

## Multitarget siRNA Inhibition of Antiapoptotic Genes (XIAP, BCL2, BCL-X<sub>L</sub>) in Bladder Cancer Cells

DOREEN KUNZE<sup>1\*</sup>, DANIELA WUTTIG<sup>1\*</sup>, SUSANNE FUESSEL<sup>1</sup>, KAI KRAEMER<sup>1</sup>, MATTHIAS KOTZSCH<sup>2</sup>, AXEL MEYE<sup>1</sup>, MARC-OLIVER GRIMM<sup>1</sup>, OLIVER W. HAKENBERG<sup>3</sup> and MANFRED P. WIRTH<sup>1</sup>

<sup>1</sup>Department of Urology and <sup>2</sup>Institute of Pathology, Technical University of Dresden, Dresden;

<sup>3</sup>Department of Urology, Rostock University, Rostock, Germany

**Abstract.** *Background: The knockdown of XIAP, BCL2 and BCL-X<sub>L</sub> by siRNAs represents a promising treatment option for bladder cancer (BCa) since the overexpression of antiapoptotic genes is often associated with tumor progression and treatment resistance. Materials and Methods: EJ28 BCa cells were transfected with siRNAs – separately and combined – followed by analysis of target expression, viability, clonogenic survival, apoptosis and cell cycle. Furthermore, a possible chemosensitization by siRNA pretreatment was investigated. Results: The siRNA-mediated inhibition of these targets – either separately or combined – reduced the targets' expression, reduced cell growth and sensitized cells to a subsequent chemotherapy. Conclusion: Since tumor cells may bypass the inhibition of a single gene by changing their expression profile, e.g. switch from BCL2 to BCL-X<sub>L</sub>, the combined knockdown of multiple genes of the same pathway might be more effective in killing cancer cells. The siRNAs used represent appropriate tools for this aim since they reduced their targets' expression significantly and long-lastingly.*

In 2007, an estimated 50,040 new cases of bladder cancer (BCa) will be diagnosed in men in the US, and approximately 9,630 men will die of the disease (1). These numbers make BCa the fourth most common malignancy and the eighth leading cause of cancer deaths in men. At the time of diagnosis, 70-80% of all BCa are superficial and will be treated by transurethral resection (TUR-B) (2). However, 50-70% of these BCa will recur and 5-20% will progress to muscle invasive cancer (2). Instillation therapies, e.g. using

bacillus Calmette-Guérin (BCG) or the chemotherapeutic agent mitomycin C (MMC), can reduce the recurrence rate, but MMC does not effect survival and BCG is not active in about one third of patients (3). Furthermore, BCG and MMC both cause local (e.g. dysuria, cystitis) and systemic (e.g. fever, malaise, nausea) side-effects (4).

To improve existing instillation therapies and to reduce their side-effects, combined treatments with specific nucleic acid inhibitors such as antisense oligodeoxynucleotides (AS-ODNs) or small interfering RNAs (siRNAs) might be suitable. siRNAs are synthetic double-stranded RNA molecules (21-23 basepairs in length) which reduce their target's expression by inducing RNA interference (RNAi) (5). Attractive targets for gene inhibition should be selectively up-regulated in tumor cells and should have an essential function in cancer-promoting pathways (6), such as the selected antiapoptotic targets XIAP, BCL2 and BCL-X<sub>L</sub> (7, 8). Furthermore, their overexpression in BCa and other tumors is often associated with poor prognosis and resistance to radiotherapy and chemotherapy (CT) (7-12).

XIAP (X-linked inhibitor of apoptosis protein, hIAP, BIRC4), an important member of the inhibitor of apoptosis protein family, directly binds to and inhibits both initiator caspase-9 and effector caspase-3 and -7. Thereby, XIAP can suppress apoptosis triggered by different stimuli such as the mitochondrial and the death receptor-mediated pathways (8). AS-ODN-mediated inhibition of XIAP reduced protein expression and sensitized T24 BCa cells to doxorubicin (13). Similar sensitization effects after XIAP down-regulation were described for several cancer types including prostate, lung and ovarian, as well as for melanoma cells (8, 14).

BCL2 (B-cell lymphoma 2) and BCL-X<sub>L</sub> (BCL2-like 1, BCL2L1), two antiapoptotic members of the BCL2 family, are mitochondrial membrane proteins which act by preventing the release of mitochondrial cytochrome *c* into the cytoplasm (15). Release of cytochrome *c* would lead to apoptosome assembly and thereby to the activation of caspase-9 (15). Studies in BCa cell lines describe AS-ODN-mediated down-regulation of BCL2 or BCL-X<sub>L</sub>, which was frequently accompanied by apoptosis induction and chemosensitization (7, 9, 11, 13, 16). In tumor cells expressing both BCL2 and BCL-X<sub>L</sub>, the prediction which

\*Both authors contributed equally to this work.

*Correspondence to:* Doreen Kunze, Department of Urology, Technical University of Dresden, Fetscherstrasse 74, D-01307 Dresden, Germany. Tel: +49 (0) 351 4584544, Fax: +49 (0) 351 4585771, e-mail: doreen.kunze@uniklinikum-dresden.de

*Key Words:* BCL2, BCL2L1, BIRC4, siRNA, chemosensitization, inhibition of apoptosis.

Table I. Sequences of siRNAs.

Target	Sequence	
	siRNA (sense)	siRNA (antisense)
BCL2	GGC GCA CGC UGG GAG AAC AdTdT	UGU UCU CCC AGC GUG CGC CdTdT
BCL-X <sub>L</sub>	GCA GCU UGG AUG GCC ACU UdTdT	AAG UGG CCA UCC AAG CUG CdAdG
XIAP	GUG GUA GUC CUG UUU CAG CdTdT	GCU GAA ACA GGA CUA CCA CdTdT
ns-siRNA	UUC UUC GAA CGU GUC ACG UdTdT	ACG UGA CAC GUU CGG AGA AdTdT

of these two proteins is more important for cell survival is difficult (10). Furthermore, tumor cells have been found to switch the expression from BCL2 to BCL-X<sub>L</sub> (17). Therefore, a combined inhibition of both targets seems to be promising.

To date, no results of siRNA-mediated inhibition of selected targets in BCa cells have been published. However, their efficacy was shown in other tumor entities, e.g. BCL2 and XIAP were down-regulated in MCF-7 human breast cancer cells using RNAi, thereby sensitizing cells to chemotherapeutics (18).

In the present study, the expression of XIAP, BCL2 and BCL-X<sub>L</sub> was reduced by siRNAs in EJ28 BCa cells. The resulting effects on cell growth were analyzed – comparing the separate inhibition of one gene with the combined reduction of two or three targets. Furthermore, effects of siRNA pretreatment on a subsequent CT were examined.

## Materials and Methods

**Cell culture, siRNAs and transfection.** The human BCa cell lines EJ28 (University of Frankfurt, Frankfurt, Germany), J82 and T24 (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 and 1% HEPES (all from Invitrogen, Karlsruhe, Germany). The target-directed siRNAs (Table I) were synthesized by Eurogentec (Seraing, Belgium) and the non-silencing-siRNA control (ns-siRNA) which was used for normalization was purchased from Qiagen (Hilden, Germany). After seeding and adherence for 24 or 72 h, cells were transfected in serum-free OptiMEM with 200 nM of one siRNA, or with combinations of two or three siRNAs (each 100 or 67 nM) using DOTAP liposomal transfection reagent (ratio 1:4, w/w) according to the manufacturer's instructions (Roche, Mannheim, Germany). Following transfection (4 h, 37°C), cells were washed with phosphate buffered saline (PBS) and incubated in serum-containing medium for 24-72 h. For further analyses, cells were harvested by trypsin treatment (0.05% trypsin/0.02% EDTA, 5 min, 37°C). Detached and adherent cells were pooled and analyzed together.

**Chemotherapeutics and treatment.** Cisplatin (CDDP; final concentration 2.1 µg/ml) and MMC (final concentration 0.9 µg/ml) were diluted in culture media. Twenty hours after siRNA transfection, cells were incubated with the chemotherapeutics for 2 h (MMC) or 24 h (CDDP) followed by PBS washing and further cultivation. The ns-siRNA+CT combination was used as control to evaluate siRNA-mediated effects of the treatment.

**Viability and proliferation assays.** Using the cell proliferation reagent WST-1 (Roche) cellular viability was examined in quadruplicates 72 h or 96 h after transfection. Colony formation assay was performed by seeding 100 cells 24 h after treatment in triplicates in 6-well plates. Cells were incubated for 9-12 days. After Giemsa staining, clonogenic survival was determined by counting macroscopically visible colonies.

**Apoptosis detection and cell cycle analysis.** Apoptosis was assessed 72 h and 96 h after transfection by staining cells with annexin V/propidium iodide and analyzed using flow cytometry (Annexin V-FITC Apoptosis Detection Kit I; FACScan; BD Biosciences, Heidelberg, Germany). Cell cycle analysis was performed 72 h and 96 h after transfection by flow cytometry using the CycleTest Plus DNA Reagent Kit (BD Biosciences).

**RNA isolation, cDNA synthesis and quantitative PCR.** Total RNA was isolated from cell lines and tissue samples according to the manufacturer's instructions (Invisorb Spin Cell/Tissue RNA Mini Kit; Invitex, Berlin, Germany) and transcribed into cDNA (SuperScript™ II Reverse Transcriptase; Invitrogen). Transcript amounts of XIAP and the reference gene TBP (TATA box binding protein) (19) were determined by quantitative PCR (qPCR) using the LightCycler (LC) FastStart DNA Master Hybridization Probes Kit and the LC instrument (both from Roche). For XIAP, the following primers and probes were used: GTGATAAAGTAAAGTGCTTTCACGT, GTAGTTCCTACCA GACA CTCCTCAA, GTGAAGAC CCTTGGGAACAACAT-fluorescein, LC Red640-ctaaatggtatccagggtgcaaatatctg-PH. BCL2 and BCL-X<sub>L</sub> were quantified by Roboscreen (Leipzig, Germany).

**Western blot.** A total of 5×10<sup>4</sup> cells per sample were lysed in loading buffer (20% glycerol, 2% SDS, 125 mM Tris pH 6.8, 5% β-mercaptoethanol, bromophenol blue), incubated at 95°C for 5 min and separated on 4-12% NuPAGE Bis-Tris gels (Invitrogen). Proteins were transferred to a Hybond-P PVDF membrane (GE Healthcare, Munich, Germany). Membranes were incubated with monoclonal primary antibodies against BCL2 oncoprotein (1:200; clone 124; Dako, Glostrup, Denmark), BCL-X<sub>L</sub> (1:100; clone 2H12; QED Bioscience Inc., San Diego, California, USA), or XIAP (1:250; clone 28; BD Biosciences). β-Actin detected by a monoclonal anti-β-actin antibody (1:15,000; Sigma, St. Louis, Missouri, USA) served as a loading control. The secondary polyclonal rabbit anti-mouse immunoglobulins horseradish peroxidase (HRP)-linked antibody (1:1,000; Dako) and the Enhanced Chemiluminescence Kit (GE Healthcare) were used for visualization. Quantification was performed using Kodak 1D image analysis software (V 3.6; Fisher Scientific, Schwerte, Germany).

**Statistics.** An unpaired Student's t-test was used to compare the

Table II. Target expression normalized to TBP (zmole/zmole) in BCa cell lines.

Cell line	XIAP/TBP	BCL2/TBP	BCL-X <sub>L</sub> /TBP
EJ28	6.85	0.18	16.5
T24	7.76	0.28	9.9
J82	7.43	0.02	21.7

differences in cell viability between target-directed-siRNA+CT and ns-siRNA+CT (\**p*≤0.05, \*\**p*≤0.01 and \*\*\**p*≤0.001).

## Results

The mRNA expression levels of *BCL2*, *BCL-X<sub>L</sub>* and *XIAP* in EJ28, T24 and J82 BCa cells are shown in Table II. Since EJ28 cells express all targets at a high or moderate level, this cell line was chosen as model for the *in vitro* inhibition of the targets.

The siRNAs used reduced the target's mRNA expression down to 54-61% compared to ns-siRNA-treated cells 48 h after transfection (data not shown). Even 96 h after transfection, a comparable significant reduction of the targeted mRNA levels to 37-59% was detected (Table III). Western blot analysis showed protein reduction of XIAP to 52%, of BCL2 to 26% and of BCL-X<sub>L</sub> to 34% in the single target treatments compared to ns-siRNA treated cells 96 h after transfection. The combined treatment of EJ28 cells with two (BCL2+BCL-X<sub>L</sub>) or three (BCL2+BCL-X<sub>L</sub>+XIAP) siRNAs caused mRNA down-regulation of all targets and showed a rate of mRNA inhibition comparable to the mono-target therapies (Table III). At the protein level, combined knockdown of *BCL2+BCL-X<sub>L</sub>* reduced BCL2 to 29% and BCL-X<sub>L</sub> to 55%. Simultaneous inhibition of *BCL2+BCL-X<sub>L</sub>+XIAP* down-regulated XIAP protein to 53%, BCL2 to 57% and BCL-X<sub>L</sub> to 28%. All siRNAs – either separately or combined – sufficiently reduced the mRNA and protein expression of their targets.

The siRNA-mediated inhibition of the three targets notably decreased cell count 96 h after transfection (Table III). This was caused by the induction of apoptosis which was already prominent 72 h after transfection (Figure 1). However, the reduction of XIAP only affected the cell count, not apoptosis. None of the siRNA treatments (mono-target or combinations) significantly influenced cell viability (Figure 2), cell cycle distribution or clonogenic survival (data not shown). Nevertheless, combined treatment with target-directed siRNAs and chemotherapeutics provoked greater antiproliferative effects than the application of ns-siRNA+CT. Specific combinations of siRNA+MMC caused a significant reduction of the cellular viability compared to the ns-siRNA+MMC control (Figure 2). The combination of target-directed siRNAs with CDDP reduced viability compared to ns-siRNA+CDDP, however, the changes were not significant (Figure 2).

Table III. Cell count and mRNA expression relative to ns siRNA control of EJ28 cells 96 h after siRNA transfection. Expression values are normalized to TBP (zmole/zmole).

siRNA targeted at	Total cell count (cell count relative to ns-siRNA)	mRNA expression		
		XIAP/ TBP	BCL2/ TBP	BCL-X <sub>L</sub> / TBP
BCL2	5.3×10 <sup>6</sup> (58%)	91%	59%	85%
BCL-X <sub>L</sub>	6.2×10 <sup>6</sup> (69%)	95%	111%	37%
XIAP	5.0×10 <sup>6</sup> (55%)	46%	93%	96%
BCL2+BCL-X <sub>L</sub>	5.1×10 <sup>6</sup> (56%)	100%	58%	69%
BCL2+BCL-X <sub>L</sub> +XIAP	6.7×10 <sup>6</sup> (74%)	62%	53%	61%
ns-siRNA	9.1×10 <sup>6</sup> (100%)	100%	100%	100%
Untreated	8.4×10 <sup>6</sup> (93%)	101%	107%	83%

Moreover, subsequent CT with CDDP after blockage of the antiapoptotic genes (single or combined, except XIAP) further increased apoptosis (Figure 1).

## Discussion

Since apoptosis is frequently dysregulated in tumor cells, for instance by up-regulation of antiapoptotic proteins, the manipulation of these pathways may represent an appropriate course in treating primary and recurrent cancer (6, 10). Overexpression of the selected targets *BCL2*, *BCL-X<sub>L</sub>* and *XIAP* is associated with treatment resistance in various tumor entities and therefore presents a limitation for existing therapies (7, 8, 11, 14). Due to the induction of natural RNAi, siRNAs represent attractive tools for sequence-specific gene inhibition. With the siRNAs directed at *BCL2*, *BCL-X<sub>L</sub>* and *XIAP*, a significant and long-lasting reduction of the target mRNA and protein expression levels was achieved. Furthermore, the tumor cell count was reduced and apoptosis increased, indicating the antiproliferative potency. Combinational treatment against *BCL2+BCL-X<sub>L</sub>* or *BCL2+BCL-X<sub>L</sub>+XIAP* with lower siRNA concentrations per target (100 or 67 nM) caused similar target-mRNA reduction and growth inhibiting rates as the mono-target therapies with 200 nM of a single siRNA. Though siRNA-monotreatment was not able to reduce cell viability, the combination of target-directed siRNAs with a subsequent CT with MMC or CDDP provoked a viability decrease. Furthermore, the apoptosis increasing effect of the target-directed siRNAs was intensified. Kim *et al.* 2007 (20) describe an enhanced radiosensitivity of grade II chondrosarcoma cells after the combined knockdown of two of the three antiapoptotic targets *BCL2*, *BCL-X<sub>L</sub>* and *XIAP* compared with the mono-target inhibition. However, not all combinations intensified the level of radiosensitivity obtained with the single target inhibitions. Lei *et al.* (21) report that a simultaneous *BCL2* and *BCL-X<sub>L</sub>* inhibition by siRNA expression vectors in human hepatoblastoma cells

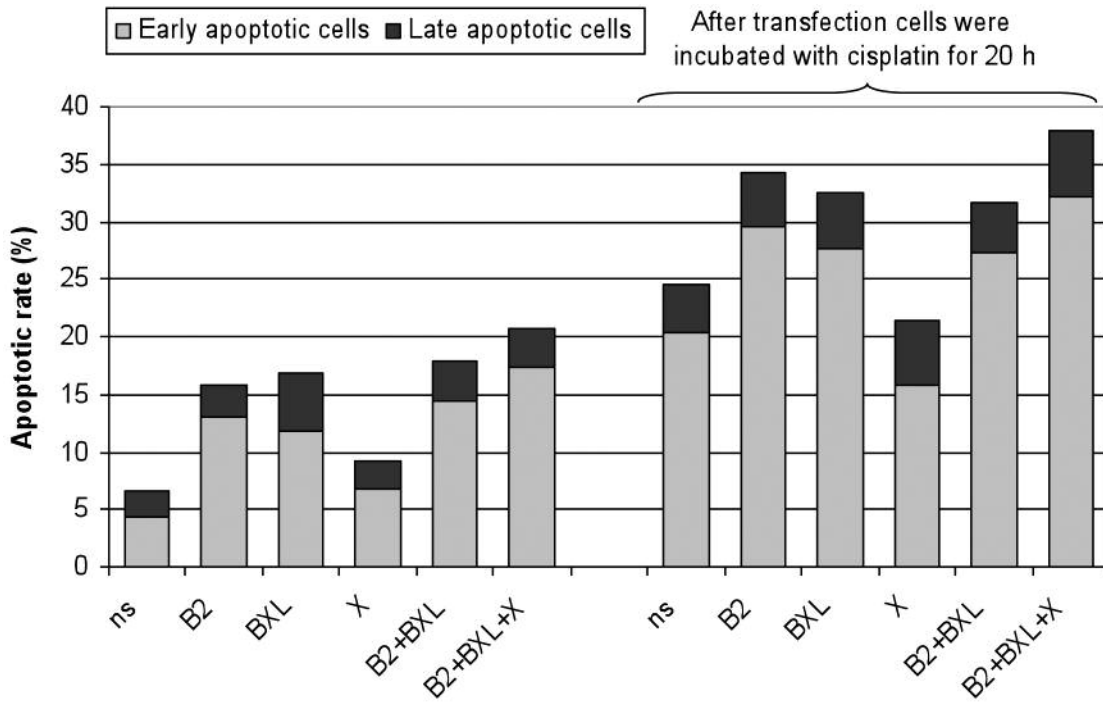


Figure 1. Rate of early and late apoptotic cells 72 h after treatment of EJ28 cells with siRNAs with or without subsequent chemotherapy with cisplatin. Abbreviations: ns, ns-siRNA; B2, BCL2; BXL, BCL-X<sub>L</sub>; X, XIAP.

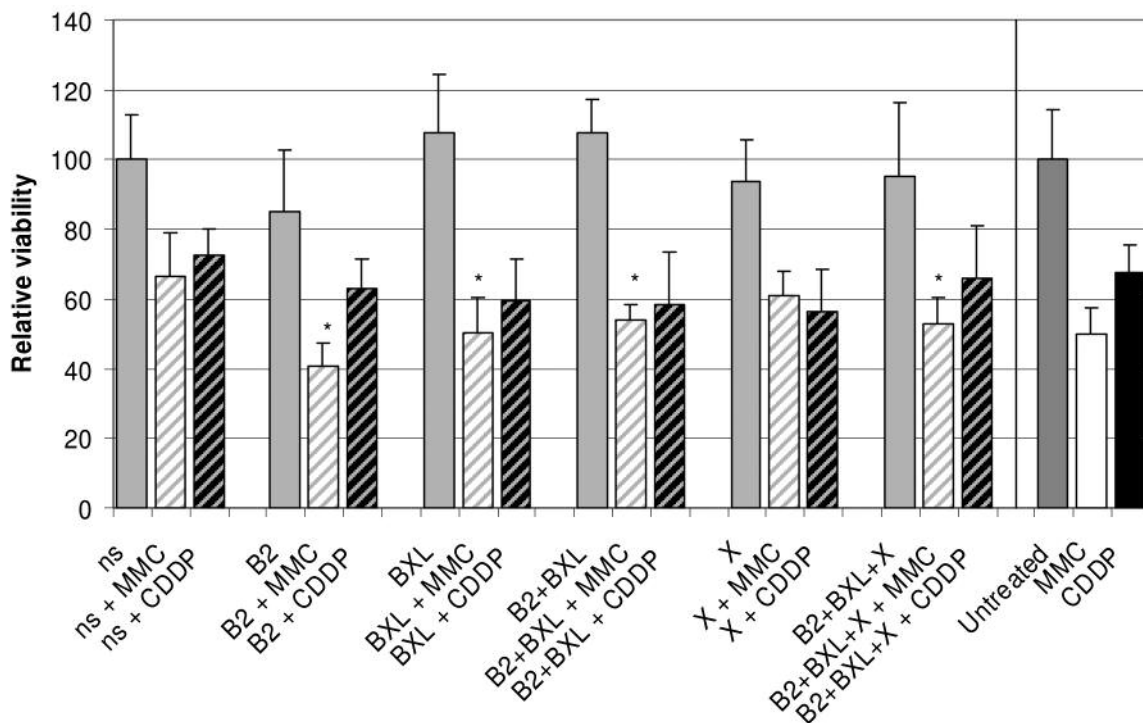


Figure 2. Relative viability of EJ28 cells 96 h after transfection with siRNA with or without subsequent chemotherapy (CT) with mitomycin C (MMC) or cisplatin (CDDP). Except for CT monotherapies (normalized to untreated cells), values are normalized to ns-siRNA treated cells (=100%). Asterisks indicate a significant difference compared to ns-siRNA+CT ( $p < 0.05$ ). Abbreviations: ns, ns-siRNA; B2, BCL2; BXL, BCL-X<sub>L</sub>; X, XIAP.

further increased the sensitivity of the cells towards 5-fluorouracil and 10-hydroxycamptothecin than the single target knockdown. These results demonstrate the potential of the simultaneous targeting of apoptosis inhibitors for anticancer treatment. Like the switch from BCL2 to BCL-X<sub>L</sub> expression in human leukemia HL-60 cells (17), a takeover of the function of one antiapoptotic protein by another, *e.g.* because of the simultaneous expression of multiple apoptosis inhibitors or changes in the expression profile, is possible. Therefore, it is important to examine the long-term effects of the combined silencing of apoptosis inhibitors in *in vivo* studies. Furthermore, if only one gene is targeted, determining its expression before treatment would be required to avoid unnecessary therapy – using siRNAs against different genes together might make this unnecessary. Of course, it would be advantageous to add more siRNAs against further inhibitors of apoptosis, *e.g.* survivin, or other tumor-associated genes which are involved in cell cycle regulation, angiogenesis or immortalization.

Achieved antiproliferative effects after the combined down-regulation of all three targets – which, despite lower siRNA concentrations used per target, were comparable to the mono-target treatments – confirm the possibility of a multitarget inhibition of antiapoptotic proteins.

## Acknowledgements

The authors would like to express their gratitude to Joerg Hofmann (Department of Urology) and Antje Zobjack (Institute of Pathology) for their excellent technical assistance. This study was supported by a grant of the Dr. Robert Pflieger-Stiftung (to S.F. & A.M.).

## References

- Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. *CA Cancer J Clin* 57: 43-66, 2007.
- Leppert JT, Shvarts O, Kawaoka K, Lieberman R, Belldegrun AS and Pantuck AJ: Prevention of bladder cancer: a review. *Eur Urol* 49: 226-234, 2006.
- Kawano H, Komaba S, Yamasaki T, Maeda M, Kimura Y, Maeda A and Kaneda Y: New potential therapy for orthotopic bladder carcinoma by combining HVJ envelope with doxorubicin. *Cancer Chemother Pharmacol* 61: 973-978, 2008.
- Shelley MD, Wilt TJ, Court J, Coles B, Kynaston H and Mason MD: Intravesical bacillus Calmette-Guerin is superior to mitomycin C in reducing tumour recurrence in high-risk superficial bladder cancer: a meta-analysis of randomized trials. *BJU Int* 93: 485-490, 2004.
- Dallas A and Vlassov AV: RNAi: a novel antisense technology and its therapeutic potential. *Med Sci Monit* 12: RA67-74, 2006.
- Fuessel S, Meye A, Kraemer K, Kunze D, Hakenberg OW and Wirth MP: Synthetic nucleic acids as potential therapeutic tools for treatment of bladder carcinoma. *Eur Urol* 51: 315-326, 2007; discussion 326-317.
- Lebedeva I, Raffo A, Rando R, Ojwang J, Cossum P and Stein CA: Chemosensitization of bladder carcinoma cells by Bcl-xL antisense oligonucleotides. *J Urol* 166: 461-469, 2001.
- Schimmer AD, Dalili S, Batey RA and Riedl SJ: Targeting XIAP for the treatment of malignancy. *Cell Death Differ* 13: 179-188, 2006.
- Duggan BJ, Maxwell P, Kelly JD, Canning P, Anderson NH, Keane PF, Johnston SR and Williamson KE: The effect of antisense Bcl-2 oligonucleotides on Bcl-2 protein expression and apoptosis in human bladder transitional cell carcinoma. *J Urol* 166: 1098-1105, 2001.
- Gleave ME and Monia BP: Antisense therapy for cancer. *Nat Rev Cancer* 5: 468-479, 2005.
- Hong JH, Lee E, Hong J, Shin YJ and Ahn H: Antisense Bcl2 oligonucleotide in cisplatin-resistant bladder cancer cell lines. *BJU Int* 90: 113-117, 2002.
- Li M, Song T, Yin ZF and Na YQ: XIAP as a prognostic marker of early recurrence of nonmuscular invasive bladder cancer. *Chin Med J (Engl)* 120: 469-473, 2007.
- Bilim V, Kasahara T, Hara N, Takahashi K and Tomita Y: Role of XIAP in the malignant phenotype of transitional cell cancer (TCC) and therapeutic activity of XIAP antisense oligonucleotides against multidrug-resistant TCC *in vitro*. *Int J Cancer* 103: 29-37, 2003.
- Amantana A, London CA, Iversen PL and Devi GR: X-linked inhibitor of apoptosis protein inhibition induces apoptosis and enhances chemotherapy sensitivity in human prostate cancer cells. *Mol Cancer Ther* 3: 699-707, 2004.
- Glackin AJ, Gray SB, Johnston SR, Duggan BJ and Williamson KE: Antisense oligonucleotides in the treatment of bladder cancer. *Expert Opin Biol Ther* 5: 67-77, 2005.
- Benimetskaya L, Miller P, Benimetsky S, Maciaszek A, Guga P, Beaucage SL, Wilk A, Grajkowski A, Halperin AL and Stein CA: Inhibition of potentially anti-apoptotic proteins by antisense protein kinase C-alpha (Isis 3521) and antisense bcl-2 (G3139) phosphorothioate oligodeoxynucleotides: relationship to the decreased viability of T24 bladder and PC3 prostate cancer cells. *Mol Pharmacol* 60: 1296-1307, 2001.
- Han Z, Chatterjee D, Early J, Pantazis P, Hendrickson EA and Wyche JH: Isolation and characterization of an apoptosis-resistant variant of human leukemia HL-60 cells that has switched expression from Bcl-2 to Bcl-xL. *Cancer Res* 56: 1621-1628, 1996.
- Lima RT, Martins LM, Guimaraes JE, Sambade C and Vasconcelos MH: Specific down-regulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. *Cancer Gene Ther* 11: 309-316, 2004.
- Schmidt U, Fuessel S, Koch R, Baretton GB, Lohse A, Tomasetti S, Unversucht S, Froehner M, Wirth MP and Meye A: Quantitative multi-gene expression profiling of primary prostate cancer. *Prostate* 66: 1521-1534, 2006.
- Kim DW, Seo SW, Cho SK, Chang SS, Lee HW, Lee SE, Block JA, Hei TK and Lee FY: Targeting of cell survival genes using small interfering RNAs (siRNAs) enhances radiosensitivity of Grade II chondrosarcoma cells. *J Orthop Res* 25: 820-828, 2007.
- Lei XY, Zhong M, Feng LF, Zhu BY, Tang SS and Liao DF: siRNA-mediated Bcl-2 and Bcl-xL gene silencing sensitizes human hepatoblastoma cells to chemotherapeutic drugs. *Clin Exp Pharmacol Physiol* 34: 450-456, 2007.

Received January 23, 2008

Revised April 4, 2008

Accepted April 21, 2008