Bcl-X<sub>L</sub> Mutations Suppress Cellular Sensitivity to Antimycin A*<sup>†</sup>†<sup>‡</sup>

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Cells expressing high levels of the BCL-X<sub>L</sub> anti-apoptotic protein are preferentially killed by the mitochondrial inhibitor antimycin A (AA). Computational modeling predicts a binding site for AA in the extended hydrophobic groove on BCL-X<sub>L</sub>, previously identified as an interface for dimerization to BAX and related pro-apoptotic proteins. Here, we identify BCL-X<sub>L</sub> hydrophobic groove mutants with normal cellular anti-apoptotic function but suppressed sensitivity to AA. The LD<sub>50</sub> of AA for cells expressing BCL-X<sub>L</sub> mutants directly correlates with the measured in vitro dissociation constants for AA binding. These results indicate that BCL-X<sub>L</sub> is a principal target mediating AA cytotoxicity.

The BCL-2 family proteins are key positive and negative regulators of apoptosis. Biochemical and subcellular fractionation studies localize the anti-apoptotic members, such as BCL-2 and BCL-X<sub>L</sub>, at outer mitochondrial membranes (OMM) and, to a lesser extent, at endoplasmic reticulum and nuclear membranes. Pro-apoptotic factors either reside at the OMM or as soluble cytosolic proteins, as with BAX, BID, and BAD. Following an apoptotic signal, these factors are translocated to mitochondria. BCL-2 and BCL-X<sub>L</sub> cancel out the activity of pro-apoptotic members at the OMM, at least partly through direct interactions (1).

The tertiary structures of pro- and anti-apoptotic BCL-2 proteins consist of all α-helical protein folds involving one or more conserved BCL-2-homology domains (BH1–4). Although all four BH domains are necessary for the proper functioning of BCL-2 and BCL-X<sub>L</sub>, the BH3 domains of pro-apoptotic members are themselves sufficient for apoptosis induction (2). BH3 domains from pro-apoptotic BH proteins also make heterodimeric contacts with related anti-apoptotic partners. The structure of the complex of a soluble BCL-X<sub>L</sub> protein (BCL-X<sub>L</sub>ΔC) and BAK-BH3 peptide from nuclear magnetic resonance (NMR) spectroscopy showed peptide bound to a hydrophobic groove bounded by the BH1, BH2, and BH3 domains of BCL-X<sub>L</sub> (3).

In addition to their ability to nullify pro-apoptotic binding partners, the anti-apoptotic BCL-2 family members may maintain cell survival through regulation of ion and metabolite gradients across mitochondrial membranes. BCL-X<sub>L</sub> and BCL-2 form pores in synthetic lipid vesicles and membranes. Interestingly, this intrinsic pore-forming activity is inhibited by a bound BAK BH3 peptide (4). It remains an unresolved question whether BCL-X<sub>L</sub> and BCL-2 affect OMM permeability directly or through interactions with other known mitochondrial channels. For example, BCL-X<sub>L</sub> may promote osmotic homeostasis by maintaining the voltage-dependent anion channel in an open state, allowing transit of metabolites across the OMM (5–7). BCL-X<sub>L</sub> also interacts with the adenine nucleotide translocator, the most abundant protein in the inner mitochondrial membrane and a major component of the permeability transition pore complex (8).

Previously, we reported that AA, an inhibitor of mitochondrial electron transport at complex III, selectively kills murine hepatocyte cell lines that over-express BCL-X<sub>L</sub> or BCL-2 (4, 9). Mitochondrial electron transport inhibition is not correlated with differential killing of BCL-X<sub>L</sub>-expressing cells, as 2-methoxy AA (2-OmAA) has similar effects despite a 1000-fold reduction in electron transport inhibition. Rather, a direct effect of AA on BCL-X<sub>L</sub> function(s) was proposed, based on fluorescent binding assays showing stoichiometric interaction of AA and BCL-X<sub>L</sub>ΔC. Computational modeling using the BCL-X<sub>L</sub>ΔC NMR structure (3) predicted a binding site for AA along the long axis of the hydrophobic groove (Fig. 1). Finally, dose-dependent inhibition of BCL-X<sub>L</sub>ΔC pores in synthetic liposomes is observed with either AA or 2-OmAA.

To validate BCL-X<sub>L</sub> as an important target for 2-OmAA cytotoxicity, we generated a series of mutant BCL-X<sub>L</sub> proteins with single amino acid substitutions at predicted contact sites for AA. Analysis of cells expressing mutant BCL-X<sub>L</sub> proteins confirms the specificity of 2-OmAA as a BCL-X<sub>L</sub> inhibitor. In addition, these data provide a detailed mapping of the AA/BCL-X<sub>L</sub> binding interface.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma unless otherwise noted. Antimycin A<sub>i</sub> stocks were prepared in dimethylsulfoxide (Me<sub>2</sub>SO) and concentrations determined using a molar absorption coefficient of 4.79 nm<sup>2</sup> M<sup>−1</sup> cm<sup>−1</sup> at 320 nm. Peptide corresponding to the BH3 domain of BAK (72-GQVGRQLAIIGDDINR-87) was synthesized by the Fred Hutchinson Cancer Research Center core facility. Fluorescein isothiocyanate (FITC)-conjugated BAK BH3 peptide was synthesized by American Peptide Company and purified to >98% by high performance liquid chromatography. Molecular weights were confirmed by matrix-assisted laser desorption/ionization mass spectrometry. 2-OmAA<sub>i</sub> was obtained from the National Cancer Institute.

Mutagenesis and Plasmid Construction—Bclx<sub>L</sub> mutants were derived from pSFFV-Bclx<sub>L</sub>-WT (4) using the Stratagene QuickChange XL site-directed mutagenesis kit. Briefly, mutagenic primers spanning each target site were used to amplify fragments containing the desired mutations. Residual wild-type template was then removed by digesting with the methylation-dependent endonuclease, DpnI. For recombinant
expression, BelxlΔ22 lacking the COOH-terminal membrane anchor sequence was generated by PCR. PCR products were digested with NdeI and XhoI and ligated into pET22b (+) (Novagen). All constructs were confirmed by sequence analysis.

Cell Transfection—TAMH cells were transfected by Lipofectin (LipofectAMINE, Invitrogen) as previously described (4). For analysis of BCL-XL expression, 20 μg of cell protein (1% Triton X-100, 5 mM Tris-HCl, pH 8.0, 150 mM NaCl) was separated by 20% SDS-PAGE and transferred (electrophoretic) to nitrocellulose membranes. Immunodetection of BCL-XL was carried out using rabbit anti-BCL-XL antibody and Protein A/horseradish peroxidase conjugate (BD Transduction Laboratories), followed by chemiluminescent detection (Amersham Biosciences). Cell Viability Assays—Cells were grown to 80% confluence in 96-well plates following addition of 100 μl of 2 × AA1 or staurosporine (STS) solution in complete medium. Cell viability was determined spectrophotometrically after 24 h treatment as the ratio of reduced and oxidized Alamar Blue (BIOSOURCE) at 570 and 600 nm, respectively. Dose responses were measured in black quartz microplates (Hellma) at room temperature. Calcein release was expressed as percentage of maximum release with detergent lysate (0.1% Triton X-100). Pore induction was calculated using cumulative dye release normalized to results obtained in absence of inhibitors, and the IC50 values were determined by non-linear regression analysis.

Crystallographic Studies—Purified WT and mutant BCL-XL(ΔC) proteins were concentrated to 1 μl and crystallized by hanging drop vapor diffusion at 4 °C. The mother liquor consisted of 50 mM MES, pH 6.0, 1.9 M ammonium sulfate. Crystals were flash-frozen in liquid nitrogen after soaking in mother liquor plus 30% trehalose (Sigma) for 1 min. Data sets were collected at 100 K with a Rigaku x-ray generator (100 mA and 50 kV) and a Raxis IV image plate. DENZO and SCALEPACK (13) were used to process the diffraction data.

The program EPMR (14) was used to find a molecular replacement solution using the BCL-XLwt structure (Protein Data Bank code 1MAZ) as a starting model. The space group for BCL-XLwt and all mutant BCL-XLwt proteins was determined to be P21212. A free R set (15) of 10% was set aside using the CCP4 program FreeRig. The Xfit 4.0 program from the CNS suite (16) was used to visualize and model the electron density maps. The CNS (17) program package was used for model refinement and simulated annealing composite omit 2Fo − Fc maps were used to guide model rebuilding. The stereochemical properties of all structures were examined by PROCHECK (18). Subsequent structural alignments, analysis, and figures were done with Swiss PDB viewer (19), with pictures rendered using POVRay (www povray.org).

Recombinant BCL-XL Purification—A pET22b (Novagen) vector coding for BCL-XL (ΔC) was transformed into Escherichia coli BL21 (DE3) cells that carried pUBS820 (encoding human Arg tRNA) and grown to an A600 of 0.6. Protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside at 30 °C. The cells were resuspended 1:5 (w/v) in PEB buffer (50 mM Tris, pH 8.0, 0.2 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM p-mercaptoethanol, 1% (v/v) glycerol), and stirred for 20 min at 4 °C. Cells were disrupted by pulse sonication, and the soluble fraction was loaded onto a nickel-nitrilotriacetic acid column (Qiagen) equilibrated with PEB buffer. The column was washed with 40 mM imidazole, eluted with 200 mM imidazole, and the protein fractions were pooled and dialyzed (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) at 4 °C overnight. The dialyzed protein was concentrated to 10 mg/ml and fractionated on a Superdex 75 gel filtration column (Amersham Biosciences). The fractions containing BCL-XL(ΔC) protein were pooled, exchanged into low-salt buffer (same as for previous dialysis buffer, except 50 mM NaCl), and loaded onto a MonoQ anion exchange column (Amersham Biosciences). Protein was eluted from the column with increasing NaCl gradient, pooled, and concentrated. Purity was >99% as determined by silver staining. BCL-XL(ΔC) protein concentrations in 6 μM guanidine HCl were determined from 280 nm absorbance using extinction coefficients of 41940 M−1 cm−1 for WT, E92L, A142L, and F146L, and 47630 M−1 cm−1 for F97V BCL-XL(ΔC) proteins.

Fluorescence Anisotropy—Fluorescence anisotropies of AA1 and FITC-labeled BAK BH-3 peptide were measured using a Fluoromax-3 spectrophotometer equipped with autoadjustors (Jobin Yvon/Spekctramatch). All reagents were prepared in 0.2 μM-filtered phosphate-buffered saline with fresh 1 mM dithiothreitol. Excitation and emission wavelengths were 340 and 420 nm for AA1, and 480 and 520 nm for FITC-labeled BAK BH3 peptide, respectively. Slit widths were set at 10 nm for both excitation and emission. AA1 (200 nM) or BH3 peptide (25 nM) were equilibrated with different concentrations of BCL-XL(ΔC) for at least 1 h at room temperature. Each data point represents the mean of three independent measurements. Fluorescence anisotropy values were corrected to fraction of ligand bound (fL) and expressed on a semi-log plot with non-linear curve fitting (10).

Pore Formation Assays—Large unilamellar vesicles composed of 60% dioleoyl phosphatidylcholine and 40% dioleoyl phosphatidylglycerol were prepared by the extrusion method of Mayer et al. (11). Lipid stocks, in chloroform, were mixed and dried under a stream of nitrogen gas. The lipid was resuspended by vortexing for 30 min in a solution of 40 μM calcine (Molecular Probes), 25 mM KCl, and 10 mM KOAc, pH 5.0. After 10 freeze-thaw cycles, the lipid suspension was extruded through two 0.1 μm pore diameter Nucleopore filters. Non-entrapped dye was removed by passage over a Sephadex G10 column (Amersham Biosciences). Lipid concentration was measured using the ammonium ferriochromate method (12). For pore assays, recombinant BCL-XL(ΔC) (500 nM) was added to large unilamellar vesicles (60 μM lipid concentration) in 100 mM KCl, 10 mM KOAc, pH 5.0, and fluorescence measured (490 nm excitation, 520 nm emission) with a Fluoromax-3 spectrophotometer. Peptide was incubated with BCL-XL(ΔC) 5 min prior to mixing with liposomes. AA1 was added to liposomes 1 min before adding BCL-XL(ΔC) protein. Samples for kinetic assays were analyzed in a thermostatted cuvette at 37 °C with constant stirring. Dose responses were measured in black quartz microplates (Hellma) at room temperature.

All results were normalized against Me2SO controls. Similar results were obtained using NaCl gradient, pooled, and concentrated. Purity was >99% as determined by silver staining. BCL-XL(ΔC) protein concentrations in 6 μM guanidine HCl were determined from 280 nm absorbance using extinction coefficients of 41940 M−1 cm−1 for WT, E92L, A142L, and F146L, and 47630 M−1 cm−1 for F97V BCL-XL(ΔC) proteins.

RESULTS

Mutations at the BCL-XL Hydrophobic Groove Reduce Cellular Sensitivity to Antimycin A—A series of point mutations were introduced to alter specific residues in the BCL-XL hydrophobic groove

FIG. 1. Position of BCL-XL hydrophobic groove mutations relative to predicted antimycin A binding site. Molecular surface of BCL-XL with modeled antimycin A molecule. The Glu-92 (brown) carbonyl oxygen and side chains of Phe-97 (magenta), Ala-142 (purple), and Phe-146 (orange) contact antimycin A in the docking model.
contact with AA from the docking model. The following single amino acid substitutions were made in human BCL-XL: E92L, F97W, A142L, and F146L. We obtained stable transfectants of TAMH murine hepatocytes for each of the mutated BclxL plasmids as well as wild-type BclxL. Mutant BCL-XL (BCL-XLmut) and wild-type proteins (BCL-XLwt) were expressed at similar levels (Fig. 2a).

To assess the effect of mutations on BCL-XL function, we tested TAMH/BCL-XLmut cells for survival during STS treatment. Dose-response curves show that each of the BCL-XLmut proteins produced equivalent levels of protection against STS-induced cell death. LD₅₀ values for STS with cells expressing BCL-XLmut were not significantly different from BCL-XLwt cells (LD₅₀ = 0.58 ± 0.1 μM) (Fig. 2a and Table I). Vector-only control cells expressed low levels of endogenous BCL-XL and were significantly more sensitive to STS (LD₅₀ = 0.11 ± 0.01 μM) than any of the BCL-XLmut or BCL-XLwt cell lines (p < 0.05) (Fig. 2b).

We next challenged BCL-XLwt and BCL-XLmut cells with AA1. In contrast to the results with STS, AA sensitivity varied substantially among the BCL-XLwt cell lines. Compared with BCL-XLmut cells (LD₅₀ = 0.47 ± 0.1 μM), the E92L and F97W BCL-XLmut cells had reduced sensitivity to AA (LD₅₀ = 1.72 ± 0.3 μM and 5.12 ± 0.9 μM, respectively), whereas the A142L and F146L BCL-XLmut cells were completely insensitive to AA (Fig. 2c and Table I).

AA-insensitive BCL-XL Mutants Have Lower Binding Affinity for AA—Recombinant BCL-XLmut(ΔC) and wild-type proteins were purified from bacterial extracts by nickel-nitritotriacetic acid affinity, gel filtration, and anion-exchange chromatography. Direct quantitative measurements of AA1 binding to BCL-XLmut(ΔC) proteins were obtained from fluorescence anisotropy under equilibrium conditions. Binding constants were calculated using non-linear regression analysis (Table I). AA1 has much weaker binding affinity with the F97W, A142L, and F146L mutants (Kₐ = 17.56 ± 5.2 μM, 41.77 ± 21.4 μM, and 20.04 ± 9.4 μM, respectively) than with BCL-XLwt(ΔC) protein (2.36 ± 1.41 μM) (Fig. 3a). The binding affinity of AA1 with the E92L mutant (Kₐ = 5.06 ± 0.86 μM) is reduced 2–3-fold relative to BCL-XLwt. Notably, the ranking of in vitro AA1 binding affinities for the BCL-XLmut(ΔC) proteins is in register with the in vitro sensitivities to AA.

The non-peptide BCL-XL inhibitors, BH3I-1 and -2, interact with Phe-97 and Ala-142 in the BCL-XL hydrophobic pocket by NMR chemical shift perturbation (21,22). We determined that BH3I-1 competes with AA1 for BCL-XL(ΔC) binding (Fig. 3c). The Kᵢ for BH3I-1 displacement of AA bound to BCL-XL(ΔC) is 1.874 ± 0.617 μM, similar to that reported for displacing BH3 peptide by Dosterev et al. (21).

We also determined the effects of the hydrophobic groove mutations on binding of BAK BH3 domain peptides. Fluorescence anisotropy measurements were conducted using a FITC-labeled 16-residue BAK BH3-peptide. The F97W, A142L, and F146L mutations resulted in substantially diminished BH3 peptide binding compared with BCL-XLwt(ΔC) (Fig. 3b and Table I). Interestingly, the relative affinities of BAK BH3 peptide with the BCL-XLmut(ΔC) proteins (BCL-XLmut > E92L > F97W > F146L > A142L) paralleled those determined for AA.

Pore-forming Activities of Mutant BCL-XL Proteins—Synthetic lipid vesicles were loaded with the self-quenching fluorescent dye, calcine, to measure membrane pore formation by recombinant BCL-XLmut(ΔC) proteins. Addition of AA1, 2-OMeAA1, or the BAK BH3 peptide inhibited BCL-XL pore-forming activity, whereas a modified antimycin bearing a bulky phenacyl substituent, a mutant BAK peptide (L78A) with low BCL-XL affinity, and BH3I-1 had no effect (Fig. 4a).

The mutant versions of BCL-XLmut(ΔC) had similar pore-forming properties as BCL-XLwt(ΔC) (Fig. 4b). We tested the sensitivity of BCL-XLmut(ΔC) pores to AA1 and to BAK BH3 peptide under similar experimental conditions (Fig. 4, c and d) and performed non-linear regression analysis of the dose-response curves to obtain IC₅₀ values (Table I). Although the clustering of AA1 IC₅₀ values in the liposome assay is tighter than ob-

Fig. 2. TAMH cells expressing BCL-XL hydrophobic groove mutants are resistant to antimycin A. a, Western blot showing relative BCL-XL expression levels in each cell line. Cell viability 24 h following treatment with staurosporine (b) and AA1 (c) was determined by Alamar Blue staining.

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**Table I.**

| AA1 IC₅₀ Values (μM) | WT | E92L | F97W | A142L | F146L |
|----------------------|----|------|------|-------|-------|
| LD₅₀                 |    |      |      |       |       |
| BCL-XLwt              | 0.11 | 1.72 | 5.12 | 0.47  | 0.58  |
| BCL-XLmut             | 0.58 | 1.72 | 5.12 | 0.47  | 0.58  |

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Bcl-X₇ Mutants Are Resistant to Antimycin A

**Table I**

Summary of AA₁ activity on WT and mutant BCL-X₇ proteins

| Cytotoxicity | Dissociation constant | Pore inhibition |
|-------------|------------------------|-----------------|
| BCL-X₇     | STS LD₅₀ | AA₁ LD₅₀ | AA₁ Kᵦ | BH₃ Kᵦ | AA₁ IC₅₀ | BAK BH₃ IC₅₀ |
| WT         | 0.58 ± 0.14 μM | 0.47 ± 0.07 μM | 2.36 ± 1.41 μM | 0.11 ± 0.029 μM | 2.03 ± 0.24 μM | 0.58 ± 0.36 μM |
| F97W       | 0.34 ± 0.10 μM | 5.12 ± 0.94 μM* | 17.56 ± 5.20 μM** | 2.89 ± 0.41 μM** | 3.45 ± 0.34 μM** | 71.61 ± 9.01 μM** |
| A142L      | 0.49 ± 0.11 μM | >10 μM | 41.77 ± 21.49 μM* | 13.00 ± 5.57 μM* | 4.10 ± 1.24 μM* | N/D |
| F146L      | 0.38 ± 0.04 μM | >10 μM | 20.04 ± 9.41 μM* | 1.26 ± 3.57 μM** | 3.60 ± 0.40 μM** | 10.62 ± 3.15 μM*** |

Asterisks denote significant differences between mutant and wild-type values as determined by Student’s t test analysis of data. * indicates p < 0.05; ** indicates p < 0.01. N/D indicates that the non-linear regression was unable to fit this curve due to a lack of inhibition.

![Diagram](image62x269.png)

**Fig. 3.** Fluorescence anisotropy binding experiments to determine affinity of interaction between BCL-X₇ΔC and AA₁ or BH₃ peptide. a, fluorescence anisotropy of AA₁ (200 nM) was measured during titration with increasing concentrations of BCL-X₇ΔC proteins. Fraction of AA₁ bound was calculated from measured anisotropies. b, fluorescence anisotropy of FITC-labeled BH₃ peptide and fraction bound was determined as in a. c, competition of AA₁ (200 nM) with increasing amounts of BH₃I-1 for binding to BCL-X₇ΔC (3.16 μM).

served for the binding affinities of AA₁ using soluble BCL-X₇ΔC, the same ordering of responses is obtained: WT > E92L > F97W > F146L > A142L. The BAK BH₃ peptide IC₅₀ values also exhibit the same ordering observed for the binding affinities obtained from the equilibrium binding assay.

**Preservation of Tertiary Fold with Mutant BCL-X₇ Proteins**—The structures of BCL-X₇ΔC (WT) and the four BCL-X₇ΔC mutants, E92L, F97W, A142L, and F146L, were solved by X-ray crystallography. Overall, the mutations produced only local effects on the WT structure. Fig. 5 shows an α-carbon overlay of WT with the mutant structures. The RMSD of the BCL-X₇ΔC mutants ranged from 0.17 to 0.49 Å (Cα atoms).

**E92L**—In the docking model, the backbone carbonyl of Glu-92 contacts the O33 atom of AA₁, whereas the side chain projects outside the hydrophobic groove with no close ligand contacts. The structure of E92L BCL-X₇ΔC at 2.1Å compared with BCL-X₇ΔC shows no main chain movement and only a minor displacement between the Leu and Glu side chains. The hydrogen bonds bridging Gln-88 and Asn-198 are missing, weakening interactions between the BH₃ α-helical domain and the COOH-terminal region of BCL-X₇ΔC. Overall, the structure is very similar to WT, with a Cα RMSD of 0.17 Å, and an all-atom RMSD of 0.40 Å.

**F97W**—The Phe-97 residue lies in close proximity to the dilactone ring of AA₁ in the docking model. Thus, the greater bulk of a Trp side chain in this position is expected to cause a steric clash with bound AA₁. The structure of F97W BCL-X₇ΔC was solved to 2.7 Å. The F97W substitution does not significantly disrupt the backbone structure (overall RMSD Cα of 0.30 Å, F97W RMSD of 0.29 Å). However, to compensate for the bulkier Trp side chain, Phe-101 rotates about 80 degrees. The maximal backbone displacement for F97W BCL-X₇ΔC occurs in this region, with residues 101 through 106 having an RMSD of 0.67, although this displacement is significantly less than in the structure of a BCL-X₇/BH₃ peptide complex (Protein Data Bank code 1BXL). Thus, the steric effects of Trp-97 on AA₁ affinity should be predominant.
The A142 residue is positioned adjacent to Phe-97 in the hydrophobic groove. The docking model for AA1 predicts a significant steric clash between the dilactone ring of AA1 and the Leu-142 side chain. The structure of A142L BCL-XL was solved to 2.2 Å resolution. The A142L BCL-XL structure is very similar to BCL-XLwt, with a RMSD (Cα) of 0.44 Å. The bulkier leucine side chain causes a compensatory chain of movement of the Phe-97 and Tyr-101 side chains. Furthermore, repositioning of the Tyr-101 backbone promotes alternative orientations of Ala-104 and Phe-105, which flip from higher energy positive backbone ϕ/ψ angles to lower energy negative ϕ/ψ angles (Fig. 5). Despite the movement of the Phe-105 main chain, the side chain orientation is conserved with BCL-XLwt. Because Ala-104 and Phe-105 also have negative ϕ/ψ angles in the BH3 peptide/BCL-XL structure, the binding pocket in the ligand-bound conformation of A142 BCL-XL should be preserved without significant structural changes.

F146L—Unlike the other BCL-XL mutations considered here, the docking model predicts a loss of van der Waals contacts to the alkyl chain of AA1 with the F146L substitution. The F146L BCL-XL structure was solved to 2.2 Å. The F146L structure shows an overall RMSD (Cα) of 0.49 Å. There is little displacement of the F146L residue compared with BCL-XLwt. The aliphatic and aromatic side chains of the α3 helix and neighboring residues to F146L adopt wild-type orientations with the exception of the rotation of Lys-108 about 2. As with the A142L BCL-XL structure, residues A104 and F105 convert from positive to negative ϕ/ψ angles. In addition, the R103 backbone has now adopted a positive ϕ/ψ configuration in F146L BCL-XL (Fig. 5).

Notably, the average B-factors across all structures, including BCL-XLwt, for residues 101–105 in the α3 helix are about twice that of the rest of the molecule. Thus, the alterations in backbone configuration at residues 103–105 in the BCL-XLmut proteins may reflect an inherent flexibility of this region. The F146L BCL-XL structure also demonstrates enlargement of an interior cavity abutted by Phe-146, which may reduce the overall stability of the protein (23, 24). In BCL-XLwt, this cavity has a calculated area of 34 Å², which increases to 54 Å² in the F146L mutant structure.

DISCUSSION

Previous work (4, 9) demonstrated that expression of the anti-apoptotic protein BCL-XL rendered cells hypersensitive to AA. AA binds directly to BCL-XL in competition with BH3 peptide ligands that occupy the hydrophobic groove, consistent with the identification of this interface as the likely AA binding site by molecular modeling. In this study, we used site-directed
mutagenesis to validate BCL-X\textsubscript{L} as a direct target for AA and map the BCL-X\textsubscript{L} binding site for AA in greater detail. Three out of four mutations in the BCL-X\textsubscript{L} hydrophobic groove (F97W, A142L, and F146L) eliminated or strongly attenuated the ability of BCL-X\textsubscript{L} to sensitize TAMH cell to AA treatment. Each of the mutants had nearly wild-type anti-apoptotic activity with staurosporine treatment, discounting loss of protein function as an explanation for the resistance to AA. However, we demonstrated reduced binding affinities of AA with the BCL-X\textsubscript{L}\textsubscript{mut} proteins, with a good correlation between binding constant and \textit{in vivo} sensitivity to AA.

The BCL-X\textsubscript{L} mutations were designed for local perturbations on ligand-protein geometry. The crystal structures of the BCL-X\textsubscript{L}\textsubscript{mut} mutants demonstrate retention of the tertiary protein fold, allowing interpretation of the binding data in terms of the molecular docking model. Our docking model for AA utilized the structure of BCL-X\textsubscript{L} bound to BAK BH3 peptide. Compared with the overall shift between ligand-bound and free BCL-X\textsubscript{L} conformations (Ca RMSD = 2.8 Å), there is minimal displacement of the residues predicted to be AA contacts in these structures (Ca RMSD for Phe-97, Ala-142, and Phe-146 = 1.3 Å). The relative AA binding affinities of the BCL-X\textsubscript{L}\textsubscript{mut} proteins were in good agreement with the docking predictions. Furthermore, we observed a good correlation between binding constant and \textit{in vivo} sensitivity to AA.

\textbf{FIG. 6. Alignments of side chain mutations on BCL-X\textsubscript{L}\textsubscript{mut} structure (1BXL) used as docking target for AA.} \textit{a}, alignments of BCL-X\textsubscript{L}\textsubscript{mut} structure in free (green) and BAK-BH3-bound (black) conformations. The modeled AA (light green) is shown in place of BAK-BH3 in binding pocket. The Ca RMSD for residues Glu-92, Phe-97, Ala-142, and Phe-146 is 1.3 Å, whereas overall Ca RMSD is 2.5 Å. \textit{b}–\textit{d}, modeling of mutations into hydrophobic groove of 1BXL. Yellow stars indicate clashing contacts. \textit{b}, F97W (purple) makes two moderate clashing contacts, each at 2.6 Å. \textit{c}, the A142L mutation (magenta) makes an extreme clashing contact with CD1 to O8 of AA at 1.2 Å. \textit{d}, although Phe-146 makes two van der Waals contacts with the C27 of AA, the F146L mutation (orange) only makes one contact from CD1 (3.4 Å).
proteins are as follows: WT > E92L > F97W > F146L > A142L. Incorporating these single mutations into the AA1 docking model allowed us to predict their effects on AA1 binding. Both F97W and A142L mutations are modeled to produce steric hindrances to the docked AA1. In the former case, the increased bulkiness of the tryptophan side chain makes close contacts (̴2.6 Å) to the dilactone ring of AA1 (Fig. 6b), whereas the A142L substitution creates a steric clash to the AA1 dilactone ring (Fig. 6c). The 8-fold and 20-fold increases in the Kd of AA1 binding for F97W and A142L BCL-XL mutants (ΔC), respectively, compared with BCL-XLwt (ΔC) are compatible with these predictions. To form a stable complex, significant compensatory movements of the BCL-XL binding pockets or AA1 from the starting docking model must occur.

The phenyl group at Phe-146 is oriented perpendicularly to the hexyl-chain of AA1 in the docking model (Fig. 6d). This predicted interaction consists of van der Waals contacts and electrostatic interactions between the partial negative charges of the phenyl ring and partial positive charge of the terminal methyl group (C27). Substitution of leucine for phenylalanine removes both types of contacts with AA1, accounting for the ~10-fold decrease in AA1 affinity with this mutant protein. Overall, the ligand-bound structure models based on the BCL-XLmut (ΔC) crystal structures provide reasonable interpretations for the measured AA1 binding constants. The E92L mutation was not expected to alter AA binding based on the docking model, as it is located at the periphery of the binding pocket and contributes only a carbonyl oxygen contact with AA1. Thus the 2–3-fold reduction of AA1 binding affinity with the E92L mutation provides an estimate of the general effects on binding for mutations at partially buried residues. The mild reduction in affinity may be due to some destabilization of the BCL-XL tertiary structure by the loss of two salt bridges.

The anti-apoptotic activities of BCL-XL and BCL-2 act through and are regulated by associations with pro-apoptotic proteins. The BCL-XLmut proteins retained normal anti-apoptotic activity despite substantially weakened binding to the pro-apoptotic BH3 peptide. BCL-XL binding affinities for BH3 peptides depend on hydrophobic interactions at the floor of the cleft with several conserved non-polar residues (Val74-Leu78, and Ile81) in the peptides. Modeling studies of the BAK BH3 peptide to the BCL-XL mutants (not shown) suggest that the F146L substitution weakens interactions with BAK Val74, whereas the F97W mutation would need to be moderately displaced to avoid a clash with the side chain of BAK Leu78. However, BCL-XL A142L requires a much larger adjustment to avoid a clash between the backbone of BAK Leu78 and the BCL-XL-Leu142 side chain. Overall, these results suggest that the mutant phenotypes discussed here are more compatible with the pro-apoptotic binding partner exerting negative control over the anti-apoptotic function of BCL-XL rather than vice versa. However, we have tested only a single pro-apoptotic BH3 domain (BAK). For example, BCL-XL and BCL-2 proteins with mutations preventing binding to BAK nevertheless strongly interacted with the BH3 only BAD protein in a previous report (25).

The hydrophobic groove mutations did not affect the pore-forming ability of purified BCL-XLmut (ΔC) in synthetic liposomes, suggesting that this property is endowed by the global protein fold and packing geometry of BCL-XLmut (ΔC). AA1 inhibited pore formation of mutant and wild-type proteins with similar IC50 values. The insensitivity of this assay for discriminating mutants with different AA binding affinities implies AA1 interacts differently with soluble versus membrane-inserted BCL-XLmut (ΔC). Using dansylated lipid in fluorescence resonance energy transfer experiments, we determined that AA1 does not interfere with the insertion of BCL-XL into lipid membranes, whereas BAK BH3 inhibits full membrane insertion of BCL-XLmut. This finding explains why the distribution of BAK BH3 IC50 values for the BCL-XLmut series reflects the range of affinities for soluble BCL-XLmut protein, because pore inhibition takes place at a pre-insertion step. The conserved ordering of mutant protein activities in AA binding and pore assays (i.e. WT > E92L > F97W > F146L > A142L) argues for similarities in the soluble and membrane-inserted binding interfaces. The AA1-BCL-XLmut (ΔC) interaction in a lipid environment appears to be significantly less constrained by the BCL-XLmut mutations considered here compared with the soluble form of the protein.

Our previous conclusion that AA selectively kills BCL-XL-expressing cells by directly targeting BCL-XL is convincingly reinforced by the mutational studies reported here. The single amino acid mutations produced minor shifts in the binding pocket geometry, allowing a high degree of confidence in extrapolating from crystal structures to the ligand-bound protein conformation. The principal basis for the AA resistance of the mutant BCL-XL proteins appears to be lower binding affinity, as reflected by the strong correlation between in vitrio binding constants and cytotoxicity. In aggregate, these results agree with the starting molecular model of how AA1 binds to the BCL-XL hydrophobic groove. This information will aid in optimizing this BCL-XL inhibitor and better understanding of its molecular action.

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