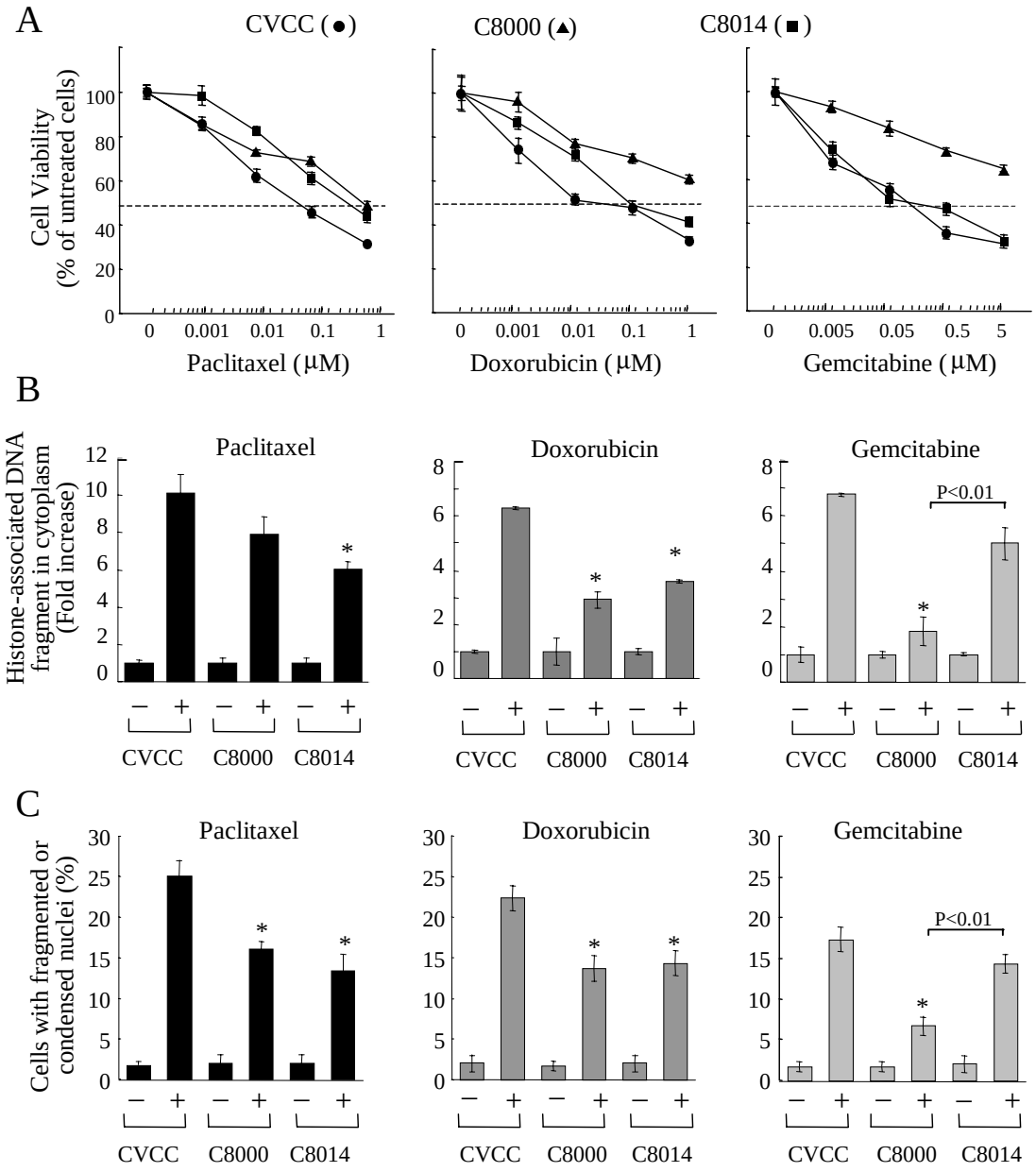

Figure 2

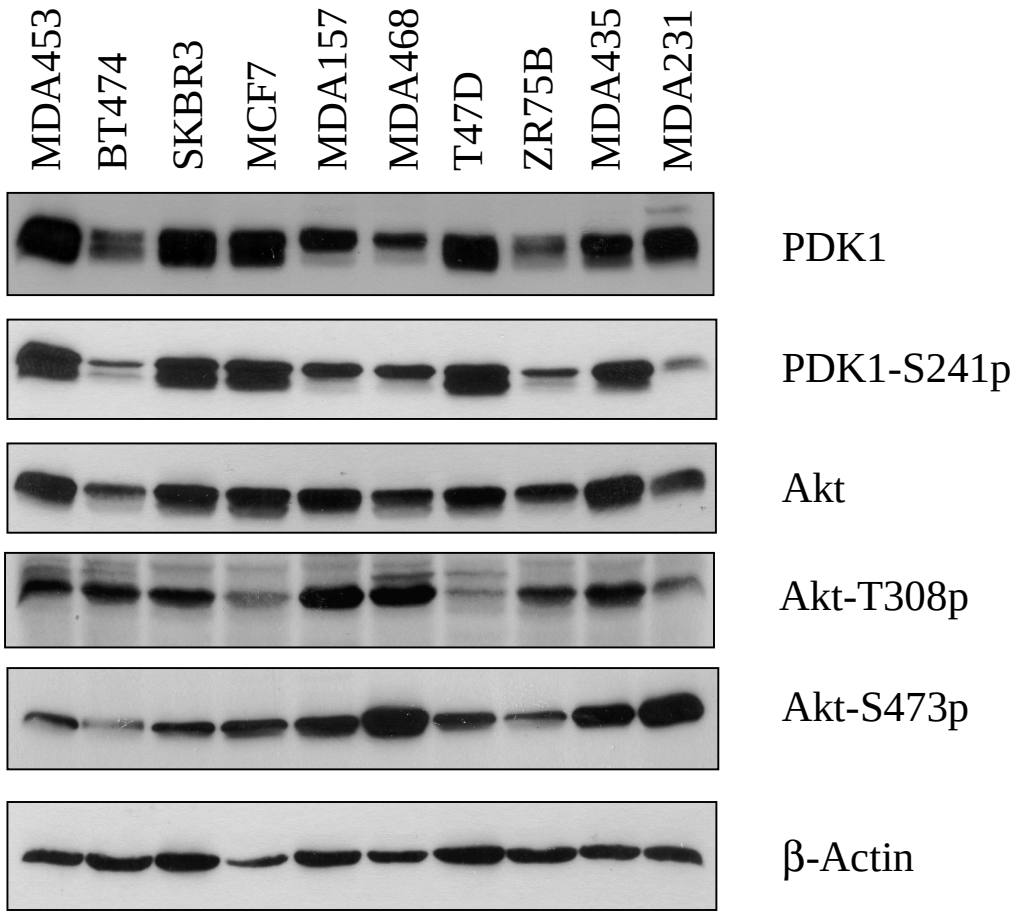

Figure 3

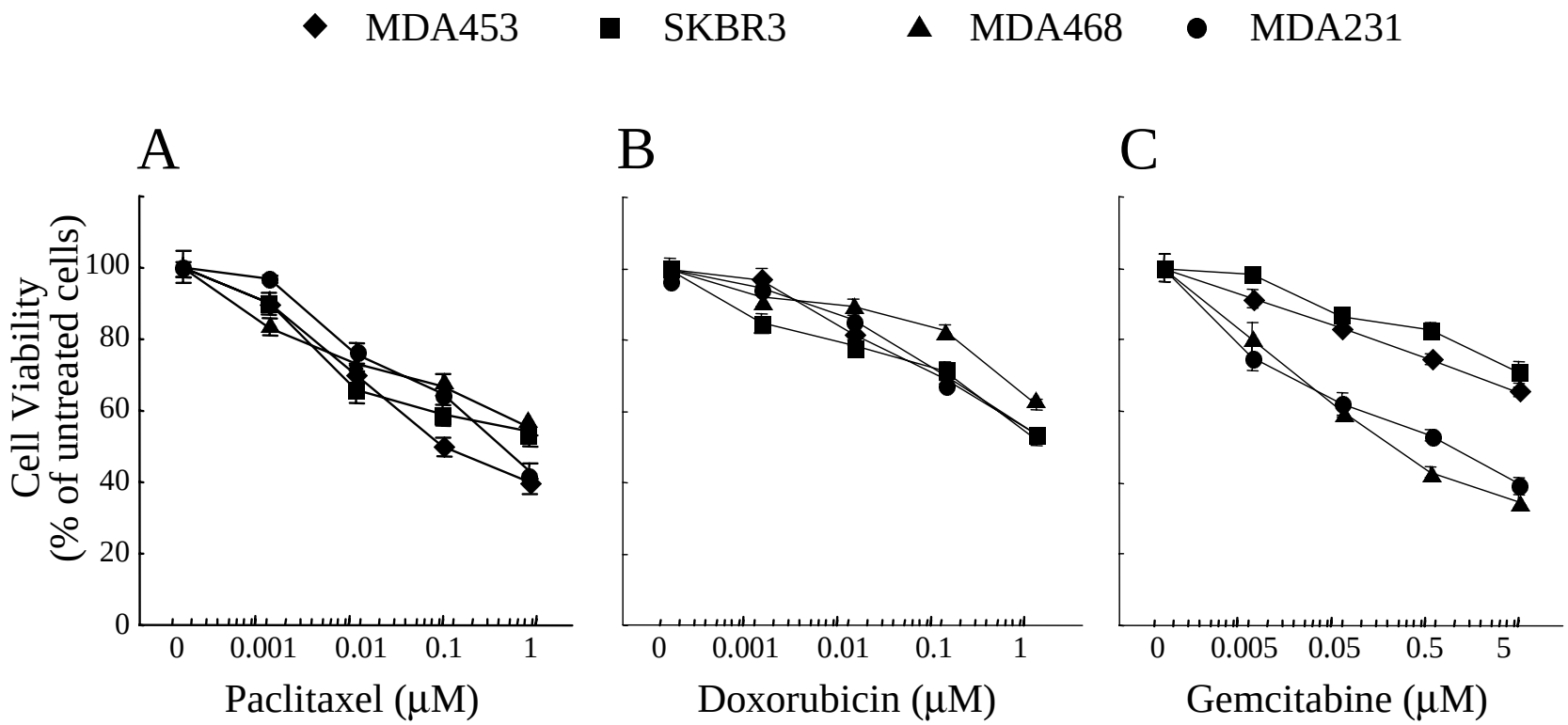

Figure 4

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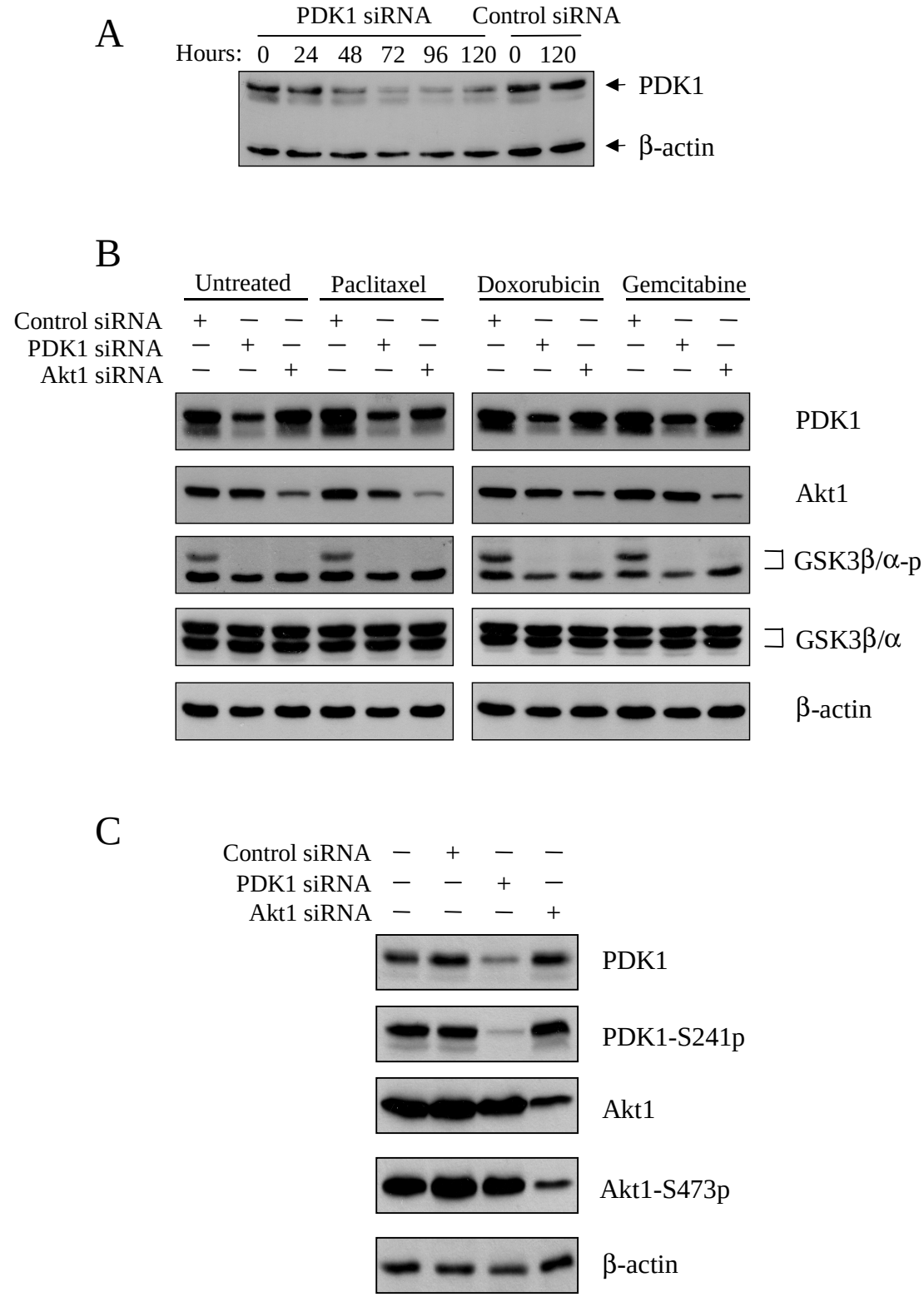

Figure 5

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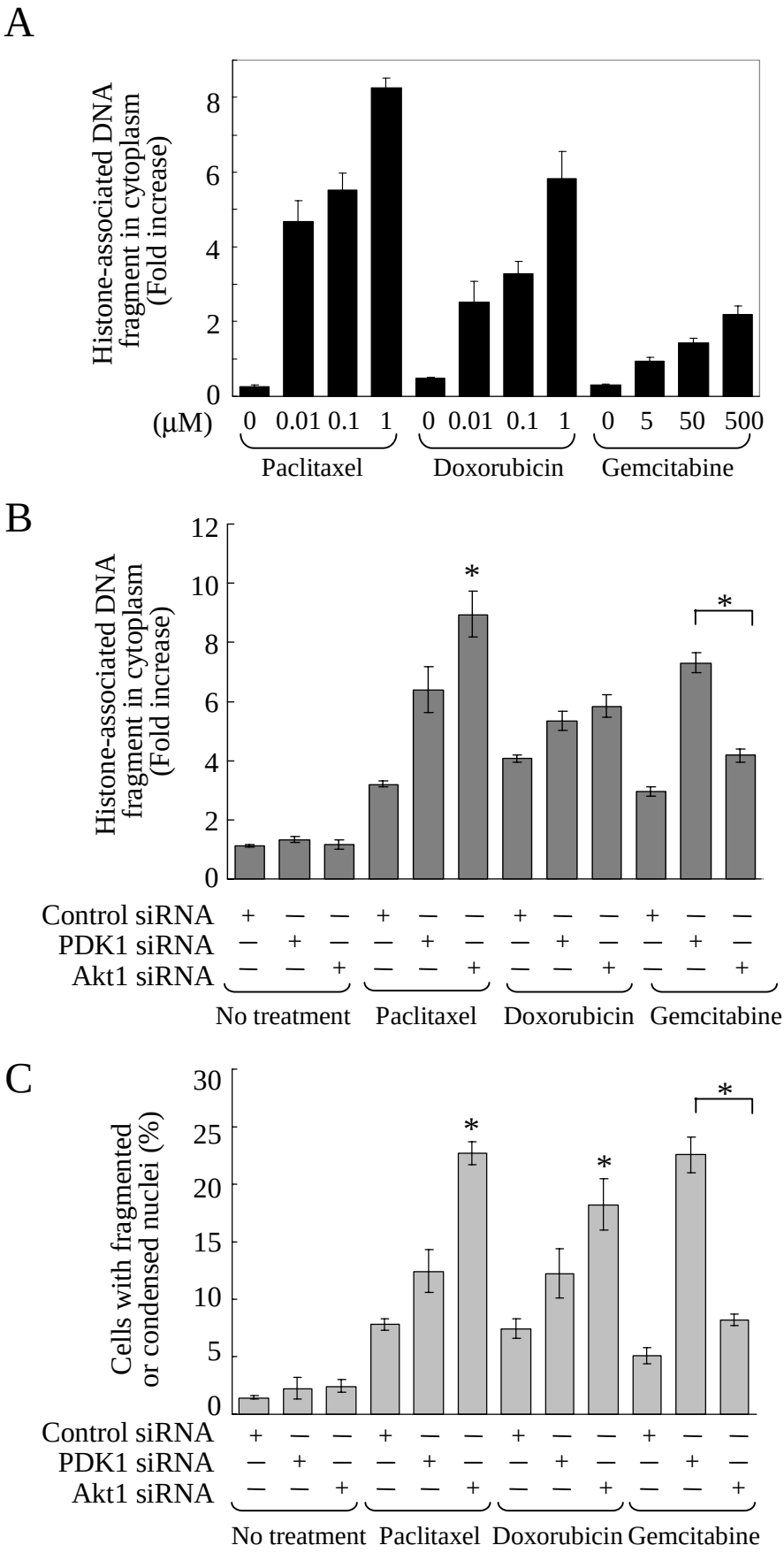

Figure 6