Sensitivity of doxorubicin-resistant cells to sorafenib: Possible role for inhibition of eukaryotic initiation factor-2α phosphorylation

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Abstract. Patients with advanced cancer including breast cancer, hepatocellular cancer and urothelial cancer frequently receive a chemotherapy regimen containing doxorubicin. However, doxorubicin-resistance is a major obstacle for cancer chemotherapy. Recently, several molecular-targeted agents have become available. Sorafenib (BAY 43-9006) is known to target multiple kinases and has demonstrated activity in renal cell and hepatocellular cancer. In this study, sorafenib was found to inhibit phosphorylation of the eukaryotic initiation factor-2α (eIF2α), induce cell cycle arrest at G2 phase and increase cellular apoptosis in doxorubicin-resistant human urothelial cell lines. An eIF2α kinase, PERK was responsible for eIF2α phosphorylation and PERK knockdown induced cellular apoptosis similar to sorafenib treatment in doxorubicin-resistant cancer cells. Furthermore, sorafenib sensitized doxorubicin-resistant cancer cells, but not their parental cells to oxidative stress exerted by both hydrogen peroxide and doxorubicin. In addition, PERK knockdown sensitized doxorubicin-resistant cancer cells to oxidative stress. In conclusion, PERK inhibition using sorafenib with or without doxorubicin might be a promising therapeutic approach for doxorubicin-resistant cancers retaining high phosphorylation levels of eIF2α.

Introduction

The majority of cancer patients with advanced stage disease receive chemotherapy. Although anticancer agents decrease patient tumor burdens and expand life expectancy, non-responsiveness or development of chemotherapy resistance are major obstacles for effective cancer treatment. We have previously investigated mechanisms of resistance to anti-cancer drugs including cisplatin, vincristine, etoposide and doxorubicin (1-11). Doxorubicin has been administered for many types of solid tumors including breast cancer, hepato-cellular cancer and urothelial cancer. Several molecules that are associated with the acquisition of doxorubicin resistance have been identified, including detoxifying enzymes, drug-efflux pumps and apoptosis-related genes (12,13). We have previously established doxorubicin-resistant cancer cells and shown that these cells expressed an increased multidrug-resistance 1 (MDR1) and MDR-related protein (MRP) mRNA and a decreased level of topoisomerase II mRNA (4). In addition, using 47 clinical samples of bladder cancer, we confirmed the significant correlation of MDR1, MRP1 and MRP3 mRNA levels with resistance to doxorubicin, and showed that the expression of MDR1, MRP1, MRP2 and MRP3 in recurrent tumors and residual tumors after chemo-therapeutic treatment was higher than that in untreated primary tumors (6). However, overcoming doxorubicin resistance remains unresolved.

Sorafenib (BAY 43-9006) is a novel bi-aryl urea that has been previously shown to inhibit Raf-1 and tumor cell line proliferation and tumor xenograft models (14,15). In addition to Raf-1 inhibition, sorafenib is a multikinase inhibitor, which targets vascular endothelial growth factor receptor (VEGFR) 2 and 3; platelet-derived growth factor receptor-ß (PDGFR-ß); FMS-like tyrosine kinase 3 (FLT-3); c-KIT; and RET-receptor tyrosine kinase (16,17). The clinical benefit of sorafenib was found in a phase II randomized trial, which indicated a lengthened progression-free survival with sorafenib compared with placebo in patients with metastatic renal cell cancer (RCC) (18). In addition, a subsequent phase III rando-mized controlled trial [treatment approaches in renal cancer global evaluation trial (TARGET)] confirmed the findings of the phase II trial (19). We have previously conducted phase II study to investigate the efficacy, safety, and pharmacokinetics of sorafenib in Japanese patients with advanced renal cell carcinoma, and revealed that sorafenib exerted encouraging efficacy and was well tolerated in Japanese patients with metastatic RCC (20).

Phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF2α) plays an essential role in regulating protein synthesis in response to a diverse range of environmental

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stresses (21-23). Four eIF2α kinases have been identified in mammals, each responding to different stresses through their unique regulatory regions. For example, phosphorylation of eIF2α by PERK (PKR-like ER kinase; also known as EIF2AK3) is induced by accumulation of malfolded proteins in the endoplasmic reticulum (ER) (24-26). Phosphorylation of eIF2α during ER stress inhibits global translation by lessening eIF2-GTP (21-23). Despite an overall reduction in protein synthesis, eIF2α phosphorylation increases the preferential translation of ATF4 mRNA, encoding a basic zipper transcription activator that is important for directing the expression of genes involved in metabolism, the redox status of cells, apoptosis and drug resistance (27-30). Decreased protein synthesis conserves energy and provides sufficient time for ATF4 and other stress-responsive transcription factors, to reconfigure gene expression that would block or ameliorate damage elicited by the underlying stress. Other members of the eIF2α kinase family include GCN2 (EIF2AK4), whose activity is enhanced by amino acid depletion, UV irradiation or proteasome inhibition; PKR (EIF2AK2), which function in an antiviral pathway; and HRI (EIF2AK1), which is regulated by heme deficiency and oxidative stress (22,23,26,31). Aberrations in these eIF2α kinase pathways are associated with a number of diseases, including diabetes, viral infection, anemia, neurological disorders and various cancers.

In this study, we investigated whether multikinase inhibitor, sorafenib could suppress the growth of doxorubicin-resistant cancer cells. Then, we attempted to elucidate a mechanism by which sorafenib inhibited the growth of doxorubicin-resistant cancer cells. Furthermore, we investigated the effects of sorafenib administration and PERK inhibition combined with hydrogen peroxide and doxorubicin.

Materials and methods

Cell culture and chemical. Human urothelial carcinoma KK47 cells, T24 cells and their doxorubicin-resistant cells were cultured in Eagle's minimal essential medium. Doxorubicin-resistant KK47/ADR and T24/ADR were established and maintained as described previously (4). Media were purchased from Invitrogen (San Diego, CA, USA) and contained 10% fetal bovine serum. Cell lines were maintained in a 5% CO_2 atmo-sphere at 37˚C. Sorafenib was obtained from Bayer Pharma-ceuticals Corporation (West Haven, CT, USA). For assay, stock solution was prepared in dimethyl sulfoxide (DMSO) (10 mM), and stored at -20˚C until use. Stock solution was diluted to appropriate concentration in culture medium before addition to the cells.

Antibodies. Antibodies against GCN2 (no. 3302), p-eIF2α (no. 9721), eIF2α (no. 2103) and p-ERK1/2 (no. 9106) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against ERK1 (sc-94), PARP (sc-1561) and PERK (sc-13073) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PKR (no. 1511-1) antibody was purchased from Epitomics (Burlingame, CA, USA). Anti-lamin B1 and anti-ß-actin antibodies were purchased from Sigma, USA. Anti-cleaved-PARP antibody was purchased from Promega (Madison, WI, USA).

Knockdown analysis using siRNAs. Knockdown analysis using siRNAs was performed as described previously (10,11,30,31). Briefly, the following double-stranded RNA oligonucleotides were commercially generated: 5'-GGUA AAAAGCAGUGGGAUUTT-3' (sense) and 5'-AAUCCCAC UGCUUUUUACCTT-3' (antisense) for PERK no. 1 (B-Bridge International, Mountain View, CA, USA); 5'-GGGAAAAGG UAAUGCGAGATT-3' (sense) and 5'-UCUCGCAUUAC CUUUUCCCTT-3' (antisense) for PERK no. 2 (B-Bridge International); 5'-CCACAUGAUAGGAGGUUUAUT-3' (sense) and 5'-UAAACCUCCUAUCAUGUGGTT-3' (antisense) for PKR (B-Bridge International); 5'-AUUAGAU GCACUCAGGACCUUAUGC-3' (sense) and 5'-GCAUA AGGUCCUGAGUGCAUCUAAU-3' (antisense) for GCN2 (Invitrogen). T24, K47 and their doxorubicin-resistant cells were transfected with 40 nM of the indicated siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Cytotoxicity analysis. Cytotoxicity analysis was performed as described previously (10,31). Briefly, T24, KK47 and their doxorubicin-resistant cells (2.5×10^3) applied to 0.5 μM of sorafenib or transfected with 40 nM of the indicated siRNA were seeded into 96-well plates. The following day, the indicated concentrations of hydrogen peroxide or doxorubicin and/or N-acetyl-L-cysteine (NAC) were applied. After 48 h, surviving cells were stained with alamarBlue Assay (TREK Diagnostic systems, Cleaveland, OH, USA) for 180 min at 37˚C. The absorbance was then measured using a plate reader (ARVO™ MX, Perkin-Elmer Inc., Waltham, MA, USA).

Flow cytometry analysis. Flow cytometry analysis was performed as described previously (10,31). Briefly, T24, KK47 and their doxorubicin-resistant cells (2.0×10^5) were seeded in 6-well plates, applied to 2.5 μM of sorafenib and cultured for 24 h, or transfected with 40 nM of the indicated siRNA and cultured for 72 h. The cells were harvested, washed twice with ice-cold PBS containing 0.1% bovine serum albumin (BSA) and resuspended in 70% ethanol. After washing twice with ice-cold PBS, cells were resuspended in PBS containing 0.1% BSA, incubated with RNase (Roche Applied Science, Indianapolis, IN, USA) and stained with propidium iodide (Sigma). Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Western blotting. Whole-cell extracts were prepared with phosphatase inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan) as previously described (10,11,30,31). The protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA), based on Bradford's method. Whole-cell extracts (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride micro-porous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The blotted membranes were incubated for 1 h at room temperature with a primary antibody. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. The bound antibody was visualized using an ECL
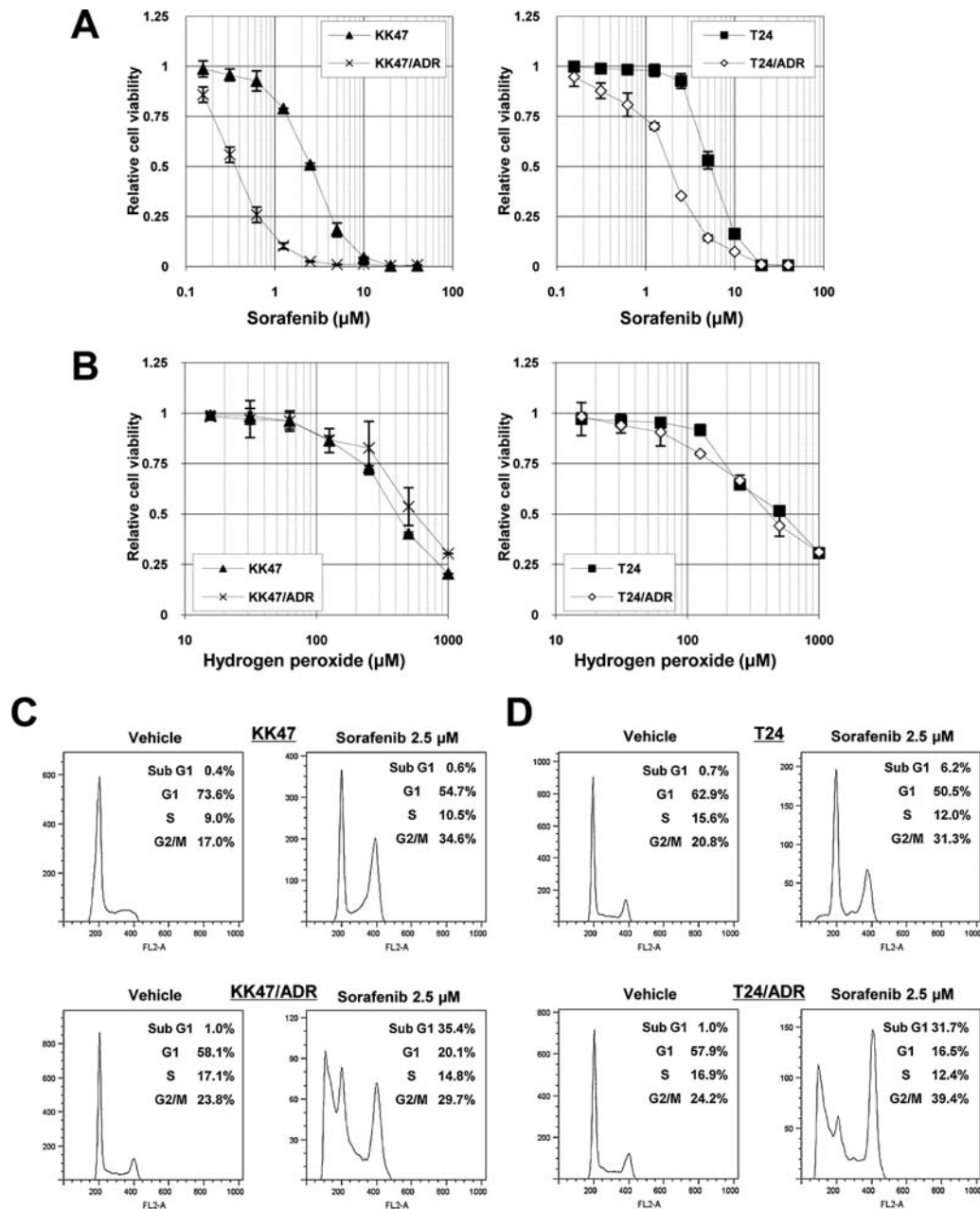
Figure 1. Sorafenib, but not hydrogen peroxide suppresses survival of doxorubicin-resistant cancer cells more effectively than their parental cells. (A) and (B) KK47, T24 and their doxorubicin-resistant cells were seeded into 96-well plates. The following day, various concentrations of sorafenib (A) or hydrogen peroxide (B) were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of sorafenib or hydrogen peroxide corresponds to 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ± s.d. (C) KK47, T24 and their doxorubicin-resistant cells were applied to vehicle or 2.5 μM of sorafenib for 24 h, and the cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle fractions are shown at the top right of each graph.

kit (GE Healthcare Bio-Science) and membranes were exposed to Kodak X-OMAT film.

Results

Sorafenib, but not hydrogen peroxide suppresses doxorubicin-resistant cancer cell survival more effectively than their parental cells. We have been investigating mechanisms of a resistance to anticancer drug and aiming to overcome anti-cancer-drug resistance (1-11). We previously established doxorubicin-resistant cells of urothelial cancer (4). To examine which anticancer agents are effective for doxorubicin-resistant cancer cells, we carried out cytotoxicity assays to

compare drug sensitivity between doxorubicin-resistant cancer cells and their parental cells using various anticancer drugs. First, doxorubicin-resistant KK47/ADR cells and their parental KK47 cells were subjected to cytotoxicity assay using sorafenib. Although doxorubicin-resistant cells were cross-resistant to various anticancer agents as previously reported (4), to our surprise, the results showed that sorafenib suppressed survival of KK47/ADR cells more effectively than KK47 cells (Fig. 1A, left panel). To examine whether this finding is applicable to other cell lines, we investigated the cytotoxicity of sorafenib using T24 cells and their doxorubicin-resistant T24/ADR cells. Sorafenib repeatedly suppressed survival of doxorubicin-resistant T24-cells more
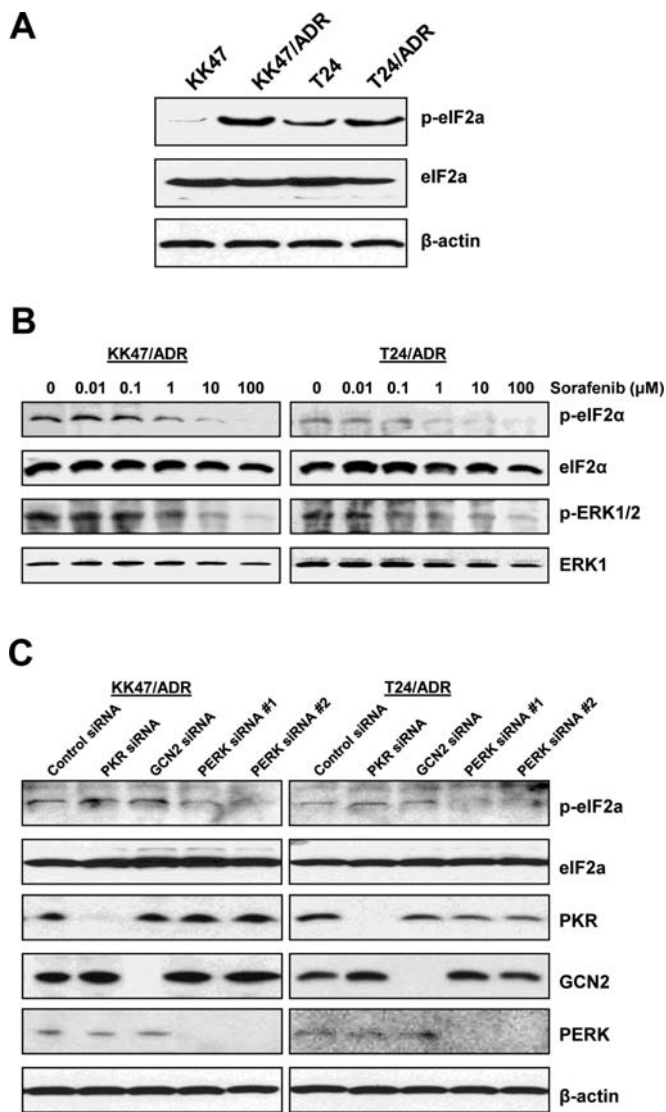
Figure 2. Sorafenib inhibits eIF2α phosphorylation PERK-dependently with higher level in doxorubicin-resistant cells. (A) Whole-cell extracts of KK47, T24 and their doxorubicin-resistant cells were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (B) KK47/ADR and T24/ADR cells were cultured with various concentrations of sorafenib for 6 h, and the cells were harvested. Whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (C) KK47/ADR and T24/ADR cells were transfected with 40 nM of control siRNA, PKR siRNA, GCN2 siRNA, PERK siRNA no. 1 or PERK siRNA no. 2. At 72 h after transfection, whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies.

2.5 μM of sorafenib which indicates cell death. Similarly, when T24 and T24/ADR cells were treated with sorafenib, T24/ADR cells were arrested at G2 phase of cell cycle and showed cell death more clearly than T24 cells (Fig. 1C).

Sorafenib inhibits eIF2α phosphorylation PERK-dependently with higher level in doxorubicin-resistant cells. To elucidate a possible mechanism of higher sensitivity of doxorubicin-resistant cells to the multikinase inhibitor sorafenib, we compared phosphorylation status of sorafenib targets between doxorubicin-resistant cells and their parental cells. Basal phosphorylation status of known targets of sorafenib such as VEGFR2 and ERK did not differ between doxorubicin-resistant cells and their parental cells (data not shown). However, phosphorylated form of eIF2α which we have found to be a target of sorafenib in RCC cells (32) was higher in both doxorubicin-resistant cell lines (KK47/ADR and T24/ADR cells) compared to their parental cells (Fig. 2A). We investigated whether sorafenib could inhibit phosphorylation of eIF2α as was shown previously in RCC cells. Expectedly, phosphorylated form of ERK1/2 as well as eIF2α was reduced by sorafenib as shown in Fig. 2B. Sorafenib concentration of 1 μM inhibited eIF2α phosphorylation to 50% of vehicle treatment in both doxorubicin-resistant cell lines. There are four members of eIF2α kinases; HRI, PKR, PERK and GCN2. To examine which form is responsible for eIF2α phosphorylation in doxorubicin-resistant urothelial cancer cells, phosphorylation status of eIF2α was investigated using eIF2α kinase-specific siRNAs (except for HRI which expression is restricted to erythroblast cells). Silencing of PERK reduced eIF2α phosphorylation whereas PKR and GCN2 knockdown did not affect phosphorylation of eIF2α (Fig. 2C).

Silencing of PERK induces cell cycle arrest at G2 phase and cellular apoptosis in doxorubicin-resistant cancer cells similar to sorafenib. Because both sorafenib and PERK inhibition could reduce eIF2α phosphorylation level (Fig. 2B and C), whether PERK knockdown using PERK-specific siRNA could affect cell cycle similar to sorafenib was investigated. Silencing of PERK induced cell cycle arrest at G2 phase and cell death in KK47/ADR cells whereas PERK knockdown induced no effect on cell cycle in KK47 cells (Fig. 3A). Similarly, suppression of PERK induces cell cycle arrest at G2 phase and cell death more effectively in T24/ADR cells than in T24 cells (Fig. 3B). These findings were confirmed by higher increase of cleaved-PARP form indicating cellular apoptosis in doxorubicin-resistant cell lines than their parental cells (Fig. 3C).

Sorafenib sensitizes doxorubicin-resistant urothelial cancer cells to oxidative stress exerted by both hydrogen peroxide and doxorubicin. Because sorafenib could sensitize cancer cells to hydrogen peroxide producing oxidative stress, whether sorafenib could also sensitize doxorubicin-resistant cells to hydrogen peroxide was investigated. As shown in Fig. 4A, sorafenib sensitized both doxorubicin-resistant cell lines to hydrogen peroxide. As expected, addition of NAC abolished the effect of hydrogen peroxide combined with sorafenib in both doxorubicin-resistant cell lines similar to

effectively than their parental cells (Fig. 1A, right panel). On the other hand, cytotoxicity to hydrogen peroxide did not differ between doxorubicin-resistant cells and their parental cells (Fig. 1B). To confirm these findings and investigate the mechanism of sorafenib cytotoxicity, flow cytometry analysis was carried out. When KK47 cells were treated with 2.5 μM of sorafenib, KK47 cells were arrested at G2 phase of cell cycle, but showed no cell death as indicated by no increase in the sub-G1 fraction of cell cycle analysis. In contrast, KK47/ADR cells were arrested at G2 phase of cell cycle and showed a marked increase in the sub-G1 fraction when treated with
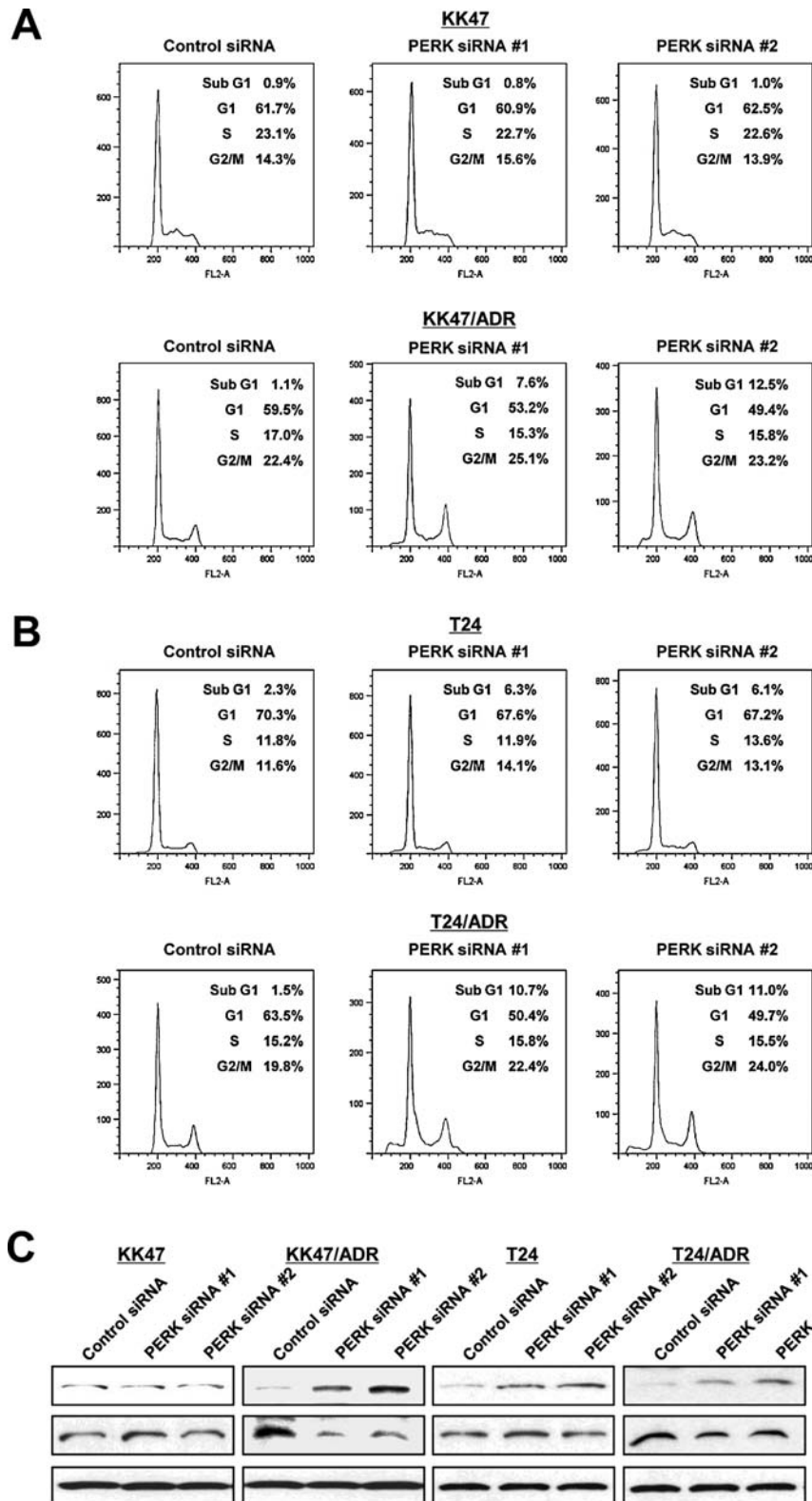
Figure 3. Silencing of PERK induces cell cycle arrest at G2 phase and cell death in doxorubicin-resistant cancer cells similar to sorafenib. (A) and (B) KK47 (A), KK47/ADR (A), T24 (B) and T24/ADR (B) cells were transfected with 40 nM of control siRNA, PERK siRNA no. 1 or PERK siRNA no. 2. At 72 h after transfection, cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle fractions are shown at the top right of each graph. (C) KK47, T24 and their doxorubicin-resistant cells were transfected with 40 nM of control siRNA, PERK siRNA no. 1 or PERK siRNA no. 2. At 72 h after transfection, whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies.

their parental cells (Fig. 4B). Doxorubicin is known to produce high-level oxidative stress to cancer cells (33). Therefore, we assayed cytotoxicity when doxorubicin plus sorafenib were applied. As shown in Fig. 4C, sorafenib sensitized both doxorubicin-resistant cell lines to doxorubicin. However, this favorable effect of doxorubicin plus sorafenib
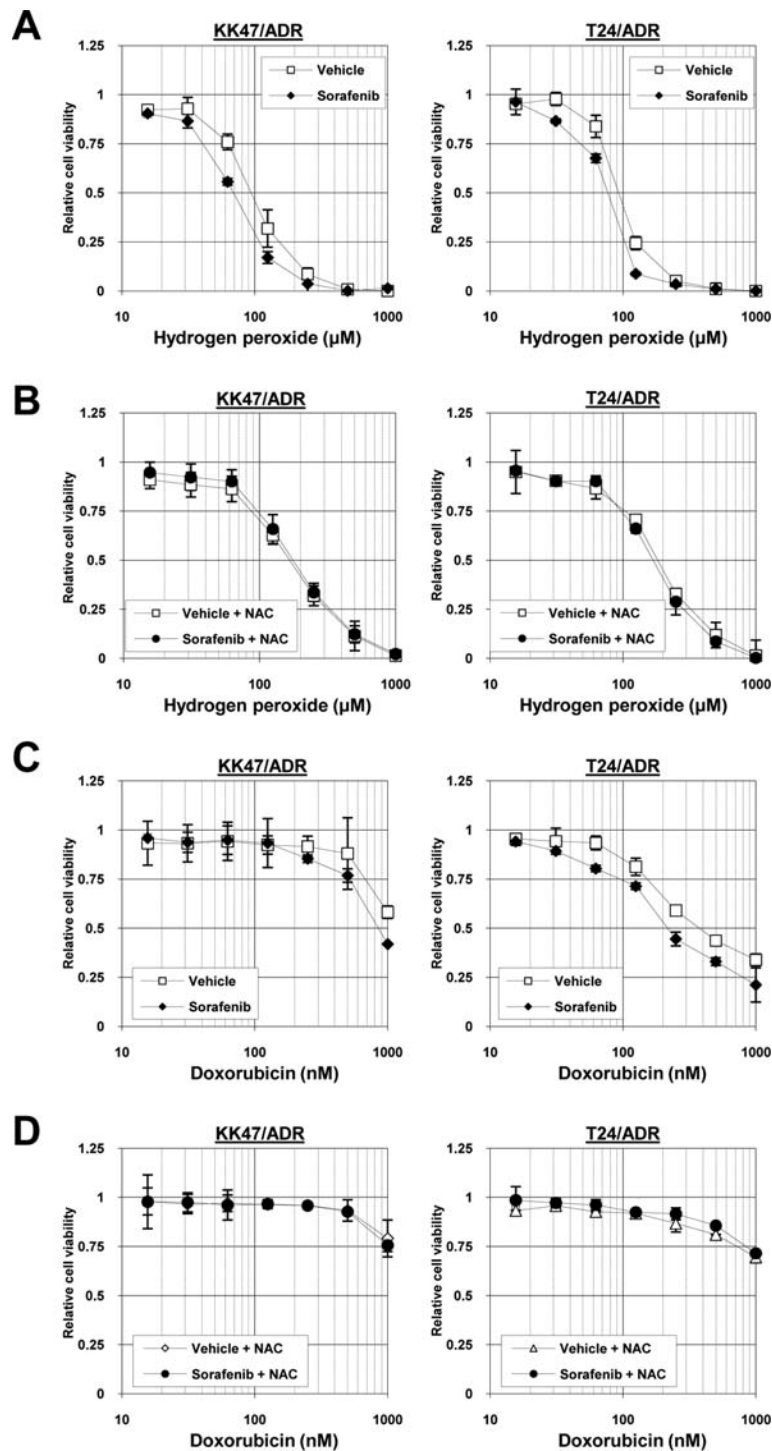
Figure 4. Sorafenib sensitizes doxorubicin-resistant cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. (A) and (C) KK47/ADR and T24/ADR cells applied to 0.5 µM of sorafenib were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (A) or doxorubicin (C) were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of hydrogen peroxide corresponds to 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ± s.d. (B) and (D) KK47/ADR and T24/ADR cells applied to 0.5 µM of sorafenib and 5 mM of NAC were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (B) or doxorubicin (D) were applied. Cytotoxicity assay was performed as described in (A) and (C). Boxes, mean; bars, ± s.d.

was abolished when NAC was added, suggesting that this combinational effect resulted from oxidative stress by doxorubicin (Fig. 4D).

PERK knockdown sensitizes both doxorubicin cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. Because both sorafenib and PERK inhibition reduced eIF2α

phosphorylation level (Fig. 2B and C), PERK knockdown using PERK-specific siRNAs instead of sorafenib was employed for cytotoxicity assay by hydrogen peroxide and doxorubicin. First, cytotoxicity by hydrogen peroxide in doxorubicin-resistant cells transfected with PERK-specific siRNAs was examined. Hydrogen peroxide with PERK knockdown suppressed survival of both doxorubicin-resistant
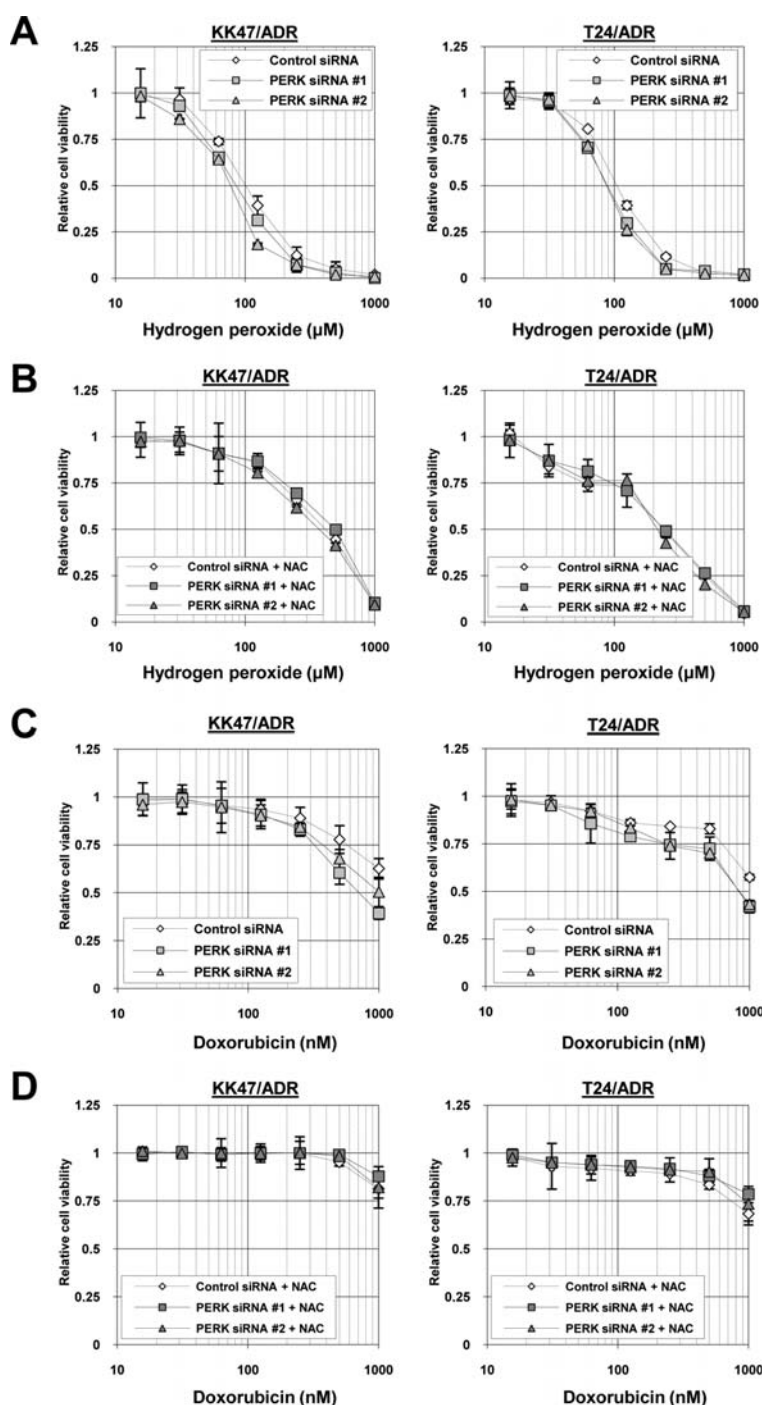
Figure 5. PERK knockdown sensitizes both doxorubicin-resistant cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. (A) and (C) KK47/ADR and T24/ADR cells transfected with 40 nM of control siRNA, PERK siRNA no. 1 or PERK siRNA no. 2 were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (A) or doxorubicin (C) were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of hydrogen peroxide corresponds to 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ± s.d. (B) and (D) KK47/ADR and T24/ADR cells transfected with 40 nM of control siRNA, PERK siRNA no. 1 or PERK siRNA no. 2, and applied to 5 mM of NAC were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (B) or doxorubicin (D) were applied. Cytotoxicity assay was performed as described in (A) and (C). Boxes, mean; bars, ± s.d.

cells more effectively compared with hydrogen peroxide alone although an increase of cytotoxicity by sorafenib was modest (Fig. 5A). To confirm a mechanism of an additional effect of PERK knockdown to cytotoxicity by hydrogen peroxide, we assayed cytotoxicity by hydrogen peroxide with PERK knockdown in the presence of NAC. When NAC was added to hydrogen peroxide with PERK knockdown, the increased cytotoxicity of hydrogen peroxide with PERK knockdown was abolished in both doxorubicin-resistant cancer cell lines (Fig. 5B).

When doxorubicin was used as an agent exhibiting oxidative stress instead of hydrogen peroxide, cytotoxicity to doxorubicin with PERK knockdown in doxorubicin-resistant cancer cell lines increased, although an increase of cytotoxicity by PERK knockdown was modest (Fig. 5C). In addition, NAC addition abolished this favorable effect of

PERK knockdown similar to when hydrogen peroxide was used (Fig. 5D).

Discussion

Sorafenib exerts anti-tumor effects through inhibition of multiple kinases, such as Raf-1, VEGFR 2 and 3, PDGFR-ß, FLT-3, c-KIT and RET-receptor tyrosine kinase (16,17). However, it remains unknown what target sorafenib acts on during cell cycle progression to inhibit cell proliferation. In this study, cell cycle analysis revealed that sorafenib induced cell cycle arrest at G2 phase, leading to cellular apoptosis in doxorubicin-resistant cancer cells. Cell cycle proceedings are controlled by modification of players involved in cell cycle progression such as phosphorylation and acetylation (34,35). Therefore, sorafenib activity could involve inhibition of kinases involved in mitotic process. There are many players which work on mitosis such as polo-like kinase (36), aurora kinase (37) and topoisomerase (38). In this study, cell cycle arrest at G2 phase was more prominent by sorafenib treatment compared with PERK knockdown. This discrepancy of cell cycle analysis between sorafenib treatment and PERK knockdown indicated that some of kinases involved in mitosis progression in addition to PERK might be responsible for cell cycle arrest at G2 phase by sorafenib treatment.

Pancreatic ß-islet cells produce large amounts of pro-insulin proteins and are continuously exposed to ER stress leading to cellular apoptosis. Therefore, pancreatic ß-islet cells regulate protein synthesis through a control of translation level. In this process of translation control, PERK plays a critical role to phosphorylate eIF2α and repress translation except for ATF4. Mutation in PERK was shown to induce diabetes mellitus through pancreatic ß-islet cells apoptosis, probably due to disorder of a mechanism of unfolded protein reaction (UPR) (39). Sorafenib has also been associated with pancreatic disorder and drug-induced pancreatitis which occurs during sorafenib administration, suggesting that multiple kinase inhibitor, sorafenib could inhibit PERK function (40). Results clearly showed that phosphorylation of eIF2α was reduced by sorafenib treatment (Fig. 2B). There are recognized to be four eIF2α kinases, that is, HRI, PRK, PERK and GCN2. In urothelial cancer cells, three eIF2α kinases except HRI are expected to be expressed. In KK47/ADR and T24/ADR cells, PERK seems to be responsible for phosphorylation of eIF2α because only PERK knockdown could repress phosphorylation of eIF2α as shown in Fig. 2C. Therefore, sorafenib is suggested to reduce phosphorylation of eIF2α through an inhibition of PERK kinase activity, at least in KK47/ADR and T24/ADR cells. In addition, phosphorylation level of eIF2α seemed to correlate with cellular sensitivity to sorafenib. Survival of doxorubicin-resistant cancer cells having high phosphorylation level of eIF2α was suppressed more effectively by sorafenib and PERK knockdown. Similar findings that sorafenib reduced eIF2α phosphorylation through PERK inhibition have been found also in RCC cells (32).

It is important task to improve the outcome of metastatic or recurrent solid tumor patients. Although so far, conventional chemotherapy using various anticancer agents such as cisplatin, doxorubicin and paclitaxel have been employed for the treatment of metastatic or recurrent solid tumors, results are not satisfactory. Furthermore, there is no effective therapy when solid tumors become resistant to conventional chemotherapy. Recently, new small molecule agents were administered for metastatic RCC patients, and one of them is sorafenib. Sorafenib in combination with doxorubicin has been reported to double the median overall survival from 6.5 months in the doxorubicin-mono therapy group to 13.7 months in the combination group in a randomized, double-blind, placebo-controlled phase II study of patients with HCC, indicating a possibility that sorafenib is effective in combination with doxorubicin (41). However, sorafenib in combination with conventional chemotherapeutic agents, paclitaxel plus carboplatin for melanoma patients failed to show improvement in overall survival (42). In this study, sorafenib *in vitro* could sensitize doxorubicin-resistant cancer cell lines possessing high eIF2α phosphorylation status to oxidative stress exerted by hydrogen peroxide and doxorubicin, indicating that combination therapy of anti-cancer agents exerting oxidative stress and sorafenib may be a beneficial therapeutic approach for cancer cells dependent on eIF2α pathway. In addition, doxorubicin-resistant cancer cells were more sensitive to sorafenib than their parental cells, suggesting that sorafenib might be very useful after progression in the chemotherapy using doxorubicin-containing regimens. However, further investigation of the effect of combination therapeutics of sorafenib and doxorubicin are needed using *in vivo* models and clinical studies. In addition, the PERK silencing and other modulators of PERK activity with or without doxorubicin would also be beneficial for doxorubicin-resistant cancer cells.

In conclusion, the multikinase inhibitor sorafenib was found to inhibit the eIF2α pathway regulating protein translation. Silencing of PERK sensitized doxorubicin-resistant cells to oxidative stress by hydrogen peroxide and doxorubicin. In addition, sorafenib sensitized to hydrogen peroxide and doxorubicin. These findings indicate that sorafenib might be a useful agent for treatment of solid tumors in combination with doxorubicin in progressive cancer after chemotherapy using doxorubicin-containing regimens.

Acknowledgements

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