Bcl-2 downregulation sensitizes nonsmall cell lung cancer cells to cisplatin, but not to docetaxel

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The antiapoptotic protein Bcl-2 contributes to a more chemoresistant phenotype of nonsmall cell lung cancer and therefore serves as an important target for novel anticancer strategies. Interestingly, docetaxel as a standard of care for treatment of nonsmall cell lung cancer has been shown to inactivate the Bcl-2 function by phosphorylation. We investigated the Bcl-2 expression status of nonsmall cell lung cancer cells in response to cisplatin or docetaxel and its effect on sensitizing nonsmall cell lung cancer cells by Bcl-2 downregulation employing a small interfering RNA approach. Bcl-2 expression was assessed by Western blotting and RT-PCR. Cell proliferation and apoptosis of nonsmall cell lung cancer cells were measured by an MTS-based assay and Annexin V/7-Aminoactinomycin, respectively. Combination treatment of Bcl-2 small interfering RNA with cisplatin resulted in a synergistic activity. By contrast, Bcl-2 downregulation did not sensitize nonsmall cell lung cancer cells to docetaxel. Of note, docetaxel treatment resulted in Bcl-2 phosphorylation of nonsmall cell lung cancer cells, whereas cisplatin increased the Bcl-2 overall expression and abrogated Bcl-2 phosphorylation. On the basis of our findings, a Bcl-2 silencing approach appears to be a suitable strategy for sensitizing nonsmall cell lung cancer to cisplatin, but not to docetaxel. *Anti-Cancer Drugs* 18:755–761 © 2007 Lippincott Williams & Wilkins.

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Introduction

Lung cancer is the leading cause of cancer-related death with overall cure rates of less than 15% [1]. Nonsmall cell lung cancer (NSCLC), the most common form of lung cancer, may only be treated by surgical resection in its early stages, but even then the 5-year survival rate remains poor [1]: recent studies report a consistent, only about 10% relative risk reduction for mortality at 5 years for adjuvant chemotherapy [2–4].

Over recent years, it has become obvious that defects in the regulation of apoptosis are of outstanding importance for the biology and treatment resistance of cancer [5]. Apoptosis can be mediated by two converging cascades referred to as the 'extrinsic' and 'intrinsic' pathway of apoptosis. One of the major antiapoptotic players of the intrinsic pathway is the Bcl-2 protein [6]. The physiological relevance of Bcl-2 as an apoptosis suppressing protein has been studied extensively, yet the detailed mechanism of action at a biochemical level still remains to be elucidated [7–9].

In NSCLC, overexpression of Bcl-2 has been reported in about 50% of all cases [10,11]. Data from clinical studies suggest that Bcl-2 contributes to a more malignant NSCLC phenotype [12] and confers resistance to chemotherapy in NSCLC [12,13]. Thus, Bcl-2 represents a promising target for novel molecular therapies to overcome the treatment resistance of NSCLC. Currently, there are several Bcl-2 targeting approaches (e.g. antisense oligonucleotides) [12,14] under clinical investigation for NSCLC in combination with standard chemotherapeutic regimens.

Among the most frequent chemotherapeutics employed for NSCLC treatment are cisplatin and docetaxel [15,16]. Both drugs mediate their antitumor effect at least in part by inducing apoptosis [17,18]. Bcl-2 overexpression confers resistance to cisplatin-induced apoptosis in many types of solid tumors and has been correlated with poor response to cisplatin chemotherapy [19].

In contrast, docetaxel has been reported to shift the apoptotic rheostat toward apoptosis via phosphorylation of Bcl-2 [20,21] and Bcl-2 overexpression enhances *in vitro* sensitivity against docetaxel in NSCLC [22]. Thus, it is not known so far whether Bcl-2 downregulation sensitizes NSCLC to cisplatin and docetaxel.

In this study, we have set out to investigate the Bcl-2 expression status of NSCLC cells following incubation with docetaxel or cisplatin and to elucidate the consequences of simultaneous Bcl-2 downregulation by employing a small interfering RNA (siRNA) approach.

Materials and methods

Cell culture

The human NSCLC cell line A549 was obtained from ATCC (CCL-185; Manassas, Virginia, USA) and cultured in DMEM 4500 (GIBCO-BRL, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL) and 1% antibiotic–antimycotic mix (GIBCO-BRL) in a humidified 5% CO_2, 95% ambient air atmosphere at 37°C.

Small interfering RNA molecules

Double-stranded 21-mer siRNA molecules with a 2-base overhang at the 3′-end of the antisense strand targeting human Bcl-2 were kindly provided by Alnylam Europe (Kulmbach, Bavaria, Germany). The human Bcl-2 mRNA target sequence was 5′-UACGAUAACCGGGAGAUAGUGAU-3′ (Bcl2 siRNA). As control, a 21-mer double-stranded siRNA targeted against luciferase (5′-CUUACGCUGAGUACUUCGAdTdT-3′) was applied.

Treatment of cells

A549 cells were seeded 24 h before siRNA treatment to allow adherent cell growth. Then, cells were incubated for 4 h with siRNA molecules precomplexed with Oligofectamin in Opti-MEM medium (both from Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. After incubation, a FBS-containing medium was added to a final FBS concentration of 10% and cells were cultivated under standard conditions. For treatment with cisplatin (Ebewe, Unterach, Austria) or docetaxel (Sanofi-Aventis, Bridgewater, New Jersey, USA), A549 cells were incubated with cisplatin or docetaxel 24 h after transfection with siRNA molecules at the concentrations as indicated.

Real-time PCR

Total RNA was isolated from cells by use of the TRI Reagent (Sigma, St Louis, Missouri, USA). Synthesis of cDNA was performed using the first-strand cDNA synthesis kit (Fermentas, Ontario, Canada). Real-time PCR was performed using TaqMan Universal Master Mix and an ABI Prism 7700 Sequence Detection system according to the manufacturer instructions (Applied Biosystems, Foster City, California, USA). Bcl-2 primers and TaqMan probes, included in the Bcl-2 assay-on-demand gene expression kit Hs00153350_m1, were purchased from Applied Biosystems. Relative gene expression was quantified using the comparative threshold cycle method and β-actin as an internal standard. Data are presented relative to untreated control samples.

Western blotting

Western blotting for Bcl-2 and actin was performed as described previously [23]. Whole-cell extracts were prepared. Afterwards, 10 µg of total protein were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted to polyvinylidine diflouride membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked for 1 h in 0.2% I-block (Tropix, Bedford, Massachusetts, USA) and 0.1% Tween 20 (Sigma) in phosphate-buffered saline, and then incubated with monoclonal antibodies recognizing Bcl-2 (Zymed, San Francisco, California, USA), pBcl-2 (Cell Signaling Technology, Danvers, Massachusetts, USA) or actin (Sigma). Incubation with second antibodies conjugated to alkaline phosphatase (Tropix) was carried out and reactive bands were detected by chemiluminescence (CSPD substrate; Tropix). The expression levels of Bcl-2, pBcl-2 and actin protein were quantified by densitometry (TotalLab, version 1.1). Signal strength of each Bcl-2 and pBcl-2 signal was normalized against the respective actin control.

Proliferation assays

For the assessment of cell growth, cells were incubated with siRNA molecules and exposed to cisplatin or docetaxel at the time points and doses indicated. Cell growth was determined by the Cell Titer 96 assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol using a Victor2 Wallac plate reader (Perkin Elmer, Boston, Massachusetts, USA).

Apoptotic cell death assessment

Apoptotic cells were quantified by Annexin V–phycoerythrin and 7-Aminoactinomycin (7-AAD) (Becton Dickinson, New Jersey, USA) staining using flow cytometrical analysis. Annexin V-positive cells were quantitated.

Cells were washed in cold phosphate-buffered saline and then resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/ml. Five milliliters of Annexin V–phycoerythrin and 5 µl of 7-AAD were added to 100 µl cell suspension. The cells were vortexed and afterwards incubated for 15 min at room temperature in the dark. A minimum of 10 000 events was analyzed on a FACScalibur flow cytometer with an argon laser tuned at 488 nm and use of the CellQuest software (Becton, Dickinson).

Statistical analysis

Statistical significance of differences among treatment groups was determined by using one-way analysis of variance and Bonferroni post-hoc test analysis using SPSS software (SPSS 10.0.7; SPSS, Chicago, Illinois, USA). P values of ≤ 0.05 were considered to be of statistical significance.

Results

Cisplatin induces Bcl-2 protein expression in human nonsmall cell lung cancer cells

The antiapoptotic protein Bcl-2 is expressed in a variety of solid tumor entities [5,7]. Notably, expression of Bcl-2 in some solid cancers can be modulated by chemotherapeutic treatment [5]. Therefore, we tested whether treatment by cisplatin or docetaxel influences the expression of Bcl-2 in NSCLC cells. As presented in Fig. 1a, we observed a clear increase in Bcl-2 protein expression to 149% (SD ± 16) after treatment with cisplatin relative to saline-treated A549 cells, whereas docetaxel did not modulate Bcl-2 expression (105%, SD ± 17).

pBcl-2 induction by docetaxel

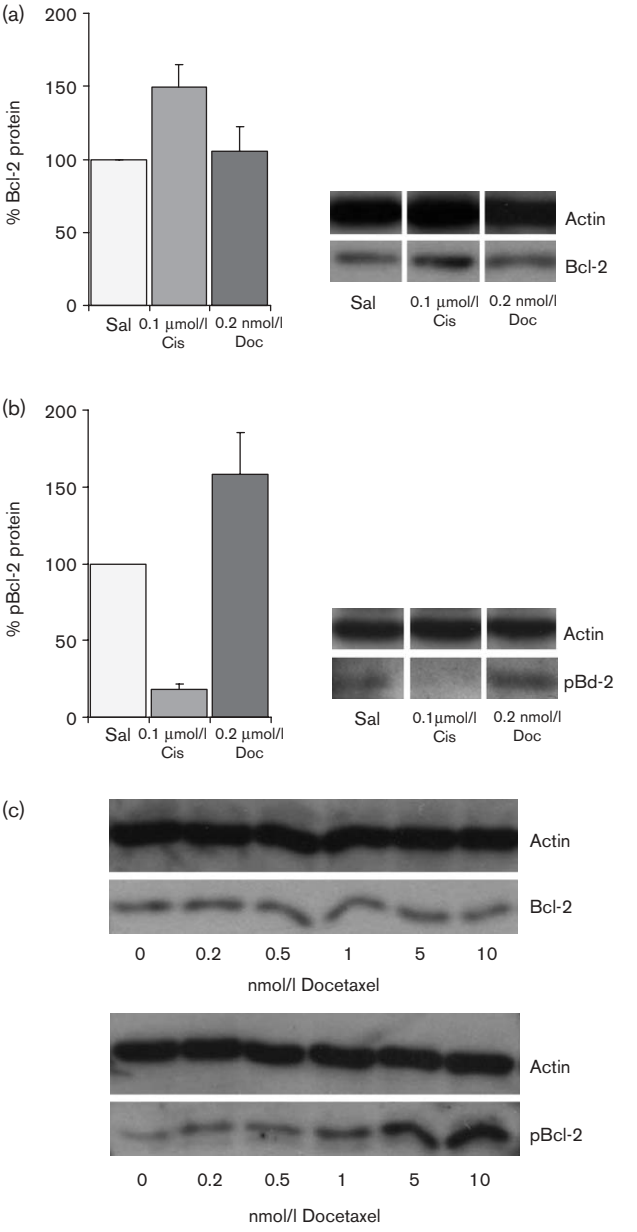
Docetaxel is able to phosphorylate Bcl-2 leading to inactivation of the antiapoptotic function of Bcl-2 [20,21]. Ser70 is postulated as the major phosphorylation site [24] and is mostly investigated as the relevant Bcl-2 phosphorylation site. In line with these findings, treatment with docetaxel resulted in an increase of the Ser70 phosphorylation site of Bcl-2 by about 60% in A549 cells compared with saline-treated cells (Fig. 1b, 158% relative to saline, SD ± 27). In dose-range experiments, treatment of A549 cells with docetaxel resulted in a dose-dependent increase in Ser70 phosphorylation, whereas Bcl-2 expression showed no relevant changes (Fig. 1c). In contrast, cisplatin nearby abrogated Ser-70 phosphorylation in A549 cells (Fig. 1b; 17% relative to saline, SD ± 4).

Dose- and time-dependent Bcl-2 downregulation by Bcl-2 small interfering RNA

Chemically synthesized siRNA allows gene-specific modulation of target gene expression [25,26]. Bcl-2 siRNA led to a dose- and time-dependent suppression of bcl-2 mRNA and protein (Fig. 2). Different siRNAs targeting bcl-2 were tested and for further analysis only the best one was used (data not shown). Bcl-2 mRNA and protein level of A549 cells were studied after treatment with increasing concentrations of siRNA ranging from 2 to 100 nmol/l (Fig. 2). Starting from 2 nmol/l siRNA, a slight downregulation of Bcl-2 mRNA was observed beginning from 24 h after transfection (1.4-fold reduction relative to saline, SD ± 0.09). At concentrations of 12.5 nmol/l or higher we found an about 2.2-fold reduction of Bcl-2 mRNA in A549 cells compared with saline. Further dose escalation to 100 nmol/l siRNA did not result in a more pronounced downregulation (Fig. 2a). Treatment with control siRNA did not alter significantly bcl-2 mRNA.

On protein level Bcl-2 downregulation was observed at siRNA concentrations of 12.5 nmol/l or higher (Fig. 2b). At 72 h, we found a maximum downregulation of Bcl-2 protein by 40–50% relative to saline or siRNA control treatment (relative to saline: 2 nmol/l, −43%; 12.5 nmol/l, −46%; 25 nmol/l, −41%, 50 nmol/l, −48%; 100 nmol/l, −66%). Treatment with control siRNA did not alter Bcl-2 protein level.

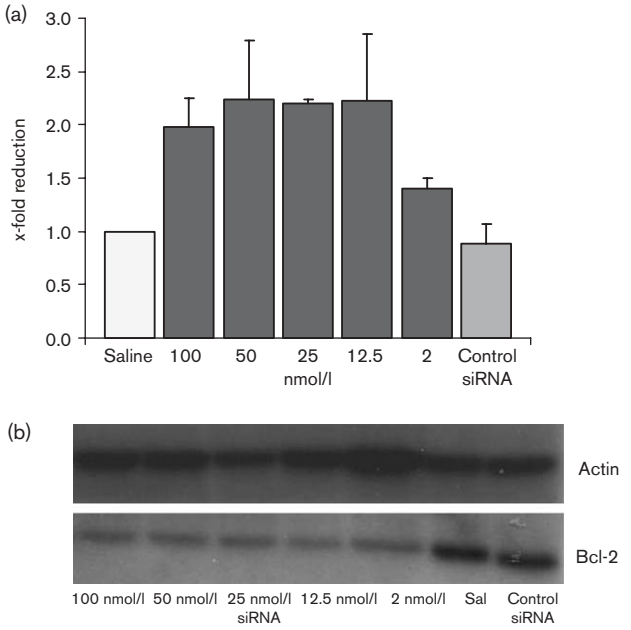
Fig. 1



(a) Bcl-2 induction by cisplatin (Cis) A549 cells were incubated with 0.1 µmol/l cisplatin, 0.2 nmol/l docetaxel or saline (Sal) for 48h. Total cell protein extracts were analysed by Western blotting. Bars = SD. (b) pBcl-2 induction by docetaxel. A549 cells were incubated with 0.1 µmol/l cisplatin, 0.2 nmol/l docetaxel or saline (Sal) for 48h. Total Phospho protein extracts were analysed by Western blotting. Bars = SD. (c) Docetaxel dose range for Bcl-2 and pBcl-2 regulation.

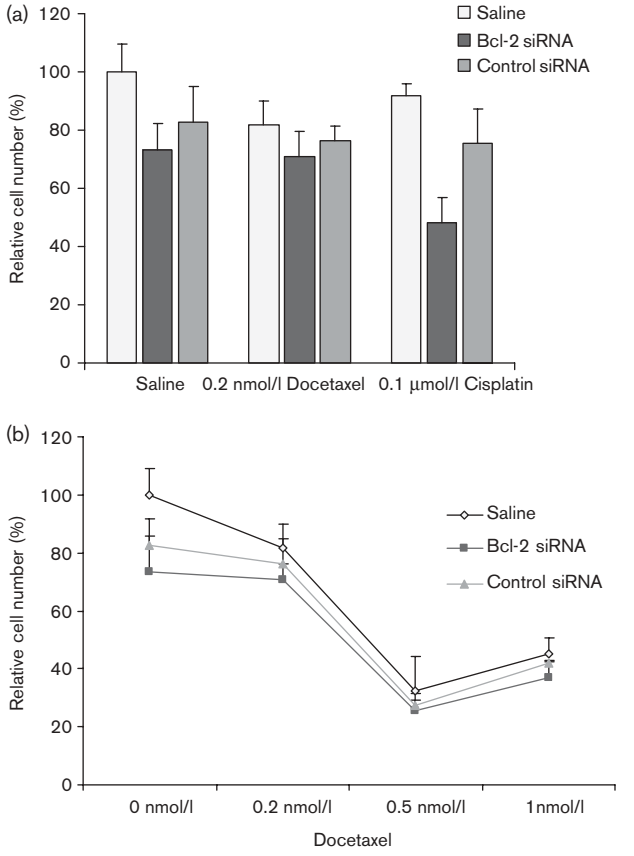
Several reports had referred to unspecific, nonsequence-dependent off-target effects caused by siRNA at higher concentrations leading to cell growth inhibition [27]. Therefore, the effect of siRNA on cell proliferation was

Fig. 2



Dose-dependent Bcl-2 downregulation by Bcl-2 small interfering RNA (siRNA). (a) RNA level and (b) protein level. A549 cells were treated with increasing concentrations of Bcl-2 SIRNA or control SIRNA. Cells were harvested for RNA and Western blot analysis at 24 and 72 hours after incubation, respectively. Data are expressed relative to saline control. Bars = SD.

Fig. 3



(a) Single agent and synergistic reduction in proliferation by Bcl-2 siRNA. (b) A549 cells were incubated with saline, control SIRNA or Bcl-2 SIRNA at a concentration of 25 nmol/l. 24 hours after transfection, 0.1 µmol/l cisplatin or 0.2 nmol/l docetaxel were added. (c) For docetaxel dose range experiments, 0.2 nmol/l-1 nmol docetaxel were added after Bcl-2 SIRNA pre-treatment Bars = SD.

assessed over the same range of siRNA as tested for Bcl-2 target modulation (2–100 nmol/l). Irrespective of the siRNA sequence, siRNA concentrations of 50 and 100 nmol/l resulted in 30% or more cell growth inhibition. Therefore, a concentration of 25 nmol/l siRNA demonstrating maximum target regulation and only minor unspecific cell growth inhibitory effects (less than 20% growth inhibition relative to saline) was defined as the optimal biological concentration for Bcl-2 downregulation in this cellular system and used for further experiments.

Synergistic reduction in proliferation by Bcl-2 small interfering RNA and cisplatin, but not docetaxel

A549 cells were pretreated with Bcl-2 siRNA to study the biological relevance of Bcl-2 as a chemoresistance factor to docetaxel or cisplatin in NSCLC cells. After a 24-h resting period allowing silencing of Bcl-2, A549 cells were exposed to either docetaxel 0.2 nmol/l or cisplatin 0.1 µmol/l. At these concentrations both drugs showed only minor activity in A549 cells (less than 20% growth inhibition), reflecting the chemoresistant phenotype of NSCLC in patients.

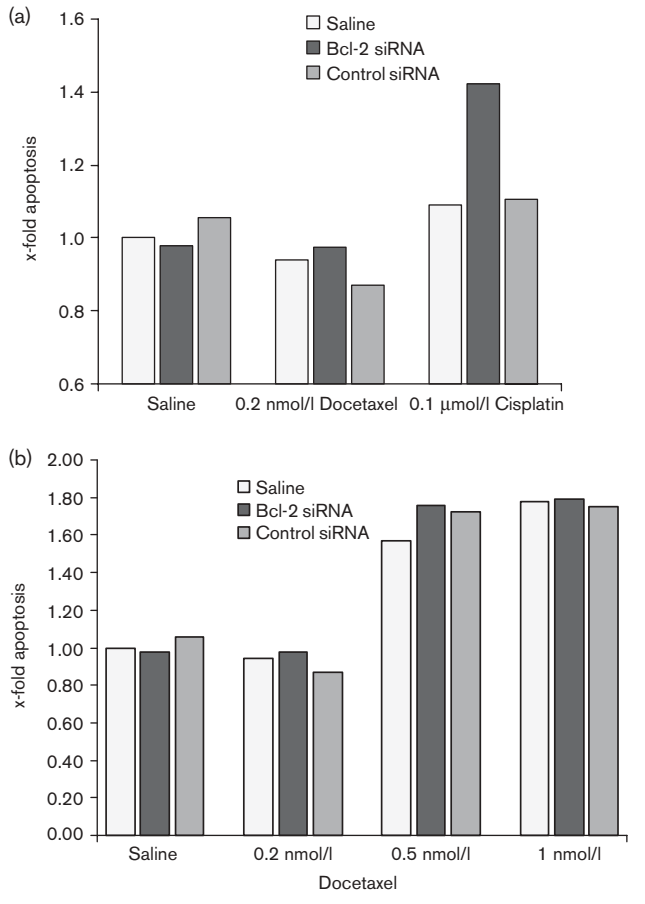
Treatment of A549 cells with 25 nmol/l Bcl-2 siRNA alone decreased the number of cells by about 30% relative to saline control (Fig. 3a). If Bcl-2 siRNA was combined with docetaxel or cisplatin, however, we observed markedly different results for both combination treatments (Fig. 3a). The combination of Bcl-2 siRNA and docetaxel revealed no significant decrease in cell viability exceeding the one observed for control siRNA combined with docetaxel (Bcl-2 siRNA + docetaxel 71% versus control siRNA + docetaxel 76%; $P > 0.05$). In contrast, pretreatment with Bcl-2 siRNA sensitized A549 cells to cisplatin. This combination treatment significantly reduced cell viability by about one-third compared with treatment with control siRNA and cisplatin or Bcl-2 siRNA treatment alone (Bcl-2 siRNA + cisplatin 48%, control siRNA + cisplatin 75%, Bcl-2 siRNA 73%; for both $P < 0.01$). The antitumor effect for the combination of Bcl-2 siRNA and cisplatin was synergistic with a combination index of 0.87 according to the calculation of Bliss [28]. Notably, the combination treatment with control siRNA and cisplatin did not differ from control siRNA treatment alone or cisplatin monotherapy (control siRNA + cisplatin 75%; control siRNA alone 83%; cisplatin alone 92%; for both $P > 0.05$).

To exclude that the investigated concentrations of 0.2 nmol/l docetaxel was chosen to low for inducing apoptosis in chemosensitization experiments, we performed dose range experiments for docetaxel (i.e. 0.2–10 nmol/l docetaxel). Docetaxel single agent treatment at concentrations above 1 nmol/l completely abrogated the viability of A549 NSCLC cells (data not shown). At docetaxel concentrations between 0.2 and 1 nmol/l, none of the combination of Bcl-2 siRNA and the docetaxel concentrations tested (i.e. 0.2, 0.5, 1.0 nmol/l docetaxel) displayed a chemosensitization effect to docetaxel (Fig. 3b).

Bcl-2 downregulation by small interfering RNA induces apoptosis in combination with cisplatin

In light of the synergistic activity of Bcl-2 siRNA and cisplatin on cell proliferation we studied the induction of apoptotic cell death. Single agent treatment by Bcl-2 siRNA, cisplatin or docetaxel resulted in no major induction of apoptosis (Fig. 4a). The combination of Bcl-2 siRNA and cisplatin, however, led to an about 40% increase in apoptotic cell death relative to saline. This induction of apoptosis corresponded to a combination index of 0.75, indicating synergistic activity for the combined treatment with Bcl-2 siRNA [28]. In contrast, treatment with Bcl-2 siRNA and docetaxel showed no obvious increase in apoptosis (Fig. 4a). Of note, no obvious differences in apoptosis induction between control siRNA and saline in combination with cisplatin or docetaxel were observed. Similar to cell proliferation experiments, dose-range experiments with docetaxel 0.2–1 nmol/l showed no increased induction of apoptosis in combination with Bcl-2 siRNA (Fig. 4b).

Fig. 4



(a) A549 cells were incubated with control siRNA, Bcl-2 siRNA and saline at a concentration of 25 nmol/l. 24 hours after transfection, 0.1 μmol/l cisplatin or 0.2 nmol/l docetaxel were added. (b) A549 cells were incubated with control siRNA, Bcl-2 siRNA and saline at a concentration of 25 nmol/l. 24 hours after transfection, 0.1 μmol/l cisplatin or 0.2 nmol/l docetaxel were added.

Discussion

At an advanced stage of disease NSCLC is an incurable malignancy with the currently available treatment options. Downregulation of the antiapoptotic protein Bcl-2 has been proposed as a promising novel strategy to resensitize cancer to standard chemotherapy. For NSCLC, it was not known so far whether Bcl-2 silencing leads to sensitization to cisplatin and docetaxel as the standard of care for NSCLC.

In our experiments, we observed a synergistic activity for Bcl-2 silencing by siRNA and cisplatin as measured by cell growth and apoptosis induction in NSCLC cells. Cisplatin enhanced the Bcl-2 overall expression level in NSCLC cells and clearly dephosphorylated Bcl-2 relative to untreated cells. Both effects may cause a more prominent chemoresistant phenotype mediated by Bcl-2 [12,19–21]. Our findings indicate that Bcl-2 expression contributes to a cisplatin-resistant phenotype of NSCLC, and provide a rationale for combination treatment with cisplatin and Bcl-2 silencing drugs as a potential therapeutic approach in NSCLC.

Bcl-2 silencing, however, did not sensitize NSCLC cells to docetaxel. Docetaxel has been reported to abrogate the antiapoptotic function of Bcl-2 by phosphorylation [29]. In line with previous reports, we observed a clear increase in phosphorylated Bcl-2 after exposure to docetaxel, whereas no change of overall Bcl-2 expression was observed in NSCLC cells. Currently, conflicting data exist about the biological relevance of Bcl-2 phosphorylation by taxanes. Some reports indicate that Bcl-2 phosphorylation may not inhibit its antiapoptotic function [30]. Moreover, docetaxel has been reported to phosphorylate Bcl-2 indeed, but cells may die by a Bcl-2-independent cell death mechanism following exposure to docetaxel [31]. In contrast, several publications report loss of the antiapoptotic effects of Bcl-2 in response to phosphorylation by taxanes [21,32] and induction of apoptosis has been associated with Bcl-2 downregulation by taxanes [21]. Controversially, in ovarian cancer cells

Bcl-2 downregulation appears to be associated with taxane resistance [20].

In line with the above-mentioned data indicating a decrease of the antiapoptotic function of Bcl-2 by phosphorylation, our findings demonstrate that Bcl-2 silencing does not provide any sensitization of NSCLC cells to docetaxel. Since docetaxel might abrogate the antiapoptotic function of Bcl-2 via phosphorylation, Bcl-2 silencing seems not to provide any further benefit. In contrast, downregulation of Bcl-2 seems to shift the apoptotic rheostat and facilitates the induction of apoptosis by cisplatin leading to synergistic activity in NSCLC cells.

We investigated the effect of Bcl-2 downregulation for sensitizing NSCLC to either docetaxel or cisplatin, because both chemotherapeutics are currently standard of care in NSCLC patients [19,33]. The concentrations of cisplatin and docetaxel employed in the experiments were demonstrated in pre-experiments to result in only minor single-agent activity in NSCLC. The rationale behind using such concentrations was to mimic the clinical situation where single-agent treatment also demonstrates only minor antitumor activity [4]. Noteworthy, even higher concentrations of docetaxel with major single-agent activity did not result in any additive or synergistic effect in combination with Bcl-2 siRNA treatment in A549 cells (Figs 3b and 4b).

Targeted downregulation of the intracellular Bcl-2 protein can be obtained by different types of nucleotide therapeutics. *In vitro*, an siRNA approach for silencing Bcl-2 expression, as applied in our experiments, provides an efficient and convenient method to evaluate the biological relevance of a protein of interest. In time-course and dose–response experiments a concentration of 25 nmol/l Bcl-2 siRNA resulted in reliable Bcl-2 downregulation, which led to the recognition of Bcl-2 as a chemoresistance factor in NSCLC cells.

Bcl-2 has also already attracted attention as a promising target to overcome chemoresistance in NSCLC patients. Currently, there is a randomized phase II/III trial ongoing with Bcl-2 antisense oligonucleotides in combination with docetaxel versus standard of treatment alone in NSCLC patients refractory to first-line chemotherapy (www.genta.com). So far, no results of this trial have been published and it remains to be seen whether the combination of Bcl-2 antisense oligonucleotides and docetaxel is a valuable therapeutic strategy for NSCLC patients.

In conclusion, our in-vitro results suggest that cisplatin – but not docetaxel – provides a promising approach for further combination studies with Bcl-2-silencing strategies for sensitizing NSCLC.

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