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Letters to the Editor

Crystal structure of ABT-737 complexed with Bcl-x_L: implications for selectivity of antagonists of the Bcl-2 family

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Dear Editor

Many cancers possess defects in their apoptotic machinery that provide them with a survival advantage.[1] Hence, one strategy for therapeutic intervention is to develop molecules that activate the cell death pathways. The Bcl-2 pro-survival proteins (e.g. Bcl-2, Bcl-x_L, Mcl-1) are attractive drug targets as their overexpression is observed in many tumours and contributes to chemo- and radio therapy resistance. Although there is controversy over how pro-survival Bcl-2 proteins function,[2,3] it is generally agreed that apoptosis is initiated by the binding of the pro-death BH3-only proteins to the pro-survival molecules. These interactions are mediated by the BH3 domain of the BH3-only proteins (Supplementary Figure 1) inserting into a hydrophobic groove on the surface of pro-survival proteins. Structural analyses of BH3 domain: pro-survival protein complexes show that four conserved hydrophobic residues (h1–h4) on one face of a helix insert into hydrophobic pockets (p1–p4) within the hydrophobic groove, while an aspartate makes a salt bridge with a conserved arginyl residue on the pro-survival molecules.[4–6] It has also been shown that BH3 domains possess selectivity for different pro-survival proteins,[7] and this has important implications for whether apoptosis occurs.[8] For example, both Bcl-x_L and Mcl-1 must be neutralized for Bak-mediated apoptosis in some cell types.[8]

Molecules that mimic the BH3 domains of BH3-only proteins have potential as anti-cancer therapeutics.[9,10] A promising candidate is ABT-737.[11] It binds to Bcl-x_L, Bcl-2 and Bcl-w with high affinity, but is unable to engage Mcl-1.[7,11] Accordingly, it is a poor killer of most cells, except those that have reduced Mcl-1 levels, or in which Mcl-1 degradation has been induced.[12–14] Because of its restricted binding profile, the range of cancers against which ABT-737 would probably be effective as a single agent is limited. Interestingly, none of the small molecule BH3 mimetics described to date bind to Mcl-1 with significant affinity (i.e. $K_D < 1\ \mu M$).[10]

Several NMR structures of Bcl-x_L bound to organic ligands have been reported,[11,15] but no X-ray structures, nor any structure for ABT-737, have been published. Information on the exact molecular pose of ABT-737 in Bcl-x_L may help explain why it cannot bind the related target Mcl-1. The Bim BH3 peptide is able to bind all pro-survival proteins equally well.[7] In contrast, Noxa BH3 is selective for Mcl-1 while Bad BH3, like ABT-737, is selective for Bcl-x_L, Bcl-2 and Bcl-w.[7] The structures of Mcl-1 bound to both Bim and Noxa BH3 domains highlighted significant differences in Mcl-1 compared to Bcl-x_L.[17] Does ABT-737 mimic features of the Bad BH3, or is its failure to bind Mcl-1 a property of other differences in the structures of Bcl-x_L and Mcl-1?

To address this, we describe here a 2.2 Å resolution crystal structure of ABT-737 bound to hBcl-x_L (PDB entry: 2YXJ). Based on insights from this, we performed saturation mutagenesis of the second and fourth hydrophobic residues (h2 and h4) in Bim BH3 (i.e. the residues that are primarily mimicked by ABT-737) to understand better the target selectivity of ABT-737.

Crystallographic details are in Supplementary Table 1. The chloro-biphenyl and thio-phenyl moieties at either end of ABT-737 (Supplementary Figure 2) engage the p2 and p4 pockets on Bcl-x_L (Figure 1a) occupied by hydrophobic residues h2 and h4 respectively when BH3 peptides bind (Figure 1a; Supplementary Figure 1). As illustrated in Figure 1a (right), the chloro-biphenyl penetrates the p2 pocket more deeply than the BH3 leucyl (h2) residue, necessitating a further opening-up of the binding groove beyond that required to accommodate BH3 peptidic ligands. A similar observation was reported in an NMR study of a related compound.[15] The acylsulphonamide of ABT-737 is far from, and does not form hydrogen bonds with Arg139 of the BH1 domain on Bcl-x_L, and thus is not a mimic of the conserved aspartyl residue present in all BH3 domains. Instead, this moiety forms a long (3.1 Å) hydrogen bond to the backbone amide of Gly138 of Bcl-x_L. The only other hydrogen bond present in the complex is between Glu96 on Bcl-x_L and the 2-dimethylaminoethyl group of ABT-737. This hydrogen bond is formed in only one of the two complexes in the crystallographic asymmetric unit.

The observation that the p2 and p4 pockets largely determine interactions with ABT-737 prompted us to investigate the role of these pockets in binding the BH3 domain of a BH3-only protein, to gain insight into ABT-737 binding selectivity. We used phage display to express Bim BH3, which binds all pro-survival molecules, and performed saturation mutagenesis at the residues at positions h2 and h4. Each position was mutated to all other amino acids except proline and cysteine. The binding affinities of all mutants for four of the five pro-survival proteins were then determined by competition ELISA.

The effects of amino-acid substitutions at the h2 position are shown in Figure 1b and c, and Supplementary Figure 3. The structural similarity of Bcl-x_L, Bcl-w and, to a lesser extent, Bcl-2 is evident from the mutant binding profiles. In contrast,

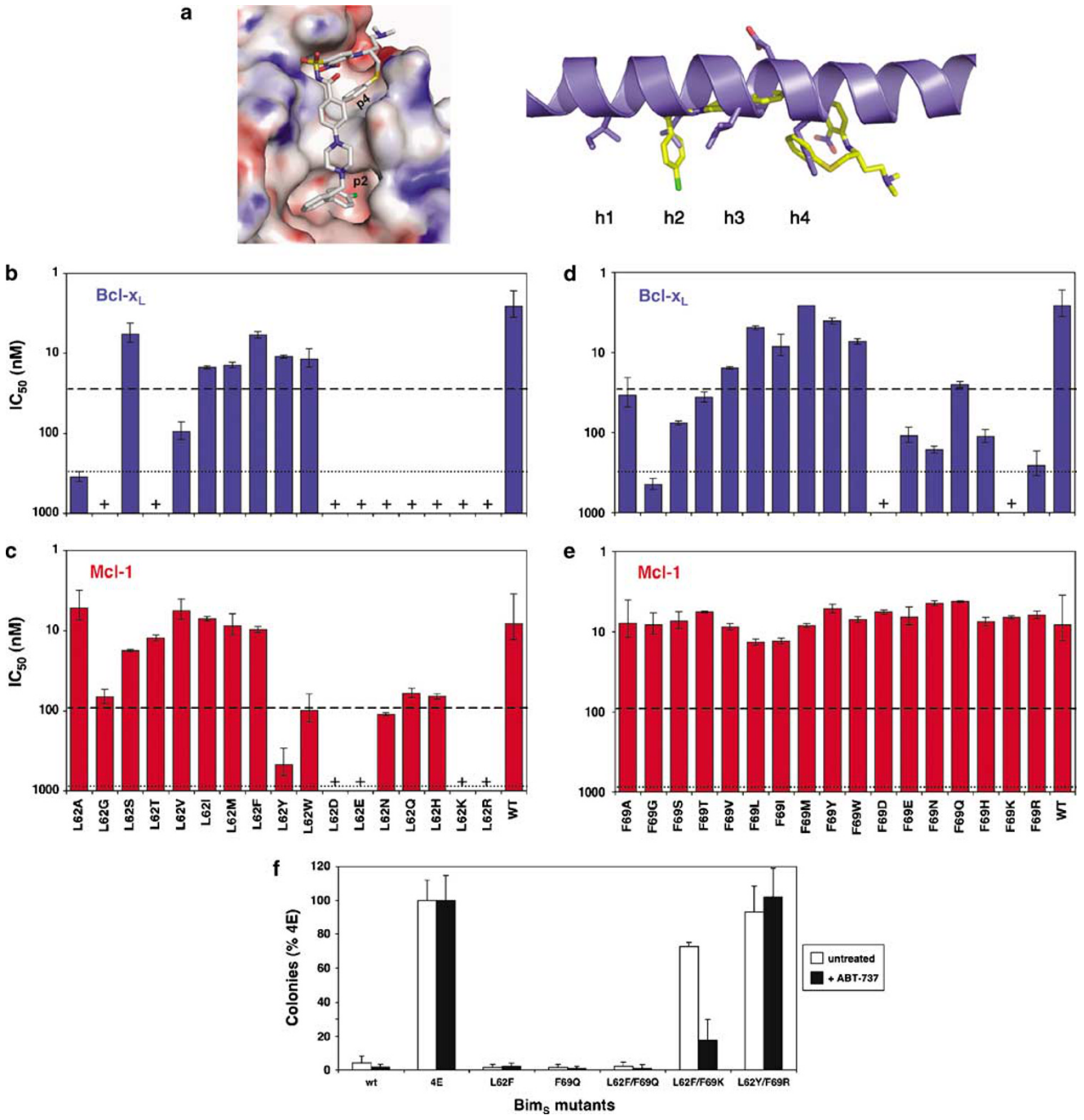

Figure 1 Crystal structure of ABT-737 in complex with Bcl-x_L and comparison with Bim BH3. (**a**) Structure of the Bcl-x_L:ABT-737 complex (right) showing the binding pockets (p2 and p4) for the chloro-biphenyl and thio-phenyl groups of ABT-737. Surface is colour-coded: blue, positive potential (15 RT); red, negative potential (−15 RT); white, zero potential. Overlay of ABT-737 and Bim BH3 (left) following alignment of Bcl-x_L from the complexes of hBcl-x_L:ABT-737 (chain A) and mBcl-x_L:Bim BH3 (1PQ1) (RMSD = 0.855). The resultant overlay of ABT-737 (yellow) on the Bim BH3 peptide (blue) is displayed. The relative locations of the four conserved hydrophobic residues of Bim are indicated h1–h4. The side chain for the conserved aspartate, Asp 99, of Bim is also displayed. (**b**–**e**) The IC_{50} values for indicated Bim BH3 mutants binding to (**b** and **d**) Bcl-x_L and (**c** and **e**) Mcl-1 were measured in solution competition assays as described.[16] The dashed line on each panel indicates an IC_{50} 10-fold weaker than the wild-type Bim BH3 while the dotted line indicates an IC_{50} 100-fold weaker. Crosses indicate interactions too weak to detect in phage-binding experiments, hence no IC_{50} was measured. WT = wild-type Bim BH3. Error bars indicate the S.D. from the mean of $n = 2$ experiments. (**f**) Killing of mouse embryonic fibroblasts by non-canonical BH3 mutants. Mutants in which h2 (L62) or h4 (F69) position, or both h2 + h4 have been substituted for highly non-canonical residues still kill potently (L62F, F69Q, L62F/F69Q). However, the L62F/F69K mutant only kills in the presence of ABT-737, consistent with its selectivity for Mcl-1, as predicted by the binding data (Supplementary Figure 3). Similarly, L62Y/F69R is unable to kill, as predicted by binding data showing that both mutations (L62Y and F69R) have significant effects on binding to all pro-survival proteins

Mcl-1-binding is less sensitive to substitutions at the h2 position: only the four charged amino acids and tyrosine at position h2 in the Bim sequence cause more than a 10-fold loss of binding. The large effect of the tyrosine substitution is particularly remarkable as replacement with phenylalanine, which differs only by a hydroxyl group, is very well tolerated. Hence, very subtle changes in the ligand have major consequences on binding, indicating that similar subtle changes in ABT-737 may alter its selectivity profile. The data, therefore, suggest that the Mcl-1 p2 pocket is significantly different from its counterparts on the other three family members. The structure of the Mcl-1:Bim BH3 complex[17] reveals that the protein backbone fold on the side of this pocket formed by the helix α-3 is very much different in Mcl-1 compared to Bcl-x_L. This difference leads to different landscapes for the molecular surface at p2 (and p1) in the two proteins.[17] Furthermore, the plasticity of the Bcl-x_L p2 pocket, which appears to be important for binding both BH3 ligands and ABT-737, may well be related to the different fold of its α-3 compared to its counterpart in Mcl-1.

Compared to the binding profiles of the h2 mutants, all pro-survival proteins were more tolerant of substitutions of the phenylalanine residue at the h4 position of Bim BH3 (Figure 1d and e; Supplementary Figure 3). This reflects the larger range of residues normally found at this position on all BH3 domain ligands (Supplementary Figure 1). Again Mcl-1 is an outlier, and binding to it is essentially unaffected by any side-chain replacements in the context of Bim BH3 (Figure 1e). Hence, the p4 pocket in Mcl-1 is probably not a critical determinant for Bim BH3 domain binding. The Mcl-1:Bim BH3 structure[17] reveals that the p4 pocket is relatively open and exposed to solvent in comparison to that seen in previously published Bcl-x_L:BH3 complexes.[4–6] Thus the p4 pocket in Mcl-1 may not be an effective anchor point for the thio-phenyl moiety of ABT-737 that is deeply buried in the equivalent pocket in the complex with Bcl-x_L.

Importantly, killing assays with various mutants reflect the binding data (Figure 1f). Full-length Bim proteins with non-canonical BH3 sequences such as Bim_SL62F or Bim_SF69Q (where the h2 and h4 residues have been mutated to phenylalanine and glutamine, respectively) are still potent killers, as predicted from our binding data, as these mutants retain high-affinity binding to all pro-survival proteins. Furthermore, mutants with a more selective binding profile, for example, Bim_SL62F/F69K which is predicted to only bind Mcl-1 with high affinity, kill in a more restricted manner, that is, only when combined with ABT-737 to neutralize Bcl-x_L. This significant tolerance for a range of non-canonical amino-acid residues at both the p2- and p4-binding sites displayed by pro-survival proteins suggests that high-affinity protein ligands for them may have been overlooked in database searches based on the canonical BH3 motif. This would particularly apply to searches where the leucine at the h2 position has been fixed owing to its absolute conservation between BH3 domains.

Therefore, with respect to binding both these mutant Bim BH3 peptides and ABT-737, Mcl-1 is an outlier among pro-survival proteins. Structural differences that distinguish Mcl-1 from Bcl-x_L, Bcl-w and Bcl-2 are manifest in the binding selectivity to both organic ligands[10] and to peptides.[7] Furthermore, ABT-737 does not seem to exploit unique features of the Bad BH3 domain to achieve its binding selectivity, because the p1 pocket, where the Bad h1 tyrosyl residue docks,[5] is not engaged by ABT-737. Thus ABT-737 is a functional but not a structural mimic of the Bad BH3 domain.

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