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Inhibition of S6K1 enhances glucose deprivation-induced cell death via downregulation of anti-apoptotic proteins in MCF-7 breast cancer cells

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ABSTRACT

Nutrient-limiting conditions are frequently encountered by tumor cells in poorly vascularized microenvironments. These stress conditions may facilitate the selection of tumor cells with an inherent ability to decrease apoptotic potential. Therefore, selective targeting of tumor cells under glucose deprivation conditions may provide an effective alternative strategy for cancer therapy. In the present study, we investigated the effects of S6 kinase 1 (S6K1) inhibition on glucose deprivation-induced cell death and the underlying mechanisms in MCF-7 breast cancer cells. PF4708671, a selective inhibitor of S6K1, and knockdown of S6K1 with specific siRNA enhanced cell death induced under glucose deprivation conditions. Moreover, inhibition of S6K1 led to apoptosis in glucose-starved MCF-7 cells via downregulation of the anti-apoptotic proteins, Mcl-1 and survivin. Further experiments revealed that sorafenib, shown to be involved in Mcl-1 and survivin downregulation via mTOR/S6K1 inhibition significantly promotes cell death under glucose deprivation conditions. These findings collectively suggest that S6K1 plays an important role in tumor cell survival under stress conditions, and thus inhibition of S6K1 may be an effective strategy for sensitizing cells to glucose deprivation.

1. Introduction

The differences between cancer and normal cells with regard to consumption and metabolism of glucose, the major caloric source, are considered a promising therapeutic target [1–3]. In particular, metabolic changes in glycolysis in cancer cells, known as the Warburg effect [4], have attracted extensive interest. Since glycolysis is far less efficient than oxidative phosphorylation in terms of ATP generation, cancer cells exhibit abnormally high glycolytic rates to maintain energy homeostasis [5,2]. This shift renders cancer cells particularly dependent on glucose and thus vulnerable to glucose deprivation, as evident from the finding that glucose depletion causes rapid and massive death of cancer cells in cell culture systems. However, cancer cells generally survive glucose deprivation better than their normal counterparts. It is proposed that the tumor microenvironment contains factors that allow cancer cells to resist glucose deprivation-induced death. Therefore, identification and targeting of these factors may aid in enhancing tumor sensitivity to glucose deprivation.

S6 kinase 1 (S6K1) is one of the most extensively characterized effectors of the mammalian target of rapamycin complex 1 (mTORC1), which regulates cell size, protein translation, and proliferation [6]. The S6K1 gene, RPS6KB1, is localized in the chromosomal region, 17q23, which is amplified in several breast cancer cell lines and about 30% of primary tumors [7–9]. S6K1 gene amplification and protein expression have been linked to poor prognosis in breast cancer patients, supporting a role in disease development and progression [7,10]. Specific targeting of S6K1 may thus present an effective novel strategy for breast cancer treatment.

In the present study, we evaluated the role of S6K1 in MCF-7 breast cancer cells under glucose deprivation conditions. Notably, inhibition of S6K1 with either PF4708671 or small interfering RNA (siRNA) enhanced glucose deprivation-induced cell death. Subsequently, we focused on the molecular mechanism underlying this enhancement of apoptosis of glucose-starved tumor cells. The promotory effects of sorafenib on cell death induced by glucose deprivation were additionally examined. Based on the collective results, we propose that S6K1 inhibition represents a promising strategy for cancer treatment under glucose-limiting conditions with minimal cytotoxicity to normal cells.

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2. Materials and methods

2.1. Cell cultures and reagents

The human breast cancer cell line, MCF-7, was obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM) (WelGEN, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C. Glucose deprivation was accomplished by washing with PBS, followed by incubation in glucose-free DMEM containing 10% FBS. PF4708671 was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Measurement of cell viability

Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a colored product. Following treatment with drugs, cell were incubated with the MTT reagent and solubilized in DMSO. The amount of converted MTT was calculated by measuring absorbance at 570 nm.

2.3. Evaluation of cell death

Cells were stained with Annexin V-FITC and Propidium Iodide (PI), according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, cells were collected, washed with cold PBS and suspended in binding buffer. After staining with 5 μl annexin V-FITC and 10 μl PI, cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.4. siRNA transfection

siRNA transfection experiments were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. S6K1 (Hs_RPS6KB1_5) and control (1022076) siRNAs were purchased from Qiagen (Valencia, CA, USA), and Mcl-1 (sc-35877), survivin (sc-29499) and control (sc-37007) siRNAs from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Measurement of caspase activation

Active caspases were detected using the CaspaTag™ Caspase 3/7 *in situ* Assay kit (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions. The green fluorescence signal directly corresponds to the amount of active caspase present in the cell at the time of reagent addition. Stained cells were analyzed with a FACScan flow cytometer.

2.6. Measurement of mitochondrial membrane potential (MMP)

MMP was measured with the voltage-sensitive lipophilic cationic fluorescence probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Briefly, cells were collected, washed with cold PBS and incubated with JC-1 for 15 min. Following another wash with PBS, cells were analyzed with a FACScan flow cytometer. Fluorescence intensities of both monomeric (FL-1, green) and aggregated (FL-2, red) molecules were observed, and the FL-2/FL-1 intensity ratio calculated. A decrease in this ratio was interpreted as reduction in MMP.

2.7. Western blot analysis

Cells were collected and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 1% SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min on ice. Insoluble components were removed from lysates by centrifugation at 13,000×*g* for 15 min, and protein concentrations determined with the Bradford method. Cell lysates were separated using SDS–PAGE and transferred to nitrocellulose membrane followed by immunoblotting with the specified primary and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent Substrates (Thermo Scientific Pierce, Rockford, IL, USA).

The following antibodies were used: p-S6 (4838), S6K (9202), and survivin (2808) were obtained from Cell Signaling Technology (Beverly, MA, USA), Mcl-1 (sc-12756) from Santa Cruz Biotechnology, and β-actin (A5316) from Sigma–Aldrich.

3. Results

3.1. S6K1 activity does not change in MCF-7 cells under glucose starvation conditions

We initially investigated whether glucose deprivation affects cell viability. MCF-7 breast cancer cells were incubated in the absence of glucose for 24 h, and cell viability determined with the MTT assay. Glucose deprivation led to a 35% reduction in cell viability (Fig. 1A). Cell death was induced in about 30% cells, assessed on the basis of Annexin V/Propidium Iodide (PI) positivity (Fig. 1B). However, glucose deprivation did not affect S6 phosphorylation (Fig. 1C), suggesting no effects on S6K1 activity in MCF-7 cells.

3.2. Inhibition of S6K1 enhances glucose deprivation-induced cell death

The downstream effector of mTORC1, S6K1, is often elevated in breast cancer, and its overexpression linked to poor prognosis in patients [10]. In particular, expression levels of S6K1 and its phosphorylated S6 substrate are considerably high in MCF-7 cells [11]. Accordingly, we examined whether S6K1 affects cell death induced by glucose deprivation in MCF-7 cells. PF4708671, recently identified as a specific inhibitor of p70 S6K1 [12], decreased S6 phosphorylation in a dose-dependent manner, indicative of effective suppression of S6K1 activity (Fig. 2A). Cell death under conditions of glucose deprivation, assessed with the MTT assay and Annexin V/PI staining, was markedly enhanced following PF4708671 treatment (Fig. 2B and C). Treatment of the cells under glucose starvation condition with PF4708671 induced the cell death via depolarization of mitochondrial membrane permeabilization (MMP) and caspase(s) activation (Fig. 2D and E). Our results demonstrate that combined glucose deprivation and PF4708671 treatment induces cell death to a more significant extent than either glucose deprivation or PF4708671 alone.

To further establish the effect of S6K1 on glucose deprivation-stimulated cell death, we transfected cells with siRNA targeting S6K1, followed by glucose deprivation. S6K1 siRNA induced considerable knockdown of S6K1 expression in MCF-7 cells (Fig. 2F) and enhanced cell death induced by glucose deprivation (Fig. 2G). Clearly, suppression of S6K1 activity effectively enhances MCF-7 sensitivity to glucose deprivation.

3.3. Sorafenib enhances glucose deprivation-induced cell death

Sorafenib, a multikinase inhibitor, has recently emerged as a promising target agent that possesses antitumor activity against

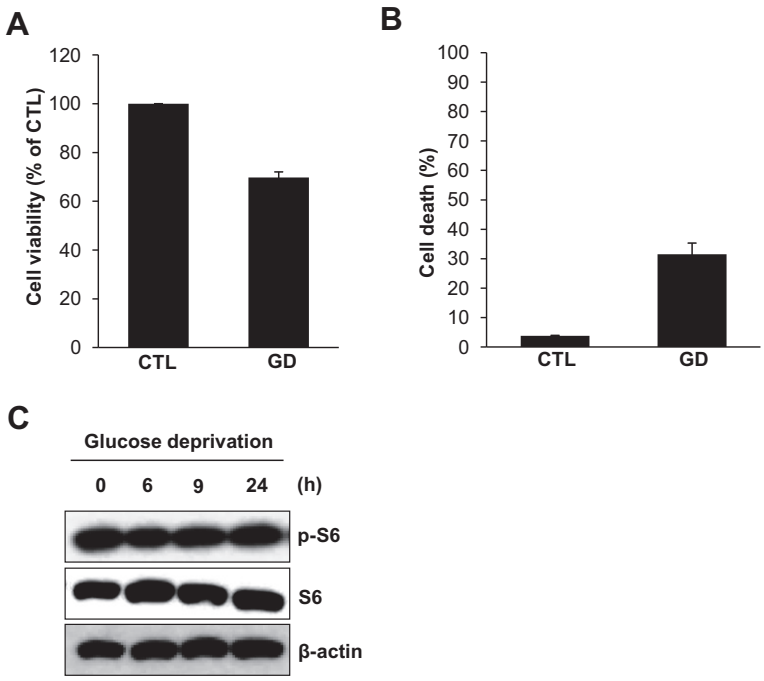

Fig. 1. Effects of glucose deprivation on MCF-7 cells. (A and B) MCF-7 cells were incubated without glucose for 24 h. Cell viability was evaluated with the MTT assay (a). Viability of control cells was set at 100%, and survival relative to the control calculated. Cell viability data are presented as means of triplicate samples, and error bars reflect S.D. Cell death was evaluated via flow cytometry after Annexin V and PI staining (b). Cell death data are presented as means of triplicate samples, and error bars reflect SD. (C) MCF-7 cells were incubated without glucose for the indicated times. The indicated protein levels were estimated using Western blot analysis. The blot is representative of three independent experiments. CTL: control; GD: glucose deprivation.

a broad range of cancers, including renal cell carcinoma, hepatoma, and leukemia [13–15]. Previous experiments by our group have shown that sorafenib exerts significant antitumor activity against lung cancer cells via suppression of mTORC1/S6K1 and downregulation of the anti-apoptotic protein, survivin [16]. In the current study, we investigated the effects of sorafenib on cell death induced with glucose deprivation. As shown in Fig. 3(A), treatment with sorafenib led to significant enhancement of cell death in MCF-7 cells subjected to glucose deprivation. MMP and caspase(s) activation were also induced upon treatment of cells with sorafenib under glucose-deprived conditions (Fig. 3B and C). Our findings indicate that combined sorafenib treatment and glucose deprivation induces cell death to a higher extent than either glucose deprivation or sorafenib alone. Based on these results, we suggest that inhibition of mTORC1/S6K1 with chemotherapeutic drugs can be effectively used to enhance glucose deprivation-induced cell death.

3.4. Downregulation of survivin or Mcl-1 promotes cell death under glucose deprivation conditions

Since Mcl-1 and survivin appear to be downregulated through a posttranscriptional mechanism mediated via blocking mTORC1/S6K1 signaling [17,18], we examined the Mcl-1 and survivin protein levels in MCF-7 cells exposed to glucose deprivation after treatment with the S6K1 inhibitor or sorafenib. As shown in Fig. 4(A) and (B), survivin and Mcl-1 protein levels were markedly decreased in cells treated with PF4708671 or sorafenib and exposed to glucose deprivation. To further confirm whether survivin or Mcl-1 downregulation enhances glucose deprivation-induced cell death, the effects of specific siRNAs were examined. Introduction of survivin and Mcl-1 siRNAs led to significant abrogation of expression of the respective proteins (Fig. 4C), and consequently enhanced glucose deprivation-induced cell death (Fig. 4D). In view of these findings, we propose that downregulation of survivin and Mcl-1 via S6K1 inhibition plays a critical role in cell sensitization to glucose deprivation-induced death.

4. Discussion

Tumor cells may be under constant stress owing to limited supplies of nutrients, compared with normal tissues. These stress conditions may facilitate the selection of tumor cells with an inherent ability to decrease apoptotic potential by modulating their energy metabolism. Therefore, selective targeting of cells exposed to these conditions may present a promising strategy to treat cancer without affecting normal cells. In this regard, it is important to identify the molecular targets involved in cancer cell survival under nutrient-limiting conditions. In the present study, we investigated the role of S6K1 in MCF-7 breast cancer cells under glucose deprivation conditions. Our experiments showed that inhibition of S6K1 with PF4708671 or knockdown with siRNA further enhances cell sensitivity to glucose deprivation. Thus, targeting of S6K1 may provide a promising strategy to treat breast cancer cells under glucose-limiting conditions.

There is extensive evidence that cancer cells are more sensitive to glucose concentrations than normal cells owing to their higher consumption ratio of energy [19,20]. Glucose restriction may therefore play an important role in cancer prevention and therapy, and considerable research interest has recently focused on identifying the basic mechanisms underlying this phenomenon. In our study, glucose deprivation reduced viability in ~35% and induced death in ~30% MCF-7 breast cancer cells. However, the S6 phosphorylation status remained unaltered, suggesting that glucose deprivation does not affect mTORC1 signaling.

Recent studies by our group have suggested that phosphorylated S6K1, a downstream effector of mTORC1, can be effectively applied as a predictive marker for breast cancer cells [21]. S6K1 expression has been associated with poor prognosis in breast can-

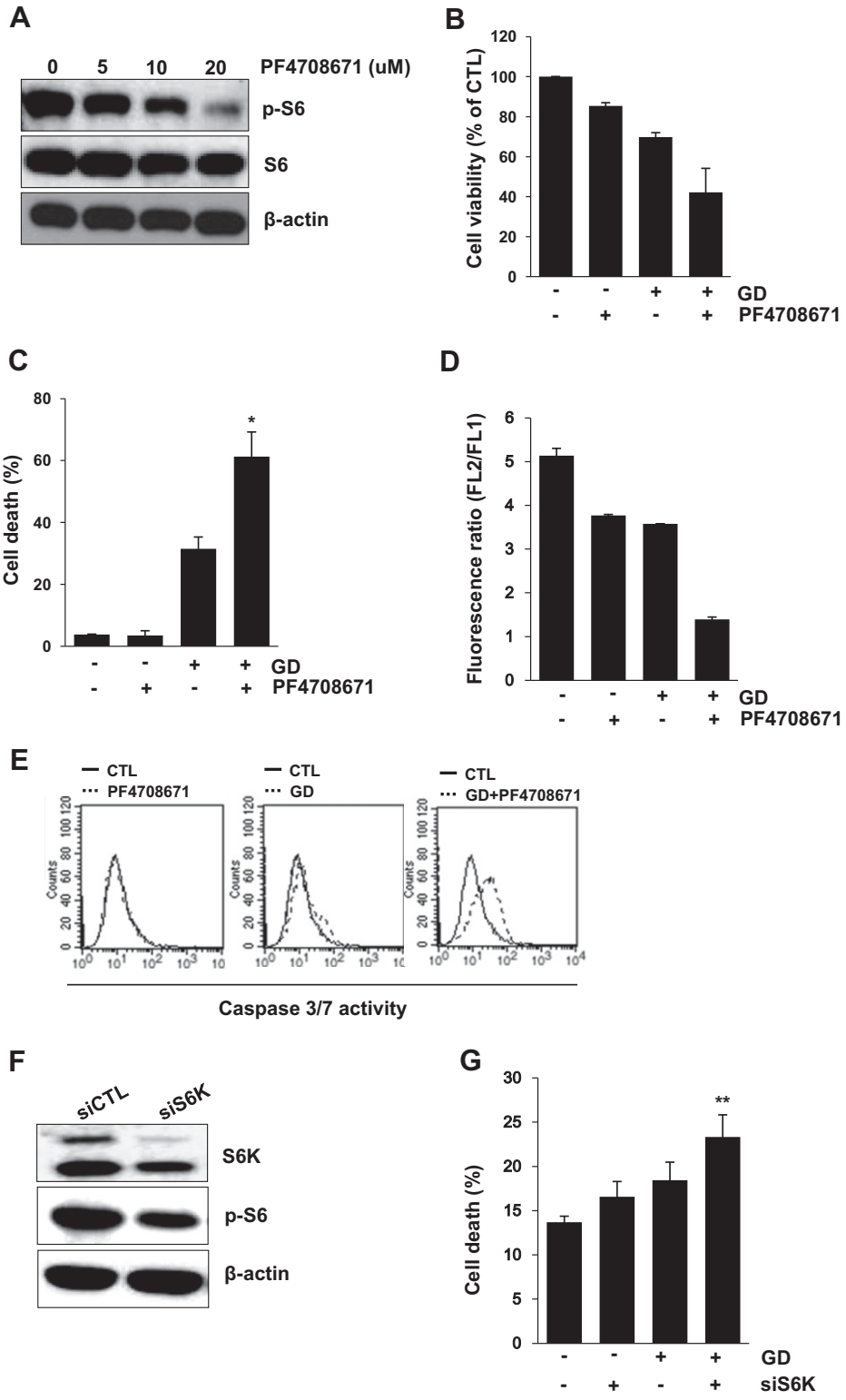

Fig. 2. PF4708671 enhances glucose deprivation induced-cell death. (A) MCF-7 cells were treated with the indicated concentrations of PF4708671 for 24 h. (B–E) MCF-7 cells were treated with 10 μM PF4708671 or/and deprived of glucose for 24 h. Cell viability was evaluated with the MTT assay (b). Viability of control cells was set at 100%, and survival relative to the control calculated. Cell viability data are presented as means of triplicate samples, and error bars reflect S.D. MMP was measured with the voltage-sensitive lipophilic cationic fluorescence probe, JC-1 (d). Fluorescence intensities of both monomeric (FL-1, green) and aggregated (FL-2, red) molecules were observed under a FACScan flow cytometer. The FL-2/FL-1 intensity ratio was calculated. A decrease in this ratio was interpreted as reduction in MMP. Caspase 3/7 activity was evaluated using the CaspaTag™ Caspase 3/7 *in situ* Assay kit (e). (F) MCF-7 cells were transiently transfected with S6K1 siRNA for 30 h. (G) MCF-7 cells were transiently transfected with S6K1 siRNA for 12 h, and incubated without glucose for 24 h. Cell death was evaluated via flow cytometry after Annexin V and PI staining (c and g). Cell death data are presented as means of triplicate samples, and error bars reflect S.D. $^{*}p<0.05$ vs. PF4708671-treated or glucose deprivation group. $^{**}p<0.01$ vs. S6K1 siRNA-treated or glucose deprivation group. The indicated protein were examined using Western blot analysis (a and f). The blot is representative of at least three independent experiments. CTL: control; GD: glucose deprivation.

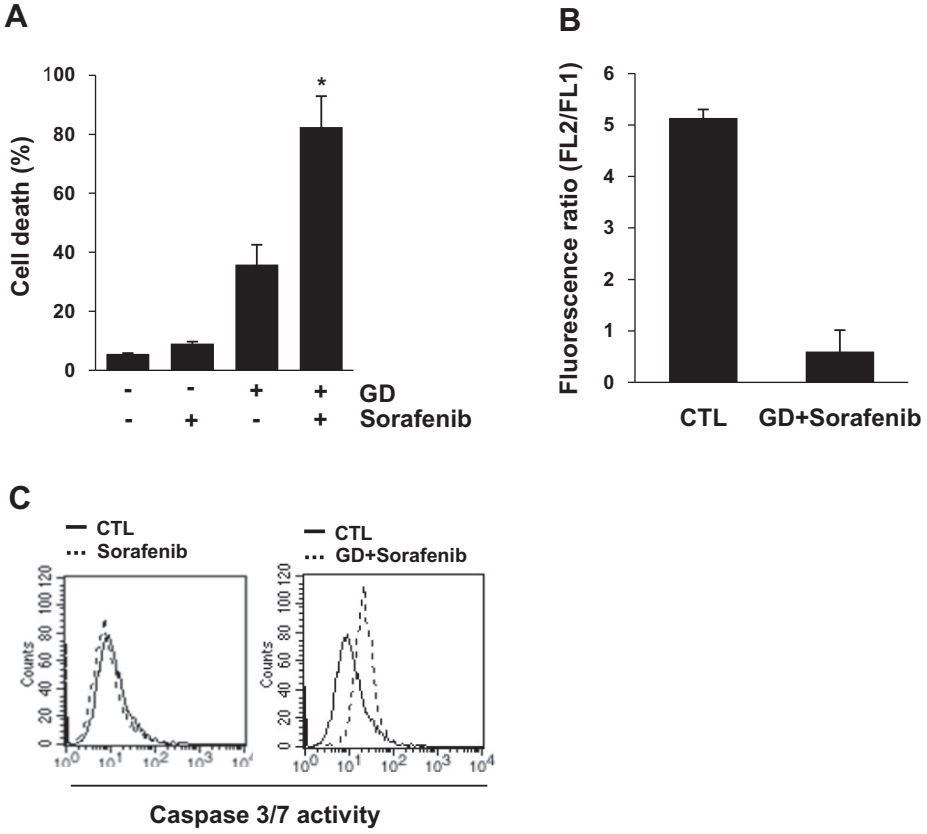

Fig. 3. Sorafenib enhances glucose deprivation-induced cell death. (A–C) MCF-7 cells were treated with 5 μM sorafenib or/and deprived of glucose for 24 h. Cell death was evaluated via flow cytometry after Annexin V and PI staining (a). Cell death data are presented as means of triplicate samples, and error bars reflect S.D. *$p<0.05$ vs. sorafenib-treated group. MMP was measured with the voltage-sensitive lipophilic cationic fluorescence probe, JC-1 (b). The fluorescence intensity of both monomeric (FL-1, green) and aggregated (FL-2, red) molecules were observed with a FACScan flow cytometer. The FL-2/FL-1 intensity ratio was calculated. A decrease in this ratio was interpreted as reduction in MMP. Caspase3/7 activity was evaluated using the CaspaTag™ Caspase 3/7 *in situ* Assay kit (c). CTL: control; GD: glucose deprivation.

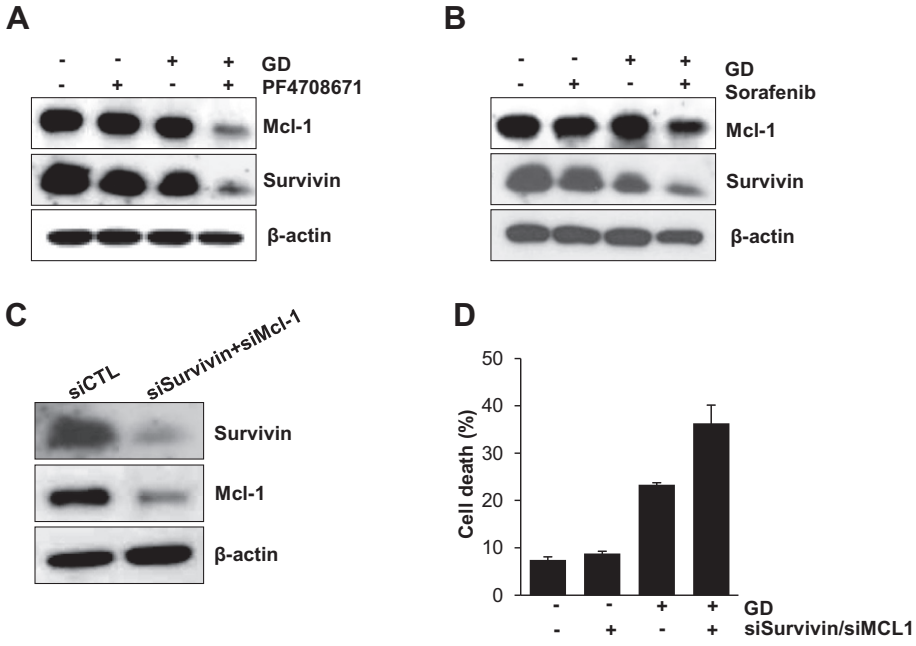

Fig. 4. Downregulation of Mcl-1 and survivin promotes glucose deprivation-induced cell death. (A) MCF-7 cells were treated with 10 μM PF4708671 or/and deprived of glucose for 24 h. (B) MCF-7 cells were treated with 5 μM sorafenib or/and deprived of glucose for 24 h. (C) MCF-7 cells were transiently transfected with Mcl-1 and survivin siRNA for 30 h. (D) MCF-7 cells were transiently transfected with Mcl-1 and survivin siRNA for 12 h, followed by glucose deprivation for 24 h. The indicated proteins were examined using Western blot analysis (a–c). The blot is representative of at least three independent experiments. Cell death was evaluated via flow cytometry after Annexin V and PI staining (d). Cell death data are presented as means of triplicate samples, and error bars reflect SD. GD: glucose deprivation.

cer patients, and shown to be amplified and overexpressed in several breast cancer cell lines (including MCF-7 and MDA-MB-431) and ~30% of primary cell lines [10,11]. PF4708671 was recently identified as a cell-permeable inhibitor of S6K1. The compound suppresses phosphorylation of the S6K1 substrates, S6, Rictor (Thr1135) and mTOR (Ser2448), but not S6K2 or other AGC kinases (Akt1, Akt2, PKA, PKCα, PKCε, PRK2, ROCK2, RSK1, RSK2, and SGK1), *in vitro* and in cell-based studies [12]. Treatment with PF4708671 inhibited S6K1 activity and enhanced cell sensitivity to glucose deprivation (Fig. 2). Similarly, transfection with S6K1 siRNA sensitized cancer cells to the cell death effects of glucose deprivation. Our data strongly suggest that resistance to glucose deprivation-cell death is mediated, at least in part, via signaling through the S6K1 pathway.

S6K1 inhibition-mediated promotion of MCF-7 cell sensitivity to glucose deprivation-induced cell death is regulated via suppressing the anti-apoptotic proteins, Mcl-1 and survivin. We propose that inhibition of S6K1 activation potentiates MCF-7 cell sensitivity to glucose deprivation by decreasing Mcl-1 and survivin translation that allows glucose deprivation to exert its toxic effects. Several studies have shown that mTOR and its direct downstream targets, S6K1 and eIF4E/4E-BP, regulate translational initiation that controls the recruitment of ribosomes to mRNA templates in response to intracellular and extracellular signaling [22]. Therefore, inhibition of these pathways appears to present a promising anti-proliferative approach. Notably, the S6K1 inhibitors, PF4708671 and sorafenib, induced a decrease in anti-apoptotic Mcl-1 and survivin protein levels in the current study. These findings indicate that Mcl-1 and survivin are downstream targets of S6K1, and dysregulation of translation machinery factor(s) of Mcl-1 and/or survivin is involved in the promotion of cancer cell death under stress conditions.

In conclusion, we have demonstrated that inhibition of S6K1 enhances breast cancer cell sensitivity to glucose deprivation. This sensitization is mediated via suppressing Mcl-1 and survivin translation that allows glucose deprivation to exert its toxic effects. Accordingly, we propose that targeting S6K1 presents an effective approach to overcome resistance of cells to glucose deprivation in breast cancer.

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