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Molecular events are associated with resistance to vinblastine in bladder cancer

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Abstract

Bladder cancer occurs in the majority of cases in males, which represents the fourth highest incident cancer in men and tenth in women. It is associated with a high rate of recurrence, and prognosis is poor once the cancer metastasizes to distant sites. Transitional cell cancer (TCC) is the most predominant histological type. Bladder cancer is highly chemosensitive. However, the presence of acquired drug resistance is one of the primary impediments to the success of chemotherapy. To differentiate and delineate the molecular events, we developed drug resistant human transitional bladder cancer T24 cells (DRC) by treating cells with the increasing concentration of vinblastine. We found that DRC was resistant to vinblastine in comparison to parental T24 cells. We analyzed the contributory factors that may be involved in the development of resistance. As expected, expression of permeability glycoprotein (P-gp) was up-regulated in DRC. In addition, levels of Caveolin-1 (Cav-1), Fatty acid synthase (FASN) and Cytochrome P450 (CYP450) were elevated in DRC. Downregulation of these proteins by respective specific pharmacological inhibitors and/or by siRNAs resensitized cells to vinblastine. These results suggested that differential levels of P-gp, Cav-1 and FASN except CYP450 play a major role in acquired resistant phenotype in bladder cancer.

Key words: Vinblastine, drug resistance, P-gp, Cav-1, FASN, CYP450, bladder cancer.

Introduction

Bladder cancer, a disease in which abnormal cells multiply without control in the bladder, is widespread malignant tumor. It is one of most common causes of cancer associated deaths in men and women. The incidence of bladder cancer is increasing. With 74 690 new cases and over 15 000 deaths estimated in the United States in 2014, bladder cancer is the fourth leading incident cancer affecting men (1). An estimated 386,300 new cases and 150,200 deaths from bladder cancer occurred in 2008 worldwide (2). Bladder cancer occurs in the majority of cases in males with a male/female sex ratio of 4:1 (1). The most common type of bladder cancer recapitulates the normal histology of the urothelium and is known as transitional cell cancer or more properly urothelial cell carcinoma. Transurethral resection of bladder tumor (TURBT) is an appropriate treatment for non-muscle invasive bladder cancer. But in case of muscle invasive cancer, the procedure is insufficient for final treatment (3). Therefore, chemotherapy remains to be the alternative treatment strategy. However, cancer cells may be intrinsically drug-resistant or acquire resistance following exposure to drug(s) (4-7), which limit effectiveness of chemotherapy. Acquired resistance is a particular problem, as tumors not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Resistance to chemotherapy is believed to cause treatment failure in over 90% of patients with metastatic cancer, and resistant micrometastatic tumor cells may also reduce the effectiveness of chemotherapy in the adjuvant setting. Clearly, if drug resistance can be overcome, the impact on survival will be highly significant.

T24 cells, derived from transitional cancers of human urine bladder with high malignancy, have been frequently used for studies of the metastasis-associated mechanisms of human bladder cancer. Vinblastine is a medication used to treat a number of types of cancer including: Hodgkin's lymphoma, non-small cell lung cancer, testicular cancer, brain cancer, and bladder cancer among others (8). It is used intravenously and works by inhibiting mitosis (8).

Cell membrane being the primary entry point for the drugs, it is likely that membrane associated proteins play a major role in the development of resistance, though multiple mechanisms may contribute to this phenomenon. One such protein, P-gp also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243) is an important protein of the cell membrane that pumps many foreign substances out of cells. It exists in animals, fungi and bacteria and likely evolved as a defense mechanism against harmful substances. Another membrane associated protein, Cav-1 is a major component of specialized plasma membrane micro domain called as caveolae, which regulates signal transduction and protein translocation in cell membrane. And it has been implicated in the development of drug resistance (9-11). Cav-1 is reported to be associated with a cytosolic protein fatty acid synthase (FASN) (12). FASN is a multifunctional enzyme involved in the synthesis of palmitate from acetyl CoA/malonyl CoA

and is reported to be associated with drug resistance in breast cancer cells (13). Yet another molecule involved in drug resistance is Cytochrome 450 (CYP450). Metabolism by CYP450 plays a major role in reduction of drugs induced pharmacological effect (14). Till now, whether the factors are involved in the development of acquired drug resistance in bladder cancer is unclear. Thus, investigation into the factors involved in the development of drug resistance is significant and necessary.

In this study, we developed acquired drug resistant T24 cells (DRC) to vinblastine. We found that P-gp, Cav-1, FASN and CYP450 levels were enhanced in DRC in comparison with the parental T24 cells. Using specific inhibitors and siRNAs, we confirmed that P-gp, Cav-1 and FASN levels were associated with acquired drug resistance in DRC. This work suggested that the cellular proteins might have to be probed into for combating drug resistance in cancer cells and their pharmacological inhibitors or siRNAs treatment in combination with chemotherapy might be a valid approach to cancer treatment.

Materials and methods

Cell culture

Human bladder cancer cells T24, obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), were grown in McCoy medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) (Invitrogen Corporation, CA, USA) at 37°C with 5% CO_2. Only DRC were routinely cultured in above mentioned media along with 10 nM vinblastine.

Development of drug resistant cells

For development of acquired drug resistant cells (DRC), T24 cells were exposed to increasing concentration of vinblastine ranging from 0.1 to 1 μM in complete medium. Briefly, T24 cells were seeded in 60 mm culture plates and allowed to grow. After 24 h incubation, 100 nM vinblastine was added for another 48 h. Subsequently, medium was removed and fresh, drug free medium was added. The cells were incubated and allowed to grow. When confluency was reached, cells were trypsizined, replated and these cells were re-exposed to double the dose of drug. This process was repeated until clones developed resistance to 1 μM vinblastine. After prolonged (3 months) exposure to increasing concentration of vinblastine, surviving clones were pooled together and termed as drug resistant cells (DRC) and these were used for subsequent experiments.

Cell survival assay

Cell survival was assessed by MTT (Sigma Aldrich, MO, USA).dye conversion. Briefly, cells were seeded 10^4 per well in a 96-well flat bottom plate. Cells were allowed to grow in a 37°C, 5% CO_2 incubator. At near confluence, the cells were treated with 1 μM vinblastine for 48 h, followed by another 4 h after 20 μL of 5 mg/mL MTT was added to each well. Cells were lysed by addition of 200 μL dimethylsulfoxide. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Morphological examination of vinblastine-resistant cells

T24 cells and DRC were seeded in 35 mm petri plates. After 24 h incubation, cells were treated with 1 μM vinblastine for 48 h and untreated cells served as control. The cells were washed twice with McCoy medium, the morphology of cells was visualized and photographed using vertical microscope (Olympus, Tokyo, Japan).

Western blotting

Cells were lysed in cell lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM $MgCl_2$, 1.5 mM EDTA, 20 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotonin, 1 μM pepstatin A, 1 mM phenylmethylsulphonyl fluoride). Protein samples (50 μg) were separated by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, MA, USA). Antibodies against P-gp, Cav-1, FASN, CYP450 and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). ECL-detecting reagent (Amersham Biosciences, Buckinghamshire, England) was used to development.

Drugs and chemicals

Vinblastine was purchased from Wako Pure Chemical Co. (Osaka, Japan). Methyl b-cyclodextrin (MCD) was purchased from Sigma-Aldrich (Sigma Aldrich, MO, USA). Verapamil and cerulenin were purchased from Calbiochem (Calbiochem, CA, USA). Working solutions were diluted in culture medium immediately before use. siRNA specific for Cav-1, CYP450 and FASN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Radiolabeled [^{3}H] vinblastine uptake was done as described previously (15).

siRNA transfection

Cell transfections were conducted using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were further grown for 24 h followed by addition of 100 nM vinblastine for another 48 h. MTT assay was performed or whole cell lysates were prepared for immunoblotting.

Statistical analysis

SPSS (version 11.0) was used to analyze the experimental data, which are presented as the mean±standard error of the mean (SEM). All of the experiments were repeated in at least three times. The value of $P < 0.05$ was considered statistically significant.

Results

Development of vinblastine-resistant bladder cancer cells

Vinblastine-resistant bladder cancer T24 cellular model was established to study the molecular mechanisms of acquired drug resistance as described in materials and methods. Characterization of DRC was performed. The cell growth curve analysis of T24 cells and DRC was carried out. The doubling time for DRC

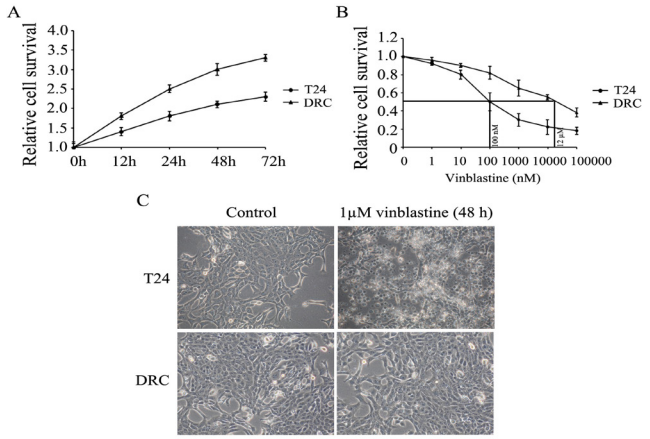

Figure 1. Characterization of human bladder cancer cell line T24 cells and DRC. (A) Cell growth curve. 4×10^3 cells were plated in 96 well plates and medium was removed at indicated time interval and cell survival was evaluated by MTT assay. (B) 1×10^4 cells were plated in 96 well plates containing 100 ml medium. After 24 h incubation, cells were treated with varying concentrations of vinblastine for 48 h and cell survival was evaluated by MTT assay. (C) Hep3B cells, DRC and SCC were treated with 1 μM vinblastine for 48 h and their morphology was observed under vertical microscope and photographed.

was calculated to be 24 h and that of T24 cells it was 36 h. (Figure 1A). Next, IC_{50} value of vinblastine was determined by treating T24 cells and DRC with varying concentration of vinblastine for 48 h. IC_{50} value were calculated to be as 100 nM for T24 cells and 12 μM for DRC at 48 h (Figure 1B). The survival capabilities of T24 cells and DRC treated with 1 μM vinblastine for 48 h were compared by observing their morphology. As expected, T24 cells exhibited apoptotic feature with membrane blebbing and they were rounded off. On the other hand, no noticeable morphological changes were observed in DRC (Figure 1C).

Molecular events involved in resistance development

Based on the above results, we speculated that the decreased sensitivity of DRC to vinblastine could be due to reduced drug uptake in comparison to T24 cells. Therefore, we performed drug uptake assay and uptake of radioactive labeled vinblastine [^{3}H] was higher in T24 cells as compared to DRC (Figure 2A). Reduced uptake in DRC may be due to overexpression of P-gp which is responsible for effluxing of drugs from the cells. To study the involvement of P-gp in DRC, protein expression of P-gp was detected using Western blotting. High P-gp expression was observed in DRC compared to T24 cells (Figure 2B). In addition, to unravel whether other drug resistance-associated proteins Cav-1, FASN and CYP450 have a role to play in the development of resistance to vinblastine in DRC, expression level of these molecules were examined. The results showed that Cav-1, FASN and CYP450 levels were all remarkably increased in DRC relative to T24 cells (Figure 2B).

Role of P-gp and CYP450 in acquired drug resistance

To verify the role of P-gp in drug resistance development, verapamil an inhibitor/blocker, was used alone or combination with vinblastine. Upto 40 μM concentration, verapamil alone did not have any effect on the cell survival (Figure 3A). Treatment of DRC with vinblastine in the presence of verapamil promoted

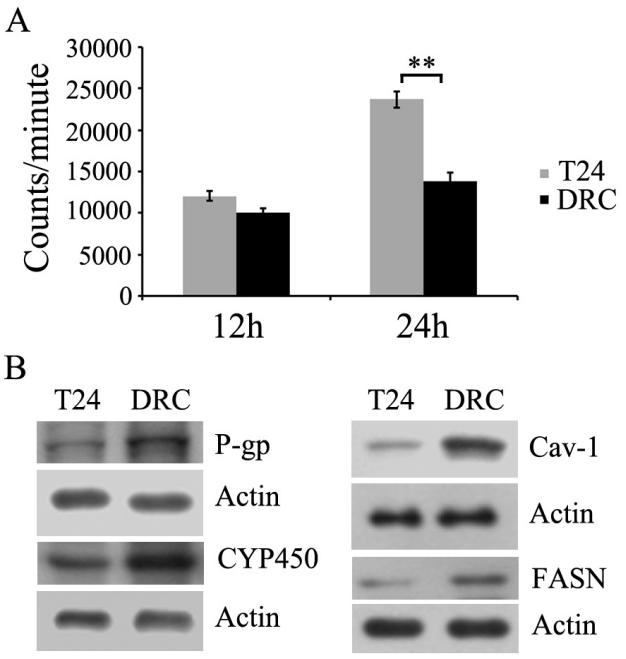

Figure 2. Molecular events involved in resistance development. (A) 1×10^5 cells were plated in 12 well plates. After 24 h incubation, 500 nCi/well of ^{3}H vinblastine was added to each well for 12 h and 24 h, respectively. Cells were lysed by adding SDS and counts were taken on Packard. (B) Western blot analysis of expression of proteins-associated with drug resistance. Cells were harvested and whole cell lysates were prepared to evaluate P-gp, Cav-1, FASN and CYP450 basal level using respective corresponding antibodies. **, $P<0.01$ versus T24.

cell death and cell survival reduced by 25% in DRC (Figure 3B). Meanwhile, we explored involvement of CYP450 by siRNA mediated knockdown. The siRNA interference efficiency was confirmed in DRC and CYP450 level was reduced by more than 4 folds (Figure 3C). Next, T24 cells and DRC were transfected with CYP450 siRNA and treated with vinblastine for additional 48 h. Cell survival was evaluated by MTT assay. We found that knockdown of CYP450 did not have significant impact on the survival of vinblastine treated T24 cells or DRC (Figure 3D).

Downregulation of FASN or Cav-1 resensitizes DRC to vinblastine

To verify the roles of FASN and Cav-1, respective molecular inhibitor and siRNA were used to inhibit protein levels. A potent inhibitor of FASN, cerulenin reduced the viability of cells in a dose dependent manner (Figure 4A). However, treatment of with 30 μM cerulenin for 48 h did not have any deleterious effect on the cell survival (Figure 4A). Combination treatment of cerulenin and vinblastine significantly inhibited cell growth in DRC by 30% in comparison with vinblastine treatment alone (Figure 4B). Further, FASN siRNA was shown to significantly decrease FASN level more than 3 folds in comparison to control siRNA transfected cells (Figure 4C). Cells transfected with siRNAs were exposed to vinblastine for 48 h and cell survival was evaluated by MTT assay. Cell survival was reduced by 20% in DRC in comparison to control siRNA transfected and paclitaxel alone treated cells (Figure 4D). In addition, a inhibitor of Cav-1, MCD decreased the cell growth in a dose-dependent manner. Treatment of with 1 mM MCD

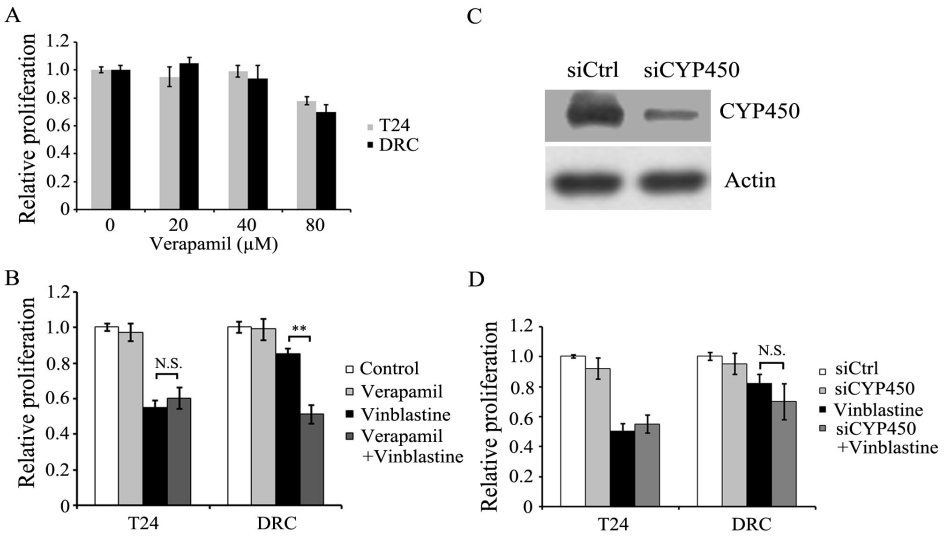

Figure 3. Involvement of P-gp and CYP450 in drug resistance. (A) Cells were treated with varying concentration of verapamil for 24 h. Thereafter medium was replaced with fresh medium and incubated for additional 48 h. Cell survival was assessed by MTT assay. (B) T24 cells and DRC were pretreated with 40 μM verapamil for 24 h and then fresh medium was added with or without 100 nM vinblastine. After 48 h treatment, medium containing drug was removed and MTT assay was performed. (C) DRC cells were transfected with control or CYP450 siRNA. After 24 h transfection, cell lysate was prepared for western blotting to evaluate CYP450 protein interference efficiency. (D) T24 cells and DRC were transfected with control or CYP450 siRNA for 24 h. Cells were washed and then fresh medium with or without vinblastine incubated for 48 h. Thereafter MTT assay was performed. **, P<0.01, N.S., no significance.

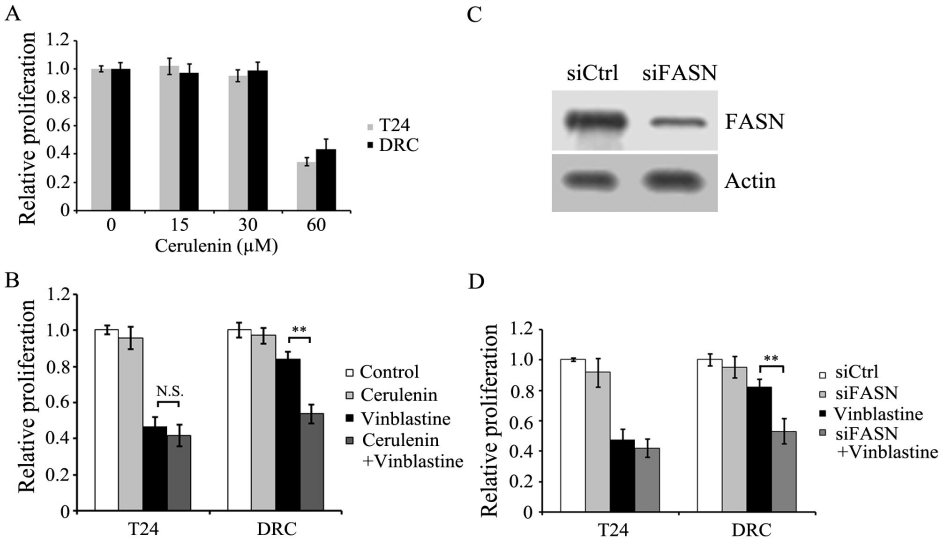

Figure 4. Knockdown of FASN enhances DRC sensitivity to vinblastine. (A) Cells were treated with varying concentration of cerulenin for 24 h. Thereafter medium was replaced with fresh medium and incubated for additional 48 h. Cell survival was assessed by MTT assay. (B) T24 cells and DRC were pretreated with 30 μM cerulenin for 24 h and then fresh medium was added with or without vinblastine. After 48 h treatment, medium containing drug was removed and MTT assay was performed. (C) DRC cells were transfected with control or FASN siRNA. After 24 h transfection, cell lysate was prepared for western blotting to evaluate protein interference efficiency. (D) T24 cells and DRC were transfected with control or FASN siRNA for 24 h. Cells were washed and then fresh medium with or without vinblastine incubated for 48 h. Thereafter MTT assay was performed. **, P<0.01, N.S., no significance.

for 48 h did not have obvious deleterious effect on the cell survival (Figure 5A). Sensitivity of T24 cells or DRC to the combination of MCD and vinblastine together was pronounced and the viability of DRC significantly reduced in comparison to individual treatments (Figure 5B). However, under identical experimental conditions viability of T24 cells was unaffected. Furthermore, Cav-1 siRNA was used to inhibit its protein expression by 3 folds in DRC (Figure 5C) and downregulation of Cav-1 enhanced DRC sensitivity to vinblastine (Figure 5D).

Discussion

Chemotherapy is widely applied to various cancers treatment. However, the effectiveness of chemotherapy is usually significantly limited by drug resistance during drug treatment. Multiple factors may act individually or synergistically and contribute to acquired chemoresistance, eventually leading to treatment below expected outcome (16-18). Additionally, cancer cells may exhibit resistance not only to the original drug used but also to many other chemotherapeutic drugs, a phenomenon known as cross resistance. Therefore, studies on mechanisms of cancer drug resistance yield great significance, providing theoretical basis about how to overcome this resistance to improve cancer chemotherapy.

Hererin, we established a cell model that mimics some of the *in vivo* phenotype of drug resistant cancer cells, called as DRC. Drug resistance phenotype was confirmed

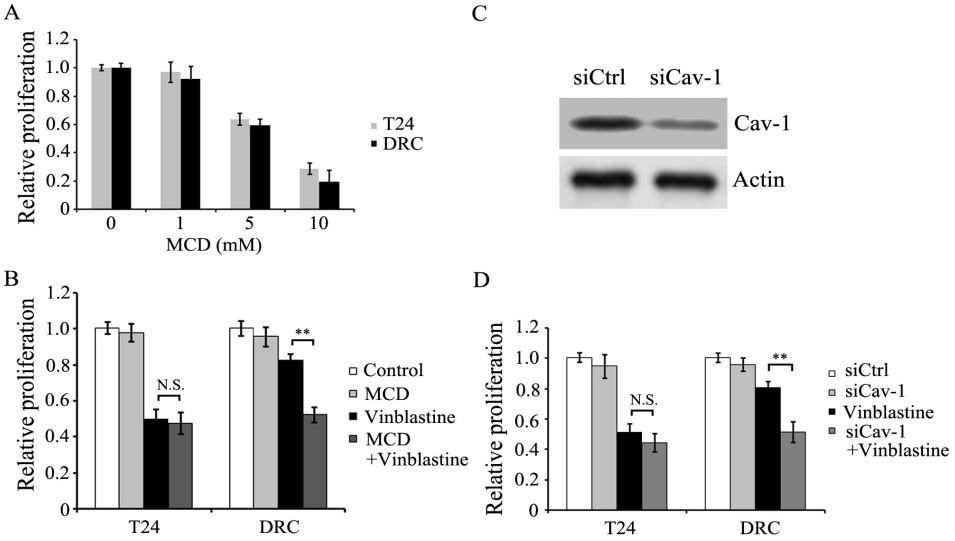

Figure 5. Downregulation of Cav-1 resensitize DRC to vinblastine. (A) Cells were treated with varying concentration of MCD for 4 h. Thereafter medium was replaced with fresh medium and incubated for additional 48 h. Cell survival was assessed by MTT assay. (B) T24 cells and DRC were pretreated with 1 mM MCD for 4 h and then fresh medium was added with or without 100 nM vinblastine. After 48 h treatment, medium containing drug was removed and MTT assay was performed. (C) DRC cells were transfected with control or Cav-1 siRNA. After 24 h transfection, cell lysate was prepared for western blotting to evaluate protein interference efficiency. (D) T24 cells and DRC were transfected with control or Cav-1 siRNA for 24 h. Cells were washed and then fresh medium with or without vinblastine incubated for 48 h. Thereafter MTT assay was performed. **, P<0.01, N.S., no significance.

by comparing the growth and survival capabilities of parental T24 cells and DRC following treatment with vinblastine. These cells significantly differed in growth properties. DRC doubled faster and was killed slower than parental cells. Also, the uptake of radiolabeled vinblastine was higher in parental T24 cells than DRC. Furthermore, P-gp expression was enhanced in DRC compared to T24 cells. Thus, initial characterization indicates that DRC cellular model becomes resistant to vinblastine eventually grows more rapidly and may eflux drug out due to P-gp overexpression. This cellular model would therefore facilitate in further investigation of drug resistance phenomena.

Next, we explored the other molecular alterations that may participate in development of chemoresistance. It was shown that Cav-1, FASN and CYP450 expression were also verified, which have been reported to be associated with resistance development. Cav-1 plays an important role in tumor progression, metastasis, invasion, cell survival and poor prognosis (9,10,19,20). It has been reported that Cav-1 expression is correlated with bladder cancer tumorigenesis and metastasis (11,21,22). Cav-1 level was elevated in DRC suggesting its association with acquired drug resistance. FASN level correlates with tumor progression and it plays an important role in tumor growth, survival and development of drug resistance in breast cancer cells (12,13). It has been reported that inhibition of FASN suppresses p-Akt and induces apoptosis in bladder cancer (23). However, there is still no report on the relevance of FASN in drug sensitivity of bladder cancer cells. Our study indicated the correlation between the two. Additionally, resistance of DRC against other chemotherapeutic drugs needs to been further explored to confirm whether cross resistance is yielded during treatment, which will provide valuable information for improvement of chemotherapy.

Pharmacological inhibitors or specific siRNA against these molecules were utilized to resensitize DRC to vinblastine, reascertaining their association with the development of chemoresistance in bladder cancer cells. Downregulation of P-gp, Cav-1 and FASN expression decreased cell survival in DRC whereas knockdown of CYP450 did not significantly alter survival in any of these cells exposed to vinblastine. In conclusion, P-gp, Cav-1 and FASN play a crucial role in acquired drug resistance caused by vinblastine exposure. This study suggests that combination therapies with novel inhibitors or siRNAs targeting key molecules such as P-gp, Cav-1 and FASN potentially represent a more effective treatment for drug resistant bladder cancer cells.

Acknowledgments

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