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ORIGINAL ARTICLE

VHL-TGFBI signaling is involved in the synergy between 5-aza-2'-deoxycytidine and paclitaxel against human renal cell carcinoma

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Summary

Purpose: *To analyse the role of von Hippel-Lindau (VHL) and transforming growth factor β-induced (TGFBI) in synergistic mechanisms of 5-aza-2'-deoxycytidine (DAC) and paclitaxel (PTX) against renal cell carcinoma (RCC).*

Methods: *To elucidate the role in the synergy between DAC and PTX against RCC cells, TGFBI expression was regulated using siRNA technology and an expression vector containing the full-length cDNA for TGFBI was also transfected into RCC cells. The proliferation of RCC cells was evaluated using the WST-1 assay and TGFBI expression was detected by real-time PCR (RT-PCR), and Western blot.*

Results: *The results indicated that the expression of TGFBI was significantly decreased by DAC or PTX alone in vitro and in vivo. Moreover, the combination of DAC and PTX caused a synergistic decrease in the expression of TGFBI in RCC cells. We also investigated the effect of VHL-TGFBI signaling on the synergy between DAC and PTX, although the synergy between the two medications was not abolished by interfering with VHL activity or TGFBI expression. RCC cells without VHL activity and RCC cells expressing high levels of TGFBI displayed an increased synergistic effect compared to control cells.*

Conclusion: *Our study suggests that VHL-TGFBI signaling is involved in the synergy between DAC and PTX against RCC cells. In addition, the synergy between DAC and PTX is more effective in VHL inactive RCC cells.*

Key words: *a5-aza-2'-deoxycytidine, paclitaxel, renal cell carcinoma, transforming growth factor beta-induced, von Hippel-Lindau*

Introduction

Human RCC is the most frequent and lethal malignancy of the kidney, and nearly 85% of RCCs are clear cell renal carcinomas [1]. Although RCC at an early stage is considered to be a local disease, 30% of RCC patients who present with limited disease at the time of diagnosis develop metastasis in 3 years [2] and the prognosis of advanced RCC is very poor [3].

Until recently, therapeutic options for advanced RCC are still limited, and RCC is usually resistant to conventional chemotherapy [4,5]. Recommended treatment options include immunotherapy, monoclonal antibodies, inhibition of signal transduction, and targeted treatments [6-9]. Some studies have indicated that DAC, a DNA methyltransferase inhibitor, is a candidate for the treatment of various tumors [10-13]. In a previous study, we confirmed the anti-proliferative effect of DAC against RCC. Moreover, DAC combined with PTX can synergistically inhibit the growth of RCC cells; however, the detailed molecular mechanisms and pathways involved in the synergy between the two medications remain unknown [14]. In a corollary study, we in-

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vestigated the synergistic mechanisms of both medications using cDNA microarray analysis, the results of which suggested that DAC and/or PTX significantly decreased the expression of TGFBI in RCC cell lines, thus indicating that TGFBI may be involved in the synergy between DAC and PTX against RCC [15].

TGFBI, a target of TGF-β1 (also known as BigH3), is a 68 kDa extracellular matrix (ECM) protein with an Arg-Gly-Asp (RGD) sequence and four fasciclin-1 (FAS1) domains [16,17]. TGFBI has been reported to play a suppressive role in the development of mesothelioma and breast cancer cells [18]. TGFBI can also reduce the metastatic potential of lung and breast tumor cells *in vitro* and *in vivo* [19]. In contrast, TGFBI expression is associated with high-grade human colon cancer, and overexpression of TGFBI enhances the aggressiveness and metastatic properties of colon cancer cells [20]. Another study suggested that TGFBI has dual functions in ovarian cancer; specifically, TGFBI functions as a tumor suppressor or promoter depending on the tumor microenvironment [21]. TGFBI is moderately expressed in normal renal tissues where TGFBI is localized predominantly in the epithelial cells of the collecting ducts, and distal and proximal tubules; however, overexpression of TGFBI has been detected in RCC specimens [22]. Although TGFBI is associated with a number of cancers [23-25], the role of TGFBI in RCC has not been fully studied. TGFBI can bind to fibronectin, collagen, and integrins, and stimulate adhesion, migration, and proliferation in renal proximal tubular epithelial cells [26]. A recent study has indicated that TGFBI is a target of von Hippel-Lindau (VHL) and TGFBI expression can be suppressed by VHL. Moreover, VHL inactivation enhances TGFBI signaling and increases the metastatic properties of RCC cells [27]. Although mRNA expression of VHL-HIF signaling is not affected by DAC and/or PTX in microarray analysis, the effect of VHL activity on the synergy of the two agents as a regulator of TGFBI remains unknown.

In the current study, we investigated the expression of TGFBI regulated by DAC and/or PTX in RCC cells, and analyzed the role of VHL and TGFBI in synergistic mechanisms of DAC and PTX against RCC. We speculated that VHL-TGFBI signaling maybe involved in the synergy between DAC and PTX against RCC and that TGFBI may serve as a therapeutic target.

Methods

Cell culture and agents

Four VHL wild-type RCC cell lines (ACHN, Caki-1, Caki-2, and NC 65) and one VHL null-type RCC cell line (789-O) were purchased from the ATCC and cultured in complete medium consisting of RPMI-1640 medium (Gibco, Bio-cult, Glasgow, Scotland) supplemented with 25 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. All cells were maintained as monolayers in 10-cm plastic dishes and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. DAC and PTX were purchased from Sigma (Sigma-Aldrich, MO, USA).

Reverse transcription-PCR and quantitative real-time PCR

Total RNA was isolated using a RNeasy mini kit (Qiagen, Frankfurt, Germany) and a first-strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, UK) was used for reverse transcription. The PCR conditions were determined according to the manufacturer's instructions and the length of the PCR products was confirmed by agarose gel electrophoresis. Quantitative real-time PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the products were quantified using a GeneAmp 5700 Sequence Detection System (Applied Biosystems). PCR reactions were performed in triplicate using the primers listed in Table 1.

Table 1. Primer and siRNA sequences used in this study.

Primer sequences	*Forword primer (5'-3')*	*Reverse primer (5'-3')*	*Length of PCR products (bp)*
TGFBI	GTGTGTGCTGTGCAGAAGGT	CATATCCAGGACAGCACTCG	124
VHL	AGAAGGTGGTGGCATTTTTG	AGCAGATGCCAATGCCTTCT	124
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226
RNAi sequences	*Sense oligonucleotide (5'-3')*	*Antisense oligonucleotide (5'-3')*	*Target gene sequence (5'-3')*
TGFBI	CCCGCUCGCAGCUUACUUAAC	UAAGUAAGCUGCGAGCGGGAG	CTCCCGCTCGCAGCTTACTTAAC (48-70 bp)
VHL	CGAGCGCGCGCGAAGACUACG	UAGUCUUCGCGCGCGCUCGGU	ACCGAGCGCGCGCGAAGACTACG (98-120 bp)
Negative control	GUACCGCACGUCAUUCGUAUC	UACGAAUGACGUGCGGUACGU	

Western blot

The following procedures were performed as previously described [28]. Protein was isolated using lysis buffer and the total protein concentration was examined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA, USA), then SDS polyacrylamide gel electrophoresis was performed. Antibodies to TGFBI and VHL were purchased from Cell Signaling Technology; an anti-β-actin monoclonal antibody (Abcam, Cambridge, UK) was used as an internal control. The immune complexes were detected with a system of enhanced chemiluminescence (ECL) combined with Western blot (Amersham, Aylesbury, UK).

Transfection and siRNA

A total of 1×10^6 RCC cells were seeded in complete medium without antibiotics and incubated to grow until cells reached 40-60% confluence. Then, the cells were transfected with siRNA oligonucleotides or scrambled siRNA controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After continuous incubation of the cells for 2 days, TGFBI or VHL expression was determined by RT-PCR and Western blot analysis. The siRNA oligonucleotide sequences were all designed using siDirect software . The target sequence, siRNA, and negative control sequences are shown in Table 1. The RCC cells were also stably transfected with an expression vector containing full-length cDNA for TGFBI or VHL by Lipofectamine 2000. Blank vectors without the gene insert were used as controls. Monoclonal antibodies were selected with G418 and confirmed by RT-PCR and Western blot analysis.

WST-1 assay

The proliferative ability of RCC cells was analyzed using the WST-1 assay. Exponentially-growing RCC cells were harvested and 1×10^3 cells were seeded in 96-well microtiter plates, then the cells were treated with DAC and/or PTX. After 3 days of continuous incubation, 10 µl of WST-1 (Roche, Penzberg, Germany) were added into each well and the incubation was continued for an additional 2 hrs. The absorbance, representing the cell count in each well, was measured using a microculture plate reader (Immunoreader; Japan Intermed Co., Ltd., Tokyo, Japan) at 450 nm.

RCC xenografts

Animal experiments were performed in the Animal Center of Beijing Friendship Hospital and in accordance with the ARRIVE guidelines. Moreover, all animal procedures that could affect animal welfare were reviewed and approved by the Institutional Animal Care and Use Committee of Beijing Friendship Hospital of Capital Medical University. Sixty BALB/C nude mice (3-4 weeks old) were randomly divided into the following 4 groups of 15 mice each: control; DAC; PTX; and DAC+PTX. A total of 1×10^7 ACHN or Caki-1 cells were injected into the backs of each mouse. When the diameter of the tumor reached 5 mm, DAC (2 mg/kg) and/or PTX (1 mg/kg) were injected into the peritoneal cavity of each mouse 3 times per week. The control mice were injected with the same volume of saline. All mice were observed continuously for 5 weeks and the volume of each tumor was recorded. After 5 weeks, the mice were sacrificed under deep anesthesia and the final volume of each tumor was measured.

Statistics

All determinations were performed in triplicate and the results were expressed as the mean ± standard deviation (SD). Statistical significance was determined using Student's t-test and a p value ≤0.05 was considered significant. Calculations of synergy were determined by isobolographic analysis, as described by Berenbaum [29]. Whether or not the combination of medications was additive, antagonistic, or synergistic was determined by the location of the point on, above, or below the straight line joining the dosages of the two medications that, when given alone, induced the same effect as the combination of medications.

Results

Decreased TGFBI expression by DAC and/or PTX in vitro

The expression of TGFBI in RCC cells was decreased by DAC or PTX alone based on microarray analysis. Furthermore, the combined treatment with DAC and PTX decreased TGFBI expression synergistically compared to either medication alone. This result suggested that TGFBI may participate in the synergy of the two medications against RCC. We further investigated the results of microarray analysis by real-time PCR (Figure 1) and Western blot analysis (data not shown). DAC (Figure 1A) and PTX (Figure 1B, 1C [results for ACHN and Caki-1 are shown]) each induced a dose-dependent down-regulation of TGFBI expression in all RCC cells. Moreover, DAC (0.5 and 1 µM) enhanced the decrease in TGFBI expression induced by PTX, and the combined treatment with DAC and PTX synergistically decreased TGFBI expression in all RCC cell lines (results for ACHN and Caki-1 are shown in Figure 1B,1C).

Decreased TGFBI expression by DAC and/or PTX in vivo

We further investigated the regulation of TGFBI expression by DAC and/or PTX in RCC xenografts of BALB/C nude mice by real-time PCR (Figure 2A), RT-PCR (Figure 2B), and Western blot analysis (Figure 2C). DAC and PTX alone significantly decreased TGFBI expression. Moreover, the combination of DAC and PTX synergistically decreased the expression of TGFBI compared to DAC or PTX alone. These results suggest that the suppression of TGFBI expression by DAC and/or PTX

is involved in the synergy of the two medications acting against RCC *in vivo*.

Effect of TGFBI on the synergy of DAC and PTX

We analyzed the effect of TGFBI on the synergy of DAC and PTX against RCC. To obtain monoclonal RCC cell lines, an expression vector containing the full-length cDNA for TGFBI was stably transfected into ACHN and Caki-1, and TGFBI expression was also transiently decreased using siRNA technology. All transfections were confirmed by RT-PCR (Figure 3A) and Western blots (Figure 3B). Compared to control cells, RCC cells expressing low levels of TGFBI exhibited a decreased sensitivity to DAC or PTX based on the WST-1 assay. In contrast, RCC cells expressing high levels of TGFBI exhibited an increased sensitivity to DAC or PTX (Figure 3C). Regardless of the level of expression of TGFBI, the synergy between DAC and PTX in all RCC cell lines was demonstrated by isobolographic analysis. Moreover, DAC enhanced the sensitivity of RCC cells to PTX effectively and a higher synergistic effect was observed in RCC cells expressing high levels of TGFBI compared to RCC cells expressing low levels of TGFBI (Figure 3D, 3E).

Effect of VHL activity on the synergy between DAC and PTX

A previous study indicated that the loss of VHL activity is correlated with increased TGFBI expression, and TGFBI is a target of VHL [27]. Therefore, we analyzed the effect of VHL activity on the synergy between DAC and PTX against RCC. VHL activity in Caki-1 was suppressed by siRNA technology, and VHL activity in 789-O was enhanced by transfection with the VHL vector. All transfections were evaluated by RT-PCR (Figure 4A) and Western blot analysis (Figure 4B). The VHL-active RCC cells had high levels of VHL ex-

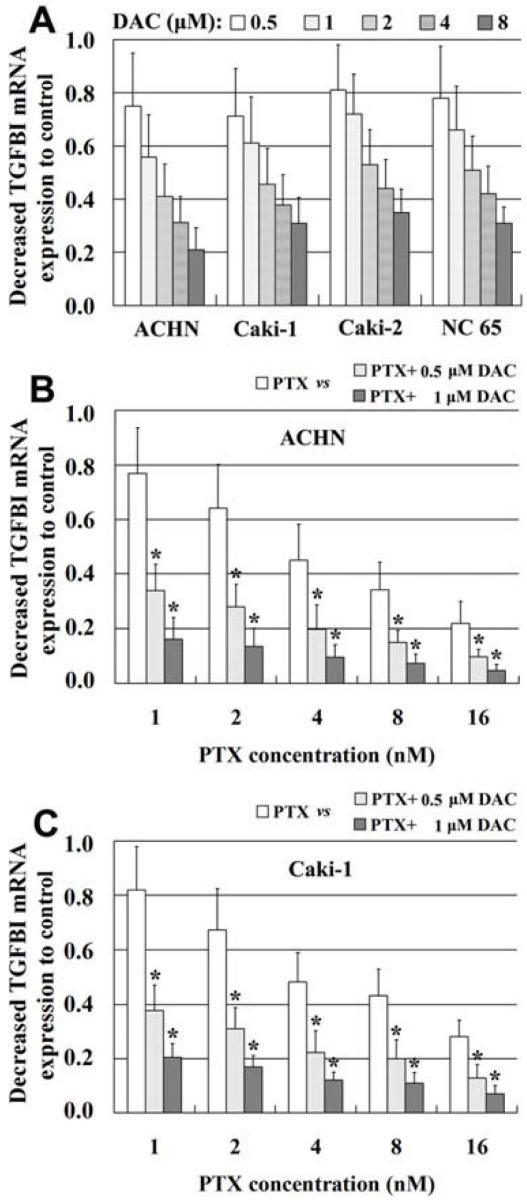

Figure 1. Decreased TGFBI expression by DAC and/or PTX was confirmed *in vitro* by real-time PCR. DAC induced a dose-dependent decrease in TGFBI expression **(A)** The combination of DAC and PTX synergistically decreased the expression of TGFBI in ACHN **(B)** and Caki-1 **(C)** All determinations were performed in triplicate and the error bar represents the SD (*p<0.05).

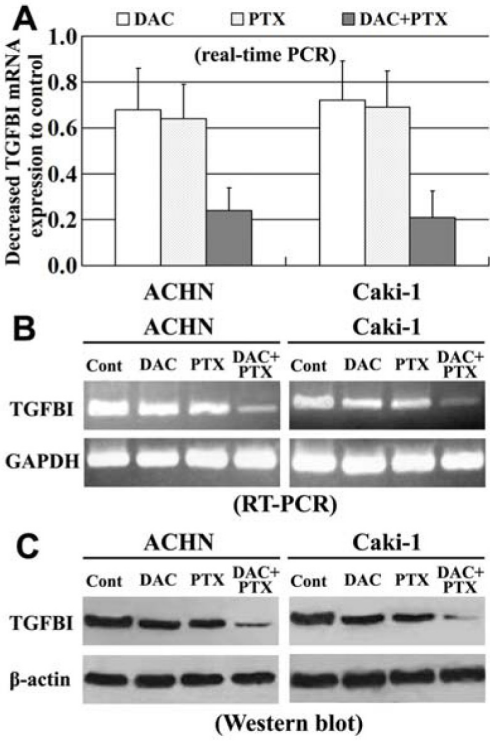

Figure 2. Decreased TGFBI expression by DAC and/or PTX *in vivo* by real-time PCR **(A)** RT-PCR **(B)** and Western blot analysis **(C)** DAC or PTX alone decreased the expression of TGFBI; the combination of DAC and PTX significantly decreased the expression of TGFBI in ACHN and Caki-1 (p<0.05). All determinations were performed in triplicate and the error bar represents the SD.

pression and low levels of TGFBI expression. In contrast, the VHL-inactive RCC cells had low levels of VHL expression and high levels of TGFBI expression. Moreover, regardless of the activity of VHL, the synergy between DAC and PTX was observed in all RCC cell lines; however, DAC more effectively increased the sensitivity of RCC cells to PTX and a higher synergistic effect was found in VHL-inactive RCC cells compared to VHL-active RCC cells (Figure 4C, 4D).

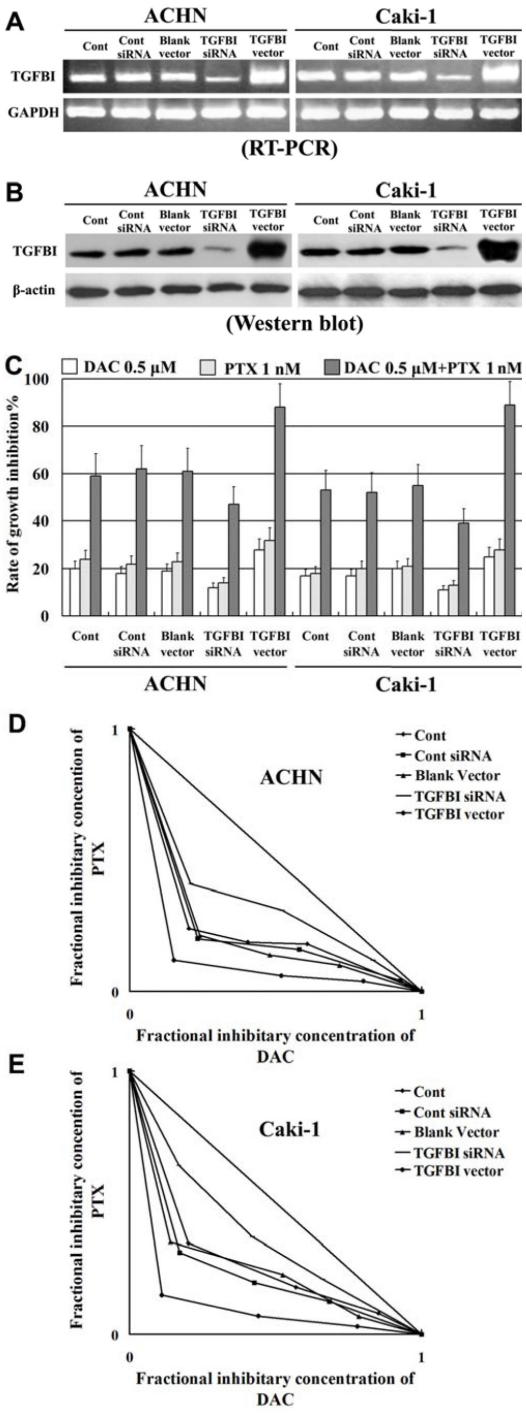

Figure 3. Effect of TGFBI on the synergy between DAC and PTX. Expression vector containing the full-length cDNA for TGFBI was transfected into ACHN and Caki-1. TGFBI expression was also decreased using siRNA technology. All transfections were confirmed by RT-PCR **(A)** and Western blot analysis **(B)** Regardless of the expression of TGFBI, the synergy between DAC and PTX was observed in all RCC cell lines and a higher synergistic effect was found in RCC cells expressing high levels of TGFBI (**C, D, E**, p<0.05).

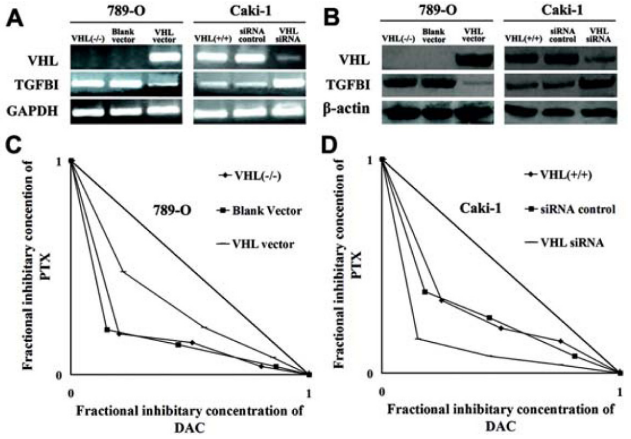

Figure 4. Effect of VHL activity on the synergy of DAC and PTX. Expression vector containing the full-length cDNA for VHL was transfected into ACHN and Caki-1. VHL activity was also decreased using siRNA technology. All transfections were confirmed by RT-PCR **(A)** and Western blot analysis **(B)** Regardless of VHL activity, the synergy between DAC and PTX was observed in all RCC cell lines and a higher synergistic effect was found in VHL-inactive RCC cells (**C, D**, p<0.05).

Discussion

TGFBI expression was first substantiated by Akhtar et al. in human lung adenocarcinoma cells after stimulation with TGF-β [30]. Until now, TGFBI expression has been detected in human organs, such as lung, bone, bladder, cornea, skin, and kidney [31]. TGFBI can provoke various changes in cellular behavior, which include modifying proliferation [32,33], differentiating epithelial cells [34,35], depositing extracellular matrix components [36], inhibiting angiogenesis [37], enhancing cellular metastasis, and altering the secretion of enzymes [38,39]. TGFBI is also involved in some human diseases, such as corneal melorheostosis, osteogenesis, dystrophies, atherothrombosis, diabetic angiopathy, and restenosis [40-42]. Although TGFBI is associated with numerous cancers, the function of TGFBI is complex, and the role of TGFBI in RCC remains unknown.

In a previous study, we showed that DAC increases the susceptibility of RCC cells to PTX by enhancing PTX-induced apoptosis and cell cycle arrest in G2/M [14]. Furthermore, to reveal the molecular mechanisms involved in the synergy of the two agents, a microarray analysis was performed and the results suggested that TGFBI expression was decreased significantly by DAC or PTX alone,

and TGFBI expression was synergistically decreased by the combination of the two medications in RCC cells [15]. Therefore, we further confirmed the results from the microarray analysis in the current study, and our results indicated that DAC and PTX synergistically decreased the expression of TGFBI compared to DAC or PTX alone *in vitro*. Moreover, the combination of DAC and PTX also significantly suppressed TGFBI expression in RCC xenografts of BALB/C nude mice, which was similar to the results obtained *in vitro*. Thus, TGFBI is involved in promoting the synergy between DAC and PTX against RCC cells. Although we demonstrated the participation of TGFBI in the synergy between DAC and PTX, interventions in TGFBI expression did not abolish the synergy between the two medications. RCC cells expressing high levels of TGFBI, however, exhibited an increased synergism compared to RCC cells expressing low levels of TGFBI, thus demonstrating that there are multiple genes and pathways participating in the synergy between DAC and PTX against RCC.

The VHL tumor suppressor is an important suppressor gene that plays a key role in RCC [43,44]. VHL recognizes the E3 ligase and ubiquitinates the hypoxia-inducible transcription factor (HIF) to regulate the response to hypoxia [45]. A high mutation frequency and loss of both copies of VHL have been observed in RCC [46]. In a previous study, we confirmed that TGFBI expression is increased by VHL inactivation and loss of VHL function activates a secondary genetic event in the TGFBI signaling pathway that may enhance the metastatic properties of RCC cells. Thus, we also investigated the effect of VHL activity on the synergy of DAC and PTX against RCC in this study. Despite VHL activity, the synergy between DAC and PTX was observed in all RCC cell lines; however, a higher synergistic effect was found in VHL-inactive RCC cells than in VHL-active RCC cells.

In conclusion, our study suggests that VHL-TGFBI signaling is involved in the synergy between DAC and PTX against RCC cells, especially in VHL-inactive and high levels of TGFBI-expressing RCC cells. These results indicate that a combination of DAC and PTX may be more effective in RCC without VHL activity and blocking TGFBI expression may provide a distinct treatment strategy for patients with advanced RCC. Finally, although TGFBI is considered to be an important gene in some malignancies and is highly expressed in RCC tissues, further research is required to elucidate the molecular mechanisms of TGFBI in human RCC.

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Conflict of interests

The authors declare no confict of interests.

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