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Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells

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Antiapoptotic genes such as bcl-2 or xIAP may be responsible for resistance to apoptosis induced by cytotoxic drugs. The aim of this study was to investigate if downregulation of bcl-2 or xIAP by RNA interference (RNAi) would sensitize MCF-7 cells to etoposide and doxorubicin. FITC-siRNAs uptake was verified by fluorescence microscopy and downregulation of Bcl-2 or XIAP was confirmed by Western Blotting. Both siRNAs reduced the number of viable cells and increased cellular apoptosis. Treatment with siRNAs followed by treatment with etoposide or doxorubicin further reduced the number of viable cells, when compared to either of the treatments alone. Therefore, downregulation of bcl-2 or xIAP by RNAi enhances the effects of etoposide and doxorubicin.
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Resistance to chemotherapeutical drugs is an important clinical problem. Most of these drugs act primarily by induction of apoptosis. Consequently, the development of resistance of cancer cells to cytotoxic drugs may be a result of resistance to apoptosis.[1] Cancer cells escape apoptosis by a number of mechanisms among which overexpression of antiapoptotic genes has been shown to play a critical role, such as overexpression of some members of the bcl-2 gene family or the IAP family.[1–4] Among the Bcl-2 protein family members, it has been repeatedly shown that Bcl-2 and Bcl-x_L overexpression delay the onset of apoptosis induced by several cytotoxic drugs.[2–5] More recently, another family of apoptosis antagonists, the inhibitor of apoptosis proteins (IAPs), has been described as acting downstream of Bcl-2 by inhibiting caspases.[4,6–8] In humans, eight members of this family have been identified to date. The most potent member of this family of proteins is XIAP.[6]

Several strategies to overcome the apoptotic resistance of neoplastic populations with potential therapeutic value have been developed. In fact, downregulation of bcl-2 expression by antisense oligonucleotides is currently in the final stages of clinical trials. More recently, considerable attention has been drawn to small organic molecules or short peptides that antagonize or mimic the function of apoptosis-regulating gene products. Promising examples of this approach are the inhibitors of Bcl-2 and Bcl-x_L[9–11] and the short peptides that substitute for the mitochondrial proapoptotic Smac protein.[12] Finally, the successful use of small interfering RNAs (siRNAs) to downregulate gene expression in several model systems[13–18] has led to many attempts to explore this methodology in potentially therapeutic settings.[19,20] Along this line, several recent studies have documented: (a) successful downregulation of BCR-ABL expression, resulting in increased apoptosis and decreased proliferation, in chronic myeloid leukemia cells;[21,22] (b) the MDR1 downregulation followed by chemosensitization of human pancreatic and gastric cell lines;[23] (c) combined inhibition of Bcl-2 and Raf-1 in human myeloid leukemia cells resulting in induction of apoptosis and chemosensitization.[24] Bearing in mind that RNAi is feasible in MCF-7 cell line[25–27] and that these cells are known to be relatively resistant to etoposide and doxorubicin,[28,29] the purpose of this study was to investigate if specific downregulation of bcl-2 or xIAP gene expression by RNAi sensitized MCF-7 cells to those chemotherapeutic drugs.

Materials and methods

siRNAs and cell lines

siRNAs targeting bcl-2 and xIAP mRNAs were designed according to the siRNA user guide (www.mpibpc.gwdg.de/abteilungen/100/105/siRNAuserguide.pdd) on 10 February, 2002. The siRNAs with the following sense

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and antisense sequences were: (a) for bcl-2 sense 5′-CCG GGA GAU AGU GAU GAA GdTdT-3′ and antisense 5′-CUU CAU CAC UAU CUC CCG GdTdT-3′ (b) for xIAP, sense 5′-GGA GAU ACC GUG CGG UGC UdTdT-3′ and antisense 5′-AGC ACC GCA CGG UAU CUC CdTdT-3′. The control (nonsilencing) siRNA used was that designed by Xeragon with the following sequences: sense 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′ and antisense 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′. All siRNAs were purchased from Xeragon and dissolved in the siRNAs buffer, as recommended by the manufacturer. MCF-7 human breast adenocarcinoma cells were routinely cultured in MEM (with Earle's salts and L-glutamine, Gibco) with 10% FBS (Gibco) and incubated in a humidified incubator at 37°C with 5% CO_2 in air.

Transfection of cells with siRNAs, verification of uptake and confirmation of downregulation of bcl-2 and xIAP

Cells were transfected 24 hours after plating at 1.5×10^4 cells/well in 24-well plates, with Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer's instructions. During the initial 3 hours of the transfection protocol, the control, bcl-2 or xIAP siRNA concentrations were 280 nM, these concentrations being reduced to 55.6 nM thereafter. Control cells were transfected with the siRNAs buffer alone. For the study on the uptake of FITC-labelled siRNAs (Xeragon), cells were cultured in four-well slides (Falcon), which were observed by fluorescence microscopy 24 hours after transfection (Olympus BH-2 microscope). For confirmation of downregulation of Bcl-2 and XIAP proteins, cells were seeded and transfected as indicated above and processed at the following time points: 24, 48 and 72 hours. Cells were lysed in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Boehringer Mannheim), proteins were quantified and separated in 10% Bis-Tris gel (Novex). Samples were transferred to a nitrocellulose membrane (Amersham) with the Novex Electrophoresis System. The membrane was incubated with one of the following primary antibodies: mouse Bcl-2 antibody (1:100, DAKO), mouse XIAP antibody (1:250, Transduction Laboratories) or goat actin antibody (1:2000, Santa Cruz Biotechnology). The membrane was then incubated with one of the following secondary antibodies: goat anti-mouse IgG -HRP (1:2000, Santa Cruz Biotechnology) or donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology). Subsequently, the membrane was incubated with streptavidin–HRP 1:1500 (Amersham) and the signal detected with the ECL-Plus Amersham kit (Amersham), the Hyperfilm ECL (Amersham) and the Kodak GBX developer and fixer twin pack (Sigma) as previously described.[30]

Cell growth and viability, programmed cell death and BrdU proliferation assay

Cell growth and viability studies were performed 96 hours following transfection, using the Trypan Blue exclusion assay. Cells were analyzed for apoptosis 48, 72 and 96 hours after transfection with the siRNAs, using the "*in situ* cell death detection kit" (Roche). In brief, cytopsins were prepared and fixed in 4% paraformaldehyde solution. Cells were permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) and incubated with TUNEL reaction mix, according to the optimized procedure recommended by the manufacturer (enzyme dilution 1:20). Cells were observed in a DM IRE 2 microscope (LEICA) and a semiquantitative evaluation was performed by counting a minimum of 500 cells per slide. For the proliferation studies, BrdU incorporation was analyzed 48, 72 and 96 hours after transfection with the siRNAs. After a pulse of BrdU (10 μM, Sigma) for 4 hours, cytopsins were fixed in 4% paraformaldehyde solution and incubated with 2 M HCl. Cells were incubated with mouse anti-BrdU (1:10, DAKO) and further incubated with anti-mouse IgG-biotinylated from Vectastain ABC Kit (1:200, VECTOR). The signal was developed with DAB chromogen solution (DAKO) and counterstained with Mayer's hemalum solution (MERCK). Similarly, for a semiquantitative evaluation, a minimum of 500 cells per slide were counted.

Treatments with chemotherapeutic agents

The chosen time at which etoposide or doxorubicin was added to the previously transfected cells was based on preliminary experiments in which different alternative times were tested. These preliminary experiments had shown that the greatest reduction in number of viable cells (Trypan blue exclusion assay) was obtained when the cytotoxic drugs were added to the cells 48 hours after transfection (results not shown). Therefore, in the experiments to investigate sensitization to etoposide or doxorubicin, cells were treated with the IC_{50} dose of either of these agents (1 μM etoposide or 40 nM doxorubicin, dissolved in DMSO), with DMSO alone or with media alone, 48 hours after transfection. Together with these treatments, further dilution of the cells was carried out by adding 140 μl of medium containing the appropriate drug concentration. Cell growth and viability studies were performed 96 hours following transfection using the Trypan Blue exclusion assay.

Statistical analysis

Differences between treatments with the xIAP or bcl-2 siRNAs and the control siRNAs were analyzed using a one-tailed or two-tailed paired *t*-test as appropriate (Statview for PC).

Results

Uptake of siRNAs and specificity of the siRNAs in gene expression downregulation

The uptake of the siRNAs for bcl-2 or xIAP was verified by transfecting cells with FITC-siRNAs and examining cells by fluorescence microscopy. Uptake was verified through the presence of green fluorescence around the

DAPI-stained nuclei, indicating a cytoplasmatic localization of the siRNAs (Fig 1). The effect of the siRNAs on the respective protein levels was assessed 24, 48 and 72 hours following transfection. Both bcl-2 (Fig 2a) and xIAP (Fig 2b) siRNAs downregulated the expression of the corresponding protein. This downregulation was greatest, in both cases, 48 hours following transfection even though it was also observed at the other time points (data not shown). No downregulation was verified with the control siRNAs, indicating a specific mode of action.

Effects of the siRNAs on cell number

To investigate if treatment with the siRNAs was capable of reducing cell number and viability, the number of viable and total cells was counted 96 hours after transfection. The results here presented (Fig 3) show that both the bcl-2 and the xIAP siRNAs significantly reduced the number of viable cells, which was consistent with a possible increase in spontaneous apoptosis with or without concomitant effects on cellular proliferation.

Effects of the siRNAs on spontaneous apoptosis

In an attempt to study if the effect of the siRNAs for bcl-2 or xIAP on MCF-7 cell number was caused by an increase in spontaneous cellular apoptosis, the TUNEL assay for detection of apoptosis at a single cell level, based on labelling of DNA strand breaks, was carried out 48, 72 and 96 hours following transfection. Results from analysis of the TUNEL assay, by fluorescence microscopy, revealed that both the bcl-2 siRNA and the xIAP siRNA treatments increased the spontaneous apoptosis of these cells (Fig 4 and Table 1) since the cell labelling was increased when compared to the control siRNA

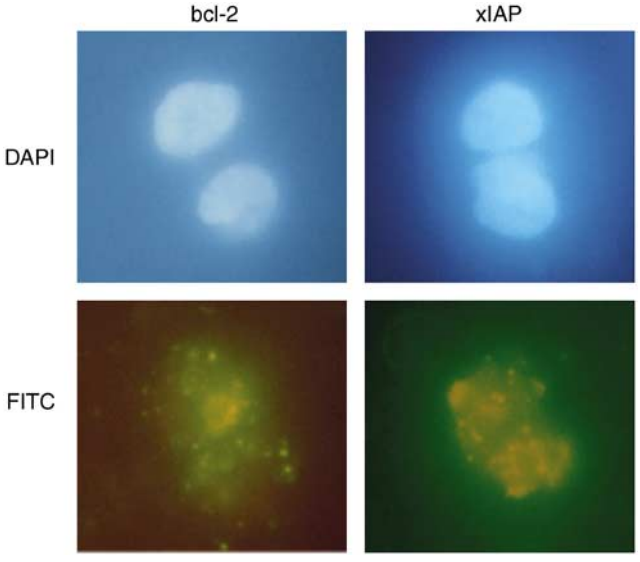

Figure 1 Uptake of FITC-labelled siRNAs for bcl-2 and xIAP in MCF-7 cells, verified by fluorescence microscopy. Slides were prepared and fixed in 4% paraformaldehyde 24 hours after transfection. Nuclei were stained with DAPI and cytoplasmic green fluorescence resulted from siRNA incorporation.

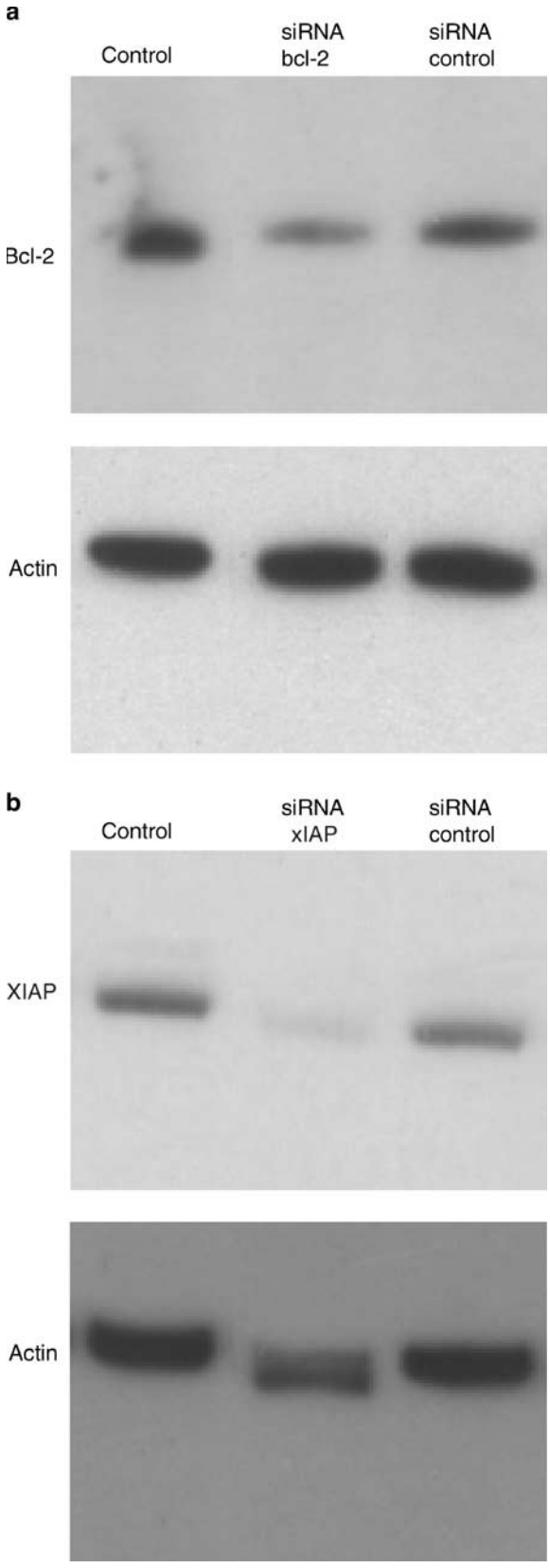

Figure 2 Specificity of the bcl-2 (**a**) and xIAP (**b**) siRNAs. It was possible to observe by Western Blotting that 48 hours after transfection, the protein levels of cells treated with bcl-2 or xIAP siRNAs were clearly decreased when compared with controls.

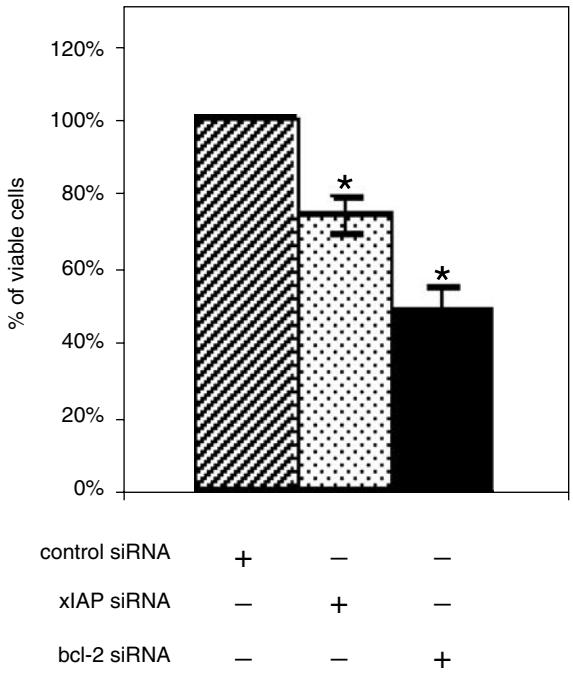

Figure 3 Effects of the siRNAs on the number of viable cells. Results are represented as a % of the control siRNA treatment, considering this as 100%. Results are the mean of three independent experiments (each with three replicates). *Represents $P \leqslant .05$ between the xIAP or the bcl-2 siRNA treatments and the control siRNA treatment.

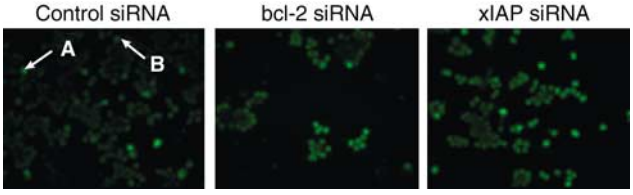

Figure 4 Programmed cell death in MCF-7 cells treated with the siRNAs for bcl-2 or xIAP, detected by the TUNEL assay. A typical apoptotic cell is indicated with the arrow A and a typical nonapoptotic cell is indicated with the arrow B. It is possible to observe increased spontaneous apoptosis in cells treated with the siRNAs for bcl-2 or xIAP.

Table 1 Cellular apoptosis determined by the TUNEL assay

Treatment (hours)	Control siRNA	siRNA bcl-2	siRNA xIAP
48	19%±1%	44%±1%*	36%±3%*
72	20%±3%[a]	29%±1%*	31%±1%*
96	23%±2%	34%±3%*	29%±4%

Quantification of the number of cells labelled apoptotic by the TUNEL assay. Cytopsins were prepared and cell death was quantified by counting at least 500 cells per experiment in 3 independent experiments, using a fluorescent microscope. *Represents $P \leqslant .05$ between the bcl-2 or the xIAP siRNA treatments and the control siRNA treatment, for each time point.
[a]A case where the results are from two experiments only.

treatment, at all time points studied. This increase in apoptosis was greatest 48 hours following transfection.

Effects of the siRNAs on cellular proliferation

In order to verify if the siRNAs for bcl-2 or xIAP encompassed effects on MCF-7 cellular proliferation, the BrdU incorporation was studied 48, 72 and 96 hours following siRNA transfections for bcl-2 or xIAP. Neither in the case of RNA interference of bcl-2 nor xIAP was an alteration in the cellular incorporation of BrdU observed, at all time points studied, when compared with the control RNAi (Table 2). The apparent reduction in cellular proliferation observed at all time points for both xIAP and bcl-2 siRNAs (Fig 5) was due to the increased cellular apoptosis verified at the same time points and was not confirmed when analyzing the results as % of proliferating cells/% of viable cells (Table 2).

bcl-2 and xIAP downregulation sensitizes MCF-7 cells to chemotherapy-induced cell death

Once it was verified that both the bcl-2 and xIAP siRNAs induced cellular apoptosis, it was possible to further evaluate its effects on drug sensitization. The number of viable cells was calculated 48 hours after treatment with the cytotoxic drugs, using the Trypan blue exclusion assay. As expected, the IC_{50} doses of etoposide or doxorubicin given to the cells was followed by a reduction in the number of viable cells to 50% of control, 48 hours after treatment with those drugs (Fig 6). When treatment with siRNAs for bcl-2 was followed by treatment with etoposide or doxorubicin, the number of viable cells was further reduced, when compared to treatment with the control siRNA and etoposide or doxorubicin, respectively ($P \leqslant .05$). This indicates that the siRNAs for bcl-2 sensitized cells to both etoposide and doxorubicin. Concerning treatment with the xIAP siRNAs, when treatment with siRNAs for xIAP was followed by treatment with etoposide or doxorubicin, the number of viable cells was also further reduced when compared to treatment with the control siRNA and etoposide or doxorubicin, respectively, but the differences observed did not reach the level of statistical significance (Fig 6).

Discussion

The results here reported demonstrate that downregulation of Bcl-2 or XIAP protein levels by RNAi is possible in MCF-7 human breast adenocarcinoma cells. This is the first report on RNAi of bcl-2 in MCF-7 cells and, to our knowledge, the first report on RNAi of xIAP in any cell line. Our study shows that downregulation of bcl-2 or xIAP gene expression by RNAi reduces the total number of viable cells by increasing spontaneous apoptosis.

In general terms, the result of downregulation of bcl-2 leading to increased spontaneous apoptosis is expected due to its prominent role in apoptosis regulation. According to the currently accepted model, the decreased levels of Bcl-2 or other antiapoptotic proteins will be

Table 2 Cellular proliferation determined by the BrdU assay

	Treatment (hours)	Control siRNA	siRNA bcl-2	siRNA xIAP
% BrdU-positive cells	48	36%±0.3%	28%±2%*	29%±2%
	72	32%±2%	27%±1%	29%±2%
	96	34%±2%	27%±2%*	29%±3%
% Viable cells	48	81%±1%	56%±1%*	64%±3%*
	72	81%±3%[a]	71%±1%*	69%±1%*
	96	77%±2%	66%±3%*	71%±4%
Proliferating cells (normalized%)	48	44%±0%	49%±3%	45%±2%
	72	42%±0%[a]	38%±1%	43%±4%
	96	44%±2%	42%±2%	41%±3%

Normalized values of BrdU incorporation. The % of cells that incorporated BrdU was corrected for the % of viable cells. Cytopsins were prepared and BrdU incorporation was confirmed by counting at least 500 cells per experiment in three independent experiments. *Represents $P \leqslant .05$ between the bcl-2 or the xIAP siRNA treatments and the control siRNA treatment, for each time point.
[a]A case where the results are from two experiments only.

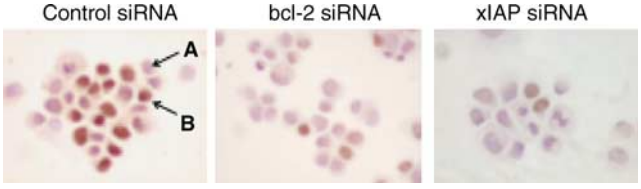

Figure 5 Cellular proliferation in MCF-7 cells treated with the siRNAs for bcl-2 or xIAP, determined using the BrdU proliferation assay. A typical BrdU-negative cell is indicated with the arrow A and a typical BrdU-positive cell is indicated with the arrow B. A lower number of BrdU-positive cells can be observed in cells treated with bcl-2 or xIAP siRNAs when compared with cells treated with control siRNAs. However, the ratio between BrdU-positive cells and viable cells is similar (see Table 2).

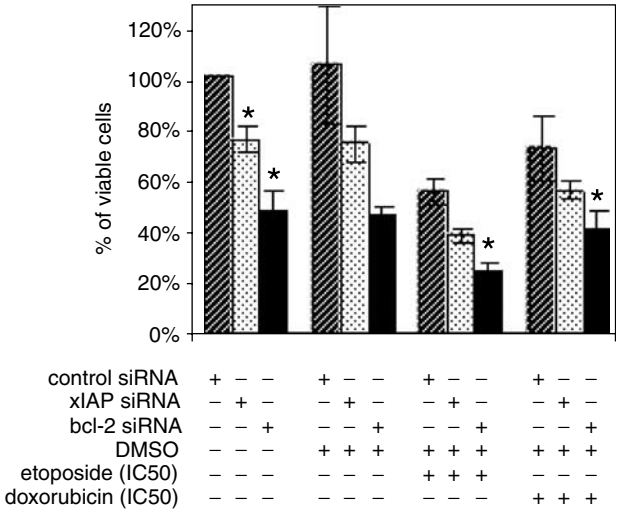

Figure 6 Effects of the siRNAs on sensitization to chemotherapy-induced cell death. The first group of columns represents the effect of the siRNAs on the number of viable cells (as in Fig 3). The remaining groups of columns represent the effects of treatment with siRNAs followed by treatment with DMSO, etoposide or doxorubicin, respectively. Results are represented as a % of the control siRNA treatment, considering this as 100%. Results are the mean of three independent experiments (with three replicates each). *Represents $P \leqslant .05$ between the xIAP or the bcl-2 siRNA treatments and the control siRNA treatment, analyzed individually for each group of columns.

reflected in an increase of free Bax or Bak with subsequent activation and mitochondrial translocation of these proapoptotic proteins.[2] Our finding that RNAi of bcl-2 in MCF-7 cells results in increased spontaneous apoptosis is in accordance with the general model. However, it is known that MCF-7 cells express Bcl-x_L at similar levels to Bcl-2, which might suggest some redundancy of its antiapoptotic functions. Our results show, in fact, that such a functional Bcl-2 and Bcl-x_L redundancy does not occur in MCF-7 cells since the decreased levels of Bcl-2 protein resulting from RNAi are not compensated by the Bcl-x_L levels and increased apoptosis does occur.

The role of XIAP in the maintenance of cell viability in the absence of an apoptotic stimulus is not yet firmly established. The current model for the antiapoptotic function of IAP relies on their capacity to block final activation of caspases and/or enzymatic activity of fully activated caspases.[31,32] Therefore, in the absence of an apoptotic stimulus leading to caspase activation, the IAP function would not occur. Consequently, a role of XIAP in the maintenance of cell viability suggests that low levels of caspase activation may occur in homeostatic conditions. Recent data on xIAP downregulation by antisense strategies in human bladder cancer cell lines[33] and in human ovarian cancer cells[34] do suggest XIAP participation in the maintenance of cell viability and our data, herein reported, strongly supports such a role. In order to fully document these results, one should analyze the pattern of activated caspases (caspases 9 and 3) in control and RNAi-treated cytosolic extracts. This would be aimed at detecting impaired processing of caspase 9 and incomplete processing of caspase 3 in control cells and full processing of these caspases in RNAi-XIAP-partially depleted cells; small differences below the level of detection of Western blotting might be uncovered through

a strategy of apoptosis induction by cytochrome *c* plus ATP in a cell free system. However, these experiments were far beyond the scope of the present work.

It is worth noting that (under the conditions tested) the levels of spontaneous apoptosis observed with siRNAs for xIAP are not much lower than those observed with siRNAs for bcl-2, at each individual time point studied (Table 1). In our study the effects on spontaneous apoptosis were more pronounced 48 hours after transfection than in the following time points studied, possibly due to degradation of the siRNAs at the latter time points.

In spite of the evidence for XIAP participation in cell viability, the siRNAs for bcl-2 exerted a greater effect on the number of MCF-7 viable cells than the siRNAs for xIAP, 96 hours following transfection (Fig 3). This could be due to the slightly higher apoptosis verified at two of the studied time points when cells were treated with bcl-2 siRNAs (Table 1), suggesting a higher number of cells ultimately killed, when compared to treatment with xIAP siRNAs. A possible explanation for the stronger effect of the siRNAs for bcl-2 than the siRNAs for xIAP on the number of viable cells could point to more compensatory mechanisms for the loss of an IAP family member than for the loss of a bcl-2 family member. Indeed, others have shown that XIAP-deficient mice were viable and showed increased levels of c-IAP1 and c-IAP2,[35] whereas only half of the bcl-$2^{-/-}$ mice were viable at 6 weeks of age.[36]

In addition to the antiapoptotic role of Bcl-2 and XIAP, both proteins have been suggested to affect cellular proliferation. Indeed, Bcl-2 overexpression resulted in decreased cellular proliferation in several mammalian cell lines,[37] and bcl-2 downregulation with antisense oligonucleotides in acute myeloid leukemia cell lines enhanced cellular proliferation.[38] Furthermore, XIAP overexpression was shown to induce cell cycle arrest in human endothelial cells.[39] However, we found that downregulation of bcl-2 or xIAP expression in MCF-7 cells had no effect on cellular proliferation. This apparent discrepancy may be accounted for by different endogenous levels of these proteins or different cellular contexts concerning cell cycle regulation. In other words, clear effects on cellular proliferation were described in experimental systems with Bcl-2 or XIAP overexpression but this does not necessarily mean that endogenous levels of these proteins are sufficient for such an effect. Along the same line of reasoning, the endogenous levels of Bcl-2 and the efficiency of bcl-2 downregulation obtained in acute myeloid leukemia cells and in our study are probably different.

The work here described shows that downregulation of bcl-2 by RNAi enhances the effects of etoposide and doxorubicin in MCF-7 human breast cancer cells. Several previous studies have shown that Bcl-2 overexpression inhibited apoptosis induced by drugs in MCF-7 cells,[40,41] and that downregulation of Bcl-2 levels in this cell line by intracellular expression of single-chain antibodies[42] or by stable expression of bcl-2 antisense transcripts[43] increased drug-induced cytotoxicity. Our work provides evidence that downregulation of bcl-2 gene expression by RNAi is an alternative strategy with potential clinical application in human breast adenocarcinoma. Even though downregulation of bcl-2 sensitized cells to both etoposide and doxorubicin, the effect was more pronounced with etoposide. This probably reflects the differences in the protective effect of Bcl-2 observed in breast cancer cells depending on the cytotoxic drug used.[44]

It is very interesting to note that the concentration of bcl-2 siRNA used in the present study was much lower than the concentration of bcl-2 siRNA used in the very recent report of Cioca et al.[24] Indeed, this report indicates that transfection with 800 nM bcl-2 siRNAs induced a minimal spontaneous apoptotic response in HL-60, U937 and THP-1 cell lines, 96 hours after transfection. In the work described here, a statistically significant increase in apoptosis was verified at that time and with a much smaller concentration of siRNAs (280 nM during the 3 hours of transfection and 55.6 nM thereafter). Several factors may contribute to this final result, including technical factors (such as design of the siRNAs, delivery systems, efficiency of the siRNAs) that may play a significant role, as well as biological differences between the tumour populations.

This work also shows that downregulation of xIAP by RNAi enhances the effects of etoposide and doxorubicin in MCF-7 human breast cancer cells. Overexpression of XIAP has been shown to decrease apoptosis following treatment with many different agents.[6] To date, antisense downregulation of xIAP has been reported to result in increased cell death following ionizing radiation of human non-small-cell lung carcinoma cell lines[45] and in enhanced cisplatin-induced cell death in chemoresistant human ovarian cancer cells.[34] Our data, together with that previously reported, strongly suggest that downregulation of xIAP may be a useful complementary anticancer therapeutic strategy.

As we have previously stated, downregulation of gene expression by antisense oligonucleotides or RNAi strategies can be considered a pathway with therapeutic potential to overcome apoptosis resistance of tumour populations, but other pathways do exist. In particular, the small inorganic molecules antagonizing Bcl-2 and Bcl-x_L[9–11] and the small peptides mimicking Smac[12] and resulting in decreased antiapoptotic effect of IAPs are promising in terms of therapeutic potential. It is too early to foresee a comparison between the different approaches under intensive investigation. The relative efficiencies of those approaches may significantly vary among different tumour populations. On the other hand, problems of cellular delivery, so far present in all strategies, may be solved differently which, by itself, may contribute useful arguments for the choice of approach used in specific clinical settings. Ultimately, one can concur that a system of simultaneous delivery of several RNAi, specifically chosen for particular tumour types, possibly involving a viral carrier, might represent a major breakthrough in "tailored" therapy.

Acknowledgments

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References

1. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer*. 2002;2:277–288.
2. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*. 2002;2: 647–656.
3. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science*. 1998;281:1322–1326.
4. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol*. 2002;3:401–410.
5. Huang Z. Bcl-2 family proteins as targets for anticancer drug design. *Oncogene*. 2000;19:6627–6631.
6. Holcik M, Korneluk RG. XIAP, the guardian angel. *Nat Rev Mol Cell Biol*. 2001;2:550–556.
7. Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol*. 2001;2:3009.1–3009.10.
8. LaCasse EC, Baird S, Korneluk RG, et al. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene*. 1998;17:3247–3259.
9. Wang J-L, Liu D, Zhang Z-J, et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci USA*. 2000;97:7124–7129.
10. Shangary S, Johnson DE. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. *Leukemia*. 2003;17:1470–1481.
11. Pei XY, Dai Y, Grant S. The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. *Leukemia*. 2003;17:2036–2045.
12. Arnt CR, Chiorean MV, Heldebrant MP, et al. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 *in situ*. *J Biol Chem*. 2002;277:44236–44243.
13. Waterhouse PM, Wang MB, Lough T. Gene silencing as an adaptative defence against viruses. *Nature*. 2001;411: 834–842.
14. Sharp PA. RNA interference-2001. *Genes Dev*. 2001;15: 485–490.
15. Tuschl T. RNA interference and small interfering RNAs. *Chembiochemistry*. 2001;2:239–245.
16. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet*. 2001;2:110–119.
17. Bosher JM, Labouesse M. RNA interference: genetic wand and genetic watchdog. *Nat Cell Biol*. 2000;2:E31–E36.
18. Bernstein E, Denli AM, Hannon GJ. The rest is silence. *RNA*. 2001;7:1509–1521.
19. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol*. 2003;4:457–467.
20. Sullenger BA, Gilboa E. Emerging clinical applications of RNA. *Nature*. 2002;418:252–258.
21. Wilda M, Fuchs U, Wossmann W, et al. Killing of leukemic cells with BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene*. 2002;21:5716–5724.
22. Scherr M, Battmer K, Winkler T, et al. Specific inhibition of *bcr-abl* gene expression by small interfering RNA. *Blood*. 2003;101:1566–1569.
23. Nieth C, Priebsch A, Stege A, et al. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). *FEBS Lett*. 2003;545: 144–150.
24. Cioca DP, Aoki Y, Kiyosawa K. RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. *Cancer Gene Ther*. 2003;10: 125–133.
25. Abdelrahim M, Samudio I, Smith III R, et al. Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. *J Biol Chem*. 2002; 277:28815–28822.
26. Abdelrahim M, Smith R, Safe S. Aryl hydrocarbon receptor gene silencing with small inhibitory RNA differentially modulates Ah-responsiveness in MCF-7 and HepG2 cancer cells. *Mol Pharmacol*. 2003;63:1373–1381.
27. Srinivasula SM, Gupta S, Datta P, et al. Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. *J Biol Chem*. 2003;278: 31469–31472.
28. Friedrich K, Wieder T, Von Haefen C, et al. Overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cell lines with acquired drug resistance. *Oncogene*. 2001;20:2749–2760.
29. Yang X-H, Sladek TL, Liu X, et al. Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. *Cancer Res*. 2001;61:348–354.
30. Vasconcelos MH, Beleza SS, Quirk C, et al. Limited synergistic effect of antisense oligonucleotides against bcr-abl and transferrin receptor mRNA in leukemic cells in culture. *Cancer Lett*. 2000;152:135–143.
31. Riedl SJ, Renatus M, Schwarzenbacher R, et al. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 2001;104:791–800.
32. Srinivasula SM, Hegde R, Saleh A, et al. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*. 2001;410:112–116.
33. Bilim V, Kasahara T, Hara N, et al. Role of XIAP in the malignant phenotype of transitional cell cancer (TCC) and the therapeutic activity of XIAP antisense oligonucleotides against multidrug-resistant TCC *in vitro*. *Int J Cancer*. 2003;103:29–37.
34. Sasaki H, Sheng Y, Kotsuji F, et al. Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. *Cancer Res*. 2000;60:5659–5666.
35. Harlin H, Reffey SB, Duckett CS, et al. Characterization of XIAP-deficient mice. *Mol Cell Biol*. 2001;21:3604–3608.
36. Nakayama K, Nakayama K-I, Negishi I, et al. Targeted disruption of Bcl-2$\alpha\beta$ in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proc Natl Acad Sci USA*. 1994;91:3700–3704.
37. Borner C. Diminished cell proliferation associated with the death-protective activity of Bcl-2. *J Biol Chem*. 1996;271: 12695–12698.

38. Konopleva M, Tari AM, Estrov Z, et al. Liposomal Bcl-2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabinoside, and induce apoptosis independent of other antiapoptotic proteins. *Blood*. 2000;95:3929–3938.
39. Levkau B, Garton KJ, Ferri N, et al. xIAP induces cell-cycle arrest and activates nuclear factor-kB. *Circul Res*. 2001;88: 282–290.
40. de Almodovar CR, Ruiz-Ruiz C, Munoz-Pinedo C, et al. The differential sensitivity of Bcl-2-overexpressing human breast tumor cells to TRAIL or doxorubicin-induced apoptosis is dependent on Bcl-2 protein levels. *Oncogene*. 2001;20:7128–7133.
41. Davis JM, Navolanic PM, Weinstein-Oppenheimer CR, et al. Raf-1 and bcl-2 induce distinct and common pathways that contribute to breast cancer drug resistance. *Clin Cancer Res*. 2003;9:1161–1170.
42. Piché A, Grim J, Rancourt C, et al. Modulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity in the breast cancer cell line MCF-7. *Cancer Res*. 1998;58: 2134–2140.
43. Teixeira C, Reed JC, Pratt MAC. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res*. 1995;55:3902–3907.
44. Del Bufalo D, Biroccio A, Trisciuoglio D, et al. Bcl-2 has differing effects on the sensitivity of breast cancer cells depending on the antineoplastic drug used. *Eur J Cancer*. 2002;38:2455–2462.
45. Holcik M, Yeh C, Korneluk RG, et al. Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene*. 2000;19:4174–4177.