Drug Repurposing of Asparaginase and Vitamin C Targeting Glutamine Synthetase Improves Anticancer Effect in Metastatic Castration-resistant Prostate Cancer

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Abstract

Background: Although in North America or Europe early dignosis of prostate cancer could sucessfully improves the therapeutic outcome. However, about 70-80% of patients still suffer from metastatic castration-resistant prostate cancer (mCRPC), because of the disproportionate medical care in China. Lutetium-177 (Lu-177) or Radium-223 (Ra-223) has been suggested as the most effective therapy for mCRPC. Unfortunately, they are either not been approved in a few countries or too expensive for patients with the financial issue. Drug repurposing has been recognized as a cost-effective and relatively low-risk alternative, gains a lot interesting recently. In this study, we explored the combined treatment with asparaginase (ASNase) and/or vitamin Cas an alternative therapeutic option for mCRPC management.

Methods: Prostate cancer cell lines PC3 and DU145 were used to observe the therapeutic effect of ASNase and/or vitamin C on mCRPC in vitro and in vivo. Change of cell proliferation, cell death as well as expression of glutamine synthetase eunder different treatment conditions were detected to analyzed anticancer effect of combined therapy with ASNase and vitamin C on mCRPC. Intracellular oxidation was also observed with NADPH and NADP+ assay. Male BALB/c nude mice bearing prostate carcinoma xenografts (PC3 or DU145) were used to assess treatment response to vitamin C with or without ASNase through tumor growth, small animal PET/CT scans as well as Immunohistochemistry in vivo.

Results: Our in vitro studies demonstrate that ASNase synergizes with vitamin C targeting expression of glutamine synthetase enhances redox imbalance and induces anticancer effect in mCRPC cells through regulation the glutamine synthetase (GS) expression. In vivo, combination of ASnase and vitamin C could provide a significant better therapeutic outcome in comparison with controls or single treated mice. 18F-FDG PET imaging illustrated that the treatment with combined therapy could significantly reduce the 18F-FDG uptake in tumor.

Conclusions: In this current study, we suggest that ASNase combined with vitamin C could be as a cost-effective strategy to manage mCRPC.18F-FDG PET/CT imaging could indicate the therapeutic response of treatment for mCRPC.

Background

Although the technology and knowledge of human disease has been fast improved, developent of effective therapeutic approach is still far slower than expected [1, 2]. High attrition rates, higher costs because of the long time spent on research and development (R&D) as well as changing regulatory requirements [3, 4], which reduces the desirableness from the pharmaceutical industry to develop new drugs [5]. Drug repurposing has been recognized as a cost-effective and relatively low-risk alternative, gains a lot interesting recently [6].

Glutamine (Gln) becomes a key feature to observe the metabolism in various types of cancers [7, 8]. Gln provides essential source for supporting biosynthesis, energy generation, modulating signaling pathways, and maintaining redox status through the maintenance of plentiful glutamine in the blood, which

influence tumor growth [9]. However, the uptake of exogenous Gln into cancer cells is limited and correlates with the metabolism of endogenous Gln. Glutamine synthetase (GS) isthe only known enzyme in an adenosine triphosphate (ATP)-dependent pathway that catalyzes the synthesis of endogenous Gln from glutamate and ammonia. The over expression of GS has also been detected in breast, prostate and pancreatic cancers. It maintains the proliferation and survival of cancer cells even under the shortage of Gln [10–12]. In the endogenous Gln-dependent cancers, the combination of GS inhibition with the Gln reduction may lead to cell death later. Therefore, targeting GS could be a suitable strategy for suppression of cancer metastasis and improvement of therapeutic outcome of patients with malignant tumors [13, 14].

The mechanism of asparaginase in anti-cancer therapy is complicated, The Ollenschläger group has demonstrated that asparaginase agent also have glutaminase like activity. The concentrations of glutamine in the plasma circulation from patients could be rapidly reduced by asparaginas [15]. The serum asparaginase activity may influence the Glutamine deamination values tightly [16]. Several studies have suggested that the activity of asparaginase may be regulated by cellular Gln [15, 17, 18]. Preclinical studies have also demonstrated that asparaginas toxicity induces the depletion of exogenous Gln [19–23]. Additionally, since more than 30 years has L-asparaginase been repurposed to treat acute lymphoblastic leukemia (ALL), ovarian cancer, human hepatocellular carcinoma and sarcomas [24–27].

Vitamin C (VC) is an essential nutrient. Recent studies have shown that the anti-cancer effect of VC may regard on its pro-oxidative function, leading to accumulation of H2O2 and labile iron pool (LIP), consequently resulting in intracellular oxidative stress and induction of cell death [28–30]. Our previous study demonstrated that VC induced cell death in endogenous Gln-dependent cancers through disrupting intracellular redox homeostasis by targeting GS. The human castration-resistant prostate cancer is reported to over express GS [31]. Since ASNase can induce the depletion of exogenous Gln and result in cell death in cancer cells [23], our current study aimed to detect the anticancer effect from combined treatment with vitamin C and ASNase on the mCRPC with over expression of GS. Our results showed that ASNase improved the anti-cancer effect of VC treatment through regulation of GS activity.

Methods

Cell culture and reagents

The Human prostate cancer cells lines PC3 and DU145, purchased from the Cell bank of the Chinese Academy of Sciences, were grown in RPMI-1640 medium and minimum essential medium (MEM). For cell culture, medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (MRC) and maintained at 37 °C in an atmosphere of 5% CO2 in air. L-ascorbic acid (Alfa aesar) was diluted in phosphate buffered saline (PBS) and neutralized with sodium hydroxide before use. ASNase was also dissolved in PBS, frozen in -20℃ refrigerator, and thawed when used [32].

WST-8 cell viability assay

The WST-8 assay was used to quantify cell viability following incubation of cells with ASNase/vitamin C. WST-8 tetrazolium salt was reduced by cell dehydrogenase to methoxypyrimidine (Orange). By measuring its absorbance (450 nm), formazan is then quantified. It indicates that the number of metabolically active (alive) cells. According to the manufacturer's instructions (DOJINDO Laboratories, Kumamoto, Japan), the WST-8 assay was carried out to monitor cell proliferation.PC3 or DU145 cells were seeded in 96-well plates with the density of 104 cells and then incubated with different drugs for the indicated time periods. Then, add 100ul WST-8 into the medium in each well and incubate the well for 1-2 hours. Finally, Absorbance was measured at 450 nm using a Multiskan FC apparatus (Thermo Fisher Scientific, Waltham, MA, USA) [31].

Flow cytometry

Cells (2-3×10^5 cells/well in 3ml) were first incubated with or without ASNase (1U/ml) for 24h at 37°C. Then, vitamin C was administered at the following concentrations:0,1,2,4,8 mM. After 16h, the cells were harvested with 0.05% trypsin solution, washed twice with PBS and centrifuged at 1,500 rpm for 5 min. Next, the cells were stained with fluorescein isothiocyanate-labeled Annexin V (BD Pharmingen, San Jose, CA, USA) and counterstained with propidium iodide (PI, BD Pharmingen), resuspended in binding solution. Finally, the cells were analyzed using flow cytometry (CytoFLEX S, Beckman Coulter, Fullerton, CA, USA), as referenced to Li et al [32].

Western blot analysis

DU145 and PC3 cells were washed, collected and lysed. Separate the proteins with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transfer onto a polyvinylidene fluoride (PVDF) membrane. First, block the sample with 5% nonfat milk powder for one hour. Rabbit anti-glutamine synthetase antibody (Abcam) were diluted to 1:10000 in blocking buffer and incubated with the membranes overnight at 4°C and use mouse anti-β-tubulin as internal reference protein. Then, wash the membranes and incubate the membranes with a secondary antibody. After incubation, ELISA determination as described previously [31].

ELISA determination

The glutamine synthetase (GS) ELISA kit was purchased from Wuhan USCN Life Science Company. The culture medium containing cells was centrifuged at 1000 rpm for 5 min, and the supernatant was taken out, and 100% was added to each well with 100μL test solution A, warm bath at 37 ℃ for 1 h, then discard the liquid in the hole, and use 350μL washing liquid for each hole. Add 100 μL solution B to each hole, and finally 90 μL TMB substrate solution and 50 μL termination solution. The optical density of each well was measured at 450 nm by microplate immediately.

NADPH/$NADP^+$ assay

NADP/NADPH Assay Kit was purchased from Abbkine Company. Cells treated with 2mM VC were collected after trypsinization. Then, the intracellular NADP+ and NADPH of PC3 and DU145 were extracted and placed on ice, adding prepared 80ul working fluid quickly. Tap plate to mix briefly and thoroughly. Read optical density at 565nm using Multiskan FC apparatus (Thermo Fisher Scientific), and after a 30-min incubation at room temperature, the NADPH/NADP+ ratio was calculated as instructions described (Thermo Fisher Scientific) [33].

Xenograft mouse model

BALB/c nude mice (5-week-old male) were purchased from the Model Animal Research Center of Nanjing University. The animal experiments were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Sun Yat-sen University and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. BALB/c male nude mice were randomly divided into four groups. Each mouse was injected subcutaneously with 5*106 PC3 or DU145 cells on both sides. Once the tumor reached a size of ~50 mm3, the mice were respectively administered with vehicle, VC (Sodium ascorbate), ASNase, or the combination of VC and ASNase; Sodium ascorbate was purchased from Sigma Aldrich (St. Louis, MO, USA). ASNase (dose=60U/ml) was administered intraperitoneally daily in all experiments. VC (dose = 4 g/kg) was administered intraperitoneally twice a day in all experiments. Tumor sizes (length×width2×0.5) were measured once every 2 days. After 16 days the tumors were excised, weighed and analyzed by immunohistochemistry. No adverse effects were observed in the animals [32].

PET imaging

13N-ammonia (Synthesis see above) was administered via tail vein (100 μ L) at an active dose of 1 mCi per mouse one day and three days after treatment initiation. After the injection, computed tomography (CT) scanning was started immediately, imaging was conducted using a micro-PET system (Inveon, SIEMENS, Germany). The mice were then imaged for a 15-min static acquisition. Regions of interest were drawn around tumors (Ts) and the contralateral normal tissues (NTs), and the tumor-to-background ratios (T/NT) were calculated.18F-FDG was administered via tail vein injection (100 μL) at an activity dose of 100 μCi per mouse one day before,one week and two weeks after treatment initiation. Different from 13N-ammonia, 18F-FDG was allowed to accumulate in the tumor for 45 min. PET-CT scans were performed as shown above [32].

Immunohistochemistry

Tumor tissues were collected for IHC at the end of treatment. Tumor tissues were cut to 4-mm thick slices and incubated with 3% hydrogen peroxide for 25 min at room temperature to block endogenous peroxidase activity. Next, the tissues were incubated with bovine serum albumin (BSA) for 30 min at room temperature to block nonspecific binding. Afterward, sections were incubated overnight at 4°C with primary antibody (purified mouse anti-GS, 1:1000), then incubated with biotinylated secondary antibody (HRP-labeled goat anti-rabbit IgG, 1:200) followed by streptavidin biotin peroxidase complex (streptavidin

biotin peroxidase complex immunohistochemical kit) for 30 minutes at room temperature. The immunoreactions were visualized using a Dolichos Biflorus Agglutinin (DBA) chromogenic reagent kit after incubation for 10 min at room temperature, followed by hematoxylin staining for 3 min as reported by Long et al [31].

Statistical analysis

Comparisons between two groups were carried out using Student's t test or analysis of variance (ANOVA) with Bonferroni post hoc test. Each experiment was repeated at least in triplicate, and mean ±SEM was calculated for each value. For all analyses, $p < 0.05$ *, < 0.01 ** or < 0.001 *** between exposure conditions were considered significant. All analyses were performed in GraphPad Prism (GraphPad Software, Inc.).

Results

L-asparaginase potentiates the cytotoxic effect of VC in prostate cancer cells

Here, we treated PC3 and DU145 prostate cancer cells with either L-asparaginase (ASNase) or VC alone or in combination to verify possible synergistic cytotoxic effects. Using a WST-8 viability assay, we can measure the effect of ASNase and VC on cell viability of metastatic prostate cancer cell lines, which was inhibited dose-dependently (VC 1mM, 2mM, 4mM and ASNase 1U/ml) in the different groups (Fig. 1A). We can see a significant decrease in cell viability in PC3 and DU145 cells following concurrent treatment with ASNase and VC. When the concentration of VC was 2 mM and ASNase was 1U/ml, exhibited significant suppressive effects on cellular growth. We can also see this phenomenon in the microscope, whether it is in PC3 or DU145 cells, it can be seen that ASNase has strong cytotoxic effects on cells, and the combination of the two drugs strengthens the effect of VC. As shown in figure 1B, a noteworthy reduction of cells can be seen after 48h treatment with 2mM VC and 1U/ml Asnase. In addition to measuring short-term cell viability, the flow cytometry experiments revealed the similar results, as shown by an obvious increase of Annexin V/PI positive cells and a remarkable decrease of Annexin V/PI negative cells (Fig. 1C). Taken together, these observations confirm that ASNase may enhance VC-induced cytotoxicity in prostate cancer cells.

ASNase consumes extracellular Gln in prostate cancer cells exhibiting high expression levels of GLUTs

Previous study has shown that ASNase, as one of the most important drugs used for acute lymphoblastic leukemia (ALL) therapy, can catalyze the hydrolysis of Gln to glutamate (Glu), resulting in depletion of extracellular Gln [31]. Many study has shown that the high GS expression in human cancers like prostate neuroendocrine [34].We hypothesized that the decrease in extracellular glutamine concentration would increase GS expression. Therefore, we first performed Western blot analysis to investigate the expression level of GS protein in PC3 and DU145 when treat with ASNase (Fig. 2A). The results showed that GS expression decreased at 3 hours after ASNase treatment, and gradually increased after 6 hours. The expression of GS in PC3 cells reached the peak at 24 hours, while that of DU145 cells peaked at 16 hours

(Fig. 2A). Furthermore, ELISA measurement was also performed to detect GS expression. When treated for 6 hours, there was no significant difference between the two groups. The expression level of GS protein increased in a time-dependent manner in ASNase-treated cancer cells, when compared with control groups (Fig. 2B).

Asnase increases the redox imbalance effect of VC through NADPH depletion

It is reported that the selective toxicity of VC in cancer cells can led to subsequent NADPH depletion. The endogenous ascorbic acid cycle mechanism is driven by the reduction equivalent of nicotinamide adenine dinucleotide phosphate (NADPH), allowing continuous production of H2O2(hydrogen peroxide). As we can see from the figure 2C, whether VC or ASNase was added, the content of NADPH decreased obviously, and the decrease degree of the two drugs was more obvious.

Combined treatment with VC and SAS suppresses tumor growth in vivo

In order to further determine the above findings, we examined the inhibitory effects of VC and/or ASNase intraperitoneal (i.p.) therapy on the growth of PC3 (Fig. 4A) and DU145 cell (Fig. 3A) xenograft tumors in BALB/c nude mice. The combined treatment with 3U/g ASNase and 4 g/kg VC for 14 days was well tolerated by athymic nude mice. We found that the growth of PC3 (Fig. 4A) and DU145 (Fig. 3A) xenograft tumors was significantly inhibited by combined drug therapy. Next, we used immunohistochemistry to investigate the change of expression of apoptosis-related proteins (including Ki-67, caspase 3, GS) in vivo(Fig. 3B and 4C).

In vivo functional imaging of the response to combined treatment with vitamin C and ASNase indicates a significant reduction in ^{18}F-FDG in the xenografts

To further evaluate the uptake of drug treatment in tumors in vivo, animal models with subcutaneous PC3 and DU145 xenografts were established. Mice bearing DU145 xenografts were intraperitoneally injected with 4g/kg VC or/and intraperitoneally injected with 3U/g ASNase for 12 days and sacrificed for IHC. Animal models with PC3 xenografts were treated with the same method as above for 15 days. ^{18}F-FDG PET imaging was performed before medication, one week after medication and 15 days after medication. For ^{18}F-FDG PET monitoring, the mice were injected intravenously with 3.7 MBq (100 μCi) ^{18}F-FDG. As we can see from Figure 4B, FDG uptake increased in control group, but inhibited in VC and ASNase groups. Combined treatment group induced a stronger reduction in cellular FDG uptake in PC3 xenografts.

Discussion

Prostate cancer is the most common malignant tumor in male urinary and reproductive system, accounting for the fifth in the world. According to the WHO global cancer epidemiology data, prostate cancer ranked the second highest incidence rate of male cancer in 2008 [35], accounting for 14% of all cancer cases in men [36]. In China's urban areas, the incidence of prostate cancer has reached its peak since the age of 60, while in the United States, the incidence of prostate cancer has reached its peak

about 10 years earlier [37]. Domestic research centers suggest that only 1/3 of the newly diagnosed patients have localized lesions, and a large number of patients have advanced or metastatic diseases. About 20% of prostate cancer patients have advanced or metastatic diseases, about 29000 men die of prostate cancer every year [38]. Androgen deprivation therapy plays an important role in the treatment of metastatic prostate cancer. However, after a period of treatment, the condition of patients with prostate cancer will enter a stagnation period, then will be insensitive to treatment [39], and recrudesces as androgen independent prostate cancer (also called mCRPC, metastatic castration-resistant prostate cancer), eventually leading to death [40]. Therefore, the treatment of androgen independent prostate cancer is a key to prove clinical outcome.

Repurposing therapy targeting glutamine depletion may be a promising treatment strategy to improve the therapeutic effect of end-stage mCRPC. In tumor cells, glutamine metabolism can be used as a substrate to supplement TCA intermediates and provides ATP for cells; More importantly, it provides raw materials for the synthesis of proteins, lipids, and nucleicacids, plays an important role in the balance of redox homeostasis, signal transduction pathways, apoptosis and autophagy [41]. Gln is involved in the synthesis of glutathione (GSH), scavenging reactive oxygen species (ROS) and maintaining intracellular redox homeostasis [42]. It is well known that a remarkable feature of cancer cells is the high Gln consumption rate that exceeds their biosynthesis and energy requirements. However, for tumor cells, their own synthesis of Gln will not meet the requirements of rapid proliferation and must be ingested from the outside of the cell. Therefore, Gln is a "conditionally essential amino acid" for cancer cells. The strong dependence of most cancer cells on this essential nitrogen substrate after metabolic reprogramming is known as “Gln addiction” [43]. As an important substance for substance, energy and homeostasis maintenance in tumor cells. The metabolic process of Gln is closely related to tumorigenesis [12], development [44] and metastasis [45]. Pathways and molecules related to Gln metabolism may become new targets for tumor therapy. It has been reported that interruption of Gln supply can make Triple-negative breast cancers more prone to apoptosis. Therefore, limiting Gln supply is also expected to become a potential anti-tumor means [46]. As an important anti-tumor drug, clinical use of L-ASNase has been mainly restricted to pediatric and adult patients with acute lymphoblastic leukemia (ALL) as well as other lymphoid malignancies. The L-ASNase deaminates asparagine and glutamine, interfering with protein synthesis and resulting in cell death [47]. It has a certain glutaminase (GLS) activity, which can catalyze glutamine (Gln) synthesis from glutamate and NH4 +, thus blocking the uptake of Gln by cells [15, 23, 25, 48]. However, the effects of asparagine and Gln depletion on anti-tumor activity of L-ASNase is still unclear [49]. Theoretically, numbers of cancer cells are dependent on the glutamine synthesized by GS activity, is followed by the up-regulation of the expression and/or activity of glutamine synthetase (GS) [50]. Prostate cancer cells were also reported that they could resynthesize glutamine under exogenous glutamine deficiency by inducing GS expression to support further proliferation of cancer cells. Therefore, drugs target GS expression, offer possibility to inhibit the cancer cell proliferation.

L-Methionine sulfoximine(MSO) is a specific and irreversible GS inhibitor, the inhibition of GS by MSO is a manifestation of severe nutritional stress in cancer cells, prevents cancer cells from successfully adapting to ASNase. Therefore, it can significantly inhibit the growth of a various cancer cells. However,

due to high toxicity is MSO not suitable for clinical use [25, 50]. A system comprised of O2, VC and trace meta has been reported that could lead to the GS degradation [51]. Therefore, we hypothesized that VC could be used as an alternative agent for MSO.VC has no toxic effect on normal human cells [52]. Beside of reducing intracellular glutamine content by inhibiting GS, VC can also cause tumor redox imbalance and excessive accumulation of reactive oxygen species (such as ROS and H2O2) [52]. Reactive oxygen species (ROS) is a by-product of cell growth. The level of ROS increased significantly, due to the vigorous proliferation of tumor cells. However, moderate increase of ROS will cause DNA damage and promote gene mutation. An excessive increase will cause cell oxidative stress and lead to cell aging or death. VC promotes tumor cell death by increasing oxidative stress, which could be analyzed by measurement of the NADPH content. NADPH is an important antioxidant in cells and plays an important role in balancing intracellular redox homeostasis [53]. Our results showed that the content of NADPH decreased significantly regardless of the addition of VC or ASNase. When prostate cancer cells were treated under combined therapy with VC and ASNase, the decrease was more obvious. Therefore, our results demonstrated that the selective toxicity of VC on cancer cells leaded to the subsequent loss of NADPH. The endogenous ascorbic acid cycle mechanism may be driven by the reduction equivalent of NADPH, allowing continuous production of H_2O_2, excessive accumulation of reactive oxygen species, increasing oxidative stress and promoting tumor cell death. Therefore, VC can consume glutamine, induce redox imbalance in prostate cancer cells. The two mechanisms jointly promote cancer cell death.

Due to the heterogeneity of cancer development, the growth state of xenograft tumor will not be judged only from the size of tumor. PET-CT is a good imaging method to provide detailed molecular information such as function and metabolism of tumoral lesions. In this study, PET-CT imaging was performed three times in nude mice implanted with DU145 and PC3. In prostate cancer, the uptake of 18F-FDG represents the invasiveness of the tumor to some extent [54]. FDG uptake in tumor under combined treatment with VC and L- ASNase inhibitor was significantly lower than that of the control group, which was only 52% of the control group. To compare with VC single or ASNase single treatment group, the FDG uptake was also significantly lower under combined treatment, which proved that the combination of ASNase and VC could significantly reduce FDG uptake and tumor invasiveness.

In conclusion, this study confirmed that ASNase combined with VC through in vivo and in vitro experiments. ASNase catalyzes the decomposition of Gln into Glu. VC consumes intracellular and extracellular Gln by targeting GS, resulting in the loss of nutritional supply of prostate cancer cells and the death of cancer cells.

Abbreviations

ALL: acute lymphoblastic leukemia

ASNase: asparaginase

FBS: fetal bovine serum

Gln: Glutamine

Glu: Glucose

GS: glutamine synthetase

LIP: labile iron pool

mCRPC: metastatic castration-resistant prostate cancer

MEM: minimum essential medium

MOS: L-Methionine sulfoximine

PVDF: polyvinylidene fluoride

ROS: reactive oxygen species

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

VC: Vitamin C

Declarations

Availability of data and materials

The data set supporting the results of this article are included within the article.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Sun Yat-sen University and conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

And our study was carried out in compliance with the ARRIVE guidelines from BMC Cancer.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zhoulei Li, Wanqing Shen and Zhifeng Chen made equal contributions to this work. They conceived and coordinated the study, designed the experiments, carried out data analysis, and wrote the paper. Gang Yuan, Peng He, Yali Long and Yue Wang designed and performed the experiments. Diachao Yue coordinated the study and made scientific contributions. Bing Zhang and Xiangsong Zhang conceived and coordinated the study, designed the experiments, carried out the data analysis and data interpretation, and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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Figures

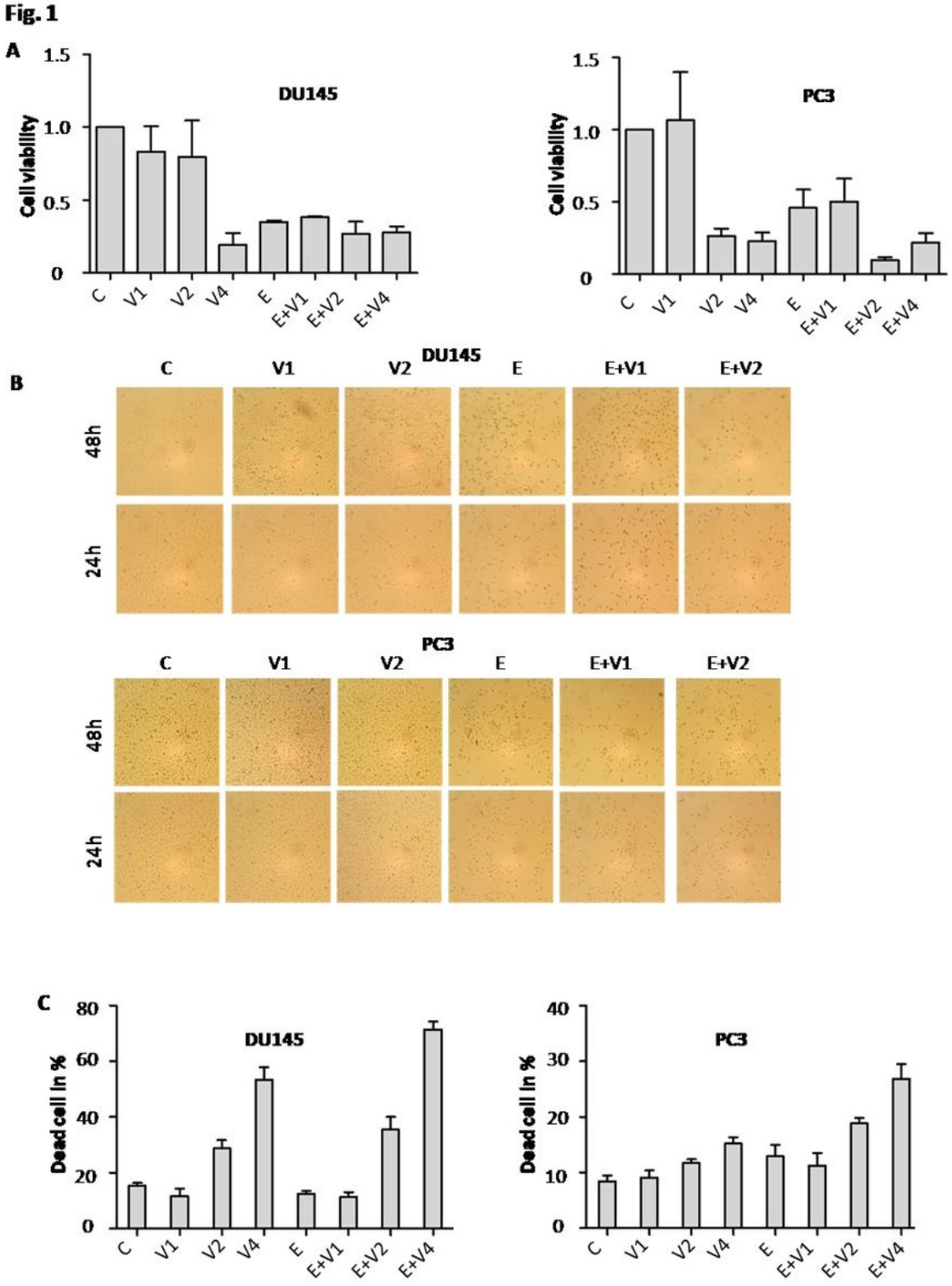

Figure 1

Asparaginase in combination with vitamin C inhibits cell proliferation and induces cell death. (A) $1x10^4$ DU145 or PC3 cells per well (96-well plate) were incubated at 37°C with vitamin C (V1: 1mM, V2: 2mM, V4; 4mM), 1U Asparaginase (ASNase: E) or the combination of vitamin C and Asparaginase (E+V) for 9 h. Vitamin C were administered to cells with pretreatment with ASNase for 12 h, cell viability was assessed by the WST-8 assay. (B) 2-3$X10^5$cell/well (6-well plate) were incubated at 37°C with vitamin C (V1: 1mM,

V2: 2mM), 1U Asparaginase (ASNase: E) or the combination of vitamin C and Asparaginase (E+V) for 24h or 48h. Vitamin C were administered to cells with (E+V1, E+V2) or without(V1, V2) pretreatment with ASNase for 12 h, cell growth situation were observed under microscope; cell death was analyzed with FACS analysis after 16h treatment (C). The bars represent the mean and SD of the mean of n≥3.

Fig. 2

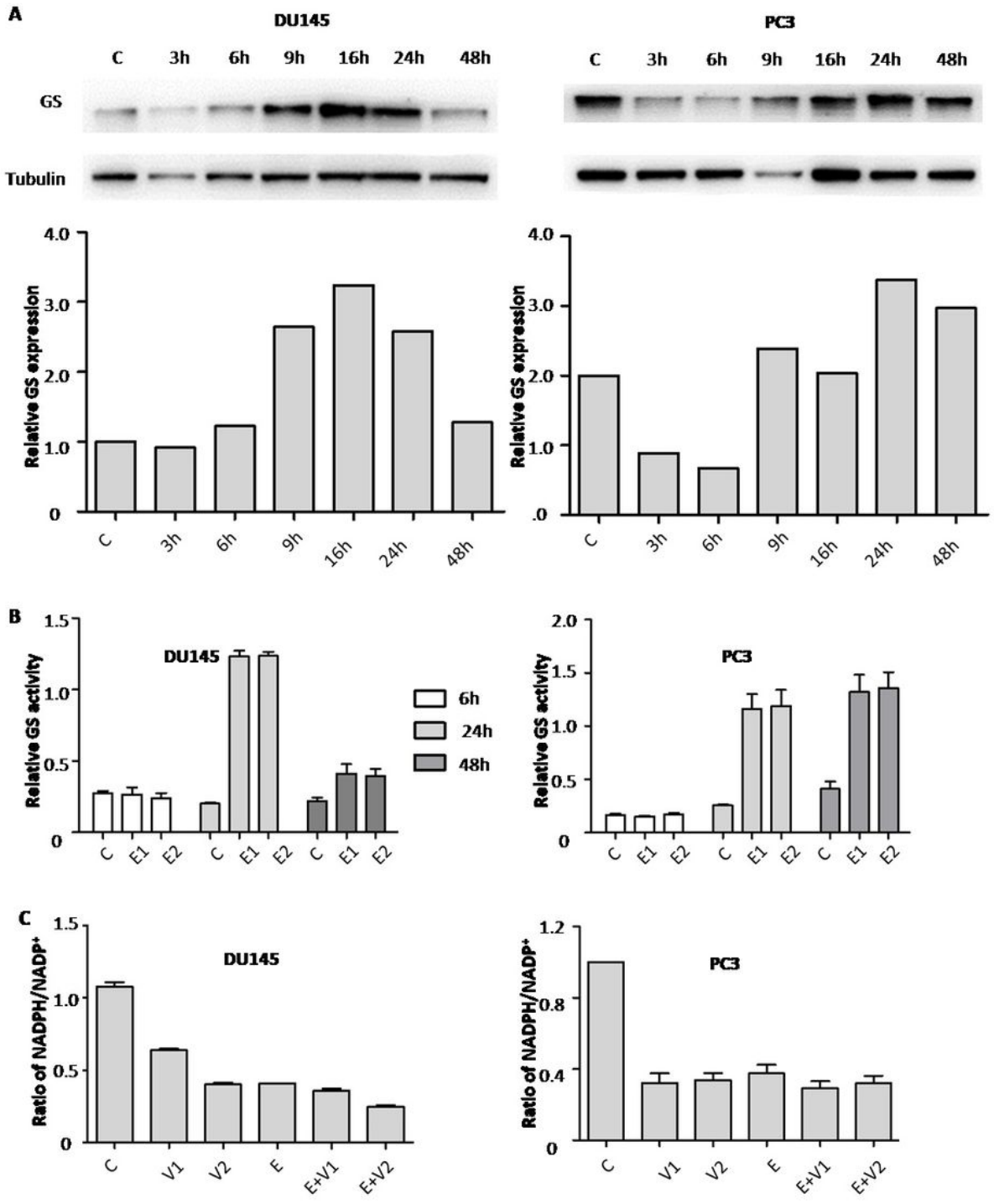

Figure 2

Asparaginase targeted GS expression enhanced the vitamin C induced imbalance of redox in mCRPC cells. (A) PC3 and DU145 PCa cells were incubated in cell culture medium under treatment 1U ASNase for 0, 3h, 6h, 9h, 16h, 24h and 48h and then lysed for Western blot assay.(B) $1X10^4$cell/well (96-well plate) were incubated at 37°C with 0.5U (E1) or 1U (E2) for 6h, 24h or 48h and then GS activity was detected using ELESA. (C) 2-$3X10^5$cell/well (6-well plate) were incubated at 37°C with vitamin C (V1: 1mM, V2: 2mM), 1U Asparaginase (ASNase: E) or the combination of vitamin C and Asparaginase (E+V) for 6h.Vitamin C were administered to cells with (E+V1, E+V2) or without(V1, V2) pretreatment with ASNase for 12 h, then cells were lysed for detection of $NADPH/NADP^+$. The bars represent the mean and SD of the mean of n≥3.

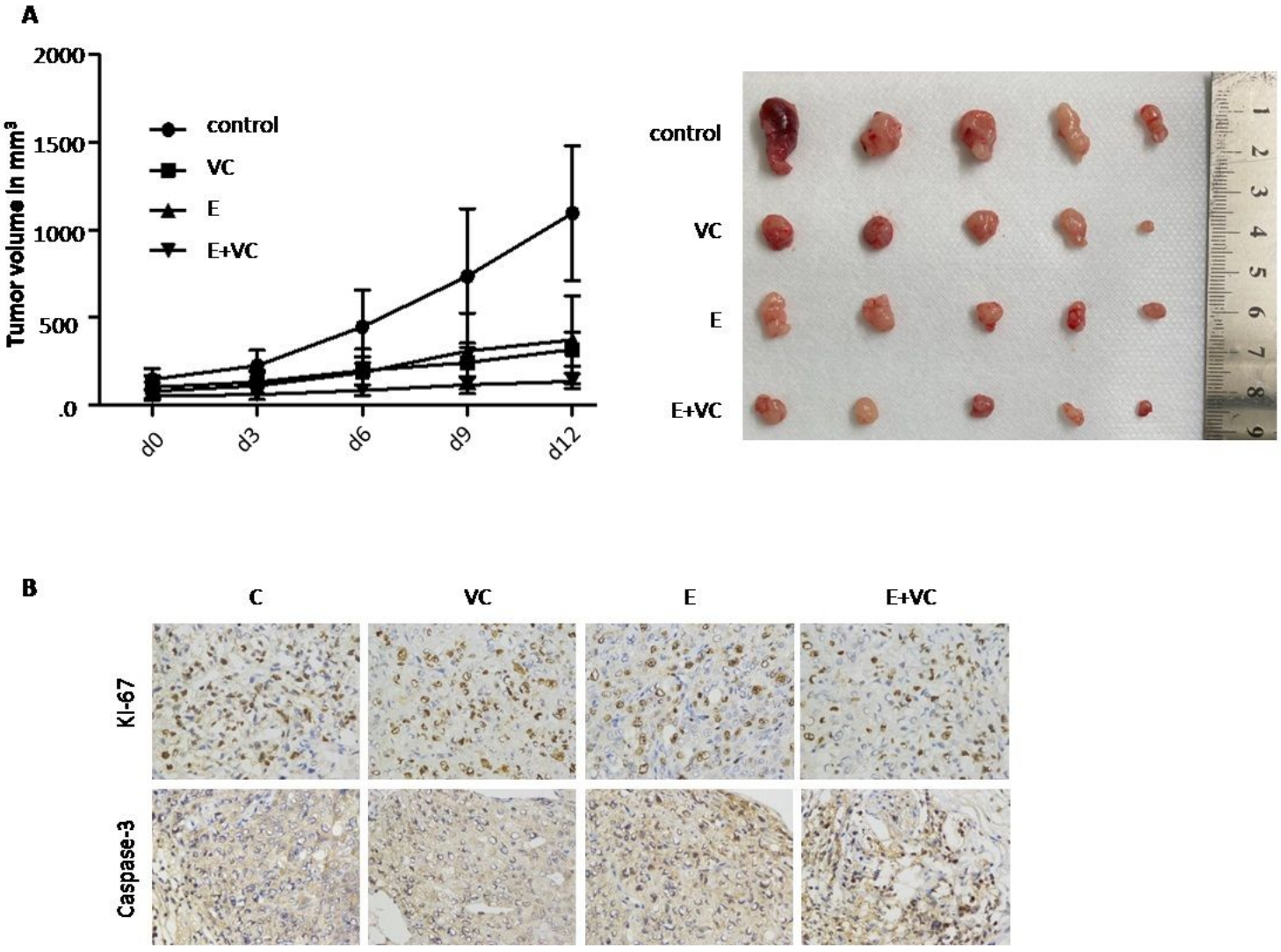

Figure 3

Asparaginase synergized the anticancer effect of vitamin C, reduced tumor growth and induced apoptotic cell death DU145 prostate cancer xenografts. $5x10^6$ DU145 cells were subcutaneously injected into male Blb/c mice. Treatment was administrated when the longest diameter of tumor was reached approximately 5mm. (A) Tumor volume measurements (left panel) and tumor were obtained two weeks

after initiation of treatment (d0 to d12, right panel). (B) Immunohistochemical analyses showing that the expression levels of cleaved caspase-3 (apoptosis marker) were increased more clearly in the combination group with ASNase and vitamin C (E+VC) than in the VC or ASNase single treatment group, whereas the expression level of Ki-67 was was downregulated under the combined treatment. Data are shown as means ± SD.

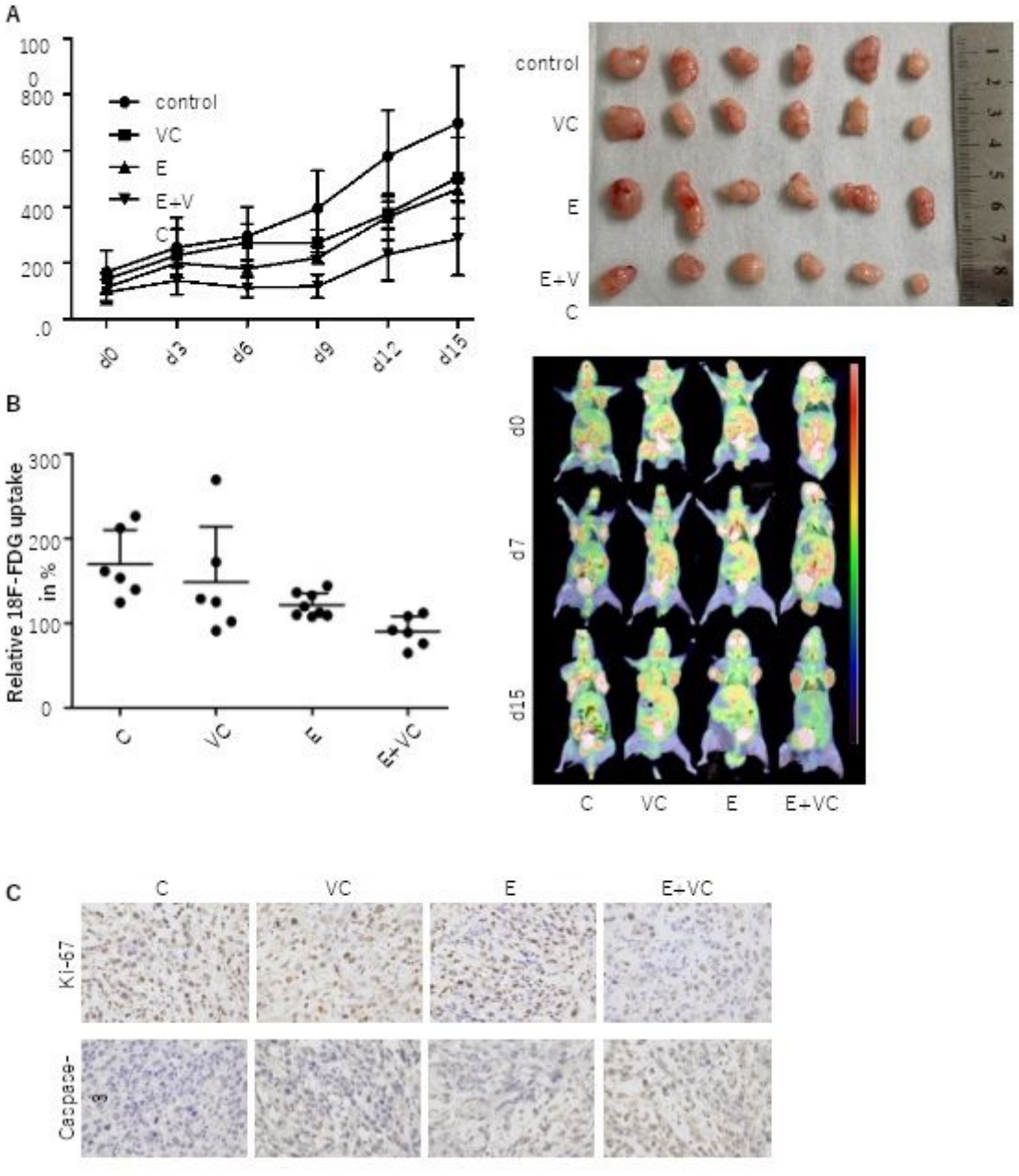

Figure 4

Functional 18F-FDG-PET/CT imaging illustrated that Asparaginase improves the anticancer effect of vitamin C in PC3 prostate cancer xenograft. 5x10^6 PC3 cells were subcutaneously injected into male Blb/c mice. Treatment was administrated when the longest diameter of tumor was reached approximately 5mm. (A) Tumor volume measurements (left panel) and tumor were obtained two weeks

after initiation of treatment (d0 to d15, right panel). (B) Uptake of ^{18}F-FDG in tumor tissue (the tumor to background ratio [TBR]) in control mice and mice treated with vitamin C (VC), ASNase (E) or both (E+VC). The TBR was calculated 15 days after treatment starting (d0). The mean TBR of ^{18}F-FDG-PET/CT was significantly reduced under combined treatment (E+VC) compared with control treatment ($p = 0.0003$), indicating a significant reduction in tumor volume. Moreover, the reduction in TBR after combined (E+VC) treatment compared to treatment with vitamin C or ASNase only was significant ($p = 0.005$ and, right panel). PET/CT scans showing the changes in tumor uptake of ^{18}F-FDG (red arrows). ^{18}F-FDG-PET/CT scans were carried out before (pre therapy, d0) and 15 days after the initiation of treatment (post therapy, d15). The red arrows indicate the sites of subcutaneous injection of PC3 tumor cells and the accumulation of ^{18}F-FDG (left panel). (C) Immunohistochemical analyses showing that the expression levels of cleaved caspase-3 (apoptosis marker) were increased more clearly in the combination group with ASNase and vitamin C (E+VC) than in the VC or ASNase single treatment group, whereas the expression level of K_i-67 was was downregulated under the combined treatment. Data are shown as means ± SD.