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RESEARCH ARTICLE

Establishment and characterization of a bladder cancer cell line with enhanced doxorubicin resistance by mevalonate pathway activation

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Abstract Resistance to chemotherapy is a major problem in the treatment of urothelial bladder cancer. Several mechanisms have been identified in resistance to doxorubicin by analysis of resistant urothelial carcinoma (UC) cell lines, prominently activation of drug efflux pumps and diminished apoptosis. We have derived a new doxorubicin-resistant cell line from BFTC-905 UC cells, designated BFTC-905-DOXO-II. A doxorubicin-responsive green fluorescent protein (GFP) reporter assay indicated that resistance in BFTC-905-DOXO-II was not due to increased drug efflux pump activity, whereas caspase-3/7 activation was indeed diminished. Gene expression microarray analysis revealed changes in proapoptotic and antiapoptotic genes, but additionally induction of the mevalonate (cholesterol) biosynthetic pathway. Treatment with simvastatin restored sensitivity of BFTC-905-DOXO-II to doxorubicin to that of the parental cell line. Induction of the mevalonate pathway has been reported as a mechanism of chemoresistance in other cancers; this is the first observation in bladder cancer. Combinations of statins with doxorubicin-containing chemotherapy regimens may provide a therapeutic advantage in such cases.

Keywords Bladder cancer · Chemotherapy · Doxorubicin resistance · Mevalonate pathway

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Introduction

Bladder cancer represents the fifth most common malignancy worldwide with an annual incidence of at least 386,000 cases and an annual mortality approaching 150,000 cases [1]. The fatal outcome results mainly from advanced muscle-invasive tumours progressing to metastatic disease; such tumours can develop either from flat dysplastic carcinoma in situ lesions or by progression of otherwise prognostically favourable superficial papillary tumours [2, 3]. Two combination chemotherapeutic regimens dominate the systemic treatment of advanced urothelial carcinoma, with methotrexate, vinblastine, adriamycin and cisplatin (M-VAC) being more and more replaced by the comparably efficient but less toxic gemcitabine and cisplatin (GC) combination [4, 5]. With both combination chemotherapy regimens, nevertheless, response rates barely exceed 50 %, implying mechanisms of primary chemoresistance operating in at least half of the tumours [6]. Moreover, clinical responses are rarely durable or even curative, which could be explained either by selection of pre-existing chemoresistant cells or by development of secondary drug resistance during the treatment. Several other chemotherapeutic combinations have been formulated to combat the failure of M-VAC or GC, like a combination of paclitaxel and doxorubicin [7] or paclitaxel and gemcitabine [8]; their clinical efficiency is, nevertheless, rather limited. The microtubule inhibitor vinflunin is the only agent to date with a proven, if moderate, clinical benefit in second-line chemotherapy [9].

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With regard to doxorubicin, which is applied either as part of the M-VAC (=adriamycin) scheme or in combination with paclitaxel, several experimental chemoresistance models have been established. Their molecular analysis yielded an enhanced drug efflux as a major chemoresistance mechanism, either alone [10–12] or in association with a partial or complete loss of the therapeutic target, DNA topoisomerase II [13, 14], or with a concomitant activation of antiapoptotic signalling pathways [15]. The analysis of clinical tumours, while corroborating the clinical significance of increased expression of drug efflux pumps like ABCB1 (MDR-1) or ABCC1 (MRP), suggested nevertheless that this is unlikely to represent the only resistance mechanism [16–19]. Here, we describe the establishment of a new cell culture model of doxorubicin resistance in bladder cancer and its molecular analysis. We observed mevalonate pathway activation that might engender substantial doxorubicin chemoresistance without the involvement of drug efflux. Importantly, pharmacologic inhibition of the pathway completely abolished this novel type of therapy resistance. In view of a recent intensive debate about the pharmacological application of mevalonate synthesis inhibitors—statins—in cancer treatment [20–22], this new experimental model could be instrumental in formulating a new therapeutic strategy in some cases of refractory chemoresistant bladder cancer.

Materials and methods

Cell culture

The human well-differentiated papillary bladder carcinoma cell line BFTC-905 [23] was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The clonally related cell lines RT112 and RT112/D21 [12] were a generous gift from Dr. M.S. Michel (Department of Urology, Mannheim Medical Center, Mannheim, Germany). The cells were cultured in high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM) (Gibco–Invitrogen, Mannheim, Germany) supplemented with 10 % foetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), penicillin (100 U/ml) und streptomycin (100 μg/ml, Biochrom AG) at 37 °C with 5 % CO_2. The clonally related cell lines UM-UC-6 and UM-UC-6dox [10, 11] were a generous gift from Dr. H.B. Grossman (MD Anderson Cancer Center, Houston, USA) and were cultured identically except that minimal essential medium (MEM) (Gibco–Invitrogen) was used instead of DMEM. Routine subculturing was performed after short-term incubation with 0.05 % trypsin in 0.02 % EDTA in phosphate-buffered saline (PBS) (Biochrom AG). The daughter cell line BFTC-905–DOXO-II was derived from a single colony obtained after intermittent doxorubicin (Calbiochem, Darmstadt, Germany) treatment, starting from 100 nM and terminating with 300 nM over a period of 2 months.

Viability and apoptosis assays

To determine cell viability after doxorubicin treatment, the CellTiter-Glo luminescent cell viability assay (Promega, Mannheim, Germany) was used. Apoptosis was followed using the Caspase-Glo 3/7 assay by the same company according to the manufacturer's instructions. The effects of doxorubicin treatment on BFTC-905 and its doxorubicin-resistant daughter cell line BFTC-905–DOXO-II were determined by two approaches. To measure acute toxicity, 3×10^3 cells were seeded in triplicate wells of a 96-well plate and allowed to adhere overnight. Growth medium containing a range of doxorubicin concentrations was applied the next day, and cell viability was assessed after a further 4 days of incubation. Drug effects were expressed as the percentage of viability loss relative to untreated control cells. The half-maximal inhibitory concentration (IC_{50}) was then determined graphically from the dose–response curve. To measure chronic toxicity, a clonogenic assay was performed after incubating the parental or the daughter cell line with doxorubicin at their respective IC_{50} dose for 8 days and further incubation for 3 weeks in doxorubicin-free culture medium. To investigate combined effects of simvastatin (Sigma Aldrich, Munich, Germany) and doxorubicin, 25,000 cells/well from BFTC905 or BFTC905 Doxo 550 were plated in 24-well plates. The next day, the medium was changed and the cells were treated with 1–30 mM simvastatin, 25–400 nM doxorubicin or a combination of both compounds. After 24 h, 50-μl MTT reagent was added and the cells were incubated for 30 min at 37 °C. The cells were then lysed with 500 μl DMSO and transferred into 96 wells. Each experiment was performed in quadruplicates with an ELISA reader at 570 nm/620 nm.

Analysis of the mechanism of chemoresistance

BFTC-905, BFTC-905-DOXO-II, RT112, RT112/D21, UM-UC-6 or UM-UC-6dox was seeded on 100-mm diameter cell culture dishes (3×10^6 cells in 10 ml) and transfected with 6 μg of the pE9ns$_2$GFP plasmid [24] on the next day. Transfections were carried out with the FuGENE 6 Transfection Reagent (Roche Applied Science, Basel, Switzerland) following the manufacturer's instructions. Transfections were left for 36 h and then split into two 60-mm cell culture dishes. One dish was left with standard growth medium, while the other one was incubated with medium containing 1 μM doxorubicin for 48 h. Green fluorescent protein (GFP) induction was then determined as the fold increase of GFP-positive cells after doxorubicin treatment, as described previously [24, 25].

Microarray gene expression profiling

Gene expression microarray analysis and evaluation were performed with independent triplicate RNA preparations from each cell line using HG U133A GeneChips (Affymetrix, Berlin, Germany) as described previously [26]. For functional annotation, the Database for Annotation, Visualization and Integrated Discovery (DAVID), version v6.7, was used [27].

Statistical analysis

The statistical significance of differences in clonogenicity and apoptotic response, respectively, was evaluated by the Wilcoxon signed-rank test. A p value of 0.05 was taken to indicate statistical significance.

Results

Derivation of the BFTC-905-DOXO-II daughter cell line

Applying a doxorubicin treatment regimen starting with 100 nM and terminating with 300 nM over a period of 2 months, five discrete colonies survived out of a nearly confluent starting culture. One of the clones was further expanded and analyzed in more detail (Fig. 1).

Acute and chronic toxicity of doxorubicin treatment

Initial analysis of acute doxorubicin toxicity revealed that doxorubicin resistance of the BFTC-905-DOXO-II daughter cell line is rather modest, with an IC_{50} value of 450 nM, as compared to 150 nM in the parental cell line. In both cell lines, doxorubicin treatment at IC_{50} for 4 days engendered a long-term culture depression, with continuous diminution of viable cell numbers over a period of the next 3 weeks. During this crisis, both apoptotic and senescent cells could readily be observed. Then, growth restoration began with morphologically distinct islands of small and very compactly arranged cells. Indeed, in clonogenicity assays of cell cultures in this phase of growth restoration, the overall clonogenicity decreased rather sharply with the parental cell line, whereas it increased by 35 % with the daughter cell line (Fig. 2), providing an additional expression of its relative doxorubicin resistance.

Atypical mechanism of resistance of BFTC-905-DOXO-II cells

In two previously published doxorubicin-resistant cell lines, RT112/RT112-D21 [12] and UM-UC6/UM-UC-6dox [10, 11], the mechanism underlying resistance was identified as classical multidrug resistance by overexpressed ABC efflux transporters. To investigate whether the same mechanism was responsible in the BFTC-905-DOXO-II daughter cell line, we employed the previously described doxorubicin-responsive reporter plasmid pE9ns$_2$GFP, which harbours the GFP reporter gene under the control of a synthetic promoter that is strongly activated by intracellular doxorubicin [24, 25]. Therefore, cells with overactive ABC efflux transporters display lower GFP fluorescence under doxorubicin treatments. Indeed, activation of the GFP reporter gene by doxorubicin treatment was substantially lower in the respective resistant daughter cell line than in the parental cell line for both control pairs RT112/RT112-D21 and UM-UC6/UM-UC-6dox (Fig. 3). Remarkably, in the BFTC-905 and BFTC-905-DOXO-II cell lines, GFP activation was actually stronger in the doxorubicin-resistant daughter cell line, excluding widespread overexpression of drug efflux pumps as a mechanism of resistance (Fig. 3). Deficient apoptosis was a logical next candidate, and indeed, we found that activation of caspase-3/7 in the wake of doxorubicin treatment was greatly compromised in BFTC-905-DOXO-II cells (Fig. 4).

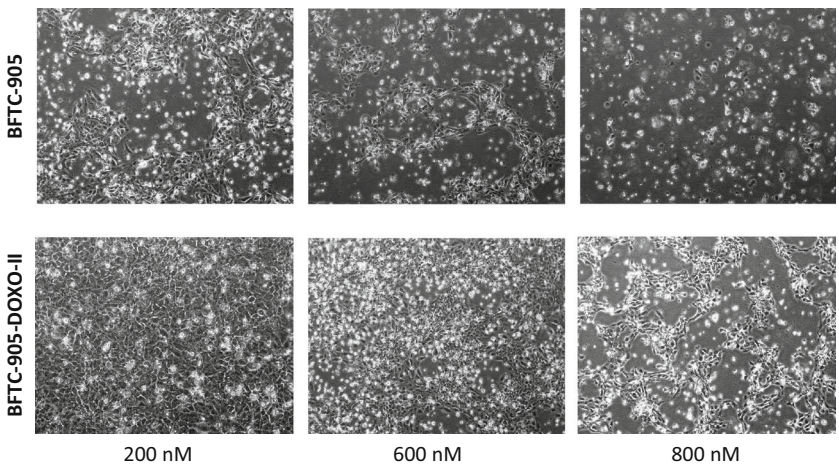

Fig. 1 Microscopic documentation of the response of BFTC-905-DOXO-II cells to doxorubicin. Doxorubicin was applied for 96 h in the concentrations indicated, and photographs were taken by Olympus IX81–CellR imaging system. Magnification ×40

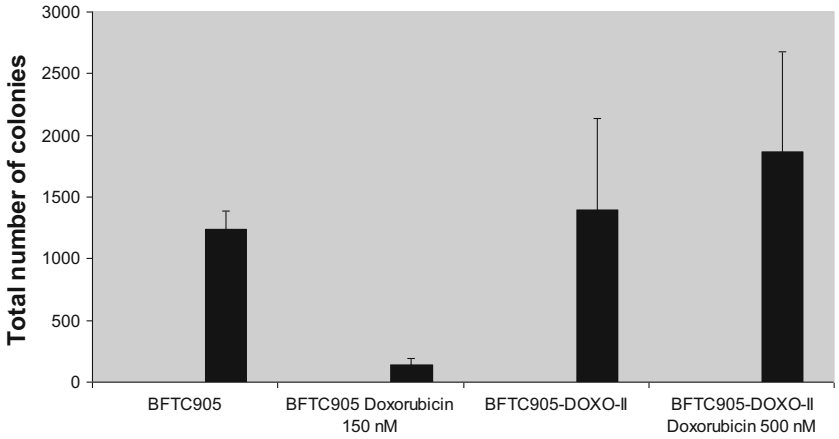

Fig. 2 Activation of clonogenic cells by doxorubicin treatment in BFTC-905-DOXO-II cells, but not in the parental cell line. Number of clones from BFTC-905 or BFTC-905-DOXO-II cells following treatment with doxorubicin. There was no statistical difference in the overall clonogenicity in untreated cultures. In the parental cell line, the clonogenicity decreased by 88 % in the doxorubicin-treated cultures (p=0.0022). Simultaneously, the overall clonogenicity increased by 35 % in the doxorubicin-selected daughter cell line (p=0.31). The difference in clonogenicity between the doxorubicin-treated parental and daughter cell lines was statistically highly significant as well (p=0.0022)

Expression profiling reveals activation of the mevalonate pathway as a probable mechanism of doxorubicin resistance in BFTC-905-DOXO-II cells

To clarify the mechanisms of resistance in more detail, we compared global gene expression profiles of BFTC905 parental cells with the doxorubicin-resistant daughter cell lines BFTC905-DOXO-II by microarray analysis using Affymetrix arrays.

Overall, 227 annotated genes were found significantly upregulated and 213 genes were downregulated in the resistant cells (Suppl. Table 1). According to GO analysis (Table 1), these genes were involved in ectoderm, epithelium or epidermis development; regulation of apoptosis, programmed cell death or cell death; sterol or steroid biosynthetic processes; and response to vitamins. An analysis by tissue distribution showed that the changes in genes involved in epithelium development reflected the downregulation of genes expressed in keratinocytes, whereas genes expressed in the urothelium were rather upregulated. Both proapoptotic and antiapoptotic genes were differentially expressed. Strikingly, all genes categorized as involved in sterol biosynthesis were upregulated, and all their products catalyze intermediate steps of cholesterol biosynthesis. The largest group of the genes categorized as "response to vitamins" is involved in responses to vitamin A (*MICB*, *BMP2*, *TFRC*, *MAP1B*, *KLF4*).

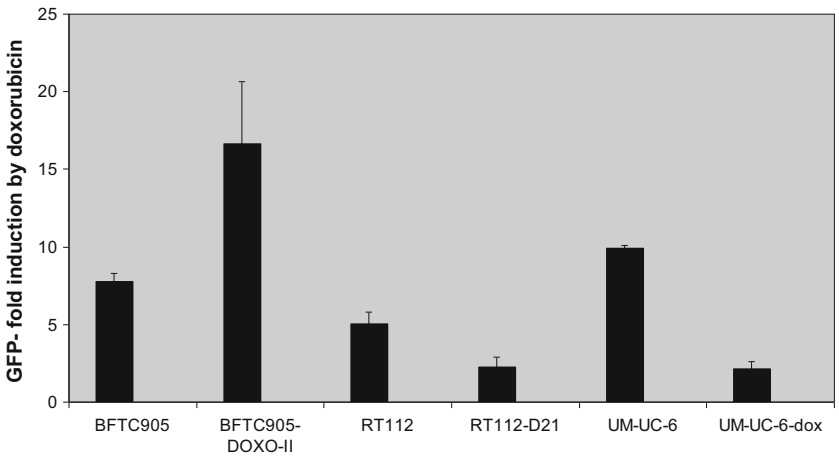

Fig. 3 Doxorubicin resistance in BFTC-905-DOXO-II cells does not involve doxorubicin efflux. The ability to actively efflux doxorubicin was assayed via a doxorubicin-activated GFP reporter, in parallel with bladder cancer cell lines with multidrug resistant (MDR) phenotype. The fold increase in GFP-positive cells after incubation with 1 μM doxorubicin is shown. Whereas GFP activation by doxorubicin was distinctly lower in MDR daughter cell lines RT112/D21 and UM-UC-6dox than in their respective parental cell lines RT112 and UM-UC-6, there was even an increase in GFP activation in BFTC-905-DOXO-II cells in comparison to that in the BFTC-905 parental cell line

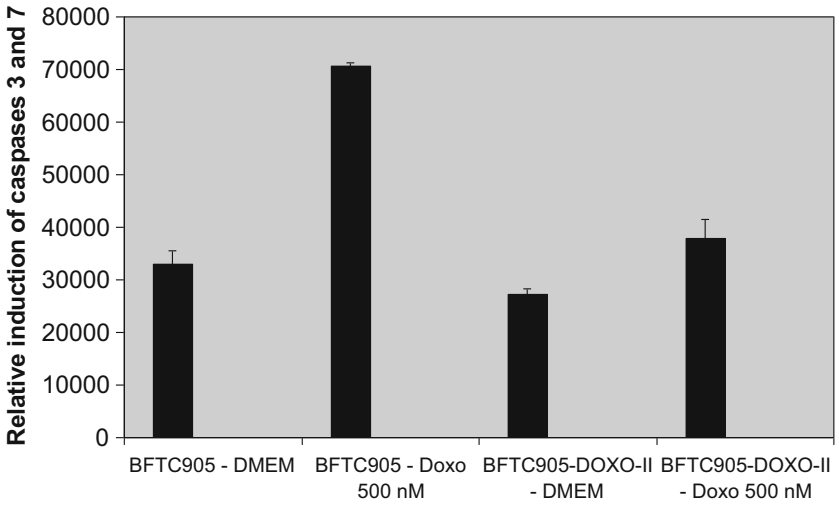

Fig. 4 Reduced apoptotic response in BFTC-905-DOXO-II cells. Activity of caspase-3/7 in doxorubicin-treated BFTC-905 cells (215 % compared with untreated cultures, $p=0.0079$) and BFTC-905-DOXO-II cells where the 39 % increase in activity was not significant ($p=0.08$).

Statin treatment reverses doxorubicin resistance in BFTC-905-DOXO-II cells

As the microarray data indicated an upregulation of the cholesterol biosynthesis pathway in the doxorubicin-resistant cell line BFTC-905-DOXO-II, we investigated its possible involvement in resistance to doxorubicin by blocking the key enzyme HMG-CoA reductase by the inhibitor simvastatin. In a short-term assay, the IC_{50} was approximately 50 nM for doxorubicin in standard BFTC-905 cells, whereas the resistant daughter cell line had an IC_{50} (DOXO) of 400 nM (Fig. 5a). Interestingly, the two cell lines differed in their IC_{50} values for simvastatin, with BFTC-905-DOXO-II being less sensitive (Fig. 5b). Combined treatment with simvastatin and doxorubicin enhanced cell death in both cell lines (Fig. 5c, d), and in the presence of simvastatin, both cell lines showed almost identical sensitivities to doxorubicin.

Table 1 Categories of genes differentially expressed in doxorubicin-resistant BFTC905 cells

Term	*p* value	Genes	FDR
GO:0007398—ectoderm development	4.30E+09	*KRT6A*, *KRT6B*, *S100A7*, *FST*, *GJB3*, *JAG1*, *SOX9*, *LAMB3*, *LAMA3*, *KRT5*, *KRT17*, *SPRR1A*, *SPRR1B*, *KRT14*, *SPRR3*, *DSP*, *EMP1*, *KLF4*	0.0075
GO:0042981—regulation of apoptosis	1.14E+11	*ITGB3BP*, *BID*, *HTATIP2*, *RBM5*, *SOX4*, *BNIP3*, *SOX9*, *TIMP3*, *MAGED1*, *PEA15*, *ERCC5*, *MYD88*, *CASP4*, *BAG1*, *ALDH1A3*, *RHOA*, *IL1B*, *HSPE1*, *HSPA5*, *CD24*, *NQO1*, *MYC*, *PHLDA1*, *IRAK1*, *HERPUD1*, *VAV3*, *ROCK1*, *EEF1A2*, *LGALS1*, *KLF10*, *MBD4*, *HBXIP*, *GAL*, *CDK5*, *DAPK3*, *PPIF*, *PLA2G4A*, *ADRB2*, *BNIP3L*, *AVEN*	0.0198
GO:0016126—sterol biosynthetic process	1.36E+11	*HMGCR*, *CYP51A1*, *SQLE*, *DHCR7*, *HMGCS1*, *IDI1*, *FDFT1*, *SC4MOL*	0.0237
GO:0043067—regulation of programmed cell death	1.41E+11	*ITGB3BP*, *BID*, *HTATIP2*, *RBM5*, *SOX4*, *BNIP3*, *SOX9*, *TIMP3*, *MAGED1*, *PEA15*, *ERCC5*, *MYD88*, *CASP4*, *BAG1*, *ALDH1A3*, *RHOA*, *IL1B*, *HSPE1*, *HSPA5*, *CD24*, *NQO1*, *MYC*, *PHLDA1*, *IRAK1*, *HERPUD1*, *VAV3*, *ROCK1*, *EEF1A2*, *LGALS1*, *KLF10*, *MBD4*, *HBXIP*, *GAL*, *CDK5*, *DAPK3*, *PPIF*, *PLA2G4A*, *ADRB2*, *BNIP3L*, *AVEN*	0.0246
GO:0010941—regulation of cell death	1.54E+10	*ITGB3BP*, *BID*, *HTATIP2*, *RBM5*, *SOX4*, *BNIP3*, *SOX9*, *TIMP3*, *MAGED1*, *PEA15*, *ERCC5*, *MYD88*, *CASP4*, *BAG1*, *ALDH1A3*, *RHOA*, *IL1B*, *HSPE1*, *HSPA5*, *CD24*, *NQO1*, *MYC*, *PHLDA1*, *IRAK1*, *HERPUD1*, *VAV3*, *ROCK1*, *EEF1A2*, *LGALS1*, *KLF10*, *MBD4*, *HBXIP*, *GAL*, *CDK5*, *DAPK3*, *PPIF*, *PLA2G4A*, *ADRB2*, *BNIP3L*, *AVEN*	0.0269
GO:0033273—response to vitamin	2.12E+10	*MICB*, *PLA2G4A*, *CCND1*, *BMP2*, *TFRC*, *MAP1B*, *IL1B*, *TIMP3*, *KLF4*, *SPP1*	0.0369
GO:0060429—epithelium development	2.44E+11	*BMP2*, *S100A7*, *DHRS9*, *JAG1*, *GREM1*, *GSTM3*, *LAMA3*, *KRT5*, *SPRR1A*, *ALDH1A3*, *SPRR1B*, *UPK1B*, *KRT14*, *SPRR3*, *DSP*, *PBX1*, *CA2*, *EMP1*	0.0425
GO:0008544—epidermis development	2.75E+11	*S100A7*, *FST*, *GJB3*, *JAG1*, *SOX9*, *LAMB3*, *LAMA3*, *KRT5*, *KRT17*, *SPRR1A*, *SPRR1B*, *KRT14*, *SPRR3*, *DSP*, *EMP1*, *KLF4*	0.0479
GO:0006694—steroid biosynthetic process	2.78E+11	*ACBD3*, *HMGCR*, *CYP51A1*, *SQLE*, *DHCR7*, *HMGCS1*, *PBX1*, *IDI1*, *SCP2*, *FDFT1*, *SC4MOL*	0.0484

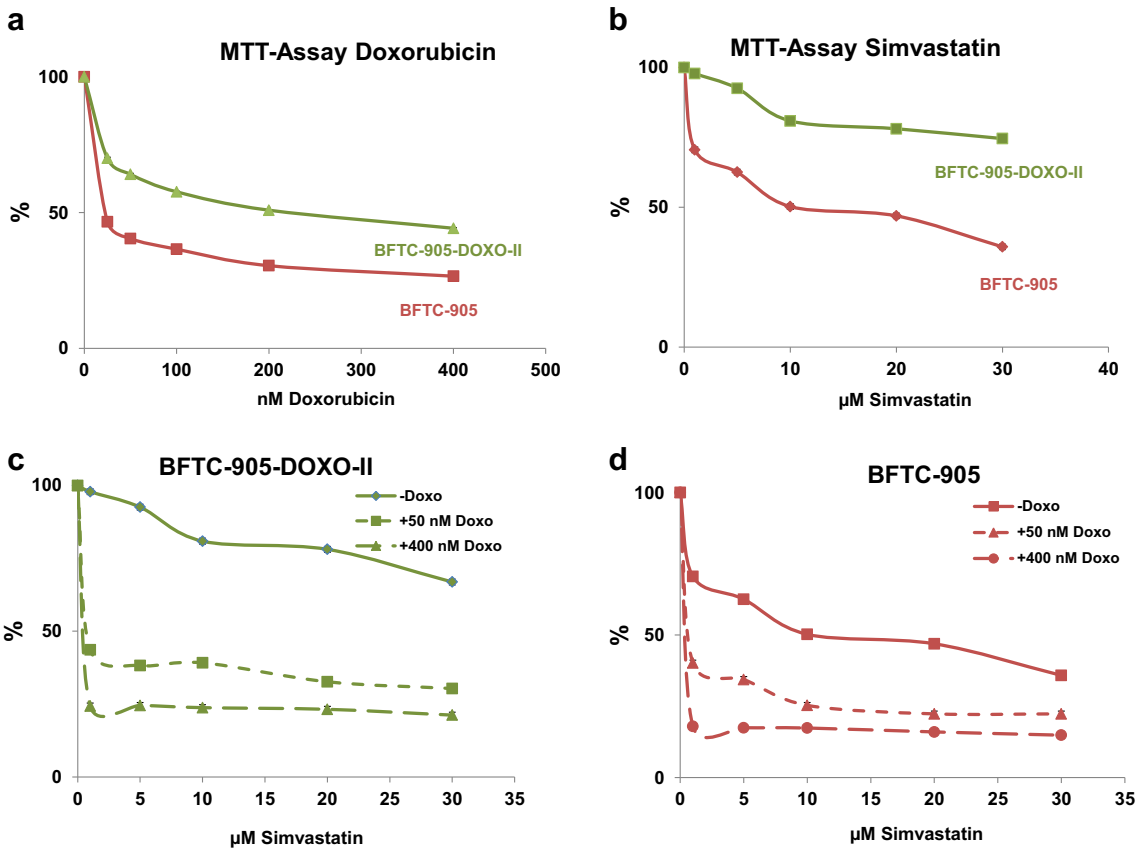

Fig. 5 Simvastatin reverts doxorubicin resistance in BFTC-905-DOXO-II cells. The simultaneous treatment with doxorubicin and simvastatin abolishes differences in chemosensitivity between the parental BFTC-905 and daughter BFTC-905-DOXO-II cells

Discussion

In the present study, we report the establishment of a new cell culture model of doxorubicin resistance in urothelial bladder carcinoma. As measured by a specific reporter gene system, the resistant daughter cell line showed a higher response to intracellular doxorubicin, excluding drug efflux as the major resistance mechanism. This is unexpected, as this was a dominant mechanism in previously established cell lines [10–15]. In accordance with other reports, however, the resistance was associated with a diminished apoptotic response to doxorubicin. To unravel the underlying molecular mechanism, we performed global gene expression profiling and discovered a permanent overexpression of multiple enzymes of the mevalonate pathway in the resistant daughter. Accordingly, simvastatin treatment restored doxorubicin sensitivity to the level of the parental cell line.

Mevalonate pathway overactivation is a frequent event in carcinogenesis [20–22], and the rate-limiting enzyme of the pathway, 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGCR), has been even classified as a metabolic oncogene [28]. The consequences of mevalonate pathway activation can be relevant not only for cancer development per se, but also for therapy response. Several mechanisms may contribute, reflecting different outputs of the pathway [20–22]. One of these is isoprenylation of Ras, Rac and Rho oncoproteins, which is crucial for their membrane localization and subsequent signal transduction. Activation of the according branch of the mevalonate pathway therefore contributes not only to cancer development, but also resistance to cytotoxic and targeted therapies [29]. Another important output of the mevalonate pathway is cholesterol, which is both a stable constituent of biological membranes and a precursor for steroid hormones. Cholesterol content can exert profound effects on physical and chemical properties of biomembranes. Mevalonate pathway overactivation can result in an increase of cholesterol content in mitochondrial membranes of hepatocellular carcinoma (HCC) cells, thereby reducing their apoptotic response to doxorubicin [30]. Indeed, the mevalonate pathway activation constitutes a sort of cell defence (immediate adaptive response) of acute leukaemia (AML) cells exposed to chemotherapeutic drugs [31]. We are not aware of any report describing mevalonate pathway overactivation in bladder cancer. In view of the limited therapeutic efficacy of current chemotherapeutic regimens [6] and the inability to explain the intrinsic and acquired urothelial carcinoma doxorubicin resistance by increased drug efflux alone [16–19], our finding calls for a more intense investigation of the function of this pathway in bladder cancer.

In particular, if mevalonate pathway activation should be a critical factor in bladder cancer development and therapy resistance, it would offer a novel pharmacological target. Statins are a group of natural and synthetic inhibitors of the HMGCR. They are widely prescribed, especially to lower cholesterol levels in serum [20–22]. Statin use for cancer treatment is intensively debated. In various circumstances, statins were found to cooperate with anticancer drugs, increasing the effects of cetuximab in colorectal cancer cells [32], of gefitinib in NSCLC cells [33] and of doxorubicin in HCC cell lines [30]. Pravastatin was even successfully incorporated into an experimental induction chemotherapy regimen of AML [34]. Interestingly, the cooperation of statins with doxorubicin may be partly based on an off-target effect on multidrug efflux pumps, as reported e.g. for neuroblastoma [35] or rhabdomyosarcoma [36] cells. With regard to urothelial bladder carcinoma cells, this is, to our knowledge, the first report showing a clear therapeutic cooperativity of a statin with a conventional anticancer drug. Which of the mechanisms outlined above is responsible for the cooperation of simvastatin and doxorubicin in our model system awaits further analysis. Our data, however, argue against an effect via multidrug efflux pump targeting, as the doxorubicin resistance of BFTC-905-DOXO-II cells is clearly not based on activated drug efflux.

Of note, despite the clear biological rationale, chronic statin use is not always cancer-protective. Especially for bladder cancer, the results are conflicting [37–39]. Another complication is a substantial interindividual heterogeneity in therapeutic response to statins [20]. Cell line collections have been used to define the biological basis of statin sensitivity in multiple myeloma [40] and breast carcinoma [41], and we believe that our pair of cell lines could be similarly helpful within the context of urothelial carcinoma. Statin use for cancer treatment thus follows the personalized treatment paradigm. With that reservation, our results may open the way to rational statin use in urothelial cancer treatment.

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