Guggulsterone inhibits prostate cancer growth via inactivation of Akt regulated by ATP citrate lyase signaling

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Keywords: Guggulsterone; prostate cancer; Apoptosis; Akt, ATP citrate lyase; xenografts

Received: *April 24, 2014* ***Accepted***: *June 24, 2014* ***Published***: *June 26, 2014*

ABSTRACT

We have shown previously that Gugggulsterone (Gug) inhibits growth of cultured LNCaP and PC-3 human prostate cancer cells by causing apoptosis induction in association with reactive-oxygen species (ROS)-dependent activation of c-Jun N-terminal kinase (JNK). The present study builds upon the novel observations and now reveals a novel mechanism of Gug-anticancer activity that ATP citrate lyase (ACLY)-regulated Akt inactivation is involved in Gug-mediated inhibition of prostate cancer growth. Oral gavage of Gug significantly retarded the growth of PC-3 xenografts in athymic mice without causing weight loss and any other side effects. The Gug-induced apoptosis was associated with remarkably down-regulation of Akt and ACLY in both cancer cells and xenografts tumor tissue of Gug-treated group. Ectopic expression of constitutively active Akt conferred significant protection against Gug-mediated apoptotic cell death in both cancer cells. Moreover, the Gug-induced apoptosis, and Akt and ACLY inactivation in PC-3 and LNCaP cells was intensified by siRNA-based knockdown of ACLY protein level and by pharmacological inhibition of ACLY, or was protected by the ectopic expression of ACLY. In conclusion, the present study reveals a novel mechanism of Gug-anticancer activity that Gug-inhibited prostate cancer growth is regulated by ACLY/Akt signaling axis.

INTRODUCTION

Despite advances in surgery, radiation, medical management and screening, prostate cancer remains a leading cause of morbidity and mortality in men and has significant treatment-associated complications [1]. Chemoprevention has the potential to decrease the morbidity and mortality of many types of cancer including prostate cancer [1-4]. Natural products have received increasing attention in recent years for discovery of novel cancer chemopreventive/therapeutic agents [2-4]. Guggulsterone [Gug, 4,17(20)-pregnadien-3,16-dione], a plant sterol derived from the gum resin (guggulu) of the Indian Ayurvedic medicinal plant *Commiphora mukul*, has been safely used for the treatment of different disorders since ancient times [5]. Previous research in our laboratory as well as by others identified Gug (Z- and E-type) as a promising agent against cancer [5-21]. The studies have shown that Gug has the potential to be used for cancer chemoprevention and chemotherapy [5-20]. We have shown previously that the Z-Gug inhibits the growth of PC-3, DU145 and LNCaP human prostate cancer cells in culture by causing apoptosis [9-12]. Interestingly, a normal prostate epithelial cell line (PrEC) is significantly more resistant to growth inhibition and apoptosis induction by Z-Gug compared with prostate cancer cells [9-11]. We also reported that Z- and E-Gug inhibits angiogenic features

(capillary-like tube formation and/or migration) of human umbilical vein endothelial cells (HUVEC) and DU145 human prostate cancer cells *in vitro* [12]. Furthermore, oral gavage of Z-Gug to male nude mice (five times/week) inhibits *in vivo* angiogenesis in DU145-Matrigel plug assay [12]. Antiproliferative and/or apoptosis-inducing effects for Gug have also been documented in other cell types including human lung, head and neck, acute myeloid leukemia, and breast cancer cells [13-21]. Even though the mechanisms underlying growth inhibitory effect of Gug against cancer are not fully understood, but several signaling molecules have been shown to be targets for the anticancer activity of Gug [5-21]. Gug is an antagonist of farnesoid X receptor [6, 20] and increases the transcription of bile salt export pump and regulates cholesterol homeostasis [6, 20]. The Z-Gug–induced cell death in PC-3 cells was not influenced by Bcl-2 protein level but correlated with the induction of proapoptotic multidomain Bcl-2 family members Bax and Bak [9-11]. The studies from our laboratory have revealed that the Z-Gug–induced apoptosis in human prostate cancer cells is initiated by reactive oxygen intermediate–mediated activation of c-*Jun* NH_2-terminal kinase [9-11]. Shishodia and Aggarwal [8, 16] have shown that Gug suppresses the activation of constitutive and inducible nuclear factor-κB (NF-κB), a transcription factor, induced by cigarette smoke condensate, tumor promoters (phorbol myristate acetate and okadaic acid), hydrogen peroxide and cytokines (interleukin-1β and tumor necrosis factor). Gug treatment blocked the NF-κB signaling pathway by targeting IκB kinase in intestinal epithelial cells and ameliorated acute murine colitis [8]. Gug-induced angiogenesis inhibition is involved in suppression of the VEGF-VEGF-R2-Akt signaling axis [12].

ATP Citrate Lyase (ACLY) is a key enzyme recently shown to be crucial for cancer cell metabolism [22-36]. It is a metabolic enzyme responsible for the conversion of mitochondria-derived citrate into acetyl CoA (ACC), a precursor for both fatty acid synthesis as well as mevalonate synthesis [22-36]. Both fatty acid synthesis and mevalonate synthesis are associated with cancer cell growth and transformation [24-25]. Activated ACLY signaling is associated with many human cancer types including prostate [24-25, 29-30]. It is believed that specific blockade of ACLY signaling may have therapeutic potential for human cancers [22-23, 26-36]. However, such anti-ACLY agents deemed suitable for and applicable to humans have yet to be developed.

Here, we reported the potent chemopreventive activity of Gug in a prostate cancer *in vivo* model. Further mechanistic investigations reveal a novel anti-cancer mechanism of Gug by inhibiting ACLY/Akt signaling in prostate cancer models.

RESULTS

Z-Gug inhibited the growth of PC-3 xenografts in athymic mice and increased apoptosis in tumors

We have shown previously that Z-Gug treatment resulted in a significant inhibition of human prostate cancer cell PC-3, LNCaP and DU145 growth *in vitro* [9-12]. Oral gavage of Z-Gug treatment (3 μmol per mouse, 5 times/week) inhibited *in vivo* angiogenesis in matrigel plug assay [12]. We raised a question of whether Z-Gug inhibits the growth of PC-3 xenografts in anthymic mice. We tested the effect of Z-Gug administration by oral gavage (3 μmol per mouse, once a day through Monday to Friday) on PC-3 xenograft growth in athymic mice. The present data strongly suggest that Z-Gug administration significantly prevented PC-3 xenograft growth without causing any side effects to the mice. The conclusion is based on our experimental results: (a) the average tumor volume in mice treated with 3 μmol Z-Gug was significantly lower than that in vehicle-treated control mice throughout the experimental protocol. For example, 31 days after treatment commenced, the average tumor volume in the Z-Gug-treated mice (266 ± 26 mm^3) was approximately 31% of the average tumor volume in the control mice (863 ± 59 mm^3, Fig. 1A); (b) the tumor weight of the control group was 615 ± 43 mg, which was much heavier than that of the Z-Gug treatment group (185 ± 23 mg, Fig 1B. resulted in a significant reduction of compared with the control group); (c) the average body weight of the control and Z-Gug-treated mice did not differ significantly throughout the experimental protocol (Fig. 1C); and (d) the Z-Gug treated mice appeared healthy and did not exhibit impaired movement and posture, indigestion, or areas of redness or swelling. Because Z-Gug induced apoptotic cell death in human prostate cancer PC-3 and LNCaP cells [9-10], we therefore examined the tumor tissues from control and Z-Gug-treated mice to determine whether Z-Gug-mediated inhibition of PC-3 xenograft growth *in vivo* is due to increased apoptosis. As shown in Fig. 1D, Z-Gug treatment caused a statistically significant increase in cleaved PARP and cleaved Caspase 3 immunoreactive bands compared with the control group. The present study indicated that Z-Gug induced apoptosis in human prostate cancer both *in vitro* and *in vivo*.

Z-Gug treatment caused inactivation of Akt in tumors and in prostate cancer cells

Akt is highly activated in human cancers including prostate cancer [40-42]. Our previous study has revealed that Z-Gug inhibited angiogenesis *in vitro* and *in vivo* by suppression of Akt signaling [12]. Gugulipid, derived from the Indian Ayurvedic medicinal plant *Commiphora mukul*

(containing 3.75% Z-Gug), significantly down-regulated Akt signaling pathway in human prostate cancer LNCaP cells [11]. We showed that Z-Gug has the potential to inhibit prostate cancer growth both *in vitro* [refs. 9-10] and *in vivo* (Fig. 1). Therefore, we speculated about whether Z-Gug treatment would result in a down-regulation of Akt protein in the PC-3 xenografts. The levels of Akt and ^{S473}phosphor-Akt proteins in tumors from control and Z-Gug-treated mice were determined by immunoblotting (Fig. 1D). Although the protein level of total Akt was comparable to that in the tumors of the Z-Gug-treated and control mice, the protein level of ^{S473}phosphor-Akt was significantly lower in tumors from Z-Gug-treated mice than that of control tumors (Fig. 1D and Fig. S1). These results indicated that Z-Gug-mediated suppression of PC-3 xenograft growth *in vivo* was accompanied by inactivation of Akt. Next, we tested whether Z-Gug can decrease the level of Akt protein in human prostate cancer cells. As shown in Fig. 2A-B, treatment with Z-Gug at 20 or 40 μM resulted in a significant decrease of the protein levels of Akt and ^{S473}phosphor-Akt in human prostate cancer PC-3 and LNCaP cells compared to those in DMSO-treated (control) cells. These observations clearly indicated that Z-Gug treatment resulted in the inhibition of Akt signaling in human prostate cancer cells.

The levels of ^{S473}phosphor-Akt protein were increased in human prostate cancer

We collected a total of 8 pairs of primary human prostate cancer samples with adjacent normal prostate tissues. Immunoblotting for Akt and ^{S473}phosphor-Akt

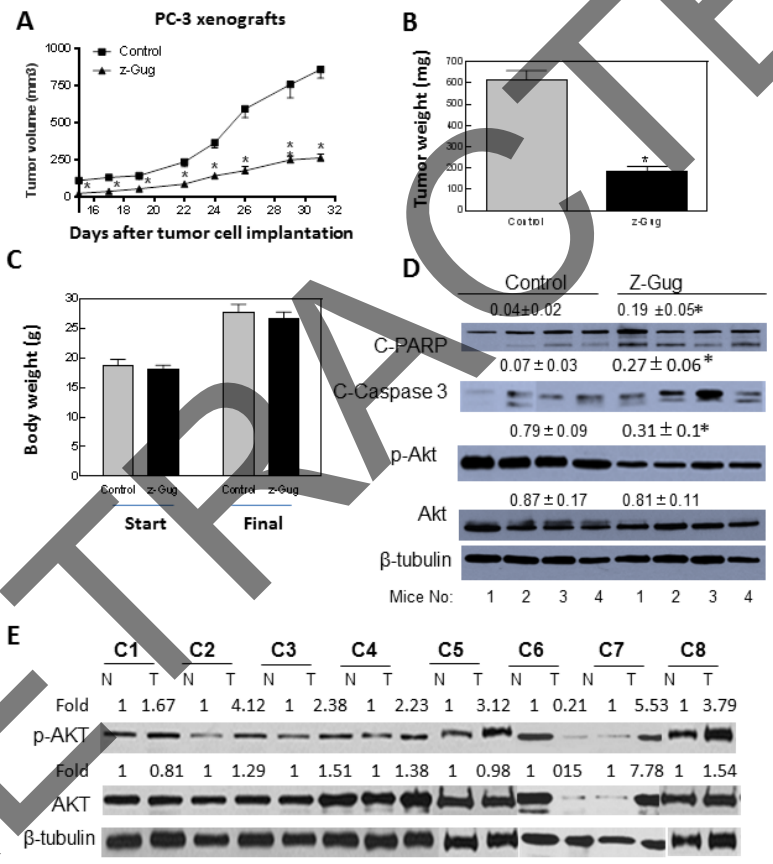


Figure 1: Average tumor volume (A), tumor weight (B) and body weight (C) in vehicle-treated control mice and mice treated with 3 μmol Z-Gug (Monday through Friday)**.** The Z-Gug administration commenced two weeks before the tumor cell injection. Points, mean (tumor; ***n*** = 20); 10 mice per group with tumor cells injected on both left and right flank of each mouse); bars, SE. *, ***P*** < 0.05, significantly different compared with control by two-way ANOVA. The body weights of the control and Z-Gug-treated mice did not differ significantly throughout the experimental protocol. Points, mean (***n*** = 10); bars, SE. D**,** Immunoblotting for cleavage of PARP, Caspase 3, Akt and ^{S473}p-Akt using lysates from tumors of control and Z-Gug-treated mice. The blots were stripped and reprobed with anti-β-tubulin antibody to correct for differences in protein loading. Densitometric scanning data for these protein levels in tumors from control and Z-Gug-treated mice were shown on top of the immunoreactive bands. Tumor tissues from four mice of each group were used for immunoblotting. Columns, mean (***n*** = 4); bars, SE. *, ***P*** < 0.05, significantly different compared with control by paired ***t*** test. E. Prostate cancer clinical cases with an increase in ^{S473}phospho-Akt protein. Human prostate cancer samples paired with tumor tissue (shown as T) and adjacent normal tissue (shown as N) were lysed. The S473phospho-Akt and Akt protein levels were compared against β-tubulin under immunoblotting. The numbers on top of the immunoreactive bands represent change in levels relative to adjacent normal tissue.

protein levels was performed and the results were shown in Fig. 1E. 7 of 8 pairs showed a significant increase of ^{S473}phosphor-Akt protein and 6 of 8 pairs showed an increase of Akt protein using β-Tubulin as a loading control (Figs. 1E). These data indicate that Akt signaling pathway is an important biomarker for prostate cancer diagnosis.

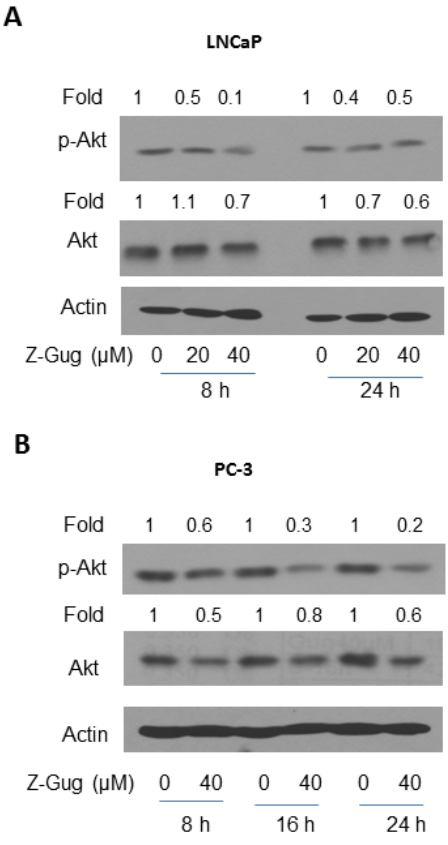
Akt signaling affected the Z-Gug-mediated apoptosis induction and growth inhibition of human prostate cancer cells

To firmly establish the role of Akt in our model, we investigated the effect of overexpression of CA-Akt on Z-Gug-mediated growth inhibition and apoptosis induction using PC-3 and LNCaP cells. Overexpression of CA-Akt in transfected PC-3 and LNCaP cells was confirmed by immunoblotting using antibodies against ^{S473}phospho-Akt (Fig. 3A and B) and total Akt (data not shown). Cells transfected with the empty vector were used as control (Fig. 3A and B). The levels of ^{Ser473}phospho-Akt were markedly higher in PC-3 and LNCaP cells transfected with CA-Akt compared with empty vector–transfected control cells (Fig. 3A and B). Similar as Z-Gug-treated PC-3 and LNCaP cells [9-10]; Z-Gug treatment resulted in a significant increase of apoptosis induction (Fig. 3B, PC-3; Fig. 3E, LNCaP) and growth inhibition (Fig. 3C, PC-3; Fig. 3F, LNCaP) of the empty vector-transfected cells. As expected, ectopic expression of CA-Akt potentiated PC-3 and LNCaP cell growth (Fig. 3C and F) and inhibition of apoptosis (Fig. 3 B and E) when compared with empty vector–transfected control cells. The Z-Gug-mediated induction of apoptotic cell death and inhibition of cell growth were statistically significantly attenuated by ectopic expression of CA-Akt compared with empty vector–transfected control cells (Fig. 3). For further providing the evidences of Akt regulation in Z-Gug-mediated cancer cell death, we tested whether knockdown of Akt in these cells affects the Z-Gug-induced apoptosis and growth inhibition. To do this, the PC-3 and LNCaP cells were transient transfected with control-siRNA (control) and Akt-siRNA. After 24 h of the transfection, these cells were treated with Z-Gug 40 μM or DMSO (control group) for 24 h. The higher levels of apoptotic cell death and lower cell viability were observed in Akt-siRNA transfected LNCaP (Fig. S2) and PC-3 (Fig. S3) cells when compared to those of the control-siRNA transfected cells. More importantly, knockdown of Akt signaling in these cells resulted in an enhanced effect of the apoptosis induction and growth inhibition caused by Z-Gug (Figs. S2-3). These results strongly indicate that Akt plays a critical role in Z-Gug-mediated inhibition of prostate cancer cell growth.

Figure 2: Immunoblotting analysis of ^{S473}phospho-Akt and Akt protein levels was performed using lysates from Z-Gug-treated or DMSO-treated LNCaP (A) and PC-3 (B) cells. The blot was stripped and reprobed with-anti-actin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control. Immunoblotting for each protein was performed at least twice using independently prepared lysates.

Z-Gug down-regulated ACLY signaling pathway in prostate cancer cells and tumors

Activated ACLY signaling is associated with many human cancer types including prostate cancer [22-36]. Inhibition of ACLY signaling can suppress tumor cell growth and induce apoptosis [22-36]. Our more recent published study [23] indicated that Cucurbitacin B, a bioactive compound from cucumber, inhibited prostate cancer growth *in vitro* and *in vivo* through inactivation of ACLY. Therefore, we tested whether Z-Gug treatment would result in a down-regulation of ACLY protein in the PC-3 xenografts and PC-3 and LNCaP cells. As shown in Figs. 4D and S1, the protein level of phosphor-ACLY was significantly lower, in tumors from Z-Gug-treated mice than in control tumors. The protein level of total ACLY in the tumors of the Z-Gug-treated was lower but not reach the statistics significances



compared to that in the tumors of control mice (Fig. 4D). These data suggested that ACLY may regulate the Z-Gug's anticancer action in PC-3 xenograft model. We confirmed the role of ACLY in the Z-Gug-mediated cell death of prostate cancer by *in vitro* experiment using PC-3 and LNCaP cells (Fig. 4B). The treatment with Z-Gug at 20 or 40 μM resulted in a significant decrease of the protein levels of phospho-ACLY and its target phospho-ACC in PC-3 and LNCaP cells compared to those in DMSO-treated (control) cells (Fig. 4B). For example, the phospho-ACLY levels in PC-3 and LNCaP cells treated for 24 h with 40 μM of Z-Gug were decreased by approximately 70 %, respectively, compared to the levels in the control group (Fig. 4B). These observations clearly indicated that Z-Gug treatment resulted in the inhibition of ACLY signaling in human prostate cancer cells. To further investigate the role of ACLY in prostate cancer; we determined the ACLY and phospho-ACLY protein levels in the pairs of primary human prostate cancer samples with adjacent normal prostate tissues (Fig. 4A). The results revealed that phospho-ACLY protein levels are significant up-regulation (7/8 cases) in the human prostate cancer samples compared to the adjacent normal prostate tissues (Fig. 4A). The data suggest that phospho-ACLY may be potential biomarker for prostate cancer diagnosis.

Inhibition of ACLY resulted in increase of cell death mediated by Z-Gug and ACLY signaling mediated the inactivation of Akt caused by Z-Gug in prostate cancer cells

Our previous study has shown that HT, a competitive inhibitor of ACLY, can induce apoptosis and growth inhibition in prostate cancer cell PC-3 and LNCaP [23]. ACLY has been reported to be an important mediator for Akt signaling in lung cancer [31]. The present data strongly indicate that the Akt signaling is involved in the Z-Gug-induced apoptotic cell death (Figs. 1-3, and S2-3). To confirm that the inhibition of ACLY and the signaling

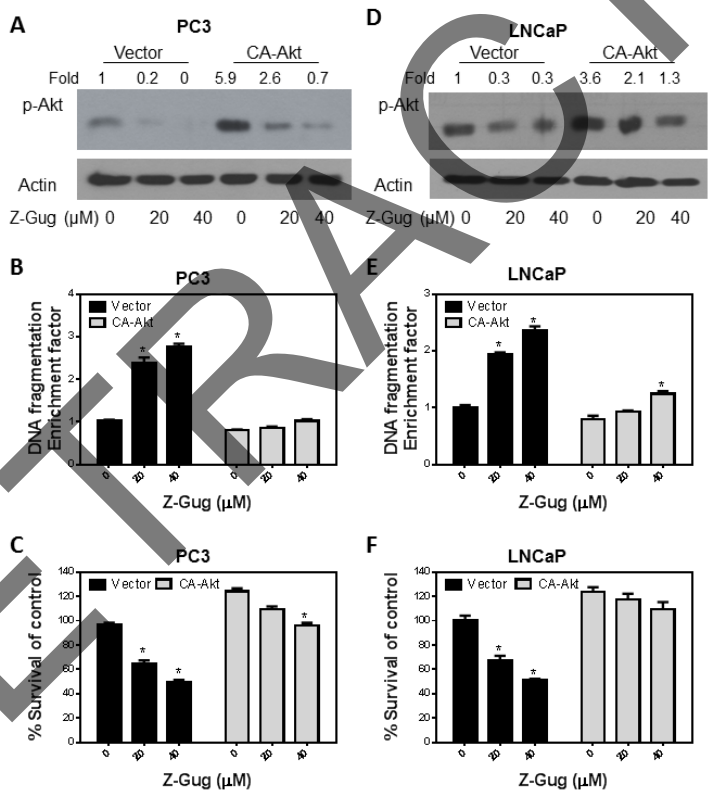


Figure 3: Immunoblotting for ^{S473}phospho-Akt using lysates from PC-3 (A) and LNCaP (D) cells transiently transfected with empty vector or expression vector encoding constitutively active Akt and treated for 24 h with DMSO or 20 or 40 μmol/L Z-Gug. The blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent changes in protein levels relative to DMSO-treated empty vector–transfected cells. Cytoplasmic histone-associated DNA fragmentation in PC-3 (B) and LNCaP (E) cells, and percent survival in PC-3 (C) and LNCaP (F) cells transiently transfected with empty vector or expression vector encoding constitutively active Akt and treated for 24 h with DMSO or 20 or 40 μmol/L Z-Gug. The results of cytoplasmic histone-associated DNA fragmentation are expressed as enrichment factor relative to DMSO-treated control cells transiently transfected with empty vector. Each experiment was done twice, and representative data from a single experiment are shown. Columns, mean (n = 3); bars, SE. *significantly different (P<0.05) between the indicated groups by one-way ANOVA followed by by Dunnett's test.

of ACLY/Akt are involved in Z-Gug-mediated inhibition of cell growth and induction of apoptosis in human prostate cancer cells, we firstly tested the effect of HT on the Z-Gug-mediated down-regulation of ACLY and Akt as well as cell death. HT treatment at 20 mM resulted in a significant decrease of phospho-ACLY and ^{S473}phospho-Akt protein expression in PC-3 and LNCaP cells (Fig. 4C). Increased down-regulation of phospho-ACLY and ^{S473}phospho-Akt protein expression (Fig. 4C) and apoptosis induction (data not shown) were observed in the combination of HT and Z-Gug-treated cells compared to these of Z-Gug-treated alone cells. These data indicate that pharmacologic inhibition of ACLY signaling can enhance the anticancer activity of Z-Gug in human prostate cancer cells. To directly test the contribution of ACLY to the regulation of Z-Gug-induced apoptosis in human prostate cancer cells, we used siRNA technology. The levels of ACLY and phospho-ACLY protein were significantly decreased by upon the transient transfection of PC-3 (Fig. 5A) and LNCaP (Fig. 5B) cells with ACLY-targeted siRNA compared with cells transfected with a nonspecific control siRNA. More importantly, knockdown of ACLY in both cells resulted in a remarkable lower protein expression of ^{S473}phospho-Akt and Akt (Fig. 5A and B) and higher apoptosis induction (Fig. 5C and D) compared with these of control-siRNA-transfected cells. Collectively, these results indicate that ACLY plays an important role in Z-Gug-induced apoptosis in human prostate cancer cells, and the Z-Gug-mediated Akt inactivation was regulated by ACLY signaling axis.

Z-Gug-induced cell death was inhibited by the ectopic expression of ACLY

To firmly establish the contribution of ACLY in the Z-Gug-induced apoptosis, we generated PC-3-ACLY

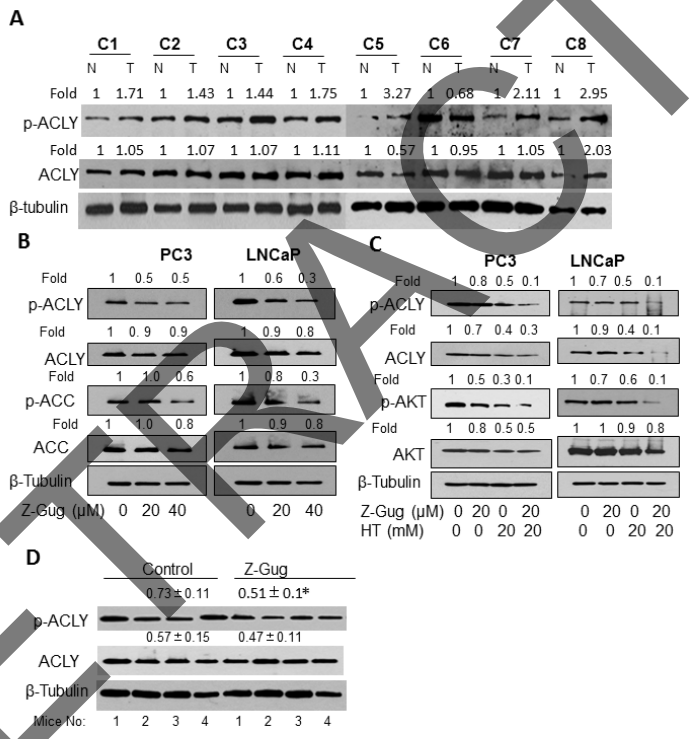


Figure 4: A. Prostate cancer clinical cases with an increase in phospho-ACLY protein. Human prostate cancer samples paired with tumor tissue (shown as T) and adjacent normal tissue (shown as N) were lysed. The phospho-ACLY and ACLY protein levels were compared against β-tubulin under immunoblotting. The numbers on top of the immunoreactive bands represent change in levels relative to adjacent normal tissue. B. Immunoblotting analysis of ACLY, phospho-ACLY, ACC and phospho-ACC was performed using lysates from Z-Gug-treated or DMSO-treated PC-3 and LNCaP cells. The blot was stripped and reprobed with-anti-β-tubulin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control. Immunoblotting for each protein was performed at least twice using independently prepared lysates. C. Immunoblotting analysis of ACLY phospho-ACLY, Ser473phospho-Akt and Akt was performed using lysates from Z-Gug-treated or DMSO-treated PC-3 and LNCaP cells with or without HT treatment. The blot was stripped and reprobed with-anti-β-tubulin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control. Immunoblotting for each protein was performed at least twice using independently prepared lysates. D. Immunoblotting for ACLY and phospho-ACLY using lysates from tumors of control and Z-Gug-treated mice. The blots were stripped and reprobed with anti-β-tubulin antibody to correct for differences in protein loading. Densitometric scanning data for these protein levels in tumors from control and Z-Gug-treated mice were shown on top of the immunoreactive bands. Tumor tissues from four mice of each group were used for immunoblotting. Columns, mean ($n = 4$); bars, SE. *, $P < 0.05$, significantly different compared with control by paired t test.

[stable cell line generation using human ACLY and the corresponding empty vector (pCMV6)] and PC-3-WT [stable cell line generation using empty vector (pCMV6)] cells. The PC-3-WT and PC-3-ACLY were characterized as Fig. S4A. ACLY overexpression led to increased *in vitro* proliferation of PC-3 cells (Fig. S4B). Our studies also showed that PC-3 and LNCaP cell growth were inhibited [23] and apoptotic cell death increased [Fig. 5C and D, ref. 23] by ACLY-targeted siRNA-transfected in the cells. Taken together, these data suggest that ACLY overexpression can facilitate oncogenic transformation in human prostate cancer cells. Eexposures of PC-3-WT cells to 20 or 40 mM Z-Gug for 24 h resulted in statistically significant increases in Caspase 3/7 activity (Fig. S5A) and in growth inhibition (Fig. S5B) over DMSO-treated control. However, the PC-3-ACLY cells are more resistance to the cell death by the Z-Gug treatment (Fig. S5). These data confirmed that the ACLY actively contributed to the Z-Gug-induced cell death of human prostate cancer.

Z-Gug treatment inhibited interaction between phosphor-ACLY and ^{S473}phospho-Akt

Next, we tested the possibility whether Z-Gug treatment affects interaction between phospho-ACLY and ^{S473}phospho-Akt. As shown in Fig. S3, the phospho-ACLY/^{S473}phospho-Akt complex was detectable in DMSO-treated control PC-3 cells as evidenced by immunoprecipitation using anti-phosphor-ACLY antibody followed by immunoblotting with the anti-^{S473}phospho-Akt antibody. The level of phosphor-ACLY/^{S473}phospho-Akt complex was decreased by ~60% on 24-hour treatment of PC-3 cells with 40 μmol/L Z-Gug (Fig. S6). These results showed that Z-Gug treatment disrupted interaction between phosphor-ACLY and ^{S473}phospho-Akt.

Z-Gug treatment caused mitochondrial ROS production in prostate cancer cells

Our previous studies have shown that Z-Gug cause apoptosis through the mediation of ROS [9-11]. It has been reported that that the inhibition of tumor growth based on ACLY inhibition is regulated by ROS generation and mitochondrial dysfunction [23, 34]. Our present results have revealed that the ACLY plays an important role in Z-Gug-induced apoptosis in human prostate cancer cells. We therefore tested whether Z-Gug-induced apoptosis was mitochondrial ROS-dependent using a cell-permeable and mitochondria-targeting chemical probe (MitoSOX Red) to measure ROS production. Z-Gug-treated PC-3 (Fig. 6A) and LNCaP (Fig.6B) cells exhibited an increase in mean MitoSOX fluorescence compared with DMSO-treated (control) cells. These observations clearly indicate that Z-Gug treatment resulted in mitochondrial ROS production in human prostate cancer cells.

NAC, an antioxidant, attenuated Z-Gug-induced mitochondrial ROS production and apoptosis in prostate cancer cells

Next, we designed experiments to determine whether Z-Gug-induced ROS generation and apoptotic cell death were attenuated by NAC, an antioxidant. The present results showed that pretreatment with NAC conferred significant protection against Z-Gug-induced ROS production (Fig. 6C and D) and apoptosis induction (Fig. 6E and F) in both cells. NAC treatment resulted in a significant inhibition of down-regulation of phosphor-

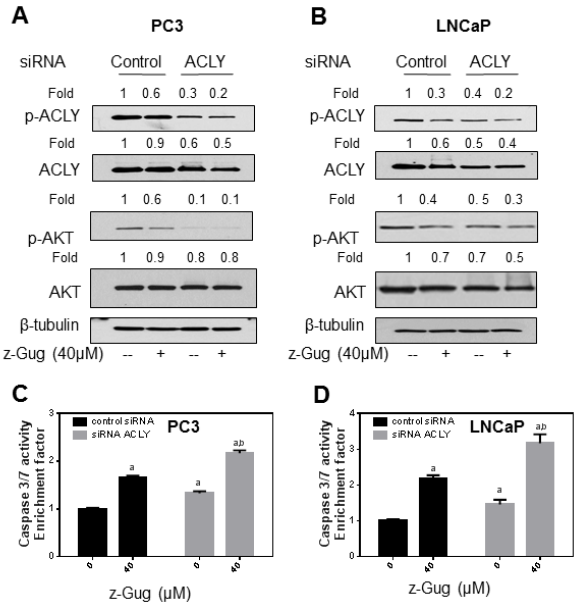


Figure 5: Immunoblotting for ACLY, phospho-ACLY, Akt and phospho-Akt using lysates from PC-3 (A) and LNCaP (B) cells transiently transfected with a control nonspecific siRNA or ACLY-targeted siRNA and treated for 24 h with DMSO or 40 μmol/L Z-Gug. The blots were stripped and reprobed with anti-β-tubulin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent changes in protein levels relative to DMSO-treated nonspecific control siRNA–transfected cells. Immunoblotting for each protein was performed at least twice using independently prepared lysates. Caspase 3/7 activity in PC-3 (C) or LNCaP (D) cells transiently transfected with a control nonspecific siRNA or ACLY-targeted siRNA and treated for 24 h with DMSO or 40 μmol/L Z-Gug. The results are expressed as enrichment factor relative to DMSO-treated control cells transiently transfected with the control nonspecific siRNA. Each experiment was done twice, and representative data from a single experiment are shown. Columns, mean (n = 3); bars, SE. *Significantly different ($^{a}P < 0.05$) compared with DMSO-treated control and ($^{b}P < 0.05$) compared with Z-Gug-treated group by one-way ANOVA followed by Dunnett's test.

ACLY and ^{S473}phospho-Akt protein expression mediated in Z-Gug-treated PC-3 and LNCaP cells (Fig. 6G and H). Taken together, these data strongly suggested that Z-Gug-induced cell death is mediated by mitochondrial ROS-dependent ACLY/Akt signaling axis inhibition.

DISCUSSION

The present study reveals that oral administration of Z-Gug results a significant prevention of human prostate cancer PC-3 xenograft growth in athymic mice without causing any side effects to the mice. Previous studies by us and others have shown that Oral gavage of Z-Gug treatment inhibited *in vivo* angiogenesis in matrigel plug assay [12] and enhanced the cetuximab activity in xenograft model of head and neck cancer [21]. Even though pharmacokinetic parameters for Z-Gug have not been determined in humans, the maximal plasma concentration of z-Gug (C_{max}) in rats was shown to be 3.3 and 18.3 μM after oral gavage with 50 mg z-Gug/kg body weight and intravenous injection with 18 mg z-Gug/kg body weight [19]. Based on these pharmacokinetic observations, it is possible that the concentrations of Z-Gug needed to inhibit cancer cell growth may be achievable in humans. In addition, Z-Gug treatment significantly induced apoptotic cell death in the Z-Gug-treated tumors (Fig 1). These data clearly suggest that the antitumor activity of Z-Gug against prostate cancer is associated with apoptosis induction.

The involvement of Akt signaling pathway in carcinogenesis and cancer prevention and therapy is well documented [12, 40-42]. An interesting observation of

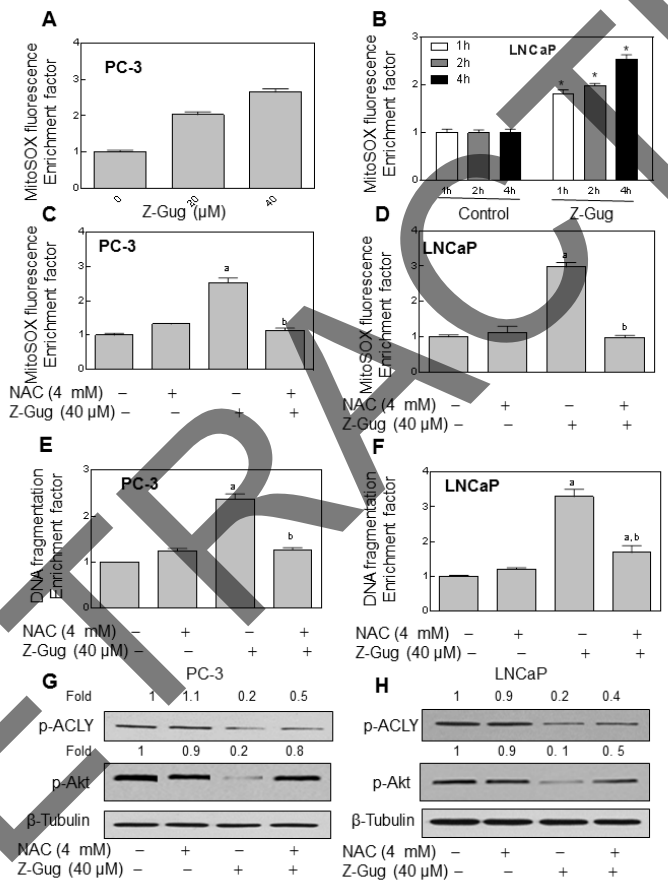


Figure 6: Anticancer effect of Z-Gug is mediated by mitochondrial ROS generation**.** Flow cytometric measurement of MitoSOX Red fluorescence in PC-3 (A) and LNCaP (B) cells treated with DMSO (control) or 20 or 40 μM Z-Gug. Results shown are mean ± S.E. total sample size is n = 4 per group. *significantly different (***P***<0.05) between the indicated groups by one-way ANOVA followed by Dunnett's test. Effect of NAC on Z-Gug (40 μM 4 h for flow cytometric measurement of MitoSOX Red fluorescence, and 24 h for cytoplasmic histone-associated DNA fragmentation in PC-3 (C and E) and LNCaP (D and F) cells treated with or without 2 h pretreatment of 4 mM NAC. The detailed treatment information was described in the section of Methods. For C to F, ***Columns,*** mean of three determinations; ***bars,*** SE. *Significantly different ($^{a}P < 0.05$) compared with DMSO-treated control and ($^{b}P < 0.05$) compared with Z-Gug-treated group by one-way ANOVA followed by Dunnett's test. Similar results were observed in two independent experiments. Representative data from a single experiment are shown. Immunoblotting analysis of ^{S473}phospho-Akt was performed using lysates from the PC-3 (G) and LNCaP (H) cells treated with or without 2 h pretreatment of 4 mM NAC. The blot was stripped and reprobed with-anti-β-tubulin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control. Immunoblotting for each protein was performed at least twice using independently prepared lysates.

the present study is that Z-Gug-mediated inhibition of cancer growth *in vitro* and *in vivo* is regulated by the Akt signaling axis (Figs. 1-3): we show that 1) Z-Gug treatment causes Akt inactivation in human prostate cancer PC-3 xenografts (Fig. 1D) and PC-3 and LNCaP cells (Fig. 2); 2) Z-Gug-mediated apoptosis induction, growth inhibition and Akt inactivation of PC-3 and LNCaP cells are statistically significantly attenuated by ectopic expression of CA-Akt (Fig. 3); 3) inhibition of Akt signaling by siRNA resulted in increase of cell death caused by Z-Gug in PC-3 and LNCaP cells (Figs. S2-3); and 4) the levels of ^{S473}phosphor-Akt protein is increased in human prostate cancer (Fig 1E).

Abnormal ACLY signaling is involved in the progress of many diseases including cancer [22-36]. Activation of ACLY signaling has been implicated in the pathogenesis of many kinds of human cancers including prostate [22-36]. The present data revealed that activated ACLY protein levels are significant up-regulation in the human prostate cancer samples compared to the adjacent normal prostate tissues (Fig. 4A). Our more recent study has shown that ACLY signaling is also involved in the prevention of prostate cancer growth *in vivo* and *in vitro* by Cucurbitacin B, a bioactive compound from cucumber [23]. It has reported recently that ACLY deficiency leads to interception of PI3K/AKT signaling to inhibit lung cancer growth [31]. We have demonstrated that z-Gug-induced apoptosis is regulated by JNKs and Akt signaling [refs. 9, 11 and the present data]. To elucidate the mechanism of Z-Gug-induced apoptosis in human prostate cancer cells, we therefore investigated the role of ACLY in the Z-Gug-induced cell death. Our present results strongly indicate that Z-Gug-induced prostate cancer cell growth inhibition and apoptosis induction are associated with inactivation of ACLY. This conclusion is based on the following observations: 1) the phospho-ACLY levels were significantly reduced in PC-3 xenograft tumors in mice treated with Z-Gug compared to those in the control mice (Fig. 4D); 2) down-regulation of the phospho-ACLY and ACLY proteins as well as its target gene ACC and phosphor-ACC proteins are observed in both prostate cancer PC-3 and LNCaP cells treated with Z-Gug compared with the DMSO-treated control cells (Fig. 4B); 3) pharmacologic down-regulation of ACLY activity by HT, a ACLY inhibitor, significantly inhibited the proliferation and induced apoptosis as well as downregulation of the protein expression of ACLY [23], and increased the inactivation of ACLY (Fig. 4C) and apoptosis induction (data not shown) by Z-Gug; 4) the inhibition of ACLY by knocked down ACLY in the cells with ACLY-siRNA resulted in a reduction of cell viability and an increase of apoptosis in human prostate cancer cells (Fig. 5); 5) the Z-Gug-induced downregulation of ACLY and induction of apoptosis were significantly enhanced by the siRNA knockdown in both cancer cell lines (Fig. 5A-D); and 6) the overexpression of ACLY significantly protected against the apoptosis induction and growth inhibition induced by Z-Gug in these cells (Figs. S4-5). Taken together, these results indicated that ACLY is the potential target for the Z-Gug-induced growth inhibition in human prostate cancer cells.

The present work indicates that either ACLY and Akt signaling pathways are involved in cellular responses to Z-Gug in our models. Crosstalk between ACLY and Akt has been reported in the progression, metastasis and drug resistance of caners including lung and colon cancers [31, 33]. Hanai et al reported that Knockdown ACLY inhibited lung cancer growth leaded to dual blockade of MAPK and PI3K/Akt pathways [31]. Our present results indeed suggest that Z-Gug-mediated inhibition of prostate cancer growth through inactivation of Akt regulated by ACLY signaling. First, the phospho-Akt protein levels were significantly reduced in PC-3 and LNCaP cells treated with ACLY inhibitor HT (Fig. 4C). HT treatment resulted in enhancement of Z-Gug-mediated downregulation of phospho-Akt protein in PC-3 and LNCaP cells. Next, down-regulation of the phospho-Akt and Akt proteins was observed in both prostate cancer PC-3 and LNCaP cells transfected with siRNA-ACLY (Fig. 5). In addition, Z-Gug treatment caused a significant reduction of interaction between phospho-ACLY and phospho-Akt in Z-Gug-treated PC-3 cells (Fig. S6). These results suggest that Z-Gug-mediated growth inhibition of prostate cancer was associated with the downregulation of ACLY/Akt signaling axis.

The inhibition of ACLY/Akt induces an anticancer effect that has been reported to be involved in mitochondrial reactive oxygen species (ROS) generation [23, 34]. The studies have shown that Z-Gug is a multitargeted cancer chemopreventive and chemotherapeutic agent [5-21]. Our studies showed that the anticancer activity of Z-Gug is involved ROS mechanism [refs. 9-11 and the present data]. Z-Gug treatment induced mitochondrial ROS generation in human prostate cancer cells (Fig. 6A and B). However, the Z-Gug-induced mitochondrial ROS production and apoptosis as well as ACLY/Akt signaling inhibition were blocked by NAC treatment (Fig. 6). These results suggest that mitochondrial ROS-dependent ACLY/Akt signaling is involved in Z-Gug-mediated inhibition of cancer growth in our models.

CONCLUSION

Our present study reveals that Z-Gug is a potent inhibitor of prostate cancer growth. The Z-Gug-mediated antitumor activity is associated with mitochondrial ROS-dependent apoptotic cell death and is regulated by ACLY/Akt signaling axis.

MATERIALS AND METHODS

Reagents

The z-(trans) isomer of guggulsterone (Z-Gug) was purchased from Steraloids. Reagents for cell culture including the medium, penicillin and streptomycin antibiotic mixture, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). The enzyme-linked immunosorbent assay kit for quantitation of cytoplasmic histone-associated DNA fragmentation was from Roche Diagnostics (Mannheim, Germany). The Caspase-Glo®3/7 activity assay kit, CellTiter-Glo® luminescent cell viability assay kit and RNase were procured from Promega (Madison, MI). The antibodies against Cleaved-poly(ADP-ribose) polymerase (PARP), Cleaved-Caspase 3, Akt, ^{S473}phospho-Akt, ACLY, phospho-ACLY, ACC, phospho-ACC and GAPDH were purchased from Cell Signaling (Danvers, MA), the antibody against β-Tubulin was from Sigma-Aldrich (St. Louis, MO). Negative nonspecific control-siRNA was from QIAGEN (Valencia, CA). Akt- and ACLY-targeted siRNA were from Life Technologies (Grand Island, NY). Lipofectamine 2000 was from Invitrogen (Grand Island, NY). pCMV6 and pCMV6-ACLY were from OriGene Technologies, Inc (Rockville, MD). Hydroxycitrate tribasic (HT) and N-acetyl-L-cysteine (NAC) were from Sigma-Aldrich (St. Louis, MO). MitoSOX Red and propidium iodide were from Molecular Probes (Eugene, OR). Protease inhibitor cocktail tablets and phosphatase inhibitors cocktail tables were from Roche (Indianapolis, IN).

Cell culture

Monolayer cultures of LNCaP and PC-3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI1640 medium supplemented with 10% (v/v) FBS and antibiotics. Each cell line was maintained in an atmosphere of 95% air and 5% CO_2 at 37^{0}C.

Xenograft study

Male athymic mice (5 weeks old) were purchased from Taconic (Germantown, NY) and housed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. The use of mice for studies described herein was approved by the IACUC (IACUC Protocol No: 1107378). The nude mice were randomized into 2 groups of 10 mice per group: PBS (control) and 3 μmol Z-Gug in corn oil per day. The groups of mice were orally gavaged with 0.1 ml of vehicle (PBS in corn oil, control group) or Z-Gug in 0.1 ml of corn oil five times (from Monday to Friday) per week for 2 weeks. Then, exponentially growing PC-3 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA) and a 0.1-ml suspension containing 3×10^6 cells was injected subcutaneously on both the left and right flanks of each mouse (n = 20). Experimental mice were continually treated by oral gavage with 0.1 ml of vehicle (PBS in corn oil, control group) or Z-Gug in 0.1 ml of corn oil 5 times (from Monday to Friday) per week. Tumor growth was monitored 3 times (Monday, Wednesday and Friday) per week as described previously by us [23]. The body weights of the control and drug-treated mice were recorded prior to the start of the experiment and every week thereafter. The mice from each group were also monitored for other symptoms of side effects including food and water withdrawal and impaired posture or movement. The mice in both the control and Z-Gug-treated groups were sacrificed at the 31 days after the tumor cell implantation. The end-point of the study was set for when the tumors reached approximately 1000 mm^3 in size. At the termination of the experiment, in accordance with the IACUC guidelines, the mice of each group were sacrificed by CO_2 inhalation. Tumors from each mouse from every group were carefully dissected out at the time of sacrifice, weighed, and frozen in liquid nitrogen, stored at -80°C, and used for immunoblotting of various proteins.

Immunoblotting

The cells and the frozen tumor tissue were lysed as described by us previously [37-38]. The lysate proteins were resolved by 6-12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto membrane. Immunoblotting was performed as described in our previous studies [37-39]. The blots were stripped and re-probed with an anti-β-tubulin or anti-GAPDH antibody to correct for differences in protein loading. Changes in protein level were determined by densitometric scanning of the immunoreactive band and corrected for the β-tubulin or GAPDH loading control. Immunoblotting for each protein was performed at least twice using independently prepared lysates to ensure reproducibility of the results.

Analysis of human PCa samples.

Eight-pair of samples (the human prostate cancer and normal adjacent prostate specimens from eight patients) were obtained from the Health Sciences Tissue Bank, University of Pittsburgh Medical Center, University of Pittsburgh with institutional review board approval. Protein isolation was done on freshly frozen material as described as us previously [37-39]. Akt, phospho-Akt, ACLY and phospho-ACLY were assessed by immunoblotting [37-39].

Ectopic Expression of Constitutively Active Akt

PC-3 and LNCaP cells were transiently transfected with pCMV6 vector encoding constitutively active Akt-1 (Myr-Akt1-HA; kindly provided by Dr. Daniel Altschuler, University of Pittsburgh, PA) or empty vector using Fugene 6 transfection regent (Roche Applied Science). Briefly, cells were seeded in six-well plates at a density of 2×10^5 cells/mL and allowed to attach by overnight incubation. Cells were transfected with the expression vector encoding constitutively active Akt or empty vector. Twenty-four hours after transfection, the cells were treated with 20 or 40 μmol/L of Z-Gug or DMSO (control) for 24 h and processed for immunoblotting of total or phosphorylated Akt levels, apoptosis and cell survival assay.

Detection of apoptosis

Apoptosis induction was assessed by analysis of cytoplasmic histone-associated DNA fragmentation, analysis of Caspase 3/7 activity and immunoblotting analysis of cleavage of PARP as described as our previous studies [23, 37].

Cell survival assays

The effect of Z-Gug on cell viability was determined by the following CellTiter-Glo® luminescent cell viability assays as described as us previous [23].

RNA interference of Akt and ACLY

The cells (1×10^5) were seeded in six-well plates and allowed to attach by overnight incubation. The cells were transfected with 200 nmol/L of control non-specific siRNA or Akt- or ACLY-targeted siRNA using lipofectamine as described by our previous studies [23, 37]. Twenty-four hours after transfection, the cells were treated with DMSO (control) or 40 μmol/L Z-Gug for 24 h. The cells were collected, washed with phosphate-buffered saline (PBS), and processed for immunoblotting or analysis of Caspase 3/7 activity and cell viability as described in our previous studies [23].

Stable cell line generation

PC3 Cells were transfected with human ACLY and the corresponding empty vector (pCMV6) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Stable cell lines were generated by subjecting cells to antibiotic selection with 1 mg/ml G418 (Life technologies, Grand Island, NY) 48 h post-transfection. Selection was typically completed within 14 days post-transfection and clones were pooled and then maintained in 400 μg/ml G418. Note that clonal cell lines were not subsequently selected from this stable population to avoid clonal variation.

Analysis of interaction between p-ACLY and p-Akt

Aliquots containing 300 μg of total lysate protein or mitochondria-enriched fractions from PC-3 cells treated with DMSO (control) or 40 μmol/L Z-Gug (24h treatment) were incubated overnight at 4°C with 3 μg of anti-phospho-ACLY antibody. Protein G–agarose beads (50 μL; Cell Signaling, Danvers, MA) were then added to each sample and the incubation was continued for an additional 3 h at 4°C. The immunoprecipitates were washed five times with lysis buffer and subjected to SDS-PAGE followed by immunoblotting using anti-^{S473}phospho-Akt antibody as described as us previously [37].

Measurement of mitochondrial ROS production

The mitochondrial ROS generation was assessed by flow cytometry after staining with MitoSOX as described in our previous study [23, 38-39]. Briefly, cells were plated, allowed to attach overnight, and treated with DMSO (control) or Z-Gug at 20 or 40 μmol/L for 1 h. Control and treated cells were rinsed with Hank's balanced salt solution supplemented with magnesium and calcium and treated with 5 μm MitoSOX Red for 30 min at 37 °C. The cells were collected by trypsinization, washed with cold phosphate-buffered saline (PBS), resuspended in Hank's solution containing 1% bovine serum albumin (BSA), and used for flow cytometric analysis.

Statistical analysis

Statistical significance of difference in the measured variables between control and treated groups was determined by t-test or one-way ANOVA. Differences were considered significant at $P<0.05$.

Disclosure of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y Gao, Y Zeng, GQ Jiang, D Xiao

Development of methodology: Y Gao, Y Zeng , J Tian, MS Islam, GQ Jiang, D Xiao

Acquisition of data: Y Gao, Y Zeng , J Tian, MS Islam, GQ Jiang, D Xiao

Analysis and interpretation of data: Y Gao, Y Zeng , J Tian, MS Islam, GQ Jiang, D Xiao

Writing and review of the manuscript: Y Gao, Y Zeng , J Tian, MS Islam, GQ Jiang, D Xiao

Study supervision: GQ Jiang, D Xiao

ACKNOWLEDGMENTS

The authors thank Drs. Joel Nelson and Zhou Wang of Department of Urology and Department of Urology Research for their support.

Grant Support

The research related to this article is supported by the National Institutes of Health National Cancer Institute and Office of Dietary Supplements Grant RO1-CA157477 to DX.

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