Effect of FAK inhibitor VS-6063 (defactinib) on docetaxel efficacy in prostate cancer

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ABSTRACT

Background: Docetaxel, the standard chemotherapy for metastatic castration-resistant prostate cancer (CRPC) also enhances the survival of patients with metastatic castration-sensitive prostate cancer (CSPC) when combined with androgen-deprivation therapy. Focal Adhesion Kinase (FAK) activation is a mediator of docetaxel resistance in prostate cancer cells. The aim of this study was to investigate the effect of the second generation FAK inhibitor VS-6063 on docetaxel efficacy in pre-clinical CRPC and CSPC models.

Methods: Docetaxel-resistant CRPC cells, mice with PC3 xenografts, and *ex vivo* cultures of patient-derived primary prostate tumours were treated with VS-6063 and/or docetaxel, or vehicle control. Cell counting, immunoblotting, and immunohistochemistry techniques were used to evaluate the treatment effects.

Results: Docetaxel and VS-6063 co-treatment caused a greater decrease in the viability of docetaxel-resistant CRPC cells, and a greater inhibition in PC3 xenograft growth compared to either monotherapy. FAK expression in human primary prostate cancer was positively associated with advanced tumour stage. Patient-derived prostate tumour explants cultured with both docetaxel and VS-6063 displayed a higher percentage of apoptosis in cancer cells, than monotherapy treatment.

Conclusions: Our findings suggest that co-administration of the FAK inhibitor, VS-6063, with docetaxel represents a potential therapeutic strategy to overcome docetaxel resistance in prostate cancer.

KEYWORDS

Docetaxel, chemoresistance, focal adhesion kinase, prostate cancer, defactinib, VS-6063

INTRODUCTION

Docetaxel remains the first-line cytotoxic treatment for metastatic castration-resistant prostate cancer (CRPC) since 2004 [1,2]. However, docetaxel confers survival and palliative benefits in only ~50% of patients [1,2], and initial responders eventually develop resistance. The addition of docetaxel to first-line androgen deprivation therapy substantially improves the survival of patients with metastatic castration-sensitive prostate cancer (CSPC), however, again this is not universal and taxane resistance remains an issue [3,4]. Overall, there is a need to understand the mechanisms of docetaxel resistance and develop new therapeutic strategies to overcome resistance.

Various docetaxel-resistance mechanisms involving perturbations in cell signalling pathways have been identified, leading to the development of potential strategies for enhancing docetaxel efficacy [5]. However, none of these findings have translated successfully to the clinical setting, as illustrated by the failure of nine Phase 3 trials to date [6].

FAK is a cytoplasmic tyrosine kinase that also functions as a scaffold, transducing signals from extracellular cues such as growth factor receptors and integrins to downstream signalling pathways to regulate cell adhesion, proliferation, survival and migration [7]. Various cancers display increased FAK expression, which is often correlated with poor prognosis and advanced disease [7]. The role of FAK signalling in tumourigenesis and tumour progression has been extensively studied, leading to the development of FAK tyrosine kinase inhibitors as potential cancer therapeutics [7].

Enhanced FAK signalling may also contribute to tumour progression in the context of drug resistance [8-11]. Previously we showed that FAK activation in docetaxel-resistant CRPC prostate cancer cell lines was implicated in docetaxel resistance, as FAK phosphorylation was increased in docetaxel-resistant cell lines and docetaxel resistance was reversed by co-treatment with docetaxel and the first generation FAK tyrosine kinase inhibitor PF-00562271 [8]. In addition to enhanced FAK phosphorylation in these docetaxel-resistant cells, the phosphorylation of other focal adhesion (e.g. BCAR1/p130Cas, paxillin) and cytoskeletal

(actinin alpha 1, vimentin, PDLIM5, caveolin-1) proteins was also increased, as was that of the upstream FAK regulator EphA2 [8], indicating that multiple inputs may underpin the elevated FAK signalling. Reversal of docetaxel resistance by inhibiting FAK phosphorylation with PF-00562271 in these cell lines suggests that co-administration of FAK tyrosine kinase inhibitors with docetaxel may overcome docetaxel resistance in CRPC patients. In light of the recent findings of the survival benefits of docetaxel in CSPC patients, there is also a need to study the effect of FAK tyrosine kinase inhibitors on docetaxel efficacy in the castration-sensitive setting.

Although PF-00562271 was well-tolerated in Phase 1 clinical trials, co-administration of PF-00562271 with docetaxel may cause significant toxicity as it is a potent inhibitor of CYP3A, the main enzyme that metabolises docetaxel [12]. The second generation FAK inhibitor VS-6063 (defactinib), previously known as PF-04554878, is a safer alternative as it is a weak inhibitor of CYP3A, has a more favourable pharmacokinetic profile, and was well-tolerated in Phase 1 trials [13].

The aim of this study was to determine if the second generation FAK inhibitor, VS-6063, overcomes docetaxel resistance in CRPC cell lines, and if VS-6063 could enhance the efficacy of docetaxel in an *in vivo* CRPC model and a patient-derived *ex vivo* CSPC model.

MATERIALS AND METHODS

Drugs and cell lines

Docetaxel (Sanofi-Aventis, Australia) and VS-6063 (Verastem, USA) (previously known as PF-4554878 [Pfizer, USA]) were obtained from their respective manufacturers. Working stocks of docetaxel was prepared with saline, whereas VS-6063 was dissolved in DMSO. PC3 and DU145 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Docetaxel-resistant sublines, referred to as PC3-Rx and DU145-Rx were established from PC3 and DU145 respectively by treatment with escalating doses of docetaxel, and maintained as previously described [8]. Experiments with cell lines were done within 10 passages. The cell lines were authenticated by CellBank Australia using Short Tandem Repeat profiling.

Cell Viability Assay

Cell viability was assessed by Trypan blue exclusion, with cell counting of viable cells using a haemocytometer as previously described [8].

Prostate cancer xenografts

Mice experiments were approved by the Garvan/St Vincent's Animal Ethics Committee according to the Animal Research Act 1985, Animal Research Regulation 2010, and the Australian code of practice for the care and use of animals for scientific purposes. Male Balb/c nude mice (BALB/c-Foxn1nu/Ausb, Australian BioResources, Moss Vale, NSW, Australia) were injected subcutaneously on the flank with 100 µL of 2 million PC3 cells suspended in 50% Matrigel (BD Biosciences) and 50% PBS (v/v). Tumours were measured twice weekly with calipers, and tumour volume calculated as 0.5 x length x width^2. When tumours reached ~100 mm^3, the mice were assigned to receive one of the following four treatments for 2 weeks: (a) control (weekly intraperitoneal injection of 100 µL saline, and twice daily oral gavage of vehicle solution of 10% DMSO, 5% Gelucire 44/14 [Gattefossé, Trapeze Associates, Clayton, VIC, Australia], and 85% water [v/w/v]); (b) VS-6063 (weekly

intraperitoneal injection of 100 µL saline, and twice daily oral gavage of 50 mg/kg VS-6063 prepared in vehicle solution); (c) docetaxel (weekly intraperitoneal injection of 10 mg/kg docetaxel in saline, and twice daily oral gavage of vehicle solution); (d) docetaxel and VS-6063 co-treatment (weekly intraperitoneal injection of 10 mg/kg docetaxel, and twice daily oral gavage of 50 mg/kg VS-6063 prepared in vehicle solution). Mice were euthanased by carbon dioxide asphyxiation followed by cervical dislocation when their tumours reached ~500 mm^3. Tumours were dissected, snap frozen in liquid nitrogen and stored at -80°C.

Ex vivo culture of patient-derived prostate tumours

Fresh tissue cores (5 mm diameter) were obtained from the surgically-resected prostate of men with primary prostate cancer undergoing radical prostatectomy at St Vincent's Private Hospital, Darlinghurst, Sydney (St Vincent's Hospital's human research ethics approval reference number 12/231). Tissue cores were dissected into ~1 mm^3 pieces, and placed on gelatine sponges (Spongostan Dental, Johnson & Johnson) individually soaked in 500 µl of culture medium in a 24-well plate as previously described [14]. For this study, the culture medium was prepared with 250 nM docetaxel and/or 200 nM VS-6063, or 0.15% DMSO solvent control. Ten tissue pieces from each patient were cultured per treatment (5 tissue pieces per sponge) at 37°C. After 72 hours of culture, the explants were formalin-fixed and paraffin-embedded, or snap-frozen in liquid nitrogen for storage at -80°C.

Immunoblotting of lysates

Immunoblotting of lysates from cell lines were performed as previously described [15]. Immunoblotting of lysates from xenografts and explants were performed as for cell lines, except that the immunoblots for explants were visualised using the digital imager Fusion-Fx7 (Vilber Lourmat, Germany). Primary antibodies used for immunoblotting were from Cell Signaling Technology (Massachusetts, USA), except the following: pY397-FAK (Invitrogen), FAK (BD Transduction Laboratories), pY576-FAK (Santa Cruz Biotechnology), β-Actin (Sigma), and GAPDH (Abcam).

Immunohistochemistry of primary prostate cancer

As described previously [16], tissue microarrays were constructed using 1.5 or 2 mm tissue core biopsies of primary prostate cancer from patients undergoing radical prostatectomy at St Vincent's Private Hospital, Darlinghurst, Sydney (St Vincent's Hospital's human research ethics approval reference number 12/231). Immunostaining of tissue microarray sections (4 μM thickness) with the FAK monoclonal mouse antibody (1:100, Clone 77, BD Transduction Laboratories) was performed on the Dako automated stainer with Dako EnVision HRP labelled polymer anti-mouse, and Dako DAB+ chromogen. The immunostaining was scored by a specialist prostate cancer pathologist (JK), and represented as the H-score which is the percentage of cancer cells with positive staining multiplied by staining intensity (graded as 0 [absent], 1 [weak], 2 [moderate] or 3 [strong]).

Immunohistochemistry of explants

Formalin-fixed paraffin-embedded sections (4 μM thickness) of explants were co-immunostained with cleaved caspase-3 rabbit antibody (1:200, Cell Signalling Technology), p63 mouse antibody (1:100, Clone DAP-p63 clone, Dako), and cytokeratin (high molecular weight) 34βE12 mouse antibody (1:200, Clone 34BETAE12, Leica Biosystems). The co-immunostaining was performed using the Leica Bond Rx automated stainer with the ChromoPlex 1 Dual Detection kit (Leica Biosystems), which results in brown staining (DAB chromogen) for primary mouse antibodies and pink staining (Fast red chromogen) for primary rabbit antibodies. The percentage of cancer cells with cleaved caspase-3 staining was determined by manual counting of cancer cells by a specialist prostate cancer pathologist (JG). Seven consecutive tissue sections were evaluated to obtain a sufficient count of cancer cells.

Statistical analyses

Statistical tests were performed using GraphPad Prism (GraphPad Software Inc). Comparisons between two groups were analysed using t-test. Comparisons between more

than two groups were analysed using ANOVA with Bonferroni post hoc correction for multiple comparisons. P-values of less than 0.05 were considered statistically significant.

RESULTS

VS-6063 and docetaxel treatment of cell lines

Previously we showed that docetaxel and PF-00562271 co-treatment overcame docetaxel resistance in the docetaxel resistant cell lines, PC3-Rx and DU145-Rx, which were derived from PC3 and DU145 respectively [8]. Similar to PF-05562271, VS-6063 reversed docetaxel resistance in PC3-Rx and DU145-Rx, with a change in docetaxel IC50 by 75- and 43-fold for PC3-Rx and DU145-Rx respectively (Figure 1). In contrast, the docetaxel sensitivity of the parental cells PC3 and DU145 was not affected by the co-treatment (Figure 1). The viability of the docetaxel-resistant or parental cells was not affected by VS-6063 treatment alone (Figure 1 inset).

FAK phosphorylation (relative to total FAK) was significantly enhanced at tyrosine residues Y397 and Y576 in PC3-Rx compared to PC3 [8]. Treatment of PC3 or PC3-Rx with either docetaxel or VS-6063 resulted in decreased FAK phosphorylation on both tyrosine residues, and this effect was enhanced upon co-treatment (Figures 2A, B, C). Interestingly, docetaxel treatment also led to reduced levels of S473-phosphorylated-AKT, and combination of docetaxel with VS-6063 led to a further reduction in AKT activation, which was particularly pronounced in PC-Rx (Figures 2A, D).

Since the batch of DU145-Rx cells used exhibited increased levels of total FAK compared to DU145 (Figure 3A), for these cell models we considered total levels of phosphorylated FAK (relative to β-actin). Total levels of phosphorylated FAK at tyrosine residues Y397 and Y576 were significantly higher in DU145-Rx compared to DU145 (Figures 3A-C). Treatment of DU145 or DU145-Rx with VS-6063 resulted in decreased FAK phosphorylation on both tyrosine residues (Figures 3A-C)

Overall, these data demonstrate that VS-6063 is able to reverse docetaxel resistance in these two cell line models, accompanied by suppression of FAK activation and signalling.

VS-6063 and docetaxel treatment of xenograft tumours

The pronounced impact of VS-6063 on docetaxel sensitivity *in vitro*, and its more favourable toxicity and pharmacokinetic profile compared to PF-00562271, strongly supported further pre-clinical testing in an animal model. The docetaxel-resistant cell lines, PC3-Rx and DU145-Rx, exhibited poor tumourigenicity when implanted in immunodeficient mice, consistent with another report of drug-tolerant cancer cells [17]. However, xenografts established from parental PC3 cells are docetaxel-resistant *in vivo* as the tumours renewed their growth when docetaxel treatment was ceased. Therefore, PC3 xenografts were used to examine the *in vivo* effects of docetaxel and VS-6063 co-treatment.

Tumour-bearing mice treated with docetaxel in combination with VS-6063 had a greater inhibition of tumour growth compared to those treated with either VS-6063 or docetaxel alone (Figure 4A). The time to reach the tumour volume endpoint was markedly delayed by the combination therapy (median 47.5 days) compared to docetaxel alone (median 29.5 days) ($p=0.003$, Figure 4B). Five of the 15 mice (33%) receiving the combination therapy experienced weight loss of up to 26% on the last day of the regimen. However, these mice regained their body weight in the following days after completing the regimen (Figure 4C). Consequently the combination therapy is not associated with significant side-effects or toxicity.

FAK phosphorylation was reduced in xenograft tumours from mice treated with docetaxel, VS-6063, or their combination, with the largest reduction for the co-treatment (Figure 5A-C). Similar to the PC3/PC3Rx *in vitro* model, docetaxel administration led to reduced AKT phosphorylation, and this effect was enhanced further with co-treatment (Figure 5A, D). Treatment with docetaxel reduced mTOR phosphorylation and p62 levels, and a greater reduction in both parameters was observed with the co-treatment (Figure 5A). Additionally, a significant increase in LC3B-I conversion to LC3B-II was observed in xenograft tumours of mice receiving the co-treatment (Figure 5A & E). This, in conjunction with the reduced mTOR phosphorylation and p62 levels, is strongly suggestive of autophagic cell death [18].

FAK expression in primary prostate cancer

Given that docetaxel treatment is now also used in the castration-sensitive setting, and FAK plays a role in docetaxel-resistance, the expression of FAK was examined in primary prostate tumours resected by radical prostatectomy from 63 patients with high risk localised prostate cancer. FAK expression was generally higher in prostate cancer cells compared to adjacent benign prostate tissue (Figure 6A). Furthermore, FAK expression was significantly higher in cancers graded as Gleason score 6, 7, and 9 compared to Gleason 5, indicating that FAK expression was higher with advanced prostate cancer grades (Figure 6B).

VS-6063 and docetaxel treatment of e*x vivo* cultures of patient-derived tumours

The *ex-vivo* culture of tumours from patients is a useful pre-clinical model that takes into account the effect of tumour stroma on the efficacy of therapeutics, as the native tissue architecture of the tumour is retained [19].

To determine if VS-6063 can enhance the efficacy of docetaxel in castration-sensitive prostate cancer, *ex vivo* cultures of prostate tumour tissue from 11 patients undergoing radical prostatectomy were cultured with docetaxel and/or VS-6063, or solvent control. Cancer cells in immunostained tissue sections were distinguished from benign prostate glands with the assistance of benign basal cell markers – p63 and cytokeratin 34βE12 (Figure 6C). Explants from two patients did not contain any malignant glands. Explants that contained cancer cells for all the treatment conditions to enable pair-wise analysis were only observed for six patients. The average number of cancer cells counted per treatment per patient was 1362 cells (minimum of 13, maximum of 3465). There was no significant difference in cleaved caspase-3 staining between explants cultured with either VS-6063 or docetaxel alone compared to solvent control (Figure 6D). However, cleaved caspase-3 immunostaining was significantly higher in explants cultured in the presence of both drugs than control (Figure 6D).

FAK phosphorylation at Y576 was significantly lower in explants cultured with VS-6063 or the co-treatment compared to control, demonstrating on-target activity of VS-6063 (Figure 6E-F). In contrast to the *in vitro* and *in vivo* PC3 models, significant changes in AKT phosphorylation were not detected with mono- or combination treatment (Figure 6G).

DISCUSSION

This study demonstrates that docetaxel efficacy in prostate cancer is enhanced by FAK tyrosine kinase inhibitor, VS-6063. Docetaxel sensitivity in docetaxel-resistant prostate cancer cell lines was restored by VS-6063 inhibition of FAK phosphorylation, consistent with the effect of the first generation FAK inhibitor PF-00562271. More importantly, the efficacy of docetaxel in inhibiting PC3 xenograft growth *in vivo* and in inducing cell death in patient-derived prostate tumour explants was enhanced by VS-6063 co-treatment.

The role of increased FAK expression and activation in cancer development and progression is well-established [7]. Accumulating evidence indicates a new role for FAK in therapeutic resistance. In addition to our previous study of FAK mediating docetaxel resistance in CRPC [8], siRNA silencing of FAK activity *in vitro* and *in vivo* was reported to enhance the efficacy of docetaxel in inhibiting the growth of taxane-resistant ovarian cancer cells and xenografts [20,21]. The role of FAK signalling in drug-resistance is not limited to taxanes, as combining *in vivo* FAK siRNA delivery with the platinum-based agent cisplatin also resulted in a greater reduction of tumour growth than either agent alone [20]. FAK also modulates sensitivity to anti-HER2 therapy, as co-treatment of VS-6063 and the HER2 inhibitor trastuzumab resulted in synergistic inhibition of the proliferation of ER+/HER2+ breast cancer cells [10]. To date, only one other study has evaluated the effect of VS-6063 in combination with a cytotoxic drug on tumour growth *in vivo* [9]. The combination of VS-6063 and paclitaxel was more effective in reducing growth of taxane-sensitive and resistant ovarian cancer cell xenografts, compared to either monotherapy. *In vitro* experiments indicated that VS-6063-mediated paclitaxel sensitisation involved inhibition of AKT pathway signalling and the transcription factor YB-1 [9].

Previously we demonstrated that the first generation FAK tyrosine kinase inhibitor PF-00562271 in combination with docetaxel caused cell death in docetaxel-resistant prostate cancer cells via autophagic cell death (type II programmed cell death), as indicated by reduced mTOR phosphorylation, LC3B-I conversion to LC3B-II, and p62 degradation (Lee *et*

al, 2014). Additionally, cell death under these conditions was blocked by either pharmacological inhibition of autophagy using the autophagosome inhibitor 3-methyladenine or ATG5 knockdown (Lee *et al*, 2014). Interestingly, similar effects on mTOR, LC3B-II and p62 were observed in the PC3 xenografts of mice receiving VS-6063 and docetaxel co-treatment, strongly suggesting that tumour growth inhibition in this model also involves autophagic cell death.

Autophagic cell death has been observed in cancer cells in response to certain chemotherapeutic agents, particularly when the cells lack essential apoptotic modulators such as BAX, PUMA or specific caspases [22,23]. This raises the possibility that certain apoptotic modulators are also lacking or non-responsive to docetaxel in our docetaxel-resistant models, thus resulting in autophagy being triggered as an alternative pathway of cell death when m-TOR phosphorylation is markedly reduced following VS-6063 treatment.

Increased AKT phosphorylation was observed in our PC3-Rx docetaxel-resistant prostate cancer cell lines, suggesting the importance of AKT signalling in taxane resistance. FAK is known to activate AKT through the interaction of FAK's phospho-Y397 with the p85 subunit of PI3K [24]. However, treatment with docetaxel alone appeared to have a greater effect on AKT phosphorylation than VS-6063 *in vitro*. In the xenografts, the only mono-therapy that affected AKT activation was docetaxel, despite similar decreases in FAK activation with docetaxel or VS-6063. In both the *in vitro* and xenograft models, the combination of docetaxel and VS-6063 resulted in a further reduction of AKT phosphorylation compared to docetaxel alone, suggesting that the influence of VS-6063 on PI3K/AKT signalling is dependent on the effect of docetaxel. While the ability of docetaxel to impact FAK activation likely reflects its effects on microtubules, which are known to interact with focal adhesions and modulate their dynamics [25], how docetaxel impacts AKT activation requires further clarification.

While we could readily detect FAK phospho-Y576 in the patient-derived explants, this was not the case for phospho-Y397. Additionally, we did not detect a decrease in AKT

phosphorylation upon co-treatment of the explants with docetaxel and VS-6063. These data may reflect a number of factors. First, the greater proportion of stroma in the explants versus xenografts may lead to a sensitivity issue with regard to detection of FAK phospho-Y397. Second, regulation of AKT in response to drug treatment may differ between the cancer cells and stroma, leading to an inability to detect changes in AKT phosphorylation in cancer cells upon drug administration. Third, crosstalk between the stroma and cancer cells may be greater in the explant model, leading to alterations in FAK and AKT regulation in this model compared to xenografts. If this is the case, we accept that there may be AKT-independent mechanisms that lead to the enhanced efficacy of the drug co-treatment in the explants.

The concentrations of VS-6063 used in our study are achieved in plasma with clinical doses [13]. VS-6063 monotherapy is well-tolerated in humans according to a Phase 1 study of patients with advanced solid tumours [13]. To date, only one clinical trial is investigating the combination of FAK inhibitors with cytotoxic agents; a phase 1/1b dose-escalation study of paclitaxel in combination with VS-6063 for advanced ovarian cancer (NCT01778803). The outcome will determine the safety of combining taxanes with VS-6063 in CRPC patients or other cancers. A Phase 2 study evaluating VS-6063 alone for mesothelioma was terminated due to lack of efficacy (NCT01870609). However, Phase 1 and 1/2a trials combining VS-6063 with the immunotherapeutic agents Avelumab or Pembrolizumab in ovarian cancer and other solid malignancies are underway, indicating that the efficacy of VS-6063 may lie in combination therapy (NCT02943317; NCT02546531, NCT02758587).

CONCLUSIONS

The findings from our study suggest that co-administration of the FAK inhibitor VS-6063 with docetaxel may enhance docetaxel efficacy in CSPC and CRPC patients. These data support the development of clinical trials to assess the efficacy of combining VS-6063 and docetaxel in advanced prostate cancer.

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FIGURE LEGENDS

FIGURE 1. Effect of VS-6063 on viability of docetaxel-sensitive and resistant prostate cancer cells. Docetaxel dose-response curves of (A) PC3 and PC3-Rx, and (B) DU145 and DU145-Rx. Cells were treated with increasing doses of docetaxel (DTX) ± 100 nM VS-6063 (VS6) for 24 hours. Inset graphs indicate that VS-6063 alone had no effect on viability of both docetaxel-sensitive and resistant cells. Data points represent the mean ± standard error for three independent experiments, with triplicate samples.

FIGURE 2. Effect of VS-6063 on FAK and AKT phosphorylation in PC3 and PC3-Rx cells. (A) Example of immunoblots of PC3 and PC3-Rx cells treated with docetaxel (DTX; 8 ng/ml) and/or VS-6063 (VS6; 100 nM) for 24 hours. (B-D) Quantitative analysis of immunoblots – FAK and AKT phosphorylation were normalised to total FAK (T-FAK) and total AKT (T-AKT) levels respectively, and expressed relative to DMSO vehicle controls. Data points represent the mean ± standard error for three independent experiments.

FIGURE 3. Effect of VS-6063 on FAK phosphorylation in DU145 and DU145-Rx cells. (A) Example of immunoblots of DU145 and DU145-Rx cells treated with docetaxel (DTX; 8 ng/ml) and/or VS-6063 (VS6; 100 nM) for 24 hours. (B, C) Quantitative analysis of immunoblots – FAK phosphorylation was normalised to β-actin, and expressed relative to DMSO vehicle controls. Data points represent the mean ± standard error for three independent experiments.

FIGURE 4. Anti-tumour efficacy of docetaxel and VS-6063 on PC3 xenografts. (A) Change in tumour volume of mice receiving weekly intraperitoneal injections of either 10 mg/kg docetaxel (DTX) or saline (days 1 and 8), and twice daily oral gavage of 50 mg/kg VS-6063 or vehicle for 14 days (arrows on x-axis). Data points indicate mean tumour volumes ±

509 standard error, with 14-15 mice per treatment group. (B) Kaplan-Meier curves of time to
510 reach tumour volume of 500 mm^3 from initiation of treatments. (C) Body weight of mice
511 during the treatments. Data points indicate mean body weight ± standard error.

512

513 **FIGURE 5. Effect of docetaxel and VS-6063 on FAK and AKT phosphorylation in PC3**
514 **xenografts.** (A) Immunoblots of individual tumour xenografts harvested on Day 9, 18 hours
515 after docetaxel (DTX) treatment and 2 hours after VS-6063 (VS6) treatment. (B-E)
516 Quantitative analysis of the immunoblots – FAK and AKT phosphorylation were normalised
517 to total FAK and AKT respectively, and LC3B-II levels were normalised to LC3B-I. All data
518 are expressed relative to vehicle (cont). Error bars indicate standard error.

519

520 **FIGURE 6. FAK expression in primary prostate cancer, and effect of docetaxel and VS-**
521 **6063 on patient-derived prostate tumour explants.** (A) Examples of primary prostate
522 cancer tissue sections (100x magnification) with different intensities of FAK immunostaining
523 – (i) absent, (ii) weak, (iii) moderate. (B) Quantitation of FAK immunostaining in primary
524 prostate cancer, H score = percentage cancer cells × staining intensity. (C) Example of
525 explant tissue section showing cancer glands with cleaved caspase-3 immunostaining (pink),
526 and basal cells of benign prostate glands with nuclear p63 and cytoplasmic cytokeratin
527 34βE12 immunostaining (brown). (D) Percentage of cancer cells in explants with cleaved
528 caspase-3 immunostaining. (E) Examples of immunoblots of explants. (F, G) Quantitative
529 analysis of immunoblots – phospho-FAK and phospho-AKT were normalised to total FAK
530 and AKT respectively, and expressed relative to control treatment. Each datapoint represents
531 a patient, and lines indicate mean ± standard error. Patient with outlier values was excluded
532 from t-test analysis and error bars (white symbols in F). Abbreviations: Cont, control (0.15%
533 DMSO); VS6, VS-6063 (200nM); DTX, docetaxel (250nM).

534

FIGURE 1

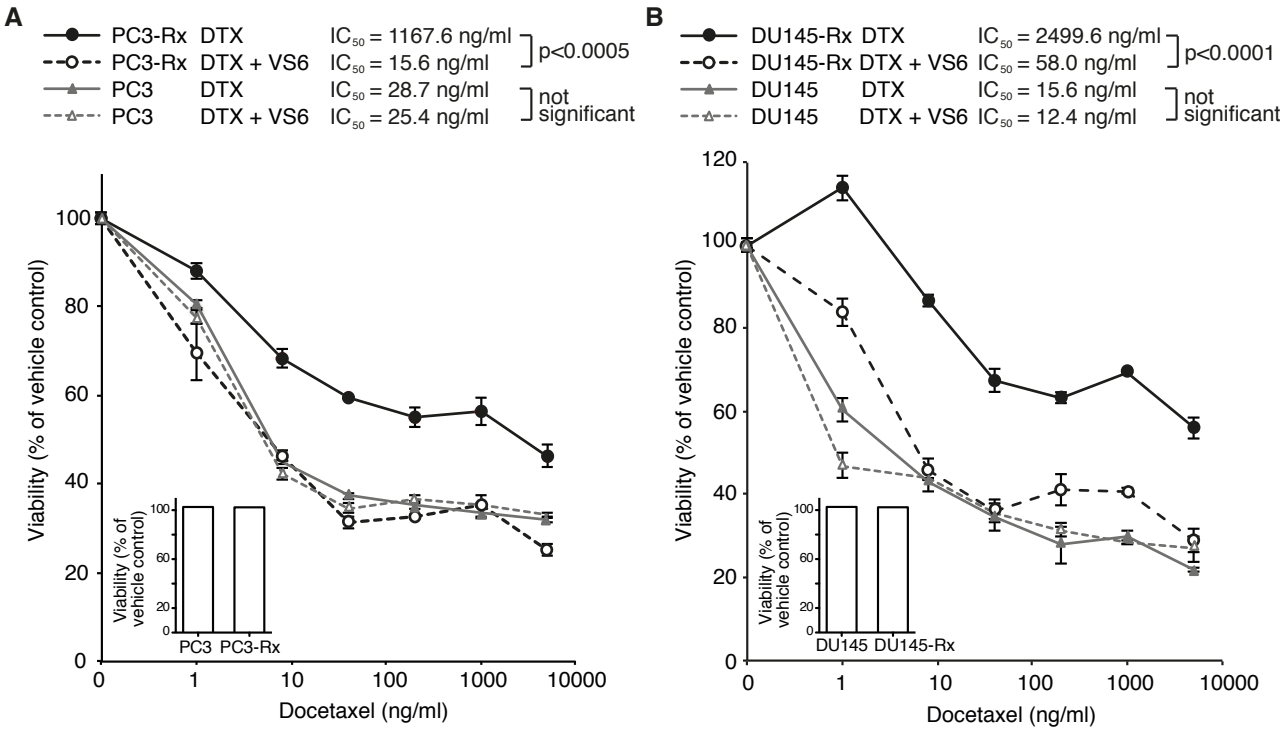

A
PC3-Rx DTX IC50 = 1167.6 ng/ml
PC3-Rx DTX + VS6 IC50 = 15.6 ng/ml
PC3 DTX IC50 = 28.7 ng/ml
PC3 DTX + VS6 IC50 = 25.4 ng/ml
p<0.0005
not significant
Viability (% of vehicle control)
Docetaxel (ng/ml)
PC3 PC3-Rx
B
DU145-Rx DTX IC50 = 2499.6 ng/ml
DU145-Rx DTX + VS6 IC50 = 58.0 ng/ml
DU145 DTX IC50 = 15.6 ng/ml
DU145 DTX + VS6 IC50 = 12.4 ng/ml
p<0.0001
not significant
Viability (% of vehicle control)
Docetaxel (ng/ml)
DU145 DU145-Rx

FIGURE 2

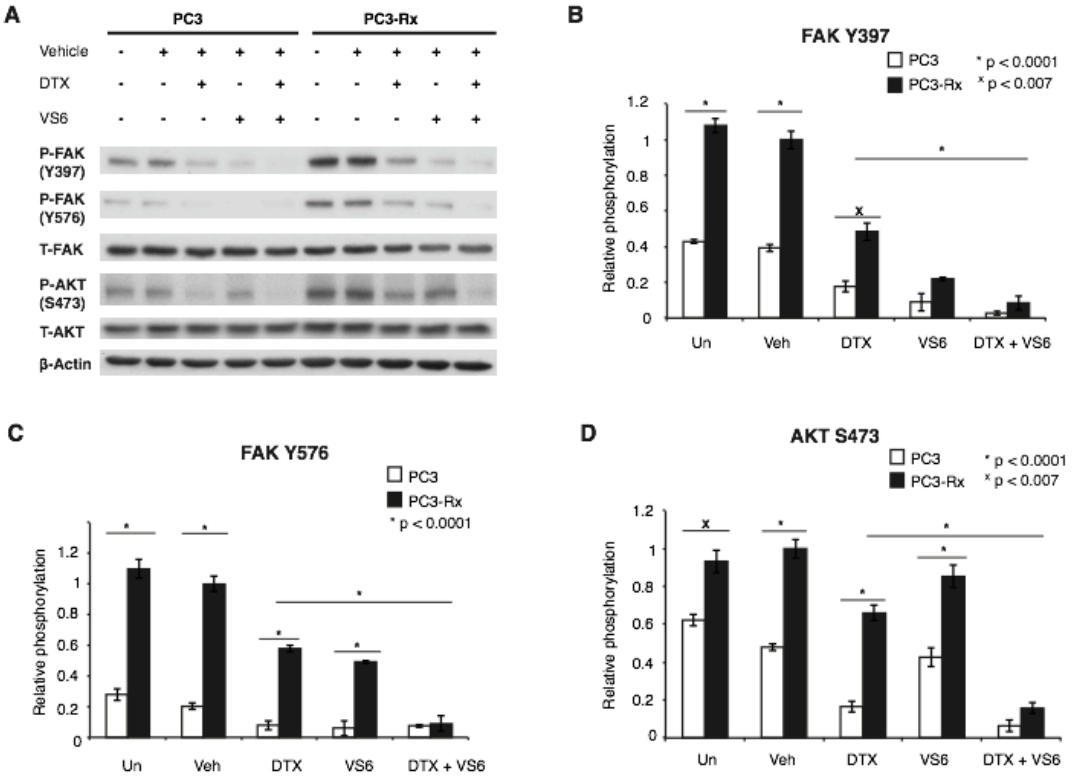

A
PC3
PC3-Rx
Vehicle
DTX
VS6
P-FAK (Y397)
P-FAK (Y576)
T-FAK
P-AKT (S473)
T-AKT
β-Actin
B
FAK Y397
PC3
PC3-Rx
* p < 0.0001
x p < 0.007
Relative phosphorylation
Un
Veh
DTX
VS6
DTX + VS6
C
FAK Y576
PC3
PC3-Rx
* p < 0.0001
Relative phosphorylation
Un
Veh
DTX
VS6
DTX + VS6
D
AKT S473
PC3
PC3-Rx
* p < 0.0001
x p < 0.007
Relative phosphorylation
Un
Veh
DTX
VS6
DTX + VS6

FIGURE 3

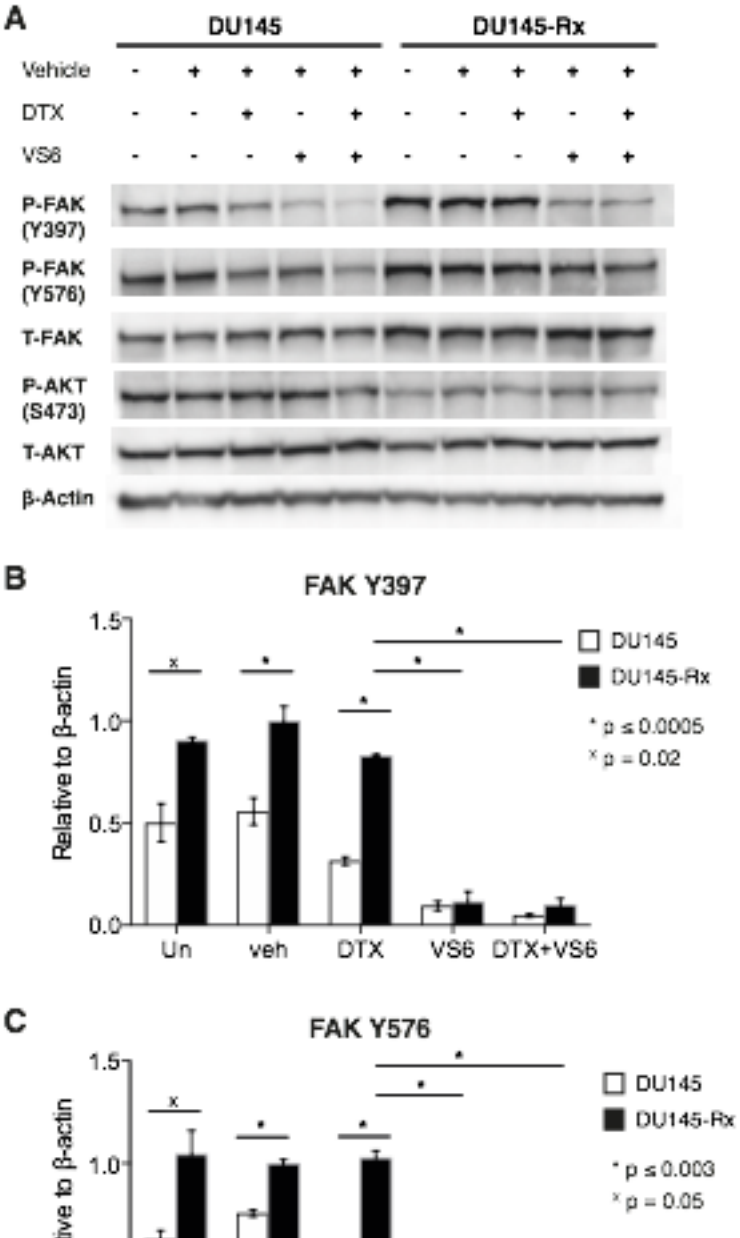

A
DU145
DU145-Rx
Vehicle
DTX
VS6
P-FAK (Y397)
P-FAK (Y576)
T-FAK
P-AKT (S473)
T-AKT
β-Actin
B
FAK Y397
Relative to β-actin
DU145
DU145-Rx
* p ≤ 0.0005
x p = 0.02
Un
veh
DTX
VS6
DTX+VS6
C
FAK Y576
Relative to β-actin
DU145
DU145-Rx
* p ≤ 0.003
x p = 0.05
Un
veh
DTX
VS6
DTX+VS6

FIGURE 4

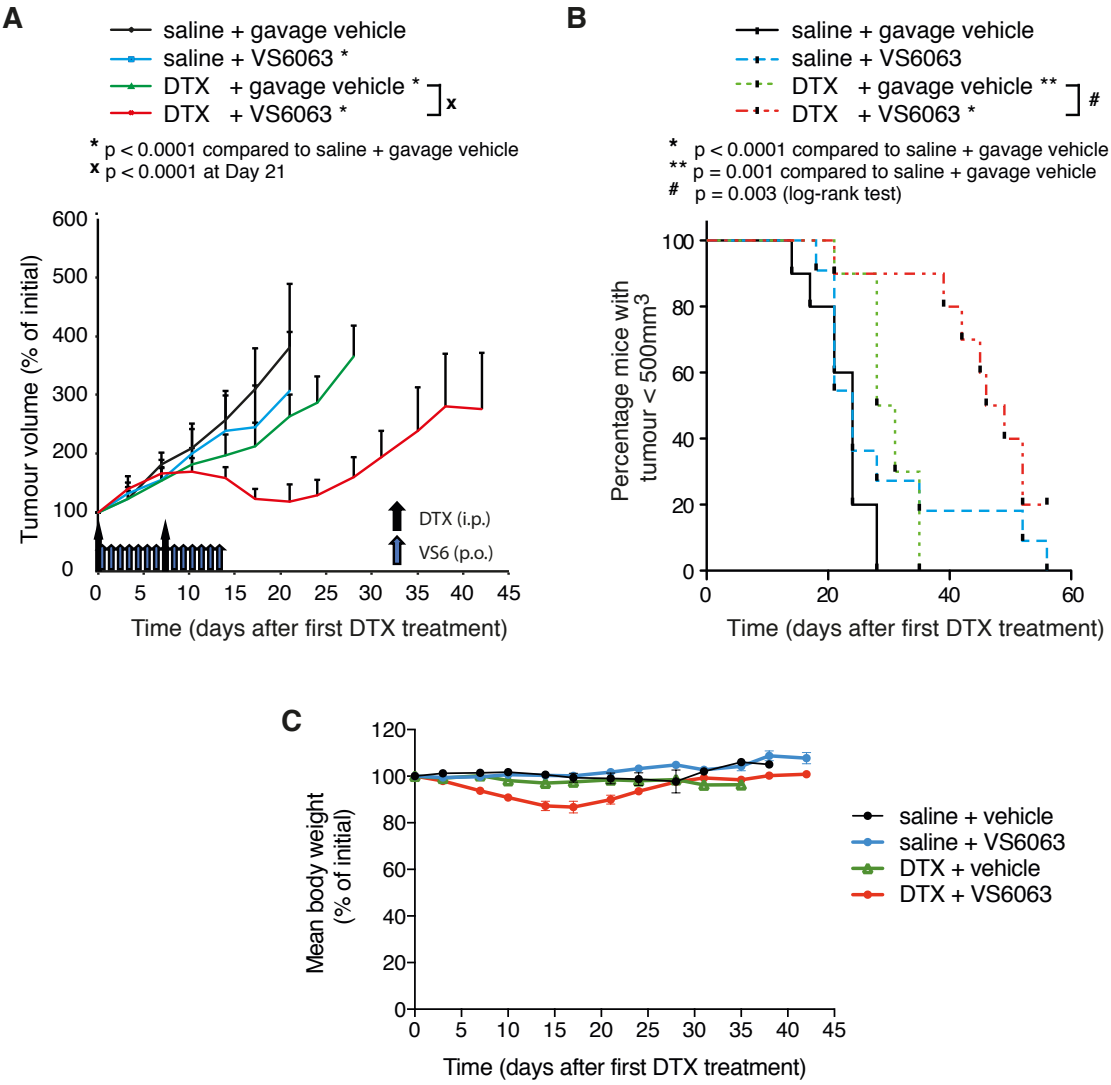

A
saline + gavage vehicle
saline + VS6063 *
DTX + gavage vehicle *
DTX + VS6063 *
x
* p < 0.0001 compared to saline + gavage vehicle
x p < 0.0001 at Day 21
Tumour volume (% of initial)
Time (days after first DTX treatment)
DTX (i.p.)
VS6 (p.o.)
B
saline + gavage vehicle
saline + VS6063
DTX + gavage vehicle **
DTX + VS6063 *
#
* p < 0.0001 compared to saline + gavage vehicle
** p = 0.001 compared to saline + gavage vehicle
# p = 0.003 (log-rank test)
Percentage mice with tumour < 500mm3
Time (days after first DTX treatment)
C
Mean body weight (% of initial)
Time (days after first DTX treatment)
saline + vehicle
saline + VS6063
DTX + vehicle
DTX + VS6063

FIGURE 5

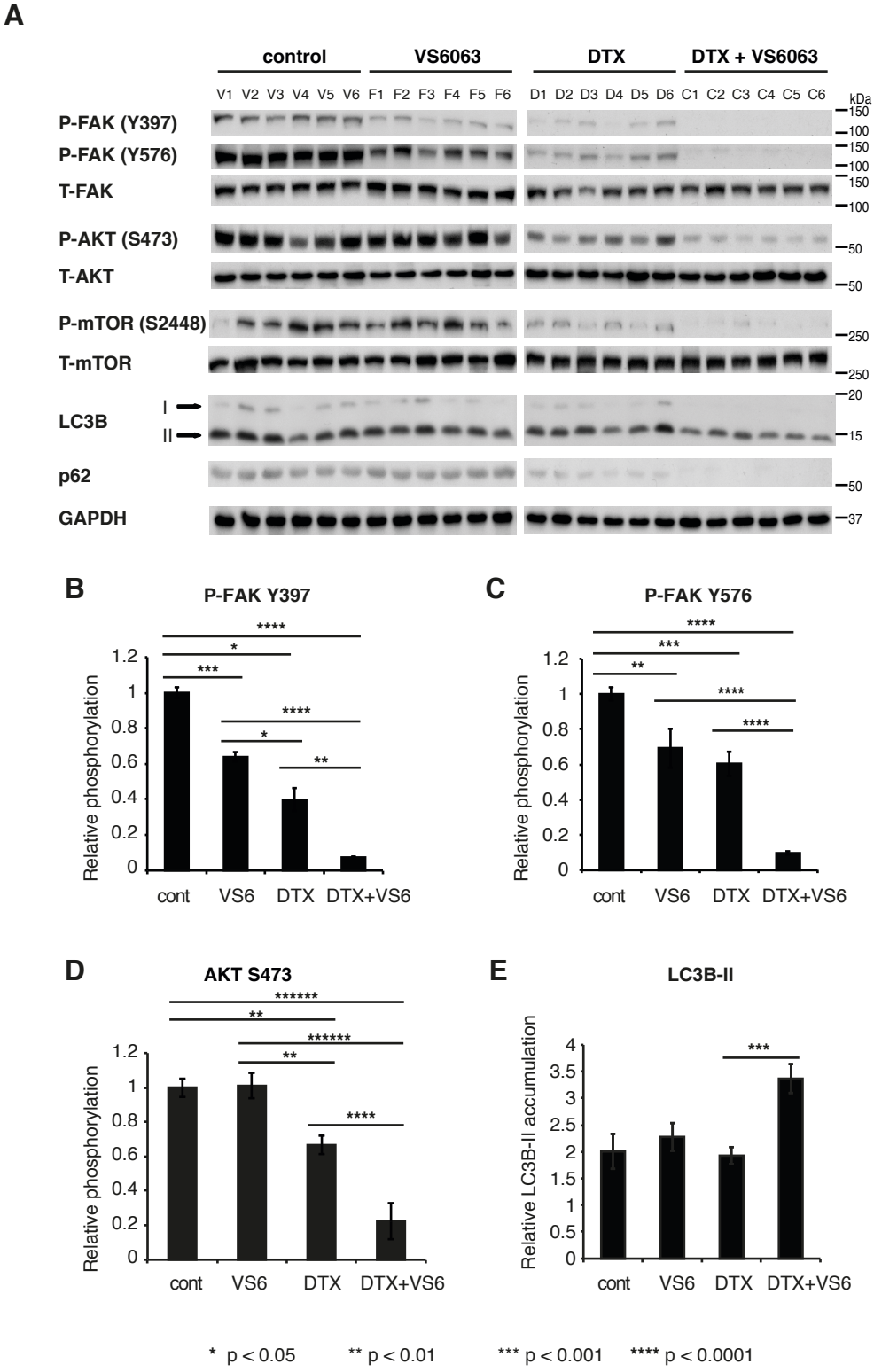
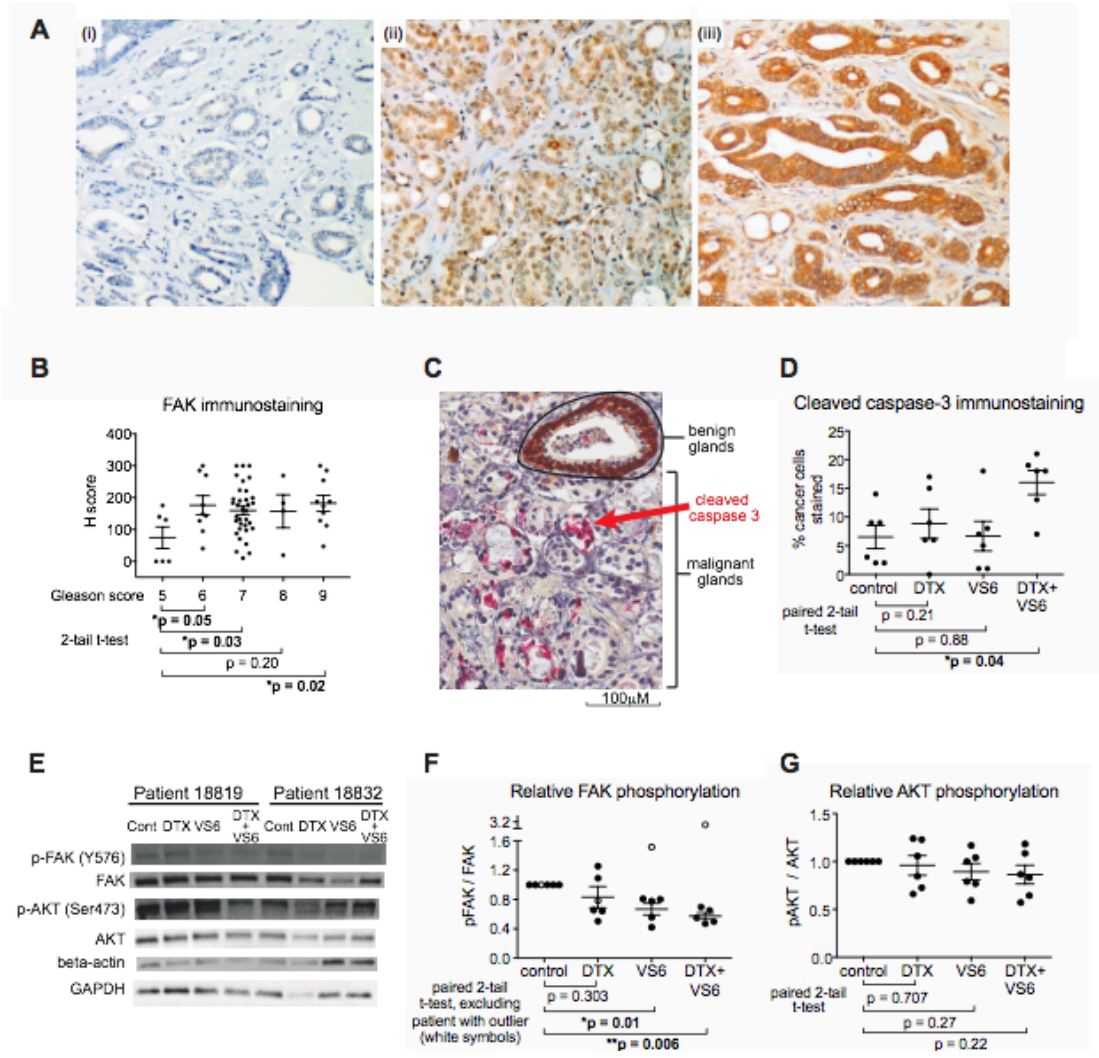

A
control
VS6063
DTX
DTX + VS6063
V1 V2 V3 V4 V5 V6 F1 F2 F3 F4 F5 F6 D1 D2 D3 D4 D5 D6 C1 C2 C3 C4 C5 C6
kDa
P-FAK (Y397)
P-FAK (Y576)
T-FAK
P-AKT (S473)
T-AKT
P-mTOR (S2448)
T-mTOR
LC3B
I
II
p62
GAPDH
150
100
150
100
150
100
50
50
250
250
20
15
50
37
B
P-FAK Y397
Relative phosphorylation
cont
VS6
DTX
DTX+VS6
C
P-FAK Y576
Relative phosphorylation
cont
VS6
DTX
DTX+VS6
D
AKT S473
Relative phosphorylation
cont
VS6
DTX
DTX+VS6
E
LC3B-II
Relative LC3B-II accumulation
cont
VS6
DTX
DTX+VS6
* p < 0.05
** p < 0.01
*** p < 0.001
**** p < 0.0001

FIGURE 6


A
(i)
(ii)
(iii)
B
FAK immunostaining
H score
400
300
200
100
0
Gleason score 5 6 7 8 9
2-tail t-test
*p = 0.05
*p = 0.03
p = 0.20
*p = 0.02
C
benign glands
cleaved caspase 3
malignant glands
100μM
D
Cleaved caspase-3 immunostaining
% cancer cells stained
25
20
15
10
5
0
control DTX VS6 DTX+ VS6
paired 2-tail t-test
p = 0.21
p = 0.88
*p = 0.04
E
Patient 18819
Patient 18832
Cont DTX VS6 DTX + VS6 Cont DTX VS6 DTX + VS6
p-FAK (Y576)
FAK
p-AKT (Ser473)
AKT
beta-actin
GAPDH
F
Relative FAK phosphorylation
pFAK / FAK
3.2
1.6
1.2
0.8
0.4
0.0
control DTX VS6 DTX+ VS6
paired 2-tail t-test, excluding patient with outlier (white symbols)
p = 0.303
*p = 0.01
**p = 0.006
G
Relative AKT phosphorylation
pAKT / AKT
1.5
1.0
0.5
0.0
control DTX VS6 DTX+ VS6
paired 2-tail t-test
p = 0.707
p = 0.27
p = 0.22