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Characterization of Peptide Aptamers Targeting Bfl-1 Anti-Apoptotic Protein

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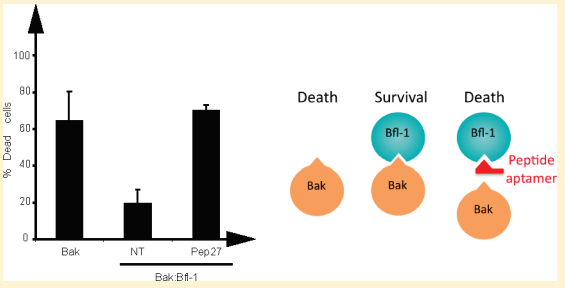
Supporting Information

ABSTRACT: Bfl-1, an anti-apoptotic protein of the Bcl-2 family, has been identified as a potential therapeutic target for B-cell malignancies. We describe herein the first characterization of peptide aptamers selected against Bfl-1. We show that most of the Bfl-1 peptide aptamers do not interact with Bcl-2, Bcl-xL, or Mcl-1 in yeast and that some of them restore the pro-apoptotic activity of Bax in yeast in which Bax and Bfl-1 proteins are coexpressed. When expressed in mammalian cells, peptide aptamers interact with Bfl-1 and sensitize B-cell lines to apoptosis induced by chemotherapeutic agents. We further demonstrate that a nonconstrained peptide derived from one aptamer variable region reverses Bfl-1 anti-apoptotic activity in HeLa cells through disruption of Bax−Bfl-1 interaction. This peptide also promotes cell death in lymphoma B-cell lines expressing a high level of Bfl-1 and sensitizes these cells to drug-induced apoptosis. Taken together, these results further validate Bfl-1 as a therapeutic target for malignant B-cells and suggest that peptide aptamers may be a useful tool for guiding the identification of small compounds that target the anti-apoptotic Bfl-1 protein.



Bcl-2 family proteins are essential regulators of apoptosis, and overexpression of anti-apoptotic members has been widely implicated in tumor development and in resistance to chemotherapy. Anti-apoptotic Bcl-2 family proteins can form heterodimers with pro-apoptotic members, sequestering them and preventing them from inducing apoptosis by disrupting outer mitochondrial membrane integrity and allowing cytochrome *c* release. The α-helix of BH3 pro-apoptotic proteins interacts with the hydrophobic groove formed by BH1, -2, and -3 domains of anti-apoptotic proteins.[1] New classes of anticancer agents targeting this hydrophobic pocket are now being developed to mimic pro-apoptotic BH3 only protein activity. The best-characterized BH3 mimetic is the small molecule ABT-737, which was identified by a nuclear magnetic resonance screen of compounds binding to Bcl-xL followed by additional chemical modifications to improve its therapeutic potential.[2] This molecule demonstrates binding efficiency in the nanomolar range toward Bcl-xL, Bcl-2, and Bcl-w but shows very poor affinity for Bfl-1 and Mcl-1. An orally bioactive molecule, ABT-263, has been developed from ABT-737.[3] Like ABT-737, ABT-263 demonstrates efficacy in vitro in killing different tumor cell lines and activity in vivo against a panel of small cell lung cancer xenograft models.[4,5] Tumor resistance to ABT-737 or ABT-263 has been associated with both Bfl-1 or Mcl-1 overexpression.[6] Combined treatment of various cancer cells with ABT-737 and drugs that downregulate Bfl-1 and/or Mcl-1 expression often restores tumor cell death, thus offering promising therapeutic options.[7−9]

The Bfl-1 protein was identified as a potential target for B-cell malignancies.[10−12] Using a short hairpin RNA strategy, we have shown that Bfl-1 downregulation induces spontaneous apoptosis in B-cell lymphoma and sensitizes these cells to apoptosis induced by various chemotherapeutic agents,[13] indicating that Bfl-1, in addition to Bcl-2, BclxL, and Mcl-1, may indeed represent a potential target for future drug development against B-cell lymphoma.

As a first step in developing strong and specific inhibitory ligands of Bfl-1 protein, we have chosen the peptide aptamer strategy. Peptide aptamers make up a class of combinatorial proteins designed to interact specifically and with a strong affinity with intracellular protein targets.[14] They are composed of a random-sequence amino acid variable region displayed in a scaffold protein. The most widely used scaffold protein is bacterial thioredoxin A (TrxA). Many studies have used a yeast two-hybrid system to isolate from combinatorial libraries peptide aptamers directed against proteins involved in human cancers such as cell cycle regulator cyclin-dependent kinase 2,[15,16] the epidermal

Received: November 18, 2010
Revised: April 1, 2011
Published: May 12, 2011

growth factor receptor,[17] transcription factors STAT3[18] and E2F,[19] the E6 and E7 papillomavirus oncoproteins,[20,21] and the RasGap positive Ras effector protein.[22] Peptide aptamers targeting the Bcl-6 transcription factor involved in different lymphomas and breast cancers have also been described and inhibit several essential Bcl-6 cellular functions.[23] More recently, a peptide aptamer targeted against the T-cell oncogenic protein LMO2 has been identified and demonstrates in vivo tumor growth inhibitory activity.[24] These peptide aptamers interact very specifically with their targets and are able to inhibit their function in vitro and in vivo. With regard to anti-apoptotic Bcl-2 family members, Nouvion et al. have isolated peptide aptamers targeting Nr-13, a chicken Bcl-2 anti-apoptotic protein involved in neoplastic transformation by the Rous sarcoma virus. These peptide aptamers are able to modulate anti-apoptotic activity of Nr-13 in vitro.[25]

We present here the first characterization of peptide aptamers selected against a human anti-apoptotic protein of the Bcl-2 family, the Bfl-1 protein.

EXPERIMENTAL PROCEDURES

Yeast Strains and Yeast Culture. EGY42 (*MATα leu2 his3 trp1 ura3*) and EGY48 strains (*MATα his3 trp1 ura3−52 leu 2: LexA6op-LEU2*) were described previously.[26]

MB226 (*MATα leu2 ura3 trp1 his3 ade2*) and MB210 (*MATα 6LexAop:LEU2 8LrxAop:ADE2 leu2 ura3 his3 trp1*) strains were developed by Aptanomics SA.[27] All reagents used for the two-hybrid assay and yeast culture have been described previously.[27]

Cell Lines and Cell Culture. All media and cell culture reagents were purchased from Invitrogen. BP3, IM9, and A549 cells were cultured in RPMI complete medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM Hepes, and 40 μg/mL gentamycin. HeLa, iBMK, and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM Hepes, and 40 μg/mL gentamycin.

Screening of the Peptide Aptamer Library. Two-hybrid screening of peptide aptamer library was performed by yeast mating as described previously.[27]

The bait Bfl-1-1−151 (Bfl-1ΔCter) was amplified by polymerase chain reaction (PCR) from the pEGZ-FLAG-Δα9-Bfl-1 template previously described[28] and cloned into EcoRI and XhoI sites of the pGILDA vector. pGILDA-Bfl-1ΔCter was cotransformed with reporter plasmid pSH18-34 in yeast strain MB226α.

A peptide aptamer library (complexity of 2×10^7) was constructed and transformed into yeast strain MB210α by Aptanomics SA. Peptide aptamers were designed by Aptanomics SA: their 13-amino acid variable region was inserted into the optimized *Escherichia coli* thioredoxin scaffold,[27] and peptide aptamers were cloned into the pJM-1 prey vector.

Briefly, after the MB226α and MB210α strains had been mated and protein expression induced with galactose, cells were selected in the absence of leucine. Positive cells were thereafter selected in the absence of adenine and stained blue in the presence of X-Gal. Following reconfirmation of the yeast two-hybrid phenotypes (absence of blue staining against empty bait vector), the variable regions of the peptide aptamer were sequenced (Genome Express).

Interaction Matrix. An interaction matrix of Bcl-2 family members with peptide aptamers in yeast was created by mating EGY42α and EGY48α strains as described previously.[27] An interaction matrix picture was taken after incubation for 48 h at 30 °C.

The Bcl-2 family members from which their C-terminal domains had been deleted were amplified by PCR from the templates described below and cloned into the EcoRI and XhoI sites of the pGILDA vector. Bfl-1mΔCter was amplified from the pEGZ-mBfl-1Δα9 template described previously,[28] Bcl-2ΔCter (Bcl-2-1−205) from the pEF-neo-Bcl-2 template (gift from L. Genestier, INSERM U851), Bcl-xLΔCter (Bcl-xL-1−212) from the pEF-neo-Bcl-xL template (gift from L. Genestier), and Mcl-1ΔCter (Mcl-1-1−329) from the pcDNA3-Mcl-1 template (gift from P. Auberger, INSERM U526, Nice, France).

Constructs pGILDA-BaxΔCter and pJG4−5-Bax1−77 (prey vector, positive control) and an irrelevant pJM-1-C peptide aptamer were gifts from Aptanomics SA. The molecular identity of all constructs was confirmed by sequencing (Genome Express).

Yeast Growth Assay. Full-length Bfl-1 and Bfl-1m were amplified by PCR from pEGZ-FLAG-FL-Bfl-1 and pEGZ-FLAG-Bfl-1mΔCter templates[28] and were inserted into the XbaI site of the pSEY vector (Gift from Aptanomics SA). Full-length Bax in the pGILDA vector was a gift from Aptanomics SA. Tested peptide aptamers were inserted into the CpoI sites of the pLE1C vector (gift from Aptanomics SA) by homologous recombination in yeast. The molecular identity of all constructs was confirmed by sequencing (Genome Express). In all these vectors, proteins are expressed under the control of the *GAL1*-inducible promoter.

The TB50α strain was transformed with either the empty pLE1-C vector or the same vector expressing peptide aptamers. The TB50α strain was cotransformed with the empty pGILDA vector or the same vector expressing Bax and with the empty pSEY vector or the same vector expressing Bfl-1 or Bfl-1m. Clones were mated and then inoculated overnight at 30 °C in selective medium containing 2% glucose.

Subsequently, clones were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 in selective medium containing 2% galactose to induce protein and peptide aptamer expression. Yeast growth was monitored for 45 h; samples were taken at different time points, and cell density was measured by determining the OD_{600}.

Immunoprecipitation of Peptide Aptamers with Anti-Apoptotic Bcl-2 Family Members in HeLa Cells. Peptide aptamers were cloned between the XhoI and NotI sites of the episomal pPER vector (gift from Aptanomics SA). FLAG-Bfl-1 in the pEGZ vector was described previously.[28] Bcl-2, Bcl-xL, and Mcl-1 were amplified by PCR from pEF-neoBcl-2, pEF-neo Bcl-xL, and pcDNA3-Mcl-1 templates, respectively, and inserted into the pEGZ vector to obtain the corresponding FLAG-tagged proteins. The molecular identity of all constructs was confirmed by sequencing (Genome Express).

HeLa cells were cotransfected with pPER constructs expressing peptide aptamers and the retroviral pEGZ vector expressing FLAG-Bfl-1ΔCter, FLAG-BCl-2ΔCter, FLAG-Bcl-xLΔCter, or FLAG-Mcl-1ΔCter using the jetPEI reagent (Polyplus transfection, Illkirch, France). Cells were harvested 48 h after transfection and lysed in NP-40 lysis buffer [200 mM NaCl, 40 mM Tris-HCl (pH 8), 2 mM EDTA, and 1% NP-40] supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, Isle d'Abeau, France). After centrifugation at 13000 rpm for 15 min, one-tenth of the supernatant was kept for Western blot analysis (total cell lysate) and nine-tenths of the supernatant was incubated with 25 μL of prewashed anti-FLAG monoclonal antibody M2 beads (Sigma) for 2 h at 4 °C. Peptide aptamer interactions were

detected by washing the beads four times in PBS followed by elution of the proteins bound to the beads in sample buffer for NuPAGE. Peptide aptamers and anti-apoptotic proteins were revealed in total cell lysate and immunoprecipitate fractions by Western blot analysis with the anti-thioredoxin antibody (Santa Cruz Biotechnology) and the anti-FLAG antibody (Sigma), respectively.

Statistical Analysis. We used a two-tailed unpaired *t* test to analyze the significance of the differences described in the different experimental conditions as indicated in the figures.

Western Blotting Analysis. Protein extracts were analyzed by Western blotting as described previously.[13]

Peptidization of Peptide Aptamers in Yeast. Peptidization allows us to test whether the variable region of the peptide aptamer interacts with the target without having its conformation constrained by the scaffold protein. Cloning of peptides into the pLP1 vector (designed by Aptanomics SA) was described by Bickle et al.[27] The molecular identity of all constructs was confirmed by sequencing (Genome Express).

The interaction matrix of Bcl-2 family members with peptides was realized with the same protocol that was used for the interaction matrix of Bcl-2 family members with peptide aptamers.

BP3 Infection with Peptide Aptamers and Sensitization to Death Induced by Chemotherapeutic Agents. Peptide aptamers Apt27, Apt50, and AptCtl were cloned into the CpoI site of retroviral vector pVRV6 (gift from Aptanomics SA). This vector was engineered to coexpress the peptide aptamer with a HA tag at the N-terminus and enhanced green fluorescent protein (GFP) as a reporter gene, permitting infected cells to be tracked by flow cytometry. The molecular identity of all constructs was confirmed by sequencing (Genome Express). Corresponding lentivirus particles were produced by the technical service of IFR128. BP3 cells were infected with peptide aptamers as described previously.[13] Peptide aptamer expression was checked 3 days after infection by Western blotting analysis (50 μg of cell lysate) by using the anti-HA antibody (Roche). Three days after infection, BP3-infected cells were treated with cisplatin (Sigma) or fludarabine (Sigma) for 48 h. Cell death was evaluated by propidium iodide staining and analyzed by FACS with CellQuest (Becton Dickinson).

Peptide Synthesis. Synthetic peptides were synthesized by Eurogentec or by the technical service of IFR128 with a purity of >98%.

Immunoprecipitation of Bcl-2 Family Members with the Synthetic Peptide. 293T cells were transfected with the retroviral pMIG vector expressing FLAG-Bfl-1ΔCter, FLAG-Bcl-2ΔCter, FLAG-Bcl-xLΔCter, or FLAG-Mcl-1ΔCter using jet-PEI reagent (Polyplus transfection). Cells were harvested 48 h later and lysed for 30 min at 4 °C in NP-40 lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). After centrifugation at 13000 rpm for 15 min, one-twentieth of the supernatant was kept for Western blot analysis (total cell lysate) and nineteen-twentieths of the supernatant was incubated for 2 h at 4 °C with 25 μL of prewashed anti-FLAG monoclonal antibody M2 beads (Sigma), previously blocked with DMEM medium containing SVF for 30 min at 4 °C and incubated for 1 h at 4 °C with synthetic biotinylated peptides (Bak BH3 or Pep27) at 1 mM for 2 h at 4 °C. Beads were then washed four times with PBS [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)], and protein−peptide complexes bound to the beads were eluted in sample buffer for NuPAGE. Anti-apoptotic proteins were revealed in the total cell lysate and immunoprecipitated fraction by Western blotting analysis with the anti-FLAG antibody (Sigma).

Bfl-1/Bax and Bfl-1/Bak Cotransfection Assay in HeLa Cells. pEYFP-N1 encoding Bax fused to YFP and pECFP-N1 encoding Bcl-xL fused to CFP at the N-terminus were kind gifts from J. Yuan. Bfl-1 cDNA was amplified by PCR from the Bfl-1pEGZ template, and EcoRI and BshtI were inserted into the pECFP-N1 vector. The molecular identity of all constructs was confirmed by sequencing (Genome Express). pRc/CMV-Bak-HA encoding Bak was obtained from A. Aouacheria.

HeLa cells were cotransfected with pRc/CMV-Bak-HA or pEYFPN-1-Bax and pECFP-N1-Bcl-xL or pECFP-N1-Bfl-1. Forty-eight hours after transfection, HeLa cells were treated with ABT-737 (gift from Abbott Laboratories) or Pep27-9R for 24 h. Cell death was evaluated by propidium iodide staining and analyzed by FACS with CellQuest (Becton Dickinson).

Bfl-1 Transfection Assay in Immortalized Baby Mouse Kidney (iBMK) Epithelial Cells. iBMK cells, wild type (WT), deficient for Bax ($Bax^{-/-}$) or Bak ($Bak^{-/-}$), or deficient for both Bak and Bax (DKO), were transfected with the retroviral pEGZ vectors expressing FLAG-Bfl-1mΔCter or FLAG-Bfl-1mΔCter. Twenty-four hours after transfection, iBMK cells were treated with staurosporine (Sigma) at the indicated concentration and/or Pep27-9R at 5 μM for 24 h. Cell death was evaluated by propidium iodide staining and analyzed by FACS with CellQuest (Becton Dickinson).

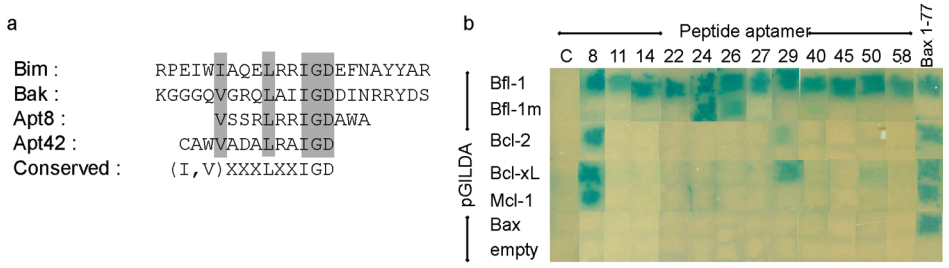
Cell Death Assay with Synthetic Peptides. IM9, BP3, A549, and HeLa cells were treated with synthetic peptide for 24 h. Cell death was evaluated by propidium iodide staining or propidium iodide and FITC-annexin V (BD Biosciences) double staining and analyzed by FACS with CellQuest (Becton Dickinson). For propidium iodide and FITC-annexin V double staining, cells were pelleted and resuspended in annexin V buffer (FITC-annexin V, 10 mM Hepes, 140 mM NaCl, and 5 mM $CaCl_2$), incubated in the dark for 15 min at room temperature, and analyzed by flow cytometry following addition of propidium iodide.

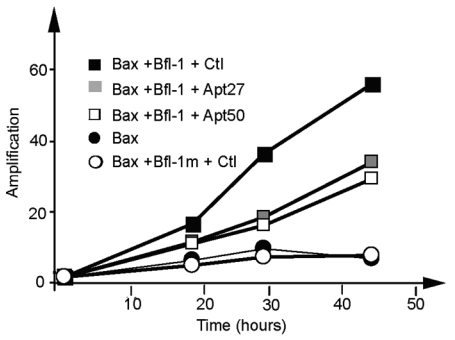
RESULTS

Isolation of Peptide Aptamers Specifically Interacting with Bfl-1. To identify inhibitors targeting Bfl-1 anti-apoptotic protein, we performed a yeast two-hybrid screen to isolate peptide aptamers that interact with Bfl-1 and disrupt its interaction with pro-apoptotic partners.

To prevent Bfl-1 from being anchored to cellular membranes, Bfl-1 with a C-terminal deletion was used as bait in the two-hybrid screen. We used a peptide aptamer library expressing 13-mer random sequence peptides displayed by the TrxA protein. The screening of 2×10^7 yeast transformants led to the identification of 67 peptide aptamers conferring a yeast two-hybrid interaction phenotype with LexA-Bfl-1ΔCter. Sequence analysis revealed that 36 peptide aptamer variable regions showed the common pattern LxxAL[I,V]xxV and five the pattern D[E]LRxI[L,V]GD (data not shown and Supporting Information). These two consensus sequences are part of the conserved sequence LxxxL[I,V]xxxLxx[I,V]GD shared by BH3 of pro-apoptotic proteins.[29] Illustrating this observation, blast analysis revealed that two of these peptide aptamers show sequence similarities with pro-apoptotic Bak and Bim protein BH3 domains (Figure 1a).

We next evaluated aptamer specificity toward other Bcl-2 family anti-apoptotic members. For that purpose, we performed





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Peptide Aptamer	Interaction		Growth inhibition
	Bfl-1	Bfl-1m	
Apt8	+	-	no
Apt11	+	-	yes
Apt14	+	-	yes
Apt22	+	-	no
Apt24	+	+	no
Apt26	+	+	yes
Apt27	+	-	yes
Apt29	+	-	yes
Apt40	+	+	no
Apt45	+	-	yes
Apt50	+	-	yes
Apt58	+	-	yes

Figure 1. Peptide aptamer identification and characterization in yeast. (a) Bak and Bim BH3 domain alignment with two peptide aptamers (Apt8 and Apt42). Conserved amino acids involved in BH3 domain definition are highlighted in gray. (b) Yeast two-hybrid interaction matrix of 12 peptide aptamers with anti-apoptotic Bcl-2 family members. Yeast transformants expressing bait proteins were mated with transformants expressing peptide aptamers as prey and containing a *lacZ* reporter gene. Prey expression was induced by the addition of galactose and incubation at 30 °C. Protein interaction was evaluated 48 h after induction by blue coloration in the presence of X-Gal. All anti-apoptotic proteins lacked their C-terminal domain. Bax was used as a negative control for peptide aptamer interaction, and Bax1−77 was used as a positive control for anti-apoptotic Bcl-2 family member interactions. C is an aptamer randomly picked up in the library during the screening, and it is used as a negative control for Bcl-2 family member interaction. (c) Peptide aptamer inhibitory activity on Bfl-1. Yeast samples were cotransformed with the indicated constructs. Protein expression was then induced in the presence of galactose, and yeast growth was evaluated by measuring OD_{600} over time. This result is representative of two independent experiments. (d) Summary of peptide aptamer characteristics: (+) interaction and (−) no interaction.

yeast two-hybrid interaction matrix mating assays using Bcl-2, Bcl-xL, and Mcl-1 protein constructs with C-terminal deletions as bait. A Bax BH3 prey construct was used as positive control and a nonrelevant aptamer, randomly picked up in the library, as a negative control. As shown in Figure 1b for 12 representative peptide aptamers, most aptamers exhibited a specific interaction with Bfl-1 (58 of the 67 aptamers identified). Only one peptide aptamer (Apt8) exhibited an interaction profile with all Bcl-2 family members tested. To locate the aptamer−Bfl-1 interaction site, we constructed a Bfl-1 protein mutated in the hydrophobic pocket (Bfl-1m G87A/R88S). This mutant did not interact with the Bax BH3 domain (Figure 1b) and was previously shown to be functionally defective.[30] We observed that only seven aptamers of the 67 identified retained their interaction phenotype with Bfl-1m (Figure 1b and data not shown), suggesting that most aptamers interact within the Bfl-1 hydrophobic groove.

It has previously been shown that Bfl-1 interacts with Bax and protects yeast from death induced by Bax overexpression.[31] We therefore evaluated the ability of our aptamers to inhibit Bfl-1 anti-apoptotic activity in yeast. For this purpose, aptamer expression was induced in yeast expressing both Bfl-1 and Bax, and yeast growth was analyzed by measuring the culture optical density at 600 nm over time. When Bax was overexpressed in yeast, no cell growth was observed. Bax's toxic effect was partly reversed when Bfl-1, but not the functionally defective Bfl-1m, was coexpressed (Figure 1c). These results suggest that Bfl-1 protects yeast from death by interacting with Bax and inhibiting its activity. As shown in Figure 1c, expression of Apt27 or Apt50 partly suppressed Bfl-1's protective effect, suggesting that Apt27 and Apt50 inhibit the Bax−Bfl-1 interaction. Results obtained with 12 representative peptide aptamers are summarized in Figure 1d. Interestingly, with the exception of Apt26, all functional aptamers interacted with the wild type but not mutated Bfl-1.

Peptide Aptamers Interact with Bfl-1 in Mammalian Cells. We next tested interactions of the peptide aptamer with Bfl-1 in mammalian cells. Bfl-1 and the peptide aptamer were therefore coexpressed in HeLa cells. Bfl-1 was then immunoprecipitated from transfected cell lysates, and binding of the peptide aptamer was revealed with the anti-TrxA antibody. Six peptide aptamers, which were efficiently expressed in HeLa cells, have been evaluated individually in this assay. As shown in Figure 2, among the six aptamers tested, only one failed to interact detectably with Bfl-1 (Apt8). We also evaluated aptamer specificity by testing coimmunoprecipitation of aptamers with other Bcl-2 family members, namely, Bcl-2, Bcl-xL, and Mcl-1. As shown in Figure 2, Apt45 and Apt11 interacted with Bfl-1 but also with the other Bcl-2 family members tested. Apt27 interacted with Bfl-1, Bcl-xL, and Mcl-1 but not with Bcl-2 in this assay, whereas Apt50 and Apt24 retained their specificity toward Bfl-1 in the mammalian system.

Peptide Aptamers Sensitize B-Cells to Chemotherapeutic Agents. We previously demonstrated that Bfl-1 knockdown using RNA interference sensitizes lymphoma B-cell lines to apoptosis

dx.doi.org/10.1021/bi101839p |*Biochemistry* 2011, 50, 5120–5129

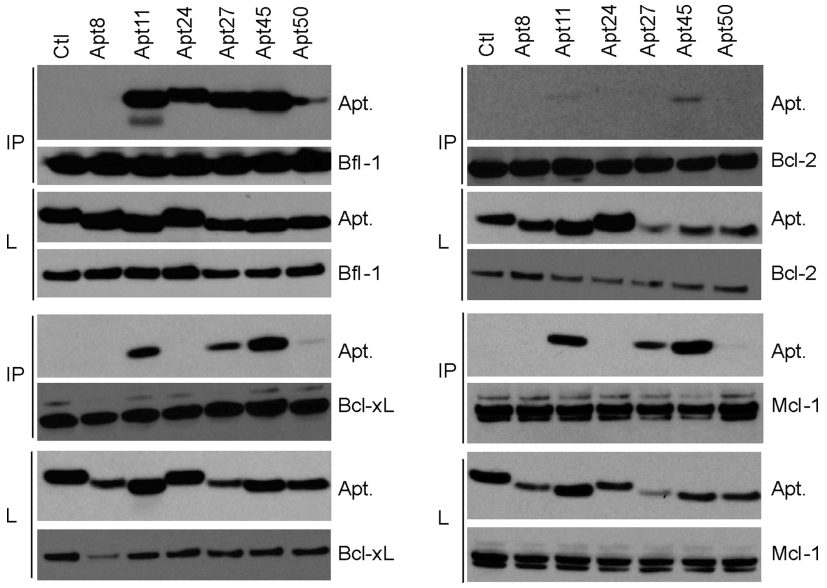

Figure 2. Interaction of the peptide aptamer with anti-apoptotic Bcl-2 family members in mammalian cells. Constructs encoding peptide aptamers and Bcl-2 anti-apoptotic proteins from which their C-terminal domains had been deleted were cotransfected in HeLa cells. Cells were lysed 48 h following transfection, and anti-apoptotic protein was immunoprecipitated with the anti-FLAG antibody and treated as described in Experimental Procedures. Peptide aptamer (Apt.) and anti-apoptotic protein expression were revealed both in immunoprecipitates (IP) and in total cell lysates (L) by Western blot analysis using specific antibodies.

induced by several chemotherapeutic agents.[13] In particular, we demonstrated that BP3 lymphoma cells express a high level of Bfl-1 and that Bfl-1 knockdown increased the sensitivity of BP3 to chemotherapeutic agents such as cisplatin and fludarabine. We thus used the BP3 cell line as a relevant cellular model to evaluate whether the expression of the two anti-Bfl-1 peptide aptamers, Apt27 and Apt50, would increase the sensitivity of BP3 to chemotherapeutic agents. BP3 cells were infected with a retrovirus encoding HA-tagged anti-Bfl-1 or control aptamers and coexpressing the green fluorescent protein (GFP). This vector allowed us to identify aptamer-expressing cells. Aptamer expression was confirmed by Western blot analysis (Figure 3). We did not observe significant spontaneous cell death of BP3 that expressed peptide aptamers interacting with Bfl-1 as compared with the control aptamer. Interestingly, we observed that BP3 cells expressing either Apt27 or Apt50 but not the control aptamer were significantly more sensitive to either cisplatin (Figure 3, left panel) or fludarabine treatment (Figure 3, right panel). These results indicate that when expressed in BP3 B-cell lymphoma cells, Apt27 and -50 increase the sensitivity of these cells to chemotherapeutic drugs.

Peptides Derived from Aptamer Variable Loops Retain the Ability To bind to Bfl-1. As illustrated above, peptide aptamer screening allows rapid identification of specific protein ligands, some of which represent potential protein−protein interaction inhibitors. However, the use of such large proteins in therapy is difficult because of stability, immunogenicity, and delivery issues. Because synthetic peptides can be derived from aptamer variable regions, we tested whether unconstrained peptides corresponding to peptide aptamer variable regions still bound to Bfl-1 and inhibited its activity. As a first approach, we used a yeast two-hybrid assay named "peptidization" previously described by Bickle and colleagues[27] to determine the capacity of peptide aptamer variable regions to interact with Bfl-1. For that purpose, the N-termini of the variable region are directly fused to

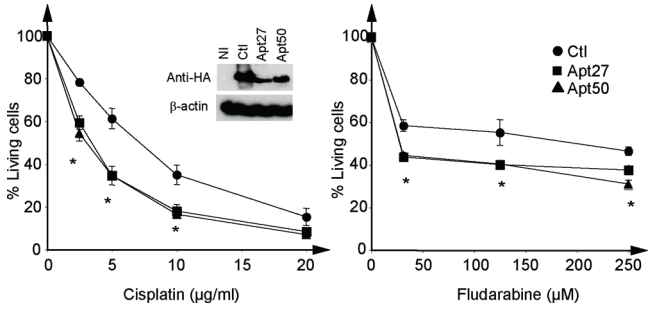

Figure 3. Peptide aptamers sensitize BP3 cells to death induced by chemotherapeutic agents. BP3 cells were infected with control aptamer (Ctl), peptide aptamer 27 (Apt27), or peptide aptamer 50 (Apt50), and cell viability was measured by propidium iodide staining and flow cytometry analysis 48 h after treatment with the indicated concentrations of cisplatin or fludarabine. Results are expressed as means ± the standard error of the mean from three independent experiments. Statistical analysis was performed as described in Experimental Procedures (*$p < 0.05$ vs control). Peptide aptamer expression was monitored by Western blotting using anti-HA antibodies 6 days following infection of BP3 cells (NI, noninfected cells).

the transcriptional activation domain, in the absence of any constraint imposed by the scaffold. We observed that variable regions derived from Apt27 (Pep27) and Apt50 (Pep50) retained a yeast two-hybrid interaction phenotype with Bfl-1 but only Pep27 showed a specific interaction phenotype, whereas Pep50 lost its specificity toward Bfl-1 and interacted with other anti-apoptotic Bcl-2 family members (Figure 4a). We thus chose to work with the peptide sequence derived from the variable region of Apt27.

Pep27 was synthesized keeping three flanking thioredoxin amino acids at its N- and C-termini and adding a biotin to its N-terminus. To confirm Pep27−Bfl-1 interaction and in mammalian

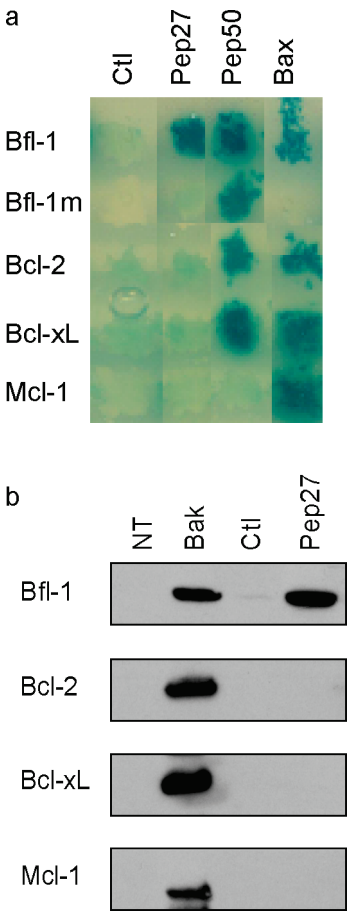

Figure 4. Interaction assays between peptides derived from aptamer variable regions and anti-apoptotic proteins. (a) Yeast two-hybrid interaction matrix of peptides derived from Apt27 and Apt50 with anti-apoptotic Bcl-2 family members. Yeast transformants expressing bait vectors were conjugated with transformants expressing unconstrained peptide aptamers (either control, as defined in Figure 1b, or Bfl-1 specific) or Bax protein as prey and containing a *lacZ* reporter gene. Prey expression was induced by the addition of galactose and incubation at 30 °C. All anti-apoptotic proteins had their C-terminal domains deleted. Protein interaction was evaluated 48 h after induction by blue coloration in the presence of X-Gal. (b) 293T cells were transfected with constructs encoding FLAG-tagged Bfl-1, FLAG-tagged Bcl-2, FLAG-tagged Bcl-xL, or FLAG-tagged Mcl-1 proteins. Forty-eight hours following transfection, cells were lysed and cell lysates were incubated with synthetic peptides (Bak BH3 or Pep27) coupled on agarose beads. Bfl-1, Bcl-2, Bcl-xL, and Mcl-1 were revealed by Western blotting using the anti-FLAG antibody. Nontransfected cells (NT) were used as a negative control.

cells, we incubated a cell lysate from 293T cells overexpressing the Bfl-1 protein with a Pep27 streptavidin agarose matrix. We used a synthetic peptide corresponding to the Bax BH3 domain as a positive control. As illustrated in Figure 4b, Pep27 bound to Bfl-1 with the same intensity as the Bak BH3 peptide. Parallel experiments realized with cell lysates from 293T cells overexpressing Bcl-2, Bcl-xL, or Mcl-1 indicated that Pep27 did not interact with Bcl-2, Bcl-xL, or Mcl-1, as observed in the yeast two-hybrid model.

Pep27 Induces B-Cell Lymphoma Cell Death. We next evaluated the capacity of Pep27 to inhibit Bfl-1 activity in cells. For that purpose, a polyarginine sequence (nine Arg residues) allowing cell membrane penetration was added to Pep27 (Pep27-9R). Peptide penetration in HeLa, BP3, and IM9 cell lines was confirmed using confocal microscopy (data not shown). We then analyzed the capacity of Pep27-9R to induce apoptosis in B-cell lymphoma cell lines BP3 and IM9 that express high levels of Bfl-1 (Figure 5a) and are sensitive to Bfl-1 downregulation.[13] We also evaluated the toxicity of Pep27-9R toward HeLa and A549 cell lines. The HeLa cell line is derived from a cervical adenocarcinoma and does not express Bfl-1, whereas the A549 cell line is derived from a lung carcinoma and expresses very low levels of Bfl-1 (Figure 5a). We observed that Pep27-9R but not a control peptide induced rapid and dose-dependent cell death in both BP3 and IM9 cells, with ~20 and ~40% dead cells, respectively, following a 24 h treatment at a peptide concentration of 10 μM (Figure 5b). On the other hand, Pep27-9R exhibited no or very low toxicity toward HeLa and A549 cells (Figure 5b).

We next evaluated the capacity of Pep27-9R to sensitize BP3 cells to apoptosis induced by chemotherapeutic agents, as previously observed following aptamer expression in these cells (Figure 3). BP3 cells were treated with different doses of cisplatin or fludarabine, in the presence of 5 μM PepCtl-9R or Pep27-9R, and viability was assessed 24 h following treatment. As described in Figure 5c, Pep27-9R treatment significantly increased the sensitivity of BP3 cells to cisplatin or fludarabine treatment compared to PepCtl-9R treatment.

Pep27-9R Inhibits Bax−Bfl-1 Interaction and Restores Cell Death in HeLa Cells. As just cited above, HeLa cells do not express Bfl-1 or Bcl-xL proteins and express very low levels of Bcl-2 and Mcl-1. As a consequence, and as previously reported, these cells were highly sensitive to apoptosis mediated by Bax overexpression (Figure 6a[32]), but this effect was inhibited by Bfl-1 overexpression. As expected, the ABT-737 molecule, which interacts with Bcl-2 and Bcl-xL but not with Bfl-1 or Mcl-1, had no effect on Bfl-1 protection, whereas gambogic acid, an antagonist of several anti-apoptotic Bcl-2 proteins, including Bfl-1,[33] prevented Bfl-1 protection (Figure 6a). The viability of nontransfected HeLa cells was almost not affected by either ABT-737 or gambogic acid compounds alone (data not shown). Bfl-1 protection toward Bax-mediated cell death was also reversed by Pep27-9R treatment (Figure 6a), suggesting that this peptide can indeed inhibit Bfl-1−Bax interaction, because it was not toxic to nontransfected HeLa cells (Figure 5b). Similar experiments conducted with Bak-overexpressing cells, showed that Pep27-9R also reversed Bfl-1 protection (Figure 6b), indicating it could also inhibit Bfl-1−Bak interaction. Because it has been recently proposed that Bfl-1 antagonizes Bak but not Bax pro-apoptotic function in living cells,[34] we tested whether Pep27-9R would reverse Bfl-1 protection in STS-treated iBMK epithelial cells that do or do not express endogenous Bak and Bax.[35] We showed that indeed WT-Bfl-1, but not Bfl-1m, very efficiently protects WT iBMK cells from STS-induced cell death (Figure 6c). We further observed that overexpression of Bfl-1 protected $Bak^{-/-}$ and $Bax^{-/-}$ iBMK cells, indicating that Bfl-1 could as previously described by Simmons et al.[34] antagonize Bak but also Bax pro-apoptotic function in those cells (Figure 6c). As shown in panels c and d of Figure 6, Pep27-9R did not significantly induce cell death in iBMK cells (WT, $Bak^{-/-}$, $Bax^{-/-}$, or DKO) but partially reversed Bfl-1 protection toward STS. Altogether, these results indicate that Pep27-9R could inhibit the interaction of Bfl-1 with endogenous Bax or Bak proteins and weaken their pro-apoptotic ability.

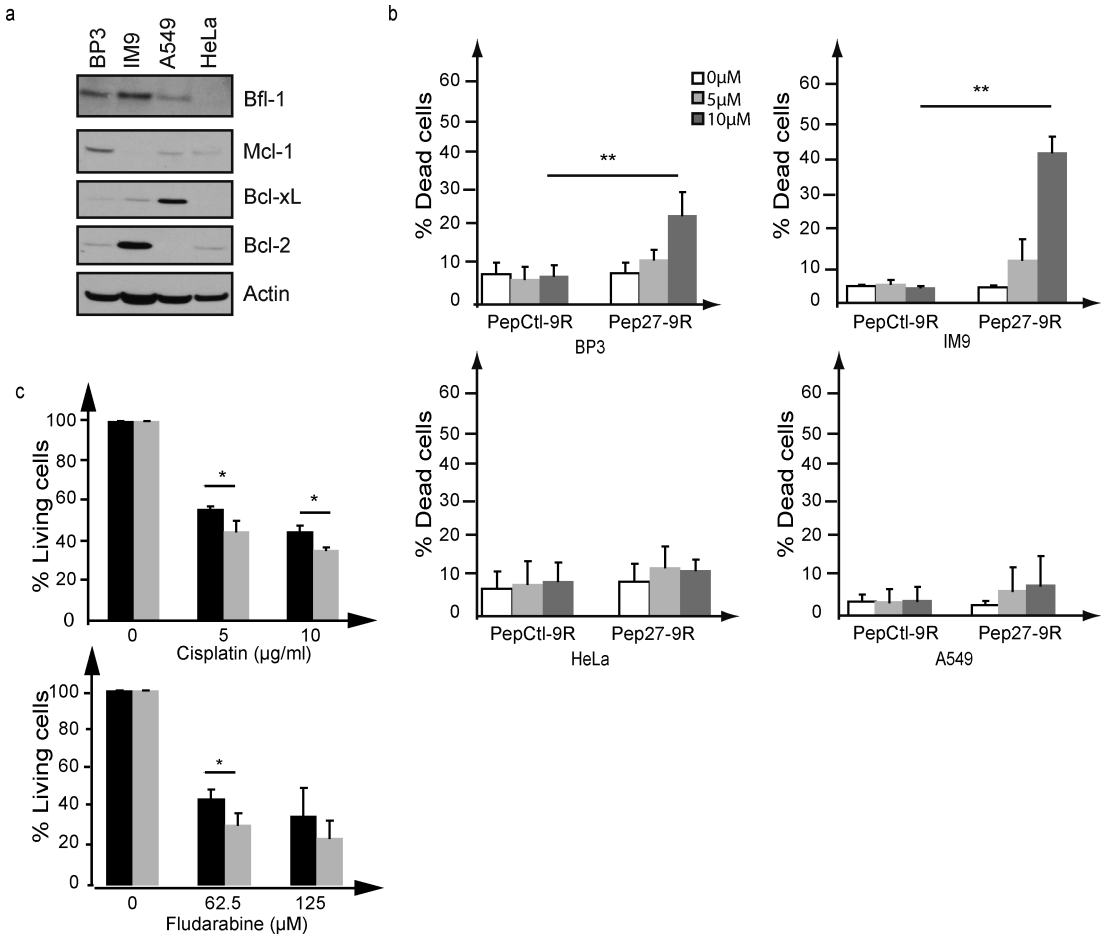

Figure 5. Activity of Pep27-9R on various tumoral cell lines. (a) BP3, IM9, A549, and HeLa cells were lysed, and anti-apoptotic protein expression was revealed by lysates by Western blot analysis using specific antibodies. (b) Cells were treated with the indicated concentrations of control peptide (Ctl) or peptide 27 (Pep27) both fused to a nine-Arg sequence allowing cell penetration at 0, 5, and 10 μM over 24 h. Cell viability was measured by propidium iodide staining and flow cytometry analysis. Statistical analysis was performed as described in Experimental Procedures (**$p < 0.01$ vs control). (c) BP3 cells were treated with the indicated doses of cisplatin or fludarabine in the presence of 5 μM PepCtl-9R (black bars) or 5 μM Pep27-9R (gray bars). Viability was measured by propidium iodide and annexin V double staining and flow cytometry analysis 24 h after treatment. Double-negative cells are considered living cells. Results are expressed as means ± the standard error of the mean from three independent experiments. Statistical analysis was performed as described in Experimental Procedures (*$p < 0.05$ vs control).

DISCUSSION

The activity of pro-apoptotic proteins of the Bcl-2 family is controlled by protein−protein interactions with their anti-apoptotic partners, and disrupting these interactions represents an interesting and innovative strategy for restoring apoptosis in cancer cells where this process is often deregulated, because of the increased level of expression of anti-apoptotic Bcl-2 family members. A peptide aptamer strategy targeting an anti-apoptotic member of the Bcl-2 family was first applied to target the chicken Nr-13 protein by Nouvion et al.,[25] who described anti-Nr-13 peptide aptamers with potential pro-apoptotic activity. In particular, the authors demonstrated that peptide aptamers interfered with Nr-13 anti-apoptotic activity in both yeast and mammalian cells, as demonstrated by their ability to restore cell death following the withdrawal of serum from COS-7 cells overexpressing Nr-13.

We describe herein the first characterization of peptide aptamers that interact with human anti-apoptotic proteins of the Bcl-2 family, and in particular with the Bfl-1 protein that was identified as a potential target for B-cell malignancies. Indeed, molecular profiling studies of large B-cell lymphoma cells have identified Bfl-1 as a gene signature in a subtype of diffuse large B-cell lymphoma and in primary mediastinal large B-cell lymphomas.[10−12] Overexpression of Bfl-1 has also been associated with chemoresistance in B-cell chronic lymphocytic leukemia (B-CLL) patients.[36]

We thus identified, by a yeast two-hybrid screen, Bfl-1-interacting peptide aptamers that also interact with Bfl-1 in mammalian cells. Importantly, even if interactions described in yeast between aptamers and Bfl-1 were most of the time confirmed in mammalian cells, we noticed that interaction profiles between peptide aptamers and anti-apoptotic Bcl-2 members could not be extrapolated from yeast to mammalian cells, suggesting that post-translational modifications may affect those protein−protein interactions.

In agreement with previous results using the RNA interference strategy,[13] we observed that Bfl-1 peptide aptamers sensitize B-cell lymphoma cell lines to apoptosis induced by chemotherapeutic agents. Taken together, these results and particularly those obtained with Apt50 that does not bind to other Bcl2

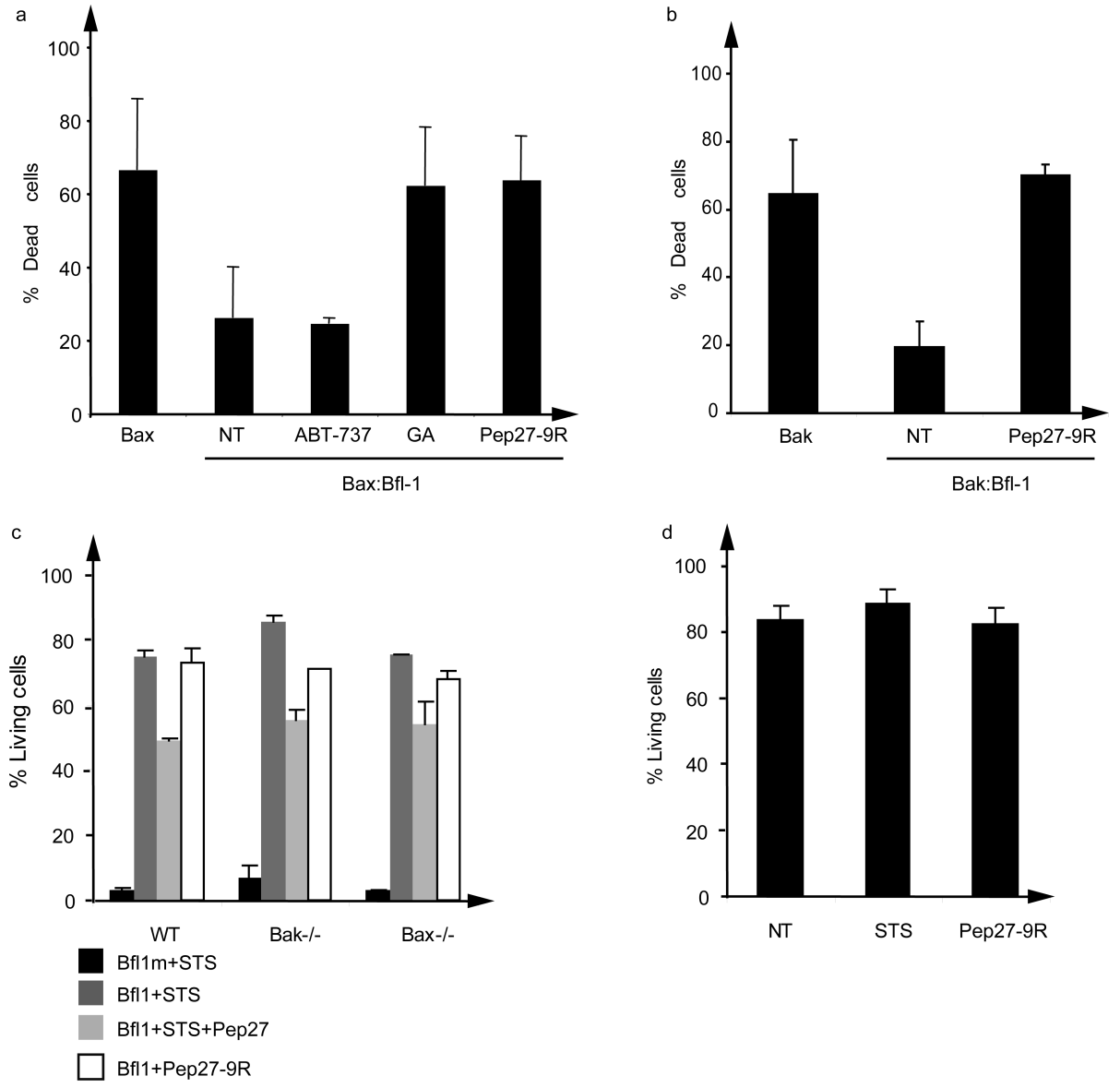

Figure 6. Pep27-9R inhibits Bfl-1 cell death rescue. (a) HeLa cells were transfected with a vector encoding Bax alone or Bax and Bfl-1. Twenty-four hours after transfection, cells were either left untreated (NT) or treated with ABT737 (10 μM), GA (2 μM), or Pep27-9R (10 μM) for 24 h. (b) HeLa cells were transfected with a vector encoding Bak alone or Bak and Bfl-1. Twenty-four hours after transfection, cells were either left untreated (NT) or treated with Pep27-9R (5 μM) for 24 h. (c) iBMK cells (WT, $Bak^{-/-}$, or $Bax^{-/-}$) transfected with a vector encoding Bfl-1-ΔCter or Bfl-1m-ΔCter were treated with STS (0.25 μM) or not treated in the presence or absence of Pep27-9R (5 μM) for 24 h. (d) DKO iBMK cells were treated with STS (0.25 μM) or Pep27-9R (5 μM) or not treated for 24 h. In all cases, cell viability was assessed by propidium iodide staining and flow cytometry analysis. Results are expressed as means $\pm$ the standard error of the mean from three independent experiments.

family members further validate Bfl-1 as a potential therapeutic target in B-cell lymphoma. We also demonstrate that a non-constrained peptide derived from one aptamer variable region exhibited inhibitory activity toward Bfl-1 in Bax- or Bak-over-expressing HeLa cells and in STS-treated iBMK cells that expressed endogenous Bax and/or Bak proteins. This peptide also efficiently promoted cell death in B-cell lymphoma cells expressing high levels of Bfl-1, but not in tumoral cell lines that do not overexpress Bfl-1, thereby establishing the specificity of its mechanism of action. Interestingly, this work also demonstrated for the first time that peptide structure constrained by insertion into the thioredoxin platform might contribute to interaction affinity and/or specificity in the yeast two-hybrid system.

To conclude, such a study clearly demonstrates the feasibility and the interest of developing tools for disrupting interactions of Bfl-1 with its pro-apototic partners in anticancer strategies. As already mentioned, specific inhibitors interacting with Bfl-1 or Mcl-1 have yet to be discovered. Recently, the natural product gambogic acid has been shown to target anti-apoptotic Bcl-2 family proteins, including Bfl-1.[33] However, gambogic acid retains cytotoxic activity against MEF Bax/Bak double KO cells, indicating that this molecule has additional cellular targets contributing to its cytotoxicity. Even if small proteins such as peptide aptamers do not represent a very straightforward therapeutic opportunity as proteins or small peptides are neither orally bioavailable nor cell permeable, aptamers should provide

dx.doi.org/10.1021/bi101839p |*Biochemistry* 2011, 50, 5120–5129

useful tools for guiding the design or screening[37] of small molecules inhibiting Bfl-1.

■ ASSOCIATED CONTENT

Supporting Information. Variable regions of Bfl-1 aptamers that have been sequenced (Genome Express). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

This work was supported by grants from INSERM and UCB Lyon 1 and additional funds from the Association pour la Recherche sur le Cancer and the Ligue contre le Cancer (07 and 26) to N.B.-B. G.B. was supported by a fellowship from the région Rhône-Alpes and by a fellowship from the Ligue Nationale contre le Cancer.

■ ACKNOWLEDGMENT

We thank the staff of the Plateforme de Cytométrie en Flux of IFR128 Biosciences Lyon-Gerland for their technical assistance. We also thank F. Hill for critical reading of the manuscript and Dr. E. White for iBMK cell lines.

■ ABBREVIATIONS

BH, Bcl-2 homology; iBMK, immortalized baby mouse kidney; GA, gambogic acid; GFP, green fluorescent protein; TrxA, thioredoxin A; STS, staurosporine; WT, wild type.

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