Molecular Targeting of Inhibitor of Apoptosis Proteins Based on Small Molecule Mimics of Natural Binding Partners†

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ABSTRACT: An assay based on a solvent-sensitive fluorogenic dye molecule, badan, is used to test the binding affinity of a library of tetrapeptide molecules for the BIR3 (baculovirus IAP repeat) domain of XIAP (*X*-linked *i*nhibitor of *a*poptosis *p*rotein). The fluorophore is attached to a tetrapeptide, Ala-Val-Pro-Cys-NH_2, through a thiol linkage and, upon binding to XIAP, undergoes a solvatochromic shift in fluorescence emission. When a molecule (e.g., a natural protein known to bind to XIAP or a tetrapeptide mimic) displaces the dye, the emission shifts back to the spectrum observed in water. As emission intensity is related to the binding of the tetrapeptide, the intensity can be used to determine the equilibrium constant, *K*, for the displacement of the dye by the tetrapeptide. The results permit residue-specific analysis of the interaction. Furthermore, we show that hydrophobic effects in the fourth position are general and can effectively increase overall affinity.

Apoptosis, or programmed cell death, is a critical process in both development and homeostasis of multicellular organisms (*1*−*3*). Alterations in apoptotic pathways can disrupt the delicate balance between cell proliferation and cell death and lead to a variety of diseases. For example, in many cancers, apoptosis is abnormally downregulated, either by the mutation of proapoptotic proteins or by the upregulation of antiapoptotic proteins, which lead to an accumulation of cells and increased resistance to proapoptotic signals. The signaling pathways of apoptosis can be divided into two components, involving either the mitochondria or death receptors. Mitochondria-mediated apoptosis occurs in response to a wide range of death stimuli, such as DNA damage, chemotherapeutic agents, or ultraviolet radiation. It is generally recognized that this pathway must be inactivated in all cancer cells (*1*).

The inhibitor of apoptosis (IAP)[1] proteins represents an important class of regulators of programmed cell death (*4*, *5*). IAPs were first discovered in baculoviruses, where they were shown to inhibit apoptosis in the host cell during viral infection. All IAPs contain at least one baculovirus IAP repeat (BIR) domain that is composed of about 70 amino acids. Human X-linked IAP (XIAP) contains three BIR domains as well as a C-terminal RING finger, and these domains exhibit distinct specificities for caspases (*6*). The third BIR domain (BIR3) selectively targets caspase-9, the initiator caspase in the mitochondrial pathway, whereas the linker region between BIR1 and BIR2 inhibits both caspase-3 (*7*) and caspase-7 (*8*, *9*). While binding to XIAP prevents the activation of all three caspases, it is apparent that the interaction with caspase-9 is the most critical for the inhibition of apoptosis (*10*). The interaction between BIR3 and caspase-9 is known to occur through binding of the amino terminus of the linker peptide on the small subunit of caspase-9 to a surface groove on the BIR3 domain (*11*).

Apoptosis is promoted by the release of Smac (second mitochondrial-derived activator of caspases) from the mitochondria into the cytosol, where it interacts with multiple IAPs and relieves their inhibitory effect on caspases (*12*, *13*). Studies have shown that Smac promotes the catalytic activity of caspase-9 by competing with the caspase for the same binding pocket on the surface of the BIR3 domain (*10*, *11*). In cancer cells, abnormally high levels of IAPs can prevent Smac from carrying out its function (*5*). Thus, the IAPs are an important target for cancer types in which IAP overexpression prevents apoptosis.

In the available NMR (*14*) and X-ray (*15*) structural analyses of Smac binding to the BIR3 domain of XIAP, the N-terminal residues of Smac (Ala-Val-Pro-Ile) appear to be critical for binding between the two proteins. The N-terminal tetrapeptide recognizes a surface groove on the BIR3 domain through hydrogen-bonding interactions and van der Waals

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[1] Abbreviations: Abu, aminoisobutyric acid; BIR, baculovirus IAP repeat; badan, 6-(bromoacetyl)-2-(dimethylamino)naphthalene; Boc, *tert*-butoxycarbonyl; DIEA, diisopropylethylamine; DTT, 1,4-dithio-DL-threitol; Fmoc, 9-fluorenylmethoxycarbonyl; IAP, inhibitor of apoptosis protein; MBHA, methylbenzhydrylamine; RING, really interesting new gene; Smac, second mitochondrial-derived activator of caspases; TFA, trifluoroacetic acid; TIS, triisopropylsilane; XIAP, X-linked inhibitor of apoptosis protein.

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Table 1: N-Terminal Amino Acids of BIR3 Binding Partners[a]

Smac/Diablo	**A**	**V**	**P**	**I**	A	Q	K	S	E
HtrA2/Omi	**A**	**V**	**P**	**S**	P	P	P	A	S
Reaper	**A**	**V**	**A**	**F**	Y	I	P	D	Q
Grim	**A**	**I**	**A**	**Y**	F	L	P	D	Q
Hid	**A**	**V**	**P**	**F**	Y	L	P	E	G
Sickle	**A**	**I**	**P**	**F**	F	E	E	E	H
hCasp-9	**A**	**T**	**P**	**F**	Q	E	G	L	R
mCasp-9	**A**	**V**	**P**	**Y**	Q	E	G	P	R
xCasp-9	**A**	**T**	**P**	**V**	F	S	G	E	G
hCasp-7	S	G	P	I	N	D	T	D	A
hCasp-3	S	G	V	D	D	D	M	A	C

[a] See ref *1*.

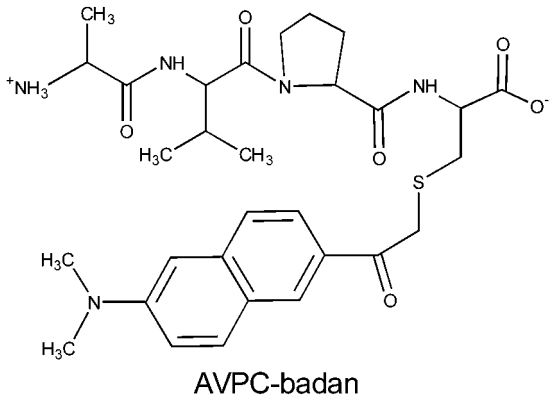
contacts. This groove is lined with both hydrophobic and negatively charged residues, and a comparison of the N-terminal residues of known binding partners of this groove displays a marked similarity in sequence (Table 1). The conserved tetrapeptide motif among the binding partners of BIR3 indicates that peptides or small molecules modeled on the binding motif might serve as prototypical drugs whose activity might complement that of Smac. Recent studies of the binding interaction between BIR3 and peptides based on the N-terminal sequences of Grim, Hid, Smac, and caspase-9 demonstrate that short peptides based on the conserved peptide motif maintain the strong binding affinities observed with the parent proteins (*16*).

In this work, a fluorescence assay is used to test the binding of a library of tetrapeptides modeled on the Smac N-terminus to the surface pocket of the BIR3 region of XIAP. The results make it possible to parse the contribution of each residue of the tetrapeptide to the total binding energy of the interaction.

MATERIALS AND METHODS

Materials. Unless otherwise stated, materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) and used without further purification. Methylbenzhydrylamine (MBHA) solid-phase peptide synthesis resin, Rink amide resin, and 9-fluorenylmethoxycarbonyl- (Fmoc-) protected amino acids were obtained from Advanced ChemTech (Louisville, KY) and NovaBiochem (San Diego, CA). 6-(Bromoacetyl)-2-(dimethylamino)naphthalene (badan) dye was obtained from Molecular Probes (Eugene, OR).

Synthesis of AVPC-badan. The peptide was synthesized by Fmoc protocol on MBHA resin (*17*). The MBHA resin was chosen because the protocol requires that the linkage to the solid support be stable under both acidic and basic conditions. The Ala-Val-Pro-Cys-NH_2 (AVPC) peptide was synthesized using a trityl group to protect the cysteine thiol. The trityl group was removed by treatment with trifluoroacetic acid (TFA), and the cysteine was derivatized with badan in the presence of diisopropylethylamine (DIEA). The Fmoc group of the alanine was removed with piperidine, and then cleavage from the resin was effected by treatment with anhydrous HF containing 10% v/v anisole as scavenger at 0 °C for 45 min. The labeled peptide was purified by HPLC on a Vydac C18 preparative column with gradient elution by solvents A (99% H_2O, 1% CH_3CN, 0.1% TFA) and B (90% CH_3CN, 10% H_2O, 0.1% TFA) and lyophilized to dryness prior to reconstitution in H_2O.



AVPC-badan

Synthesis of N-Fmoc-N-methylamino Acids. N-Methylamino acids were synthesized according to the methods of Freidinger et al. (*18*). *N*-Fmoc-*N*-methylisoleucine and *N*-Fmoc-*N*-methylphenylalanine were chromatographed over silica gel (5% methanol in chloroform as eluent); *N*-Fmoc-*N*-methylvaline was used without further purification.

Synthesis of Tetrapeptide Libraries. With the exception of the position 1 library, all of the library molecules were synthesized on an Advanced ChemTech 396 MPS automated peptide synthesizer by Fmoc protocol on Rink amide resin (*17*). For the AVPX and the AXPI libraries, the X positions were substituted with all 20 naturally occurring amino acids. The side chains of the amino acids that are sensitive to side reactions were protected as follows: cysteine, histidine, asparagine, and glutamine were protected using a trityl group; aspartic acid, glutamic acid, serine, threonine, and tyrosine were *tert*-butyl protected; lysine and tryptophan were protected by Boc groups; and a pentamethyldihydrobenzofuran group was used to protect the arginine. After alanine was added, deprotection and cleavage of the tetrapeptides from the resin were effected by adding 1 mL of a 95% TFA, 2.5% water, and 2.5% triisopropylsilane (TIS) solution to each well and shaking for 1 h. The cleavage solution was collected, and a further 0.5 mL of the cleavage solution was added to each well and mixed for another hour. The combined cleavage solutions were added to 20 mL of water, lyophilized to dryness, then taken up in 5 mL of water before being filtered through syringe filters (0.2 μm), and lyophilized again.

The position 1 tetrapeptides were synthesized on a hand shaker, also by Fmoc protocol on Rink amide resin (*17*). Cleavage and workup were done as described above. The presence of the desired tetrapeptide molecules was confirmed by mass spectroscopy.

The tetrapeptides were reconstituted in water, and test solutions were made that were approximately 200 μM in the tetrapeptides. Exact concentrations were determined for 10 representative test solutions by ^{1}H NMR using a dioxane solution of known concentration as an external reference. The concentrations of the other test solutions were taken to be the average value of the known solutions from the same library synthesis.

Expression and Purification of BIR3. Recombinant XIAP-BIR3 (residues 238−358) was overexpressed as a GST fusion protein using pGEX-2T (Amersham Biosciences). The

soluble fraction of the GST-BIR3 in the *Escherichia coli* lysate was purified over a glutathione−Sepharose column and further purified by anion-exchange chromatography (Mono-Q, Amersham Biosciences). The fusion protein was cleaved by thrombin, and the GST portion was removed by the glutathione−Sepharose column. The BIR3 protein was further purified over a gel filtration column (Superdex 30, Amersham Biosciences).

Fluorescence Experiments. Luminescence spectra were recorded using a Photon Technologies, Inc., fluorometer with a Xe arc lamp and a PMT detector. The absorbance of all solutions was less than 0.2 at the excitation wavelength (387 nm). The buffer used in all of the fluorescence experiments was 50 mM potassium phosphate, 100 mM NaCl, and 2 mM 1,4-dithio-DL-threitol (DTT), pH 7.

Determination of the AVPC-badan Binding Constant to BIR3. Two milliliters of a 2 μM AVPC-badan stock solution (buffer same as above) was titrated with a BIR3 stock solution from 0 to 10 μM in 15 μL increments. The dissociation constant for AVPC-badan and BIR3 was determined from the intensity observed at 470 nm after each addition of the protein.

Assay of Tetrapeptide Libraries. The samples were prepared in a 96-well plate lined with glass tubes, to prevent adsorption of the dye to plastic. The plate was stored on ice in the dark between measurements. A small volume cuvette, with a path length of 2 mm, was used to collect the emission spectra. A 44 μM aqueous solution (2.5 mL) of AVPC-badan, 1.75 mL of a 63 μM BIR3 solution, and 15.25 mL of buffer were mixed to give a stock solution which was 5.6 μM in both AVPC-badan and BIR3. Three hundred ninety microliters of this stock solution was added to 50 wells of the 96-well plate. Fifty microliters of the test tetrapeptide solutions was added and mixed immediately prior to taking the emission spectra. The final solutions were 5 μM in both badan and BIR3 and approximately 20−30 μM in the tetrapeptide solutions. Fifty microliters of water was added to three of the wells by way of controls to determine the intensity observed when the AVPC-badan was bound to BIR3. One hundred ninety microliters of AVPC-badan and 1020 μL of buffer were mixed and added to three wells in 390 μL aliquots. Fifty microliters of water was added to these wells, again as controls, to determine the intensity of the unbound dye. Equilibrium constants were determined by relating the observed intensity of the test solution at 470 nm to the average values obtained from the control experiments.

RESULTS

The binding of various tetrapeptide mimics to the BIR3 domain of XIAP was determined using a fluorescence-based competition assay. The assay is based on an environment-sensitive fluorogenic dye molecule, badan (Figure 1A). Badan is a dye whose sensitivity to environmental changes has previously been used to probe protein binding interactions (*19*−*21*). A tetrapeptide based on the Smac binding motif, Ala-Val-Pro-Cys-NH_2 (AVPC), was derivatized with the badan molecule to create a binding interaction with BIR3. When AVPC-badan binds to the surface groove of BIR3, changing the environment of the dye from water to the hydrophobic interior of the protein, the result is a large shift in both fluorescence maximum and intensity (Figure 1B and

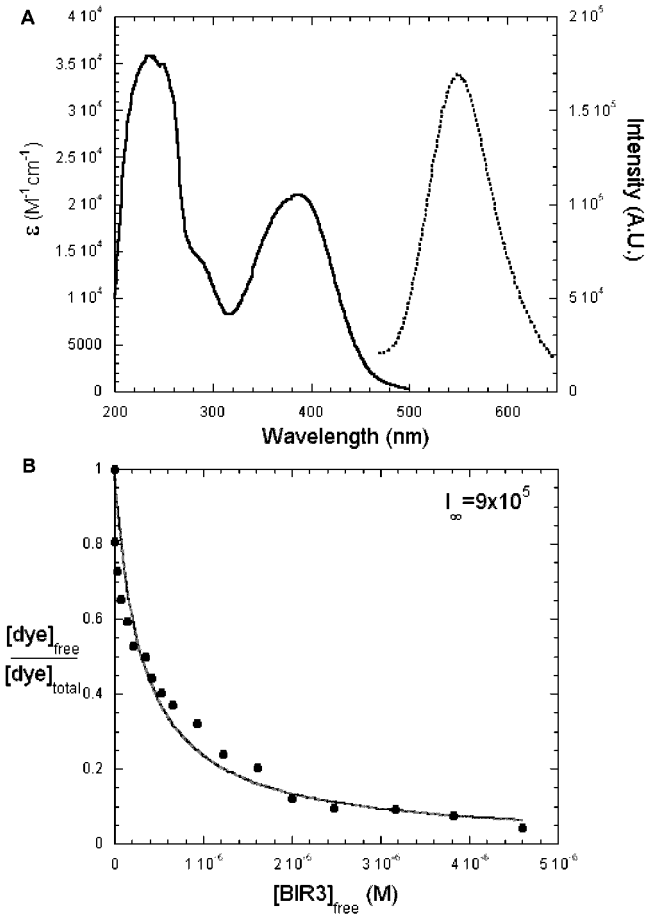


FIGURE 1: (A) Absorption (—) and emission (---) spectra of AVPC-badan in water (excitation at 387 nm). (B) Titration of AVPC-badan with BIR3. The fraction of free AVPC-badan was determined by relating the difference of the observed fluorescence intensity and a maximum intensity where all of the dye is assumed to be bound, I_∞, to the difference between the intensity of the unbound dye and I_∞.

Figure 2A). The K_D for the AVPC-badan/BIR3 complex, as determined from a fluorescence titration, is 0.31 ± 0.04 μM (Figure 1B).

AVPC-badan can be displaced from the binding pocket of the protein by any competing molecule. As the dye is displaced from the binding pocket by the test molecule, the emission shifts back toward the aquated spectrum. Thus, the observed emission intensity of the dye can be related to the degree of displacement of AVPC-badan by the test molecules. This allows the most promising inhibitors to be quickly determined, and structural information about effective inhibitors can be incorporated into the design of candidates for the next round of testing.

Using the four N-terminal residues of Smac as a starting point, six libraries of related tetrapeptides were synthesized (Scheme 1) and evaluated in terms of their ability to displace AVPC-badan from the peptide binding groove on the surface of BIR3. The tetrapeptide libraries were designed to deconvolve the contribution of each amino acid to the binding of Smac to BIR3 (Scheme 1). The position 1 library only consisted of three members, reflecting the critical role that Ala1 plays in the recognition of the binding element by BIR3. The role of position 3 was explored using a tetrapeptide based on the N-terminal sequence of Reaper, one of the few natural binding partners without a proline in position 3 (Table 1).

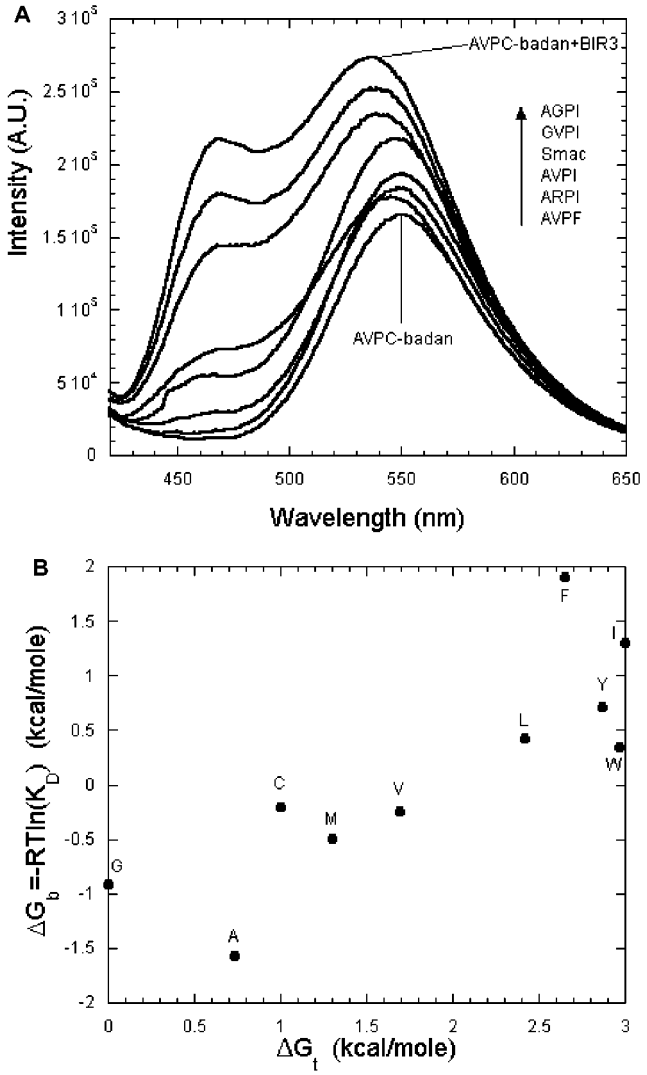


FIGURE 2: (A) Emission spectra of AVPC-badan, AVPC-badan in the presence of BIR3 and AVPF, AVPC-badan in the presence of BIR3 and ARPI, AVPC-badan in the presence of BIR3 and AVPI, AVPC-badan in the presence of BIR3 and GVPI, AVPC-badan in the presence of BIR3 and AGPI, and AVPC-badan in the presence of BIR3, in order of increasing emission intensity. (B) Correlation of hydrophobic interaction expressed as ΔG_t (EtOH−H_2O) (*23*) with ΔG_b for a range of nonpolar amino acids (polar amino acids are not shown in this graph).

Libraries of positions 2 and 4, over all 20 naturally occurring amino acids, were synthesized. The tetrapeptide ARPF was synthesized to investigate the possibility of additivity by modifying both positions simultaneously.

There are two bonds in the tetrapeptide that are vulnerable to proteolysis: the peptide bond between position 1 and position 2 and the peptide bond between position 3 and position 4. One means of rendering these bonds more resistant to proteolysis is to replace the hydrogen on the amide with a methyl group. Several tetrapeptide homologues were synthesized with *N*-methylamino acids to explore the effect that such modifications have on the affinity of these compounds for BIR3.

The dissociation constants (K_D) for the library members are listed in Table 2. The tetrapeptide mimics displace badan from BIR3 with varying facility (Table 2, Figure 2A). The K_D values ranged from 0.02 μM to greater than 100 μM. The conservation of sequence of the binding motif observed across the range of protein binding partners suggests that nature has optimized the appropriate sequence to some extent,

Scheme 1

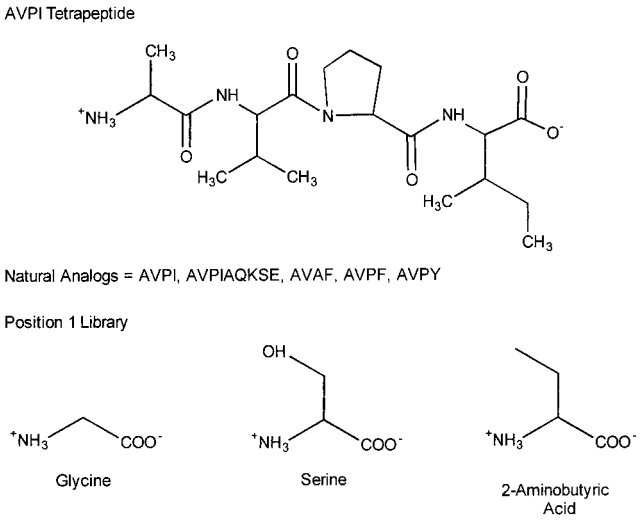


Table 2: K_D for Tetrapeptide Homologues[a]

	K_D (μM)		K_D (μM)
natural analogues		position 4	
Smac (full length)[b]	0.80	AVPW	0.11
AVPI	0.48	AVPL	0.49
AVPIAQKSE	0.40	AVPC	1.4
AVAF	0.56	AVPV	1.5
AVPF	0.04	AVPT	2.1
AVPY	0.30	AVPM	2.3
position 1		AVPS	4.4
AbuVPI	0.24	AVPG	4.7
GVPI	9	AVPP	5.7
SVPI	27	AVPD	7.3
position 2		AVPH	7.3
ARPI	0.18	AVPA	14
ALPI	0.29	AVPK	28
AHPI	0.33	AVPE	93
AIPI	0.39	AVPR	>100
AKPI	0.57	AVPN	>100
AYPI	0.59	AVPQ	>100
ACPI	0.65	positions 2 and 4	
AMPI	0.73	ARPF	0.02
AFPI	0.79	*N*-methyl analogues	
AQPI	0.94	ARP(*N*-Me)F	0.71
AWPI	0.99	AVP(*N*-Me)F	0.89
ATPI	1.2	AVP(*N*-Me)I	174
ASPI	1.4	ARP(*N*-Me)I	190
ANPI	1.5	A(*N*-Me)VPF	1358
AEPI	2.7	A(*N*-Me)VP(*N*-Me)F	2369
AAPI	2.8		
ADPI	17		
AGPI	46		
APPI	>100		

[a] Many of these values were determined from single measurements. Tetrapeptides for which multiple measurements were made had errors ranging from 2.5% to 16%. [b] At very high Smac concentrations, a Smac/AVPC-badan complex can be observed with a K_D of approximately 66 μM. We did not explicitly correct for this complex since, under the conditions of BIR3/AVPC-badan competition (K_D = 0.31 μM), less than 1% Smac/AVPC-badan complex would be formed.

but the variety of tetrapeptides tested in this assay explores the specific contribution made at each position to the overall binding interaction.

DISCUSSION

Residue 1. In previous studies, it was noted that mutations of the N-terminal amino acid of Smac completely abrogated the binding interaction between Smac and BIR3 (*14*, *22*). The recognition between Smac and the surface groove of the BIR3 is based on a combination of eight intermolecular hydrogen bonds and van der Waals contacts (*15*). The necessity of the N-terminal alanine is obvious from the crystal structure. Ala1 donates three hydrogen bonds to nearby residues in the surface groove of BIR3, and its carbonyl group makes two additional contacts. The methyl group of Ala1 fits tightly into a hydrophobic pocket, and any modification of the alanine residue must be carefully designed to avoid steric hindrance in this pocket or disruption of any of these essential hydrogen bonds. Although the next three residues contribute to the positioning of Ala1 in the binding pocket, their identity does not appear to be as critical as that of the Ala1. The position 1 library members demonstrate how sensitive the binding interaction is to any modification at this position. Binding is greatly diminished with GVPI, consistent with an earlier report (*14*), and SVPI is also a diminished binder, but a slight enhancement in binding was observed with the unnatural amino acid, 2-aminobutyric acid (Abu).

Residue 3. AVAF has a binding affinity similar to that observed for the other natural analogues, AVPI and AVPI-AQKSE. However, this affinity is diminished by greater than a factor of 10 relative to that observed for the AVPF tetrapeptide from the position 2 library. Previous studies have also noted a decrease in binding affinity when proline is replaced by alanine (*14*, *16*). On the basis of that observation, and the relative homogeneity observed in the natural binding partners at position 3 (Table 1), it would seem that replacing proline will diminish the binding affinity of the test tetrapeptide.

Residue 2. As stated earlier, nature has already optimized the appropriate sequence to some extent. However, the position 2 library gives some surprising results. The high affinity of tetrapeptides such as ARPI and AHPI relative to the natural sequence of AVPI would seem to indicate that positive charge at position 2 would increase the binding affinity of the peptide. This is not an unexpected result given the negatively charged residues that line the binding pocket of BIR3. Nonetheless, none of the natural binding partners of IAP listed in Table 1 has positively charged residues at position 2. All of the natural IAP interacting motifs that have been observed so far contain β-branched amino acids at position 2, such as valine, threonine, and isoleucine (Table 1). This result indicates that the natural sequence can be improved upon and gives a basis for the structural design of the next set of potential binding partners.

Residue 4. The X-ray structure of Smac binding to BIR3 indicates that there are no intermolecular hydrogen bonds to residue 4, and of the four residues of the binding motif, residue 4 is the least sterically hindered (*15*). This would seem to make position 4 least sensitive to modification. Indeed, the K_D that is observed for the AVPC tetrapeptide (Table 2) is greater than that of the AVPC-badan, which indicates that binding is slightly enhanced by the presence of the dye. However, a much wider range of K_Ds is observed for the position 4 library than for the position 2 library. Although modification at this position can lead to the greatest enhancement in binding affinity that is observed, it can also essentially destroy the binding interaction.

The AVPF tetrapeptide was by far the most strongly binding library member, closely followed by AVPW. AVPY was also determined to have a binding affinity slightly greater than that of the natural analogue, AVPI. These results indicate that an aromatic group side chain on the amino acid at position 4 substantially enhances the binding affinity of the tetrapeptide for BIR3. This result is consistent with phylogenic data: other proteins that interact with IAPs have phenylalanine or tyrosine at position 4 (Table 1).

When high-affinity substitutions at positions 2 and 4 were probed simultaneously using the ARPF tetrapeptide, the effects were found to be additive. Consequently, the detrimental effect on binding affinity observed with the N-methylated tetrapeptides could be somewhat counteracted by the increased affinity gained from the appropriate choice of amino acid.

N-Methyl Analogues. N-Methylation at the peptide bond between residues 1 and 2 disrupts a structurally defined hydrogen bond and has a correspondingly large effect on binding. By contrast, N-methylation of residue 4 has a much smaller effect, consistent with structural data, which show no hydrogen bond to this amide. From a standpoint of molecular design, this relieves an important design constraint. Consideration of side chain contributions to the free energy of binding, ΔG_b, using the free energy of transfer from ethanol to water, ΔG_t (EtOH$-$H$_2$O) (*23*), to approximate the energy contribution of the side chain for hydrophobic amino acids, follows a clear general trend. More hydrophobic amino acids clearly bind more strongly, as indicated in Figure 2B. The obvious correlation indicates that there is little specificity of interaction but also suggests that the full hydrophobic effect is not realized. For example, the ΔG_t of W is greater than that of F, but the ΔG_b of AVPF is greater than that of AVPW. A more detailed analysis can be obtained by modeling the various peptides onto the known structure and determining the solvent-exposed surface area within the model.

IAPs such as XIAP are widely expressed in human cancers and are likely to play a role in both disease progression and resistance to therapy (*5*). Therefore, IAPs represent attractive potential targets for drug treatments of the disease. The information presented here about the roles of functional groups in modulating the binding affinity of the tetrapeptide binding motif for the BIR3 domain of XIAP should aid in the design of small molecules intended to mimic the function of Smac and may be useful for treating cancers in which IAPs are overexpressed.

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