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Glutaminase 1 inhibition reduces thymidine synthesis in NSCLC

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ABSTRACT

We found that non-small cell lung cancer (NSCLC) is remarkably sensitive to the regulation of glutamine supply by testing the metabolic dependency of 11 cancer cell lines against regulation of glycolysis, autophagy, fatty acid synthesis, and glutamine supply. Glutamine is known as a key supplement of cancer cell growth that is converted to α-ketoglutarate for anabolic biogenesis via glutamate by glutaminase 1 (GLS1). GLS1 inhibition using 10 μM of bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) showed about 50% cell growth arrest by SRB assay. By testing the synergistic effects of conventional therapeutics, BPTES combined with 5-fluorouracil (5-FU), an irreversible inhibitor of thymidylate synthase, significant effects were observed on cell growth arrest in NSCLC. We found that GLS1 inhibition using BPTES reduced metabolic intermediates including thymidine and carbamoyl phosphate. Reduction of thymidine and carbamoyl-phosphate synthesis by BPTES treatment exacerbated pyrimidine supply by combination with 5-FU, which induced cell death synergistically in NSCLC.

1. Introduction

Tyrosine kinase inhibitors for targeted therapy against lung cancer showed a therapeutic limit of progression in overall survival [1]. The major reason for this limit in targeted therapy appears to be based on over half a million somatic mutations for cancer progression [2]. In the last decade, a growing number of reports have initiated a discussion about the benefits of metabolic regulation in cancers (see reviews in Refs. [3,4]). Therefore, understanding the metabolic character of non-small-cell lung cancer cell (NSCLC) may provide a chance to improve therapeutic approaches to drug resistance.

In this study, we tested the metabolic dependency of a cancer cell panel composed of 11 different cancer cell lines, namely NSCLC, renal, breast, stomach, liver, prostate, colon, ovarian, CNS, leukemia, and melanoma, for four types of metabolic pathways, including glycolytic inhibition using 2-deoxyglucose (see review in Ref. [5]), autophagy inhibition using chloroquine (see review in Ref. [6]), fatty acid oxidation inhibition using etomoxir (an irreversible inhibitor of carnitine palmitoyltransferase-1, see review in Ref. [7]), and glutaminolysis inhibition using glutamine-depleted media or bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) [8,9]. NSCLC showed more dependency on glutamine supply than other cancers (Fig. 1). In glutaminolysis, the first metabolic reaction is converting glutamine to glutamate by glutaminase for the anaplerotic reaction in cancer [10]. Glutamine is considered to support cancer cell anabolism building TCA intermediates through conversion to α-ketoglutarate (α-KG) [11]. Recently, glutamine metabolism regulated by cancer-specific glutaminase (EC 3.5.1.2, glutaminase 1, L-glutaminase, and glutamine aminohydrolase) has been gaining attention in cancer biology as it has been reported that high levels of kidney-type glutaminase (glutaminase 1, GLS1, KGA) are associated with oncogenic activation [12–14]. Furthermore, diminishing tumorigenesis by GLS1 knockdown or inhibition using small molecules was demonstrated in a hepatocellular carcinoma xenograft model [15]. Here we explored the best combination of anti-cancer therapeutics with GLS1 inhibition using BPTES and the related mechanism in NSCLC.

Abbreviations: NSCLC, non-small cell lung cancer; GLS1, glutaminase 1, kidney-type glutaminase; BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; 5-FU, 5-fluorouracil; TYMS, thymidylate synthetase.

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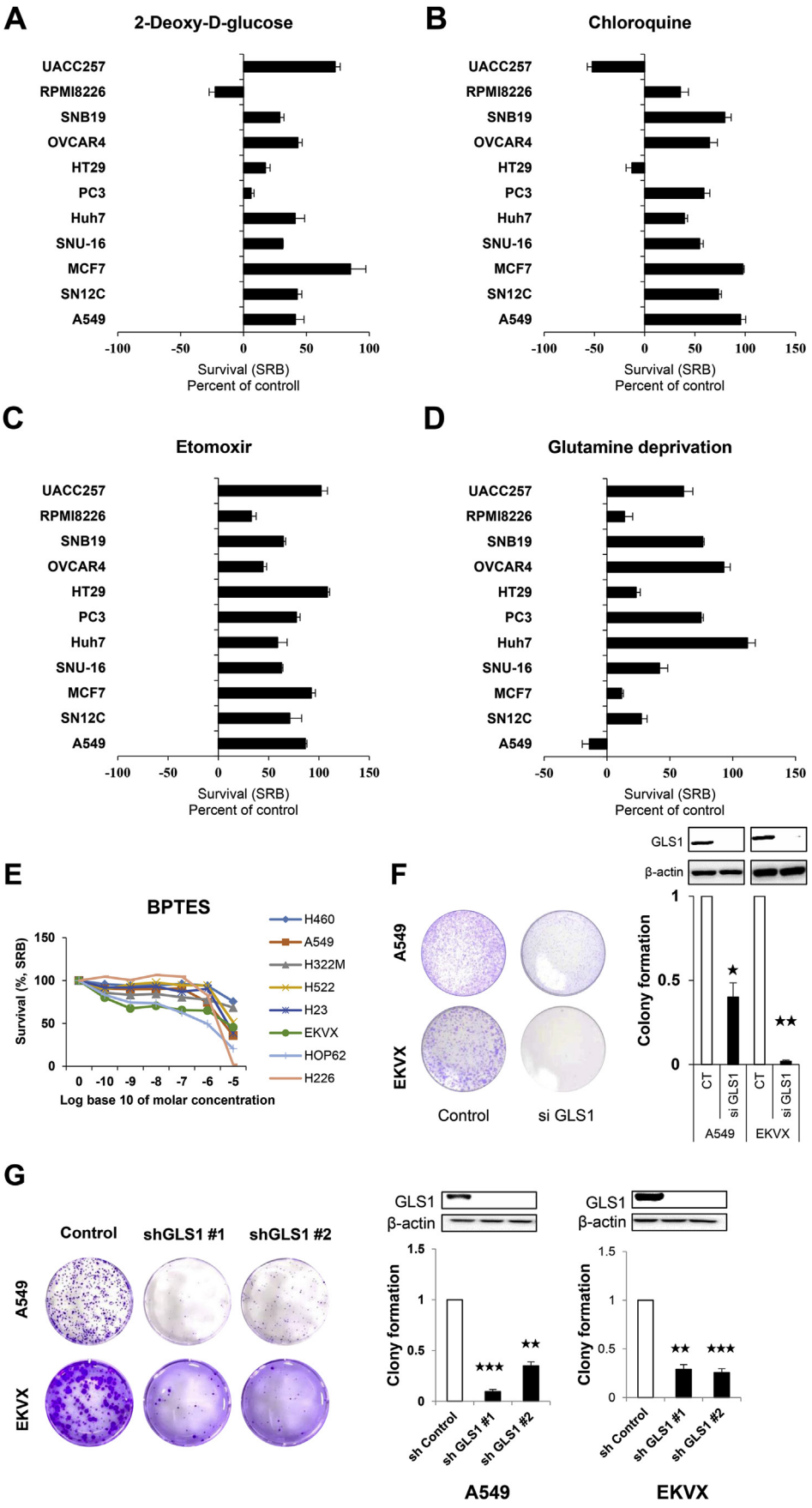
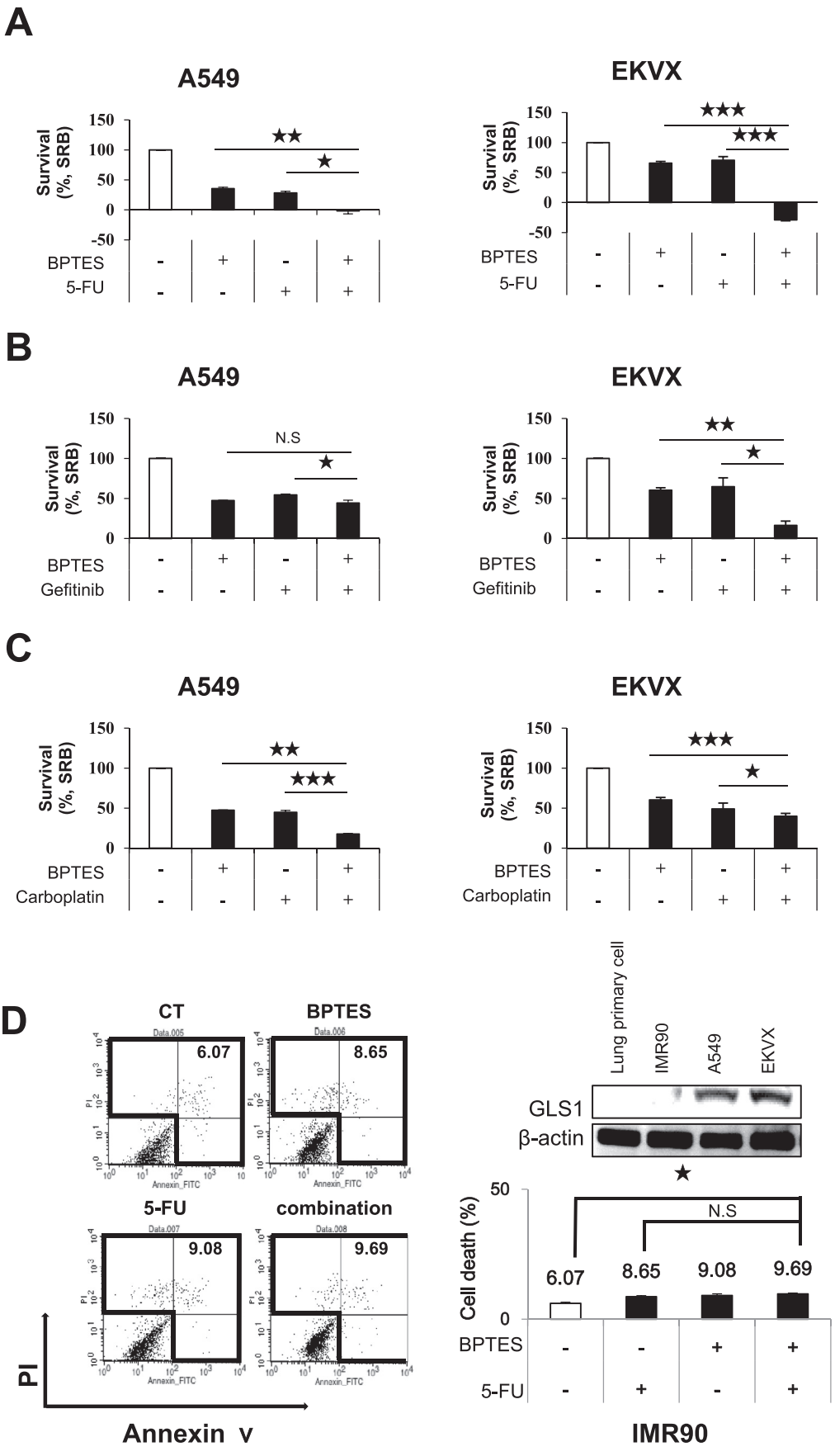


Fig. 1. Glutamine Metabolism is Critical for NSCLC Proliferation. Eleven types of cancer cells, including NSCLC, ovarian, CNS, hematopoietic, colon, renal, melanoma, breast, prostate, stomach, and liver, were treated with (A) 2-deoxy-D-glucose (2-DG, 5 mM), (B) chloroquine (100 μM), (C) etomoxir (100 μM), and (D) glutamine-free medium for 48 h, and cell proliferation was tested by the SRB assay. (E) Survival of NSCLC cells by BPTES treatment was tested by the SRB assay in various concentrations (0.1 nM−10 μM). (F) Clonogenic assay was performed using A549 and EKVX cells with siRNAs of control and GLS1 (20 nM) for 2 weeks. (G) A549 and EKVX cells stably transduced with a control shRNA or GLS1 shRNA were cultured, and stained with crystal violet after 2 weeks. *p*-Values were determined using two-tailed Student's *t*-tests (ns, not significant; ★$0.01 < p < 0.05$; ★★$0.001 < p < 0.01$; ★★★$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


A
A549
EKVX
Survival (%, SRB)
150
100
50
0
-50
★★
★
★★★
BPTES
5-FU
B
N.S
Gefitinib
C
Carboplatin
D
CT
BPTES
5-FU
combination
6.07
8.65
9.08
9.69
PI
Annexin v
Lung primary cell
IMR90
A549
EKVX
GLS1
β-actin
Cell death (%)
IMR90

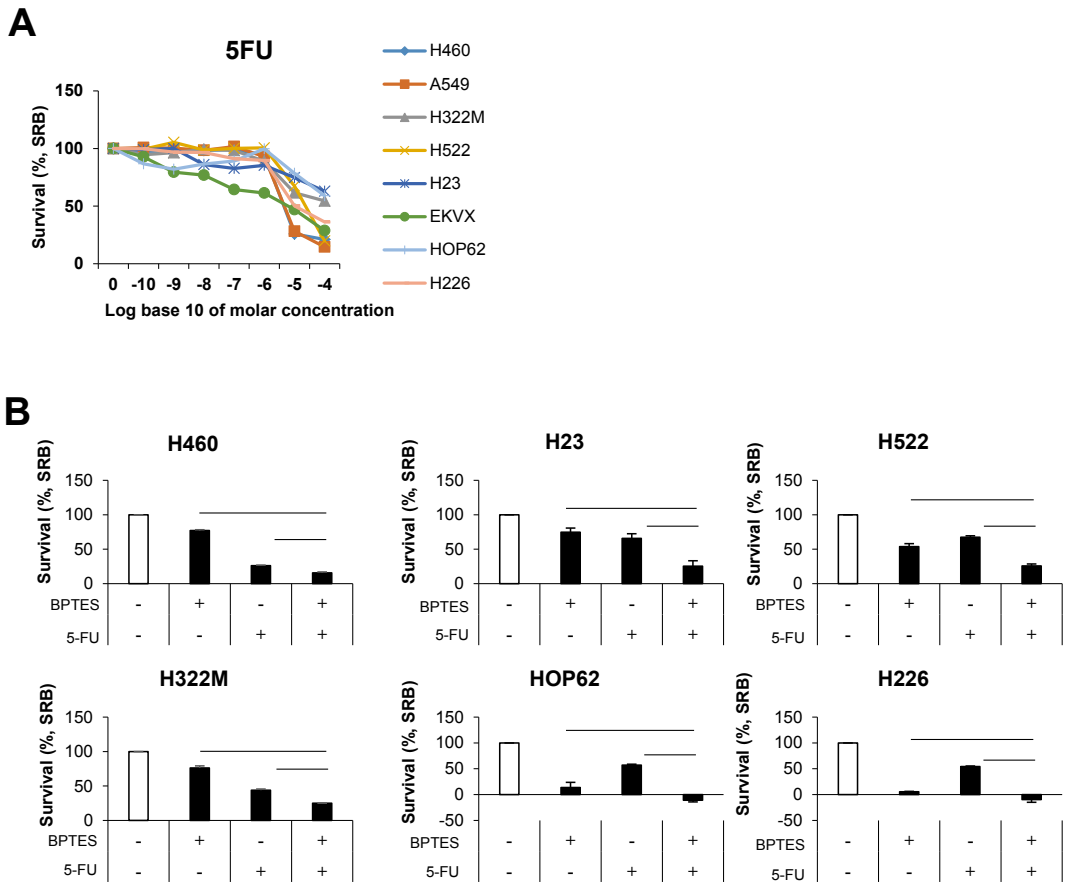


Fig. 3. Cell growth arrest is induced by combined BPTES and 5-FU treatment. NSCLC cells were treated with 10 μM of BPTES, 10 μM of 5-FU, or a combination of 10 μM of BPTES and 10 μM of 5-FU for 48 h. (A) The inhibitory effect of 5-FU on NSCLC proliferation was measured by the SRB assay after cells were treated with serial concentrations of BPTES for 48 h (B) NSCLC survival is remarkably reduced by combined BPTES and 5-FU treatment. Cell proliferation was measured by the SRB assay after cells were treated with 10 μM of BPTES alone, 10 μM of 5-FU alone, or a combination of the two for 48 h in NSCLC cell lines. *p*-Values were determined using two-tailed Student's *t*-tests (ns, not significant; ★$0.01 < p < 0.05$; ★★$0.001 < p < 0.01$; ★★★$p < 0.001$).

2. Materials and methods

2.1. Cell culture and cells with stable knock down of GLS1

All cell lines were obtained from the US National Cancer Institute (MTA 1-2702-09). All cells were incubated at 37 °C and maintained at 5% CO_2. IMR90 was obtained from ATCC and was grown in DMED medium (Hyclone, Logan, UT, USA). Cells were grown in RPMI 1640 medium (Hyclone) plus 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin, and streptomycin. GLS1 shRNA vectors were obtained from sigma-aldrich. The sequences and RNAi Consortium clone IDs for each shRNA are as follows. shGLS #1: GCA CAG ACA TGG TTG GTA TAT (TRCN0000051135), shGLS #2: GCC CTG AAG CAG TTC GAA ATA (TRCN0000051136). A549 and EKVX cells were transfected with shRNA of GLS1 and two clones of each selected with puromycin for further study.

2.2. SRB assay

SRB was performed as previously described [16]. Cells (100 μl) were inoculated into 96-well microtiter plates at plating densities ranging from 5000 to 20,000 cells/well depending on the doubling time of the individual cell line. After cell inoculation, the microtiter plates were incubated for 24 h prior to the addition of the experimental drugs. The drugs were prepared at the appropriate concentrations (10^{-10}–10^{-4} M) and 100 μl was added to each well; the plates were then incubated in CO_2 incubator. The assay was terminated by the addition of cold TCA. The cells were fixed *in situ* by gently adding 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and then air dried. Sulforhodamine B solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and the plates were then left for 10 min at room temperature. After staining, the unbound dye was removed by washing five times with 1% acetic acid; the plates were then air dried. The bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was recorded using an automated plate reader at a 515 nm.

2.3. Western blot

Western blots were performed as previously described [17]. Briefly, the cells were harvested, washed in phosphate-buffered

Fig. 2. NSCLC survival is reduced by a combination of GLS1 knockdown and 5-FU treatment. (A) Cell proliferation was measured by the SRB assay after cells were treated with 10 μM of BPTES alone, 10 μM of 5-FU alone, or combined BPTES and 5-FU for 48 h in A549 and EKVX. (B) The SRB assay was performed using 1 μM of gefitinib in A549 and EKVX. (C) The SRB assay was performed using 100 μM of carboplatin in A549 and EKVX. (D) Cell death was measured with FACS in IMR90 as a normal control using the same treatment as above. *p*-Values were determined using two-tailed Student's *t*-tests (ns, not significant; ★$0.01 < p < 0.05$; ★★$0.001 < p < 0.01$; ★★★$p < 0.001$).

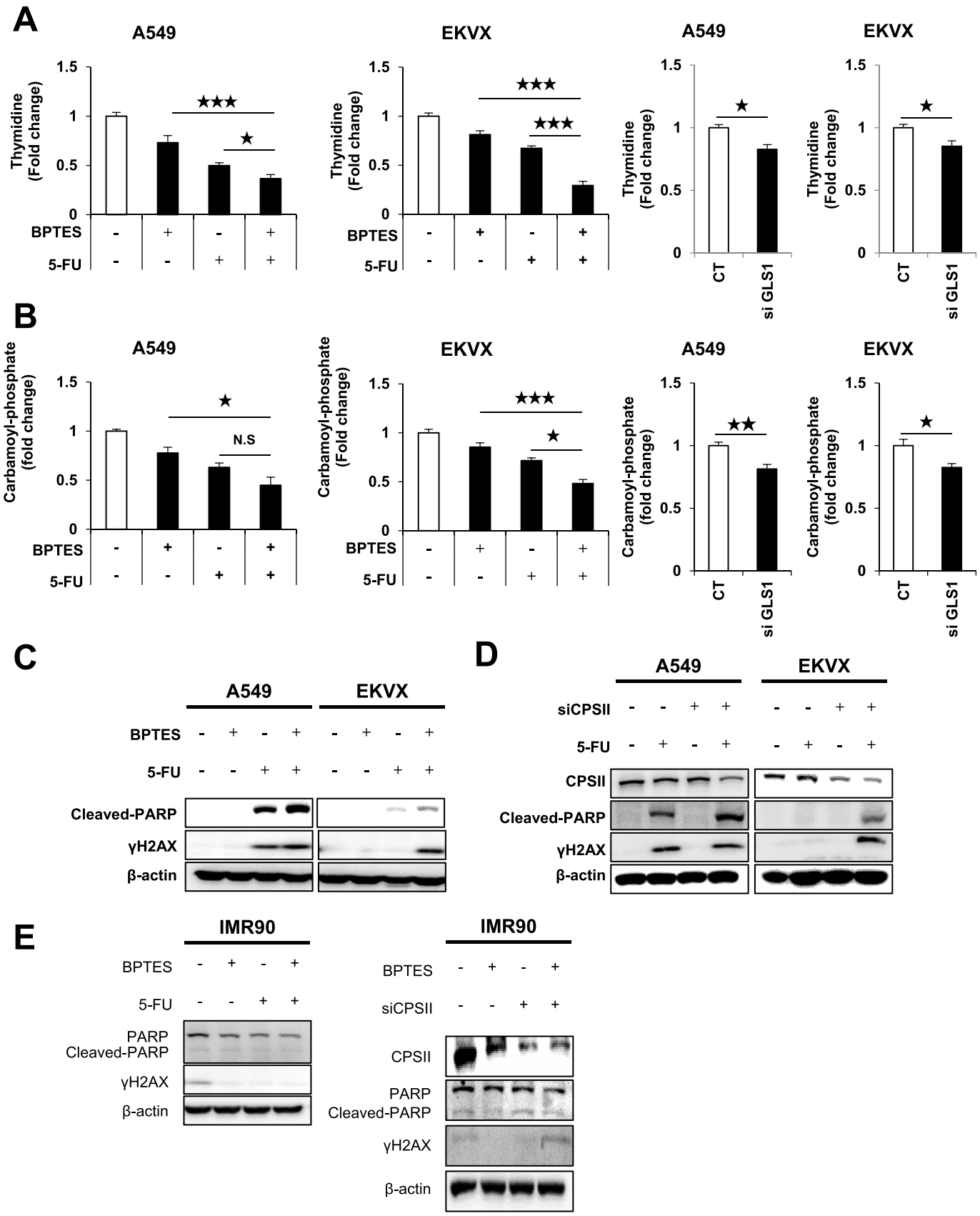


Fig. 4. Dual treatment with BPTES and 5-FU induced cell death through impairment of pyrimidine synthesis. (A) Thymidine levels were measured by LC-MS/MS after treatment with 10 μM of BPTES, 10 μM of 5-FU, a combination of the two, or siRNA of GLS1 for 48 h in A549 and EKVX. (B) Carbamoyl phosphate levels were measured by LC-MS/MS after treatment with 10 μM of BPTES, 10 μM of 5-FU, a combination of the two, or siRNA of GLS1 for 48 h in A549 and EKVX. (C) Immunoblotting of PARP and γ-H2AX were performed in A549 and EKVX after treatment of 10 μM of BPTES, 10 μM of 5-FU, or a combination for 48 h (D). Immunoblotting of PARP and γ-H2AX were performed in A549 and EKVX after

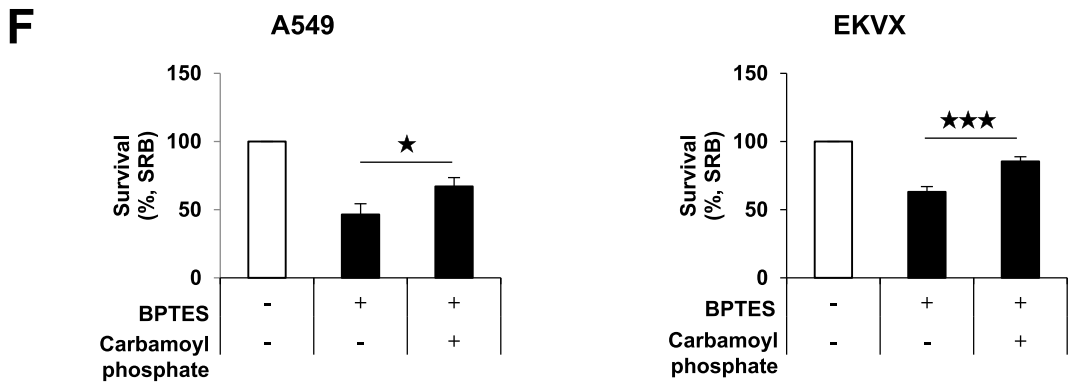


Fig. 4. (*continued*).

saline (PBS) and lysed in lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, and protease inhibitors]. The lysates were analyzed using western blot.

2.4. Clonogenic assay

Cells were plated in 60 mm plates at 1000 cells per well in 3 ml media. Media was not changed throughout the course of the experiment. After 14 days, colonies were stained with 0.005% crystal violet staining solution.

2.5. FITC annexin V apoptosis detection

The cells were incubated with or without 10 μM BPTES, 10 μM 5-FU, or both for 48 h. The cells were then collected, washed with cold PBS, centrifuged at 1400 rpm for 3 min, and resuspended in 1× binding buffer at concentration of 1 × 10^6 cells/ml. 100 μl of the solution was transferred to a 5-ml culture tube (1 × 10^5 cells), and 5 μl each of annexin V-FITC and PI were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Then, 400 μl of 1× binding buffer was added to each tube, and the samples were analyzed by FACS flow cytometry (BD Falcon).

2.6. Quantitative measurement of carbamoyl phosphate and thymidine

Standard metabolites and $^{13}C_5$ glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). U-$^{13}C_{10}$, U-$^{15}C_2$ Thymidine was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The cell number was ~1 million, and cells were harvested using 1.4 mL of cold methanol/H_2O (80/20, v/v) after sequential washing with PBS and H_2O. Then, the cells were lysed by vigorous vortexing with 80% methanol, and 100 μl of the internal standard solution was added. The internal standard solution consisted of 100 nM U-$^{13}C_{10}$, U-$^{15}C_2$ thymidine for thymidine and 5 μM $^{13}C_5$

10 μM of 5-FU treatment for 48 h following to 40 nM of CPSII siRNA treatment for 24 h. (E) Immunoblotting of CPSII, PARP and γ-H2AX were performed in IMR-90 after treatment of BPTES, 5-FU and siCPSII under the same conditions as above. (F) The effect of carbamoyl phosphate on survival was measured by treatment with 100 μM of carbamoyl phosphate together with or without 10 μM of BPTES for 48 h (G) A schematic diagram of possible synergistic inhibition of BPTES and 5-FU is suggested, which blocks pyrimidine synthesis in cancer cell growth. The role of GLS1 remains to be elucidated. *p*-Values were determined using two-tailed Student's *t*-tests (ns, not significant; ★$0.01 < p < 0.05$; ★★$0.001 < p < 0.01$; ★★★$p < 0.001$).

glutamine for the other metabolites. The metabolites were extracted from the aqueous phase by liquid–liquid extraction after adding water and chloroform. The aqueous phase was dried using a vacuum centrifuge and was reconstituted with 50 μl of 50% methanol or acetonitrile prior to LC–MS/MS analysis.

For carbamoyl phosphate and thymidine, calibration curves were generated using authentic standards, and the calibration range was 1–10,000 nM ($r^2 > 0.99$). The quantitation results were normalized to the amount of protein in each sample. For thymidine analysis, a HILIC column (HILIC plus 3.5 μm, 100 × 2.1 mm; Agilent) was used.

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test as appropriate.

3. Result

3.1. NSCLC cell lines are selectively sensitive to glutamine depletion

We tested the metabolic dependency of a cancer cell panel composed of 11 different cancer cell lines under normal nutrition media and normoxia, namely NSCLC (A549), renal (SN12C), breast (MCF7), stomach (SNU16), liver (Huh7), prostate (PC3), colon (HT29), ovarian (OVCAR4), CNS (SNB19), leukemia (RPMI8226), and melanoma (UACC257), for four types of metabolic pathways, including glycolytic inhibition using 2-deoxyglucose (2-DG), autophagy inhibition using chloroquine, fatty acid oxidation inhibition using etomoxir (an irreversible inhibitor of carnitine palmitoyltransferase-1), and glutaminolysis inhibition using glutamine-depleted media (Fig. 1). Leukemia showed selective dependency on glucose metabolism compared to other cancers (Fig. 1A), and melanoma showed selective sensitivity on autophagy inhibition (Fig. 1B). Single treatment of etomoxir did not show selective dependency among cancers (Fig. 1C). Especially A549 human lung adenocarcinoma cell line showed selective glutamine dependency for growth compared to the other cancer cells among the metabolic challenges (Fig. 1D). We tested whether NSCLC shows a common dependency on glutamine depletion or GLS1 inhibition using BPTES. Interestingly, eight different NSCLC cell lines regardless of oncogenic differences showed universal dependency on glutamine as well as GLS1 inhibition using BPTES (Fig. 1E). We tested whether GLS1 knock down induce growth arrest in colony formation assay using NSCLC cell lines such as A549 and EKVX (Fig. 1F). GLS1 knock down using siRNA of GLS1 resulted in over 50% growth arrest of colony formation in NSCLC (Fig. 1F). To verify the clonogenic assay using stable knockdown of GLS1, we have developed GLS1 stable knock down cell lines of A549 and EKVX using shRNA of GLS1 (Fig. 1G). Two sub-clones were selected in each cell line of A549 and EKVX, and GLS1 knock down was confirmed by immune-blotting. Clonogenic assay with GLS1 stable knock down resulted in over 50% growth arrest of colony formation in A549 and EKVX, which concurred with results from siRNA treatment of GLS1 (Fig. 1F).

3.2. GLS1 inhibition using BPTES combined with 5-FU potentiates cell growth arrest

It is clear that single inhibition of GLS1 reduces cancer cell growth but does not exhibit an adequate anti-cancer effect. Therefore, we explored whether BPTES has a sensitizing effect when combined with conventional lung cancer therapeutics, such as 5-fluorouracil (5-FU) as an irreversible inhibitor of thymidylate synthetase (TYMS) [18] (Fig. 2A), gefitinib as an EGFR inhibitor (see review [19]) (Fig. 2B), and carboplatin as a platinum-based antineoplastic agent (see review [20]) (Fig. 2C). Interestingly, 10 μM of BPTES showed a clear synergistic anti-cancer effect with 10 μM of 5-FU in A549 and EKVX cell lines among the three therapeutics (Fig. 2A). In comparison with the NSCLC cancer cell lines, the IMR90 normal immortalized cell line did not show any cell growth arrest or cell death induction (Fig. 2D). This suggests that BPTES treatment combined with 5-FU affects more efficiently on fast growing cancer cells such as NSCLC than on slow growing normal cells.

3.3. Combination treatment of BPTES and 5-FU significantly induces growth arrest with thymidine reduction in NSCLC

In Fig. 1E, eight different NSCLC cell lines showed significant dependency on GLS1 inhibition using BPTES. Although single treatment with either BPTES (Fig. 1A) or 5-FU (Fig. 3A) resulted in the reduction of cell growth in NSCLC, it is not sufficient to induce significant cell death. Here, a dual treatment with 5-FU and BPTES resulted in a growth reduction response not only in EKVX and A549 but also in most of the NSCLC cell lines, including NCI-H23, NCI-H522, NCI-H226, HOP62, NCI-H322M and NCI-H460, in a synergistic manner (Fig. 3B).

We tested whether GLS1 inhibition regulates pyrimidine synthesis because BPTES showed synergistic effect with pyrimidine synthesis inhibition of 5-FU. We analyzed changes in level of thymidine after BPTES treatment or GLS1 knock down in A549 and EKVX (Fig. 4A). We found that BPTES effectively reduced the level of thymidine as well as synergistically reduced the thymidine level with 5-FU treatment (Fig. 4A). Dual BPTES and 5-FU treatment reduced the thymidine levels to approximately 20%–30% compared with those in the control cells (Fig. 4A). As in Fig. 1F, GLS1 knock down using specific siRNA also reduced the thymidine levels to approximately 20% compared to the control in A549 and EKVX (Fig. 4A). In the A549 and EKVX cell lines, BPTES treatment reduced the levels of carbamoyl phosphate by 18% and 15%, whereas dual BPTES and 5-FU treatment synergistically reduced these levels by 55% and 52% compared with 5-FU treatment (Fig. 4B). GLS1 knock down using specific siRNA also reduced the level of carbamoyl phosphate to approximately 20% compared to the control in A549 and EKVX (Fig. 4B). Carbamoyl phosphate synthetase II (CPSII) is an enzyme that catalyzes reactions that produce carbamoyl phosphate in the cytosol and serves as the rate-limiting enzyme in pyrimidine synthesis [21]. To test whether GLS1 affects the level of carbamoyl phosphate as a precursor of orotate that converts to dTMP through dUMP, carbamoyl phosphate level was measured after BPTES treatment (Fig. 4B). Data revealed that BPTES reduced the level of carbamoyl phosphate as well as synergistically reduced the level combined with 5-FU treatment (Fig. 4B). 5-FU reduces thymidine synthesis through inhibiting thymidylate synthetase (TYMS). Carbamoyl phosphate synthesis is a precursor of dUMP as TYMS substrate. Therefore decrease of carbamoyl phosphate production by 5-FU treatment may be associated with cell death (Fig. 4C). Immunoblotting of poly ADP ribose polymerase (PARP) and γ-H2AX demonstrated that combined BPTES and 5-FU treatment increased the levels of cleaved PARP and γ-H2AX (Fig. 4C). This suggests that the dual treatment is associated with cell death through DNA damage. To test whether CPSII contributes to survival of NSCLC, the CPSII knock down was tested with or without 5-FU in NSCLC (Fig. 4D). Immunoblotting of PARP and γ-H2AX demonstrated that CPSII knock down combined with 5-FU treatment increased the levels of cleaved PARP and γ-H2AX (Fig. 4D). This suggests that CPSII is critical in NSCLC survival. In order to compare the effect of combination treatment or CPSII knock down on apoptosis in normal immortalized cell line, IMR90 cells were employed (Fig. 4E). Combination of BPTES and 5-FU or CPSII knock down combined

with 5-FU treatment did not affect the levels of cleaved PARP and γ-H2AX in slow growing normal cell such as IMR90 (Fig. 4E). Therefore blocking thymidine synthesis by combination of BPTES and 5-FU appears to be effective on growth arrest of fast growing cancer. To test whether GLS1 inhibition is significant in growth arrest through carbamoyl phosphate synthesis, cell growth and survival was assayed after 10 μM of BPTES was supplied to NSCLC cells together with or without 100 μM of carbamoyl phosphate for 48 h (Fig. 4F). Carbamoyl phosphate reversed the BPTES mediated decrease of NSCLC survival from about 50% to 80% of survival (Fig. 4F). This implies that BPTES plays an important role in carbamoyl phosphate synthesis in NSCLC. This suggests that BPTES and 5-FU synergistically blocks thymidine synthesis (Fig. 4G).

4. Discussion

Conventional treatment for NSCLC is a combination of 2 chemo drugs. A common first line therapeutic against NSCLC is carboplatin that interacts with DNA to interfere with DNA repair. Carboplatin is now popular in clinical treatment due to reduced side effects compared to the previous compound cisplatin [22,23]. The advanced targeted drug in NSCLC is Gefitinib (Iressa), approved by The US Food and Drug Administration for the primary treatment of NSCLC which contains specific mutations in the EGFR gene [24]. However, although NSCLC is the most common type of lung cancer, only about 10% of cases have EGFR gene mutations. Therefore, the vast majority of lung cancer patients are waiting for specific targeted drugs. Yet, elaborated sequencing of NSCLC tumors have revealed that over half a million somatic mutations cannot be grouped with common mutations. NSCLC patients are desperately looking for common therapeutic targets as a result. GLS1 levels increase substantially in NSCLC, and this increase is inversely correlated with the overall survival rate in NSCLC (data not shown). Here we found that GLS1 knockdown or inhibition using BPTES induces cell death, which potentiates an anti-cancer effect when it is combined with TYMS inhibition using 5-FU in NSCLC.

Blocking the glutamate supply with BPTES alone was not sufficient to induce critical cell death. However, dual BPTES and 5-FU treatment demonstrated a great synergistic effect in cell death induction. We propose that the synergistic mechanism of the dual therapy against GLS1 and TYMS is connected with the inactivation of CPSII. The activation of CPSII depends on ATP levels [25] and requires two ATPs for the catalysis of carbonate to carbamoyl phosphate. Therefore, GLS1 induced decrease of carbamoyl phosphate synthesis may be associated with ATP level connected with glutamate metabolism. The upstream regulation of CPSII by GLS1 remains to be answered in the near future (Fig. 4G).

It is established theory that the increased levels of GLS1 can be used in anaplerotic reactions in cancer [26,27]. The rapid turnover of glutamate leads to the synthesis of nucleotides and amino acids [26]. Some groups have demonstrated that MYC regulates metabolic gene expression via glycolytic genes including lactate dehydrogenase A and glutaminolysis genes including GLS1 [28]. This suggests that the oncogene activated glutaminolysis supports a significant proportion of the biosynthetic needs of the cells. However, MYC mutation or activation significantly correlates neither with the increase of GLS1 in NSCLC.

In summary, inactivation of CPSII through GLS1 inhibition may synergize DNA damage with 5-FU that blocks pyrimidine synthesis in proliferative cancer cells.

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