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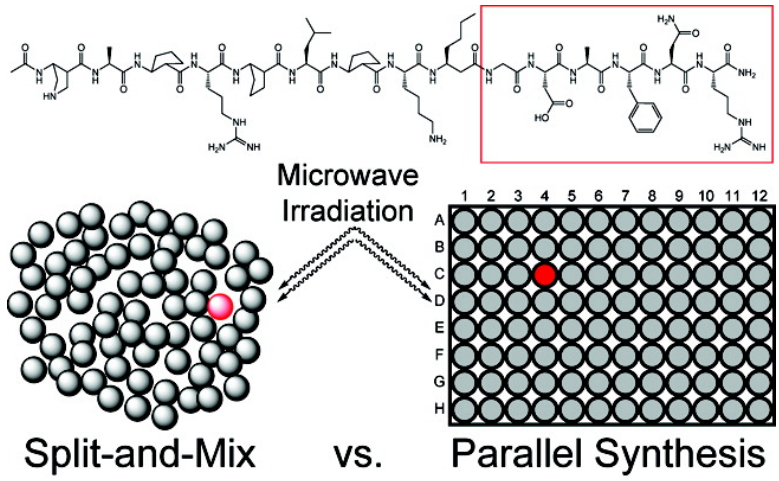

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Exploration of Structure−Activity Relationships among Foldamer Ligands for a Specific Protein Binding Site via Parallel and Split-and-Mix Library Synthesis

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We describe the use of parallel and split-and-mix library synthesis strategies for exploration of structure−activity relationships among peptidic foldamer ligands for the BH3-recognition cleft of the anti-apoptotic protein Bcl-x_L. This effort began with a chimeric ($\alpha/\beta+\alpha$)-peptide oligomer (composed of an α/β-peptide segment and an α-peptide segment) that we previously identified to bind tightly to the target cleft on Bcl-x_L. The side chains that interact with Bcl-x_L were varied in a 1000-member one-bead-one-compound library. Fluorescence polarization (FP) screening identified four new analogues with binding affinities similar to that of the lead compound but no analogues with enhanced affinity. These results suggested that significant improvements in affinity were unlikely in this series. We then used library synthesis to examine backbone variations in the C-terminal α-peptide segment of the lead compound. These studies provided an opportunity for direct comparison of parallel and split-and-mix synthesis formats for foldamer libraries with respect to synthetic variability and assay sensitivity. We found that compounds from both the parallel and one-bead-one-compound libraries could be reliably screened in a competition FP assay without purification of library members. Our findings should facilitate the use of combinatorial library synthesis for exploration of foldamers as inhibitors of protein–protein interactions.

Introduction

Protein–protein interactions are involved in many aspects of cell signaling and growth. Deregulation of cell signaling pathways is often associated with disease. Although protein–protein interactions have emerged as an important class of therapeutic targets, a general approach to the development of suitable inhibitors has not yet been identified.[1] Antibodies and other proteins may be useful as antagonists but are expensive to manufacture.[2] Small molecule strategies have proven to be effective for inhibition of only a limited subset of protein–protein interactions.[3] Peptide inhibitors of protein–protein interactions are often discovered, but obstacles to development of these compounds include poor pharmacokinetic properties, such as proteolytic sensitivity.[4] The challenges associated with inhibition of protein–protein interactions have created the need to explore new classes of molecules as potential antagonists.

Several groups have examined "foldamers", unnatural oligomers with specific folding propensities,[5] as potential sources of protein–protein interaction antagonists. Specifically, we and other groups have focused on β-amino acid oligomers (β-peptides)[6] or oligomers containing both α- and β-amino acid residues (α/β-peptides).[7] Helical conformations of β-peptides or α/β-peptides can be achieved by following simple design rules,[5,8] and these unnatural scaffolds have been used to create functional mimics of α-helical peptides or α-helical fragments of proteins.[9] Both split-and-mix[10] and parallel[11] synthesis methods have been employed to prepare foldamer libraries, but there has been no direct comparison of these synthetic formats. Here we provide such a comparison in the context of our efforts to develop foldameric ligands for the BH3 recognition cleft of Bcl-x_L, a member of the Bcl-2 protein family.

Interactions between members of the Bcl-2 family regulate the programmed cell death (apoptotic) pathway. Anti-apoptotic Bcl-2 family members include Bcl-x_L, Bcl-2, Bcl-w, Mcl-1, and A1. These proteins feature a deep hydrophobic cleft that can bind to an α-helical domain on pro-apoptotic family members, the Bcl-homology 3 (BH3) domain.[12] This binding mechanism is supported by several high-resolution structures of anti-apoptotic Bcl-2 family proteins (e.g., Bcl-x_L) in complex with pro-apoptotic BH3-derived peptides (e.g., Bak^{BH3}, Bad^{BH3}, or Bim^{BH3}).[13]

Several strategies have been pursued to discover inhibitors of BH3 domain interactions with Bcl-x_L and other anti-apoptotic proteins; such inhibitors might prove to be valuable as tools that can be used to dissect the complex signaling activities within this family, as anticancer drug leads, or both.[12,14] Naturally derived BH3 peptides and related peptide analogues can bind with high affinity and pronounced

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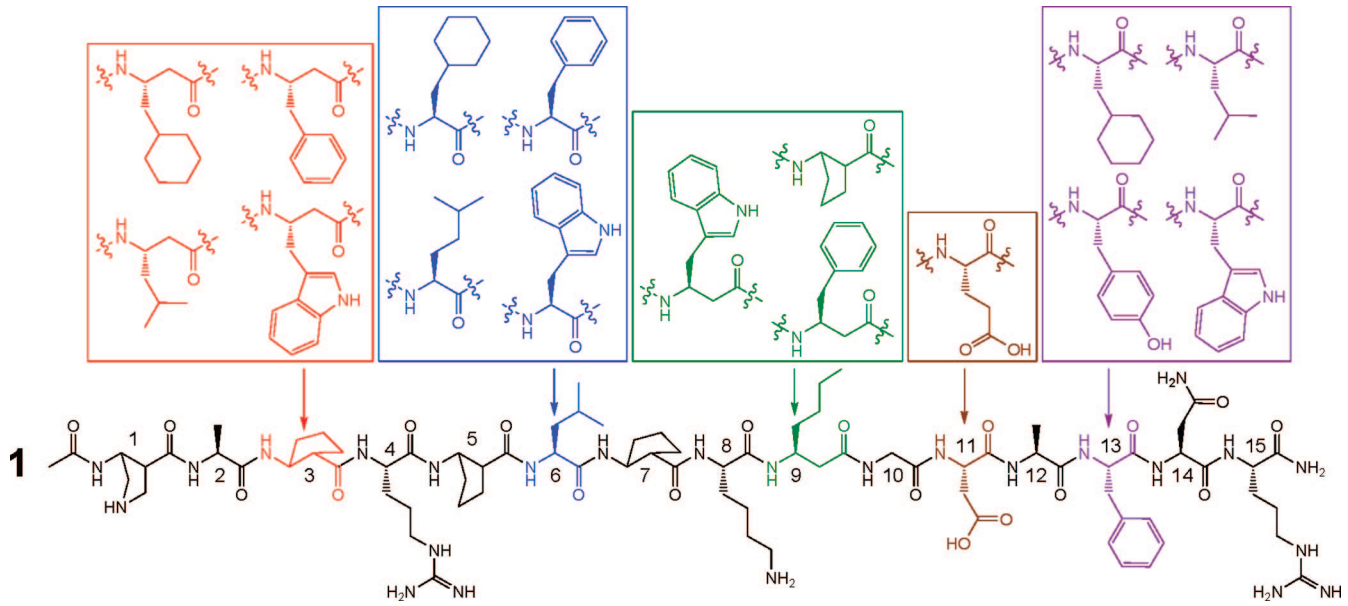

Figure 1. ($\alpha/\beta + \alpha$)-Peptide one-bead-one-compound library based on **1** and produced via split-and-mix synthesis on PS macrobeads with microwave irradiation. Four or five different residues were incorporated at positions 3, 6, 9, and 13; two different residues were installed at position 11 ($5 \times 5 \times 4 \times 2 \times 5 = 1000$ members).

selectivity to anti-apoptotic Bcl-2 family members. Korsmeyer, Verdine, and co-workers[4a] and Arora and co-workers[15] have developed relatively potent α-peptide ligands that are conformationally stabilized by macrocyclization via alkene metathesis. By contrast, most small molecule antagonists have displayed modest affinity for anti-apoptotic Bcl-2 proteins; although a few recent examples have been quite successful. Specifically, Fesik and co-workers have used a fragment-based drug-discovery approach to identify extremely potent small molecule inhibitors of Bcl-2 family proteins.[3] Alternative proteomimetic strategies have also been employed; Hamilton and co-workers used terphenyl, terephalamide, and tris-pyridyl amide scaffolds as α-helix mimics to develop ligands for the BH3-recognition cleft of Bcl-x_L and Bcl-2.[16]

We recently described ($\alpha/\beta+\alpha$)-peptide foldamers that bind to Bcl-x_L, Bcl-2, and Bcl-w with nanomolar dissociation constants and block BH3 domain recognition by these proteins.[7] The ($\alpha/\beta+\alpha$)-peptides we discovered are composed of an N-terminal α/β-peptide segment (alternating α- and β-amino acids) and a C-terminal α-peptide segment. We chose to evaluate such "chimeric" peptides for binding to Bcl-x_L only after screening several hundred β- and α/β-peptide foldamers that showed weak or no detectable affinity for Bcl-x_L. Our results with Bcl-x_L and the results of others lead to the prediction that, while foldamers may offer a source of protein–protein interaction antagonists, it will likely be essential to prepare and screen large foldamer libraries or many focused foldamer libraries with diverse backbones to find the most potent inhibitors. Thus, it is important to address technical hurdles that currently limit the pace and efficiency of oligomer library synthesis and evaluation.

Peptidic foldamers may be prepared using the solid-phase peptide synthesis (SPPS) procedures developed for the production of conventional α-peptides.[17] However, because of their oligomeric nature (for example, 30 reaction steps with a 99% yield for each step would result in a 15-residue peptide with 74% initial purity) and sequence-dependent synthetic challenges,[18] the crude products typically have a low initial purity (≪70% total peak area based on analytical reverse-phase (RP) HPLC analysis monitored via UV absorbance at 220 nm). The requirement for HPLC purification of crude products prior to screening can place a practical limit on the number of members in a foldamer library.[19] For example, ($\alpha/\beta+\alpha$)-peptide **1** (Figure 1) was identified as a high-affinity ligand for the BH3-recognition cleft of Bcl-x_L in a process that involved manual solid-phase synthesis of oligomers in sets of ≤48. Each oligomer was synthesized at room temperature in an individual reaction vessel and purified by HPLC. Overall, the time required to prepare and purify a 48-member oligomer library using the "standard" methodology is typically about 2 weeks in our experience.

We have recently reported that microwave irradiation facilitates the synthesis of β-peptide foldamers in terms of both initial product purity and synthesis time.[20,21] Microwave irradiation has been successfully applied to a large number of organic reactions with impressive results,[22] but it has been a challenge to harness this method for the preparation of large libraries.[23] On the basis of microwave reaction conditions that we had optimized for specific β-peptides on polystyrene (PS) macrobeads, we used a split-and-mix strategy to prepare a small β-peptide one-bead-one-compound library that was screened for inhibition of the p53-MDM2 protein–protein interaction.[10a] We further enhanced the utility of microwave heating by developing methods for the parallel synthesis of β-peptide libraries using 96-well polypropylene filter plates in combination with a multimode microwave reactor.[11] Here, we apply the microwave-assisted synthesis approach to preparation of ($\alpha/\beta+\alpha$)-peptide ligand candidates for Bcl-x_L. Through these studies, we were able to identify structure–activity relationships among ($\alpha/\beta+\alpha$)-peptide ligands. Furthermore, these results, when compared with recently published findings from our group, have allowed us to make a direct comparison between split-and-

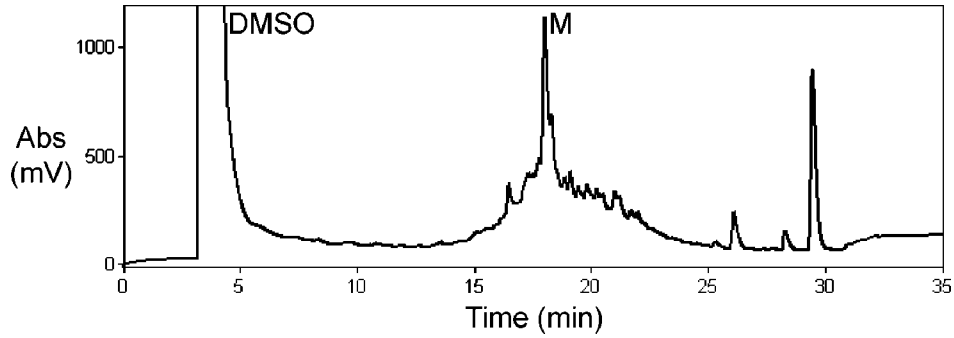

Figure 2. Analytical RP-HPLC trace (UV detection at 220 nm) of a representative library member. "M" indicates the identification by MALDI-TOF MS of an oligomer with a molecular weight corresponding to a predicted library member.

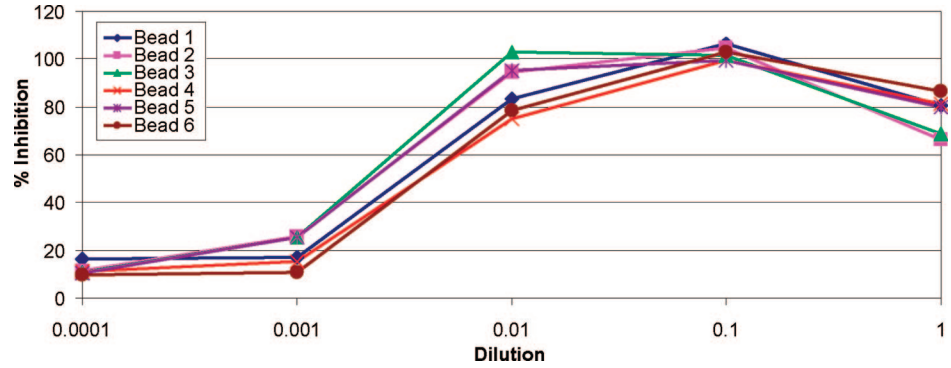

Figure 3. Activity of crude (α/β+α)-peptide **2** from six different PS macrobeads in the competition FP assay.

mix and parallel synthesis formats in terms of their ability to identify and rank order potent and moderate-affinity ligands based on screening without HPLC purification.

Results and Discussion

One-Bead-One-Compound Library for Side-Chain Optimization. We first asked whether we could improve upon the already high affinity of (α/β+α)-peptide **1** for Bcl-x_L by combinatorially varying the side chains that interact directly with Bcl-x_L in the bound state. Previous mutational studies of (α/β+α)-peptide ligands identified the *trans*-aminocyclopentanecarboxylic acid (ACPC) residue at position 3, Leu6, β^3-homonorleucine (β^3-hNle) at position 9, and Phe13 (Figure 1) as the hydrophobic residues that make key contacts with the Bcl-x_L surface.[24] The charged residues Arg4 and Asp11 are also important. We designed a 1000-member library that varied the size and shape of the side chains at each of the four key hydrophobic positions discussed above (residues 3, 6, 9, and 13 in **1**; Figure 1). ACPC was included as a possibility at position 9 to increase the helical propensity of the α/β-portion of the oligomer near the junction with the α-peptide segment.[8] Glutamic acid was included in the library as a possible substitution for Asp11 in **1** to allow compensation for disturbances of the electrostatic interaction of the key carboxylate side chain with the Bcl-x_L surface that could be caused by substitutions at neighboring positions in the sequence.

This library of side-chain variants was constructed in a one-bead-one-compound format to allow testing of every combination of the above substitutions. Library preparation via split-and-mix synthesis was performed in three days in a multimode microwave reactor using conditions originally developed for β-peptide synthesis.[10] Multiple cycles of microwave irradiation were used to ensure complete reaction on the PS macrobeads during both the removal of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group and coupling of the activated amino acids.[10,25] Approximately 4800 PS macrobeads were used (an average of 4.8 beads per theoretical library member) to provide reasonable statistical coverage of the library members.[26] At the end of the synthesis, beads were arrayed (one bead per well) into fifteen 384-well polypropylene plates. Treatment with trifluoroacetic acid cleaved the peptide product mixture from the resin with simultaneous global side-chain deprotection. After concentration by centrifugal evaporation, the crude product mixtures were dissolved in DMSO. Material from 50 of the beads was analyzed by reversed-phase (RP) HPLC. The products were not exceptionally pure (Figure 2), but most contained a major peak with a mass corresponding to an expected library member as determined by MALDI-TOF MS.

Before screening the side-chain library, we wanted to ensure that crude peptides could be reliably evaluated with a competition FP assay for binding to the BH3-binding cleft of Bcl-x_L. To the authors' knowledge, interfacing one-bead-one-compound peptide libraries with FP screening has not previously been reported. A potent Bcl-x_L ligand previously reported by our laboratory, (α/β+α)-peptide **2** (the Lys**8** → Ile analogue of **1**; IC_{50} = 150 nM in the competition FP assay)[24] was synthesized on PS macrobeads. The crude product cleaved from six individual macrobeads bearing **2** was evaluated for binding to Bcl-x_L (by the competition FP assay). As shown in Figure 3, FP binding results obtained with samples of **2** cleaved from different macrobeads showed only slight variability. The approximate IC_{50} for each of these

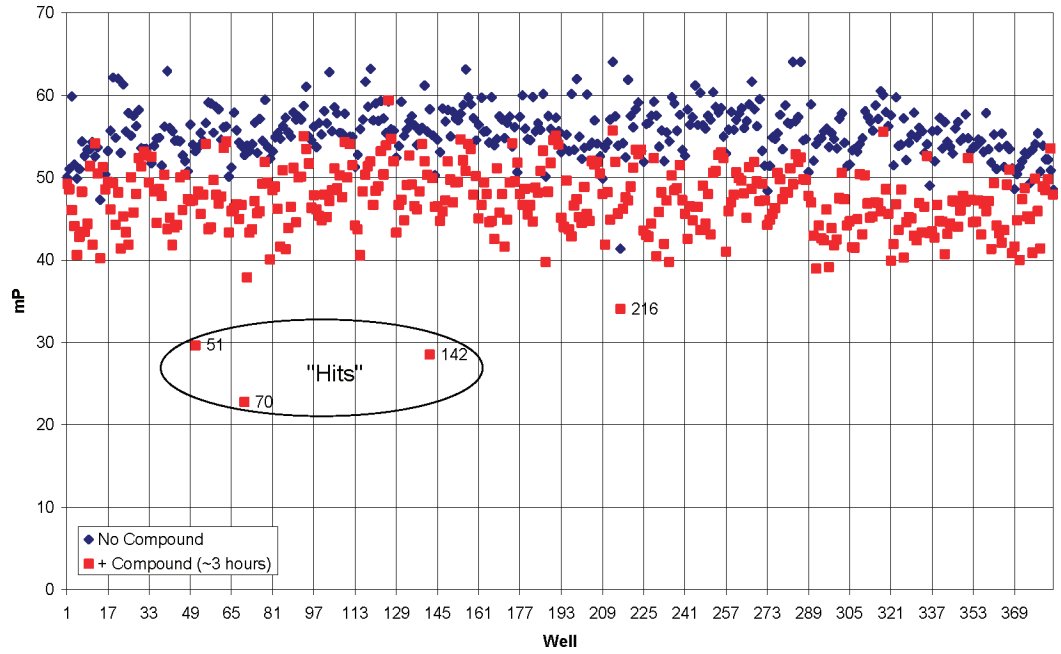

Figure 4. Representative initial screening data (one of fifteen 384-well plates with one compound per well) for the 1000-member one-bead-one-compound library. In blue is the polarization (mP = millipolarization units) measured for each well without compound to provide a reference for 0% inhibition of fluorescently labeled Bak BH3 peptide probe binding to the Bcl-x_L protein. The polarization for each well with added compound after a 3 h equilibration is in red. The lower the mP value with compound, the greater the extent to which the Bcl-x_L/Bak BH3 peptide interaction is inhibited. Compounds with mP = 50 ± 10 were considered to have no effect on Bak BH3 peptide binding to Bcl-x_L.

Table 1. (α/β + α)-Peptide Hits from Library Screening and Sequencing As Validated by Resynthesis, Purification, and Retesting[a]

	sequence																		initial screen			
no.	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	C	av mP	times identified	IC_{50} (μM)	IC_{50} ratio (IC_{50} analogue/IC_{50} **1**)
1	Ac	APC	Ala	ACPC	Arg	ACPC	Leu	ACPC	Lys	β³-hNle	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	ND	0	0.060	1
2	Ac	APC	Ala	ACPC	Arg	ACPC	Leu	ACPC	**Ile**	β³-hNle	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	NA	NA	0.15	3
3	Ac	APC	Ala	ACPC	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	24.9	3	0.048	1
4	Ac	APC	Ala	ACPC	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hNle	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	26.9	1[b]	0.050	1
5	Ac	APC	Ala	ACPC	Arg	ACPC	**Cha**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	28.1	1	0.053	1
6	Ac	APC	Ala	ACPC	Arg	ACPC	Leu	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	27.7	2	0.056	1
7	Ac	APC	Ala	ACPC	Arg	ACPC	**Phe**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	25.5	5	0.089	1
8	Ac	APC	Ala	β³-hLeu	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	21.2	2	0.12	2
9	Ac	APC	Ala	ACPC	Arg	ACPC	**Trp**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	21	4	0.22	4
10	Ac	APC	Ala	β³-hLeu	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hNle	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	21.7	1	0.24	4
11	Ac	APC	Ala	ACPC	Arg	ACPC	**Trp**	ACPC	Lys	β³-hNle	Gly	Asp	Ala	Phe	Asn	Arg	NH_2	29.2	2	0.28	5
12	Ac	APC	Ala	β³-hLeu	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	**Tyr**	Asn	Arg	NH_2	27.1	1[b]	0.29	5
13	Ac	APC	Ala	ACPC	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hTrp	Gly	Asp	Ala	**Trp**	Asn	Arg	NH_2	27.1	1[b]	1.2	20
14	Ac	APC	Ala	β³-hCha	Arg	ACPC	**Trp**	ACPC	Lys	**ACPC**	Gly	Asp	Ala	**Cha**	Asn	Arg	NH_2	26.9	1[b]	26	433
15	Ac	APC	Ala	ACPC	Arg	ACPC	**Phe**	ACPC	Lys	β³-hPhe	Gly	**Glu**	Ala	**Trp**	Asn	Arg	NH_2	23.5	1	31	517
16	Ac	APC	Ala	ACPC	Arg	ACPC	**hLeu**	ACPC	Lys	β³-hPhe	Gly	Asp	Ala	**Tyr**	Asn	Arg	NH_2	N.D.	0	0.53	9

[a] mP = millipolarization units; NA = compound **2** was not prepared as a member of the library. Positions 3, 6, 9, 11, and 13 were varied in the library; residues in bold differ from compound **1** at the indicated position. The uncertainty associated with the IC_{50} values was ± 10% (see Supporting Information). [b] Two beads found in one well, a common artifact of bead arraying.

samples was reached between the 100- and 1000-fold dilution of the crude product.

We screened the entire one-bead-one-compound library at the concentration resulting from a 1:1000 dilution to search for compounds that inhibit the Bcl-x_L interaction with greater potency than that of **2** (samples of **2** itself display no inhibition at this concentration). Thus, we intended to search for Bcl-x_L ligands with IC_{50} values below 150 nM. On the basis of our inspection of the data, we chose a fluorescence polarization value of <30 millipolarization units (mP) to indicate "hits" (Figure 4). Compounds from the 23 wells meeting this criterion (0.4% hit rate) were sequenced by μLC-MS/MS, as described previously for β-peptides.[10,27] This process identified 13 unique (α/β + α)-peptide Bcl-x_L ligands (oligomers **3–15**), six of which were identified on more than one bead. Manual resynthesis of the hits, followed by HPLC purification and testing in the competition FP assay, gave the results in Table 1. Four oligomers, **3–6**, were found to have IC_{50} values of approximately 50 nM (about 3-fold more potent than oligomer **2** and comparable in affinity to oligomer **1**). The sequences of **3–6** differed from **1** at one or two positions (residue 6, 9, or both). (α/β+α)-Peptide **1** was a theoretical member of the side-chain library but was not identified as a hit during the screen, possibly representing a false negative result. The weaker hits (**7–15**) had IC_{50} values ranging from 90 to 31 000 nM and may be considered false positives. We wondered whether some amino acid-deletion side product was contributing to the seemingly false activity

Table 2. Comparisons among Resynthesized, Purified Library Members Arranged to Compare Directly the Effects of Variation at Positions 3, 6, 9, and 13[a]

	sequence						
no.	3	6	9	11	13	IC_{50} (nM)	IC_{50} ratio (IC_{50} analog/IC_{50} **1**)
3	**ACPC**	hLeu	β^3-hPhe	Asp	Phe	48	1
8	β^3-hLeu	hLeu	β^3-hPhe	Asp	Phe	120	3
4	**ACPC**	hLeu	β^3-hNle	Asp	Phe	50	1
10	β^3-hLeu	hLeu	β^3-hNle	Asp	Phe	240	5
3	ACPC	**hLeu**	β^3-hPhe	Asp	Phe	48	1
5	ACPC	**Cha**	β^3-hPhe	Asp	Phe	53	1
6	ACPC	**Leu**	β^3-hPhe	Asp	Phe	56	1
7	ACPC	**Phe**	β^3-hPhe	Asp	Phe	89	2
9	ACPC	**Trp**	β^3-hPhe	Asp	Phe	220	5
6	ACPC	Leu	β^3-hPhe	Asp	Phe	56	1
1	ACPC	Leu	β^3-hNle	Asp	Phe	60	1
3	ACPC	hLeu	β^3-hPhe	Asp	Phe	48	1
4	ACPC	hLeu	β^3-hNle	Asp	Phe	50	1
3	ACPC	hLeu	β^3-hPhe	Asp	**Phe**	48	1
16	ACPC	hLeu	β^3-hPhe	Asp	**Tyr**	530	11

[a] The uncertainty associated with the IC_{50} values was ± 10% (see Supporting Information).

seen for **7–15** in the initial screen.[28] Examination of the LC-MS/MS sequencing data for **11** showed that an Arg4-deletion side-product was present in a significant amount (see Supporting Information). However, synthesis and testing of the Arg4-deletion mutants of **7–15** revealed that these compounds were not active ($IC_{50} > 1$ μM; data not shown). Overall, the synthesis and screening were successful in the identification of new potent oligomeric antagonists, but the synthetic variability (i.e., purity and yield) of the crude products lowered the sensitivity of the assay to the point where subtle changes in binding affinity resulting from side-chain substitutions were largely obscured in the initial screen and were discovered only after evaluation of the resynthesized hits.

Useful SAR information was gleaned from comparison of the activity of the resynthesized hits from the side chain library (Table 2). Among the four most active oligomers (**3–6**), ACPC3, Asp11, and Phe13 were constant. Substitution of β^3-hLeu for ACPC at position 3 resulted in a 3- to 5-fold increase in IC_{50} value relative to **1**, reflecting diminished Bcl-x_L affinity (**8** and **10**). There was considerable variation of the residue at position 6 among the hits from the library; the most active oligomers contained leucine, homoleucine (hLeu), or cyclohexylalanine (Cha) at this position, whereas analogues bearing aromatic side chains (Phe or Trp) at position 6 were somewhat less potent. Substitution of β^3-hPhe for β^3-hNle at position 9 had a negligible effect on the inhibitory potency of the oligomer when comparing oligomer **6** to **1** and oligomer **3** to **4**. Substitution of Tyr for Phe at position 13 caused an 11-fold loss of inhibitory activity (**16**).

α → β Libraries. The results described above suggest that affinity for the BH3-recognition cleft of Bcl-x_L among our (α/β+α)-peptide foldamers cannot be dramatically improved, relative to **1**, by side chain modification. We therefore turned our attention to optimizing another property of oligomer **1**, proteolytic stability of the C-terminal segment; in **1** itself, the C-terminal α-peptide portion is rapidly cleaved in the presence of several different proteases. We previously described our approach to reduce the susceptibility of oligomer **1** to proteolysis.[29] This approach was based on the known positive effects on α-peptide proteolytic stability of replacing α-amino acids with β-amino acids.[30] We designed a combinatorial "α → β" library to incorporate either the original α-amino acid or its β^3-amino acid analogue at positions 10 through 14 of **1**; the library also included a flexible "linker" residue (Gly or β-hGly) that could be inserted between the α- and α/β-peptide segments (between β^3-hNle9 and Gly10). Design of the 96-member library encompassing all combinations of these substitutions is shown in Figure 5.

Previously, we prepared the α → β oligomer library via microwave-assisted parallel synthesis in a 96-well plate, and each library member was purified by HPLC prior to evaluation in the competition FP assay for binding to Bcl-x_L.[29] Here we focus on the issue of library quality by comparing the previous screening results from the purified parallel library with results obtained here from the same library members without HPLC purification. In addition, we have resynthesized the α → β library using split-and-mix methodology on polystyrene macrobeads, and we compare screening results from this library (no HPLC purification) with those from the parallel library (prior to HPLC purification). As previously reported, one oligomer in the α → β library (oligomer **1**, with an all-α C-terminus) is clearly superior to all other library members in terms of Bcl-x_L affinity ($IC_{50} = 60$ nM); four additional library members, all single α → β^3-amino acid variants (Figure 6), display modest activity ($IC_{50} = 1-6$ μM), and the remaining 91 library members are essentially inactive (several oligomers deemed "inactive" in the initial library screen were resynthesized and screened in the FP assay, only to show IC_{50} values >25 μM). Thus, screening various versions of the α → β library provides an excellent opportunity to assess the robustness of the FP assay (e.g., ability to detect Bcl-x_L ligands of varying affinities) as a function of synthesis/purification format.

After parallel synthesis of the α → β library, initial HPLC and MS analysis of the crude library members showed that the desired oligomer represented the single major peak in the product mixture for approximately 50% of the samples, but in many other samples there were significant side products. (See Supporting Information. Further optimization of microwave-assisted parallel synthesis of (α/β+α)-peptides has been described in ref 29.) The library was initially screened in the FP assay without purification. Figure 7 compares FP assays at three successive 10-fold dilutions of the parallel α → β library before and after HPLC purification of library members. In both cases, FP assay results at 1:1000 dilution clearly indicate (α/β+α)-peptide **1** as the most potent library member. Some among the four moderately active oligomers (**17–20**) are identified in the 1:100 dilution data. Interestingly, more of these moderately active oligomers are found by analysis of the parallel library before purification versus analysis of the library after purification, perhaps because the concentrations of these library members were decreased by sample loss during purification (e.g., **19**).

A comprehensive comparison of the results obtained before and after HPLC purification of library members is shown in Figure 8. Overall this comparison suggests that strong and

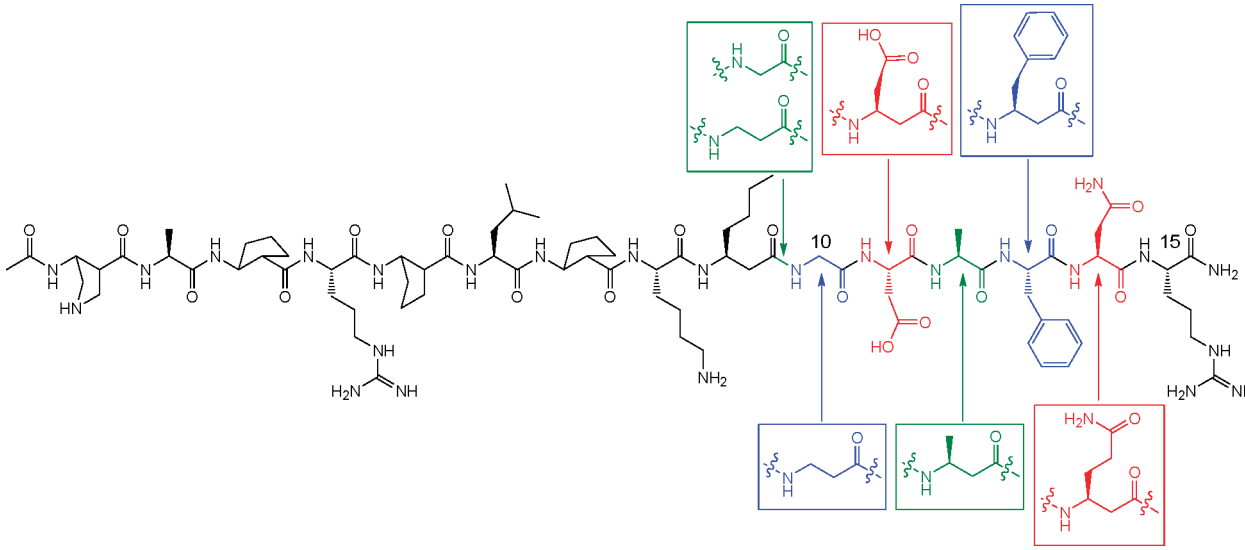

Figure 5. (α/β + α)-Peptide library synthesized with microwave irradiation both via split-and-mix techniques and in parallel. Two different residues (either the natural α-amino acid or its β^3-amino acid analogue) were incorporated at positions 10 through 14; Gly or β-hGly was optionally installed between β^3-hNle9 and Gly10 ($3 \times 2 \times 2 \times 2 \times 2 \times 2 = 96$ members).

moderate inhibitors of BH3 peptide binding to Bcl-x_L (IC_{50} values below 6 μM) can be reliably identified with the competition FP assay in a parallel library without purification of the individual library members, although the concentration at which the library members are evaluated must be carefully chosen to avoid extensive weak positive results. Ideally, crude parallel libraries should be screened in FP-based assays at several dilutions of the library members.

The α → β oligomer library was synthesized in a one-bead-one-compound format for comparison with the results obtained using parallel library synthesis. Members of the one-bead-one-compound library were not purified prior to screening in the Bcl-x_L/Bak FP assay. Crude material from nineteen beads showed ≥18% inhibition in the FP assay at a 1:1000 dilution of the solution obtained upon cleavage of the compounds from the beads (Figure 9). Analysis of these hits by MALDI-TOF MS showed that the five most-active samples (>60% inhibition) all had masses corresponding to (α/β+α)-peptide **1**. The masses of the remaining hits corresponded to oligomers resulting from single α → β replacements (**17–20**, 10 beads; 19–53% inhibition) or an insertion of a Gly residue (**21**, 1 bead) or β-hGly residue (**22**, 1 bead) between the α/β- and α-peptide segments of **1**. Thus, screening the one-bead-one compound library accurately and efficiently identified the single compound that is significantly more active than all other library members, achieving the objective for which split-and-mix library approaches are typically intended. Furthermore, we could reliably identify several modest-affinity ligands for Bcl-x_L (IC_{50} = 1–6 μM) using a split-and-mix library approach to oligomer synthesis and FP-based screening. These modest-affinity Bcl-x_L ligands had been previously characterized as being approximately 10-fold less potent than compound **1** in our previous screening of the parallel library (e.g., compounds **17–20** showed approximately 40% inhibition at the 1:100 dilution, but compound **1** showed approximately 40% inhibition at the 1:1000 dilution). Indeed, after **17–20** had been resynthesized individually and purified, these compounds displayed IC_{50} values more than an order of magnitude higher than the IC_{50} value of **1**.[29] As a control, we had included resin beads bearing peptide **2** in our postsynthesis processing of the one-bead-one-compound library. Compound **2** was found to be only slightly less active than peptide **1** in the screen even though its IC_{50} value is 2.5-fold higher than that of **1** in the purified form. Comparison of the results for (α/β+α)-peptides **1**, **2**, and **17–20** indicates reduced assay sensitivity for screening crude peptide products from split-and-mix synthesis relative to parallel synthesis, which complicates interpretation of the SAR within the one-bead-one-compound library. The lower sensitivity also increases the potential for false negative results; in two instances compound **1** actually showed lower inhibition levels than compounds with an α → β substitution that were 10-fold less potent than **1** after resynthesis and purification.

Finally, we considered the incidence of false positives in the two library formats. Compounds **21** and **22** were identified as hits in both the parallel (before purification) and one-bead-one-compound libraries. Both compounds differ from compound **1** only by having an additional residue inserted into the sequence; however, incomplete coupling of that additional residue would result in a small amount of the highly active compound **1** as a contaminant in the product mixture. The activity of compounds **21** and **22** was greatly reduced after purification of the parallel library, which more closely reflects their IC_{50} values after individual resynthesis and purification (46 and 570 μM for **21** and **22**, respectively).

Conclusions

The comparative studies presented here show that split-and-mix and parallel methods for library synthesis can be interfaced with an FP assay for exploration of peptidic foldamer structure–activity relationships. Our findings show that judicious choice of analysis conditions enables one to identify high-affinity ligands for the BH3-recognition cleft on Bcl-x_L without purifying library members prior to screening in a competition FP assay. Even moderate-affinity ligands can be identified via this approach, although both

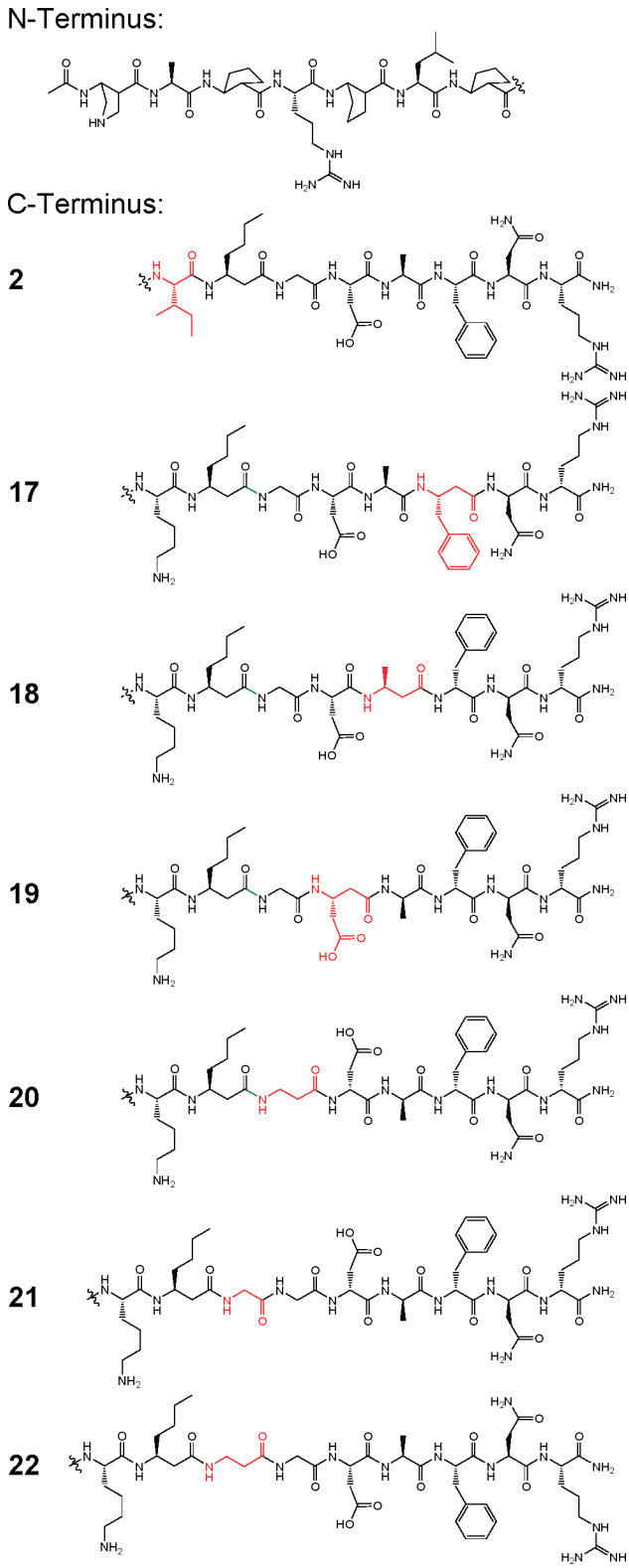

Figure 6. Structures of hits from α → *β* library described in Figure 5.

false positive and false negative results become problematic in the moderate affinity range. Both library synthesis methods provided rapid access to large numbers of oligomers in acceptable purities through application of microwave irradiation. In the one-bead-one-compound format, the FP assay was capable of identifying a hit that was approximately 10-fold more active than the other members of the library (i.e., compound **1** in the α → *β* library). Only if the library was synthesized in parallel could oligomers with activities differing by less than 10-fold be distinguished. The FP assay's sensitivity was somewhat reduced when screening material from single beads, perhaps because of the lower purity of the products relative to those prepared in parallel, as judged by HPLC analysis. We found that parallel synthesis, which avoids LC-MS/MS sequencing and resynthesis before purification and validation of active compounds, is more time-efficient for elucidation of the SAR of a lead compound series.

The combinatorial chemistry described here allowed us to evaluate relatively quickly the effects of side-chain and backbone modifications on Bcl-x_L affinity of α/*β*-peptide oligomers. These efforts did not lead to an oligomer with greater Bcl-x_L affinity than starting compound **1**, presumably, because of the extensive prior structure–activity studies that led to the identification of **1**[7,29] and the relatively limited modifications that were explored in the libraries discussed above. We suspect that ligand discovery for new protein targets will require oligomer libraries with considerable backbone and side chain diversity. The comparative studies reported here should be useful for other foldamer-based efforts to identify antagonists of protein–protein interactions, in particular the use of parallel synthesis for the rapid preparation of focused foldamer libraries based on different backbones.

Experimental Section

General Procedures. Fmoc-(*S*,*S*)-ACPC and Fmoc-(*R*,*S*)-APC(Boc)-OH were prepared by the method of Lee et al.[31] Fmoc-(*S*)-β^3-hNle-OH, Fmoc-(*S*)-β^3-hPhe-OH, Fmoc-(*S*)-β^3-hTrp(Boc)-OH, Fmoc-(*S*)-β^3-hLeu-OH, Fmoc-(*S*)-β^3-hCha-OH, Fmoc-(*S*)-β^3-hAsp(*t*Bu)-OH, Fmoc-(*S*)-β^3-hAla-OH, and Fmoc-(*S*)-β^3-hGln(Trt)-OH were prepared from the corresponding Fmoc-L-α-amino acids (Novabiochem and SynPep) as described previously[18a] or were purchased from Peptech. Methanol, CH_2Cl_2, and acetonitrile were purchased from Burdick & Jackson. Piperidine, 1-hydroxybenzotriazole hydrate (HOBt), *i*Pr_2EtN, trifluoroacetic acid (TFA), triethylsilane, triisopropylsilane, and DMSO were purchased from Aldrich. NovaSyn TGR resin (0.25 mmol/g loading), Fmoc-*β*-Gly OH, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and Fmoc-α-amino acids were purchased from Novabiochem or SynPep. Polystyrene A RAM macrobeads (500–560 *μ*m diameter, 0.55 mmol/g loading) were purchased from Rapp Polymere. DMF (biotech grade solvent, 99.9+ %) was purchased from Aldrich and stored over Dowex ion-exchange resin. CH_2Cl_2 and *i*Pr_2EtN were distilled from calcium hydride. Deep-well polypropylene filter plates for parallel peptide synthesis were purchased from Artic White. Costar black polystyrene 96-well plates for FP assays were purchased from Corning. The Bcl-x_L construct used in FP studies lacked the C-terminal transmembrane domain and a nonessential loop and was expressed in *Escherichia coli* as previously described.[32]

Split-and-Mix Library Synthesis on PS Macrobeads with Microwave Irradiation. The side chain optimization library was synthesized using split-and-mix techniques on

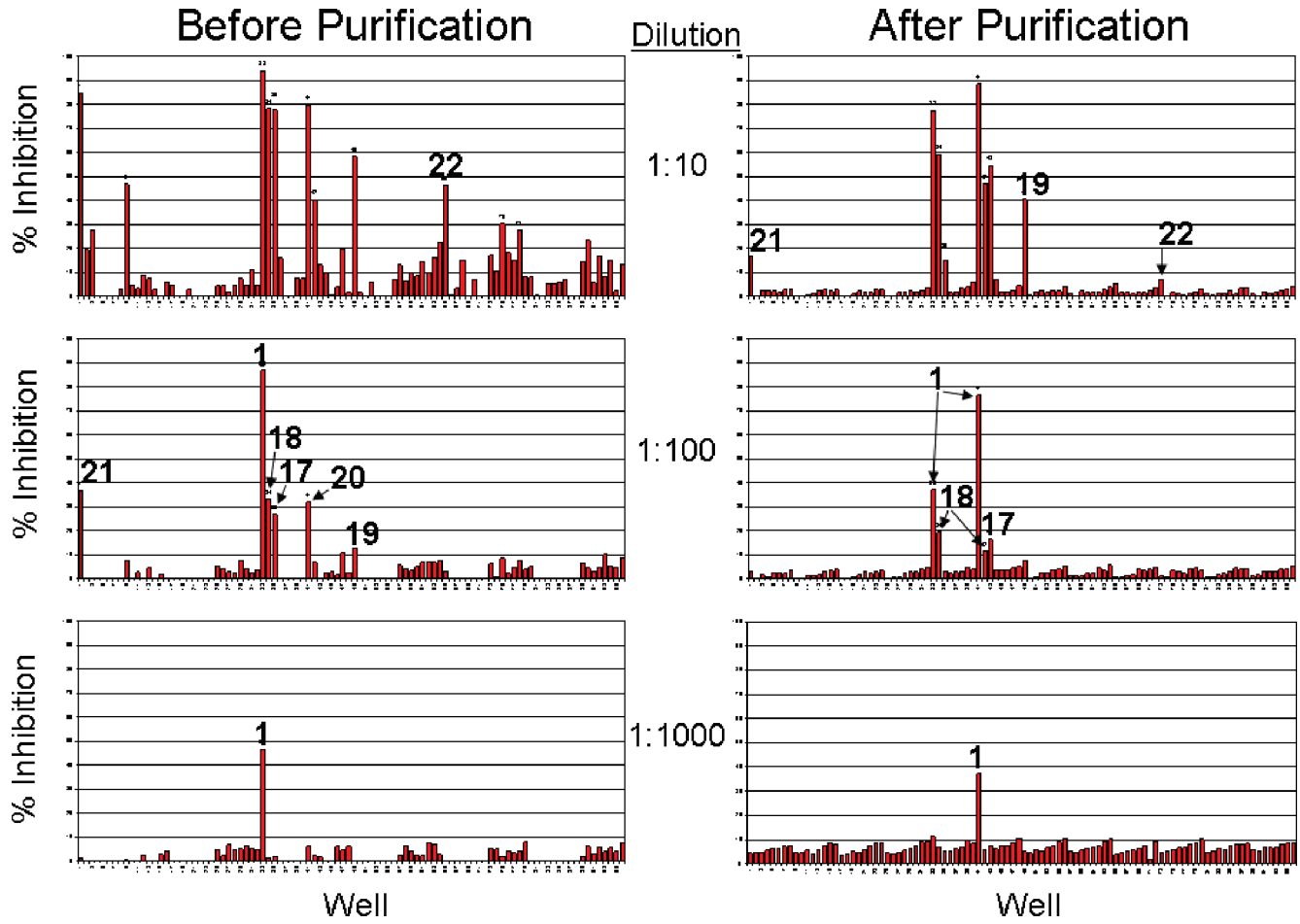

Figure 7. Screening of parallel β-scan library both before (left) and after (right) purification. Only one of the two plates into which the purified compounds were reformatted is shown. The second post-purification plate contained compound **20**. Compounds **1** and **18** each appear twice in the screening data for the plate of purified compounds because the large volume of the collected fractions resulting from the HPLC purification of these two compounds exceeded the 2 mL per well capacity of the 96-well deep well plate used for rotary concentration. Therefore, the eluent was split between two wells in the reformatted plate for concentration and screening.

PS macrobeads using microwave irradiation in a multimode microwave reactor. PS A RAM macrobeads (661 mg, 364 μmol, ~4800 beads) were placed in a polypropylene solid-phase extraction (SPE) tube (25 mL, Alltech) and swelled with DMF for ~10 min. The resin was washed (5 × DMF, 5 × CH_2Cl_2 and 5 × DMF). Deprotection solution (20 mL of 20% piperidine in DMF (v/v)) was added to the resin, and the tube was capped and placed on a shaker for 2 h. The resin was washed as before. In a separate vial, Fmoc-Arg(Pbf)-OH (708.5 mg, 1092 μmol) was activated by adding HBTU (2184 μL of 0.5 M solution in DMF), DMF (16 mL), HOBt (2184 μL of 0.5 M solution in DMF), and iPr$_2$EtN (2184 μL of 1.0 M solution in DMF). The mixture was vortexed and allowed to stand for 1 min before being added to the resin. The tube was capped and placed on a shaker overnight. The resin was washed, and deprotection solution was added (20 mL). The vessel was placed in one slot of a 52-position turntable inside the multimode microwave reactor (CEM MARS). The fiber optic temperature sensor was suspended in the reaction mixture by pressing it through a small hole (made with a needle) in the plastic top cap of the SPE tube and placing the cap loosely on the reaction vessel. The sample was subjected to three cycles of irradiation in the microwave reactor (600 W maximum power, 90 °C, ramp 2 min, cool-down 10 min). All microwave irradiations were conducted at atmospheric pressure. The tube was removed from the microwave reactor, and the resin was washed. Fmoc-Asn(Trt)-OH was activated as before and added to the resin. The sample was subjected to six cycles of irradiation in the microwave reactor (600 W maximum power, 80 °C, ramp 2 min, cool-down 10 min). The tube was removed from the microwave reactor, and the resin was washed, and Fmoc was removed with microwave irradiation as before. After it was washed, the resin was partitioned into five aliquots of approximately equal volume using a spatula with care not to crush the swollen beads. Each resin sample was placed in a 15 mL SPE tube (Alltech). Fmoc-Phe-OH, Fmoc-Cha-OH, Fmoc-Leu-OH, Fmoc-Trp-(Boc)-OH, and Fmoc-Tyr(tBu)-OH (218 μmol of each) were each activated in separate vials by adding HBTU (437 μL of 0.5 M solution in DMF), DMF (3.2 mL), HOBt (437 μL of 0.5 M solution in DMF), and iPr$_2$EtN (437 μL of 1.0 M solution in DMF) and vortexing. One coupling solution was added to each aliquot of resin. The samples were evenly distributed around a turntable within the multimode microwave reactor, the fiber optic probe was inserted into one sample, and the samples were irradiated (6 cycles, 600 W maximum power, 80 °C, ramp 2 min, cool-down 10 min). (We found later that heating coupling solutions of different amino acids simultaneously in the multimode microwave

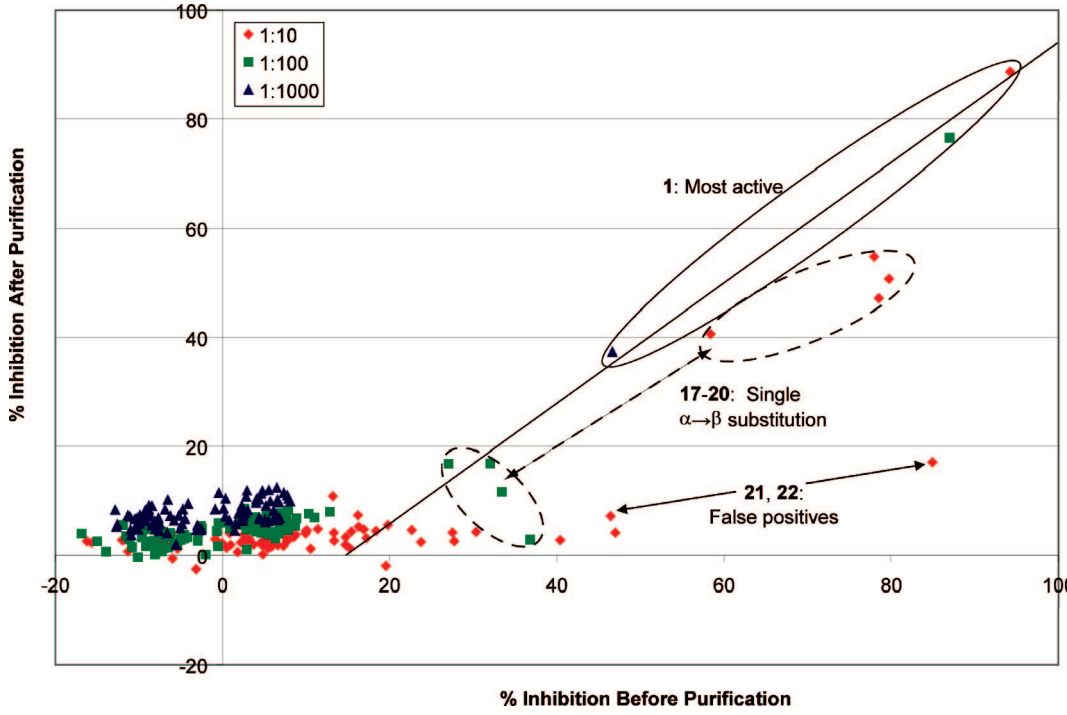

Figure 8. Correlation of the percent inhibition of parallel library members (Figure 5) screened before and after HPLC purification. The line is a manual fit of the data for compound **1**.

reactor while monitoring the temperature of one sample led to differences in reaction temperatures among the different reaction vessels. This artifact was avoided in the future by performing sequential couplings.) The resin was washed, combined in the 25 mL SPE tube, suspended in DMF, and thoroughly mixed. Fmoc removal and coupling of Fmoc-Ala-OH were performed. The resin was split into two equal portions. Fmoc-Asp(*t*Bu)-OH (546 μmol) was coupled to one aliquot of resin, and Fmoc-Glu(*t*Bu)-OH was coupled to the other aliquot. The resin was combined; Fmoc was removed, and Fmoc-Gly OH was coupled. The resin was split into four equal portions. Fmoc-(*S*)-β^3-hNle-OH, Fmoc-(*S*)-β^3-hPhe-OH, Fmoc-(*S*)-β^3-hTrp(Boc)-OH, or Fmoc-(*S*,*S*)-ACPC-OH (273 μmol) were each coupled to a different aliquot of resin. The resin was recombined and subjected to two deprotection/coupling cycles to add Fmoc-Lys(Boc)-OH and Fmoc-(*S*,*S*)-ACPC-OH. After Fmoc-deprotection, the resin was split into five equal portions. Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-hLeu-OH, or Fmoc-Cha-OH (218 μmol) were each coupled to a different aliquot of resin. The resin was recombined and subjected to two deprotection/coupling cycles to add Fmoc-(*S*,*S*)-ACPC-OH and Fmoc-Arg(Pbf)-OH. After Fmoc removal, the resin was split into five equal portions. Fmoc-(*S*,*S*)-ACPC-OH, Fmoc-(*S*)-β^3-hPhe-OH, Fmoc-(*S*)-β^3-hTrp(Boc)-OH, Fmoc-(*S*)-β^3-Cha-OH, and Fmoc-(*S*)-β^3-Leu-OH were each coupled to a different aliquot of resin. The resin was recombined and subjected to two deprotection/coupling cycles to add Fmoc-Ala-OH and Fmoc-(*R*,*S*)-APC(Boc)-OH. After Fmoc removal and washing (5 × DMF, 5 × CH_2Cl_2, 5 × DMF, and 5 × CH_2Cl_2), the peptides were N-terminally acetylated by adding 20 mL of a 14:5:1 solution of CH_2Cl_2/acetic anhydride/triethylamine and shaking for 30 min. After it was washed (5 × CH_2Cl_2 and 5 × MeOH) and dried under a stream of N_2, the resin was arrayed (one bead per well) into 15 384-well polypropylene plates (Costar) using tweezers and a bead arrayer. The bead arrayer had a pinhole at each position of a 384-well plate and was connected to both a vacuum aspirator and a N_2 line. A sample of beads was poured into the trough with a vacuum being pulled on the device. The resin was maneuvered until one bead was positioned at each pinhole and held there with the vacuum. Excess resin was removed. A 384-well plate was inverted and placed in the trough over the beads. While being held firmly together, the bead arrayer and plate were inverted, and the valve was switched from the vacuum source to the N_2 line. The arrayer and plate were tapped smartly on the benchtop to transfer the beads from the arrayer to the plate. The vacuum was reapplied, and the bead arrayer was removed. Any beads remaining in the arrayer were transferred with tweezers. The 384-well plate was scanned visually to ensure that each well contained only one bead.

The material on each bead was cleaved from the solid support with simultaneous side-chain deprotection (80 μL, 45:45:5:5 TFA/CH_2Cl/triethylsilane/water, 2 h, room temperature (RT)), with orbital shaking; the plates were covered with aluminum foil. At the end of the reaction, the cleavage solutions were concentrated by centrifugal evaporation (RT, 4 h, SpeedVac, Thermo Savant). The crude peptide mixtures were dissolved in 80 μL of DMSO; 10 μL of this stock solution was used for the FP assay, while the remaining solution was reserved for analytical characterization and compound (hit) identification. The crude oligomer products from 50 beads were analyzed by HPLC (Shimadzu); 30 μL was injected on a C_4-silica reversed-phase analytical column (5 μm, 4 mm × 250 mm, Vydac) and eluted with a gradient of acetonitrile in water (10–60%, 25 min, 0.1% TFA in each) at a flow rate of 1 mL/min. The chromatograms typically showed a major peak riding on a mound of impurities. The major peak in each HPLC run was collected and oligomer

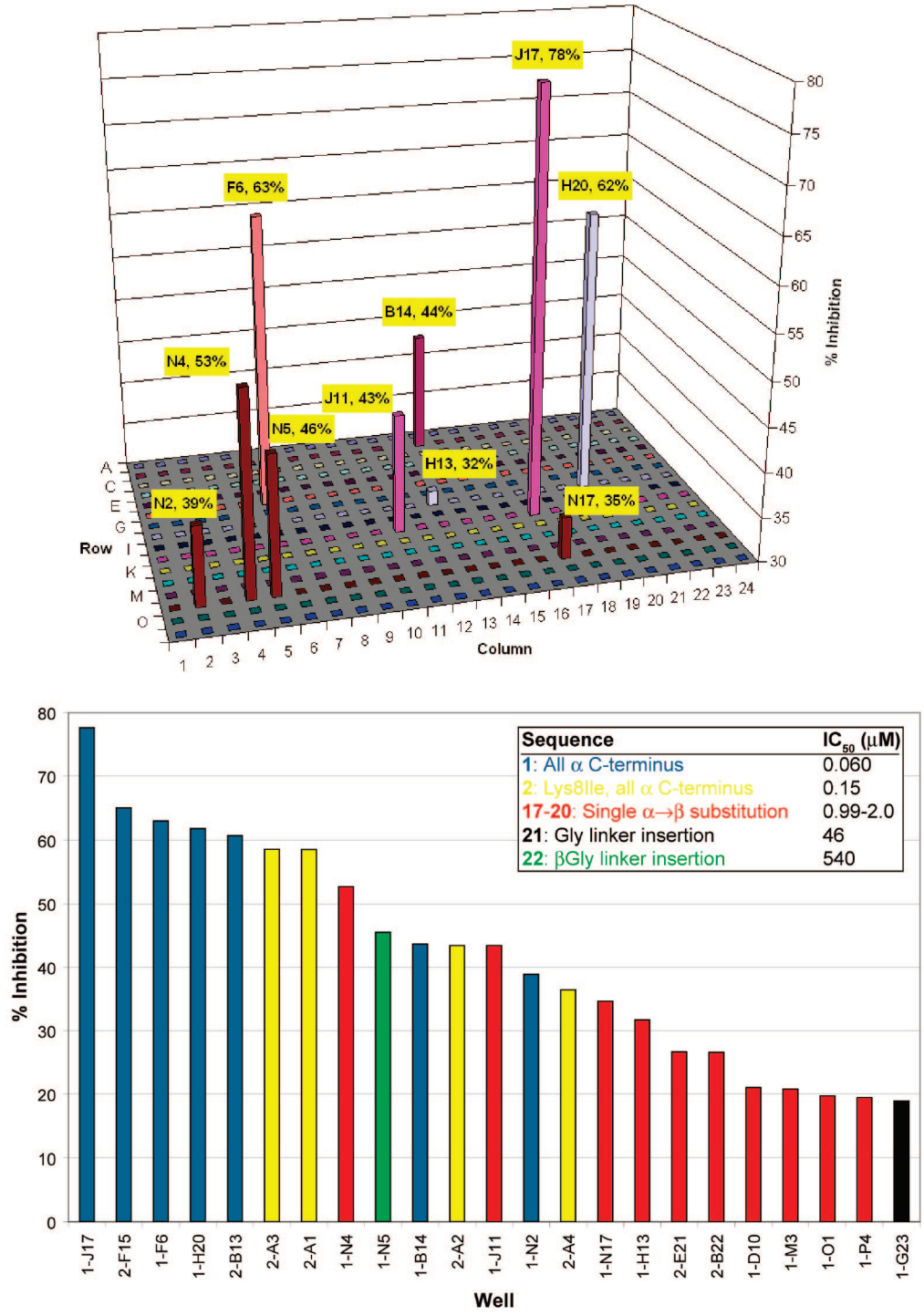

Figure 9. Screening of the one-bead-one compound β-scan library. (Top) Plate 1 with a 30% inhibition lower limit. (Bottom) Ranked hits as identified by MALDI-TOF MS.

masses were measured by MALDI-TOF-MS (Bruker Reflex II, α-cyano-4-hydroxycinnamic acid matrix).

The $\alpha \rightarrow \beta$ library was synthesized using split-and-mix techniques on PS macrobeads using microwave irradiation in a monomode microwave reactor (see Supporting Information). The parallel synthesis and purification of the $\alpha \rightarrow \beta$ library has been described previously.[29]

Fluorescence Polarization Binding Assays. Fluorescence polarization experiments were performed with a PerkinElmer EnVision multilabel plate reader (Wellesley, MA) with polarized filters and optical modules for the BODIPY-TMR fluorophore ($\lambda_{excitation}$ = 531 nm, $\lambda_{emission}$ = 595 nm). The G-factor for all FP experiments was set to 1. The BODIPY^{TMR}-labeled Bak^{BH3} peptide used as the probe in competition FP experiments was synthesized as previously described.[7] The binding dissociation constant (K_d) of BODIPY^{TMR}-Bak^{BH3} for Bcl-x_L, determined by a direct-binding FP assay, was 4 ± 1.8 nM. Competition FP assays were conducted in black 96-well polystyrene plates with final assay concentrations of Bcl-x_L and BODIPY^{TMR}-Bak^{BH3} probe fixed at 20 nM and 33 nM, respectively, in assay buffer (20 mM phosphate, 1 mM EDTA, 50 mM NaCl, 0.2 mM NaN_3,

0.5 mg/mL Pluronic F-68, pH 7.4). To obtain IC_{50} values for oligomers **1–22**, these compounds were added as stock solutions in DMSO (5 nM−5 mM) to 96-well plates containing Bcl-x_L and BODIPY^{TMR}-Bak^{BH3} in assay buffer; final assay concentrations of oligomers **1–22** ranged from 0.0002 to 400 μM. All oligomers were assayed in duplicate. To assay libraries, a stock solution of the library member in DMSO was added to a 96-well plate containing Bcl-x_L and BODIPY^{TMR}-Bak^{BH3}. In all FP assays, the final concentration of DMSO was 4% (v/v). Assay plates were incubated in the dark for ~3 h at room temperature before being analyzed by the plate reader. Raw competition FP data were converted to percent inhibition of the BODIPY^{TMR}-Bak^{BH3}/Bcl-x_L interaction. For oligomers **1–22**, binding data were fit in GraphPad Prism 4.0 (San Diego, CA) by using the one-site competition binding model to determine IC_{50} values and associated 95% confidence intervals. Binding inhibition constant (K_i) values were calculated from IC_{50} values according to ref 33 (see Supporting Information).

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Supporting Information Available. Full experimental procedures, characterization of oligomer library members (including LC-MS/MS sequencing), and FP assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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