

Evaluation of cytotoxic effects induced by bcl-2 and bcl-x_L antisense-oligodeoxynucleotides in normal urothelium and transitional cell carcinoma

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Received April 9, 2008; Accepted July 22, 2008

DOI: 10.3892/or_00000161

Abstract. We report on cytotoxic effects of bcl-2 and bcl-x_L antisense-oligodeoxynucleotides (AS-ODNs) in benign urothelial and transitional cell carcinoma (TCC) cell lines. The benign urothelial cell line (UROtsa) and four TCC cell lines (UM-UC-3, RT 112, HT 1197 and T 24/83) were incubated with bcl-2 and bcl-x_L AS-ODNs and cell mortality rates were assessed. Bcl-2 and bcl-x_L AS-ODN treatment resulted in low toxicity in UROtsa cells (6% and 10% cell mortality, respectively). After bcl-2 AS-ODN treatment, cell mortality rates in TCC cell lines were significantly higher than in UROtsa cells (mean values: 33% vs. 6%, respectively). Bcl-2 AS-ODN treatment also caused significantly higher cell death rates in the majority of TCC cell lines when compared to bcl-x_L-AS-ODN therapy (mean values: 33% vs. 11%, respectively). In conclusion, bcl-2 AS-ODNs show significantly higher cell mortality in TCC cells, whereas toxic effects on normal urothelium seem to be minor. Our results suggest favourable characteristics for the clinical application of AS-ODN in intravesical chemotherapy of TCC.

Introduction

Transitional cell carcinoma (TCC) is the second most common malignancy of the genito-urinary tract. Despite intravesical chemotherapy, recurrence and progression of non-muscle-invasive TCC occur in up to 40 and 15% of all patients, respectively (1,2). Patients with advanced stages (T2-T4)

under adjuvant systemic chemotherapy have an average five-year progression-free rate and overall survival rate of 11.3 and 15.3%, respectively (3).

Anti-apoptotic proteins such as bcl-2 and bcl-x_L have been reported to promote resistance to chemotherapy in TCC cell lines, in particular when overexpression is detected (4,5). Antisense-oligodeoxynucleotides (AS-ODNs) are small DNA-sequences. For biological stabilisation, the chemical structure is usually modified, for example by phosphorothioate substitutions in the backbone (6). Three main intracellular mechanisms are reported for AS-ODNs to inhibit target protein synthesis: hybridization with target mRNA activates endogenous RNase H, which then degrades the complex (7). Hybridization to an mRNA-AS-ODN complex, as a second mechanism, also blocks translation at the ribosome. Furthermore, AS-ODNs may proceed to the nucleus, form complexes with double strand DNA sequences encoding the target mRNA and inhibit its transcription.

We previously investigated the effects of AS-ODNs targeting bcl-2 and bcl-x_L in preclinical settings to optimize chemotherapy (8,9). AS-ODNs promise a potentiated effect in chemotherapy, and combined therapy with AS-ODNs may become an interesting tool for intravesical treatment in TCC in the near future (8-11).

Besides the target-specific mechanisms, unspecific off-target effects induced by AS-ODNs have been observed (7,12-14). Potential toxicity on benign urothelial cells during intravesical treatment in TCC have to be assessed before clinical use of AS-ODNs can be suggested.

The aim of our study was to evaluate cytotoxicity and unspecific effects induced by bcl-2 and bcl-x_L AS-ODN in TCC and benign urothelial cells.

Materials and methods

Cell lines and cell culture conditions. Four human TCC cell lines [UMUC-3 (American Tissue and Cell Collection, ATCC), RT 112, (German Collection of Microorganisms and Cell Cultures, DSMZ), HT 1197 and T 24/83 (European Collection of Cell Cultures, ECACC)] and the benign urothelial cell line UROtsa (provided by Professor R. Knüchel-Clark, Universitätsklinikum Regensburg, now RWTH

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Key words: transitional cell carcinoma, chemotherapy, gene therapy, oligodeoxyribonucleotides, toxicity test

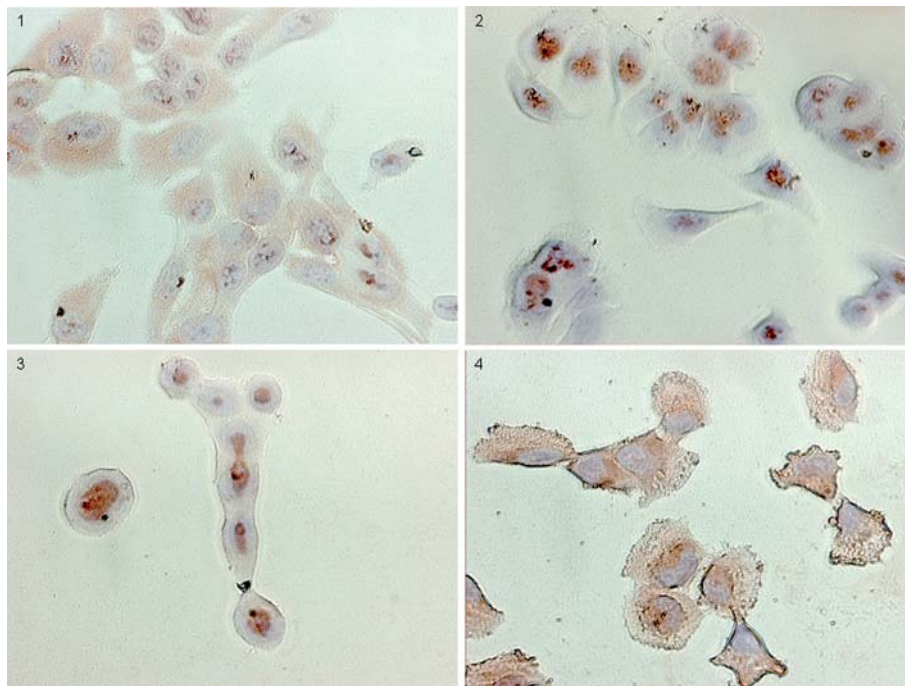


Figure 1. Immunohistochemical staining of bcl-x_L expression in UM-UC-3 (1), RT 112 (2), HT 1197 (3) and T 24/83 cells (4), magnification, x40.

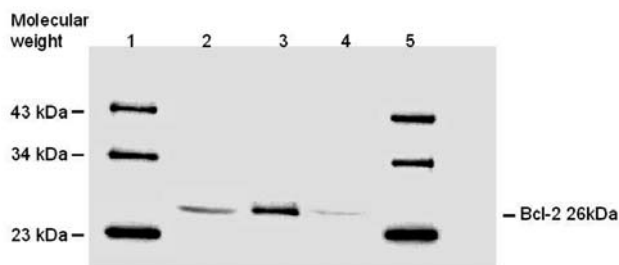


Figure 2. Western blotting of (1 and 5) molecular weight marker, (2) native T 24/83 cells, (3) T 24/83 cells after 48 h of treatment with 5 µg/ml Mitomycin C, (4) T 24/83 cells after 48 h of treatment with 5 µM bcl-2 antisense-oligodeoxynucleotides. Bcl-2 expression can either be induced or suppressed in TCC cell line.

Aachen) were routinely maintained in 75 cm² cell culture flasks at standard conditions (37°C humid atmosphere of 5% CO₂). We used different culture media [Dulbecco's modified essential medium (D-MEM) with 10% FCS and 4 mM L-glutamin for RT 112 and UMUC-3, RPMI 1640 medium with 5% FCS and 4 mM L-glutamin for UROtsa (both Invitrogen, Carlsbad, CA, USA), Eagle's MEM with 10% FCS, 2 mM L-glutamin and 0.1 mM non-essential amino acids for HT 1197 and McCoy's modified 5A medium with 10% FCS and 2 mM L-glutamine for T 24/83 (both PAN, Biothech GmbH, Aidenbach, Germany).

Antisense oligonucleotides and therapy. AS-ODNs were fully phosphorothioated in order to provide better protection against nuclease mediated degradation (15). The sequence of the bcl-2 AS-ODNs was 5' AAT CCT CCC CCA GTT CAC CC 3', while the bcl-x_L AS-ODNs had a 5' AAA GTA TCC CAG CCG CCG TT 3' sequence, as already implemented

in our laboratory and described in other publications (5,9,10). Modified AS-ODNs were received from Trilink Biotechnologies (San Diego, CA, USA) and purified by double RP-HPCL. The purity of the AS-ODNs was determined by using a 15 % PAGE gel with 7 M urea and bromophenol blue marker.

Therapy on TCC and benign cells was simulated by applying bcl-2 and bcl-x_L AS-ODN to the cell cultures at a concentration of 5 µM in the cell culture medium.

Immunohistochemical staining. Immunohistochemical staining was performed on all cell lines in order to verify the expression of Bcl-2 and Bcl-x_L. After incubation, the cells were fixed in methanol. Primary anti-Bcl-2 (N19, rabbit polyclonal, Santa Cruz) and anti-Bcl-x_L (A20, goat polyclonal, Santa Cruz) antibodies were added in a 1:50 dilution. The biotinylated secondary antibody was added (Vectastain ABC kit, Linaris, Wertheim-Bettingen, Germany), and the cells were incubated with AEC (Dako AEC high sensitive, Dako, Carpinteria, CA, USA) as a coloring substrate. Sections were counterstained with hematoxylin.

Western blot analysis. Bcl-2 and Bcl-x_L protein expression was identified in UMUC-3, RT 112, HT 1197, T 24/83 and UROtsa cells by Western blot analysis in native, untreated cells, after 48 h of treatment with 5 µM bcl-2- bcl-x_L AS-ODN, and, as positive control, after 48 h of treatment with 5 µg/ml MMC. The protein concentration in all samples was determined initially by a BCA protein assay (Pierce, Rockford, IL, USA). The amount of protein used for PAGE in each lane was 100 µg. Cell lysates from JURKAT cells (T-cell leukaemia cell line) served as a positive control. Cell reaction to externally-induced cytotoxic effects was evaluated by applying bcl-2 and bcl-x_L AS-ODN (5 and 10 µM).



Cell line	Cell survival rates (%)		Differences in cell survival rates after bcl-2- and bcl-x _L AS-ODN treatment
	Bcl-2 AS-ODN (5 μ M)	Bcl-XL AS-ODN (5 μ M)	
UROtsa (benign)	94.02	90.07	0.43
RT 112 (TCC)	55.72	92.57	<0.001
HT 1197 (TCC)	60.78	87.50	<0.001
UMUC-3 (TCC)	63.62	81.98	<0.001
T24/83 (TCC)	88.61	93.73	0.066

AS-ODN, antisense-deoxyoligonucleotides and TCC, transitional cell carcinoma.

Molecular weight markers (Cruz Marker™), anti-bcl-2 and anti bcl-x_L polyclonal antibodies, positive controls, secondary IgG-HRP antibodies and Luminol reagent solution were provided by Santa Cruz, CA, USA.

Neubauer haemocytometry. Cell vitality and numbers were determined by trypan blue staining and consecutively counting the cells in a Neubauer haemocytometer. Cell numbers of each sample were independently determined two times.

Cell death rate calculation. The cell death rate was calculated in relation to the untreated control group according to the formula: $[1 - (\text{survived treated cells} / \text{survived untreated cells})] \times 100\% = \text{cell death rate } (\%)$.

Statistical analysis. All statistical calculations were performed with SAS software, release 8.02. A p-value of <0.05 was considered to be significant. TCC cell lines were compared as one group to the normal urothelial cell line for each treatment by linear contrast. A Dunnett-test was used to compare cell mortality of each TCC cell line individually to the normal urothelial cell line after therapy. To compare the two different therapies in each cell line a t-test was applied, assuming a Gaussian distribution.

Results

Bcl-2 and bcl-x_L expression. Immunohistochemical staining of the native, untreated UMUC-3, RT 112, HT 1197, T 24/83 and UROtsa detected the expression of bcl-2 and bcl-x_L in all cell lines (Fig. 1). Western blot protein analysis identified bcl-2 and bcl-x_L expression in the native, untreated UMUC-3, RT 112, HT 1197, T 24/83 and UROtsa cells. Bcl-2 and bcl-x_L AS-ODN treatment decreased bcl-2 and bcl-x_L protein expression in all cell lines. In the positive control with Mitomycin C chemotherapy bcl-2 and bcl-x_L protein expression increased in all cell lines (Fig. 2).

Bcl-2-AS-ODN treatment. Monotherapy with bcl-2 showed significantly higher cell death rates in all TCC-cell lines compared to the normal urothelial cell line UROtsa

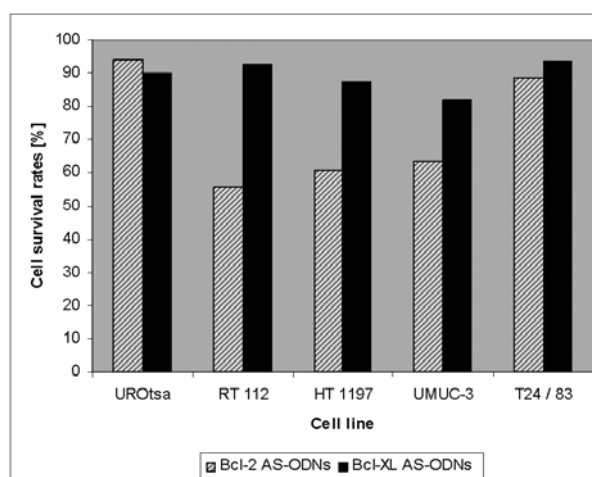


Figure 3. Median cell survival rates after 5 μ M-antisense-oligodeoxynucleotide (AS-ODN) monotreatment.

($p < 0.001$) (Table I). The RT-112 cell line was most sensitive to the treatment with a cell survival rate of only 56%. The T24/83 line was most resistant to bcl-2 AS-ODN treatment with 89% cell survival (Fig. 3). Monotreatment with 10 μ M bcl-2 AS-ODN concentration in TCC-cell lines resulted in ~5% lower cell survival rates (data not shown).

Bcl-x_L-AS-ODN treatment. Bcl-x_L-AS-ODN treatment of the TCC-cell lines showed partly higher cell death rates in the TCC-lines compared to the normal urothelial cell line UROtsa, which was not significant ($p = 0.691$) (Table I). Bcl-x_L treatment showed the highest cytotoxic effect on UMUC-3 TCC cells with a survival rate of 82%. RT-112 and T24/83 cells were the least affected with 93 and 94% cell survival, respectively. The normal urothelial UROtsa showed 90% cell survival (Fig. 3).

Cell line influence on bcl-2 and bcl-x_L-AS-ODN treatment. Monotherapy with bcl-2 and bcl-x_L AS-ODN increased cell mortality in TCC-cell lines and in the normal urothelial cell line UROtsa. Cell mortality in the normal urothelial cell line

was 6% after bcl-2-AS-ODNs compared to 10% after bcl-x_L AS-ODN treatment, but the difference was not significant ($p=0.427$). In TCC cells bcl-2-AS-ODNs caused higher cell mortality than bcl-x_L AS-ODN-therapy. This difference was highly significant for the cell lines HT 1197, RT 112 and UMUC-3 ($p<0.001$), and a tendency was observed for T24/83 ($p=0.066$) (Table I). Furthermore, cell mortality after bcl-2 treatment was significantly higher in all TCC cells compared to the UROtsa cells ($p<0.01$).

Discussion

Our study has demonstrated low toxic effects of bcl-2- and bcl-x_L AS-ODN treatment in benign urothelial cells. Minor toxicity was observed after bcl-2 AS-ODN monotreatment, suggesting bcl-2 AS-ODNs to be advantageous over bcl-x_L AS-ODNs in TCC treatment. Bcl-2- and bcl-x_L-proteins belong to the bcl-2 family of the inhibitor of apoptosis protein (IAP) family. The bcl-2 family of proteins and related phosphorylating enzymes are the major regulators of mitochondrial apoptotic homeostasis through cytochrome c and apaf-1 down-regulation into the cytoplasm. Overexpression of bcl-2 and bcl-x_L in cancer cells after chemotherapy has been associated with resistance to chemotherapy (4,5). In bladder TCC bcl-x_L expression relates to high-grade and advanced tumours (16) and bcl-2 expression is associated with increased recurrence and progression (17). AS-ODN are short DNA-strands with modified backbone that interact with target mRNA of a cell (7). We and other authors already reported on the synergistic effects of AS-ODN when combined with chemotherapy in TCC cells (4,8,9). Bcl-2 and bcl-x_L AS-ODNs hybridize specifically with the mRNA that encodes for bcl-2 or bcl-x_L, resulting in down-regulation of their expression levels (5,15). In addition unspecific effects induced by AS-ODNs have been discussed (7). These so-called off-target effects induce unspecific immuno-reactions of unspecific RNA interaction. Phosphorothioate substitution stabilises AS-ODN against enzymatic degradation but increases unspecific cell toxicity and activity including immune stimulation and protein interaction (6,7).

To our knowledge, effects of bcl-2 and bcl-x_L AS-ODN monotreatment on benign urothelial cells have not been previously evaluated. In the present study, we verified the expression of bcl-2 and bcl-x_L in four different TCC and one normal urothelial cell line by Western blot analysis and immunohistochemical staining. We then applied bcl-2 and bcl-x_L AS-ODNs to one benign and four malignant bladder cell lines and analysed the cell mortality. Cell mortality is neither associated distinctly with specific inhibition of the corresponding mRNA nor with unspecific off-target effects but both are most likely to contribute to cell mortality. From our findings, however, we conclude relevant differences in specific and off-target effects between bcl-2 and bcl-x_L AS-ODNs.

Bcl-2 AS-ODN treatment caused significantly lower cell mortality rates in benign urothelial cells compared to TCC cells, and the same tendency could be seen after bcl-x_L AS-ODN treatment. This may be explained by the fact that bcl-2 and bcl-x_L are not over-expressed in normal urothelial

cells and AS-ODNs consecutively cause lower specific down-regulation. Furthermore, in UROtsa cells, toxicity was lower for bcl-2 AS-ODNs indicating lower off-target effects when compared to bcl-x_L AS-ODN treatment. The lower toxicity in benign bladder cells would be highly desired in potential intravesical therapy thus preventing unspecific toxicity to cause undesired urothelial damage. In the majority of TCC cell lines we found significantly higher cell mortality after bcl-2- compared to bcl-x_L AS-ODN treatment. We conclude that bcl-2 AS-ODNs are more specific than bcl-x_L AS-ODNs. The overall comparison of bcl-2 and bcl-x_L AS-ODNs again suggests advantage of bcl-2 AS-ODNs for potential intravesical therapy in TCC.

Limitations to our study include the lack of total quantification of protein expression. Bcl-2 and bcl-x_L showed higher expression in TCC cell lines when compared to the normal urothelial cells in Western blot analyses, but this was not separately quantified and remains a hypothesis. Our results are preliminary and have to be further evaluated on protein and transcription level for more detailed insight.

In conclusion, Bcl-2- and bcl-x_L AS-ODN treatment in benign urothelial cell lines results in low cytotoxicity. According to our findings no major cytotoxic effects in benign urothelium is expected during potential bladder instillation, confirming AS-ODNs as suitable candidate agents for targeted intravesical gene therapy. Bcl-2 AS-ODNs seem to be advantageous over bcl-x_L AS-ODNs in TCC treatment, as higher cell mortality was observed in TCC cells and less cytotoxicity was detected in benign urothelial cells. Studies on protein and transcription levels as well as *in vivo* studies have to further evaluate our findings. Based on the preliminary *in vitro* results we encourage the use of AS-ODNs in intravesical chemotherapy.

Acknowledgements

This work was supported by a grant from the H.W. & J. Hector Stiftung, Weinheim, Germany.

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