Promiscuous and selective: how intrinsically disordered BH3-proteins interact with their pro-survival partner MCL-1.

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Highlights

• Despite differences in residual helicity BH3 peptides bind equally fast to MCL-1.

• Differences in stabilities results from off-rates, altering complex lifetimes.

• This difference arises from variation in interactions at MCL-1 binding interface.
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Abstract

The BCL-2 family of proteins plays a central role in regulating cell survival and apoptosis. Disordered BH3-only proteins bind promiscuously to a number of different BCL-2 proteins, with binding affinities that vary by orders of magnitude. Here we investigate the basis for these differences in affinity. We show that eight different disordered BH3 proteins all bind to their BCL-2 partner (MCL-1) very rapidly, and that the differences in sequences result in different dissociation rates. Similarly, mutation of the binding surface of MCL-1 generally affects association kinetics in the same way for all BH3 peptides but has significantly different effects on the dissociation rates. Importantly, we infer that evolution of homologous, competing complexes has resulted in producing complexes with significantly different lifetimes.

1 BCL-2 : B cell lymphoma-2
MCL-1 : Myeloid cell leukemia 1
A1: BCL-2-related gene A1
BAK: BCL-2 antagonist killer 1
BAX: BCL-2 associated X protein
CD: Circular Dichroism
wt: wild-type
Introduction

The physiological process of cell death, or apoptosis, is a highly regulated process and the B cell lymphoma-2 (BCL-2) family of proteins are important molecular arbitrators of the process\(^1,2\). To date, five anti-apoptotic (BCL-2, BCL-xl, BCL-w, Myeloid cell leukemia 1 (MCL-1) and BCL-2-related gene A1 (A1)) and at least two pro-apoptotic BCL-2 proteins (BCL-2 antagonist killer 1 (BAK) and BCL-2-associated X protein (BAX)) have been identified\(^3,4\). A third group of ten pro-apoptotic proteins (termed BCL-2 homology domain (BH3)-only) are thought to interact with both pro-apoptotic BAK and BAX and also the anti-apoptotic proteins\(^5-7\). The BH3-only proteins can be divided into two groups; Activators (BIM, BID and PUMA) and Sensitizers (NOXA A, NOXA B, BMF, BIK, BAD, HRK)\(^8\). Several models have been proposed to understand the role of these pro and anti-apoptotic BCL-2 proteins\(^9\). In all these models, differences in affinity between the anti-apoptotic proteins and the pro-apoptotic proteins are predicted to determine their mode of action. These BCL-2 family proteins have a homologous BH3 domain, which is intrinsically disordered in isolation, but form a contiguous helical segment upon binding to its partner BCL-2 proteins\(^9-13\). This disordered nature is likely be the source of promiscuity as well as selective binding, thus increasing the diversity and flexibility of this intricate network.

The sequence alignment of eight BH3 peptides calculated using Clustal Omega\(^14,15\), shows that there are only two absolutely conserved residues, although they all fold to the same structure upon binding - the calculated RMSD values show that the complexes of PUMA, NOXA A, NOXA B, BIM, BID and BAX with MCL-1 have high structural homology (Fig.1 A and C). Previous studies have shown that BH3 peptides bind to anti-apoptotic MCL-1 with different affinities\(^2,16,17\).
Here, we compare the interaction of eight BH3 peptides with MCL-1, by using kinetic and thermodynamic analyses to understand the biophysical nature of these interactions. Our results show that the association rates are within the same order of magnitude but there are large differences in the dissociation rates. We further investigate two mutants of MCL-1 that have previously been shown to increase the association rate constant of the PUMA peptide and find similar results for all the BH3 peptides investigated in this study\textsuperscript{18}. Our results are in line with our previous hypothesis that the BH3 binding groove of MCL-1 opens up to allow binding of the BH3 peptides\textsuperscript{18}. Interestingly, we see larger differences in dissociation rate constants but the effect on off-rates is different for different peptides. Hence, the affinity of BH3 peptides to MCL-1 is controlled by dissociation rate, which is a result of differences in the specific interactions of the BH3 peptides at the MCL-1 binding interface. The results presented here provide insight into the underlying molecular basis for both the promiscuity and the selectivity afforded by these structurally homologous complexes of BH3 peptides with pro-survival MCL-1.

**Results**

**Different sequences impart different degrees of disorder**

Circular dichroism (CD) spectra reveal that the BH3 peptides investigated are mainly disordered under our experimental conditions. However, the percentage helicities of the BH3 peptides calculated from MRE value at 222 nm\textsuperscript{19}, ranges between approximately 9-63\% (Fig 1B). Percentage helicity for the BH3 peptides estimated from CD does not correlate with predicted percentage helicity from Agadir\textsuperscript{20-22}, or the predicted percentage disorder from PONDR VSL2\textsuperscript{23-30}, ESPRIT (X-Ray)\textsuperscript{31-35} and ANCHOR\textsuperscript{36, 37} (Fig S1).
Differences in affinity is due to differences in $k_{\text{off}}$

We investigated the kinetic signatures of seven different peptides (BID, BIM, BMF, NOXA A, NOXA B, BAK, BAX) and compared it with previously published data for PUMA interacting with wild-type (wt) MCL-1 (Table 1). The association rate constants, $k_{\text{on}}$, vary by only an order of magnitude for all the peptides investigated (Fig. 2A, Fig. 3A). On the other hand, the dissociation rate constants, $k_{\text{off}}$, differ by approximately four or more orders of magnitude (Fig. 2B, Fig. 3B). The equilibrium dissociation constants, $K_d$, also differ by four-five orders of magnitude and reflect the pattern of $k_{\text{off}}$ (Fig. 3C).

Kinetic effects of mutation of MCL-1 binding interface

To investigate the role of partner protein in the interaction we studied two MCL-1 mutations (V234A and T247A) in the binding groove (Table 2). We had previously shown that these mutations, although they weaken binding, actually slightly increase the association rate constant ($k_{\text{on}}$) of wild-type PUMA. Similarly, all BH3 peptides (except BAX) in our experiments have somewhat enhanced association rates to the MCL-1 mutants, with $k_{\text{on}}$ increased by up to 4-fold, compared to wt (Fig. 4, Fig. S2). However, the effect of mutation in the binding groove of MCL-1 on dissociation rates differs by up to ~40-fold for different BH3 peptides, with some seeing a faster and some slower off-rates compared to wt MCL-1 (Fig.4).

Discussion

The $K_d$ values calculated from the kinetics data are presented in Tables 1 and 2. The activator BH3 peptides (PUMA BID and BIM) all have sub-nM affinities. The sensitizers are known to display more specificity in their binding, so NOXA B, known to bind MCL-1 specifically also has sub-nM affinity whereas NOXA A and, in particular, BMF, bind more weakly. As expected, the effector, pro-apoptotic peptides BAK and BAX bind less tightly than the activator proteins (Table 1). These data are more-or-less consistent with
previously reported affinities for BH3 proteins interacting with wt MCL-1; given the differences in experimental conditions \(^2, ^{16}, ^{17}\).

Due to the abundance of charged residues and their disordered nature, IDPs are susceptible to changes in ionic strength and solvent environments\(^{38}, ^{39}\). Patterning of charges is also known to affect the overall conformational ensembles of IDPs\(^{27}, ^{40}, ^{41}\). As increased residual structure in free IDP has been seen to increase affinity of IDPs to partner proteins, we investigated whether there is a relationship between residual helicity of the peptides and our kinetic and thermodynamic data\(^{42}, ^{43}\). As shown in Supplementary Fig. S3, although differences in sequence result in differences in residual helicity of the BH3 peptides, we see no correlation between residual helicity and \(k_{\text{on}}, k_{\text{off}}\) or \(K_d\) (Fig. S3).

\(k_{\text{on}}\) values observed for all the BH3 peptides are within the range of association kinetics reported for most IDP:protein interactions (\(~10^5 - 10^8\ \text{M}^{-1}\text{s}^{-1}\)\(^{44}, ^{45}\). However, despite differences in sequences, all the BH3-only peptides bind to wt MCL-1 with a similar association rate (Note that the scale in Fig 3A is very different to that of Figs. 3B or 3C). Since, the BH3 peptides have only 6% sequence identity, the estimated charges of the peptides are varied, and we see that the \(k_{\text{on}}\) is related to the estimated charge on the peptide (Fig. S4). Note that our experiments were conducted in a buffer of ionic strength = 100mM. Previous studies of the PUMA : MCL-1 interaction (PUMA has an estimated charge of \(~3\) in our experimental conditions) have shown that increasing ionic strength slows the association of PUMA\(^{46}\). The fold-change from an ionic strength of 0 to infinite ionic strength is some 20-fold (although this is also ion-type dependent)\(^47\). However, variations in the physiological range (100-200 mM) affect the association rate by only approximately 2-fold\(^{47}\). Thus, these marginal differences in \(k_{\text{on}}\) observed for the BH3 peptides binding to MCL-1 may simply be due largely to differences in the charges
of the peptides, as was seen for a similar comparative study of different IDP ligands binding to KIX\textsuperscript{48}.

However, we find that the dissociation rate constants (k\textsubscript{off}) of the BH3 peptides from MCL-1 differ by 4-orders of magnitude. The results clearly reveal that differences in affinity reflect differences in the rate at which BH3 peptides dissociate (Fig. 5). This behavior has been observed for other IDP systems where the effect of post-translational modification (phosphorylation in pKID/KIX) and conserved helix flanking prolines (conserved prolines in MLL/KIX and p53/MDM2) were investigated\textsuperscript{49,50}. The results from these papers and the results presented here, suggest that different characteristic of IDPs primarily allow modulation of lifetimes of the IDP/partner complexes. This behavior of stability in signaling proteins being controlled by dissociation rate has been previously proposed to be functionally relevant\textsuperscript{50-52}. Perhaps being disordered allows IDPs involved in complex network and signaling pathways, such as BH3 proteins, to adapt their kinetics allowing them to bind/unbind to many target proteins with different kinetics; that is, allowing promiscuous binding. This may also be why disorder is conserved and abundant in proteins involved in cell signaling processes\textsuperscript{53-55}.

A structural alignment of the available complex structures of BH3 peptides (PUMA, BID, BIM, NOXA A, NOXA B and BAX) with MCL-1 (Fig 1C) reveals a close structural homology of these complex structures, with RMSDs 1.2 - 3.5 Å (Fig 1C). All BH3 peptides bind to the same ‘BH3-binding’ groove in MCL-1 and only minor structural differences are observed in the structural alignment. Analysis of the contacts formed, however, show that each peptide binds MCL-1 using a different subset of binding residues, similar to that suggested for A1\textsuperscript{56} (Fig. S5). Previous NMR chemical shift analysis indicated similar changes in MCL-1 conformation upon BH3 peptide binding, suggesting that MCL-1 accommodates each BH3 ligand in a similar manner\textsuperscript{11,57}. Our results for the two conservative MCL-1 interface mutants (V234A and T247A), show that
although the effect on association kinetics is the same for all the BH3 peptides (i.e. we see marginal increase in association rates compared to wt MCL-1), the effects on the affinity of the complex (and thus the off-rates) is very different. Mutating the residues of MCL-1 causes changes in affinity by affecting the dissociation rate constant. In some cases, the effect is marginal, but in others the affinity changes by orders of magnitude. Furthermore, while most complexes are destabilized by mutation of interface residues in MCL-1, in others affinity is increased by several orders of magnitude (Fig 4). Interestingly, although the loss in affinity (ΔΔG) is correlated with the number of contacting residues in the peptide for the MCL-1 mutant V234A, there is no such correlation for the T247A mutant (Fig. S5, Table 2). We infer that there may be small adjustments of the structure of the peptide when binding to a mutant MCL-1. Thus, the differences in affinity are regulated by differences in specific contacts with the BCL-2-like partner (in our case MCL-1).

**Concluding Remarks**

We find that varying the dissociation rates essentially controls the affinity of a BCL-2 protein, MCL-1, with different BH3 peptides. These differences in affinity are largely due to different interactions formed at the MCL-1 binding interface once the complex structure matures. Evolution of these homologous and competing complexes has resulted in different stabilities by modulation of the off-rates of these complexes. We infer that it is the lifetime of the complex that may be the most important biophysical property in a competing network, such as the BH3:BCL-2 system.
Material and Methods

Biophysical buffer

All biophysical experiments were carried out in 50mM sodium phosphate buffer (0.05% Tween20), pH 7.0.

Protein expression and purification

Wild-type MCL-1 and mutants thereof (*Mus musculus*, 152-308 residues, Uniprot P97287) were expressed as described previously. The harvested cells after expression were resuspended in 1*PBS 25mM Imidazole, pH 7.5 and sonicated. The sonicate was centrifuged and the supernatant incubated with Ni$^{2+}$- agarose resin for 1 hour at 4ºC. The protein bound to the Ni$^{2+}$- agarose resin was then eluted with 1*PBS 500mM Imidazole (pH 7.5-8) and 2mM ethylenediaminetetraacetic acid (EDTA) was added to the elution to prevent precipitation. The protein solution was then buffer exchanged to 1*PBS 25mM Imidazole, pH 7.5 using a 5kDa molecular weight cut off spin column. 600 units of thrombin was then added to the protein solution and left overnight at room temperature to cleave off the HisTag. Thrombin cleaved supernatant supplemented with 2mM CaCl$_2$ to sequester the EDTA, was incubated with Ni$^{2+}$- agarose resin for 1 hour to remove uncleaved protein. Cleaved protein was loaded into 5mL HiTrap™ SP HP ion-exchange pre-equilibrated with 10mM HEPES, pH 7.5, and eluted using 1M NaCl buffer gradient. The protein was then loaded into Superdex G75 gel filtration column to elute with the biophysical buffer, 50mM Sodium phosphate (0.05% Tween20) pH7.0. Eluted protein was confirmed to be MCL-1 after analysis by gel electrophoresis and mass spectroscopy and stored at 4ºC.

PUMA peptide with 35 residues (*Mus musculus*, 127-161 residues, Uniprot Q99ML1) with M144A mutation was expressed as described previously. The purification was also carried out as described before except that after binding to the Ni$^{2+}$ agarose resin,
the resin was washed with 20mM Tris pH7.0, 10 mM imidazole buffer and cleaved with Factor Xa in 20mM Tris, 50mM NaCl, 5mM CaCl$_2$, pH 8.0 buffer. The cleaved supernatant was then purified by ion-exchange using 10mM Tris, pH8 and a linear gradient of 1M NaCl was applied and the protein eluted at 12-17%. The peptide was then further purified using Superdex G30 gel filtration column and eluted with the biophysical buffer, 50mM Sodium phosphate (0.05% Tween20) pH7.0. PUMA peptides were then frozen using liquid N2, and stored at -80ºC.

**Peptides**

All BH3 peptides (Uniprot code: PUMA - Q99ML1, BID - P70444, BIM - O54918, BMF – Q91ZE9, NOXA A – Q9JM54, NOXA B – Q9JM54, BAK – Q16611, and BAX – Q07812; 35 amino acids, labeled with TAMRA dye at the N-terminus) were purchased from Biomatik. Peptides were dissolved in the biophysical buffer as required and filtered using a 0.22 mm cutoff filter. The stock solutions of peptides were frozen using liquid N2 and stored at -80ºC.

**Estimated parameters for peptides and PDB complex structures**

Estimated peptide charges were calculated using pK$_a$ values of amino acids for model peptides$^{59}$ and corrected for addition of TAMRA dye at the N-terminus. Agadir % helicity predictions were made for pH 7.0, 25 ºC and 100mM ionic strength$^{20-22}$. Percentage (%) disorder predictions were generated using the tools PONDR-VSL2$^{23-25, 28-30}$, ESpritz (X-Ray - chosen as the peptides are only 35 residues long and most have X-ray structures of bound complex with their partners available in the PDB)$^{31-35}$ and ANCHOR$^{36, 37}$. To create the contact map, contacting residues of BH3 peptides PUMA, NOXA A, NOXA B, BIM, BID and BAX with MCL-1 were plotted from PDB structures 2ROC, 2ROC, 2JM6, 2PQK, 2KBW and 3PK1. Contacts between the peptides and MCL-1 were defined between all non-hydrogen atoms within 4Å. Structural alignment for PDB structures
(2ROC, 2ROD, 2JM6, 2PQK, 2KBW and 3PK1) were built using PyMOL and RMSD values reported for each.

**Concentration measurements**

The concentrations of purified protein, MCL-1 (wild-type and mutants) and PUMA peptide were measured by absorbance at 280nm and calculated using extinction coefficients $22157 \text{ M}^{-1}\text{cm}^{-1}$ and $7113 \text{ M}^{-1}\text{cm}^{-1}$ respectively. The extinction coefficients were previously determined using amino acid analysis.

TAMRA labeled peptide concentrations were determined from absorbance at 555nm, using extinction coefficient $83000 \text{ M}^{-1}\text{cm}^{-1}$. The extinction coefficient was again calculated by amino acid analysis of multiple peptide samples.

**Circular Dichroism (CD) and residual helicity**

Samples were prepared at different concentrations using the biophysical buffer. CD spectra (190-260 nm) were recorded in a 2mm path-length cuvette at 25ºC using a ChiraScan CD spectrometer from Applied Photophysics. All spectra were buffer subtracted and estimate for percentage helicities were calculated using average mean ellipticity at 222nm using the methods described previously.

**Kinetic measurements**

For all kinetic experiments, an excitation wavelength of 555nm was used and emission above 570nm was measured.

Association kinetics experiments were performed using a SX18 or SX20 fluorescence stopped-flow spectrometer from Applied Photophysics with the temperature maintained at 25ºC. Experiments were performed with 10-fold or larger excess of the folded partner (wild-type and mutant MCL-1) over TAMRA labeled BH3 peptides. An average of 30-40 traces were fitted to an equation describing a single exponential process to extract the observed rate ($k_{obs}$) for each concentration of the partner protein. The dependence of
$k_{\text{obs}}$ on the concentration of the partner protein was fit to a straight line, and the gradient of the line used to determine the association rate constant ($k_{\text{on}}$). Fluorescence polarization accessory was used to measure anisotropy change instead of fluorescence change where signal change was poor.

Out-competition dissociation kinetics experiments at 25ºC were carried out using SX18 and SX20 fluorescence stopped-flow spectrometer from Applied Photophysics for $k_{\text{obs}} > 0.03 \text{s}^{-1}$ or a Varian Cary Eclipse spectrophotometer for $k_{\text{obs}} < 0.03 \text{ s}^{-1}$. A pre-formed complex of the partner protein and the TAMRA labeled BH3 peptides was mixed with different concentrations of unlabeled PUMA peptide, used as a competitor in these experiments. A change in fluorescence upon dissociation of the TAMRA labeled peptides was monitored and fitted to a single exponential equation to determine the $k_{\text{obs}}$.

In some cases, the change in fluorescence signal upon dissociation of the TAMRA labeled peptides was very small to monitor, therefore a fluorescence polarization accessory was used to monitor the change in anisotropy instead. As a large excess of the competitor was used the $k_{\text{obs}}$ represents the dissociation rate ($k_{\text{off}}$).

**Acknowledgements**

The authors would like to thank Dr Joseph M. Rogers for use of data describing PUMA interacting with wild-type and mutants of MCL-1. We would also like to thank Dr Sarah L. Shammas for helpful discussions.

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References

BAK            PLEPNSILGQVRQLALIGDDINRRYDTEFQNLLE
BAX            QPPQDASTKKLSEALRRIGRIGDEDSMLQRLMADV
BID            SESQEEIIHNIARHLAQIGDEMNHIQPTLVRQLA
BIM            EPEDLRPEIRIAQELRIGDEFNETYTRVFANDY
BMF            QFLQHRAEVQIARLQAIADQFHRHLHTQHHQQRND
NOXA_A         NPTRAELPPEFAAQLRKIGDKVYATWSAPDITVVL
NOXA_B         GTRVPADLKDAAAQLRRLRIGDKVNLQRKLLNLESKL
PUMA           RVEEEWAREIGAQLRRAADDLNAQYERRRQEEQH

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**Figure A**

- $k_{\text{obs}}$ (s$^{-1}$)
- [wtMCL-1] (µM)

**Figure B**

- $k_{\text{obs}}$ (s$^{-1}$)
- [unlabelled competitor] µM
**A**

$k_{on}$ (μM$^{-1}$ s$^{-1}$)

wt MCL-1

**B**

$k_{off}$ (s$^{-1}$)

wt MCL-1

**C**

$K_d$ (M)

wt MCL-1
$k_{on}$

$V234A$  

$T247A$

$F_{old \ change}$

$V234A/wt$

$T247A/wt$

$K_{off}$

$V234A/wt$

$T247A/wt$

$K_d$

$V234A/wt$

$T247A/wt$
**Figure Legends**

**Fig. 1.** BH3 peptides have different sequences and intrinsic helicities but fold to the same structure. (A) Sequence alignments of eight BH3 peptides (PUMA, BID, BIM, BMF, NOXA A, NOXA B, BAK and BAX). For each BH3 peptides the sequences from *Mus musculus* were aligned using Clustal Omega. Absolutely conserved residues are indicated by *, residues where the character of the residue is conserved denoted by a period (.). (B) Percentage residual helicity calculated from mean residual ellipticity (MRE) data obtained from Circular Dichorism (CD), see Methods; is shown for the eight N-terminally TAMRA labeled BH3 peptides. The peptides show different degrees of disorder with BAX being least helical and BMF the most helical. (C) Structural alignment of available bound structures of MCL-1 (grey cartoon) with different BH3 peptides (colored cartoon; PUMA - dark blue (Protein Data Bank (PDB) entry: 2ROC\textsuperscript{11}, *Mus musculus*), NOXA A - red (PDB entry: 2ROD\textsuperscript{11}, *Mus musculus*), NOXA B - light green (PDB entry: 2JM6\textsuperscript{57}, *Mus musculus*), BIM – purple (PDB entry: 2PQK\textsuperscript{60}, *Homo sapiens*), BID- yellow (PDB entry: 2KBW\textsuperscript{61}, *Homo sapiens*), and BAX- dark green (PDB entry: 3PK1\textsuperscript{62}, *Homo sapiens*), built using PyMol. RMSD values calculated from PyMOL for each structural alignment are reported alongside.

**Fig. 2.** Kinetics data for interactions of BH3 peptides (PUMA, BID, BIM, BMF, NOXA A, NOXA B, BAX and BAK) with wild-type MCL-1. (A) Observed rate constants for BH3 peptides associating with wild-type MCL-1 under pseudo-first-order conditions with MCL-1 in excess. (B) Observed rate constants for dissociation of BH3 peptides from wild-type MCL-1 obtained by mixing with an excess of out-competing peptide. Colors of the data points represent each BH3 peptides as shown in the legend at the top of the Figure.
Data for kinetics of PUMA interacting with MCL-1 was obtained from Rogers et al. 2014\textsuperscript{18}. Note: unlike other BH3 peptides investigated in this study PUMA is not labeled with TAMRA or any other extrinsic dye\textsuperscript{18}.

**Fig. 3.** Representation of kinetic and thermodynamic parameters. (A) Association rate constant \( (k_{\text{on}}) \) (B) Dissociation rate constant \( (k_{\text{off}}) \) and (C) Equilibrium dissociation constant \( (K_d) \) for BH3 peptides binding to wt MCL-1. Larger differences in \( k_{\text{off}} \) than \( k_{\text{on}} \) are observed (note differences in scale of y-axes). The differences in \( K_d \) reflect the difference in \( k_{\text{off}} \). Colors for BH3 peptides as shown at the top of the figure. Data for PUMA are taken from Rogers et al. \textsuperscript{18}.

**Fig. 4.** Fold-change in thermodynamics and kinetic parameters for BH3 peptides binding to MCL-1 mutants (V234A, left column and T247A, right column). Top: Fold-change in association kinetics \( (k_{\text{on}}) \). Although most BH3 peptides bind slightly faster to mutant MCL-1, changes in \( k_{\text{on}} \) upon mutation of MCL-1 are <5-fold for all the BH3 peptides investigated. Middle: Fold-change in association kinetics \( (k_{\text{off}}) \) (Note different scale compared to top row). Change in \( k_{\text{off}} \) upon mutation of MCL-1 for all the BH3 peptides investigated are more significant and very different, with a maximum change of 40-fold. Bottom: Fold-change in Equilibrium constant \( (K_d) \). Changes in \( K_d \) upon mutation of MCL-1 for all the BH3 peptides investigated reflect changes in \( k_{\text{off}} \) with maximum changes of ~40-fold. The color for BH3 peptides as shown at the top of the figure. Data for PUMA are taken from Rogers et al. \textsuperscript{18}.

**Fig. 5.** Correlation between kinetic and thermodynamic parameters studied for all BH3 peptides interacting with wild-type and mutants of MCL-1. A.) \( k_{\text{off}} \) vs \( k_{\text{on}} \); no co-relation is observed between association \( (k_{\text{on}}) \) and dissociation kinetics \( (k_{\text{off}}) \). B) \( K_d \) vs \( k_{\text{on}} \); no co-
relation is observed between equilibrium constant ($K_d$) and association kinetics ($k_{on}$). C) $K_d$ vs $k_{off}$: a clear co-relation is observed between equilibrium constant ($K_d$) and dissociation kinetics ($k_{off}$). Note that the plots shown are in log scale.
**Tables**

**Table 1.** Kinetic and thermodynamic parameters of wild-type MCL-1 interacting with different BH3 peptides

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<th>BH3 peptides</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$) (x 10$^6$)</th>
<th>$k_{off}$ (s$^{-1}$) (x 10$^3$)</th>
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$K_d$ is calculated from kinetic rate constants ($K_d = k_{off}/k_{on}$). Errors reported in $k_{on}$ and $k_{off}$ are errors from the linear fit of observed rate constants. Errors for $K_d$ were propagated using standard equations. Data for PUMA was obtained from Rogers et al. 2014$^{18}$
Table 2. Kinetic and thermodynamic parameters of mutant forms of MCL-1 interacting with different BH3 peptides

<table>
<thead>
<tr>
<th>Protein</th>
<th>BH3 peptides</th>
<th>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{off}}$ (s$^{-1}$)</th>
<th>$K_d$ (M)</th>
<th>$\Delta \Delta G$ (kcal.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(x \times 10^6)$</td>
<td>$(x \times 10^{-3})$</td>
<td>$(x \times 10^{-9})$</td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td></td>
<td>10.1 ± 0.05</td>
<td>16 ± 0.04</td>
<td>1.6 ± 0.01</td>
<td>1.3 ± 0.03</td>
</tr>
<tr>
<td>BID</td>
<td></td>
<td>12.7 ± 0.6</td>
<td>13 ± 0.4</td>
<td>1.0 ± 0.06</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>NOXA A</td>
<td></td>
<td>4.2 ± 0.04</td>
<td>750 ± 30</td>
<td>179 ± 7</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>NOXA B</td>
<td></td>
<td>1.2 ± 0.04</td>
<td>0.37 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.31 ± 0.1</td>
</tr>
<tr>
<td>V234A MCL-1</td>
<td></td>
<td>1.8 ± 0.04</td>
<td>690 ± 10</td>
<td>383 ± 9</td>
<td>0.52 ± 0.08</td>
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<tr>
<td>BMF</td>
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<td>6.5 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>0.40 ± 0.02</td>
<td>1.6 ± 0.06</td>
</tr>
<tr>
<td>BIM</td>
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<td>4.0 ± 0.3</td>
<td>730 ± 60</td>
<td>183 ± 21</td>
<td>1.3 ± 0.07</td>
</tr>
<tr>
<td>BAX</td>
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<td>4.8 ± 0.1</td>
<td>0.18 ± 0.02</td>
<td>0.038 ± 0.004</td>
<td>-2.1 ± 0.08</td>
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<tr>
<td>BAK</td>
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<td>8.9 ± 0.6</td>
<td>5.3 ± 0.3</td>
<td>0.60 ± 0.05</td>
<td>0.71 ± 0.06</td>
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<tr>
<td></td>
<td></td>
<td>13.6 ± 0.3</td>
<td>3.8 ± 0.4</td>
<td>0.28 ± 0.03</td>
<td>-0.11 ± 0.07</td>
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<tr>
<td>NOXA A</td>
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<td>4.7 ± 0.1</td>
<td>67 ± 2</td>
<td>14.3 ± 0.5</td>
<td>0.21 ± 0.04</td>
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<tr>
<td>NOXA B</td>
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<td>1.4 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>-0.07 ± 0.10</td>
</tr>
<tr>
<td>MCL-1</td>
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<td>3.0 ± 0.03</td>
<td>580 ± 70</td>
<td>193 ± 21</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>T247A</td>
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<td>7.4 ± 0.5</td>
<td>0.084 ± 0.001</td>
<td>0.011 ± 0.0008</td>
<td>-0.49 ± 0.06</td>
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<tr>
<td>BMF</td>
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<td>4.5 ± 0.3</td>
<td>23 ± 1</td>
<td>5.1 ± 0.4</td>
<td>-0.87 ± 0.05</td>
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<tr>
<td>BIM</td>
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<td>7.1 ± 0.2</td>
<td>0.062 ± 0.001</td>
<td>0.008 ± 0.0002</td>
<td>-3.06 ± 0.04</td>
</tr>
</tbody>
</table>

$K_d$ is calculated from kinetic rate constants ($K_d = k_{\text{off}} / k_{\text{on}}$) and $\Delta \Delta G = RT \ln (K_d_{\text{mutant}} / K_d_{\text{wt}})$. Errors reported in $k_{\text{on}}$ and $k_{\text{off}}$ are errors from the linear fit of observed rate constants. Errors for $K_d$, $\Delta \Delta G$ were propagated using standard equations. Data for PUMA was obtained from Rogers et al. 2014$^{18}$. 
Supporting Information

Promiscuous and selective: how intrinsically disordered BH3 proteins interact with their pro-survival partner MCL-1.

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Supplementary Figures

**Fig. S1.** No correlation was observed between estimated helicity (%) calculated from CD experiments and predicted helicity (%) or predicted disorder (%) calculated from online tools. Left: Estimated helicity (%) calculated from CD¹ vs predicted helicity (%) using Agadir²⁻⁴ (black). Right: Estimated helicity (%) calculated from CD¹ vs predicted disorder (%) using online tools PONDR VSL²⁵⁻¹² (green), ESPript (X-Ray)¹³⁻¹⁷ (red), and ANCHOR¹⁸,¹⁹ (blue).
Fig. S2. Comparing association kinetics data for all BH3 peptides (PUMA – dark blue, BID – yellow, BIM – purple, BMF – black, NOXA A – red, NOXA B – light green, BAX – dark green and BAK – orange) interacting with wt and mutants of MCL-1. Observed rate constants for BH3 peptides associating with MCL-1 under pseudo-first-order conditions with MCL-1 in excess. Closed circles with dotted line fit represent data obtained for wild-type MCL-1. Open circles represent data obtained for V234A MCL-1 and closed squares represent data for T247A MCL-1. Compared to wt, faster association kinetics is observed for all BH3 peptides interacting with mutants of MCL-1 except for BAX. Data for kinetics of PUMA interacting with MCL-1 was obtained from Rogers et al. 2014.
Fig. S3. No correlation observed between percentage (%) residual helicity and kinetic and thermodynamic parameters studied for all BH3 peptides interacting with wild-type and mutants of MCL-1. Left: $k_{on}$ vs % residual helicity; Centre: $k_{off}$ vs % residual helicity; Right: $K_d$ vs % residual helicity.
Rate constants for association between wt MCL-1 and BH3 peptides correlate with peptide charge. Left: $k_{on}$ vs estimated charge, the straight line fit includes data for all BH3 peptides; Right: $k_{on}$ vs estimated charge, the straight line fit includes data points for all BH3 peptides except BAK (shown in orange) which seems to be an outlier. The charge on each peptide was calculated using pK$_a$ values reported for each amino acid for model peptides and corrected for the addition of TAMRA dye at the N-terminus$^{21}$. 

Fig. S4.
Fig. S5. Contact map for interactions between six BH3 peptides and MCL1. The contacts with MCL-1 for PUMA (blue) was obtained using PDB structure 2ROC\textsuperscript{22}, for NOXA A (red) 2ROD\textsuperscript{22}, for NOXA B (light green) 2JM6\textsuperscript{23}, for BIM (purple) 2PQK\textsuperscript{24}, for BID (yellow) 2KBW\textsuperscript{25} and for BAX (dark green) 3PK1\textsuperscript{26}. Contacts between BH3 peptide and MCL-1 was defined between any non-hydrogen atom within 4Å distance. Note that three of these are for Murine peptides and proteins (2ROC, 2ROD, 2JM6) and three for human complexes (2PQK, 2KBW, 3PK1).
References


