

## siRNA-mediated Inhibition of Antiapoptotic Genes Enhances Chemotherapy Efficacy in Bladder Cancer Cells

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**Abstract.** *Background: The up-regulation of antiapoptotic B-cell CLL/lymphoma 2 (BCL2), BCL2-like 1 (BCLXL), X-linked inhibitor of apoptosis (XIAP) and survivin is one mechanism by which cancer cells develop resistance towards chemotherapeutics. Therefore, the knockdown of these four genes could sensitise bladder cancer (BCa) cells towards chemotherapy. Materials and Methods: BCL2, BCLXL, XIAP and survivin were inhibited using siRNAs – either one target-alone or all four targets simultaneously – in EJ28 and J82 BCa cells. After 24 h, cells were treated with mitomycin C or cisplatin. Treatment effects were analysed regarding cell viability, cell count and apoptosis induction. Results: Knockdown of BCLXL and survivin, as well as the simultaneous inhibition of all four antiapoptotic genes, sensitised EJ28 and J82 cells towards mitomycin C and cisplatin. Conclusion: Since the contribution of one antiapoptotic gene to chemotherapy response can vary between BCa cell lines, the simultaneous knockdown of multiple inhibitors of apoptosis might represent a more promising option for enhancing chemotherapy efficacy in BCa treatment.*

In Europe, bladder cancer (BCa) accounted for approximately 139,500 new cases and 51,300 cancer-related deaths in 2008 (1). Worldwide, BCa represents the ninth most common malignancy (2). Chemotherapy is an important part of BCa treatment. Patients with non-muscle-invasive BCa will be treated by transurethral resection and further intravesical instillation of a chemotherapeutic [e.g. mitomycin C (MMC) or epirubicin], or by an immunotherapeutic agent (3). However, despite these therapies, 37-53% of BCa cases will recur and about 8% will progress to muscle-invasive disease (4). If the

tumour has already invaded the bladder muscle, radical cystectomy with neoadjuvant cisplatin-containing combination chemotherapy is recommended (5). In metastatic disease, combination chemotherapy, e.g. with gemcitabine and cisplatin, is the standard of treatment. However, the median survival of these patients is less than 13 months (6). Therefore, the improvement of chemotherapy efficacy is a priority objective in BCa research.

Alterations in apoptotic signalling represent one mechanism by which cancer cells develop chemotherapy resistance (7). In particular, increased expression of antiapoptotic genes such as B-cell CLL/lymphoma 2 (*BCL2*), BCL2-like 1 (*BCLXL*), X-linked inhibitor of apoptosis (*XIAP*) and *survivin* can desensitise tumour cells to chemotherapy (7). *BCL2* and *BCLXL* are two members of the *BCL2* family and impede apoptosis by preventing cytochrome c release from the mitochondria and in consequence, caspase activation (8). *XIAP* and *survivin* represent the most important members of the inhibitor of apoptosis protein (IAP) family. *XIAP* directly binds and inhibits caspases, while *survivin* can enhance the stability and activity of *XIAP* (9, 10).

Patients with invasive BCa and with negative tumour *BCL2* protein staining had a better response towards neoadjuvant cisplatin therapy (11). Moreover, a high amount of *survivin* protein in tissue or urine samples from patients with BCa is associated with a poor response to radio- and chemotherapy (12, 13). *In vitro* studies showed an inverse correlation between chemotherapy efficacy and expression of *BCL2*, *BCLXL*, *XIAP* and *survivin* in BCa cell lines. For example, in cisplatin-resistant T24 BCa cells, increased *BCL2* protein content was found and knockdown of *BCL2* by siRNAs in these cells reversed cisplatin-resistance (14). Stable overexpression of *BCLXL* in T24 cells desensitised these cells to different cytotoxic agents, and antisense oligonucleotide-mediated *BCLXL* inhibition in T24 and 5637 BCa cells improved chemotherapy efficacy (15). Furthermore, knockdown of *XIAP* in T24 cells, as well as inhibition of *survivin* in EJ28 and 5637 cells, increased the sensitivity of these BCa cell lines towards cytotoxic agents (16, 17). Since tumour cells are able to bypass the knockdown of one antiapoptotic gene by the up-regulation

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of another, *e.g.* *BCL2* down-regulation can be compensated by increased expression of *BCLXL* (18), the simultaneous knockdown of *BCL2*, *BCLXL*, *XIAP* and *survivin* might be a more promising approach for enhancing chemotherapy efficacy than the single inhibition of one target. Therefore, we examined the effects of single and simultaneous siRNA-mediated inhibition of these four important antiapoptotic factors in combination with MMC and cisplatin therapy in BCa cell lines.

## Materials and Methods

**Cell culture.** The human BCa cell lines EJ28 (University of Frankfurt, Frankfurt, Germany) and J82 (ATCC, Manassas, VA, USA) were cultured under standard conditions (37°C, humidified atmosphere containing 5% CO<sub>2</sub>) without antibiotics in Dulbecco's modified Eagle's medium (4.5 g/l glucose) containing 10% fetal calf serum, 1% MEM non-essential amino acids and 1% HEPES (all from Invitrogen, Karlsruhe, Germany).

**Combined treatment with siRNAs and chemotherapy.** The designations and sequences of the target-directed siRNAs (Eurogentec, Seraing, Belgium) are shown in Table I. As a control and for normalisation, the negative control siRNA (ns-si, reference: SR-CL000-005; Eurogentec) was used. Twenty-four or 72 h after seeding, cells were transfected for 4 h in serum-free OptiMEM (Invitrogen) with the siRNAs using N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) liposomal transfection reagent (ratio 1:30, w/w) according to the manufacturer's instructions (Roche, Mannheim, Germany). The siRNAs were transfected either separately with 40 nM of one construct (single-target treatments) or with siRNA combinations (M4-1, M4-2 or M8). In all combination treatments, the final siRNA concentration was kept constant at 40 nM. In the combination M4-1, the siRNAs B2-1, BX-1, X-1 as well as S-1 (10 nM per siRNA) were incubated simultaneously. Accordingly, the constructs B2-2, BX-2, X-2 and S-2 were used in the treatment M4-2. In the combination M8 all eight target-directed siRNAs (5 nM per siRNA) were mixed. After 4 h, the transfection medium was replaced with fresh culture medium and cells were incubated for 20 h. Subsequently, chemotherapeutics were added. For EJ28 cells, final concentrations of cisplatin and MMC were 2.1 µg/ml and 0.9 µg/ml, respectively. For J82 cells, 1.2 µg/ml cisplatin and 1.0 µg/ml MMC were used. Cells were incubated with MMC for 2 h and with cisplatin for 24 h. Afterwards, cells were washed with phosphate buffered saline and cultivated with fresh culture medium. For analyses, cells were harvested by trypsin treatment (0.05% trypsin/0.02% EDTA for 5 min at 37°C). Detached and adherent cells were pooled and analysed together. The ns-si plus chemotherapy (CT) combination was used as control to evaluate siRNA-mediated effects of the treatment.

**Cell counts and viability.** Cells were counted using the Coulter Z2 Particle Count & Size Analyser (Beckman Coulter, Krefeld, Germany). Cellular viability was analysed in quadruplicates using the cell proliferation reagent WST-1 (Roche). Cells were seeded in 96-well plates and treated as previously described. WST-1 reagent (10 µl per well) was added to the cells 96 h after transfection start. Colour development was monitored for up to 4 h by measuring the absorbance at 450 nm and 620 nm (reference) with a spectrophotometer (Anthos labtec, Krefeld, Germany).

Table I. Designations and target sequences of the siRNAs.

siRNA	Target	siRNA target sequence
B2-1	<i>BCL2</i>	CGGUGGUGGAGGAGCUCUU
B2-2	<i>BCL2</i>	GCAUGCGGCCUCUGUUUGA
BX-1	<i>BCLXL</i>	GGGACAGCAUACAGAGCU
BX-2	<i>BCLXL</i>	CAGCUGGAGUCAGUUUAGU
X-1	<i>XIAP</i>	CGAGCAGGGUUUCUUUAUA
X-2	<i>XIAP</i>	CUGGGCAGGUUGUAGAUAU
S-1	<i>survivin</i>	GAAGCAGUUUGAAGAAUUA
S-2	<i>survivin</i>	CCAACAAUAGAAGAAAGA

All siRNAs have 3'-dTdT overhangs.

**Apoptosis detection.** Apoptosis was assessed by annexin V/propidium iodide (PI) staining 48 h and 72 h after transfection start, using flow cytometry (FACScan; BD Biosciences, Heidelberg, Germany), according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences). The percentage of early (annexin V-FITC-positive, PI-negative) and late (annexin V-FITC-positive, PI-positive) apoptotic cells was determined by quadrant analysis of annexin V-FITC/PI plots using the WinMDI2.8 software (<http://facs.scripps.edu/software.html>).

**Statistics.** An unpaired Student's *t*-test was used to compare the differences in cell viability between target-directed-siRNA plus CT- and ns-si plus CT-treated cells.

## Results

Treatment with cisplatin or MMC reduced EJ28 and J82 BCa cell viability by 33%-74% 72 h after start of chemotherapy treatment (Figure 1). Pre-treatment with the control siRNA ns-si did not change the chemotherapy efficacy and caused reductions in cell viability comparable to those of chemotherapy-alone, *e.g.* for ns-si plus MMC by 39% in J82 and by 61% in EJ28 cells (Figure 1). To evaluate the specific enhancement of chemotherapy effects, treatments with target-specific siRNA plus CT were compared to ns-si plus cisplatin or ns-si plus MMC treatments, respectively. Out of the single target treatments, the siRNA-mediated inhibition of *BCL2* and *XIAP* did not change or only marginally changed the efficacy of cisplatin and MMC in BCa cells (data not shown). In contrast, the knockdown of *BCLXL* and *survivin* sensitised EJ28 and J82 cells towards subsequent chemotherapy (Figure 1). For example, cell viability decreased by 77% and 75% after inhibition of *BCLXL* and subsequent MMC treatment in EJ28 cells, whereas ns-si plus MMC mediated a reduction of only 61%. The differences were statistically significant ( $p < 0.001$ ). Likewise, J82 cell viability was reduced by 57% after *survivin* knockdown in combination with MMC, which is significantly different from the value after ns-si plus MMC treatment (40%;  $p < 0.001$  for S-1 and  $p = 0.006$  for S-2). The simultaneous knockdown of *BCL2*, *BCLXL*, *XIAP* and *survivin* by the siRNA combinations M4-1,

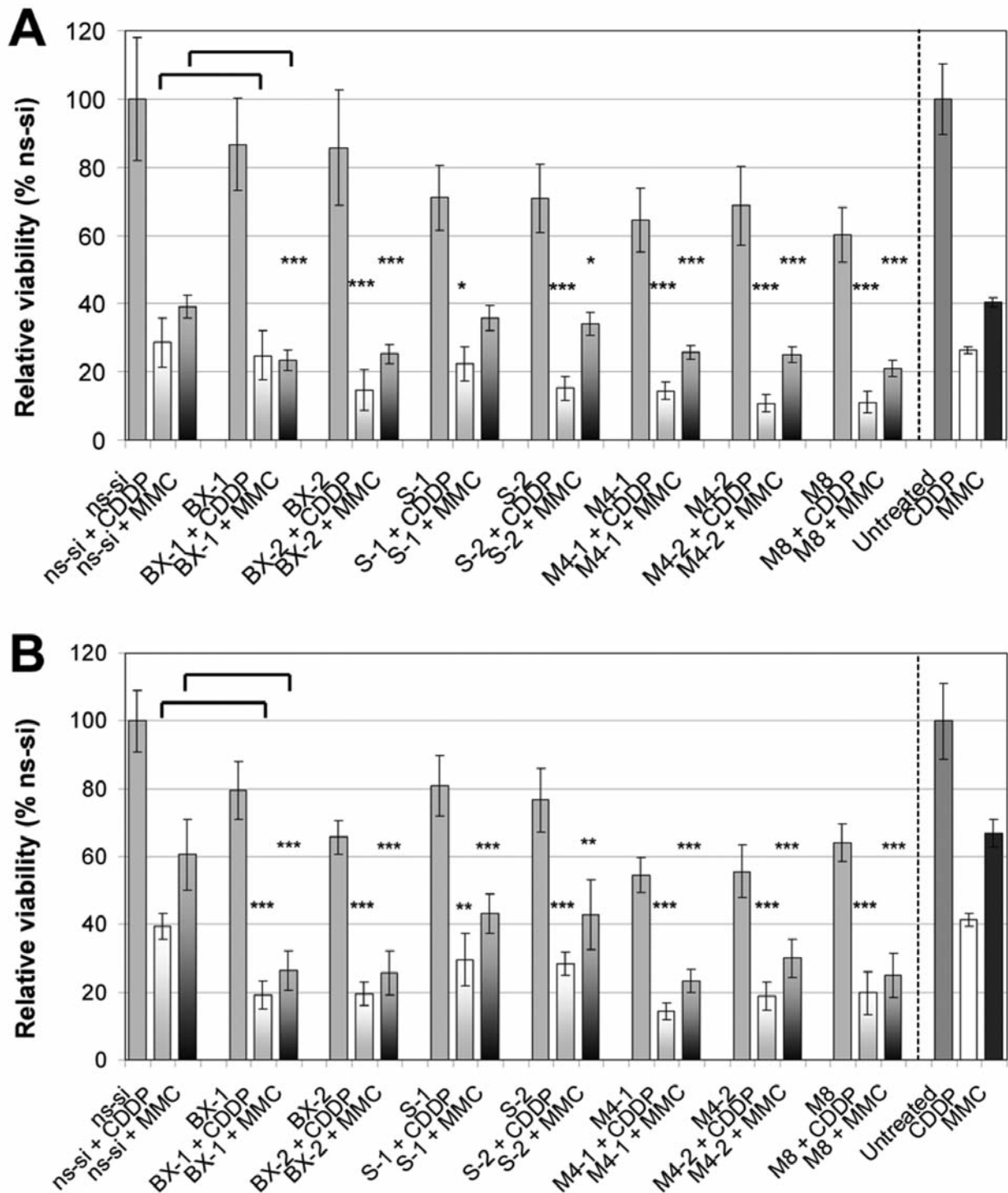


Figure 1. Viability of EJ28 (A) and J82 (B) BCa cells after combined treatment with siRNAs and chemotherapy. Cells were transfected with the appropriate siRNAs for 4 h. Twenty-four hours after transfection, cells were treated with cisplatin (CDDP; 2.1  $\mu\text{g/ml}$  for EJ28 and 1.2  $\mu\text{g/ml}$  for J82) for 24 h or with mitomycin C (MMC; 0.9  $\mu\text{g/ml}$  for EJ28 and 1.0  $\mu\text{g/ml}$  for J82) for 2 h. The cell viability was examined 96 h after transfection. Values shown are relative to the control siRNA ns-si (=100%, for all siRNA treatments) or relative to untreated cells (only for CDDP and MMC single-treatments) and are averages of a fourfold determination. Error bars represent the 95% confidence interval. An unpaired Student's *t*-test was used to compare the differences in cell viability between target-directed-siRNA plus chemotherapy and ns-si plus chemotherapy-treated cells (exemplarily indicated for BX-1; \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ).

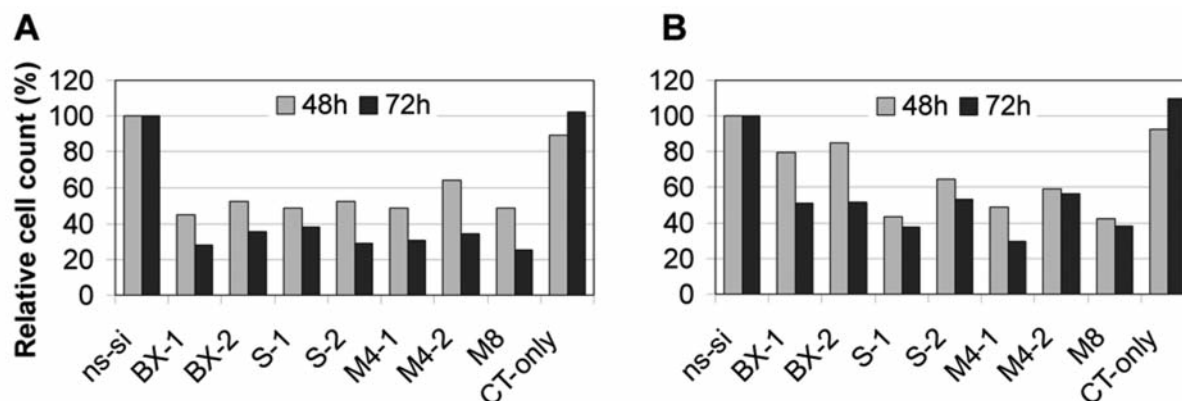


Figure 2. Growth-inhibitory effects mediated by combined treatment with siRNAs and chemotherapy (CT) in EJ28 BCa cells. Cells were transfected with the appropriate siRNAs for 4 h. CT-only cells were treated with serum-free OptiMEM medium during transfection. Twenty-four hours after transfection, cells were treated with 2.1  $\mu$ g/ml cisplatin for 24 h (A) or with 0.9  $\mu$ g/ml mitomycin C for 2 h (B). The cell count was determined 48 h and 72 h after transfection. Values are shown relative to the control treatment (ns-si plus CT=100%).

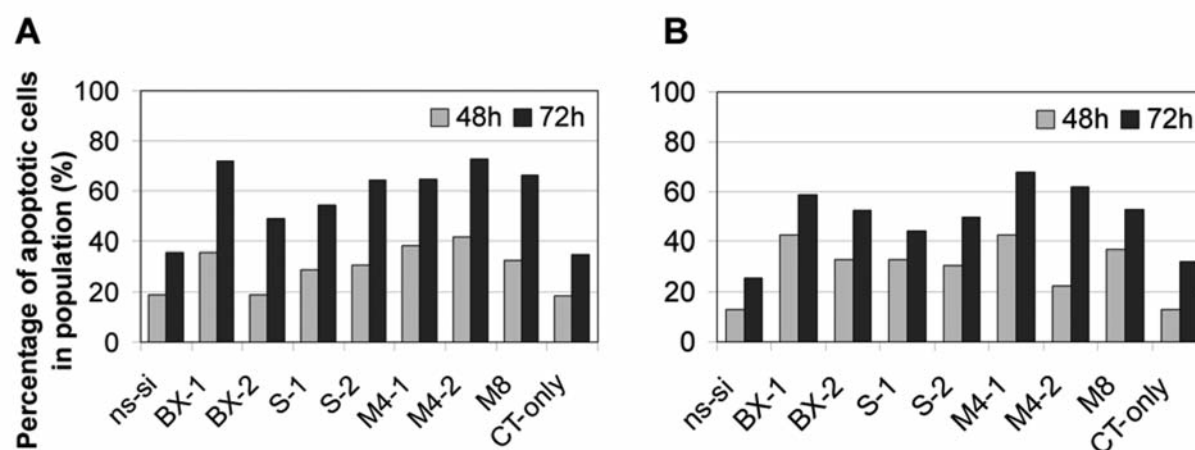


Figure 3. Percentage of apoptotic cells after combined treatment with siRNAs and chemotherapy (CT) in EJ28 BCa cells. Cells were transfected with the appropriate siRNAs for 4 h. CT-only cells were treated with serum-free OptiMEM medium during transfection. Twenty-four hours after transfection, cells were treated with 2.1  $\mu$ g/ml cisplatin for 24 h (A) or with 0.9  $\mu$ g/ml mitomycin C for 2 h (B). The rate of apoptosis was determined 48 h and 72 h after transfection start.

M4-2 or M8 plus subsequent chemotherapy mediated stronger decreases in cell viability in both BCa cell lines than the inhibition of just one antiapoptotic gene. For example, the treatment with M8 plus MMC reduced EJ28 and J82 cell viability by 79% and 75%, respectively (Figure 1).

Besides changes in cell viability, siRNA-mediated inhibition of *BCLXL* and *survivin*, as well as the simultaneous knockdown of *BCL2*, *BCLXL*, *XIAP* and *survivin*, in combination with subsequent chemotherapy reduced cell counts and induced apoptosis in EJ28 cells. As soon as 48 h after transfection, cell counts decreased by 36% (M4-2) to 55% (BX-1) in combination with cisplatin (Figure 2A). Twenty-four hours later, treatment with target-specific siRNA and cisplatin further diminished the cell count by up to 75% (M8, Figure 2A). In the

M4-2 and BX-1 treatments, cell counts decreased by 66% and 72%, respectively (Figure 2A). The percentage of apoptotic cells in the population rose from 19% and 36% in the ns-si plus cisplatin control to up to 42% and 73% in the M4-2 plus cisplatin treatment 48 h and 72 h after transfection, respectively (Figure 3A). For MMC in combination with siRNAs targeted to *BCLXL*, *survivin* or all four genes, similar changes in cell counts and apoptosis rates were observed (Figures 2B and 3B). In J82 cells, inhibition of *BCLXL* and simultaneous knockdown of all four target genes in combination with MMC or cisplatin strongly increased apoptosis 72 h after transfection (Figure 4). The highest percentage of apoptotic J82 cells was seen after M8 plus MMC treatment with 57% apoptotic cells in comparison to 20% in the control.

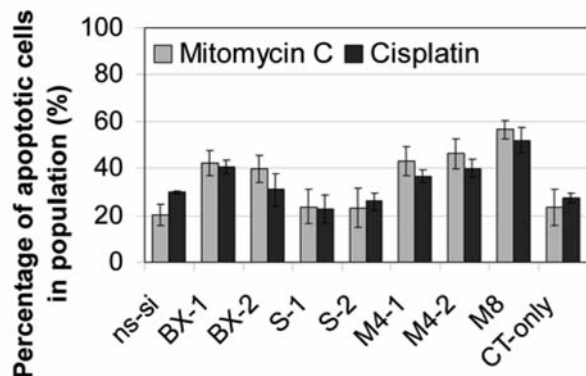


Figure 4. Rate of apoptosis after combined treatment with siRNAs and chemotherapy (CT) in J82 BCa cells. Cells were transfected with the appropriate siRNAs for 4 h. CT-only cells were treated with serum-free OptiMEM medium during transfection. Twenty-four hours after transfection, cells were treated with 1.0 µg/ml mitomycin C for 2 h or with 1.2 µg/ml cisplatin for 24 h. The rate of apoptosis was determined 72 h after transfection. Values shown are averages of two independent experiments. Error bars represent the mean deviation.

## Discussion

The up-regulation of antiapoptotic genes, such as *BCL2*, *BCLXL*, *XIAP* and *survivin*, is one mechanism by which cancer cells develop resistance towards chemotherapeutic agents (7). Consequently, the inhibition of these factors might improve chemotherapy response. It was shown that the siRNA-mediated knockdown of one of the four antiapoptotic targets *BCL2*, *BCLXL*, *XIAP* and *survivin* is able to sensitise different cancer cell lines to chemotherapeutics (19-22). In the present study, we analysed cellular effects of siRNA-mediated knockdown of *BCL2*, *BCLXL*, *XIAP* and *survivin*, both one target-alone and all four target genes simultaneously, on the efficacy of MMC and cisplatin in EJ28 and J82 BCa cells. As shown previously, the selected siRNAs induce an efficient and long-lasting reduction of the mRNA and protein levels of their corresponding target gene (23). Also in the combination treatments M4-1, M4-2 and M8, considerable target knockdown was achieved. For example, *BCL2* was reduced to 43%, *BCLXL* to 38%, *XIAP* to 59%, and *survivin* to 22% in J82 BCa cells 48 h after transfection with the siRNA combination M8 (23).

Out of the four selected antiapoptotic factors, only knockdown of *BCLXL* and *survivin* significantly sensitised EJ28 and J82 BCa cells towards treatment with MMC and cisplatin. The combined treatment of these BCa cells with siRNAs targeted to *BCLXL* or *survivin* and chemotherapy mediated strong reductions in cell viability and cell counts (Figures 1 and 2). This is in accordance with previously published results (see below). For *survivin*, increased response of EJ28 and 5637 BCa cells to three different

cytotoxic agents was shown after siRNA- and antisense oligonucleotide-mediated target inhibition (17). In addition, antisense oligonucleotide-mediated *BCLXL* knockdown sensitised T24 and 5637 cells to chemotherapeutics (15). According with the present results, another siRNA targeted to *XIAP* did not increase MMC and cisplatin activity in EJ28 cells (24). However, in T24 BCa cells, inhibition of *XIAP* by antisense oligonucleotides and siRNAs improved doxorubicin and MMC efficacy (16, 25). Out of the four BCa cell lines, only one was sensitised towards MMC by antisense oligonucleotide-mediated *BCL2* decrease (26). Likewise, an siRNA targeted to *BCL2* improved chemotherapy efficacy only in one of the two analysed hepatoblastoma cell lines (27). Since the contribution of one antiapoptotic factor to tumourigenesis and chemoresistance can vary between cancer cells and since the inhibition of one antiapoptotic gene might be bypassed by the tumour cells by the up-regulation of another, the simultaneous knockdown of multiple inhibitors of apoptosis could represent a more promising option for BCa treatment.

Combined inhibition of *BCL2*, *BCLXL*, *XIAP* and *survivin* was analysed in the three different siRNA combinations M4-1, M4-2 and M8. All three siRNA combinations together with subsequent chemotherapy mediated reductions in cell counts, as well as induction of apoptosis, 48 h and 72 h after transfection that were comparable with the most effective single-target treatment (Figures 2-4). However, decreases in BCa cell viability in the combination treatments 96 h after transfection were stronger than after the single-knockdown of the targets (Figure 1). Since the cell colony formation assay is not applicable after chemotherapy, the long-term effects of combined *BCL2*, *BCLXL*, *XIAP* and *survivin* knockdown together with subsequent chemotherapy need to be analysed further in an *in vivo* study. In particular, the question whether knockdown of *BCL2* and *XIAP*, which did not improve chemotherapy activity in the single-target treatments in the two selected BCa cell lines, might contribute to therapy efficacy after long-term and repeated treatments *in vivo* needs to be answered. Moreover, the addition of further targets, such as other antiapoptotic genes, as well as other important tumour-related genes, could improve therapy response. For example, the combined inhibition of the three IAPs, livin, *XIAP* and *survivin* strongly sensitised T24 cells towards MMC (25). Altogether, the simultaneous knockdown of genes that contribute to chemoresistance is a promising approach for enhancing chemotherapy efficacy in the treatment of BCa. Since the contribution of one apoptosis inhibitor to chemotherapy response varies between different BCa cell lines the simultaneous targeting of multiple antiapoptotic genes promises success of the therapy in a higher number of cell lines and could consequently improve chemotherapy in a multitude of patients with BCa.

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