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Ligand Specificity in Fragment-Based Drug Design

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Fragment-based drug design consists of identifying low-molecular weight compounds that weakly bind to a target macromolecule and will then be modified or linked to yield potent inhibitors. The specificity of these low-complexity and low-affinity molecules has rarely been discussed in the literature. To address this question, NMR spectroscopy was used to investigate the interactions of 150 fragments with five proteins: three proteins from the Bcl-2 family (Bcl-x_L, Bcl-w, and Mcl-1), human peroxiredoxin 5, for which very few ligands have been reported, and human serum albumin, which is known to bind a large number of ligands. Our results show that the fragments are rather versatile binders and able to identify binding hot spots in very different targets. Despite the different hit rates observed related to the druggability of the proteins, two scaffolds appear as preferred binders for all proteins. Low specificity was observed between homologous proteins or unrelated poorly druggable proteins, while higher specificity could be achieved with highly druggable targets.

Introduction

The growing popularity of fragment-based drug design (FBDD[a]) shows that this methodology is more and more recognized as a tangible alternative to high throughput screening (HTS).[1,2] Fragment-based drug design was first proposed in 1996 by Abbott Laboratories and is now implemented in most big pharmaceutical companies.[3,4] This drug discovery process consists of identifying small low-molecular weight compounds (fragments) that weakly bind to the target macromolecule, typically proteins. The fragments are very simple molecules with few chemical functional groups; therefore, their affinity for the target is usually low compared to the bigger, more complex HTS molecules (high micromolar to low millimolar). However, the fragments are highly efficient ligands and display a high binding affinity per heavy atom.[5] The small size and weak complexity of the fragments provide several advantages to FBDD over HTS, as described below. Since the sizes of fragment and druglike chemical spaces are estimated to be around 10^8 and 10^{63}, respectively,[6,7] a small (10^2) fragment library samples a much bigger portion of the chemical space than even a large (10^6) HTS library, thus allowing a better exploration of the fragment chemical space. Also, there is an inverse relationship between the molecular complexity of the compound screened and the probability that the compound possesses good complementarity to the target protein.[8] A small fragment will have more chances to bind to the target protein than a larger druglike molecule, which is more likely to suffer from steric hindrance due to the multiple chemical functionalities. This leads to higher hit rates for FBDD. In addition, optimization of big, druglike molecules into more potent compounds tends to actually reduce their druglikeness and therefore the bioavailability of the final optimized compound,[8−10] whereas the small size of the fragments provides more latitude for their optimization into lead compounds, thus enhancing the chances of the molecules to make their way through the drug design process and finally to the clinic. These characteristics of FBDD account for the growing interest for this drug discovery method, which has already yielded impressive results as largely demonstrated in the literature.[11−19]

Still, little is known about protein−small molecule low affinity interactions, and it is legitimate to ask whether such small and weakly binding fragment molecules can actually display specificity toward the target protein. In fact, the high hit rates observed in fragment screenings may be due to the low specificity of fragment molecules. To our knowledge, this issue has rarely been discussed in the literature. A statistical analysis of binding data for 104 fragments to 11 protein targets, from Abbott Laboratories, showed that specificity could be achieved with these small molecules.[20] In a recent paper from Chen and Shoichet,[21] a fragment library was screened against the CTX-M β-lactamase, and the hits were then tested against another β-lactamase. The results suggest that the fragment molecules may be characterized by low specificity. Higher specificity was achieved only when optimizing the initial fragments into more complex molecules. On the other hand, the analysis of output from multiple screening campaigns from Vernalis suggests that fragments can exhibit quite a high target specificity.[22] Only 0.6% of the library appeared to be rather versatile binders. Thus, it is not clear yet whether the fragment molecules behave as specific ligand, or if specificity cannot be achieved at this very early stage of the drug discovery process. This question is particularly important when dealing with structurally similar proteins. Here, we

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[a] FBDD, Fragment-Based Drug Design; HAC, Heavy Atoms Count; HSA, Human Serum Albumin; HSQC, Heteronuclear Single Quantum Coherence; HTS, High Throughput Screening; NMR, Nuclear Magnetic Resonance; PDB, Protein Data Bank; PRDX5, human peroxiredoxin 5; STD, Saturation Transfer Difference; WaterLOGSY, Water-Ligand Observed via Gradient SpectroscopY.

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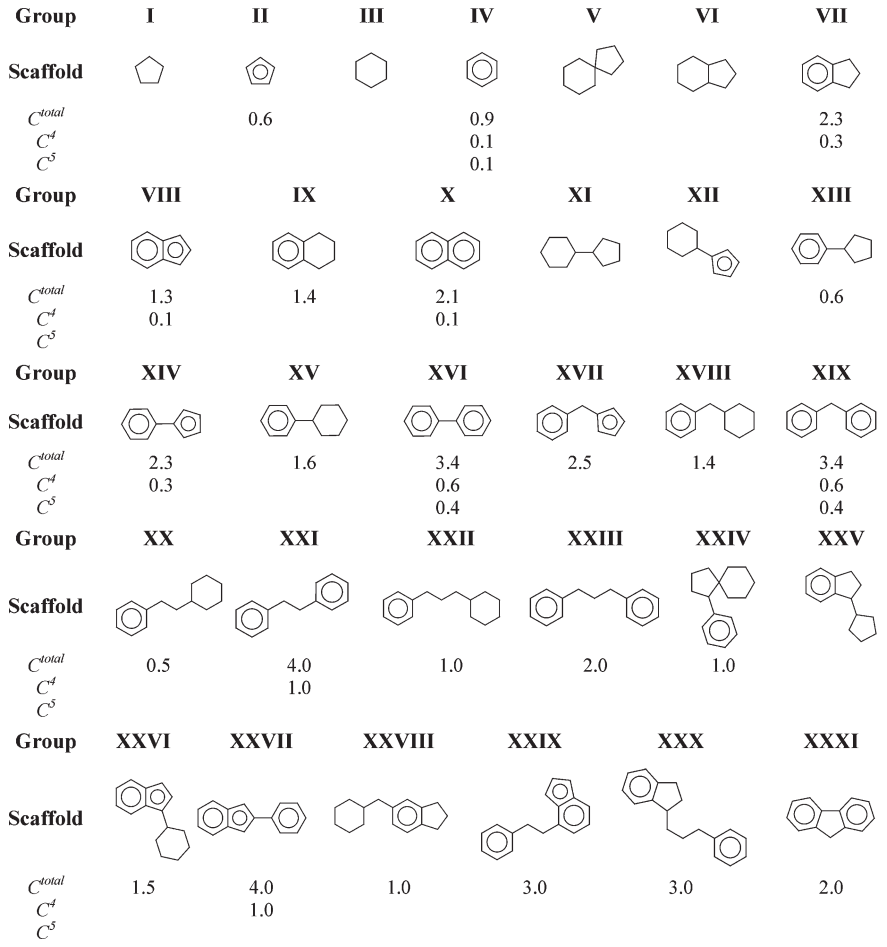


Figure 1. Scaffolds of the 150-fragment library. Heteroatoms and side chains are not represented. For each scaffold, three coefficients are indicated. The coefficient C^{total} indicates the number of hits observed to any of the five protein targets (HSA, Bcl-x_L, Bcl-w, Mcl-1, and PRDX5) divided by the number of fragments sharing this scaffold in the library. The coefficient C^4 refers to the 22 fragments that are able to bind four proteins (HSA, Bcl-x_L, Bcl-w, Mcl-1), whereas coefficient C^5 refers to the 7 fragments that interact with all five targets.

present a study of 150 fragment molecules tested against five target proteins including three paralogues from the well-characterized Bcl-2 family of proteins, Bcl-x_L, Bcl-w, and Mcl-1. These three proteins are involved in apoptosis,[23,24] and their antiapoptotic activity is regulated by protein−protein interactions[25] through a shallow groove on the protein surface, referred to as the binding cleft region.[26,27] A large number of inhibitors competing with the natural proapoptotic partners have been discovered against these Bcl-2 proteins; among these, some are currently in clinical trials.[28−36] The fourth target protein used in this study is the human peroxiredoxin 5 protein PRDX5, which belongs to the peroxiredoxin family of proteins, for which very few inhibitors or ligands have been reported to date.[37,38] PRDX5 is a 36 kDa dimer involved in the detoxification of cells, through the reduction of hydroperoxides into alcohols.[39] We recently discovered the first small molecule inhibitor of this protein using fragment screening.[40] In addition, we have analyzed the interactions of the fragment molecules with the well-characterized protein, human serum albumin (HSA), and compared the screening results to the four other proteins. HSA (a 66 kDa monomer) is a very abundant protein in human plasma involved in the transportation of various molecules such as hormones, fatty acids, and drugs. Several binding sites for different substrates have been identified on its surface, and a very large number of ligands have been characterized for this protein.[41]

To analyze the binding properties of the fragments toward the different proteins, a library of 150 fragments was tested by 1D ligand-observed NMR experiments (saturation transfer difference (STD[42]) and WaterLOGSY[43]) and 2D protein-observed NMR experiments with all the proteins except HSA. Fragment hits of the different targets were identified and their physicochemical properties compared to the properties of the ensemble 150-fragment library. The binding factors from STD and WaterLOGSY experiments were compared across the series of hits to better characterize the binding specificity of the fragments. This study shows that despite the low affinity of the fragments, (i) the hit rates of the fragment-based screenings are highly protein-dependent,[22,44] (ii) the probability of identifying specific fragment hits depends on the druggability of the protein, and (iii) the fragments bind to particular binding sites, efficiently highlighting the protein hot spots.[44] Thus, we find that fragments are rather versatile binders, highly adaptable to various protein binding sites, with some scaffolds showing higher hit rates for all proteins.

Results

Elaboration of a Fragment Library. The fragment library was elaborated from commercial compounds characterized by physicochemical properties defined by the rule of three.[45] A search for 31 different substructures (Figure 1) was performed to build a diverse library in terms of size, shape,

and chemical functionalities. Filters were applied for molecular weight (< 300 Da) and log P (< 3). Side chains of the molecules were chosen to allow the polarity required for compound solubility; compounds with unwanted functions were rejected.[46−48] All selected compounds were dissolved in DMSO-d_6 stock solutions and tested for aqueous solubility (600 μM) and purity by 1D ^{1}H NMR spectra. WaterLOGSY experiments were recorded to detect possible aggregation, as LOGSY effects are characteristic of high molecular weight compounds in water.[49] Then to optimize the experimental time and to reduce the protein quantity required for the NMR screening experiments, fragments were pooled as mixtures of three to six molecules with minimum spectral overlap. NMR spectra of the mixtures were rerecorded after 3 months to confirm the absence of degradation or evolution of the compounds inside the mixture. Figure 1 illustrates the different chemical scaffolds present in the final 150-fragment library, and Figure 2 shows the distribution of the molecular weight, number of rings, heavy atom count, number of heteroatoms, and log P for the ensemble. The library is characterized by an average molecular weight of 166.9 Da, an average log P of 1.02, and an average number of rings, heavy atoms, and heteroatoms of 1.8, 12.2, and 2.9, respectively.

Probing the Fragment−Protein Interactions by 1D NMR of the Ligand. All the NMR screening experiments were recorded with the same experimental conditions including buffer, pH, temperature, NMR spectrometer, and NMR parameters. The 150-fragment library was tested against Bcl-x_L, Bcl-w, Mcl-1, PRDX5, and HSA by ^{1}H NMR, using both STD[42] and WaterLOGSY[43] experiments. To allow an accurate comparison between the Bcl-x_L, Bcl-w, and Mcl-1 proteins, the C-terminal α-helix 8 that obstructs the binding pocket in the Bcl-w protein was removed and all the NMR experiments were performed on a construction lacking the additional transmembrane C-terminal helix.[50] A fragment was considered a hit (ligand) if it gave rise to both WaterLOGSY and STD signals (Figure 3). First, hits were identified from the fragment mixtures, and then the individual fragments were tested alone to confirm binding. Figure 4 illustrates the STD spectra obtained for one fragment mixture on the five proteins. As shown, different results are observed depending on the target protein. As for the entire library, very different hit rates are obtained from one protein to another, with values ranging from 7 hits (5%) for PRDX5 to 72 hits (48%) for HSA. Regarding the three homologous proteins, different hit rates are also observed: 71 fragments bound to Bcl-x_L, 51 bound to Bcl-w, and 29 bound to Mcl-1, leading to hit rates of 47%, 34%, and 19%, respectively.

Probing the Fragment−Protein Interactions by Protein-Observed 2D NMR. The fragments that were detected as ligands in the 1D NMR screening experiments were then tested by protein-observed 2D NMR experiments (heteronuclear single quantum correlation, ^{1}H−^{15}N HSQC). As previously observed,[40,51] not all the fragments that gave WaterLOGSY and STD signals induced chemical shift perturbations on the protein spectra. The absence or presence of chemical shift perturbations was correlated with the strength of the STD signals;[51] the weakest ligands were not detected by 2D NMR methods. For the four proteins, about two-thirds of the 1D NMR-detected hits induced specific chemical shift changes in the 2D spectra.

For the fragments that induced changes, the interaction regions were determined by mapping the chemical shift changes onto the surface of the proteins (Bcl-x_L, Bcl-w, Mcl-1, and PRDX5). Interestingly, the residues perturbed upon the fragment addition are similar from one fragment to another, suggesting that all four proteins contain hot spots, particular binding sites on the surface that bind multiple ligands (Figure 5). For PRDX5, all fragments bound in the region of the active site. For the Bcl-2 proteins, all fragments bound within the BH3 (Bcl-2 homology domains) binding pocket that is common to all three proteins.[24]

Comparison of the Hits across the Different Proteins. Figure 6A illustrates the distribution of the fragment hits across the three homologous proteins from the Bcl-2 family. A total of 83 compounds out of the 150 fragment library were shown to bind at least one of the Bcl proteins. About one-fourth of the 83 hits (22 fragments, 27%) were detected as ligands of all three proteins, while 40 molecules (48%) were shown to bind both the Bcl-w and Bcl-x_L proteins. Only 1, 7, and 29 fragment hits were observed to bind to Mcl-1, Bcl-w, and Bcl-x_L, respectively. We found 76% of the Mcl-1 hits also bind Bcl-w and Bcl-x_L, and 78% of the Bcl-w fragments hits also bind Bcl-x_L. In Figure 6B, the repartition of the fragment hits of the HSA and Bcl-x_L proteins is indicated. A total of 104 hits were observed for those proteins, among them 39 hits that bound both HSA and Bcl-x and 32 fragments that were shown to bind Bcl-x_L but not HSA. Such screening results could be used in the drug discovery process. Binding to HSA is known to control the free active concentration of a drug, affecting the drug absorption, distribution, and elimination. Therefore, the knowledge of substructures that are capable of binding the receptor target but not HSA can be very useful in the lead optimization phase. Interestingly, the 32 fragments that do not interact with HSA share some structural features, with four substructures identified as privileged nonbinding motifs for HSA, as reported in Figure 7. These positively charged substructures do not bind the HSA binding sites, likely because of the hydrophobic character and the positive patches located at the entrance of the binding pockets of the two major HSA binding sites.[52,53] Those findings are in agreement with the features observed for the hits of HSA identified from the library (see below).

The physicochemical properties of the fragment hits are shown in Figure 8 in comparison with the physicochemical properties of the entire library. Most of the properties of the hits are quite similar to the properties of the whole library. The variations were generally smaller than 13% between the average values for the library and ligands. A larger difference (up to 24%) is observed for the number of heteroatoms. Finally, the log P average value appears to be clearly different between the entire library and the different hit groups, as a significant increase (up to 109%) is observed from the average value of the library (1.02) to the average values of the ligands (1.61 for HSA to 2.13 for PRDX5; see Figure 8). This result was previously observed in a recent analysis of screening campaign results where a clear separation was observed between hits and nonhits when considering the log P value, with hits shown to be much more hydrophobic.[22] If one looks more closely at the different profiles, one can notice that the lower the hit rate is, the more hydrophobic the hits are. This behavior is obvious when looking at the PRDX5 and Mcl-1 proteins, for which most of the hits have a heavy atom number in the 14−17 range and log P > 2.

Fragment Ranking by Measurement of STD and WaterLOGSY Factors. We previously measured the dissociation constant for the best ligand of Bcl-x_L, fragment **1** (2-phenylphenol), using chemical shift variations observed upon fragment addition on the 2D protein spectrum, and a value of 2.7 ± 0.5 mM was

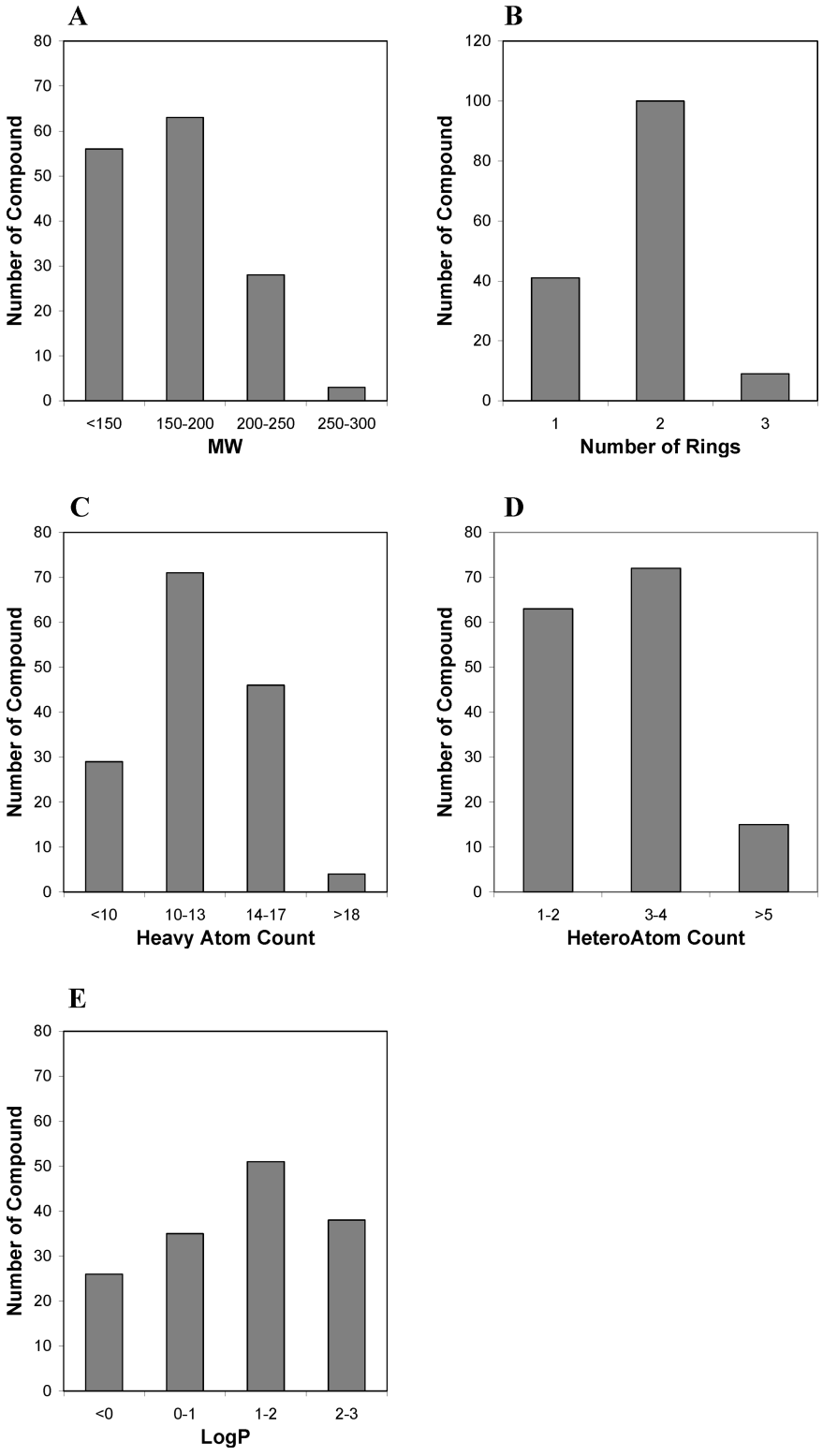


Figure 2. Repartition of (A) molecular weight, (B) number of rings, (C) heavy atom count, (D) heteroatom count, and (E) log *P* values for the fragments of the library used in this study.

obtained.[51] Because of the poor solubility of the fragments in water and the low affinity, it was not possible to obtain reliable measurements and to determine the dissociation constants of the other fragment hits. Although STD and WaterLOGSY factors are not directly correlated to the affinity of the ligand,[54] these data can be used to rank a series of ligands. So, to further analyze the specificity of the fragment hits, their STD and WaterLOGSY factors have been measured to compare the hits across the proteins of the Bcl family.

We first measured the STD and WaterLOGSY factors (f_{STD} and $f_{WaterLOGSY}$) of 29 Bcl-w hits composed of the 22 fragments that bind all three Bcl proteins plus 7 other fragments that bind exclusively Bcl-w (Figure 6A). Average values of 11% and 57% are calculated for the f_{STD} and

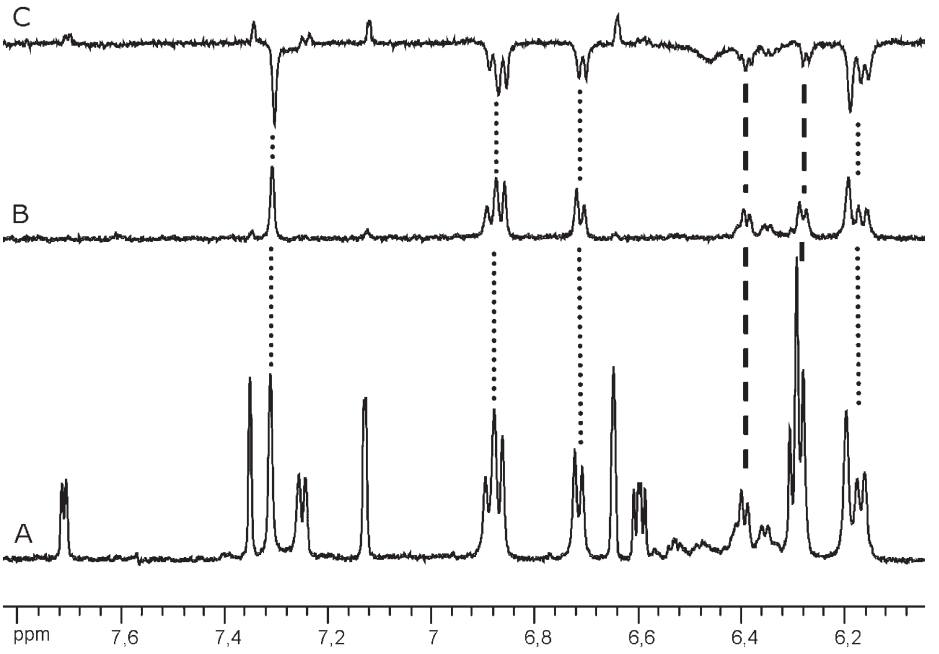


Figure 3. 1D ligand-observed NMR experiments recorded on the HSA protein: (A) 1D spectrum, (B) STD spectrum, and (C) WaterLOGSY spectrum of a fragment mixture composed of five fragments. Dotted lines highlight two different HSA hits.

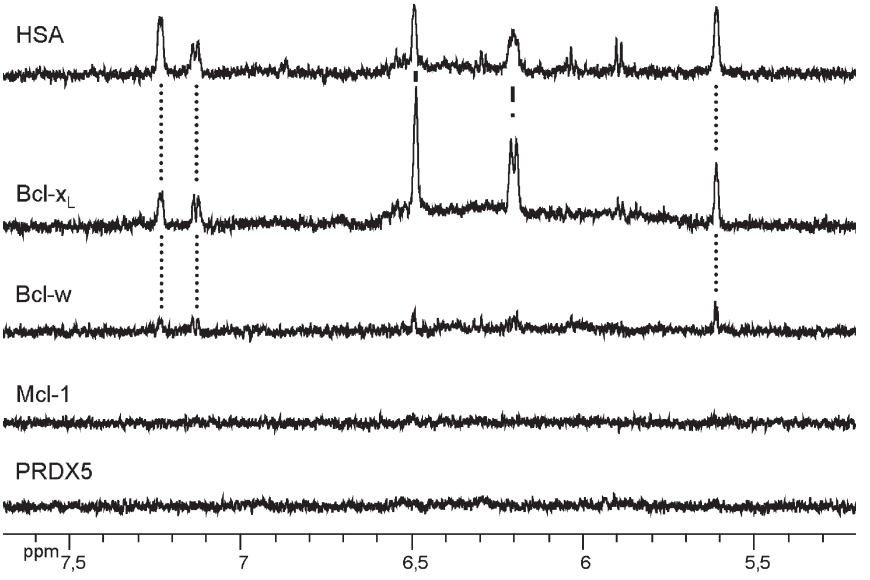


Figure 4. STD spectra of a fragment mixture recorded on the five proteins. All experiments were recorded in the same conditions.

$f_{WaterLOGSY}$ of the seven specific ligands, respectively, whereas the 22 nonspecific ligands display average values of 30% and 187% for the f_{STD} and $f_{WaterLOGSY}$, respectively (Supporting Information Figure S1). These NMR data clearly indicate that the best ligands of Bcl-w (in terms of affinity) are the hits common to the three proteins of the Bcl-2 family and are not specific Bcl-w ligands. Then the f_{STD} values of 13 fragments that were shown to bind the three homologous proteins Bcl-x_L, Bcl-w, and Mcl-1 were measured (Table 1). Measurements are not reported for Mcl-1 because of a poor S/N ratio that induced large errors in the f_{STD} calculations. The comparison of the f_{STD} values obtained for Bcl-x_L and Bcl-w clearly shows that the ranking of the 13 fragments is very similar for both homologous proteins (Table 1).

Discussion

In this study, we report the binding properties of 150 fragments toward five different proteins: human peroxiredoxin 5 PRDX5, human serum albumin HSA, and three proteins from the well-characterized Bcl-2 family of proteins, Bcl-x_L, Bcl-w, and Mcl-1. The binding specificity of these small low affinity ligands was measured by 1D and 2D NMR experiments. These results were used to identify the ligands of each protein, to rank the ligands by their affinity, and to analyze the ligand binding sites.

The first observation of this work is that the hit rates observed for the five proteins, using the 150 fragment library as described in Figures 1 and 2 and using identical detection methods, strongly vary from one protein to another, from 5% for PRDX5 to 48% for HSA (Figure 8F). This result shows

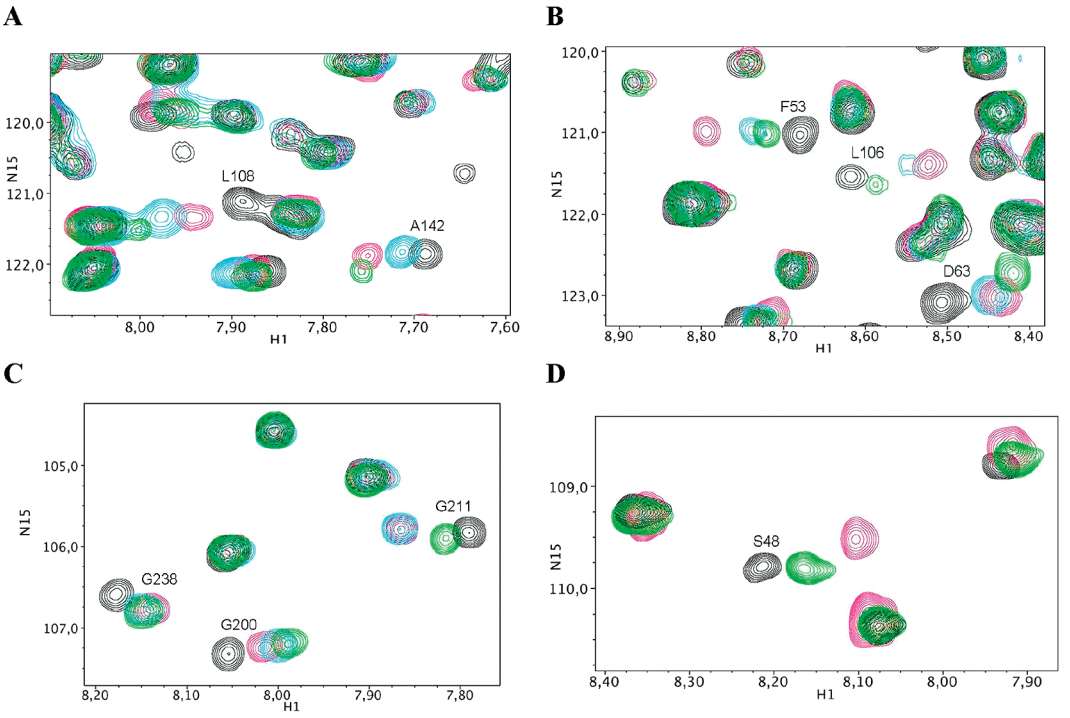


Figure 5. Superposition of the 2D ^{15}N-HSQC spectra of the protein in the absence and in the presence of fragments **1** (2-phenylphenol) (red), **2** (2-benzylbenzyl alcohol) (cyan), and **4** (2,4,4′-trihydroxybenzophenone) (green). The reference spectrum is displayed in black. Protein concentration is 80 μM, and ligand concentration is set to 1 mM: (A) Bcl-x_L; (B) Bcl-w; (C) Mcl-1; (D) PRDX5. For PRDX5, no chemical shift variation was observed in the presence of **2**.

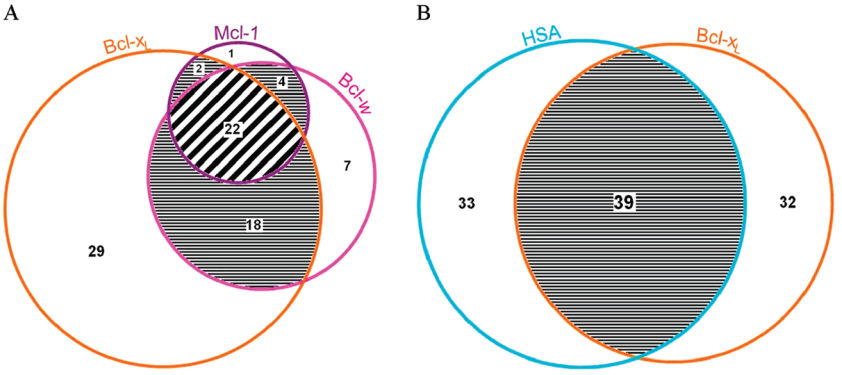


Figure 6. Repartition of the fragment hits: (A) overlap of the Bcl-x_L, Bcl-w, and Mcl-1 hits; (B) overlap of the Bcl-x_L and HSA hits. Numbers indicate the number of hits observed for each group. Horizontal lines indicate the portion of common hits to two proteins, and tilted lines indicate the portion of common hits to all three proteins of the Bcl-2 family.

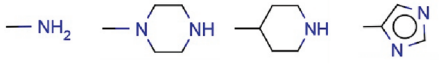
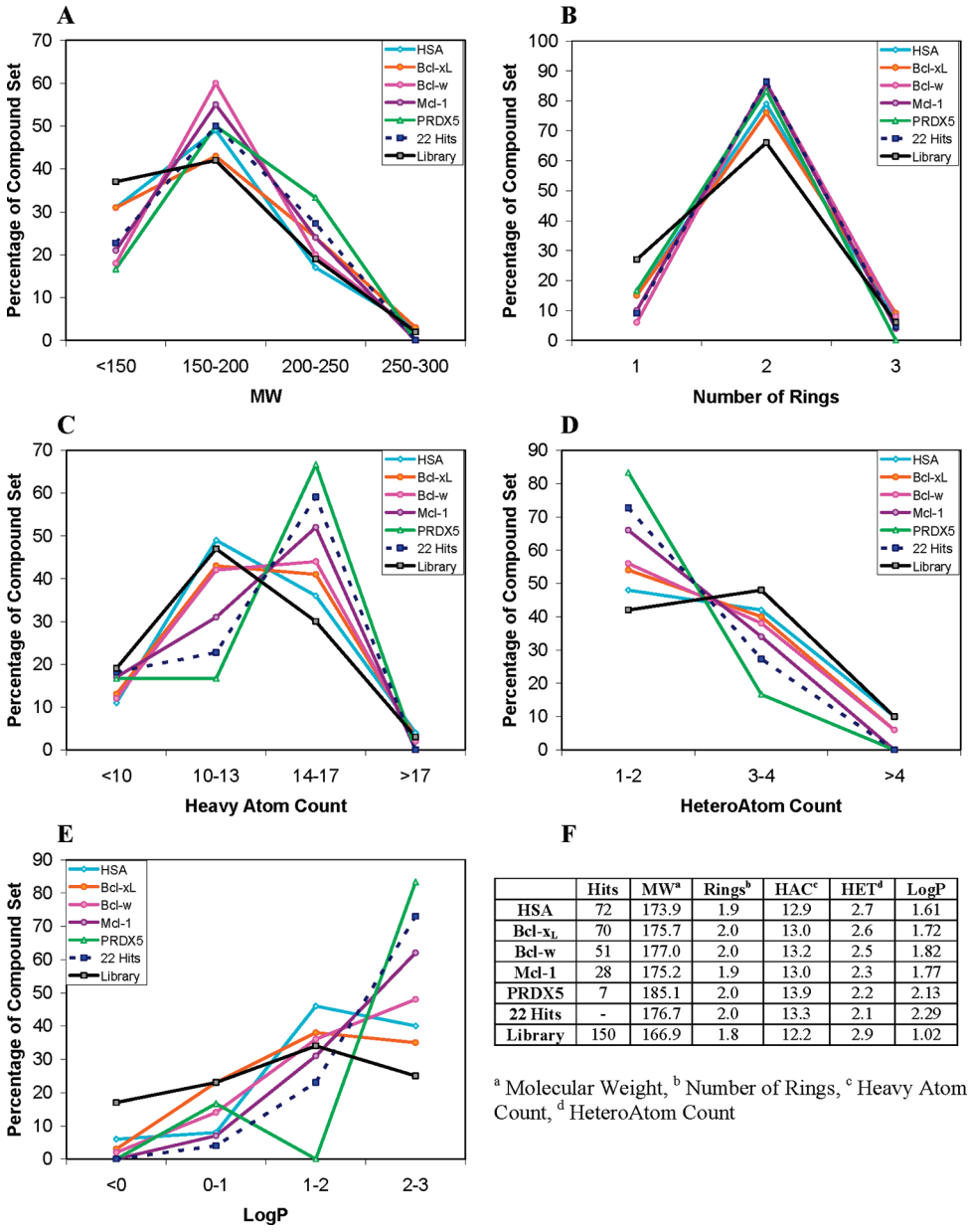


Figure 7. Privileged substructures identified in the 32 fragments that bind Bcl-x_L but not HSA. The percentage of compounds containing each substructure is indicated.

that in spite of the low affinities of the fragments (100 μM to 10 mM), the number of molecules that are detected as ligands is highly protein dependent, reflecting the protein's inherent druggability (see below). Notably, the hit rates we report here are higher than the hit rates published by Abbott,[44] most probably because of the different detection techniques. The 1D ligand-observed NMR experiments detect a larger number of ligands than 2D protein-observed NMR methods. The theoretical explanation of this sensitivity difference has been recently reported.[55] The hit rate can also be strongly related to some physicochemical parameters of the library compounds, such as the log *P* coefficient.[22] Here, the hits were observed to be more hydrophobic than the nonhits (Figure 8). Finally, to be compared, the hit rates should be associated with a binding affinity range, since the hit rates will dramatically decrease if weak affinities (K_d > 1 mM) are not detected. In this study, hits identified through STD and WaterLOGSY NMR experiments comprise ligands with affinities as weak as 10 mM.

Hit rates were previously proposed to be related to the druggability of the protein.[44] The druggability concept, which refers to the probability of finding high-affinity, druglike leads for a protein, was developed by Hajduk and co-workers, and classifies the proteins as highly druggable to poorly druggable according to the hit rate obtained in a 2D heteronuclear NMR screen.[44] Recently, Hubbard suggested that 1D NMR could also be used to determine the druggability of proteins.[22]



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	Hits	MW[a]	Rings[b]	HAC[c]	HET[d]	LogP
HSA	72	173.9	1.9	12.9	2.7	1.61
Bcl-x$_L$	70	175.7	2.0	13.0	2.6	1.72
Bcl-w	51	177.0	2.0	13.2	2.5	1.82
Mcl-1	28	175.2	1.9	13.0	2.3	1.77
PRDX5	7	185.1	2.0	13.9	2.2	2.13
22 Hits	-	176.7	2.0	13.3	2.1	2.29
Library	150	166.9	1.8	12.2	2.9	1.02

[a] Molecular Weight, [b] Number of Rings, [c] Heavy Atom Count, [d] HeteroAtom Count

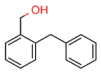
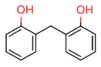
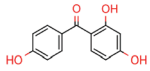
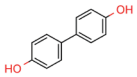
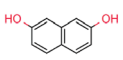
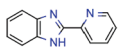
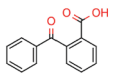
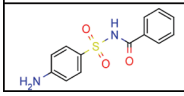
Figure 8. Physicochemical parameters of the hits compared to the physicochemical parameters of the library: distribution of (A) molecular weight, (B) number of rings, (C) heavy atom count, (D) heteroatom count, and (E) log *P* values for the HSA, Bcl-x$_L$, Bcl-w, Mcl-1, and PRDX5 hits, the 22 hits common to HSA, Bcl-x$_L$, Bcl-w, Mcl-1, and the whole library. Averages values are indicated for each parameter in (F).

Our results are clearly in accordance with such a proposition. As 2D heteronuclear NMR screen is not easily applicable to protein targets with molecular weights above 30 kDa, 1D NMR screening performed using a restrained library (100 fragments is sufficient) appears to be a very fast and robust experimental method to assay the druggability of a new target and to compare proteins.

The second observation inferred from this work is that 2D protein-observed NMR experiments, when feasible, represent a very attractive experimental method to identify the "hot spots" on a protein surface.[44] These hot spots correspond to energetic focal points on protein surfaces that are the major contributors to the binding energy.[44] Here we observe that all the PRDX5 fragment hits bind into the enzyme active site, while the ligands of the Bcl-2 family of proteins tested all interact within the BH3-binding groove (Figure 5). More precisely, we recently showed that if most known Bcl-x$_L$ inhibitors span the whole BH3 binding cleft, the fragments all bind into a preferred subsite, the so-called "site 1".[51] Hence, this study confirms that despite their low complexity and low affinity, the fragments have a particular binding site and are able to highlight these "hot spots" on a protein surface.

In agreement with the fact that very few inhibitors have been described for the peroxiredoxin family of proteins,[40,56] a low hit rate was observed for PRDX5. This result is consistent with the small size of the PRDX5 active site hot spot and with the fact that the protein surface of the peroxiredoxin does not offer other pockets for compounds to bind.[39,40] In contrast, the multiple pockets on the surface of HSA likely account for the high number of hits observed for this protein. Regarding the three homologous proteins of the Bcl-2 family, the hit rates vary among them, and Mcl-1 exhibits the smallest number of ligands (19% in comparison with 47% for Bcl-x$_L$). This observation is interesting considering the features that distinguish this protein

Table 1. NMR-Based Ranking of Bcl-x_L and Bcl-w Hits[a]

Fragment		Bcl-x_L		Bcl-w	
Chemical Structure	Molecule Name	f_{STD}	Ranking	f_{STD}	Ranking
	**1**	100	1	100	1
	**2**	100	1	91	3
	**3**	78	2	91	3
	**4**	78	2	95	2
	**5**	65	5	43	6
	**6**	61	6	50	5
	**7**	56	7	41	7
	**8**	43	8	39	9
	**9**	39	9	41	7
	**10**	39	9	27	10
	**11**	30	11	7	12
	**12**	13	12	5	13
	**13**	4	13	11	11

[a] For each fragment, the STD factor f_{STD} is indicated.

from the other prosurvival proteins: Mcl-1 does not share binding properties toward certain BH3 domains and is also insensitive to some Bcl-x_L antagonists, among them ABT-737.[27,57] Those dissimilarities originate from several differences both in sequence and 3D structure between Mcl-1 and Bcl-x_L.[24,58] The differences we observed in fragment-based screening appear to confirm the fact that Bcl-x_L and Bcl-w are more easily targeted and druggable than Mcl-1.[32,57,59]

If the hit rates differ from a protein to another, do the hits behave as specific ligands or are they rather versatile binders? The overlap of the fragment hits of the three homologous proteins of the Bcl-2 family is illustrated in Figure 6A. As shown, only one Mcl-1 hit binds specifically to this protein and a large majority of the Mcl-1 hits (76%) also bind to both Bcl-x_L and Bcl-w. Similarly, among the 51 Bcl-w hits, only 7 fragments (14%) appear as specific Bcl-w ligands while 22 fragments are shown to bind also Bcl-x_L and Mcl-1 (see the hatched area in Figure 6A). To further investigate the fragment specificity, we analyzed the hits by measurement of NMR-based affinities. According to the NMR ranking based on f_{STD} and $f_{WaterLOGSY}$ values, the best hits for Bcl-w do not specifically bind to this protein but also bind the two other homologous proteins. Therefore, the 7 hits specific for Bcl-w are only weak Bcl-w ligands. A similar conclusion can be drawn for the 29 specific Bcl-x_L hits (data not shown). If we compare the f_{STD} values of common Bcl-x_L and Bcl-w hits, the ranking is very similar for both proteins (Table 1). Thus, it is clear from these results that little or no specificity is observed across the homologous proteins. These observations are concordant with a recent study where little specificity was observed in fragments that were tested against two different β-lactamases, the class A β-lactamase CTX-M and the class C β-lactamase AmpC.[21]

Do we observe similar results when hits are compared across unrelated proteins? The repartition of the hits of Bcl-x_L and HSA is shown in Figure 6B. A total of 33 and 32 fragments bind specifically the HSA and Bcl-x_L proteins, respectively, while 37% of the hits are shared by both proteins. For Bcl-w and Mcl-1, a majority of the hits also bind HSA (78% and 80%, respectively). All seven PRDX5 hits also bind the four other proteins. These results suggest that the specificity of the fragments is rather low, even between unrelated proteins, supporting the idea that high hit rates reflect the low specificity of the fragments. We want to emphasize that such an observation is obtained with fragments that display very weak affinities (>1 mM), and we anticipate that specificity would be observed for higher affinity hits, as others have reported.[20,21] Nevertheless, one observes specific ligands for the proteins showing the highest hit rates, Bcl-x_L and HSA (Figures 6B and 7). Interestingly, good ligands of HSA ($f_{STD} > 80$) that do not bind any of the four other proteins were identified in the fragment library (Supporting Information Table S2). The results show that the best HSA ligands are highly hydrophobic fragments with hydroxyl or carboxylate polar groups. These results are in agreement with the fact that a large majority of Bcl-x_L ligands that do not bind HSA contain positively charged groups (Figure 7). As a whole, these results suggest that (i) the specificity of the fragments is very low when dealing with homologous proteins, (ii) the lower the hit rate, the higher is the versatility of the fragment hits, and (iii) fragment specificity can be observed for highly druggable targets.

Are there common properties shared by the versatile fragments? Among the 150 fragments, 22 were shown to bind four out the five proteins and a further 7 fragments were able to interact with all the proteins studied. The physicochemical properties of these hits are shown in Figure 8. The nonspecific fragments tend to be more hydrophobic, with $\log P > 2$, a heavy atom count in the 14−17 range, and less than 3 heteroatoms. But their most interesting feature is certainly their scaffold. For each scaffold in the library, a coefficient C was calculated as the number of hits divided by the number of fragments with this scaffold in the library (Figure 1). The coefficient C^{total} is calculated for all the protein hits, and the coefficients C^4 and C^5 are indicated for the 22 and 7 fragments common to the 4 proteins (Bcl protein and HSA) and all the 5 proteins, respectively. As illustrated, five scaffolds are clearly overrepresented among the fragment hits. Moreover, if we analyze the 22 fragments that are capable of binding Bcl-x_L, Bcl-w, Mcl-1, and HSA and the 7 fragments shown to bind to all five proteins, two different scaffolds, the biphenyl (scaffold XVI) and the bicyclic (scaffold XIX), appear as preferred substructures. This is in agreement with the study of Hajduk and co-workers that showed that the

biphenyl motif is a preferred substructure for protein binding.[20] Those scaffolds afford hydrophobic interactions that are important for the ligand−receptor association but not for the specific ligand recognition, which enable them to bind to a very diverse set of proteins.

Conclusion

In this study, we have investigated the behavior of 150 fragment molecules toward three protein paralogues and two unrelated proteins. NMR methods were used to identify and rank the hits and to characterize the fragment binding sites. Our results illustrate the tendency of the fragment to act as low affinity ligands and show that (i) fragment hits are more hydrophobic than nonhits, (ii) the biphenyl and the bicyclic scaffolds are preferred substructures that are able to bind very different proteins, (iii) in spite of the different hit rates obtained, due to the druggability properties of the proteins, little or no specificity is observed toward structurally related proteins, (iv) low specificity is observed between unrelated poorly druggable proteins, (v) higher specificity is observed with the most druggable target. However, even if the specificity of individual fragments is low, fragment-based hit identification represents a very powerful lead-design method, since it enables one to identify a *series* of fragment hits for one target. What will differentiate one protein from another is the *ensemble* of the hits, the relative affinity of the hits, and the *binding mode* of the hits, which contains the key information necessary to improve both the affinity and specificity of the initial hits.

Materials and Methods

Library of Fragments. The compounds of the library were chosen from Aldrich or Acros online catalogs with a search for the scaffolds displayed in Figure 1. All molecules had molecular weight of less than 300 Da and a log *P* of less than 3.[45] Aqueous solubility was checked for the compounds by recording 1D ^{1}H NMR spectra and WaterLOGSY[43] spectra at 600 μM. Molecules for which autoassociation was observed from the WaterLOGSY signals were rejected. The selected compounds were stocked in 110 mM DMSO-d_6 solutions and conserved at −20 °C. The 1D ^{1}H NMR spectra were recorded to check that no degradation occurs over 3 months. Compounds were mixed by 3 to 6 to decrease the NMR experimental time as well as the protein quantity. The criterion for the compound selection for the mixtures was the absence of overlapping resonances in the 1D NMR spectra. The library contained 150 validated fragments.

Protein Samples. Protein targets used for the NMR screening experiments are three proteins of the Bcl-2 family of proteins (Bcl-x_L, Bcl-w, and Mcl-1), the human peroxiredoxin 5 PRDX5, and the human serum albumin HSA. Bcl-x_L, Bcl-w, and Mcl-1 were prepared as described below, and PDRX5 was prepared as previously published.[40] Human serum albumin was directly purchased from Sigma (CAS 70024-90-7).

Protein Production and Purification of Bcl-x_L, Bcl-w, and Mcl-1. Human Bcl-x_L containing deletions in the C-terminus (197−233) and the internal loop between α-helix 1 and α-helix 2 (45−84) and human Bcl-w lacking the last C-terminal α-helix 8 (1−154) were expressed as 6xHis-tagged proteins in *Escherichia coli* strain BL21 (DE3). Mouse Mcl-1 containing deletions in the C-terminus and N-terminus (151−308) was expressed as glutathione *S*-transferase protein in *Escherichia coli* strain BL21 (DE3). *E. coli* were grown at 37 °C either in LB medium to produce unlabeled protein or in M9 minimal medium supplemented with thiamine (20 mM) and containing $^{15}NH_4Cl$ (1 g/L) or $^{15}NH_4Cl$ (1 g/L), [$^{13}C_6$]D-glucose (4 g/L), and 70:30% (v/v) D_2O/H_2O to produce ^{15}N-labeled protein or $^{15}N/^{13}C$/50% ^{2}H-labeled protein, respectively. Protein synthesis was induced with 1 mM IPTG for Bcl-w and Mcl-1 and with 0.5 mM IPTG for Bcl-x_L. The 6xHis-tagged Bcl-x_L and Bcl-w were purified in one step by Ni^{2+}-affinity chromatography and exchanged into 90% H_2O/10% D_2O, 25 mM Na_2HPO_4, 0.5 mM EDTA, and 2 mM dithiothreitol (DTT), pH 6.8 and 7.0, respectively. Mcl-1 was purified by glutathione affinity chromatography. The GST tag was cleaved overnight by prescission protease, and the Mcl-1 protein was exchanged into 90% H_2O/10% D_2O, 50 mM, 70 mM NaCl, pH 6.7.

Screening by 1D Ligand-Observed NMR Experiments. All spectra were acquired at 20 °C with a Varian Inova 600 MHz NMR spectrometer equipped with a standard 5 mm triple-resonance inverse probe with a *z*-axis field gradient, actively shielded, and with an autosampler robot. The NMR samples were prepared with a robot TECAN Miniprep 60. For all protein but HSA, the NMR tubes were prepared with 20 μM protein and 600 μM fragments. For the HSA, the protein concentration was set to 10 μM. Samples contained one to six fragments, and the total concentration of DMSO-d_6 did not exceed 4% in the NMR tubes. Control 1D normal and WaterLOGSY ^{1}H spectra preceded all experiments to assess the purity and stability of the fragments. NMR screening was achieved using 1D STD[42] and WaterLOGSY[43] experiments. The parameters used were the same as previously described.[60] The number of scans was set to 500 and 1000 for the WaterLOGSY and STD experiments, respectively. All NMR spectra were processed with the Varian VnmrJ software.

STD Factor Measurement. For quantitative analyses of STD spectra, the STD amplification factors f_{STD} were derived from the following equation:

$$f_{STD} = \frac{I_{STD}}{I_0}\frac{[L]_{tot}}{[P]_{tot}}$$

where I_{STD} and I_0 are peak integrals in the STD and 1D experiments, respectively, and $[L]_{tot}$ and $[P]_{tot}$ are the total concentrations of the ligand and protein, respectively. 1D and STD experiments were performed in the same experimental conditions (spin lock, interscan delays), and parameters for the STD experiments (saturation frequency, saturation time) were identical for all samples. STD signals were measured for aromatic protons. To easily compare the fragments, the STD factors have been scaled, using 100 for the strongest STD factor measured.

WaterLOGSY Factor Measurement. Quantitative LOGSY effects[61] were measured as the relative percentage differences, $[(I_p - I_f) \times 100]/I_f$, between the NMR line intensities of a ligand in the presence (I_p) or the absence (I_f) of the protein. In the absence of binding the two intensities are equal, leading to a null LOGSY effect (0%).

Backbone Resonance Assignment of the Proteins. We have recently achieved the backbone resonance assignment of Bcl-x_L and PRDX5.[40,51] For the Mcl-1 protein, the backbone assignment is available in the Biological Magnetic Resonance Bank (BMRB).

Backbone resonances of human Bcl-w lacking the last C-terminal α-helix 8 (1−154) was assigned using triple-resonance experiments recorded on a 0.5 mM uniformly $^{15}N/^{13}C$ labeled Bcl-w sample. Experiments were recorded at 28 °C on a 600 MHz spectrometer. The following experiments from the Varian Protein Pack were recorded: HNCA, HN(CO)CA, HNCACB. A ^{15}N-HSQC spectrum was collected before and after each 3D experiment to check the protein stability. A 3D $^1H-^{15}N$ NOESY-HSQC experiment was also recorded with a mixing time of 150 ms. All NMR spectra were processed with the NMRPIPE software[62] and analyzed using NMRView.[63]

Fragment−Protein Interactions Studied by 2D Protein-Observed NMR Experiments. All NMR samples contained 80 μM uniformly ^{15}N-labeled Bcl-x_L, Bcl-w, Mcl-1, and PRDX5 protein and 0.2−2 mM ligand (depending on solubility and affinity for the protein). Control 1D ^{1}H spectra preceded all experiments to

assess the purity and stability of the fragments. All HSQC spectra were recorded at 28 °C using 64 t_1 increments. All NMR spectra were processed with the Varian VnmrJ software and the NMRPIPE software[62] and analyzed using NMRView.[63]

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Supporting Information Available: Figure showing the 1D ^{1}H spectrum, the STD spectrum, and the WaterLOGSY spectra (in absence or presence of the protein Bcl-w) for a strong ligand and a weak ligand; table listing the ranking of the 10 best HSA ligands of the library. This material is available free of charge via the Internet at http://pubs.acs.org.

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