

GSK1838705A, an insulin-like growth factor-1 receptor/insulin receptor inhibitor, induces apoptosis and reduces viability of docetaxel-resistant prostate cancer cells both in vitro and in vivo

Fayou Zhou^{1,2}
 Xianguo Chen¹
 Song Fan¹
 Sheng Tai¹
 Changqin Jiang¹
 Yifei Zhang¹
 Zongyao Hao¹
 Jun Zhou¹
 Haoqiang Shi¹
 Li Zhang¹
 Chaozhao Liang¹

¹Department of Urology, First Affiliated Hospital of Anhui Medical University, Hefei, ²Department of Urology, Traditional Chinese Medical Hospital of Wuhu City, Wuhu, People's Republic of China

Abstract: Prostate cancer is the leading malignancy and the second most common cause of cancer-related death in men. Despite high cure rates with surgery and/or radiation, 30%–40% of patients eventually develop advanced cancer. Docetaxel is one of the most effective and well established chemotherapeutic agents for prostate cancer. However, docetaxel resistance often develops within months. Combination therapies have been proposed to improve the therapeutic efficacy of docetaxel in prostate cancer, and there is an urgent need to identify agents that are effective for treatment of the disease, especially docetaxel-resistant prostate cancer. In this work, we investigated the activity of GSK1838705A, a potent insulin-like growth factor-1 receptor (IGF1R)/insulin receptor (IR) inhibitor, in prostate cancer, especially docetaxel-resistant prostate cancer. We found that GSK1838705A could effectively reduce the viability of both docetaxel-sensitive and docetaxel-resistant prostate cancer cells. GSK1838705A induced marked apoptosis in docetaxel-resistant cells, and also dramatically inhibited migration of these cells. Further, GSK1838705A significantly inhibited phosphorylation of IGF1R/IR. Importantly, GSK1838705A significantly suppressed docetaxel-resistant PC-3R tumor growth in vivo. This is the first study of GSK1838705A in prostate cancer. Our results indicate that GSK1838705A is a promising compound for the treatment of prostate cancer, especially for those who develop resistance to docetaxel, and might shed new light on treatment for prostate cancer.

Keywords: prostate cancer, GSK1838705A, insulin-like growth factor-1 receptor/insulin receptor inhibitor, viability, resistance, migration

Introduction

Prostate cancer is one of the most common noncutaneous malignancies and the second most common cause of cancer-related death. Prostate cancer accounts for 28% of cancer diagnoses and 10% of cancer deaths in men.^{1–4} Docetaxel is one of the most effective chemotherapeutic agents available for prostate cancer; however, development of resistance to docetaxel soon after the start of treatment is common, and leads to disease relapse.^{5–7} Resistance to docetaxel can be either intrinsic or acquired by adopting various mechanisms that are strongly associated with genetic alterations, decreased influx, and increased efflux of drugs.^{5–7} Novel therapeutic strategies, such as combination therapies, have been proposed to improve the therapeutic potential of docetaxel in resistant prostate cancer.^{8,9}

Correspondence: Chaozhao Liang
 Department of Urology, First Affiliated
 Hospital of Anhui Medical University,
 Hefei, Anhui 230022, People's Republic
 of China
 Tel/Fax +86 0551 6292 3440
 Email liang_chaozhao@163.com

Insulin-like growth factor-1 receptor (IGF1R)/insulin receptor (IR)-mediated signaling pathways are implicated in proliferation and survival of many tumor types. IGF1R and IR are overexpressed in prostate cancers, and the signaling mediated by these receptor tyrosine kinases promotes proliferation and survival of prostate cancer cells.^{10–13} These signaling pathways have been linked to an increased risk of prostate cancer as well as development of resistance in prostate cancer cells.^{14,15} IGF1R and IR are important receptor tyrosine kinases. A well-known problem in the treatment of prostate cancer is acquisition of drug resistance.^{8,9} Inhibition of single, dual, or multiple receptor tyrosine kinases might provide a therapeutic strategy for prostate cancer, especially when resistance develops. In this work, we investigated the anticancer activity of GSK1838705A, a potent IGF1R/IR inhibitor,¹⁶ in prostate cancer, especially docetaxel-resistant disease, both *in vitro* and *in vivo*.

Materials and methods

Materials

GSK1838705A (molecular weight 532.57) and docetaxel (molecular weight 807.88) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Rabbit anti-phospho-IGF1R, anti-phospho-IR, anti-IGF1R, anti-IR, anti-cleaved caspase-3, mouse anti-glyceraldehyde-3-phosphate dehydrogenase, and secondary horseradish peroxidase-conjugated antibody were sourced from Cell Signaling Technology (Danvers, MA, USA). Other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cells and cell culture

A PC-3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and a docetaxel-resistant (PC-3R) cell line was obtained by culturing PC-3 cells with gradually increasing doses of docetaxel (12.5–400 nM). All cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in an atmosphere comprising 95% air and 5% CO₂.

Cell viability assay

PC-3 and PC-3R cells were plated in 96-well white plates at 5×10³ cells/well. Docetaxel (12.5–400 nM) and GSK1838705A (0.0625–2 µM) were added, followed by incubation for 72 hours. A CellTiter-Glo kit (Promega, Madison, WI, USA) was then used to assess cell viability. Briefly, a cell lysis/ATP detection reagent provided by the CellTiter-Glo kit was added (30 µL/well), the cells were

shaken for 10 minutes, and the resulting luminescence was assessed using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All IC₅₀ values were determined using Compusyn software.

Transwell assay

To measure cell migration, 24-Transwell Boyden chambers (Costar, Bedford, MA, USA) containing a polystyrene membrane were plated with PC-3R cells. The cells were placed in the upper compartment at a density of 5×10⁴ cells/well in medium without serum. Next, 600 µL of serum-free medium containing 20 µg/mL fibronectin were added to the lower compartment. The cells were incubated for 8 hours, after which they were fixed and stained with 0.1% crystal violet. Cells that failed to migrate were discarded, and all cells that crossed to the lower chamber were imaged under a microscope (Nikon, Tokyo, Japan). These migrating cells were then lysed with 10% acetic acid and their absorbance was measured at 595 nm.

Staining of cell nuclei

PC-3R cells were seeded onto coverslips and incubated with GSK1838705A (0.25–2 µM) for 48 hours. After incubation, the cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 for 20 minutes. After blocking with 5% normal goat serum for 30 minutes, the cells were stained with Hoechst for 20 minutes, and then imaged using a Nikon microscope.

Flow cytometry analysis

PC-3R cells were plated in six-well plates at a density of 4×10⁵ cells/well, and treated with GSK1838705A (0.25–2 µM) for 48 hours. The cells were then collected, fixed with 70% ethanol, and stained with propidium iodide in the presence of RNase (1 g/L), 1 g/L sodium citrate, and 0.5% Triton X-100 (v/v) for 30 minutes in the dark. A FAC-SCalibur sorter (BD Biosciences, Franklin Lakes, NJ, USA) was used to collect cells for further analysis of apoptosis. The percentage of hypodiploidy was taken as a measure of apoptosis and quantified using ModFIT LT software.

Immunohistochemistry

Samples of tumor tissue were fixed in phosphate-buffered formalin and embedded in paraffin. Paraffin slices were then taken at a thickness of 4 µm before mounting onto slides. The slides were then deparaffinized by three 5-minute xylene washes before being rehydrated through the following series of alcohol washes: two washes with 100% ethanol for 10 minutes each, two washes with 95% ethanol for

10 minutes each, and a final deionized water wash for 1 minute with gentle agitation. The slides were incubated with 10 mM sodium citrate buffer (pH 6.0) and heated for 1 hour. The slides were then washed three times in deionized water for 5 minutes per wash before a 30-minute block in 5% normal goat serum. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was performed using a FragEL™ kit (Calbiochem Billerica, MA, USA) according to the manufacturer's instructions. Following TUNEL staining, the slides were stained with Hoechst for 15 minutes before imaging by fluorescent microscopy.

Tumor growth suppression in vivo

Nud/nud mice were purchased from the Shanghai Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, People's Republic of China). PC-3R cells were injected subcutaneously into the axillary regions of selected nud/nud mice (4×10^6 cells/100 μ L/mouse). Cells were allowed to grow to a volume of 50 mm³ before the mice were randomized to one of the following experimental groups: GSK1838705A (20 mg/kg), GSK1838705A (60 mg/kg), or control. All groups had six animals per condition. Tumors were measured on alternating days with a microcaliper along with body weight. Total tumor volumes were calculated as follows: (mm³) = width \times width \times length \times 0.5. Mice were euthanized 2 weeks later, at which point the tumors were collected and saved for later analysis. All of the studies on animals were conducted according to the protocol approved by the Animal Research Ethics Committee of Anhui Medical University (Approval No LLSC2013013).

Statistical analysis

The Student's *t*-test and analysis of variance were performed using StatView (SAS Institute, Cary, NC, USA). $P < 0.05$ was

considered to be statistically significant. The data shown are representative of at least three independent experiments with similar results, and are the mean values of measures taken in triplicate. Error bars indicate the standard deviation.

Results

GSK1838705A reduces the viability of both docetaxel-sensitive and docetaxel-resistant prostate cancer cells

In order to generate a docetaxel-resistant cell line, we cultured PC-3 cells with gradually increasing doses of docetaxel (5–50 nM). After 6 months of culture, we examined and compared the sensitivity of the PC-3 and PC-3R lines toward docetaxel. The results showed an approximately 16-fold increase in the IC₅₀ for PC-3R compared with PC-3 ($P < 0.01$), suggesting that a docetaxel-resistant PC-3R cell line was successfully generated (Figure 1A).

The IGF1R and IR are known to be important mediators of viability in prostate cancer cells.^{14,15} Therefore, we then determined how GSK1838705A, an IGF1R/IR inhibitor, altered the viability of docetaxel-sensitive and docetaxel-resistant cells. As shown in Figure 1B, GSK1838705A significantly reduced the viability of both docetaxel-sensitive (PC-3) and docetaxel-resistant (PC-3R) lines to a similar extent in a concentration-dependent manner.

GSK1838705A induces apoptosis of PC-3R cells

IGF1R/IR-mediated signaling pathways play a crucial role in inhibiting apoptosis and increasing cell viability,^{10–15} so we next examined GSK1838705A-induced apoptosis in PC-3R cells. As shown in Figure 2A and B, the subG1 DNA content in PC-3R cells significantly increased after treatment with GSK1838705A when compared with the control group ($P < 0.01$). We also observed the nuclear morphology in PC-3R cells after treatment

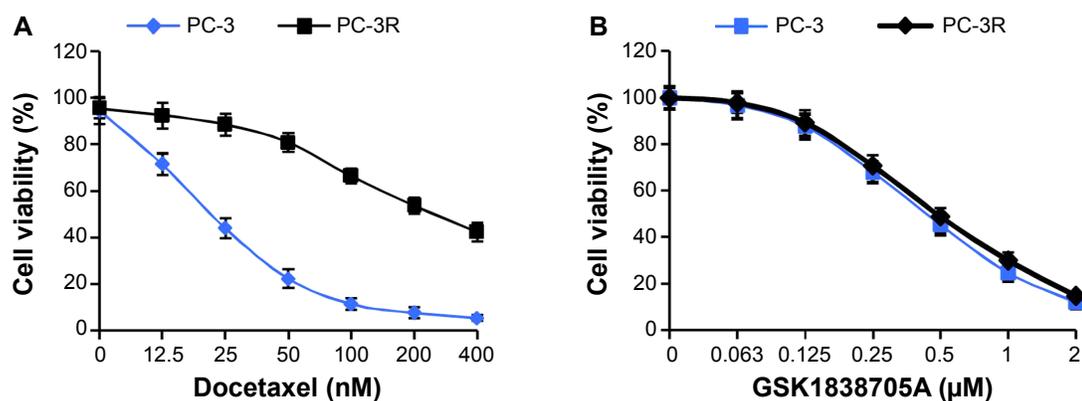


Figure 1 GSK1838705A suppresses viability of docetaxel-sensitive and docetaxel-resistant prostate cancer cells.

Notes: (A) PC-3 cells were induced to become docetaxel-resistant (PC-3R cells) by incubation with gradually increasing doses (5–50 nM) of docetaxel. Docetaxel sensitivity was then examined by cell viability assay. (B) PC-3 and PC-3R cells were treated with GSK1838705A (0.063–2 μ M) for 72 hours, followed by measurement of cell viability.

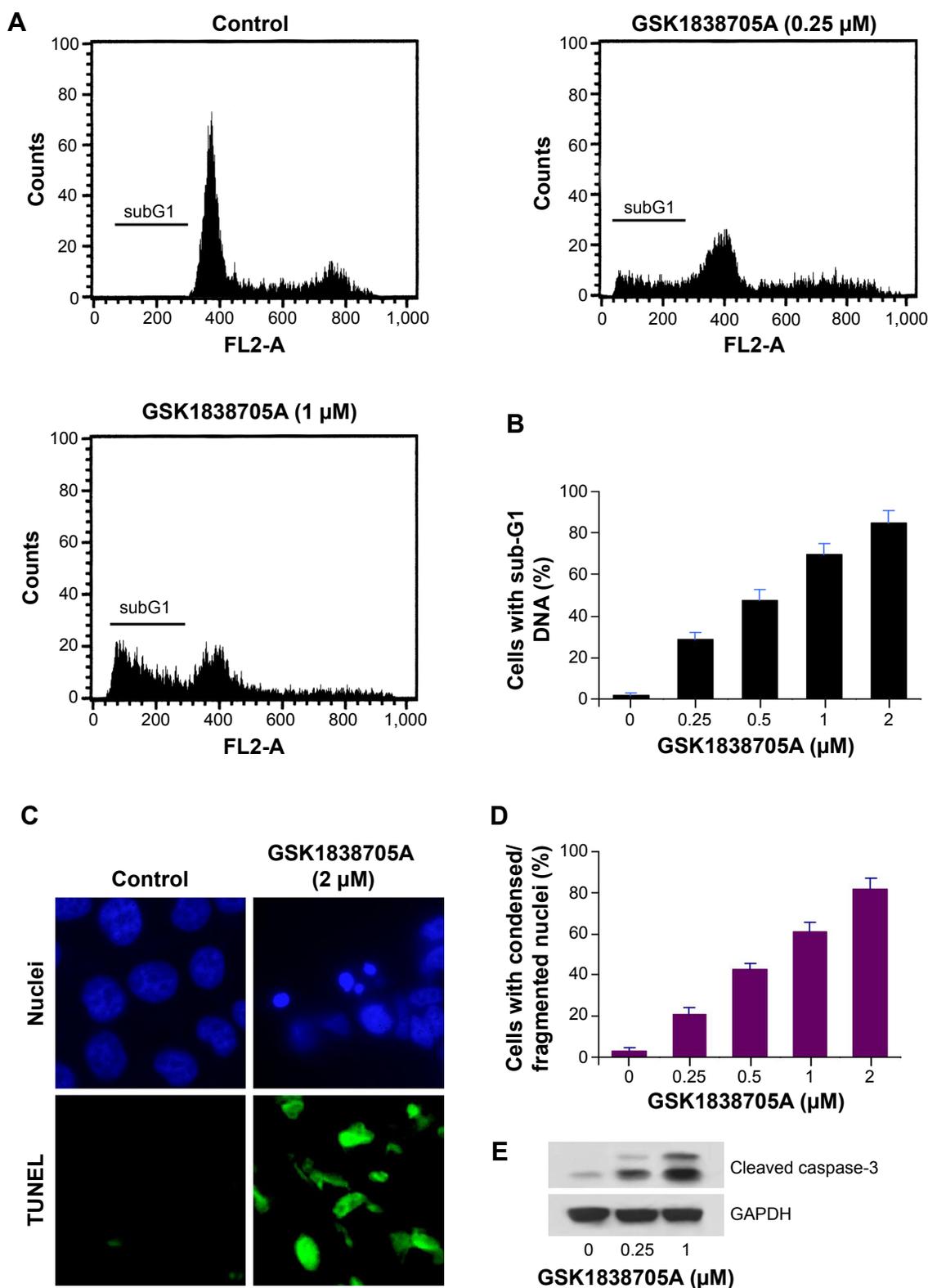


Figure 2 GSK1838705A induces apoptosis in docetaxel-resistant cells.

Notes: (A) PC-3R cells were treated with GSK1838705A (0.25–2 μM) for 48 hours, followed by propidium iodide staining and flow cytometry analysis. (B) Analysis of results of flow cytometry data. (C) PC-3R cells were incubated with GSK1838705A (0.25–2 μM) for 48 hours. The nuclei were stained with Hoechst or a TUNEL staining kit, and analyzed by fluorescent microscopy. Representative images are shown. (D) The number of cells with condensed/fragmented nuclei was quantitated by counting in five random fields and inhibition was calculated. (E) Western blot analysis of cleaved caspase-3 after treatment with GSK1838705A (0.25 and 1 μM) for 48 hours.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

with GSK1838705A. After treatment with GSK1838705A, the PC-3R cell nuclei showed consistent morphological changes, including chromatin condensation and nuclear fragmentation, both of which are indicators of cell apoptosis (Figure 2C and D). GSK1838705A-induced cell apoptosis was also confirmed by TUNEL staining assay (Figure 2C). These structural alterations indicate that treatment with GSK1838705A generated apoptosis in docetaxel-resistant PC-3R cells. Further, Western blot analysis showed that treatment with GSK1838705A significantly increased levels of cleaved caspase-3 in resistant cells (Figure 2E), further confirming GSK1838705A-induced apoptosis in resistant cells. These data are consistent with the viability suppression results reported above.

GSK1838705A inhibits phosphorylation of IGF1R and IR in PC-3R cells

We next observed the effect of GSK1838705A on phosphorylation of IGF1R and IR in PC-3R cells. We found that GSK1838705A could significantly inhibit phosphorylation of IGF1R and IR, while there were no significant changes in total protein levels (Figure 3).

GSK1838705A reduces PC-3R cell migration

IGF1R/IR-mediated signaling plays a critical role in migration of cancer cells,^{17,18} so we sought to examine the effect of GSK1838705A on migration of docetaxel-resistant PC-3R cells. As shown in Figure 4A and B, GSK1838705A dramatically reduced the migration of PC-3R cells in a Transwell model when compared with the control group ($P < 0.01$), suggesting that in addition to viability-suppressing activity, GSK1838705A could also suppress migration of prostate cancer cells, especially those that were docetaxel-resistant.

GSK1838705A suppresses PC-3R tumor growth in vivo

To further validate our in vitro work, nud/nud mice were treated with GSK1838705A (20 and 60 mg/kg) to evaluate the effect of GSK1838705A (intraperitoneal injection) on docetaxel-resistant PC-3R tumor growth in vivo. The results show that a low dose of GSK1838705A (20 mg/kg) culminated in an intermediate degree of tumor growth suppression ($P < 0.05$). In contrast, a high dose of GSK1838705A (60 mg/kg) led to significant inhibition of PC-3R tumor growth when compared with the control group (Figure 5A, $P < 0.01$). These results were further confirmed by TUNEL and nuclear staining data, which showed that treatment with GSK1838705A resulted in

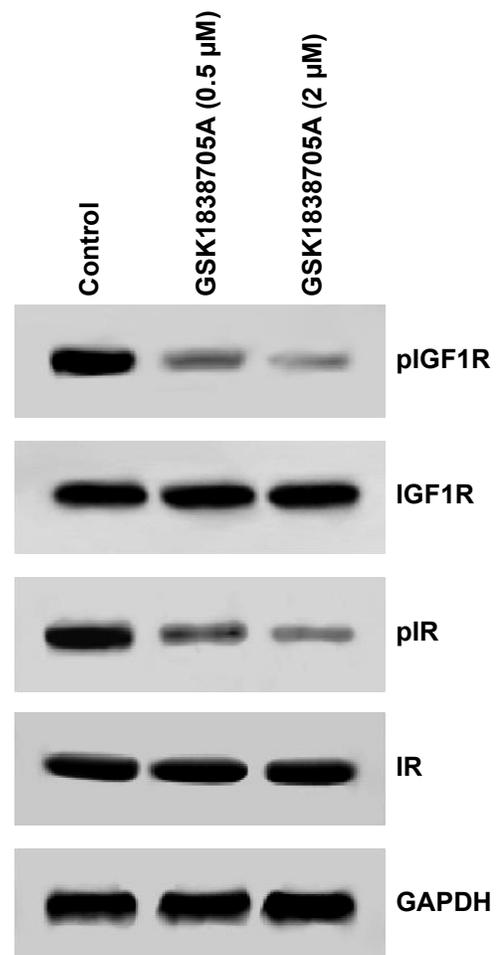


Figure 3 GSK1838705A inhibits phosphorylation of IGF1R and IR in docetaxel-resistant cells. PC-3R cells were seeded in six-well plates. After 24 hours, the medium was replaced with serum-free medium, and GSK1838705A (0.5 and 2 μ M) was added and incubated for 4 hours, then cells were stimulated with 30 ng/mL IGF1 or 3 μ g/mL insulin for 20 minutes, followed by Western blot analysis with the indicated antibodies.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF1, insulin-like growth factor-1; IGF1R, insulin-like growth factor-1 receptor; IR, insulin receptor; p, phosphorylated.

morphological changes indicative of apoptosis (Figure 5B). It should be noted that administration of GSK1838705A was well tolerated by the mice, which showed no measurable signs of overt toxicity or decreases in body weight ($P > 0.05$, Figure 5C). In summary, our data indicate that GSK1838705A could reduce the viability of both docetaxel-sensitive and docetaxel-resistant prostate cancer cells in vitro and suppress tumor growth in vivo.

Discussion

Prostate cancer is the most common malignancy in men, occurring at a rate of 62 per 100,000 adult men, and is the second most common cancer in men worldwide. Docetaxel is one of the most effective compounds for the treatment of

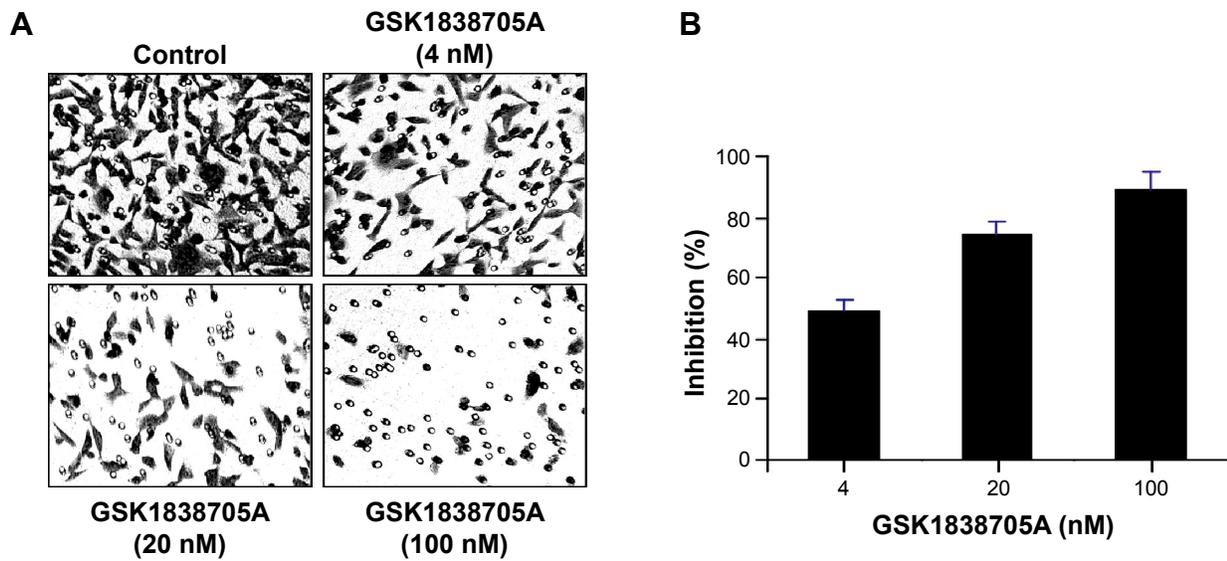


Figure 4 GSK1838705A inhibits cell migration of docetaxel-resistant cells. **Notes:** (A) PC-3R cells were treated with GSK1838705A (4, 20, or 100 nM) for 8 hours. The nonmigrated cells on the upper surface of the filter were removed, and the migrated cells on the lower side were stained and photographed. Representative images are shown. (B) The migrated cells were lysed and colorimetric determination was done at 595 nm and the quantitation result for inhibition is shown.

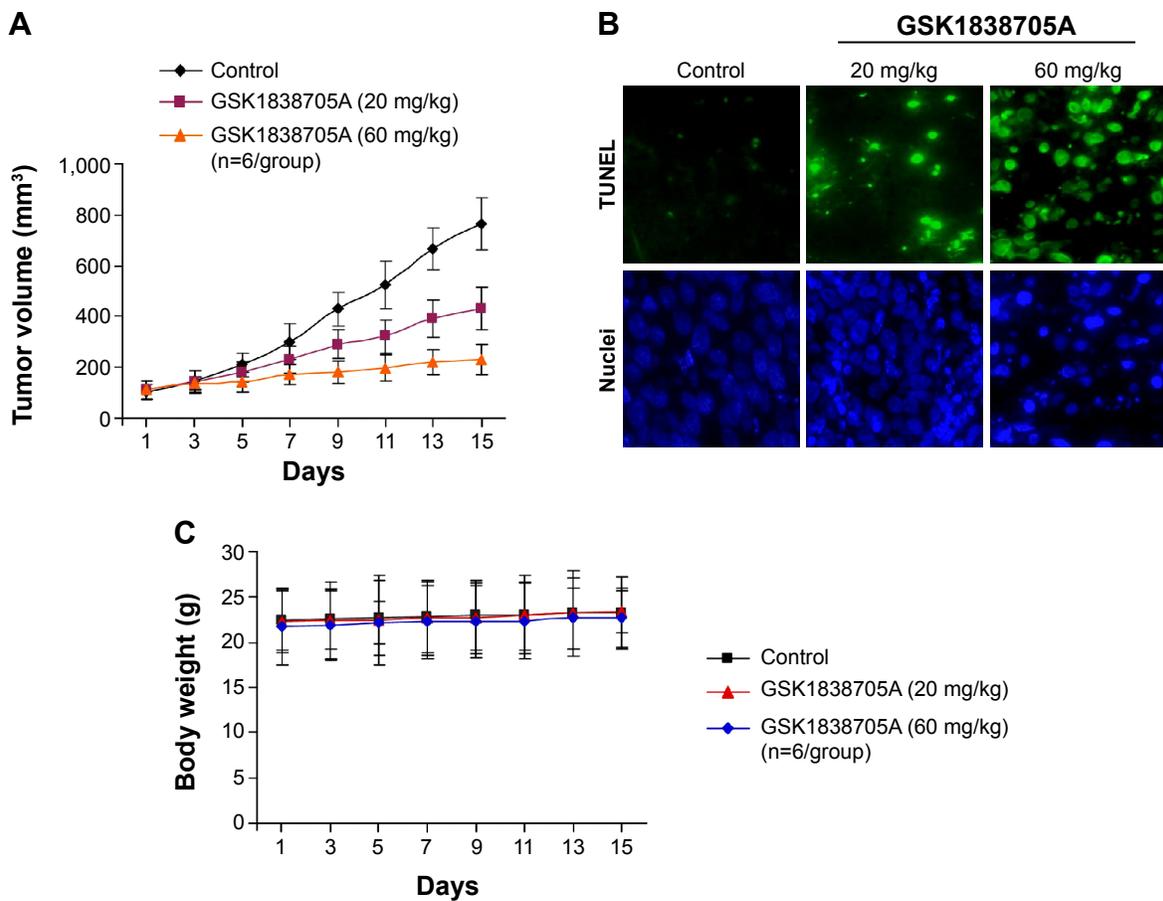


Figure 5 GSK1838705A suppresses PC-3R tumor growth in vivo. **Notes:** (A) After inoculation of PC-3R cells, GSK1838705A (20 and 60 mg/kg) was injected into the mice every day. The tumors were measured every other day, and the tumor volumes are shown. (B) GSK1838705A induced apoptosis of PC-3R tumor cells in vivo as determined by TUNEL assay (green), and the nuclei were stained with Hoechst (blue). (C) GSK1838705A had no significant cytotoxic effect on body weight during treatment. **Abbreviation:** TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

prostate cancer.^{5–7} However, resistance to docetaxel develops in patients with prostate cancer rapidly within months,^{8,9} so overcoming or attenuating docetaxel resistance is an urgent issue. Combination therapies have been proposed to improve the therapeutic potential of docetaxel in prostate cancer.^{14,15,19} Therefore, much research effort is focused on identifying effective agents for the treatment of prostate cancer, especially docetaxel-resistant disease.

IGF1R/IR-mediated signaling pathways promote proliferation and survival of a number of cancer cells, including prostate cancer cells,^{10–13} and inhibition of IGF1R/IR kinases represents a therapeutic opportunity. These signaling pathways have been linked to an increased risk of prostate cancer, as well as development of resistance in patients with the disease.^{14,15} Numerous studies have shown that inhibitors of IGF1R/IR or antibodies against IGF1R/IR have potential therapeutic efficacy in a number of types of cancer, including docetaxel-resistant prostate cancer.^{14,15,20–23} For example, Tang et al found that lycopene could enhance the effect of docetaxel in castration-resistant prostate cancers associated with changes in IGF1R levels.¹⁵ Considering the critical role of IGF1R and IR in prostate tumor growth, metastasis, and development of resistance,^{14,15,17,18,20–23} we investigated the activity of GSK1838705A, an IGF1R/IR inhibitor,¹⁶ in the disease, especially docetaxel-resistant prostate cancer, both in vitro and in vivo. Sabbatini et al reported that GSK1838705A could inhibit IGF1R and IR and block the in vitro proliferation of cancer cells derived from solid and hematological malignancies, including multiple myeloma and Ewing's sarcoma, and suppress growth of tumor xenografts in vivo. In addition to the inhibitory effect of GSK1838705A on IGF1R and IR, Sabbatini et al also found that GSK1838705A could inhibit the anaplastic lymphoma kinase in anaplastic large-cell lymphomas, some neuroblastomas, and a subset of nonsmall cell lung cancers.¹⁶ Herein, we focused on the effect of GSK1838705A in docetaxel-resistant prostate cancer both in vitro and in vivo. We found that GSK1838705A could effectively suppress viability of both docetaxel-sensitive and docetaxel-resistant prostate cancer cells. Consistent with this, treatment with GSK1838705A resulted in significant apoptosis in docetaxel-resistant cells. Further, GSK1838705A effectively inhibited phosphorylation of IGF1R and IR in these resistant cells, significantly reduced migration of docetaxel-resistant cells, and dramatically suppressed docetaxel-resistant PC-3R tumor growth in vivo.

In summary, this is the first report of the effect of GSK1838705A, an IGF1R/IR inhibitor, on prostate cancer cells and tumors. The anticancer activity of GSK1838705A in docetaxel-resistant cells make it a promising agent for treating

prostate cancer, especially when it has developed resistance to docetaxel. Moreover, GSK1838705A has strong inhibitory activity on migration of docetaxel-resistant prostate cancer cells. All these findings might open up another avenue for overcoming docetaxel resistance in prostate cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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