Resistance to nanoparticle albumin-bound paclitaxel is mediated by ABCB1 in urothelial cancer cells

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Abstract. Nanoparticle albumin-bound (nab)-paclitaxel appears to exhibit better response rates in patients with metastatic urothelial cancer of the bladder whom are pretreated with nab-paclitaxel compared with conventional paclitaxel. Paclitaxel may induce multidrug resistance in patients with cancer, while the mechanisms of resistance against paclitaxel are manifold. These include reduced function of pro-apoptotic proteins, mutations of tubulin and overexpression of the drug transporter adenosine 5'-triphosphate-binding cassette transporter subfamily B, member 1 (ABCB1). To evaluate the role of ABCB1 in nab-paclitaxel resistance in urothelial cancer cells, the bladder cancer cell lines T24 and TCC-SUP, as well as sub-lines with acquired resistance against gemcitabine (T24^{r}GEMCI^{20} and TCC-SUP^{r}GEMCI^{20}) and vinblastine (T24^{r}VBL^{20} and TCC-SUP^{r}VBL^{20}) were examined. For the functional inhibition of ABCB1, multi-tyrosine kinase inhibitors with ABCB1-inhibiting properties, including cabozantinib and crizotinib, were used. Additional functional assessment was performed with cell lines stably transduced with a lentiviral vector encoding for ABCB1, and protein expression was determined by western blotting. It was indicated that cell lines overexpressing ABCB1 exhibited similar resistance profiles to nab-paclitaxel and paclitaxel. Cabozantinib and crizotinib sensitized tumor cells to nab-paclitaxel and paclitaxel in the same dose-dependent manner in cell lines overexpressing ABCB1, without altering the downstream signaling of tyrosine kinases. These results suggest that the overexpression of ABCB1 confers resistance to nab-paclitaxel in urothelial cancer cells. Additionally, small molecules may overcome resistance to anticancer drugs that are substrates of ABCB1.

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Introduction

Patients with locally advanced or metastatic urothelial cancer of the bladder exhibit poor prognoses, with an overall survival of 12-14 months subsequent to first-line chemotherapy with the combination of gemcitabine and cisplatin as standard of care. Treatment failure is commonly caused by resistance acquisition to chemotherapy subsequent to the primary response (1,2).

In Europe, vinflunine is the only approved second-line chemotherapy, with only moderate response rates (3). There is currently no USA Food and Drug Administration (FDA)-approved treatment subsequent to first-line chemotherapy in the USA. However, taxanes are commonly used for palliative chemotherapy based on modest response rates in several small, nonrandomized phase II trials (4).

Nanoparticle albumin-bound (nab)-paclitaxel, also termed Abraxane®, is already widely used in the clinical treatment of breast cancer with FDA approval in 2005, non-small cell lung cancer (NSCLC) with FDA approval in 2010 and pancreatic cancer with FDA approval in 2013 (5,6). Nab-paclitaxel achieved a marked overall response rate of 27.7% in patients with metastatic urothelial cancer of the bladder who were pretreated with cisplatin, and may be more effective than conventional paclitaxel (7). At present, a randomized phase II trial [National Clinical Trials (NCT) no., 02033993] comparing nab-paclitaxel and paclitaxel is ongoing.

It has been postulated that the increased efficacy of nab-paclitaxel compared with that of paclitaxel is based on an increased transendothelial glycoprotein (gp)60-mediated transport and enhanced intratumoral accumulation as a result of the secreted protein acidic and rich in cysteine (SPARC)-albumin interaction (5,8,9). Dong *et al* (10) demonstrated that paclitaxel-loaded lipid-based nanoparticles containing the Brij 78 surfactant may overcome adenosine 5'-triphosphate-binding cassette transporter subfamily B, member 1 (ABCB1)-mediated drug resistance. By contrast, other studies hypothesized

that resistance to nanoparticle-bound paclitaxel may also be ABCB1 mediated (11,12). However, it remains unclear whether albumin-bound paclitaxel nanoparticles may overcome drug resistance caused by ABCB1 (5).

The present study evaluated whether ABCB1 transporters affect the antitumoral activity of nab-paclitaxel in a panel of urothelial cancer cell lines. The results demonstrate that ABCB1 overexpression mediates resistance to nab-paclitaxel. Resistance to nab-paclitaxel may be overcome by inhibitors of ABCB1 transporters, including cabozantinib and crizotinib, two FDA-approved small molecule inhibitors being tested at present as second-line therapy for urothelial carcinoma (NCT nos., 02612194, 01688999 and 02496208).

Materials and methods

Drugs. Cisplatin was purchased from Gry-Pharma GmbH (Dresden, Germany), gemcitabine from Lilly Germany GmbH (Bad Homburg, Germany), paclitaxel from Bristol-Myers Squibb (New York, NY, USA), vinblastine from Teva Pharmaceutical Industries Ltd., (Petah Tikva, Israel), and cabozantinib and crizotinib from Selleck Chemicals (Houston, TX, USA). Nab-paclitaxel was purchased from Celgene International (Boudry, Switzerland) and was stored at 4°C subsequent to preparation.

Cell lines and lentiviral transduction. The urothelial bladder cancer T24 and TCC-SUP cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Drug-resistant sublines were established by continuous exposure to increasing drug concentrations as described previously (13) and are part of the Resistant Cancer Cell Line collection (Institute of Medical Virology, University Hospital Frankfurt, Frankfurt, Germany): T24[r]GEMCI[20] (gemcitabine-resistant, 20 ng gemcitabine/ml), T24[r]VBL[20] (vinblastine-resistant, 20 ng vinblastine/ml), TCC-SUP[r]GEMCI[20] and TCC-SUP[r]VBL[20] (vinblastine-resistant, 20 ng vinblastine/ml).

The cell lines TCC-SUP[ABCB1] and T24[ABCB1] with ectopic overexpression of ABCB1 (University Medical Center Hamburg-Eppendorf, Hamburg, Germany), and the corresponding control cell lines with empty vector TCC-SUP[CER2] and T24[CER2] (University Medical Center Hamburg-Eppendorf), were established by lentiviral transduction using the Lentiviral Gene Ontology Vector technology as described previously (14,15).

All cell lines were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell line authentication was performed by short tandem repeat profiling.

Cell viability assay. Cell viability was determined by the MTT dye reduction assay after 120 h of incubation, as described previously (13). Drug resistance was determined according to resistance factors defined as half maximal inhibitory concentration (IC_{50}) drug in resistant cells/IC_{50} drug in parental cells. The cell lines were considered to be resistant to a drug if the resistance factor was >2 (16). Sensitization to a drug was determined according to sensitization factors defined as IC_{50} drug in the tested cell line without tyrosine kinase inhibitor (TKI)/IC_{50} drug in the tested cell line plus TKI. To evaluate the *in vitro* stability of nab-paclitaxel, efficacy factors were defined as IC_{50} of 7 or 28 days-old nab-paclitaxel/IC_{50} of freshly prepared nab-paclitaxel.

Western blotting. Cells were lysed on ice in Triton X-100 sample buffer, centrifuged at 4°C at 14,000 x *g* for 5 min, and supernatant was stored at -20°C. Protein concentration was determined using BioRad DC protein assay (catalog no. 5000112, Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the proteins (concentration 1 mg/ml; 15 *µ*l per lane) were separated by 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (catalog no. 88,018; Thermo Fisher Scientific, Inc.) and blocked with 3% bovine serum albumin (Carl Roth, Karlsruhe, Germany) in Tris buffered saline containing 0.05% (v/v) Tween®-20 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 40 min at room temperature. Membranes were incubated overnight at 4°C with specific primary antibodies against β-actin (1:1,000; catalog no. A2228; Sigma-Aldrich; Merck KGaA), protein kinase B (Akt; 1:1,000; catalog no. 9272; Cell Signaling Technology, Inc., Danvers, MA, USA) and phosphorylated (p)-Akt (T308; 1:1,000; catalog no. 2965; Cell Signaling Technology). Membranes were washed in Tris buffered saline containing 0.05% (v/v) Tween®20 (Sigma-Aldrich; Merck KGaA) for 40 min at room temperature, blocked and incubated for 1 h with fluorescence-labeled secondary antibodies IRDye 800CW goat anti-rabbit immunoglobulin G (IgG; heavy and light chains; 1:20,000; catalog no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA) and IRDye 800CW goat anti-mouse IgG (heavy and light chains; 1:20,000; catalog no. 926-32210; LI-COR Biosciences) at room temperature. Fluorescence of the secondary antibody was measured with the Odyssey CLx Imaging system and the Image Studio software (version 3.1; LI-COR Biosciences).

Statistical analysis. For statistical analyses, unpaired Student's t-test, analysis of variance and Student-Newman-Keuls test were performed for comparison of IC_{50} values following treatment of tumor cell lines with various anti-cancer compounds. Statistical analyses were performed with GraphPad Prism® (Version 5.0c; GraphPad Software Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

In vitro stability studies of nab-paclitaxel. As the instability of nab-paclitaxel has been examined previously *in vivo* (17), The present study sought to evaluate for how long freshly prepared nab-paclitaxel may be used *in vitro*. Therefore, functional MTT assays were conducted with nab-paclitaxel on days 0, 7, and 28 subsequent to drug preparation. A slight loss of the antitumoral efficacy of nab-paclitaxel after 7 days was observed, with efficacy factors of 0.95-1.64 (efficacy factor of day 7=IC_{50} at day 7/IC_{50} at day 0) compared with that revealed by MTT assays at day 0. On day 28, a marked loss of antitumoral efficacy with efficacy factors of 4.91-19.90 (efficacy factor of day 28=IC_{50} day at 28/IC_{50} at day 0) was observed (Fig. 1). A significant time-dependent loss of efficacy of nab-paclitaxel was also observed. Therefore, nab-paclitaxel was used only on the day of preparation for additional experiments.

Table I. IC$_{50}$ values of paclitaxel, nab-paclitaxel, gemcitabine and vinblastine in TCC-SUP, TCC-SUP^{r}GEMCI^{20}, TCC-SUP^{r}VBL^{20}, TCC-SUP^{CER2} and TCC-SUP^{ABCB1} cells in the presence of cabozantinib.

| Cell line | Cabozantinib, μM | IC$_{50}$ (sensitization factor)[a], ng/ml | | | |
		Paclitaxel	Nab-paclitaxel	Gemcitabine	Vinblastine
TCC-SUP	0	1.14±0.12	0.78±0.34	1.20±0.39	0.36±0.14
TCC-SUP	0.5	1.07±0.07 (1.07)	0.93±0.31 (0.84)	0.92±0.01 (1.3)	0.47±0.02 (0.77)
TCC-SUP	1.25	1.18±0.14 (0.97)	0.79±0.14 (0.99)	2.27±0.30 (0.53)	0.31±0.16 (1.16)
TCC-SUP^{r}GEMCI^{20}	0	4.98±1.50	0.69±0.45	42.56±0.90	0.69±0.23
TCC-SUP^{r}GEMCI^{20}	0.5	2.01±0.35 (2.48)	0.56±0.15 (1.23)	37.72±0.60 (1.13)	0.60±0.07 (1.15)
TCC-SUP^{r}GEMCI^{20}	1.25	1.23±0.36 (4.05)	0.31±0.17 (2.22)	43.40±13.60 (0.98)	0.35±0.07 (1.97)
TCC-SUP^{r}VBL^{20}	0	159.60±24.50	395.40±65.80	1.24±0.30	35.29±6.50
TCC-SUP^{r}VBL^{20}	0.5	66.33±13.50 (2.41)	79.52±6.90 (4.97)	1.19±0.40 (1.04)	8.19±1.16 (4.31)
TCC-SUP^{r}VBL^{20}	1.25	20.86±3.90 (7.65)	22.64±1.90 (17.46)	1.92±0.21 (0.65)	3.44±0.69 (10.26)
TCC-SUP^{CER2}	0	1.96±0.44	2.43±0.25	1.84±0.18	0.43±0.19
TCC-SUP^{CER2}	0.5	1.88±0.58 (1.04)	2.11±0.23 (1.15)	1.71±0.11 (1.08)	0.58±0.09 (0.74)
TCC-SUP^{CER2}	1.25	1.42±0.42 (1.38)	1.77±0.26 (1.37)	1.57±0.02 (1.17)	0.31±0.14 (1.39)
TCC-SUP^{ABCB1}	0	93.14±18.40	140.0±91.30	1.46±0.22	11.33±3.70
TCC-SUP^{ABCB1}	0.5	19.12±7.10 (4.87)	25.96±17.30 (5.39)	1.53±0.01 (0.95)	2.00±0.83 (5.67)
TCC-SUP^{ABCB1}	1.25	7.81±1.60 (11.93)	5.03±2.50 (27.83)	1.61±0.23 (0.91)	0.87±0.35 (13.06)

[a]Sensitization factor=IC$_{50}$ of the drug in the tested cell line without cabozantinib/IC$_{50}$ of the drug in the tested cell line plus cabozantinib. Values are means ± standard deviations. IC$_{50}$, half maximal inhibitory concentration; nab, nanoparticle albumin-bound.
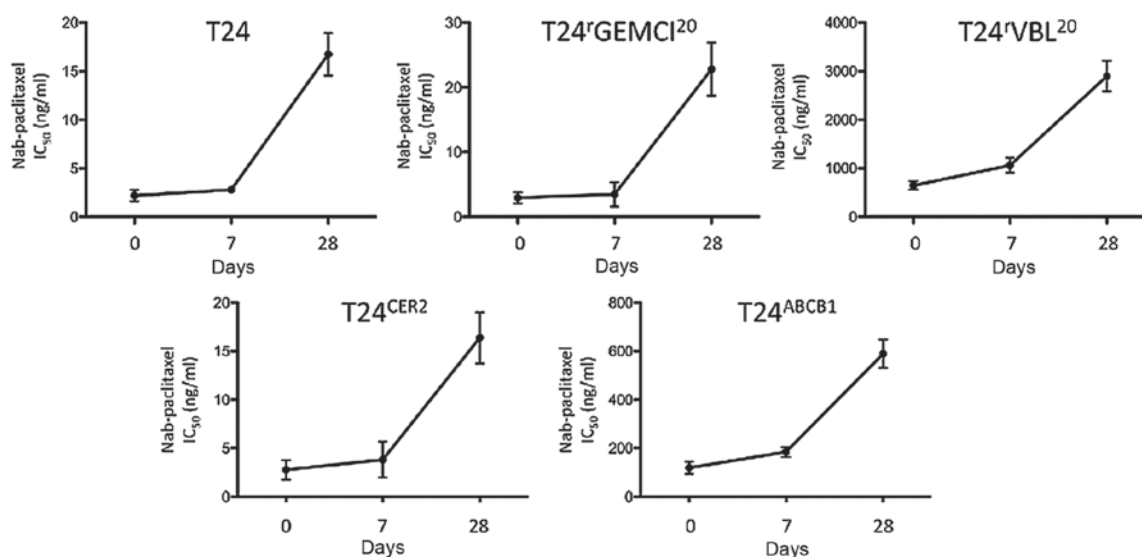

Figure 1. *In vitro* stability of nab-paclitaxel. IC$_{50}$ values of MTT assays with nab-paclitaxel on days 0, 7 and 28 in T24, T24^{r}GEMCI^{20}, T24^{r}VBL^{20}, T24^{CER2} and T24^{ABCB1} cells. Values are means ± standard deviations of at least three independent experiments. Nab, nanoparticle albumin-bound; IC$_{50}$, half maximal inhibitory concentration.

Cell viability assays. There was no significant difference between the antitumoral activity of nab-paclitaxel and paclitaxel in chemosensitive urothelial cancer cells or their chemoresistant sublines (Tables I-IV). The ABCB1-overexpressing cell lines were similarly resistant to these two compounds. Cabozantinib and crizotinib sensitized the ABCB1-overexpressing tumor cell lines T24^{r}VBL^{20}, T24^{ABCB1}, TCC-SUP^{r}GEMCI^{20}, TCC-SUP^{r}VBL^{20} and TCC-SUP^{ABCB1} (18) to nab-paclitaxel and paclitaxel in a dose-dependent manner (Tables I-IV). In addition, cabozantinib sensitized these ABCB1-overexpressing tumor cell lines to vinblastine therapy in a dose-dependent manner (Tables I and III). The IC$_{50}$ values of cabozantinib and crizotinib monotherapy were in the low-micromolar range. Resistance against gemcitabine, vinblastine or stable transduction with a lentiviral vector encoding for ABCB1 did not appear to significantly change the sensitivity profiles to cabozantinib or crizotinib (Table V).

Table II. IC$_{50}$ values of paclitaxel, nab-paclitaxel, gemcitabine and vinblastine in TCC-SUP, TCC-SUP^{r}GEMCI^{20}, TCC-SUP^{r}VBL^{20}, TCC-SUP^{CER2} and TCC-SUP^{ABCB1} cells in the presence of crizotinib.

Cell line	Crizotinib, μM	IC$_{50}$ (sensitization factor)^a, ng/ml			
		Paclitaxel	Nab-paclitaxel	Gemcitabine	Vinblastine
TCC-SUP	0	0.95±0.21	0.60±0.22	0.72±0.14	0.43±0.08
TCC-SUP	0.5	0.79±0.18 (1.20)	0.47±0.10 (1.28)	0.76±0.04 (0.95)	0.52±0.13 (0.83)
TCC-SUP	1.25	0.51±0.09 (1.86)	0.36±0.07 (1.67)	1.11±0.80 (0.65)	4.94±2.61 (0.09)
TCC-SUP^{r}GEMCI^{20}	0	4.40±1.86	0.94±0.85	39.98±7.20	1.00±0.38
TCC-SUP^{r}GEMCI^{20}	0.5	1.19±0.25 (3.70)	0.35±0.05 (2.69)	45.20±3.14 (0.88)	0.47±0.22 (2.13)
TCC-SUP^{r}GEMCI^{20}	1.25	0.29±0.03 (15.17)	0.39±0.13 (2.41)	41.50±36.30 (0.96)	0.63±0.40 (1.59)
TCC-SUP^{r}VBL^{20}	0	150.20±35.10	358.17±81.70	1.34±0.14	35.07±9.20
TCC-SUP^{r}VBL^{20}	0.5	90.38±37.60 (1.66)	210.67±19.94 (1.70)	1.23±0.08 (1.09)	17.98±0.72 (1.95)
TCC-SUP^{r}VBL^{20}	1.25	40.81±38.10 (3.68)	62.16±18.23 (5.76)	1.46±0.15 (0.92)	5.40±3.53 (6.49)
TCC-SUP^{CER2}	0	2.00±0.61	2.11±0.56	2.02±0.13	0.53±0.14
TCC-SUP^{CER2}	0.5	1.42±0.18 (1.41)	1.25±0.31 (1.69)	1.90±0.13 (1.06)	0.47±0.20 (1.13)
TCC-SUP^{CER2}	1.25	0.89±0.80 (2.25)	0.39±0.16 (5.41)	2.06±1.40 (0.98)	0.46±0.13 (1.15)
TCC-SUP^{ABCB1}	0	73.44±19.60	125.49±71.00	1.76±0.20	12.73±3.90
TCC-SUP^{ABCB1}	0.5	51.36±10.80 (1.43)	74.63±39.48 (1.68)	1.47±0.06 (1.20)	8.71±3.87 (1.46)
TCC-SUP^{ABCB1}	1.25	58.64±9.40 (1.25)	40.85±8.17 (3.07)	1.79±0.32 (0.98)	15.27±3.42 (0.83)

^{a}Sensitization factor=IC$_{50}$ of the drug in the tested cell line without crizotinib/IC$_{50}$ of the drug in the tested cell line plus crizotinib. Values are means ± standard deviations. IC$_{50}$, half maximal inhibitory concentration; nab, nanoparticle albumin-bound.

Table III. IC$_{50}$ values of paclitaxel, nab-paclitaxel, gemcitabine and vinblastine in T24, T24^{r}GEMCI^{20}, T24^{r}VBL^{20}, T24^{CER2} and T24^{ABCB1} cell viability in the presence of cabozantinib.

Cell line	Cabozantinib, μM	IC$_{50}$ (sensitization factor)^a, ng/ml			
		Paclitaxel	Nab-paclitaxel	Gemcitabine	Vinblastine
T24	0	5.48±1.15	4.65±0.75	3.60±0.80	0.62±0.16
T24	0.5	4.89±0.52 (1.12)	4.78±0.85 (0.97)	3.10±0.04 (1.16)	0.73±0.16 (0.85)
T24	1.25	5.05±0.88 (1.09)	3.87±0.32 (1.20)	6.40±0.24 (0.56)	0.60±0.11 (1.03)
T24^{r}GEMCI^{20}	0	7.05±0.90	8.36±0.50	54.28±1.60	0.56±0.22
T24^{r}GEMCI^{20}	0.5	4.49±0.52 (1.57)	4.76±0.60 (1.76)	56.37±0.90 (0.96)	0.67±0.05 (0.84)
T24^{r}GEMCI^{20}	1.25	4.22±0.35 (1.67)	4.09±0.20 (2.04)	78.48±0.40 (0.69)	0.47±0.11 (1.19)
T24^{r}VBL^{20}	0	576.00±81.50	1,174.00±278.00	1.31±0.50	117.28±13.00
T24^{r}VBL^{20}	0.5	155.20±49.90 (3.70)	257.50±48.20 (4.56)	1.20±0.05 (1.09)	32.86±2.19 (3.57)
T24^{r}VBL^{20}	1.25	61.99±3.90 (9.29)	72.76±7.10 (16.14)	2.30±0.15(0.60)	9.44±1.09 (12.42)
T24^{CER2}	0	4.68±1.60	7.28±1.97	4.90±1.60	0.67±0.29
T24^{CER2}	0.5	4.54±1.50 (1.36)	6.52±1.76 (1.12)	3.40±0.06 (1.44)	0.93±0.35 (0.72)
T24^{CER2}	1.25	3.34±0.50 (1.40)	5.04±1.17 (1.44)	6.40±0.09 (0.77)	0.52±0.12 (1.29)
T24^{ABCB1}	0	105.80±28.70	139.30±27.10	3.30±2.50	11.62±1.37
T24^{ABCB1}	0.5	26.15±4.80 (4.05)	32.30±3.95 (4.31)	3.10±0.07 (1.06)	6.99±1.20 (1.66)
T24^{ABCB1}	1.25	8.97±2.01 (11.97)	12.80±2.58 (10.88)	3.80±0.16 (0.87)	5.54±0.89 (2.10)

^{a}Sensitization factor=IC$_{50}$ of the drug in the tested cell line without crizotinib/IC$_{50}$ of the drug in the tested cell line plus crizotinib. Values are means ± standard deviations. IC$_{50}$, half maximal inhibitory concentration; nab, nanoparticle albumin-bound.

Western blot analysis. Akt is a well-known downstream marker of the tyrosine-protein kinase Met (c-MET) and vascular endothelial growth factor receptor 2 inhibitor cabozantinib, and of the anaplastic lymphoma kinase, hepatocyte growth

Table IV. IC$_{50}$ values of paclitaxel, nab-paclitaxel, gemcitabine and vinblastine in T24, T24[r]GEMCI[20], T24[r]VBL[20], T24[CER2] and T24[ABCB1] cells in the presence of crizotinib.

Cell line	Crizotinib, μM	IC$_{50}$ (sensitization factor)[a], ng/ml			
		Paclitaxel	Nab-paclitaxel	Gemcitabine	Vinblastine
T24	0	4.92±1.07	4.31±0.48	3.82±1.10	0.61±0.28
T24	0.5	3.31±0.47 (1.49)	2.54±0.33 (1.70)	3.03±0.05 (1.26)	0.86±0.30 (0.71)
T24	1.25	2.29±0.10 (2.15)	19.92±4.30 (0.22)	4.46±0.74 (0.86)	4.65±2.32 (0.13)
T24[r]GEMCI[20]	0	7.09±1.24	9.33±2.54	51.54±3.20	0.62±0.13
T24[r]GEMCI[20]	0.5	2.54±0.55 (2.79)	2.73±0.37 (3.42)	52.60±0.03 (0.98)	0.45±0.07 (1.38)
T24[r]GEMCI[20]	1.25	8.60±2.86 (0.82)	7.50±0.01 (1.24)	47.42±6.60 (1.09)	11.20±4.39 (0.06)
T24[r]VBL[20]	0	575.30±46.30	1,006.60±106.00	1.97±0.64	118.82±15.20
T24[r]VBL[20]	0.5	305.80±82.70 (1.88)	565.62±33.20 (1.78)	1.58±0.03 (1.25)	87.76±8.16 (1.35)
T24[r]VBL[20]	1.25	118.50±80.10 (4.85)	168.70±41.80 (5.96)	1.94±0.51 (1.02)	58.13±8.85 (2.04)
T24[CER2]	0	4.51±1.56	7.02±1.97	5.40±2.16	0.66±0.11
T24[CER2]	0.5	2.56±0.66 (1.76)	3.83±1.55 (1.83)	3.44±0.10 (1.57)	0.83±0.20 (0.80)
T24[CER2]	1.25	1.52±0.35 (2.97)	5.32±0.75 (1.32)	10.31±1.30 (0.52)	0.96±0.27 (0.69)
T24[ABCB1]	0	100.80±23.40	129.99±15.60	4.43±1.80	13.24±2.48
T24[ABCB1]	0.5	64.80±17.50 (1.56)	77.07±16.20 (1.69)	2.81±0.04 (1.58)	13.92±3.30 (0.95)
T24[ABCB1]	1.25	36.20±3.97 (2.78)	68.22±21.70 (1.91)	5.99±0.90 (0.74)	10.32±4.25 (1.28)

[a]Sensitization factor=IC$_{50}$ of the drug in the tested cell line without cabozantinib/IC$_{50}$ of the drug in the tested cell line plus cabozantinib. Values are means ± standard deviations. IC$_{50}$, half maximal inhibitory concentration; nab, nanoparticle albumin-bound.

Table V. IC$_{50}$ values of cabozantinib and crizotinib in TCC-SUP, TCC-SUP[r]GEMCI[20], TCC-SUP[r]VBL[20], TCC-SUP[CER2], TCC-SUP[ABCB1], T24, T24[r]GEMCI[20], T24[r]VBL[20], T24[CER2] and T24[ABCB1] cells.

Cell line	IC$_{50}$ (resistance factor)[a], μM	
	Cabozantinib	Crizotinib
TCC-SUP	5.08±0.17	0.90±0.02
TCC-SUP[r]GEMCI[20]	8.45±1.25 (1.66)	2.87±0.33 (3.12)
TCC-SUP[r]VBL[20]	6.67±0.21 (1.31)	1.55±0.15 (1.72)
TCC-SUP[CER2]	5.80±0.10 (1.14)	0.74±0.01 (0.82)
TCC-SUP[ABCB1]	7.70±1.43 (1.52)	1.80±0.09 (2.00)
T24	11.02±0.27	6.16±0.74
T24[r]GEMCI[20]	11.64±1.68 (1.06)	3.33±0.34 (0.54)
T24[r]VBL[20]	7.16±0.09 (0.65)	1.50±0.13 (0.24)
T24[CER2]	10.21±0.04 (0.93)	3.54±0.31 (0.57)
T24[ABCB1]	8.91±0.63 (0.81)	5.32±0.21 (0.86)

[a]Resistance factor=IC$_{50}$ of the drug in the resistant sub-line/IC$_{50}$ of the drug in the parental cell line. Values are means ± standard deviations. IC$_{50}$, half maximal inhibitory concentration.

factor receptor (MET) and proto-oncogene tyrosine-protein kinase ROS inhibitor crizotinib (19,20). Therefore, differences in Akt/pAkt expression subsequent to TKI treatment were evaluated. There were no significant differences in the basal expression of Akt or pAkt (T308) observed in TCC-SUP, TCC-SUP[r]GEMCI[20], TCC-SUP[r]VBL[20], TCC-SUP[CER2] or TCC-SUP[ABCB1] cells. In addition, treatment with 5 μM cabozantinib or 2.5 μM crizotinib did not appear to affect Akt or pAkt (T308) expression (Fig. 2).

Discussion

Acquired taxane resistance is considered to be mediated by multiple mechanisms, including overexpression of drug pumps such as ABCB1, variations in tubulin structure, altered signal transduction and apoptotic pathways (21). One of the most important factors for taxane chemoresistance appears to be ABCB1 overexpression, which has been frequently detected subsequent to the administration of taxanes (22). Conventional paclitaxel is usually solved in a Cremophor® EL/ethanol vehicle, due to the very low aqueous solubility of the compound, which may cause severe anaphylactic reactions (23-25).

Nab-paclitaxel is a colloidal suspension of 130-nm particles homogenized in albumin and bound to paclitaxel (26). The superior antitumor activity of nab-paclitaxel compared with that of paclitaxel was demonstrated to be caused by increased transendothelial gp60-mediated transport and increased intratumoral accumulation as a result of the SPARC-albumin interaction (5,8,9). Zhang et al (23) revealed that paclitaxel-relapsed tumors are responsive to nab-paclitaxel treatment, and Dong et al (10) demonstrated that paclitaxel-loaded lipid-based nanoparticles containing the Brij 78 surfactant may overcome ABCB1-mediated drug resistance. By contrast, there are studies postulating that ABCB1 overexpression is a possible reason for resistance to nanoparticle-bound paclitaxel (11,12). Therefore, the role of
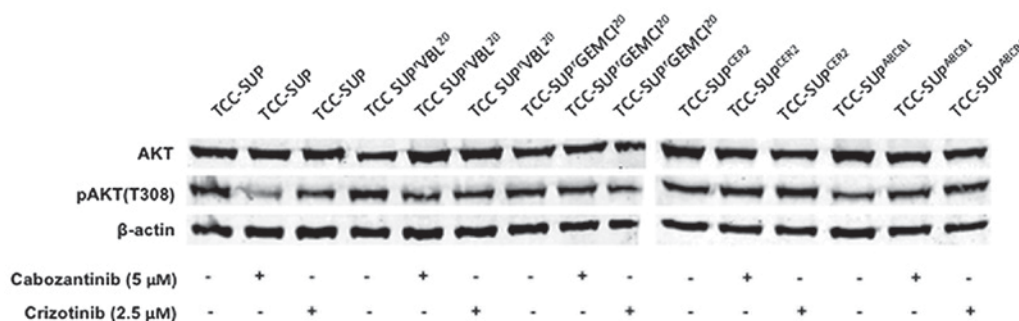
Figure 2. Effect of cabazantinib and crizotinib treatment on Akt/pAkt expression. A representative western blot analysis of at least three independent experiments in TCC-SUP, TCC-SUP[r]VBL[20], TCC-SUP[r]GEMCI[20], TCC-SUP[CER2] and TCC-SUP[ABCB1] cell lines subsequent to treatment with 5 or 2.5 μM crizotinib compared with untreated cells. The cells were lysed, and proteins were detected using specific antibodies against Akt, pAkt (T308) and β-actin. Akt, protein kinase B; p, phosphorylated.

ABCB1 as a resistance mechanism to nab-paclitaxel remains unclear (5).

In the present study, a well-established panel of urothelial cancer cell lines with acquired resistance to gemcitabine was used as a part of the standard chemotherapy of patients with metastasized urothelial bladder cancer (18). In addition, cell lines with acquired resistance to vinblastine, a well-known substrate of ABCB1 that forms part of the alternative bladder cancer chemotherapy regimen methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) (27), were used. Furthermore, cell lines with stable ectopic expression of ABCB1 were used to elucidate the resistance mechanisms against nab-paclitaxel treatment. ABCB1 overexpression and functional drug transport were previously demonstrated in the T24[r]VBL[20], TCC-SUP[r]GEMCI[20] and TCC-SUP[r]VBL[20] cell lines with acquired chemoresistance, and in the T24[ABCB1] and TCC-SUP[ABCB1] cell lines with stable ectopic expression of ABCB1 (18).

Nano-sized drug carrier systems have been demonstrated to circumvent ABC transporter-mediated drug efflux (28,29), and the increased efficacy of nanoparticle-bound paclitaxel was explained by a decreased efflux rate through the inhibition of ABCB1 (10). By contrast, in the cell line model of the present study, a similar cross-resistance profile in ABCB1-overexpressing cell lines to paclitaxel and nab-paclitaxel was demonstrated (Tables I-IV). Zhao *et al* (12) identified that an NSCLC cell line with acquired resistance to nab-paclitaxel was also resistant to ABCB1 substrates. The authors suggested that the overexpression of ABCB1 serves an important role in resistance to nab-paclitaxel in NSCLC, similarly to the common resistance mechanism for paclitaxel (12). They hypothesized that paclitaxel, being the active component in nab-paclitaxel, is responsible for the development of drug resistance (12). Additionally, they concluded that paclitaxel is likely to be dissociated from albumin inside the cell and pumped out by ABCB1 as a free molecule, as the albumin is too large to be transported by ABC drug pumps (12). Therefore, the reason for ABCB1-mediated resistance to nab-paclitaxel may be the aforementioned dissociation of paclitaxel from albumin subsequent to endocytosis, and nab-paclitaxel may cause the same cytotoxicity to tumor cells as unbound paclitaxel (12,30). This would suggest that tumor cells *in vitro* are sensitive or resistant to nab-paclitaxel and paclitaxel at the same level.

Numerous studies have been conducted to develop inhibitors for ABC transporters to circumvent ABCB1-associated resistance. At present, several clinical trials are ongoing to evaluate the clinical role of ABCB1 inhibitors to prevent drug resistance (31-34). However, none of the tested compounds had been approved for clinical use until 2010 (34). In the present study, the TKI crizotinib reversed ABCB1-mediated drug resistance of paclitaxel and nab-paclitaxel without changing Akt/pAkt expression. The phosphorylation of Akt and extracellular signal-related kinase (ERK) 1/2 are known downstream markers of crizotinib (19). These molecules may be used to test the targeted activity of crizotinib (19). The results of the present study are in accordance with the study of Zhou *et al* (33). In that study, the authors demonstrated that crizotinib reversed multidrug resistance in different cancer cell lines by inhibiting the function of ABCB1 without incurring significant changes to the expression of Akt, ERK or c-MET (33). Additionally in the present study, the second tested TKI, cabozantinib, re-sensitized chemoresistant cancer cell lines to ABCB1 substrates without affecting the expression of downstream molecules. These results are consistent with the study of Xiang *et al* (20), who revealed that cabozantinib treatment in hepatocellular cancer cells reversed ABCB1-mediated chemoresistance with no significant change to the levels of the downstream molecules Akt, ERK1/2 or MET. As promising overall response rates have been demonstrated for nab-paclitaxel treatment in patients with metastatic platinum-refractory urothelial cancer (7), a combination of nab-paclitaxel and cabozantinib or crizotinib should be clinically evaluated to avoid the development of resistance against nab-paclitaxel and to extend the antitumoral effect of the drug.

In the present study, the cytotoxic effects of vinblastine were demonstrated to be increased when vinblastine was administered in combination with cabozantinib. The *Vinca* alkaloid vinblastine binds to tubulin and inhibits the assembly of microtubules, similarly to the mechanisms of paclitaxel and nab-paclitaxel (35). Furthermore, ABCB1 serves a similar role in the development of vinblastine resistance (36). Vinblastine, as a part of the alternative bladder cancer chemotherapy regimen MVAC, is administered as first-line therapy, or subsequent to the failure of gemcitabine/cisplatin treatment (27,37). Therefore, a combination of vinblastine with cabozantinib may be a reasonable option for the treatment of patients with

metastatic urothelial bladder cancer. However, additional *in vivo* studies are required to evaluate the efficacy of a combination therapy of TKI with vinblastine.

In conclusion, resistance to nab-paclitaxel in ABC transporter-expressing urothelial cancer cells appears to be mediated by ABCB1. The data of the present study suggest that the previously identified beneficial clinical effects of nab-paclitaxel compared with those of paclitaxel are possibly due to improved pharmacokinetics and decreased systemic toxicity. In addition, ABCB1 inhibition by the small molecule inhibitors cabozantinib or crizotinib may improve clinical response to chemotherapy.

Acknowledgements

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