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Role of the microtubule-targeting drug vinflunine on cell-cell adhesions in bladder epithelial tumour cells

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Abstract

Background: Vinflunine (VFL) is a microtubule-targeting drug that suppresses microtubule dynamics, showing anti-metastatic properties both *in vitro* and in living cancer cells. An increasing body of evidence underlines the influence of the microtubules dynamics on the cadherin-dependent cell-cell adhesions. E-cadherin is a marker of epithelial-to-mesenchymal transition (EMT) and a tumour suppressor; its reduced levels in carcinoma are associated with poor prognosis. In this report, we investigate the role of VFL on cell-cell adhesions in bladder epithelial tumour cells.

Methods: Human bladder epithelial tumour cell lines HT1376, 5637, SW780, T24 and UMUC3 were used to analyse cadherin-dependent cell-cell adhesions under VFL treatment. VFL effect on growth inhibition was measured by using a MTT colorimetric cell viability assay. Western blot, immunofluorescence and transmission electron microscopy analyses were performed to assess the roles of VFL effect on cell-cell adhesions, epithelial-to-mesenchymal markers and apoptosis. The role of the proteasome in controlling cell-cell adhesion was studied using the proteasome inhibitor MG132.

Results: We show that VFL induces cell death in bladder cancer cells and activates epithelial differentiation of the remaining living cells, leading to an increase of E-cadherin-dependent cell-cell adhesion and a reduction of mesenchymal markers, such as N-cadherin or vimentin. Moreover, while E-cadherin is increased, the levels of Hakai, an E3 ubiquitin-ligase for E-cadherin, were significantly reduced in presence of VFL. In 5637, this reduction on Hakai expression was blocked by MG132 proteasome inhibitor, indicating that the proteasome pathway could be one of the molecular mechanisms involved in its degradation.

Conclusions: Our findings underscore a critical function for VFL in cell-cell adhesions of epithelial bladder tumour cells, suggesting a novel molecular mechanism by which VFL may impact upon EMT and metastasis.

Keywords: Microtubule, Cell-cell contacts, E-cadherin, Vinflunine, Bladder cancer

Background

Bladder cancer is a common malignancy affecting the genitourinary system that represents the fifth most common cancer in the world. Transitional cell carcinoma (TCC) represents 95% of these tumours [1]. Most bladder cancers (70%-80%) present non-muscle invasive or superficial disease confined to the bladder mucosa (Ta)

or lamina propria (T1), and the remaining (20%-30%) are muscle-invasive at the time of diagnosis (T2-T4) [2]. Although both bladder cancers originate from urothelium in the urinary bladder (the epithelium that lines the urinary tract), they have different clinical characteristics. Muscle invasive TCC of the bladder is associated with a high frequency of metastasis, resulting in poor prognosis for patients [3]. Therefore, an effective strategy for preventing the progression of bladder cancer is clearly needed.

Epithelial cells bind to each other, forming a strong adhesive cell layer with important barrier functions.

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Cell-cell contacts comprise different types of junctions, but adherens junctions are the major cell-cell junctions that mediate cell recognition, adhesion, morphogenesis, and tissue integrity. Adherens junctions are linked to the actin cytoskeleton, establishing molecular communication with other cell-cell junctions and cell-substratum adhesions, and are involved in the organization and movement of the cells within the epithelium and in the transmission of information to the interior of the cell. The most important mediators of cell-to-cell adhesion are the transmembrane proteins called cadherins. E- and N-cadherin were the first cadherins identified [4]. E-cadherin is the prototype and best-characterized member of adherens junctions in mammalian epithelial cells. It contains an extracellular domain that forms homophilic interactions in a calcium-dependent manner and is responsible for cell-to-cell adhesions, and a cytoplasmic domain linked to the actin cytoskeleton through its interaction with several catenins [5,6]. E-cadherin is regarded as a tumour suppressor and its loss is associated with poor prognosis in carcinoma.

E-cadherin is considered a hallmark of epithelial-to-mesenchymal transition (EMT). EMT is an early step during carcinoma metastasis characterized by the loss of epithelial morphology and the acquisition of mesenchymal and motile characteristics, resulting from the loss of apical-basal polarity, the loss of cell-cell contacts, and the reorganization of the actin cytoskeleton [7-9]. Numerous studies suggest that EMT is associated with cancer cell invasion, recurrence, progression and metastasis in various malignancies, including bladder cancer [10]. However, the EMT is a reversible transitional process, as the cells can return to their epithelial phenotype: a process that is known as mesenchymal-to-epithelial transition [11]. The loss of E-cadherin expression may also have a pivotal role in tumour progression characterized by increased mobility and invasiveness in bladder cancer [12-14]. Indeed, several studies on the prognostic role of E-cadherin in bladder cancer have shown that its aberrant expression is associated to tumour progression and poor prognosis [15]. A key change that occurs during EMT is the "cadherin switch", in which the normal expression of E-cadherin is replaced by the abnormal expression of N- or P-cadherin [16,17]. Another important marker frequently used in cells undergoing EMT during metastatic progression is vimentin. Vimentin is an intermediate filament protein that is also upregulated during EMT. Vimentin expression induces cell changes including mesenchymal cell shape, increased cell motility, and loss of adhesion in epithelial cells during EMT [18]. Other studies have also suggested that transcriptional and posttranscriptional regulators are involved in the control of EMT [19,20]. E-cadherin is also regulated at posttranslational level; Hakai was the first posttranslational

regulator of E-cadherin stability [19,21]. Hakai is a RING finger-type E3 ubiquitin-ligase for the E-cadherin complex that mediates E-cadherin ubiquitination, endocytosis and degradation; in consequence, it disrupts cell-cell contacts. Moreover, many articles have described the emerging biological functions for Hakai protein pointing out its influence on tumour progression during EMT, proliferation, and oncogenesis [21-28].

The microtubule system, a major component of the cytoskeleton, was identified as a suitable target for cancer therapy, primarily based on their biological importance in coordinating chromosome segregation during mitosis. Microtubules are macromolecular filaments composed of tubulin. The clinical efficacy of the first-generation vinca alkaloid has prompted further research for novel analogues with improved clinical efficacy and safety. Such efforts have led to the development of vinflunine (VFL), a third-generation, semi-synthetic vinca alkaloid that, similar to other microtubule-targeting drugs, suppresses microtubule dynamics both *in vitro* and in living cancer cells [29,30]. In contrast to other vinca alkaloids, VFL shows superior antitumor activity and an excellent safety profile. VFL was approved by the European Medicines Agency (EMA) as a second-line treatment for patients with urothelial carcinoma resistant to first-line platinum-containing chemotherapy [31,32]. VFL has shown anti-angiogenic, anti-vascular and anti-metastatic properties *in vitro* and *in vivo* [33]. Some potential underlying mechanisms of the anti-angiogenic property of microtubule targeting-agents have been reviewed [34,35]. Interestingly, in endothelial cells, it was shown that microtubule-targeting agents, including VFL, may produce their anti-migratory/anti-angiogenic effects through an increase in interphase microtubule dynamics. In endothelial cells, at low and non-cytotoxic concentrations, VFL inhibits cell motility [36].

Although cadherins are best understood to cooperate with the actin cytoskeleton, there is increasing evidence supporting a role of the microtubules in regulating cadherin biology. Indeed, the cross-talk between microtubule networks and cell-cell adhesion sites profoundly impact upon these structures and is essential for proper cell organization, polarization and motility [37-42]. In the current study, we wanted investigate the possible impact of the microtubule-targeting drug VFL on E-cadherin-based cell-cell adhesion, and to determine the possible influence on the EMT transition markers in epithelial bladder tumour cell lines. We describe that VFL induces cell death in bladder cancer cells and activates epithelial differentiation of the remaining living cells. It has an impact on cell-cell contact, leading to an increase E-cadherin dependent cell-cell adhesion, while reducing vimentin and N-cadherin mesenchymal markers. Moreover, the levels of the E3 ubiquitin-ligase

Hakai were significantly reduced by VFL treatment in all cell lines tested. Moreover, this reduction in Hakai protein levels was recovered in presence of the proteasome inhibitor MG132 in 5637 cell line, suggesting that Hakai could be, at least, partially degraded in a proteasome-dependent manner. Our data suggest that VFL may be involved in a cross-talk between microtubule networks and cell-cell adhesion sites by its function as a microtubule-targeting drug, suggesting a novel molecular mechanism by which VFL may impact upon EMT and metastasis.

Methods

Cell culture and treatments

Human bladder epithelial tumour cell lines HT1376, 5637, UMUC3, SW780 and T24 were used. HT1376 cell line was obtained from American Type Culture Collections (Manassas, VA). UMUC3 and SW780 were generously donated by Dr. F. Garcia (Pharmamar S.A., Madrid) and 5637 and T24 by Dr. F. Real (Spanish National Cancer Research Centre from Madrid, Spain). HT1376 and UMUC3 cells were cultured in DMEM medium (Gibco, LifeTech), 5637 was cultured in RPMI medium (Gibco, LifeTech), SW780 was cultured in Leibovitz's medium (Gibco, LifeTech) and T24 cell line was cultured in McCoy's 5A (Gibco, LifeTech); each media was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine and 10% foetal bovine serum. Cultures were maintained at 37°C with 5% CO₂ in a humidified incubator. HT1377 cells were grown in the indicated medium additionally supplemented with non-essential aminoacids (Gibco, LifeTech). A stock solution of vinflunine was prepared in distilled water. Cells were treated with VFL at the indicated final concentrations and for the times shown. MG132 was obtained from Sigma-Aldrich (St Louis, USA) and was added to the medium at final concentration of 20 µM for 2 hours.

Antibodies and reagents

Antibodies were used that recognized the cytoplasmic portion of E-cadherin (Invitrogen, California, USA), Hakai (Hakai-2498, kindly provided by Dr. Yasuyuki Fujita [14]), N-cadherin (Abcam, Cambridge, UK), vimentin (Cell Signaling Technology, Massachusetts, USA), cyclin D1 (Santa Cruz Biotechnology, Texas, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Invitrogen, California, USA). HRP-rabbit and mouse polyclonal antibodies were from GE Healthcare (Uppsala, Sweden) and Alexa Fluor 488 secondary antibody was from Invitrogen (UK). All antibodies were used at dilutions of 1:1000 for Western blot analysis, except for HRP-rabbit, mouse polyclonal antibodies, and anti-GAPDH antibodies that were used at 1:2000, 1:2000, and 1:10000 respectively. E-cadherin antibody (BD Bioscience, California, USA) was used for

immunofluorescence at a dilution of 1:500 and Alexa Fluor 488 secondary antibody was used at a dilution of 1:100.

Viability assay

For cytotoxicity assays, 1×10^4 cells were plated per well into a 96-well plate and cultured for 24 h before treatment with VFL for 48 h. Serial dilutions of VFL dissolved in fresh medium were added to the cells in fresh medium. Growth inhibition of the epithelial tumour bladder cell lines was measured by using a MTT colorimetric cell viability assay kit (Sigma Aldrich, St Louis, MO) according to the manufacturer's instructions. To measure absorbance at 570 nm, a Multiskan Plus Reader (Thermo Fisher, MA, USA) was used. The half-maximal inhibitory concentration (IC₅₀) and the corresponding 95% confidence interval (95% CI) values were calculated from dose-response curves constructed using GraphPad Prism software. The data presented are the average of three independent experiments performed six times.

Phase contrast microscopy

For phase-contrast images, 2×10^5 cells were plated per well in a 6-well plate and treated with the indicated final concentrations of VFL (VFL) during 48 h. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Phase-contrast images were acquired using a Nikon Eclipse-Ti microscope.

Protein analysis

Protein was isolated using TriPure Reagent (Roche, Germany) according to the manufacturer's instructions. Cell lysates (20 µg of proteins) were obtained by lysing cells in a buffer containing 1% Triton X-100 (20 mM Tris/HCl pH 7.5, 150 mM NaCl and 1% Triton X-100), a protease inhibitor cocktail (Sigma Aldrich, St Louis, USA), and 50 mM PMSE. Western blot analysis was performed as described previously [43].

Transmission electron microscopy

For transmission electron microscopy, 5×10^4 cells of 5637 bladder tumour cell line were plated into 0.4 µm-pore culture inserts (Corning 353095, USA) placed on a 24-well plate. After treatment with 5 µM of VFL during 48 hours, cells were fixed with 2.5% cold glutaraldehyde (Panreac, Spain) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, Germany) pH 7.4, for 16 h at 4°C. Inserts were postfixated in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h at room temperature, following by several washes with 0.1 M sodium cacodylate buffer and distilled water. Inserts were dehydrated in increasing concentrations of acetone and embedded in Spurr's resin (Taab, Berkshire, UK). Ultra-thin sections of 70- to 80-nm thickness were cut using an Ultracut-E ultramicrotome (Leica) and collected on

formvar-coated copper mesh grids. Samples were examined with a JEOL JEM 1010 transmission electron microscope at 80 kV.

Immunofluorescence and TUNEL assay

For immunofluorescence and TUNEL assay, 3×10^4 cells were plated in chambers slides (Millipores, USA), fixed in 4% paraformaldehyde for 10 min, and then permeabilized in 0.5% Triton X-100-phosphate buffered saline (PBS) for 15 min. Cell death was measured by using Click-it TUNEL Alexa Fluor® 594 Imaging Assay (Invitrogen, UK) according to manufacturer's instructions followed by blocking with BSA 3% in PBS for 1 h. Incubation with E-cadherin primary antibody for 1 h was followed by incubation in Alexa-Fluor 488-conjugated secondary antibody solution for 1 h. To visualize nuclei, it was used 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, LifeTech, UK). Finally, the mounting media used was ProLong Gold antifade reagent (LifeTech, USA). Epi-fluorescence images were taken in Olympus microscope.

RNA analysis

Total RNA was isolated using TriPure Reagent (Roche, Germany) according to manufacturer's instruction. The immunoprecipitated RNA pellet was washed by following an alternative protocol described for small RNAs in RiboPure (Life Technologies, UK). The quality and quantity of the obtained RNA was determined by using Nanodrop ND-spectrophotometer (Thermo Fisher Scientific, MA, USA). For reverse transcription (RT), random hexamers and SuperScript first-strand Synthesis System for RT-PCR (Invitrogen, UK) were used. For mRNA analysis, real-time quantitative (q) PCR analysis was performed using gene-specific primers 5'-CGCAGACGAATTCCTATAAAGC-3' and 5'-CCTTCTTCATCACCAGGTGG-3' for human Hakai and 5'-TGACCTTGATTTATTTGCATACC-3' and 5'-CGAGCAAGACGTTTCAGTCCT-3' for HPRT. PCR was performed by using Light Cycler 480 SYBR Green I Master (Roche, Germany); amplification and quantification were carried out using a LightCycler 480 real-time lightcycler (Roche, Germany).

Statistical analysis

Unless indicated, all experiments were analysed by using Students t-test to evaluate differences between treatments at the indicated significance levels.

Results

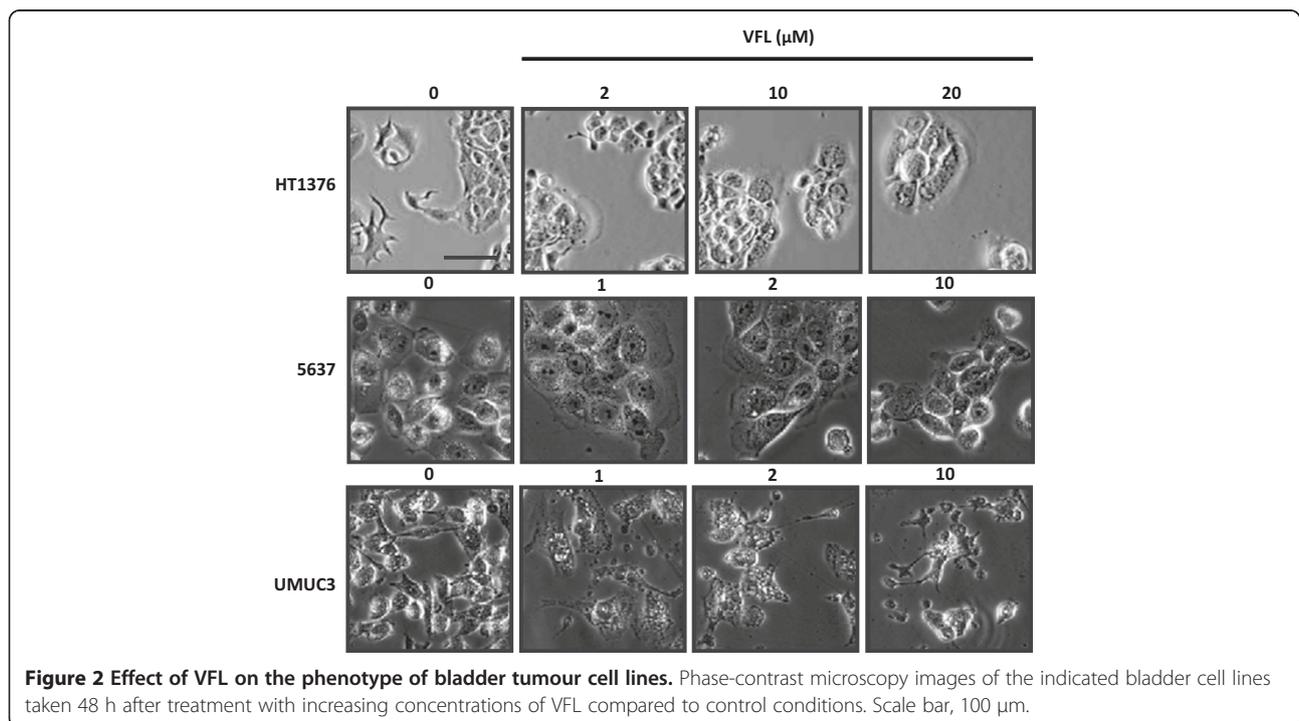
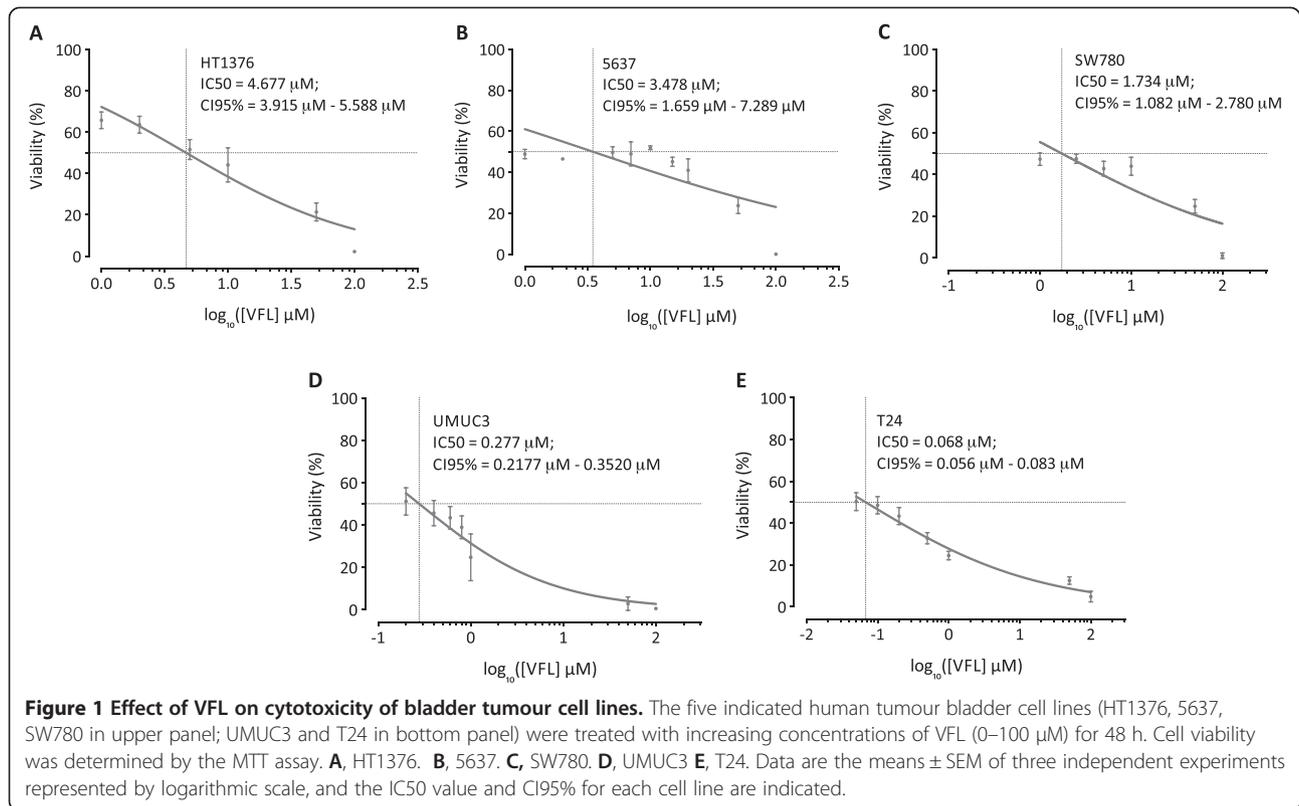
Vinflunine induces epithelial phenotype in bladder tumour cells

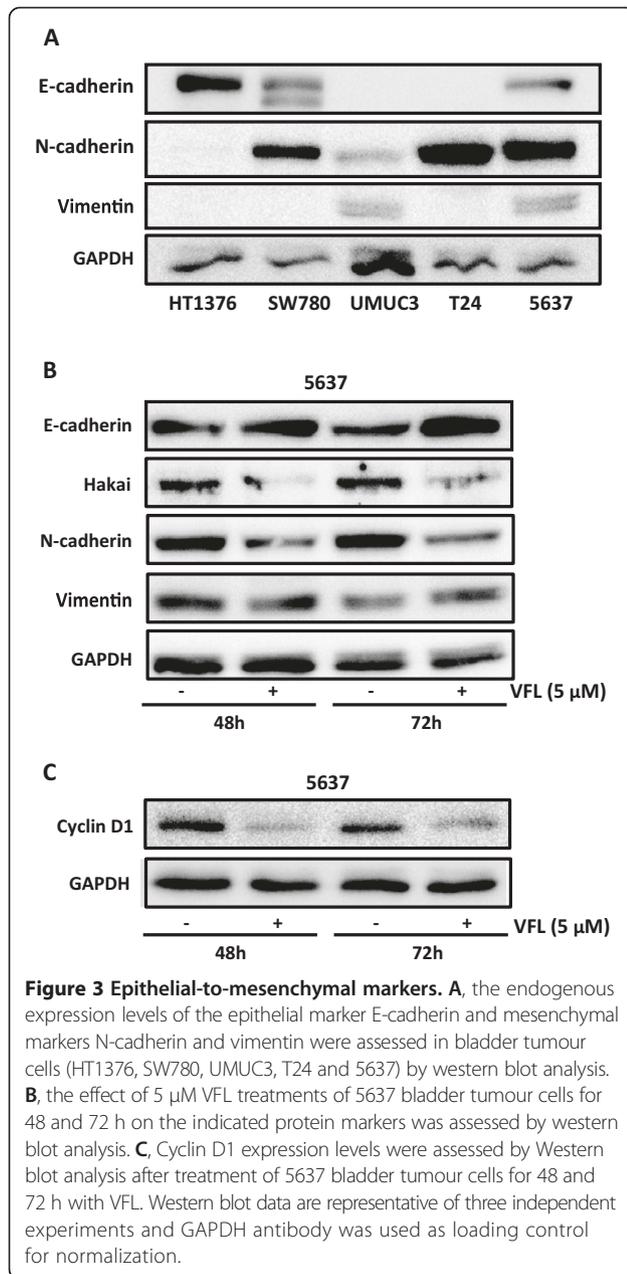
VFL, a microtubule-targeting drug, is used in monotherapy for treatment of advanced or metastatic urothelial cancers in adults. Given the rising evidence of crosstalk

between microtubule networks and cell-cell adhesion sites, we sought to investigate the possible impact of VFL on cell-cell adhesions in bladder epithelial tumour cells. To this end, we first examined the effect of VFL on cell viability of HT1376, 5637, SW780, T24 and UMUC3 bladder epithelial tumour cells by using increasing concentrations of VFL (0–100 μ M) treatment for 48 h. Figure 1 shows the dose-dependent inhibition of cell growth observed: $IC_{50} = 4.677 \mu$ M for HT1376, $IC_{50} = 3.478 \mu$ M for 5637 cells, $IC_{50} = 1.734 \mu$ M for SW780, $IC_{50} = 0.277 \mu$ M for UMUC3 cells and $IC_{50} = 0.068 \mu$ M for T24, the latest cell lines showing the highest sensitivity to VFL. The cellular morphology following VFL treatment was analysed by phase-contrast microscopy in the indicated cell lines (Additional file 1 and Figure 2). As shown, HT1376 and 5637 showed drastic changes, with a morphology resembling that of epithelial cells, at VFL doses ranging between 1–20 μ M, and tighter cell-cell contacts, as compared to control cells, which displayed a fibroblast-like morphology with decreased cell-cell contacts and increased numbers of membrane protrusions (Figure 2). This effect was also observed in SW780 tumor bladder cell line (data not shown). However, the fibroblastic morphology of UMUC3 and T24 cell lines was not affected by VFL treatment, showing an increased cell death, even in the presence of lower VFL concentrations (Figure 2 and data not shown). In conclusion, VFL affects the fibroblastic phenotype in HT1376, 5637 and SW780 bladder epithelial tumour cells, but not in UMUC3 and T24 cells.

VFL effect on epithelial-to-mesenchymal transition markers

Given the different impact of VFL upon the presence of cell-cell contacts among the analysed bladder tumour cell lines, we set out to examine the endogenous levels of several EMT markers in the different bladder epithelial tumour cell lines. As shown in Figure 3A, important differences were found between the analysed cell lines. First, E-cadherin, a major epithelial marker that mediates cell-to-cell adhesions, was only detected in HT1376, 5637 and SW780 cells (Figure 3A), precisely the cell lines that were switched to a more epithelial-like phenotype and are more resistant to VFL treatment. Non E-cadherin expression was found in the cell lines that experienced the most cytotoxicity in response to VFL (Figure 3A), such as UMUC3 and T24. Interestingly, phenotypical changes under VFL treatments were not detected in UMUC3 and T24 cell lines (Figure 1). It was also analysed the expression level of N-cadherin and vimentin mesenchymal markers, which are frequently expressed in carcinoma cells that have undergone EMT. Together, these data suggest that E-cadherin expressing bladder tumour cells are more resistant to VFL and respond better to the VFL-triggered switch from mesenchymal-to-epithelial

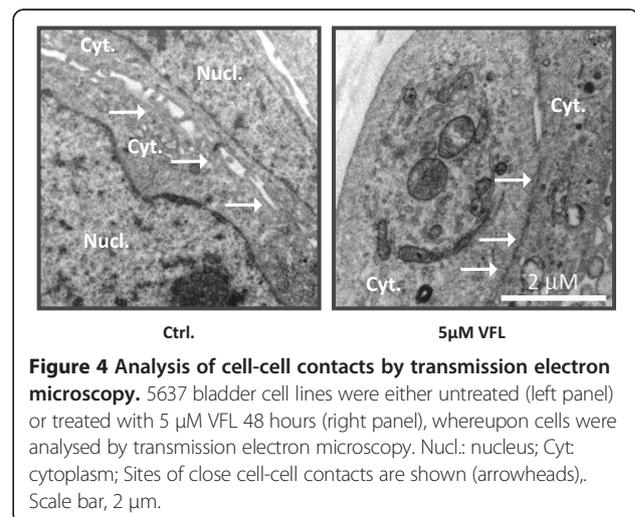




phenotype. Therefore, our results suggest that VFL can modulate cell death and epithelial cell differentiation.

VFL has an anti-metastatic property *in vitro* and *in vivo*; *in vitro* invasion assays showed an inhibitory effect of VFL treatment on invasion ability in a transitional cell carcinoma of the bladder. Moreover, in an orthotopic murine model of transitional cell carcinoma of the bladder, VFL showed potent high antitumor activity [44]. Since the initiation of metastasis requires invasion, which is enabled by EMT, we were interested in determining whether VFL might regulate the levels of EMT protein markers. A key change that occurs during EMT is the “cadherin switch”, in which the normal expression

of E-cadherin is replaced by the abnormal expression of N-cadherin [16,17]. Downregulation of E-cadherin, responsible for the loss of cell-cell adhesions, and upregulation of mesenchymal-related proteins, such as vimentin or N-cadherin, define the EMT process [9]. As shown in Figure 3B, VFL treatment (5 μ M) modestly increased protein expression of E-cadherin after 48 and 72 hours in 5637 bladder tumour cells; instead, the mesenchymal N-cadherin marker was reduced under the treatment. Moreover, the E3 ubiquitin-ligase Hakai for the E-cadherin complex was significantly reduced under these conditions, suggesting that the disappearance of Hakai protein could influence the recovery of E-cadherin expression. Hakai was also proposed to be involved in the regulation of both cell-cell contacts and cell proliferation. It was suggested that cyclin D1, a member of the cyclin protein family involved in the regulation of the cell cycle progression, was one of the substrate effector proteins through which Hakai might regulate cell proliferation [25]. Indeed, VFL treatment of 5637 cells caused a reduction in cyclin D1 protein levels compared to control conditions, while Hakai was also decreased (Figure 3C). In addition, transmission electron microscopy indicated that neighbouring VFL-treated E-cadherin expressing 5637 cells had very closely apposed cell-cell contacts compared to control cells (Figure 4). We extended this study in other bladder tumour epithelial cells. As shown in Figure 5A, in HT1376, VFL treatment modestly increases E-cadherin protein levels while Hakai is reduced; these cells do not express the mesenchymal markers vimentin or N-cadherin. By immunofluorescent staining, the VFL-elevated E-cadherin was detected at cell-cell contacts in epithelial cells (Figure 5B) while a reduction of E-cadherin protein at cell-cell was observed in cells undergoing apoptosis (Figure 5C). Finally, in UMUC3 cells, which do not express E-cadherin, it was shown that Hakai, vimentin,



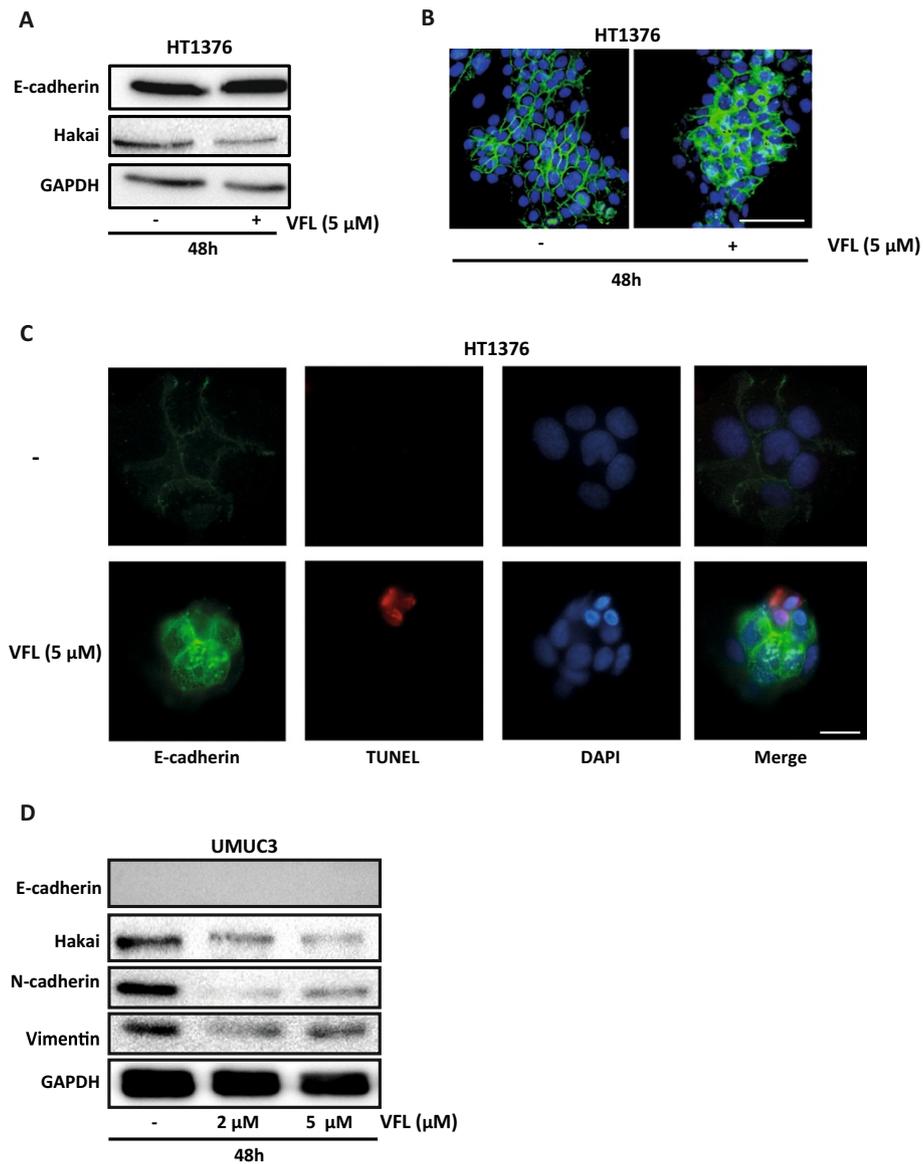


Figure 5 Effect of VFL on epithelial differentiation and apoptosis. A, Western blot analysis of E-cadherin and Hakai expression levels in HT1373 bladder tumour cells treated with 5 μM VFL for 48 h. **B,** immunofluorescence analysis of E-cadherin expression in HT1376 cells treated with 5 μM VFL for 48 hours. Scale bar, 200 μM. **C,** TUNEL staining for the analysis of apoptosis was performed following by immunofluorescence of E-cadherin in HT1376 cells treated with 5 μM VFL for 48 hours, as indicated in material and methods. Scale bar, 50 μM. **D,** Western blot analysis of the levels of E-cadherin, Hakai, N-cadherin and vimentin in UMUC3 bladder tumour cells treated with 5 μM VFL for 48 hours. Western blots are representative of three independent experiments and GAPDH was assessed as loading control.

and N-cadherin levels were reduced after 48 h of vinflunine treatment (Figure 5D). Taken together, these data suggest that VFL causes cell death and epithelial cell differentiation in the E-cadherin-expressing cells.

VFL promotes proteasome-mediated Hakai degradation

Since VFL causes a reduction in Hakai protein levels, we examined whether VFL affects Hakai mRNA levels using reverse transcription (RT) followed by real-time, quantitative (q) PCR. In contrast with Hakai protein levels,

Hakai mRNA levels were not downregulated by VFL treatment in 5637, HT1376 and UMUC3 (Figure 6A), suggesting that VFL lowers Hakai protein levels without decreasing Hakai mRNA abundance. Previous studies demonstrated that in all tissues, the majority of intracellular proteins are degraded by the ubiquitin proteasome pathway [45]. However, extracellular proteins and some cell surface proteins are taken up by endocytosis and degraded within lysosomes. Given that Hakai is an intracellular protein, we investigated whether the reduced Hakai

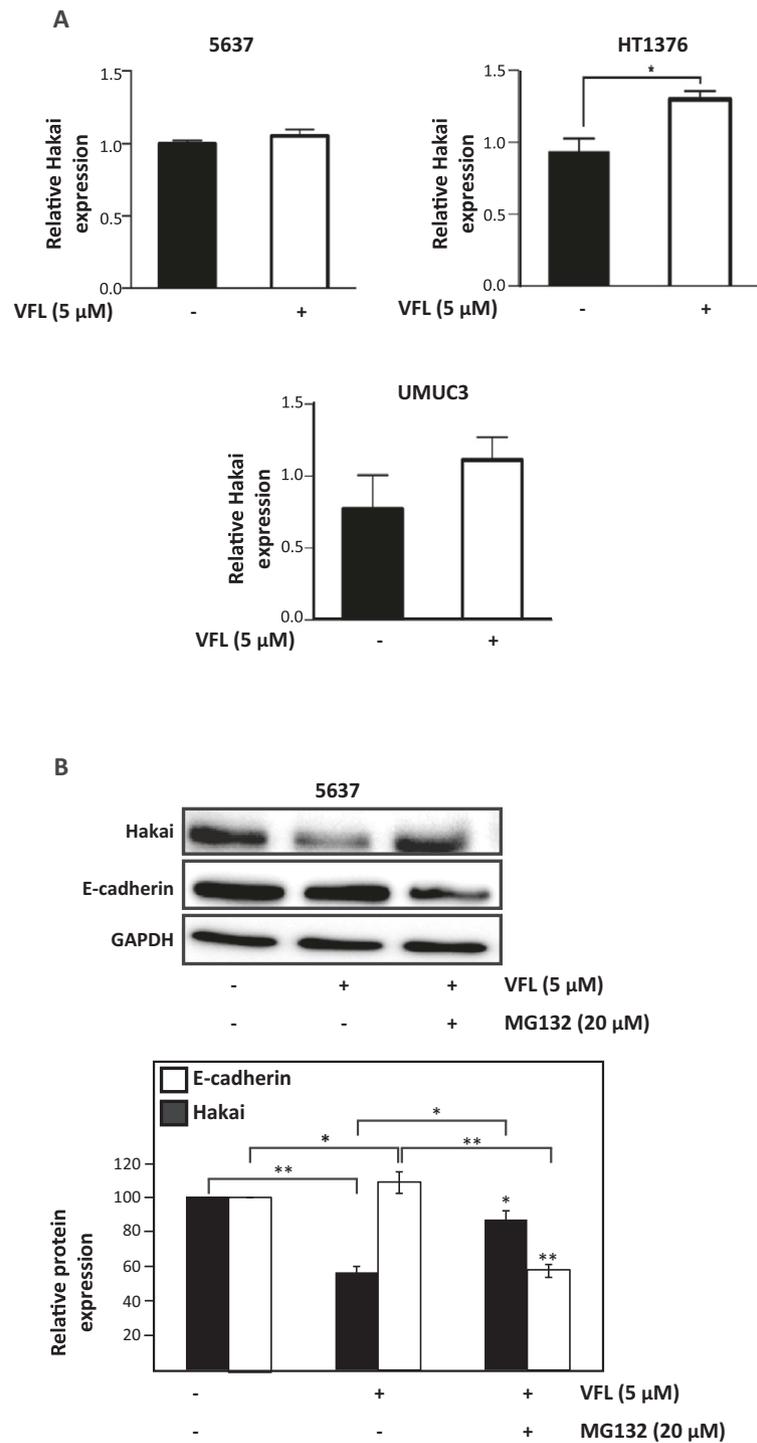


Figure 6 VFL is involved in Hakai degradation via proteasome-mediated in 5637 cell line. **A**, the levels of Hakai mRNA in 5637 (upper panel, left), HT1376 (upper panel, right), and UMUC3 (lower panel) cells following treatment with 5 μM VFL for 48 hours were determined by RT-qPCR analysis. HPRT mRNA levels were measured for normalization. The means ± SEM are represented from three independent experiments (* $p < 0.05$, $n = 3$). **B**, effect of proteasome inhibitor, MG132, on Hakai expression in 5637. Cells following treatment with 5 μM VFL for 48 hours were incubated for 2 h in the absence or presence of proteasome inhibitor (20 μM MG132) and cell lysates were prepared for Western blot analysis to detect Hakai, E-cadherin and normalization control GAPDH. Western blot data are representative of three independent experiments and quantification by densitometry was represented (lower panel, * $p < 0.05$, ** $p < 0.01$).

levels in VFL-treated cells could be affected by the increased degradation via proteasome of Hakai protein. We analyzed the effect of the proteasome inhibitors MG132 in VFL-treated 5637 cells compared to control conditions. As shown in Figure 6B, treatment of 5637 bladder cancer cells with VFL consistently reduced Hakai protein levels; however, the addition of MG132 inhibited this VFL-mediated-Hakai down-regulation. As expected, given that Hakai protein levels are restored by MG132 treatment, E-cadherin is reduced under these conditions. In conclusion, in 5637 cell lines Hakai reduction can be recovered by using proteasome inhibitors, MG132, further supporting the notion that Hakai down-regulation induced by VFL can be at least partially controlled in a proteasome-dependent mechanism.

Discussion

The transitional cell carcinomas of the bladder that invade muscle are associated with high frequency of metastasis, which is the major cause of death from cancer. Microtubules, a major component of the cytoskeleton, are one of the best established targets for cancer therapy. Indeed, the microtubule-targeting drug VFL, which suppresses microtubule dynamics *in vitro* and *in vivo*, is the recommended option treatment of metastatic transitional cell carcinoma of the urothelial tract which has progressed after treatment with platinum-containing chemotherapy. Given the increasing body of evidence supporting that microtubules regulate cadherin biology, and the well-established role of E-cadherin in the EMT during bladder cancer progression and metastasis, here we have studied the effect of VFL on E-cadherin cell-cell adhesions. Using bladder cancer cell lines, we have shown that VFL treatment induces cell death in bladder cancer cells and activates epithelial differentiation in the remaining cells, leading to increased E-cadherin-dependent cell-cell adhesions and to reduced levels of mesenchymal markers, such as N-cadherin or vimentin. Moreover, we have demonstrated that Hakai, a post-translational regulator of E-cadherin stability, was significantly reduced in a VFL-dependent manner in 5637 suggesting that the proteasome pathway is at least partially involved in its diminution; however, other post-translational mechanisms are waiting to be investigated. In conclusion, we have demonstrated a novel molecular mechanism of VFL to explain its anti-invasive effect.

In the last few years, differentiation therapy came out as a novel strategy for treating cancers. This approach is based on the concept that cancer cells arise from tissue stem cells and share the stemness and plasticity with normal stem cells. Differentiation therapy aims to induce cancer cells to differentiate by treatment with differentiation-inducing agents [46,47]. Most of the differentiation agents can inhibit proliferation and

induce cells to differentiate and then undergo apoptosis [48,49]. It is well established that VFL blocks mitosis at the metaphase/anaphase transition, leading to apoptosis [50]. It is still necessary to elucidate how VFL could modulate epithelial cell differentiation and cell death; however, it appears to be an important therapeutic strategy for transitional cell carcinomas by its influence on these processes. Understanding the differentiation mechanisms and the fate of the treated cells may eventually lead us to gain insights into cancer therapy by differentiation.

Cadherins are key mediators of cell-to-cell adhesion in epithelial tissues. The roles of these proteins in bladder cancer-related EMT have been investigated extensively. Bryan and Tselepis summarized the patterns of P-cadherin and N-cadherin expression in the bladder during EMT [51]. In the normal urothelium, P-cadherin, but not N-cadherin, is expressed in the basal layer. However, during the EMT, P-cadherin expression in bladder cancer cells is upregulated along with N-cadherin expression; these events occur either independently or synchronously. E-cadherin expression in bladder cancer cells is lost after changes in P- and/or N-cadherin expression levels, as invasion and metastasis increase. This cadherin switching event is an important process that occurs late in the molecular pathogenesis of bladder cancer; although the precise timing and nature of these events remain unknown. On the other hand, the mesenchymal intermediate filament, vimentin, often increases its expression in carcinoma cells that have undergone an EMT. However, under VFL treatment, vimentin downregulation is observed in UMUC3 cell line but not in 5637. Vimentin levels are not always affected during the reversion of mesenchymal-to-epithelial transition. Indeed, partial EMT has been suggested to occur in some metastatic cancers, where distant metastasis can retain much of the epithelial differentiation of the original tissue of the cancer [52]. For instance, under silibinin treatment, a natural agent that reverses EMT, vimentin mesenchymal marker was not always influenced by this agent. Indeed, it was observed that vimentin was not always regulated by silibinin during MET and this modulation was dependent on the type of prostate epithelial tumor cells [53,54]. Various lines of evidence clearly indicate that the EMT is strongly associated with aggressive bladder cancer behavior, such as recurrence, progression, and metastasis, an observation that raises the possibility that the EMT may be a target for bladder cancer treatment. As mentioned above, E-cadherin is the first and most important regulator of the EMT. Moreover, in bladder cancer, loss of E-cadherin expression is a marker of poor response to the monoclonal antibody cetuximab, which blocks EGFR binding and represses bladder cancer cell proliferation [55]. Thus, E-cadherin expression levels could constitute an

important predictive marker for the responsiveness to microtubule-targeting VFL therapy.

Several studies have demonstrated the influence of the microtubules on cadherin-dependent cell-cell adhesions. Kitase et al. demonstrated that RhoA is implicated during neurodetermination, where it influences cell-cell contact and cadherin levels [56]. They used the P19 cell model of neuronal differentiation to show that RhoA affects cadherin protein level and cell-cell contacts during neuroinduction. RhoGTPases have an important role during neurite growth, axonal guidance, and synaptogenesis [57-59]. The cellular effects of RhoA are mediated by ROCK and seem to involve microtubules, pointing, for the first time, at the existence of a potential complex cross-talk between RhoA/ROCK, N-cadherin, and microtubules. The effect on cadherin level occurred in the timing that corresponds to the switch of E- to N-cadherin that trigger neurodifferentiation [60]. N-cadherin level appears to be critical for cell fate determination during morphogenesis. Advanced induction of Cdc42 had similar effects to RhoA inactivation, underscoring the importance of correct timing of RhoGTPases during neurodifferentiation [56].

Similar to VFL, another microtubule destabilizing agent, nocodazole, influenced cell-cell adhesions. The study reporting these findings examined the role of microtubules on the transcriptional regulation of cell adhesion proteins, providing evidence that the microtubule cytoskeleton critically affects EMT by regulating TGF- β /SMAD2 signaling during palatal fusion and prevents E-cadherin repression [61]. During palatal fusion, the midline epithelial seam (MES) degrades to achieve mesenchymal confluence. EMT is one of the mechanisms that function during MES degradation. TGF- β induces EMT in medial edge epithelium (MEE) by down-regulating the epithelial marker E-cadherin. Microtubule disassembly impaired palatal fusion leading to a multi-layered MES in the mid-region and inhibited palatal fusion accompanied by the development of a multi-layered MES in the mid-palatal region [62]. The authors further showed that treatment with nocodazole led to the accumulation of cell-cell adhesion proteins at intercellular junctions in medial edge epithelium [61]. Microtubule disruption by nocodazole triggered the aberrant accumulation of E-cadherin adhesion at intercellular junctions in MEE. Due to the aberrant expression of both negative (Snail and Zeb) and positive (c-MYC) E-cadherin transcriptional regulators when the TGF- β /SMAD2 signaling pathway was blocked, resulted in failure for EMT to progress. These data also support the important role of the microtubule cytoskeleton in mediating TGF- β /SMAD2 signals to control E-cadherin expression in MEE during palatal fusion [61].

Several lines of evidence support that the interaction of the microtubules with cadherin affects cadherin

biology [63]. First, the junctional integrity of cadherin is perturbed by drugs such as nocodazole and VFL, which disrupt microtubules. Second, specific targeting of microtubule-binding proteins found at junctions also impairs broad aspects of cadherin biology. For example, Nezha-bound microtubules at their minus ends and tethered them to the zonula adherens. In cultured mammalian epithelial cells, depletion of either Nezha or PLEKHA7, which is responsible for recruiting Nezha to p120-ctn, disrupted the ability of cells to concentrate E-cadherin to the apical junction of the zonula adherens. The functional impact of these junctional microtubule-binding proteins further supports the idea that microtubules that interact with cadherin adhesions are responsible for regulating junctional integrity. Still, how microtubules influence these diverse aspects of cadherin biology is complex and poorly understood. Microtubules are commonly implicated in directing intracellular traffic of membrane-bound vesicles or molecular complexes; accordingly, it was postulated to influence cadherins through their intracellular traffic. Indeed, a number of microtubule-based motors have been identified that support intracellular transport of cadherins [64].

The EMT is regarded as a key process that allows cancer cells to migrate to adjacent organs or metastasize to distant sites. In bladder cancer, EMT is closely associated with grave clinical characteristics, such as recurrence, progression, metastasis, and reduced survival. However, metastases of the most common human cancers (well- to moderately-differentiated carcinomas) often show a re-differentiation in the sense of a mesenchymal-epithelial transition (MET). Therefore, strategies to induce reversal EMT (MET), such as VFL, might be able to suppress cancer cell migration and metastasis. However, this issue is controversial, as several lines of evidence support that transient dedifferentiation (EMT) and re-differentiation (MET) processes are a driving force in metastasis [65]. Although many clinical reports fostered the concept of transient EMT-MET switches in metastasis, only little experimental evidence is available [66]. Chaffer et al. support the role of an EMT in dissemination and the need of a MET for efficient metastasis in bladder cancer metastasis [66]. Two reports support the need of re-differentiation (MET) for the colonization and metastasis of differentiated carcinomas and implicate EMT-associated growth arrest in these events [67-69]. Therefore, MET has a key clinical impact for future therapeutic strategies against metastasis. On one hand, EMT-targeting therapy may be useful as a personalized medicine approach that complements conventional bladder cancer treatments, while on the other hand, the induction of differentiation and targeting EMT alone might be counterproductive by activating the proliferation of disseminated cells. It is possible that therapeutic

approaches directed against EMT can benefit cancer patients diagnosed at early stages of the disease to prevent invasion and dissemination, while anti-MET drugs could be potentially benefit patients with established metastasis. Still, a better understanding of the molecular mechanisms of the EMT in bladder cancer is crucial to the development of new therapeutic modalities for this cancer [69,70].

Conclusions

Our data suggest that VFL, a microtubule-targeting drug, may be involved in a cross-talk between microtubule networks and cell-cell adhesions. We suggest a novel molecular mechanism by which VFL may impact upon EMT and metastasis. VFL impacts on E-cadherin-based cell-cell adhesion and influence on the EMT markers in epithelial bladder tumour cell lines. We have shown that VFL induces cell death in bladder cancer cells and activates epithelial differentiation of the remaining living cells. VFL increases E-cadherin dependent cell-cell adhesion, and reduces vimentin and N-cadherin mesenchymal markers. Moreover, the levels of the E3 ubiquitin-ligase Hakai were reduced by VFL in 5637 suggesting that proteasome-dependent mechanism could be one of the molecular mechanisms implicated in this reduction. Our findings suggest the existence of a new mechanism of VFL-microtubule targeting drug to cell-cell contacts with potential functional implications in the maintenance of epithelial cell phenotype.

Additional file

Additional file 1: Figure for reviewer 3. Effect of VFL on the phenotype of 5637 and HT1376 bladder tumour cell lines. Phase-contrast microscopy images, showing larger fields from images of Figure 2, of the indicated bladder cell lines taken 48 h after treatment with the indicated concentrations of VFL compared to control conditions.

Abbreviations

EMT: Epithelial-to-mesenchymal transition; MEE: Medial edge epithelium; MET: Mesenchymal-to-epithelial transition; TCC: Transitional cell carcinoma; VFL: Vinflunine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RC, MHC and MR contributed to experimental research. AF contributed to the design, the analysis of the results and writing the manuscript. All the authors have given the discussion, critical reading of the manuscript and final approval of the version to be published.

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