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Modulation of Nr-13 antideath activity by peptide aptamers

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Tumor cells are characterized by deregulated proliferation and resistance to proapoptotic stimuli. The Bcl-2 family of antiapoptotic proteins is overexpressed in a large number of chemoresistant tumors. Downregulation or inhibition of antiapoptotic proteins might result in the sensitization of cancer cells to chemotherapeutic agents. In the present study, we took advantage of the peptide aptamer strategy to target Nr-13, a Bcl-2 antiapoptotic protein involved in neoplastic transformation by the Rous sarcoma virus. We isolated peptide aptamers that behave as Nr-13 regulators, *in vitro* and in mammalian cells in culture. Some of these aptamers have potential proapoptotic activities. These data suggest that peptide aptamers targeting the Bcl-2 family of apoptosis inhibitors may be useful for the development of anticancer molecules.

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Introduction

A number of tumor cells are characterized by deregulated proliferation and resistance to proapoptotic stimuli, including chemotherapeutic agents (Hanahan and Weinberg, 2000). Strategies aimed at sensitizing tumors to anticancer treatments are thus of critical importance. Proteins that are overexpressed or structurally affected in cancers could conceivably be promising drug targets. Indeed, overexpression of *bcl-2*-related genes is often correlated with chemoresistant phenotypes, including multidrug resistance (Pommier *et al.*,

2004). The Bcl-2 family of proteins is a key regulator of apoptosis, determining cell fate in response to developmental cues and various stress signals (Cory *et al.*, 2003). Various approaches are being used to inactivate these proteins in tumor cells, for example, antisense oligonucleotides (Cotter *et al.*, 1999), small synthetic inhibitors obtained from computer-based or functional screening of libraries (Wang *et al.*, 2000; Jiang *et al.*, 2003; Oltersdorf *et al.*, 2005) or peptides mimicking the BH3 domain of apoptosis accelerators (Degterev *et al.*, 2001; Walensky *et al.*, 2004). Clinical trials are currently underway with a number of these potential new anticancer agents (Reed, 2003; Juin *et al.*, 2004).

Peptide aptamers are a powerful technology to isolate specific inhibitors against intracellular targets. They are made of short peptides of random sequences presented into a scaffold protein, which constrains their conformation. The most widely used platform protein is the bacterial thioredoxin A (TrxA). A number of peptide aptamers have been isolated, which act as inhibitors of proteins involved in human cancers such as the cell cycle regulator cyclin-dependent kinase (Cdk)2 (Colas *et al.*, 1996), the transcription factor E2F (Fabbizio *et al.*, 1999), the G-protein Ras (Kurtz *et al.*, 2003), the epidermal growth factor (EGF) receptor (Buerger *et al.*, 2003) and transforming growth factor- β (Cui *et al.*, 2005). Two studies have shown an induction of apoptosis in cancer cells by peptide aptamers targeting the E6 and E7 papillomavirus oncoproteins (Butz *et al.*, 2000; Nauenburg *et al.*, 2001). To our knowledge, peptide aptamers have not yet been isolated against apoptosis regulators of the Bcl-2 family.

The chicken Nr-13 protein is a member of the Bcl-2 family of apoptosis inhibitors. Nr-13 and orthologous genes (zebrafish *nrz*, murine *boo/diva* and human *nrh*), together with *mcl-1* and *bfl-1*, belong to a subgroup among the antiapoptotic genes (Lanave *et al.*, 2004). Like Bcl-2 and related death inhibitors, Nr-13 inhibits apoptosis at least in part via heterodimerization with the death accelerator Bax (Lalle *et al.*, 2002; Girard-Egrot *et al.*, 2004). There is evidence that Nr-13 plays a role in cells infected by the Rous sarcoma virus (Mangeny *et al.*, 1996; Aouacheria *et al.*, 2002). This raises the possibility that the inactivation of the Nr-13 protein may impair tumor progression.

In this report, we present the characterization of peptide aptamers raised against the Nr-13 protein. One of these aptamers is able to bind the N terminus of the

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protein, whereas the other binds the more conserved C-terminal moiety. To evaluate their effects on Nr-13 activity, first we co-transfected plasmid constructs encoding peptide aptamers together with Nr-13 expression vectors into yeast or mammalian cells. Second, we tested the effect of synthetic peptides corresponding to the variable sequence of the aptamers using an *in vitro* caspase assay. We show that one of the aptamers is a potent inhibitor of Nr-13, whereas the other one behaves as an agonist of Nr-13. Our data show for the first time that peptide aptamers can modulate the antideath activity of a Bcl-2 protein, either negatively or positively. Interestingly, these aptamers do not share sequence homology with any of the BH3-derived peptides known to target the typical hydrophobic cleft found in Bcl-2 family members, which have been used to date to isolate Bcl-2 antagonists.

Results

Isolation of peptide aptamers specifically interacting with the Nr-13 protein

In order to isolate peptide aptamers that specifically bind the Nr-13 protein, a yeast two-hybrid screen was performed as described previously (Colas *et al.*, 1996). Nr-13 belongs to a large family of apoptosis regulators sharing homology domains in the C-terminal moiety, including the conserved hydrophobic cleft, which is the binding site for BH3 peptides and related molecules (Juin *et al.*, 2004). Thus, in order to maximize the chances of obtaining specific Nr-13 ligands, we focused on the N-terminal moiety of Nr-13 that shows poor homology with other Bcl-2-related proteins (Lalle *et al.*, 2002). The first 63 amino acids of Nr-13, comprising the BH4 and BH3 domains, fused to the LexA protein served as bait (in the pGILDA vector). As prey constructs, we used the pJM1 vector to express a library in which 20-mer peptides of randomized sequence were inserted into the active loop of the *Escherichia coli* TrxA. The interaction screen was performed using the EGY48 strain co-transfected with the two constructs and the reporter plasmid pSH18-34. The screening of 2×10^6 transfected yeasts led to the isolation of 51 transformants. One of them, named C6-14, was confirmed after rescuing, retransformation of the plasmids and reselection.

A simultaneous two-hybrid screen directed against the murine Bax protein (P Colas, unpublished data) allowed us to isolate another peptide aptamer, B16, able to target both Bax and the C-terminal moiety of Nr-13. Random mutagenesis was performed on C6-14 and B16 to isolate derivatives with higher affinity for Nr-13. Random mutagenesis of C6-14 led to the isolation of M10b1 and M2a2, the latter no longer able to recognize Nr-13. B16 derivative B16m3 lost its Bax binding capacity but still recognized Nr-13, whereas B16m39 no longer bound Bax or Nr-13 (Table 1 and Figure 1).

Figure 1 Interaction matrix of Nr-13 and Bcl-2 family members with peptide aptamers in yeast. Yeast clones expressing various truncations of Nr-13, Nr-13, Bcl-2 (human), Bfl-1 (human), BodL (rat), Bax (mouse), Bak (human) and full-length Bid (mouse) in bait vectors pGILDA or pEG202 were mated with clones expressing peptide aptamers in prey vector pJG4-5. An interaction matrix picture was taken after 48 h incubation at 30°C. Aptamers M10b1 and C6-14 interact specifically with the 1–63 amino-acid region of Nr-13 (containing the BH4 and BH3 domains of Nr-13), but not with the other truncations of Nr-13 or other Bcl-2 family members. B16 aptamer interacts with Bax, whereas its derivative B16m3 interacts with the 58–157 amino-acid region of Nr-13 (containing the BH1 and BH2 domains of Nr-13). Another B16 derivative, B16m39, lost the ability to interact with both Bax and Nr-13. Expression of the different proteins was checked by Western blotting (not shown).

Table 1 Sequences of peptide aptamers

Aptamer	Isolated by	Nr-13 binding	Bax binding	Sequence of the variable region
C6-14	Anti-chicken Nr-13 screening	+	–	TrxA – RRGSPVLEASKAVDVLFLVL – TrxA
M10b1	C6-14 mutagenesis	+	–	TrxA – RRGSPVMEASKAVDVLFLAL – TrxA
M2a2	C6-14 mutagenesis	–	–	TrxA – RRGSPVLEASRAVDVSFLVL – TrxA
B16	Anti-murine Bax screening	+	+	TrxA – PRGAPMWMRWVCQMLETMFL – TrxA
B16m3	B16 mutagenesis	+	–	TrxA – PRGAPLWLCWVWQMLETMLP – TrxA
B16m39	B16 mutagenesis	–	–	TrxA – PWVAPMRMRRLMLETMFL – TrxA

Abbreviation: TrxA, thioredoxin A.

BLAST searches did not reveal sequence similarities between any of these peptides and known proteins.

To confirm the specificity of the interaction with Nr-13, we used an interaction mating assay (Finley and Brent, 1994) with different bait constructs, including both pro- and antiapoptotic members of the Bcl-2 family. Expression of all proteins was checked using an anti-LexA antibody and was found equivalent. Nr-13 was the only protein recognized by B16m3, C6-14 and its derivative M10b1 among a series of Bcl-2 family members (Figure 1). M10b1 harbors a slightly stronger blue coloration than C6-14 (Figure 1). In addition, this coloration is detected significantly earlier than in C6-14 colonies (not shown), suggesting a higher affinity for Nr-13. In summary, these aptamers recognize different regions of Nr-13. B16m3 binds the C-terminal moiety, containing the conserved BH1 and BH2 domains, whereas C6-14 and its derivative M10b1 recognize the N terminus moiety.

Interactions of the selected peptide aptamers were confirmed using co-immunoprecipitations. Different Bcl-2 proteins were overexpressed in COS-7 cells transiently transfected with the pSG5-FLAG vector. Cell lysates were incubated with purified aptamers, followed by immunoprecipitation using anti-FLAG antibody. The presence of the aptamer in the immunoprecipitates was analysed by Western blotting with an anti-TrxA antibody (Figure 2a). Significant binding was observed between Nr-13 and C6-14 (not shown) or M10b1, but not with TrxA (not shown) or the negative control M2a2. The other Bcl-2 proteins used in this experiment do not bind M10b1 under these conditions. These observations perfectly matched the yeast two-hybrid results (Figure 1). Together, these data indicate that the selected peptide aptamers that specifically interact with Nr-13 *in vivo* in yeast also interact with Nr-13 *in vitro*. This rules out the possibility of nonspecific interactions mediated by an endogenous third protein in yeast.

An enzyme-linked immunosorbent assay (ELISA)-binding assay demonstrated that fluid-phase aptamers bound to recombinant Nr-13 that was passively adsorbed to wells of a microtitration plate. After washing such coated plates, aptamers were added in incremental amounts and the extent of binding was quantified by the sequential addition of anti-TrxA-peroxidase-conjugated antibody. Binding was expressed as the enzymatic activity of the bound peroxidase ($\Delta A_{405\text{nm}}$) (see Figure 2c). Complex formation was saturable with estimated apparent K_D of 0.2 μM (B16m3) and 4.5 μM (M10b1). By comparison, BH3 peptides typically bind to the prosurvival proteins with K_D of 5–100 nM (Chen *et al.*, 2005).

Peptide aptamers interfere with Nr-13 antideath activity in yeast

We previously showed that Nr-13 behaves as an anti-Bax protein (Lalle *et al.*, 2002). In particular, we showed that Nr-13 efficiently counteracts Bax cytotoxicity in yeast. We thus tested the ability of the peptide aptamers

Figure 2 Confirmation and characterization of Nr-13/peptide aptamers binding. (a) The interaction of peptide aptamers with four antiapoptotic proteins was checked by co-immunoprecipitation experiments using recombinant proteins. Purified peptide aptamers M10b1 and M2a2 were incubated with COS-7 cell extracts overexpressing or not the FLAG-tagged antiapoptotic proteins, and then immunoprecipitated using anti-FLAG antibody. Immunocomplexes were analysed by Western blotting using anti-TrxA and anti-FLAG antibodies. M10b1 but not M2a2 interacts with Nr-13, whereas no interaction is detected with the three other Bcl-2 family members, thus confirming the specificity of the interaction found in the two-hybrid experiments. (b) Analysis of samples before immunoprecipitation. Western blots were revealed with anti-TrxA antibody, showing that the two peptide aptamers were present in equivalent amounts before immunoprecipitation. (c) Concentration dependence of the Nr-13/peptide aptamer interaction determined in an ELISA-style ligand binding assay. Different concentrations of M10b1 or B16m3 were incubated with 6His-Nr-13-ATM-6His coated onto a 96-well plate. Binding was detected in an ELISA-style manner using an antibody directed against the TrxA scaffold. Intensity of the signal obtained with TrxA used as a negative control was subtracted from each experimental point.

to modulate Nr-13 activity in this model (Figure 3). As expected, control aptamers M2a2 and B16m39 did not alter the inhibitory effect of Nr-13 on Bax-induced cell death (data not shown), whereas in contrast, C6-14 and M10b1 significantly reduced the growth rate of transfected yeasts, suggesting that these latter aptamers actually inhibited Nr-13 anti-Bax activity. M10b1 exhibited the strongest effect as expected by the apparent higher affinity observed in the two-hybrid assay. C6-14 and M10b1 did not affect Bax-induced cell death in the absence of Nr-13. The third aptamer, B16m3, showed no effect in this system (not shown).

Peptide aptamers modulate cell death in vertebrates

Co-transfection experiments were carried out to assess the biological activity of the most efficient antagonist aptamer (M10b1) in mammalian cells. Plasmid constructs expressing aptamers and Nr-13 or Bcl-xL proteins were transiently transfected in COS-7 cells. The two C6-14 derivatives M10b1 and M2a2 were

Figure 3 Effect of peptide aptamers on Nr-13 activity. The EGY40 strain was co-transformed with pGILDA-Bax and pJG4-5 (with or without Nr-13) vectors. In this system, aptamers were expressed using the pBC103 vector (Sun *et al.*, 1997). After overnight growth in the presence of glucose, yeast cells were cultured in the presence of 2% galactose to induce the expression of Bax alone (empty vectors) or together with Nr-13 and/or the indicated aptamer. Bax totally abolishes yeast growth (open diamonds); this effect is prevented by Nr-13 (black diamonds). C6-14 (black circles) and M10b1 (black triangles) aptamers behave as inhibitors of Nr-13. In the absence of Nr-13, C6-14 (open circles) or M10b1 (open triangles) have no effect on Bax activity. Expression of B16m3 does not affect yeast growth, whether or not Nr-13 is expressed or not (curves are not shown for clarity).

expressed as green fluorescence protein (GFP) fusions so that their expression could be easily monitored by fluorescence (not shown). Nr-13 and Bcl-xL were expressed as FLAG-tagged recombinant proteins. Expression of the proteins of interest was checked by Western blotting (Figure 4a). Eight hours after transfection, serum was withdrawn to induce apoptosis. As demonstrated previously, serum withdrawal in these cells induced cell death as shown by phosphatidyl serine exposure and propidium iodide labeling (Aouacheria *et al.*, 2001). Nuclear changes typical of apoptosis were observed by Hoechst 33258 staining. As shown in Figure 4b, the appearance of pycnotic nuclei was largely prevented by the expression of Nr-13 or Bcl-xL antiapoptotic proteins. Under these conditions, expression of the M2a2 aptamer that does not bind Nr-13 had no effect, whereas M10b1 fully abolished the effect of Nr-13, but not of Bcl-xL. Together, these data suggest that in both yeast and vertebrate, M10b1 is a potent antagonist of Nr-13.

Peptides corresponding to the variable region of the aptamers modulate Nr-13 activity in a cell-free system

In the assays performed so far, variable regions of the peptide aptamers were conformationally constrained owing to their insertion into the TrxA expression platform. To determine whether the unconstrained 20-mer variable regions alone would display the same

Figure 4 M10b1 aptamer specifically modulates Nr-13 activity in mammalian cells. COS-7 cells were transiently transfected to express Nr-13 or Bcl-xL and the indicated aptamer, using pSG5-FLAG and pEGFPC2 vectors, respectively. Expression of both aptamers and antiapoptotic proteins was verified by Western blotting 24h post-transfection using anti-GFP and anti-FLAG antibodies, respectively (a). Vectors and expressed proteins are listed in the upper part of the figure; '-' means that cells were transfected with empty vectors. (b) Assay for inhibitory effects of aptamers: transfected cells were serum starved for 48h. The percentage of pycnotic nuclei was determined using Hoechst 33258 staining. Histograms represent the mean value of three independent experiments. Standard error of the mean is shown. Nr-13 and Bcl-xL efficiently inhibit cell death when co-transfected with either empty pEGFPC2 or the negative control pEGFPC2-M2a2. In contrast, Nr-13 antideath activity is entirely suppressed by M10b1 aptamer co-expression, whereas Bcl-xL activity is not.

effects as the corresponding full-length aptamers, we synthesized the peptides corresponding to the variable regions of the two Nr-13-specific aptamers C6-14 and B16m3. Structure predictions using SOPMA software (Geourjon and Deléage, 1995) suggested an alpha-helical conformation for these peptides. Circular dichroism analyses confirmed these predictions (not shown). We also synthesized the variable region of the B16m3-negative control. We then tested the capacity of these three peptides to interfere with the activity of Nr-13 using *Xenopus* egg extracts, in which caspases activate spontaneously. Caspase activity was detected via the cleavage of a specific substrate and subsequent fluorescence emission. We previously showed that the addition of purified Nr-13 protein delays caspase activation in these extracts (Moradi-Améli *et al.*, 2002). Addition of the synthetic peptides alone in this experiment does not significantly alter caspase activation. As expected, co-incubation of the B16m39 peptide with Nr-13 does not alter Nr-13 activity, whereas the addition of the C6-14 peptide together with Nr-13 efficiently inhibits the anticaspase activity of Nr-13, confirming the antagonistic effect of the C6-14 aptamer (Figure 5). This suggests

that the C6-14 peptide can spontaneously adopt an active conformation and that the TrxA scaffold protein is dispensable. Interestingly, the B16m3 peptide was able to potentiate the activity of Nr-13 in this assay. To confirm this unexpected effect, the experiment was carried out using different B16m3/Nr-13 ratios and time points. The results depicted in Figure 5b confirm that B16m3 behaves as an agonist of Nr-13 in a dose-dependent manner.

Finally, to both explore the mode of action of B16m3 and confirm this result with the entire aptamer, we tested if the B16m3 aptamer was able to modulate Bax/Nr-13 interaction in a three-hybrid experiment. For this, we expressed in yeast a truncated Bax (amino acids 1–169) that does not induce yeast's death. We previously reported that Nr-13 binds Bax via its C terminus part (amino acids 58–157), whereas its N terminus part

(amino acids 1–63) was unable to do so (Lalle *et al.*, 2002). In Figure 5c, we show that a stronger Bax/Nr-13 interaction is detected using the β -galactosidase reporter when B16m3 is co-expressed in the same cells. This result suggests that B16m3 enhances or favors Bax/Nr-13 binding.

Discussion

The Bcl-2 family of proteins plays a major role in the control of apoptosis. The activity of the Bcl-2-like proteins is largely determined by their interactions with specific partners. Specific disruption of these interactions could be essential for studying and understanding the regulation of cell death at a molecular level, but also for the design of new drugs targeting the apoptosis machinery. In this study, we employed a variation of the yeast two-hybrid system to isolate one peptide aptamer (C6-14) selectively interacting with Nr-13. A simultaneous screen against murine Bax allowed one to identify another aptamer (B16) able to bind both Bax and Nr-13. Such crossreactivity, although rarely reported, is not unique. Using the serine threonine kinase Cdk2 as bait, Colas *et al.* (1996) isolated a number of aptamers that also interacted with other Cdk family members. Not surprisingly, the Nr-13 region recognized by B16 corresponds to the most conserved region between chicken Nr-13 and murine Bax. A set of additional aptamers with distinct specificities was obtained by random mutagenesis of C6-14 and B16 (Table 1). The apparent K_D values as determined by ELISA-based assay were 0.2 and 4.5 μ M for B16m3 and M10b1, respectively. The fact that B16m3 exhibits significantly higher affinity for Nr-13 is not unexpected. Indeed, B16m3 may well interact with the hydrophobic cleft of

Figure 5 C6-14 linear peptide antagonizes Nr-13 activity, whereas B16m3 peptide behaves as an activator. The activity of peptides derived from peptide aptamers was tested with an acellular assay. *Xenopus* egg extracts were incubated in the presence or absence of purified Nr-13 protein, with or without the indicated peptides. Kinetics of caspase activation was followed by monitoring the increase in the emission of fluorescence at 460 nm upon excitation at 380 nm. The results shown in (a) correspond to a time point when 50% of caspase activity is inhibited by recombinant Nr-13 protein. Under these conditions, the peptides alone have no significant effect. C6-14 peptide counteracts the inhibitory effect of Nr-13 on caspase activation. In contrast, the B16m3 peptide enhances this inhibitory effect. The ratio of peptides to Nr-13 is 1 in all cases. These data are representative of three independent experiments. Evaluation of the residual DEVDase activity was carried out using different B16m3/Nr-13 ratios (b), ranging from 0.42 to 2.66, and different time points (1 and 3 h). The effect of B16m3 on caspase activation is clearly dose-dependent. (c) To perform a mating assay, the EGY42 strain was co-transformed with pGILDA-Nr-13 and pSH18-34, whereas EGY48 was co-transformed with pJG4-5 (with or without Bax 1–169) and pBC103 expressing or not an aptamer. In this assay, Bax is able to bind only the C terminus part of Nr-13. In the presence of the B16m3 aptamer that binds the C terminus part of Nr-13, a stronger interaction is reported as shown by the dark blue color. 'Ø' means that cells were transformed with an empty vector.

Nr-13 (like BH3 peptides do) as it recognizes the C terminus half of Nr-13 (see Figure 1), encompassing this hydrophobic cleft. In contrast, M10b1 binds to the N terminus region of Nr-13, encompassing the BH4 domain, which is devoid of such hydrophobic pocket, giving rise possibly to a less stable interaction.

In a yeast mating assay, the aptamers showed a highly specific interaction with Nr-13 as shown by testing a collection of Bcl-2 family members, including both pro- and antiapoptotic proteins harboring a similar three-dimensional (3D) structure (Chou *et al.*, 1999; McDonnell *et al.*, 1999; Suzuki *et al.*, 2000; Zhang *et al.*, 2000; Petros *et al.*, 2001; Lalle *et al.*, 2002). Random mutagenesis of C6-14 allowed a derivative (M10b1) to be obtained with higher apparent affinity for Nr-13. Co-immunoprecipitation experiments confirmed that the selected aptamers interacted directly with Nr-13 and do not bind three other antiapoptotic Bcl-2 proteins, including the human Nr-13 ortholog Nrh (Figure 2a).

The ability of peptide aptamers to bind to Nr-13 does not necessarily imply that they would have an effect on its activity. To assess this point, we first took advantage of our previous studies on Nr-13 activity in yeast. Bax expression is lethal in yeast; this cytotoxic effect can be counteracted by several antiapoptotic proteins such as Bcl-xL, Bcl-2, Mcl-1, A1/Bfl-1, Nrh and Nr-13 (Hanada *et al.*, 1995; Tao *et al.*, 1997; Zhang *et al.*, 2000; Aouacheria *et al.*, 2001; Lalle *et al.*, 2002). In this model, C6-14 and its derivative M10b1 partly inhibited Nr-13 activity, whereas B16m3 had no effect. Sequence alignment between C6-14 and M10b1 showed that the most severe change might have been the Leu to Met substitution at position 7 (Table 1). The higher efficacy of M10b1 might be owing to this mutation.

The most active Nr-13 antagonist, M10b1, was further investigated in COS-7 mammalian cells. These cells are sensitive to serum withdrawal, showing typical chromatin condensation of apoptotic cells that is largely prevented by Nr-13 and Bcl-xL (this report), or Nrh (Aouacheria *et al.*, 2001). We show here that the antideath effect of Nr-13 is completely annihilated by M10b1, but not by another peptide aptamer unable to bind to Nr-13. In the same assay, M10b1 is unable to modulate the Bcl-xL-protective effect. These results show that peptide aptamers directed against Bcl-2 family of apoptotic inhibitors may have proapoptotic properties.

We demonstrate here that peptide aptamers targeting an apoptotic inhibitor are actually functional in vertebrate cells. This is an important step toward the *in vivo* application of the peptide aptamer strategy. To our knowledge, the only *in vivo* study reported today was performed with anti-Cdk2 aptamers. These anti-human protein aptamers were also shown to inhibit related cell cycle regulators in *Drosophila* (Kolonin and Finley, 1998). Most of the other peptide aptamers that have been identified so far have not yet been tested *in vivo* probably because a prerequisite to their intracellular action is their delivery into cells. This could be a limiting step as largely shown for other gene therapy strategies. From this perspective, finding linear active

peptides outside the context of the scaffold protein is of major interest. Several protein transduction domains have been developed over the past few years and used to transport various molecules into cells, including peptides (Wender *et al.*, 2000). Success of these tools depends largely on the size of the product to transport, small peptides up to 20 kDa being more efficiently transported.

Therefore, the description of peptides still active after removal from their scaffold is clearly a bonus when developing aptamer-derived drugs. To this aim, we decided to explore the functionality of the variable regions of C6-14 and B16m3. We chemically synthesized the two corresponding peptides and used them in an acellular caspase assay. The two peptides were found to be active in this assay: C6-14 behaves as a potent antagonist of Nr-13, whereas B16m3 showed agonist properties (Figure 5a). This observation could explain the lack of effect of B16m3 shown in yeast: as Nr-13 by itself fully restored the yeast exponentially growing phase (Lalle *et al.*, 2002), a possible potentiation of Nr-13 effect by B16m3 could not be visualized in this assay.

Our data confirm previous reports describing that in some cases the variable regions of peptide aptamers are not dependent on the TrxA scaffold, suggesting that they could adopt a functional 3D structure on their own (Fabrizio *et al.*, 1999; Klevenz *et al.*, 2002; Schmidt *et al.*, 2002; Buerger *et al.*, 2003; Kurtz *et al.*, 2003). In contrast, other peptides show a drastic decrease in their efficiency outside the TrxA scaffold (Cohen *et al.*, 1998; Klevenz *et al.*, 2002).

This report is the first demonstration that apoptosis can be modulated by targeting a Bcl-2 family member with peptide aptamers. Among the three selected aptamers, two of them interact with the N-terminal moiety of Nr-13. This part of the protein encompasses the BH4 and BH3 domains. The BH4 domain is usually considered as a general feature of antiapoptotic proteins of the Bcl-2 family. Nevertheless, the definition of the BH4 domain is not as stringent as for the other Bcl-2 homology domains. Indeed, some apoptosis inhibitors may lack BH4, whereas a BH4 domain has been described in some apoptosis accelerators (Lanave *et al.*, 2004). The BH4 domain corresponds to the most N-terminal amphipathic helix, which is supposed to stabilize the structure of the hydrophobic groove formed by the BH3, BH1 and BH2 domains, which serves as a receptor for BH3 amphipathic helices of proapoptotic proteins. A simple explanation for the antagonist effect of the aptamers in our assays is that they could prevent the Nr-13/Bax binding.

Recently, Cartron *et al.* (2005) reported that helix 1 of Bax could interact with BH3 via an electrostatic bond and that this interaction is essential to regulate Bax activation and addressing to mitochondria. As we described previously, a similar electrostatic interaction exists between the BH4 and BH3 domains of Nr-13 that is able to modulate its antiapoptotic function, although the mechanism of this regulation is still unknown (Lalle *et al.*, 2002). Interestingly, two peptide aptamers that we isolated are able to target the first 63 amino acids of

Nr-13 encompassing the BH4 and BH3 domains. Therefore, these aptamers could be valuable tools for exploring the conformational changes occurring in this region of the protein, the regulation of its binding to Bax and its antiapoptotic activity.

The third aptamer that we isolated binds to the more conserved C-terminal part of Nr-13. Surprisingly, it is able to potentiate its antiapoptotic activity. To our knowledge, this is the first aptamer to behave as an agonist. This is all the more surprising as all Bcl-2 ligands described to date act as antagonists (Aouacheria *et al.*, 2003). Thus, this aptamer could stabilize the Nr-13-active conformation and favor the formation of Nr-13 dimers or its association with proapoptotic partners. Indeed, as shown in Figure 5c, B16m3 appears to increase Nr-13/Bax interaction in yeast, which may be owing to Nr-13 stabilization. Interestingly, it has been recently reported that ligands that bind to the C terminus moiety of A1, a Bcl-2 family member sharing homologies with Nr-13, are able to increase the half-life of the A1 protein (Herold *et al.*, 2006). Resolution of the 3D structure of Nr-13-aptamer complexes will help to solve their precise mechanisms of action.

Nr-13 displays several domains whose relative contributions to the function of the protein are not always understood. Developing inhibitors that target a precise domain represents an alternative strategy to the more classical knockout approach that affects the activity or the expression of the entire gene product. Here, we report such an inhibitor that is a potent and specific inhibitor of Nr-13 activity in live cells.

Finally, their small size and the knowledge of the TrxA scaffold 3D structure should help to elucidate the aptamer structure and to develop non-peptide mimetics with optimal efficiency. Such molecules would have important applications in basic research and cancer therapy. Future studies will focus on the efficiency of their *in vivo* delivery for therapeutic purposes. The present study could serve as a first step in the design of new anticancer molecules targeting Bcl-2 family members.

Materials and methods

Yeast strains and construction of two-hybrid plasmids

EGY40 (MAT α leu2 his3 trp1 ura3), EGY42 (MAT α leu2 his3 trp1 ura3) and EGY48 strains (MAT α his3 trp1 ura3–52 leu2::LexA6op-LEU2) have been described previously (Gyuris *et al.*, 1993; Estojak *et al.*, 1995). Expressions of all fusion proteins in pEG202 and pGILDA were checked by Western blotting using an anti-LexA horseradish peroxidase (HRP) conjugate antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-7544, 1:100). To allow nuclear localization of Bcl-2 proteins, C-terminal putative transmembrane domains were systematically removed. In all cases, subcloning of the coding sequences was performed between *Eco*RI and *Xho*I sites.

Screening of the peptide aptamer library

Aptamer screening was carried out in EGY48 as described previously (Colas *et al.*, 1996). Briefly, as bait, the N-terminal part of the Nr-13 protein, containing amino acids 1–63, was fused to the LexA protein into the pGILDA vector.

A randomized 20-mer peptide aptamer library in the pJM1 vector was used. EGY48 yeasts co-transformed with the bait construct and the library were selected for growth in the absence of uracil, histidine, tryptophan and leucine. Prey-bait interaction was verified by retransformation and interaction mating assay as described, using the pSH18-34 reporter plasmid (Finley and Brent, 1994).

Random mutagenesis

Variable regions of the C6-14 and B16 aptamers were amplified from the original library vectors following a mutagenic polymerase chain reaction protocol (Cadwell and Joyce, 1992). A pool of pJM-1 vectors harboring random mutations into the variable region were transformed into EGY48 containing pGILDA-Nr-13 1–63 or pGILDA-Bax and pRB18–40 (Estojak *et al.*, 1995). Yeasts were plated on Ura[–] His[–] Trp[–] glucose. Transformants were replica plated onto Ura[–] His[–] Trp[–] glucose/XGal and Ura[–] His[–] Trp[–] galactose/XGal plates. After 2 days at 30°C, colonies that showed a blue color were transferred onto Ura[–] His[–] Trp[–] glucose plates. After a new replica plating onto Ura[–] His[–] Trp[–] glucose/XGal and Ura[–] His[–] Trp[–] galactose/XGal, colonies that still displayed intense blue color were selected. The aptamer-encoding plasmids were recovered. After retransformation and mating, clones that still conferred an interaction phenotype were sequenced and used for further experiments.

Yeast growth assays

The EGY40 strain was co-transformed with pGILDA-Bax, pJG4-5 (Nr-13 or empty) and pBC103 (expressing or not an aptamer) (Sun *et al.*, 1997). In these vectors, proteins are expressed under the control of the *GAL1*-inducible promoter. Selective medium containing 2% glucose was inoculated with a single colony of transformants and incubated overnight at 30°C. Subsequently, cells were washed three times with water. Typically, 10 ml of selective medium with 2% galactose was inoculated at optical density (OD)_{600 nm} = 0.1. Growth was monitored for 60 h. Samples were taken at different time points, and cell density was measured by determining the OD_{600 nm}. Appropriate dilutions were performed regularly to maintain cells in an exponential growth phase.

Three-hybrid experiment

EGY42 was transformed with pGILDA-Nr-13 and pSH18-34, whereas EGY48 was transformed by pJG4-5–Bax 1–169 and pBC103 (coding or not for an aptamer). Yeasts were mated as described previously (Finley and Brent, 1994).

Production and purification of recombinant Nr-13, peptide aptamers and TrxA

The expression of recombinant Nr-13 with an hexahistidine tag at its C terminus (Nr-13- Δ TM-6His) has been described previously (Moradi-Améli *et al.*, 2002). Peptide aptamers and TrxA were produced as glutathione S transferase (GST) fusion proteins; the GST moiety was removed by cleavage with thrombin. Peptide aptamer or TrxA cDNAs were subcloned into a GST expression vector (pGEX4T1, Amersham Biosciences, Orsay, France). The GST-fusion proteins were expressed in the BL21 *E. coli* strain, lysed in 20 mM Tris (pH 7.5), 250 mM NaCl, 0.1% Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 g/l lysozyme, 250 U/ml benzonase and a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics, Meylan, France). Bacterial lysates were clarified by centrifugation for 30 min at 14 000 r.p.m. and loaded onto a 1 ml glutathione affinity column (GSTrap, Amersham Biosciences) equilibrated

in Tris 20 mM (pH 7.5) and NaCl 150 mM buffer. The column was washed with 10 column volumes of equilibration buffer; then, 50 U thrombin (Amersham Biosciences) re-suspended in cold phosphate-buffered saline (PBS) were loaded on the column that was then incubated for 16 h at 4°C. The cleaved protein was eluted from the column with PBS and thrombin was eliminated through a *para*-aminobenzamidine affinity column (Amersham Biosciences). Eluted fractions were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and those containing the wanted protein were pooled and dialysed overnight against 20 mM Tris (pH 8). The dialysate was then loaded onto an anion exchange column (Mono Q HR5/5, Amersham Biosciences) equilibrated in 20 mM Tris (pH 8) and proteins were eluted with a NaCl gradient (100–500 mM). Eluted fractions were analysed by SDS–PAGE. Aptamer- or TrxA-containing fractions were pooled, concentrated and buffer exchanged using Vivaspin 4 devices with a 5 kDa cutoff. Aptamer and TrxA concentrations were determined by both Bradford and UV_{280 nm} absorbance. Analysis of the different aptamers and TrxA was carried out by mass spectrometry (API 16S Sciex, Perkin-Elmer, Courtaboeuf, France) and secondary folding was checked by circular dichroism (Jobin Yvon CD6 spectropolarimeter).

Co-immunoprecipitations

COS-7 cells (African green monkey kidney cells) were maintained at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Biowest, Nuailles, France), 1 mM sodium pyruvate and penicillin/streptomycin (Biomedix, Boussens, France). Transfections were performed with pSG5-FLAG coding or not for a Bcl-2 family member using Eugene 6 reagent (Roche Diagnostics) according to the manufacturer's instructions. COS-7 lysates were prepared 24 h post-transfection by lysing in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetate, 1% Triton X-100, 1 mM PMSF and a cocktail of protease inhibitors. Lysate samples (40 µg protein) were mixed with 1 µg of the purified aptamers (C6-14, M10b1 or M2a2) or TrxA devoid of the GST moiety. The volume was adjusted to 500 µl with lysis buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors) containing 0.1% Triton X-100 and 1 mM DTT. The mix was then incubated for 1 h at room temperature. After the addition of 40 µl of anti-FLAG antibody covalently linked to agarose beads (Sigma, Saint-Quentin-Fallavier, France, A2220), samples were further incubated for 16 h at 4°C. After four washes in 50 mM Tris (pH 7.5), 150 mM NaCl, immunocomplexes were re-suspended in 80 µl of Laemmli buffer, boiled and 30 µl were resolved on SDS–PAGE. After transfer onto nitrocellulose membrane (Hybond, Amersham), Bcl-2 proteins and aptamers were detected by using monoclonal anti-FLAG (Sigma, M2, 1:250) and polyclonal anti-TrxA antibodies (Santa Cruz, sc-801, 1:250), respectively. Detection was performed with anti-mouse HRP-conjugated immunoglobulin (IgG) (DAKO, Trappes, France, 1:3000) or anti-rabbit HRP-conjugated IgG (DAKO, 1:2000), then chemoluminescent revelation (ECL, Amersham).

Quantification of cell death

COS-7 cells were co-transfected with pSG5-FLAG coding or not for a Bcl-2 family member and pEGFP2 constructs coding or not for aptamers. To check the expression of proteins by SDS–PAGE, total cell lysates were prepared 24 h after transfection by lysing cells in 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors. Samples

(50 µg protein) were loaded on gels, transferred onto nitrocellulose membrane and immunoblotted with anti-GFP polyclonal antibody (Invitrogen, Cergy-Pontoise, France, 1:5000) or anti-FLAG monoclonal antibody (Sigma, M2, 1:250, M2). Detection was performed using HRP-conjugated secondary antibodies and chemoluminescent detection. For quantification of cell death, cells were serum starved 8 h after transfection for 48 h. Nuclei were then labeled with Hoechst 33258 fluorescent dye to detect pycnotic nuclei as described (Aouacheria *et al.*, 2001). Three independent experiments were performed for each experimental point, with 100–250 GFP-expressing cells randomly counted in different areas.

ELISA-style ligand binding assays

Purified recombinant Nr-13-ΔTM-6His was diluted in 15 mM Na₂CO₃, 35 mM NaHCO₃ and 5 mM DTT (pH 9.6), and coated at 600 nM (1.1 µg/well) overnight at room temperature onto 96-well plates (Nunc, Wiesbaden, Germany). After washing with PBS, plates were blocked twice for 30 min with PBS and 2% bovine serum albumin (BSA) (Sigma). Various amounts of recombinant aptamers or TrxA protein dissolved in PBS 0.2% BSA 3 mM DTT were added for 50 min. After extensive washing with PBS, 400 ng of polyclonal anti-TrxA antibody (Santa Cruz, sc-801) were added in PBS 0.2% BSA for 35 min at room temperature. After three washing steps, 15 ng of polyclonal goat anti-rabbit IgG-HRP conjugated (DAKO, P0448) were added in PBS 0.2% BSA for 30 min. After three washes with PBS–Tween 20 0.01%, plate-bound peroxidase was detected using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic) acid peroxidase substrate (Sigma, P1888) by measuring absorbance at 405 nm. Apparent *K_D* was determined as described (Mann *et al.*, 2004).

Caspase inhibition assay in *Xenopus* egg extracts

The caspase inhibition assay was originally designed to mimic the cleavage of poly(ADP-ribose) polymerase by caspase-3 in the *Xenopus* egg extracts (Cosulich *et al.*, 1997; Moradi-Améli *et al.*, 2002). Caspase-3 activity was measured by assaying the cleavage of a fluorogenic peptide, Ac-DEVD-AMC, based on the known cleavage site of poly(ADP-ribose) polymerase (DEVD/G). *Xenopus* extracts were incubated in the presence or absence of Nr-13 (20 µM) with or without B16m39, C6-14 or B16m3 peptides. Equimolar amounts of the peptide and Nr-13 protein (peptide/Nr-13 ratio: 1) were used in all experiments, except for B16m3 (ratio from 0.42 to 2.66 depending on the experiments). All the peptides used in this assay were synthesized by the solid-phase method (Merrifield, 1964), purified by reverse-phase chromatography. Molecular masses were checked by mass spectrometry. For B16m3, only the first 19 amino acids of the variable region were synthesized.

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References

- Aouacheria A, Arnaud E, Venet S, Lalle P, Gouy M, Rigal D *et al.* (2001). Nr13, a human homologue of Nr-13 associates with Bcl-Xs and is an inhibitor of apoptosis. *Oncogene* **20**: 5846–5855.
- Aouacheria A, Gillet G, Lalle P. (2003). Manipulating the Bcl-2 network. Experimental strategies and clinical applications. *Recent Res Dev Mol Cell Biol* **4**: 59–84.
- Aouacheria A, Ory S, Schmitt J-R, Rigal D, Jurdic P, Gillet G. (2002). p60(v-src) and serum control cell shape and apoptosis via distinct pathways in quail neuroretina cells. *Oncogene* **21**: 1171–1186.
- Buerger C, Nagel-Wolfrum K, Kunz C, Wittig I, Butz K, Hoppe-Seyler F *et al.* (2003). Sequence-specific peptide aptamers, interacting with the intracellular domain of the epidermal growth factor receptor, interfere with Stat3 activation and inhibit the growth of tumor cells. *J Biol Chem* **278**: 37610–37621.
- Butz K, Denk C, Ullmann A, Scheffner M, Hoppe-Seyler F. (2000). Induction of apoptosis in human papilloma-virus-positive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. *Proc Natl Acad Sci USA* **97**: 6693–6697.
- Cadwell RC, Joyce GF. (1992). Randomization of genes by PCR mutagenesis. *PCR Methods Appl* **2**: 28–33.
- Cartron PF, Arokium H, Oliver L, Meflah K, Manon S, Vallette FM. (2005). Distinct domains control the addressing and the insertion of Bax into mitochondria. *J Biol Chem* **280**: 10587–10598.
- Chen L, Willis SN, Wei A, Smith BJ, Fletcher JJ, Hinds MG *et al.* (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17**: 393–403.
- Chou JJ, Li H, Salvesen GS, Yuan J, Wagner G. (1999). Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell* **96**: 615–624.
- Cohen BA, Colas P, Brent R. (1998). An artificial cell-cycle inhibitor isolated from a combinatorial library. *Proc Natl Acad Sci USA* **95**: 14272–14277.
- Colas P, Cohen B, Jessen T, Grishina I, McCoy J, Brent R. (1996). Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature* **380**: 548–550.
- Cory S, Huang DC, Adams JM. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* **22**: 8590–8607.
- Cosulich SC, Worrall V, Hedge PJ, Green S, Clarke PR. (1997). Regulation of apoptosis by BH3 domains in a cell-free system. *Curr Biol* **7**: 913–920.
- Cotter FE, Waters J, Cunningham D. (1999). Human Bcl-2 antisense therapy for lymphomas. *Biochim Biophys Acta* **1489**: 97–106.
- Cui Q, Lim SK, Zhao B, Hoffmann FM. (2005). Selective inhibition of TGF- β responsive genes by Smad-interacting peptide aptamers from FoxH1, Lef1 and CBP. *Oncogene* **24**: 3864–3874.
- Degterev A, Lugovskoy A, Cardone M, Mulley B, Wagner G, Mitchison T *et al.* (2001). Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. *Nat Cell Biol* **3**: 173–182.
- Estojak J, Brent R, Golemis EA. (1995). Correlation of two-hybrid affinity data with *in vitro* measurements. *Mol Cell Biol* **15**: 5820–5829.
- Fabrizio E, Le Cam L, Polanowska J, Kaczorek M, Lamb N, Brent R *et al.* (1999). Inhibition of mammalian cell proliferation by genetically selected peptide aptamers that functionally antagonize E2F activity. *Oncogene* **18**: 4357–4363.
- Finley Jr RL, Brent R. (1994). Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators. *Proc Natl Acad Sci USA* **91**: 12980–12984.
- Geourjon C, Deléage G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Comput Appl Biosci* **11**: 681–684.
- Girard-Egrot A, Chauvet J-P, Gillet G, Moradi-Améli M. (2004). Specific interaction of the antiapoptotic protein Nr-13 with phospholipid monolayers is prevented by the BH3 domain of Bax. *J Mol Biol* **335**: 321–331.
- Gyuris J, Golemis E, Chertkov H, Brent R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**: 791–803.
- Hanada M, Aimé-Sempé C, Sato T, Reed JC. (1995). Structure–function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J Biol Chem* **270**: 11962–11969.
- Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. *Cell* **100**: 57–70.
- Herold MJ, Zeitz J, Pelzer C, Kraus C, Peters A, Wohlleben G *et al.* (2006). The stability and anti-apoptotic function of A1 are controlled by its C-terminus. *J Biol Chem* **281**: 13663–13671.
- Jiang X, Kim HE, Shu H, Zhao Y, Zhang H, Kofron J *et al.* (2003). Distinctive roles of PHAP proteins and prothymosin- α in a death regulatory pathway. *Science* **299**: 223–226.
- Juin P, Geneste O, Raimbaud E, Hickman JA. (2004). Shooting at survivors: Bcl-2 family members as drug targets for cancer. *Biochim Biophys Acta* **1644**: 251–260.
- Klevenz B, Butz K, Hoppe-Seyler F. (2002). Peptide aptamers: exchange of the thioredoxin-A scaffold by alternative platform proteins and its influence on target protein binding. *Cell Mol Life Sci* **59**: 1993–1998.
- Kolonin MG, Finley Jr RL. (1998). Targeting cyclin-dependent kinases in *Drosophila* with peptide aptamers. *Proc Natl Acad Sci USA* **95**: 14266–14271.
- Kurtz SE, Esposito K, Tang W, Menzel R. (2003). Inhibition of an activated Ras protein with genetically selected peptide aptamers. *Biotechnol Bioeng* **82**: 38–46.
- Lalle P, Aouacheria A, Dumont-Miscopein A, Jambon M, Venet S, Bobichon H *et al.* (2002). Evidence for crucial electrostatic interactions between BCL-2 homology domains BH3 and BH4 in the anti-apoptotic NR-13 protein. *Biochem J* **368**: 213–221.
- Lanave C, Santamaria M, Saccone C. (2004). Comparative genomics: the evolutionary history of the Bcl-2 family. *Gene* **333**: 71–79.
- Mangenev M, Schmitt JR, Leverrier Y, Thomas J, Marvel J, Brun G *et al.* (1996). The product of the v-src-inducible gene nr-13 is a potent anti-apoptotic factor. *Oncogene* **13**: 1441–1446.
- Mann HH, Ozbek S, Engel J, Paulsson M, Wagener R. (2004). Interactions between the cartilage oligomeric matrix protein and matrilins. Implications for matrix assembly and the pathogenesis of chondrodysplasias. *J Biol Chem* **279**: 25294–25298.
- McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ, Cowburn D. (1999). Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell* **96**: 625–634.
- Merrifield RB. (1964). Solid-phase peptide synthesis. 3. An improved synthesis of bradykinin. *Biochemistry* **14**: 1385–1390.

- Moradi-Améli M, Lorca T, Ficheux D, Di Pietro A, Gillet G. (2002). Interaction between the antiapoptotic protein Nr-13 and cytochrome *c*. Antagonistic effect of the BH3 domain of Bax. *Biochemistry* **41**: 8540–8550.
- Nauenburg S, Zwerschke W, Jansen-Durr P. (2001). Induction of apoptosis in cervical carcinoma cells by peptide aptamers that bind to the HPV-16 E7 oncoprotein. *FASEB J* **15**: 592–594.
- Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al.* (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**: 677–681.
- Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K *et al.* (2001). Solution structure of the anti-apoptotic protein bcl-2. *Proc Natl Acad Sci USA* **98**: 3012–3017.
- Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW. (2004). Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* **23**: 2934–2949.
- Reed JC. (2003). Apoptosis-targeted therapies for cancer. *Cancer Cell* **3**: 17–22.
- Schmidt S, Diriong S, Mery J, Fabbrizio E, Debant A. (2002). Identification of the first Rho-GEF inhibitor, TRIPalpha, which targets the RhoA-specific GEF domain of Trio. *FEBS Lett* **523**: 35–42.
- Sun LJ, Peterson BR, Verdine GL. (1997). Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1. *Proc Natl Acad Sci USA* **94**: 4919–4924.
- Suzuki M, Youle RJ, Tjandra N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* **103**: 645–654.
- Tao W, Kurschner C, Morgan JI. (1997). Modulation of cell death in yeast by the Bcl-2 family of proteins. *J Biol Chem* **272**: 15547–15552.
- Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD *et al.* (2004). Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. *Science* **305**: 1466–1470.
- Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM *et al.* (2000). Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci USA* **97**: 7124–7129.
- Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci USA* **97**: 13003–13008.
- Zhang H, Cowan-Jacob SW, Simonen M, Greenhalf W, Heim J, Meyhack B. (2000). Structural basis of BFL-1 for its interaction with Bax and its anti-apoptotic action in mammalian and yeast cells. *J Biol Chem* **275**: 11092–11099.