Structures of BCL-2 in complex with venetoclax reveal the molecular basis of resistance mutations

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Venetoclax is a first-in-class cancer therapy that interacts with the cellular apoptotic machinery promoting apoptosis. Treatment of patients suffering chronic lymphocytic leukaemia with this BCL-2 antagonist has revealed emergence of a drug-selected BCL-2 mutation (G101V) in some patients failing therapy. To understand the molecular basis of this acquired resistance we describe the crystal structures of venetoclax bound to both BCL-2 and the G101V mutant. The pose of venetoclax in its binding site on BCL-2 reveals small but unexpected differences as compared to published structures of complexes with venetoclax analogues. The G101V mutant complex structure and mutant binding assays reveal that resistance is acquired by a knock-on effect of V101 on an adjacent residue, E152, with venetoclax binding restored by a E152A mutation. This provides a framework for considering analogues of venetoclax that might be effective in combating this mutation.

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The connection between aberrant cell death and cancer sparked a detailed understanding of the mitochondrial pathway to apoptosis and led to the first BCL-2 antagonist approved for cancer therapy, venetoclax. BCL-2 is the founding member of the pro-survival class of proteins that includes BCL-X, BCL-W, A1/BFL-1 and MCL-1. They exert their pro-survival function by binding and restraining related members of a family of pro-apoptotic proteins—the sensors of cellular stress (the BH3-only proteins) and the effectors of apoptosis (BAX and BAK). This restraint is exerted through interactions between the helical BCL-2 Homology 3 (BH3) motifs of pro-apoptotic molecules and a cognate groove on the surface of pro-survival proteins. Four or more hydrophobic amino acids on successive helical turns within the BH3 motif engage complementary pockets (P1 through P4) in this surface groove of pro-survival proteins. Blocking this interaction by targeting the binding groove with organic ligands has long held promise for cancer therapy, and venetoclax is the first realisation of that promise.

The first bona fide BH3 mimetics, ABT-737 and ABT-263 targeted multiple pro-survival family members BCL-2, BCL-X, and BCL-W, engaging with the P2 and P4 pockets in their BH3-binding groove. Treatment with these compounds results in thrombocytopenia, an on-target toxicity due to BCL-X inhibition, which limits its use as effective chemotherapeutics. This led to a revised strategy of selectively targeting BCL-2 that resulted in ABT-199 (venetoclax). Venetoclax spares platelets but retains the ability to promote apoptosis in malignant cells dependent on BCL-2. It is among the first approved small molecule cancer therapeutics that directly blocks a protein-protein interaction. Other BH3 mimetics are in development, including another BCL-2 selective compound, S55746.

Mutation of drug-binding sites is a common mechanism by which malignant cells evade therapies, as typified by resistance to the ABL1 tyrosine kinase inhibitors. Resistance to some tyrosine kinase inhibitors can be conferred through mutation of threonine 315 to isoleucine (T315I). Rational drug design strategies have been successful in developing approved therapeutics that are effective in cases with the T315I mutation. Venetoclax was approved in 2016 and as a consequence relatively few patients have been treated to date and there is a limited understanding of potential resistance mechanisms. To predict potential resistance mutants a mouse model was used to induce venetoclax tolerance in cancer cells. This study identified that mutation of phenylalanine 104 (human numbering) to either leucine (F104L) or cysteine (F104C), located within BCL-2’s BH3-binding groove, rendered the lymphoma cell line resistant to venetoclax. Subsequent work confirmed that these mutations can also confer resistance in models of human leukaemia and lymphoma, but have not yet been observed in patients. Recently a novel BCL-2 mutation was described exclusively in patients undergoing treatment with venetoclax. This mutant, G101V, was found in chronic lymphocytic leukaemia (CLL) patients from the clinical trials who had initially responded to treatment but developed CLL-type clinical progression after 19–42 months. Tellingly, the presence of the mutation in patient samples was predictive of clinical progression. The BCL-2 G101V mutation reduces the affinity for the drug to BCL-2 by some 180-fold. On the other hand BCL-2 G101V maintains affinity for the BH3 motif of pro-apoptotic proteins, such as BAX and BIM, and thus can still function to suppress apoptosis. By selectively reducing affinity to venetoclax the BCL-2 G101V mutation provides resistance to the therapy. To understand how the G101V and other resistance mutations can selectively decrease the affinity for venetoclax whilst retaining anti-apoptotic activity we searched for a structural rationalisation.

The first disclosure of venetoclax (ABT-199) in 2013 included several structures of compounds bound to BCL-2, including the structure of ABT-263 (PDB:4LVT) and an analogue of ABT-199 (PDB:4MAN), but no structure of venetoclax itself with BCL-2 has yet been published.

Here we describe crystal structures of venetoclax bound to wild type BCL-2 and the BCL-2 mutants G101V and F104L. We also characterise the binding profiles of the drug to various BCL-2 mutants by surface plasmon resonance. Through these analyses we reveal the molecular mechanisms by which these mutants compromise drug-binding and, in the case of G101V, enable disease progression. These structures pave the way for rational optimisation of the venetoclax scaffold to counter this BCL-2 mutation.

**Results**

Crystal structure of BCL-2 bound to venetoclax. Crystal structures of BCL-2 with ABT-263 and various analogues of venetoclax have been deposited in the PDB and described in the literature (Fig. 1a, b). One of those analogues is 4-[(4’-chloro-3-[(2-[1H-indol-5-yloxy])biphenyl-2-yl]methyl)piperazin-1-yl]-2-(1H-indol-5-yl)-N-[(3-nitro-4-[(tetrahydro-2H-pyran-4-yl)methyl]amino)phenyl]sulfonamido benzamide, hereafter referred to as compound 1. We obtained crystals of BCL-2 in complex with venetoclax that diffracted to high resolution (1.62 Å) in the space group P2₁2₁2₁ with one molecule in the asymmetric unit (Fig. 1a, Table 1). The electron density for the drug was well defined (Supplementary Fig. 1a, b) and the binding pose was in general agreement with the published structures of ABT-263 and compound 1, with the 4-chlorophenyl (CP) bound in the BCL-2 P2 pocket, the piperazine bridging the P2 and P4 pockets over residue F104 and the azaindole substitution bound in the BCL-2 P4 pocket. It was possible to model two distinct conformations with the 4-4-dimethylcyclohex-1-ene (4DM) ring flipping at the 4 and 5 positions above the BCL-2 P2 pocket and the benzamide (BA) acyl oxygen adopting two conformations above the BCL-2 P4 pocket (Fig. 1b). An interesting difference was the positioning of the venetoclax 4DM moiety, which was further from the α helix and more central over the P2 pocket than the equivalent rings from ABT-263 and 4MAN compounds (Fig. 1c, d, Supplementary Fig. 2, Supplementary Table 1). This small deviation in the positioning of the 4DM was unexpected as the equivalent six membered ring systems from ABT-263 and compound 1 are similar, differing only by the positioning of the gem-dimethyl group in ABT-263 (5 position) and the lack of a methyl and a phenyl ring in compound 1 (Fig. 1a).

Structures of BCL-2 mutants bound to venetoclax. To understand how these BCL-2 mutations compromise drug binding we solved crystal structures of both complexes (Table 1 and Fig. 2). The G101V mutation resides on the BCL-2 α2 helix packing against the α3 helix and is within the BCL-2 BH3 motif. The glycine is a conserved, defining feature of the motif. Adjacent residues A100 and D103 define the boundaries of the P4 pocket so the mutation was expected to alter drug binding by changes to this region (Fig. 2a). The BCL-2 G101V:venetoclax complex crystallised in P2₁ spacegroup with two molecules in the asymmetric unit diffracting to 2.2 Å, with well-defined electron density for both copies of the drug (Supplementary Fig. 1). The overall structure of BCL-2 G101V was similar to WT with no significant deviations in α2–α5 core helices or α6–α8 (Fig. 2b, c). There was a minor change in orientation of the α1 helix resulting in a ~1 Å deviation at either end of the helix, but this was far from the drug binding site. The binding pose of venetoclax is conserved between WT and the G101V mutant (Fig. 2b). The P2 pocket volume was...
maintained at 478 Å³ (480 Å³ for WT) as was the volume of the P4 pocket at 379 Å³ (380 Å³ for WT). Interestingly, the G101V mutation did not alter the positioning of the α2 helix relative to α5 or impact the residues defining the P4 pocket. Instead the bulk of the valine sidechain was accommodated by a deviation of the sidechains of Y18 on α1 and E152 on α5 (Fig. 2c). In the BCL-2 G101V:venetoclax structure E152 had a 60° rotamer change relative to WT (mm-40 for WT to tp10 for G101V), placing the sidechain Cγ in van der Waal’s contact with the chlorine atom of the venetoclax chlorophenyl moiety. This conformational change in E152 in the BCL-2 G101V structure causes a small repositioning of the venetoclax 4DM and chlorophenyl moieties in the P2 pocket, moving on average 0.25 Å closer to L137 in the α4 helix (Fig. 2b, Supplementary Fig. 2, Supplementary Table 1), i.e. more similar to the BCL-2 WT complexes with ABT-263 and compound 1. Additionally, we obtained a structure of BCL-2 G101A bound to venetoclax (Table 1), representing a milder introduction of bulk at the G101 position than the valine substitution. The BCL-2 G101A:venetoclax and BCL-2 WT:venetoclax crystals were isomorphous and there were no significant deviations in venetoclax positioning or E152 (Supplementary Fig. 2, 3, Supplementary Table 1). Despite the closer proximity of the azaindole moiety to the mutation site, its orientation in the P4 pocket was conserved between BCL-2 WT and G101V structures. Therefore, the G101V mutation appears to modulate venetoclax affinity more through its interactions with the P2 pocket than the P4 pocket.

The crystals of venetoclax complexed with BCL-2 F104L and BCL-2 WT are isomorphous (Table 1). Well-defined electron density for the drug in the mutant complex structure (Supplementary Fig. 1) suggests two conformations for the 4DM and acyl group of the BA moiety as in WT (Fig. 2d). The side chain of F104 separates the P2 and P4 pockets of BCL-2. The P4 pocket volume was maintained between WT and F104L structures (P4 pocket volume 380 Å³ for both WT and F104L). In contrast the volume of the P2 pocket increased with the F104L mutation as the leucine sidechain occupies a smaller volume than phenylalanine (Fig. 2d–f). In this structure two conformers for F112 on α3 are evident, one like WT and a second occupying some of the volume vacated by the F104L mutation. (Fig. 2c, e and f). The new F112 conformation extends into the P2 pocket, packing against L104. This inserted F112 conformation compensates for the loss of P2 pocket volume in the BCL-2 F104L mutant and is comparable to the BCL-2 WT P2 pocket volume—P2 pocket volumes of 480 Å³ (BCL-2 WT), 475 Å³ (F104L inserted conformation) and 596 Å³ (F104L conserved conformation). The occupancy of F112 refined to 0.48 for the conserved conformation and 0.52 for inserted conformation, indicating that the compensation in P2 pocket volume only occurs 50% of the time and the vacated space is unfavoured. The consequence of the F104L mutation is to alter the packing environment of the chlorophenyl moiety of the drug.

**Binding of BH3 peptides and venetoclax to BCL-2 mutants.** SPR experiments were performed using a BIMBH3 or BAXBH3 immobilised sensor surface with BCL-2 mutants as the analyte and determining venetoclax affinity by competition experiments17,25, (Fig. 3, Table 2 and Supplementary Fig. 4, 5). We have previously reported BIMBH3 and BAXBH3 affinities for WT, G101V and F104L17, and these were comparable to F104C, with less than 10-fold change relative to WT (Table 2). In contrast, the affinities for venetoclax differed by 25 to ~1500-fold with Kᵢ values 0.018, 3.2, 0.46 and 25 nM for WT, G101V, F104L and F104C, respectively (Table 2). This indicates that the BCL-2 mutants maintain tight binding to BH3 domains, allowing their overexpression to prevent apoptosis, whilst selectively reducing the affinity for the drug and thus providing resistance to therapy.

**The role of E152 in venetoclax affinity.** E152 moved into the base of the P2 pocket in the BCL-2 G101V:venetoclax structure (Fig. 2b, c). To test the role of E152 in reducing affinity we generated a BCL-2 G101V/E152A double mutant. Alanine does not have a Cγ or Cδ to impact the base of the P2 pocket and would allow the chlorophenyl to insert unimpeded into the P2 pocket in the G101V mutant. We repeated SPR experiments with the BCL-2 G101V/E152A double mutant and a BCL-2 E152A single mutant (Table 2 and Fig. 3c, d). The E152A single mutant had comparable binding to WT and when combined with G101V
KI competition SPR experiments become less accurate as the ligand venetoclax that binds principally to the P2 and P4 pockets that E152A mutation rescues high affinity from G101V (Table 2 and Supplementary Fig. 4). This indicates BIMBH3 and BAXBH3 was largely unaltered compared to WT revealed binding to the P1, P2 and P3 pockets in contrast to disclosed crystal structure of BCL-2 WT bound to S55746 selective antagonist that has progressed to the clinic. The recently disclosed binding to the P1, P2 and P3 pockets of WT conformation, not the G101V. The G101A mutant bound to formation in a BCL-2 G101A:venetoclax structure matched the rotamer change observed in the G101V mutant. Note also that an increase in affinity was 10-fold higher than WT, however LC50 concentrations were determined as 0.32 ± 0.15 μM with the G101V mutation. To further investigate this we solved the structure of BCL-2 G101V/E152A and BCL-2 E152A for interactions with BH3 domains, which typically display a significantly tighter or weaker than the affinity venetoclax binding, with WT binding at 18 pM, BCL-2 E152A at 27 pM and BCL-2 G101V/E152A affinity was 10-fold higher than WT, however competition SPR experiments become less accurate as the ligand Kᵢ becomes significantly tighter or weaker than the Kᵢ for the competing BimBH3 peptide; as such it is unclear whether this increase in affinity is significant. Furthermore, the E152 conformation in a BCL-2 G101A:venetoclax structure matched the WT conformation, not the G101V. The G101A mutant bound to venetoclax with a Kᵢ of 110 pM comparable to WT but distinct from G101V (Table 2 and Supplementary Fig. 4). This indicates that E152A mutation rescues high affinity for venetoclax when combined with G101V and confirms the importance of the E152 rotamer change observed in the G101V mutant. Note also that the affinity of BCL-2 G101V/E152A and BCL-2 E152A for BIMBH3 and BAXBH3 was largely unaltered compared to WT (Table 2 and Supplementary Fig. 3).

### Table 1 Crystal structure data collection and refinement statistics

| PDB id | BCL-2 WT: Ven | BCL-2 G101V: Ven | BCL-2 G101A: Ven | BCL-2 F104L: Ven | BCL-2 G101V: S55746 |
|--------|---------------|------------------|------------------|------------------|---------------------|
| PDB id | 600K          | 600L             | 600P             | 600M             | 6000                |
| Wavelength (Å) | 0.9537    | 0.9537           | 0.9537           | 0.9537           | 0.9537              |
| Resolution range (Å) | 42.4-1.62  | 47.51-2.2 (2.28-2.20) | 43.24-1.8 (1.86-1.80) | 43.9-1.75 (1.81-1.75) | 43.24-1.8 (1.86-1.80) |
| Space group | P2,2,1 | P2₁ | P2₁,2,1 | P2₁,2,1 | P2₁,2,1 |
| Unit cell (Å, °) | 33.73, 80.11, 87.32 | 33.15, 82.01, 47.51, 90 | 33.40, 48.95, 86.47 | 33.69, 48.27, 87.80 | 37.68, 68.58, 64.49 |
| Total reflections | 128406 (8348) | 49434 (4673) | 82474 (5028) | 87816 (6285) | 148201 (14601) |
| Unique reflections | 18474 (1508) | 12699 (1270) | 13559 (1200) | 15022 (1427) | 22155 (468) |
| Multiplicity | 7.0 (5.5) | 3.8 (3.7) | 6.1 (4.2) | 5.8 (4.4) | 6.7 (6.6) |
| Completeness (%) | 97.77 (80.73) | 98.84 (99.53) | 98.45 (86.85) | 99.72 (97.25) | 74.31 (21.24) |
| Mean I/σ(I) | 12.57 (2.16) | 7.60 (1.56) | 9.56 (0.82) | 10.58 (1.58) | 7.29 (0.90) |
| Wilson B-factor (Å²) | 15.5 | 36.24 | 30.55 | 21.39 | 33.19 |
| R-meas | 0.1054 (0.9667) | 0.1432 (0.9676) | 0.1129 (2.024) | 0.1154 (1.119) | 0.148 (2.474) |
| R-free | 0.03963 (0.3948) | 0.07165 (0.4947) | 0.04476 (0.9436) | 0.04728 (0.5198) | 0.05719 (0.9544) |
| CC₁₀₀ | 0.998 (0.604) | 0.991 (0.646) | 0.997 (0.292) | 0.996 (0.436) | 0.997 (0.596) |
| Reflections used in refinement | 18462 (1500) | 12924 (1266) | 13525 (1176) | 15011 (1417) | 16499 (468) |
| Reflections used for R-free | 905 (69) | 628 (60) | 672 (56) | 743 (69) | 816 (19) |
| R-work | 0.1633 (0.2301) | 0.1952 (0.2530) | 0.1929 (0.3681) | 0.1732 (0.2908) | 0.2167 (0.2937) |
| R-free | 0.2013 (0.2848) | 0.2278 (0.2963) | 0.2314 (0.4223) | 0.2181 (0.3273) | 0.2666 (0.3437) |
| Number of non-hydrogen atoms | 1512 | 2502 | 1313 | 1511 | 2409 |
| Macromolecules | 1264 | 2328 | 1195 | 1275 | 2288 |
| Ligands | 150 | 152 | 82 | 150 | 106 |
| Solvent | 98 | 22 | 36 | 86 | 15 |
| Protein residues | 141 | 279 | 141 | 141 | 273 |
| RMS(bonds) | 0.018 | 0.003 | 0.003 | 0.004 | 0.005 |
| RMS(angles) | 1.41 | 0.59 | 0.57 | 0.72 | 0.91 |
| Ramachandran favored (%) | 99.27 | 99.26 | 100 | 100 | 98.49 |
| Ramachandran outliers (%) | 0 | 0 | 0 | 0 | 0 |
| Rotamer outliers (%) | 0.75 | 0.41 | 0 | 0.75 | 1.26 |
| Clashscore | 5.67 | 8.05 | 4.94 | 7.1 | 4.39 |
| Average B-factor (Å²) | 22.23 | 50.02 | 39.44 | 25.35 | 50.2 |
| Macromolecules | 21.2 | 50.34 | 39.44 | 24.28 | 49.9 |
| Ligands | 22.4 | 46.14 | 38.12 | 28.02 | 58.21 |

Statistics for the highest-resolution shell are shown in parenthesis

as a double mutation restored high affinity venetoclax binding, with WT binding at 18 pM, BCL-2 E152A at 27 pM and BCL-2 G101V/E152A at 2 pM (Table 2 and Fig. 3c, d). The BCL-2 G101V/E152A affinity was 10-fold higher than WT, however Kᵢ was overexpressed in the KMS-12-PE cells and S55746 complexPDB ID 6GL8(Fig. 4b, c)12. The P2 pocket is key agreement with the published structure of the BCL-2 WT: Ven BCL-2 G101V: Ven BCL-2 G101V: S55746 (Table 1). We obtained diffraction to 2.0 Å in a P₂₁ spacegroup with two molecules in the asymmetric unit. The BCL-2 G101V:S55746 structure was in general agreement with the published structure of the BCL-2 WT: S55746 complex PDB ID 6GL8 (Fig. 4b, c)12. The P2 pocket is key for interactions with BH3 domains, which typically display a leucine residue engaging this pocket. S55746 inserts a 4-hydroxyphenyl moiety into the P2 pocket similar to the chlorophenyl of venetoclax. In that case the chlorophenyl inserts deeply into the P2 pocket causing F112 to change rotamer (venetoclax rotamer t80, S55746 rotamer m-85) and exposing chlorine to the base of the P2 pocket (Fig. 4d). In the S55746 structure F112 seals the P2 pocket shielding the S55746 hydroxyphenyl from E152 (Fig. 4d). In the BCL-2 WT:S55746 structure E152 is
Currently venetoclax is approved for treatment of patients with previously treated chronic lymphocytic leukemia. Venetoclax selectively inhibits BCL-2, thereby promoting apoptosis in cells refractory to conventional apoptotic cues. No crystal structure for BCL-2 binding to venetoclax has been described, although analogues have been published. We have now crystallised and determined the structure of venetoclax bound to BCL-2 at high resolution. The structure reveals subtle differences between the binding of the drug relative to the published analogues (Fig. 1), which would not have been predicted based on the previous structures. Notably, the orientation of the moiety inserted into the P2 pocket is subtly different in venetoclax compared to ABT-263 and compound 1 structures. The P2 pocket is an important determinant in selectivity of BH3 peptides and BH3 mimetics, and here it emerges as the critical feature conferring resistance to a drug-selected BCL-2 mutant, G101V.

The BCL-2 G101V mutation was exclusively identified in CLL patients with disease progression on venetoclax clinical trials, but only after many months of continuous treatment. Early detection of BCL-2 G101V by highly sensitive ddPCR assays predicted subsequent clinical disease progression. The mutation reduces the affinity of the drug for BCL-2 by ~180-fold. In contrast, it only moderately reduces the affinity for BH3 peptides, allowing the mutant to still function normally as a pro-survival protein. Interestingly, G101 is part of the BCL-2 BH3 sequence. The BH3 motifs of BH3-only proteins or of BAX and BAK are ligands for a binding groove on pro-survival BCL-2 proteins, but the role of the BH3 motif in pro-survival proteins is unclear. Notably, the motif consensus sequence of φ1-x-x-x-φ2-x-x-φ3-G-D-x-φ4, where φ1-4 are hydrophobic amino acids, includes the largely conserved G101 of BCL-2. There are examples of BCL-2 family proteins that have either alanine or serine instead of glycine at this position, but not valine. In the multi-BH domain BCL-2 proteins the BH3 motif is in the α2 helix, with the small glycine packing against the BH1 domain in the α5 helix. The G101V mutation is adjacent to but not directly part of the P4 pocket that engages a leucine residue (φ4) when the BAX BH3 motif binds (Fig. 5). We could not obtain crystals for BCL-2 G101V bound to BAXBH3 peptide so cannot comment on how the mutation affects BAXBH3 interactions in this region. The azaindole ring of venetoclax, one of the key features of its selectivity for BCL-2, occupies this pocket. However, there were no differences in the P4 pocket with venetoclax bound to BCL-2 or BCL-2 G101V. Instead the additional bulk from the valine sidechain is accommodated by movements in the positions of Y18 (α1) and E152 (α5), with a distinct rotamer change for the glutamate. The E152 rotamer change in the BCL-2 G101V:venetoclax complex places the Cy sidechain atom in contact with the chlorophenyl introducing a subtle change in the orientation of that moiety in the P2 pocket. To illustrate the connection between these small structural movements and the ~180-fold reduction in affinity for the G101V mutant, we introduced the E152A mutation on the G101V mutant and restored near-wildtype affinity for venetoclax. Additionally, introduction of an alanine at the G101 position did not provide sufficient bulk to displace E152 in the crystal structure and affinity for venetoclax was maintained for the G101A mutant. Thus the functional consequences of the G101V mutant are felt in the P2 pocket via the ‘knock-on’ effect of E152 repositioning forced by the additional bulk of the valine sidechain.

The BCL-2 F104L and F104C mutations were observed as venetoclax-resistance mutations in a mouse tumour model, and both induce drug tolerance in human cell lines. Interestingly, in the initial study, human cell lines did not acquire resistance through mutation to BCL-2, instead truncation of BAX occurred preventing translocation to the mitochondria. The BCL-2 F104L and F104C mutations were observed as venetoclax-resistance mutations in a mouse tumour model and both induce drug tolerance in human cell lines. Interestingly, in the initial study, human cell lines did not acquire resistance through mutation to BCL-2, instead truncation of BAX occurred preventing translocation to the mitochondria.
binding decreased by ~10–500-fold (for F104L and F104C, respectively) relative to WT BCL-2. The structure of BCL-2-F104L:venetoclax presented here reveals that the P2 pocket increases in volume with the F104L mutation relative to WT (480 Å³ and 596 Å³ for WT and F104L, respectively). This increase in pocket volume decreases the surface complementarity between the drug and its target, likely contributing to the decrease in affinity. Binding to the F104C mutant is weaker still, and this may have thwarted attempts to obtain a crystal structure of the BCL-2-F104C:venetoclax complex despite extensive efforts. This further decrease in affinity is likely due to an even larger P2 pocket volume, as cysteine occupies a smaller volume than leucine, though other structural features may also come into play.

Pro-survival proteins prevent apoptosis by binding and sequestering the pro-apoptotic proteins. Venetoclax competes with pro-apoptotic BH3 motifs for BCL-2 binding, releasing pro-apoptotic proteins and allowing apoptosis in cells primed for death. It is therefore required that drug-resistant mutants of BCL-2 retain the ability to bind pro-apoptotic BH3 motifs to maintain the tumour’s viability. The G101V, F104L and F104C BCL-2 mutants all have this property. The critical difference between venetoclax and a BH3 helical peptide is the greater penetration of the drug into the P2 pocket, with the G101V mutant this compromises drug-binding but not BH3-binding. This is also a feature of ABT-737, one of the earliest precursors of venetoclax. Analogues of venetoclax that may retain binding to

Fig. 3 Steady-state competition SPR with BCL-2 mutants, BIMBH3 and venetoclax or S55746. SPR chip surfaces were immobilised with BIMBH3 peptide and analytes with the indicated BCL-2 mutants pre-combined with either a–e venetoclax (Ven) or f–h S55746 at the indicated concentrations (circles 0 nM, squares 20 nM, triangles point up 40 nM, triangles point down 60 nM). Response at steady-state is plotted against BCL-2 concentration, with fits from a steady-state competition SPR model shown (see methods and Supplementary Figs. 5, 6 for further details), that were used to derive the indicated mean $K_I$ values for venetoclax or S55746 binding to BCL-2. Data are representative of at least $n = 2$ independent experiments.
BCL-2 G101V include those that lack the chlorine of the chlorophenyl moiety or have it replaced with a smaller atom. S55746 binds BCL-2 via the P1, P2 and P3 pockets and our SPR data show it binds to BCL-2 WT with 10-fold lower affinity than venetoclax. S55746 inserts a 4-hydroxyphenyl moiety into the P2 pocket but does not insert as deep into the pocket as the corresponding chlorophenyl from venetoclax. This shallow insertion allows the BCL-2 F112 on the α3 helix to insert into the BCL-2 groove extending the P3 pocket. We show here that S55746 also loses potency (~100-fold) against the BCL-2 G101V mutant and we sought a structural explanation for this. The orientation of S55746 in the BCL-2 G101V:S55746 complex structure is conserved relative to the WT structure. In both BCL-2 WT and BCL-2 G101V structures compared with S55746 E152 is shielded from the base of the P2 pocket by the insertion of F112 into the groove. Consistent with this the combination of the G101V mutation with the E152A mutation did not fully restore WT binding to S55746, in contrast to venetoclax, suggesting additional structural features underlying affinity in this case. Those features may include changes in the structure and dynamics of BCL-2 G101V prior to engaging drug, or the distribution of conformers in the ensemble of BCL-2 G101V:drug structures in solution compared to WT that are not readily detectable crystallographically.

Venetoclax is the first FDA-approved drug that directly targets the mitochondrial apoptotic pathway. We have solved the crystal structure for BIMBH3 and BAXBH3 peptides and BCL-2 selective compounds binding to BCL-2 mutants in Table 2

Table 2 SPR affinity values for BH3 peptides and BCL-2 selective compounds binding to BCL-2 mutants

| BCL2 protein | BIMBH3 $K_D$ (nM ± SD, $n \geq 2$) | BAXBH3 $K_D$ (nM ± SD, $n \geq 2$) | Ven $K_I$ (nM ± SD, $n=3$) | S55746 $K_I$ (nM ± SD, $n \geq 2$) |
|--------------|---------------------------------|---------------------------------|-----------------------------|---------------------------------|
| WTa          | 0.29 ± 0.17                     | 1.4 ± 0.2                       | 0.018 ± 0.014               | 0.36 ± 0.02                     |
| G101V        | 0.84 ± 0.04                     | 12 ± 2                          | 3.2 ± 1.1                   | 36 ± 12                         |
| G101A        | 0.23 ± 0.04                     | ND                              | 0.11 ± 0.03                 | ND                              |
| F104C        | 0.17 ± 0.10                     | 4.0 ± 0.2                       | 25.1 ± 6.3                  | ND                              |
| F104La       | 0.039 ± 0.01                    | 6.1 ± 1.0                       | 0.46 ± 0.12                 | ND                              |
| E152A        | 0.36 ± 0.10                     | 3.6 ± 0.9                       | 0.027 ± 0.008               | ND                              |
| G101V/E152A  | 0.63 ± 0.39                     | 5.5 ± 0.3                       | 0.0020 ± 0.0027            | 5.3 ± 4.1                       |

Affinity for BIMBH3 and BAXBH3 peptides were determined by direct binding using kinetic fitting (see Supplementary Table 2 for detailed binding fit parameters). Venetoclax and S55746 by steady-state competition against a BIMBH3 peptide SPR surface with the indicated BCL-2 mutant. Mean values from at least two independent experiments with one standard deviation indicated.

*previously reported17
structure of the drug bound to its target, BCL-2, revealing a pose subtly different to that observed with structural analogues. We also describe the molecular basis for resistance to venetoclax observed in patients on treatment displaying the BCL-2 mutant G101V. These structures provide a basis to further optimise the venetoclax scaffold, for binding both to BCL-2 WT and the G101V mutant.

Methods

Compounds, peptides and primers. All peptides were custom synthesized by Mimotopes Pty Ltd (Australia). S55746 was purchased from Probetch (Cat#PC-63502). Venetoclax was purchased from ActiveBiochem (#A-1231). All other chemicals, unless specified, were obtained from Sigma-Aldrich (Australia). Primer sequences are detailed in Supplementary Table 3.

Protein expression and purification. BCL-2 WT construct, expression and purification was reported previously31 and the G101V and F104L mutants were previously reported17. Briefly, mutants were introduced by PCR using primers with the desired mutation. Bacterial E. coli BL21 cells were transformed with appropriate plasmids and proteins expressed as N-terminal GST fusions by IPTG induction in Superbroth. Recombinant GST-BCL-2 fusions were purified from cellular proteins by glutathione-agarase resin (Genescript; Cat#LD0206) and eluted with 10 mM reduced glutathione. GST-BCL-2 fusions were cleaved overnight at 4 °C with Prescision Protease to remove the GST-fusion and purified to homogeneity by size exclusion chromatography using a Superdex 75 10/300 (GE healthcare) equilibrated in 20 mM Tris pH 8 and 150 mM NaCl.

Crystallisation. BCL-2 proteins were incubated with 3 molar excess of the desired compound in DMSO. DMSO was removed by buffer exchange in an Amicon® Ultra-4 10 kDa cut of spin concentrator (Millipore; Cat#UFC801096). Initial crystals were obtained with BCL-2 WT and the desired compound by the hanging drop vapour diffusion method at 291 K with a precipitant solution consisting 5% PEG400, glycerol or ethylene glycol, then cooled in liquid nitrogen. Crystallisation conditions were optimised by diluting precipitant conditions (P1 through P4) indicated for each compound and hydrophobic (φ) 1–4 residues (L, I, L) from the BaxBH3 peptide. BCL-2 surface contacts with venetoclax are coloured in orange and with BaxBH3 peptide in slate highlighting the increased surface contact area of BaxBH3 relative to venetoclax (PDB id 2XA0) and scaled in either XDS or Aimless34. The phase problem was solved by molecular replacement using Phaser35 and the BCL-2 WT:venetoclax structure chain A with all waters and ligands removed (PDBe 4LVT) as a search model39. The model was refined by iterative reciprocal and real space refinement using PHENIX refine36 and Coot37, respectively with at least one round of cartesian simulated annealing refinement in PHENIX prior modelling ligands to avoid phase bias. Ligand initial models and restraints for venetoclax and S55746 were generated using the Grade web server (version 1.2.9, Global Phasing Ltd.). For atoms with multiple conformations initial occupancies were set to 0.5 modelling each conformation into appropriate density prior to multiple rounds of refinement in phenix using the refine occupancy function in addition to standard refinement procedures. X-ray data to geometry weights or atomic displacement factors were determined automatically using the optimize X-ray/stereochemistry weight and optimise X-ray/ADP weight respectively. Model validation was performed in Coot and MolProbity38. Data statistics were calculated in PHENIX using the generate Table1 for journal function. Stereo images with electron density are displayed in Supplementary Fig. 7.

The BCL-2 G101V:venetoclax data processed and solved in the orthorhombic spacegroup P212121 with a single protein chain in the asymmetric unit. However, during refinement Rmerge and Rfree increased in successive refinement cycles, giving refined values that were unreasonable when compared to similar resolution structures from the protein data bank. This did not occur when the structure was modelled in the monoclinic spacegroup P21 with two protein chains in the asymmetric unit. As a consequence, the monoclinic model and data were used. The BCL-2 G101V:S55746 structure processed in the monoclinic spacegroup P21 with two protein chains in the asymmetric unit. The data were anisotropic with data extending to 2.7 Å in the α and 2.0 Å in the β* and c* directions, and were ellipsoidally truncated and scaled by the diffraction anisotropy server39 without applying B-factor sharpening. After applying ellipsoidal truncation the data completeness in high resolution shells dropped, with completeness dropping below 70% from 2.5–3.0 Å. Furthermore, the electron density for S55746 in one of the protein chains was more poorly resolved. This copy of S55746 was modelled into the electron density according to the S55746 orientation from the BCL-2 WT: S55746 structure (PDB id 6CL8)32. To avoid phase bias no coordinates from the original WT:S55746 structure were used in refinement and orientations were matched by protein alignments and visual inspection only. The analysis presented here relates to the protein chain without this problem.

Structural analyses. All crystal structure representations were made using MacPyMOL version 1.8.0.3 (Schrödinger LLC). BCL-2 pocket volumes were calculated in a multistep process. Initially PDBe models were stripped of all non-protein atoms and pockets were filled with water molecules using the hollow program (version 1.2)40 with a 12 Å sphere radius from residue BCL-2 104 and 0.2 grid spacing. BCL-2 pockets were then defined as any water molecule within 5 Å of the venetoclax chlorophenyl (atoms Cl and Cl0) for the P2 pocket or 2 Å from atoms in the venetoclax azaindole (atoms C37–43, N5–6). Water atom selections were visually inspected to remove atoms that may be included in selection criteria but not connected to the desired pocket. The volume of each pocket selection was then determined using the 3eye web server volume assignor function (http://3eye.molmovdb.org/volumeCalc.php)32 with a 5 Å probe radius and grid resolution set to high. Alignments to compare structures were performed in MacPyMOL version 1.8.0.3 (Schrödinger LLC) aligning Ca carbons from the a2–5 helices residues

Fig. 5 BCL-2 surface contacts with either venetoclax, S55746 or BaxBH3 peptide. Structure of BCL-2 G101V with a Venetoclax (Ven) or b S55746, or c BCL-2 WT with a BaxBH3 peptide. BCL-2 surface contacts are coloured according to interactions with venetoclax (orange), S55746 (magenta) or BaxBH3 peptide (slate). Key BCL-2 binding pockets (P1 through P4) indicated for each compound and hydrophobic (φ) 1–4 residues (L, I, L) from the BaxBH3 peptide. BCL-2 surface contacts with venetoclax are coloured in orange and with BaxBH3 peptide in slate highlighting the increased surface contact area of BaxBH3 relative to venetoclax (PDB id 2XA0) and scaled in either XDS or Aimless34. The phase problem was solved by molecular replacement using Phaser35 and the BCL-2 WT:venetoclax structure chain A with all waters and ligands removed (PDBe 4LVT) as a search model39. The model was refined by iterative reciprocal and real space refinement using PHENIX refine36 and Coot37, respectively with at least one round of cartesian simulated annealing refinement in PHENIX prior modelling ligands to avoid phase bias. Ligand initial models and restraints for venetoclax and S55746 were generated using the Grade web server (version 1.2.9, Global Phasing Ltd.). For atoms with multiple conformations initial occupancies were set to 0.5 modelling each conformation into appropriate density prior to multiple rounds of refinement in phenix using the refine occupancy function in addition to standard refinement procedures. X-ray data to geometry weights or atomic displacement factors were determined automatically using the optimize X-ray/stereochemistry weight and optimise X-ray/ADP weight respectively. Model validation was performed in Coot and MolProbity38. Data statistics were calculated in PHENIX using the generate Table1 for journal function. Stereo images with electron density are displayed in Supplementary Fig. 7. The BCL-2 G101V:venetoclax data processed and solved in the orthorhombic spacegroup P212121 with a single protein chain in the asymmetric unit. However, during refinement Rmerge and Rfree increased in successive refinement cycles, giving refined values that were unreasonable when compared to similar resolution structures from the protein data bank. This did not occur when the structure was modelled in the monoclinic spacegroup P21 with two protein chains in the asymmetric unit. As a consequence, the monoclinic model and data were used. The BCL-2 G101V:S55746 structure processed in the monoclinic spacegroup P21 with two protein chains in the asymmetric unit. The data were anisotropic with data extending to 2.7 Å in the α and 2.0 Å in the β* and c* directions, and were ellipsoidally truncated and scaled by the diffraction anisotropy server39 without applying B-factor sharpening. After applying ellipsoidal truncation the data completeness in high resolution shells dropped, with completeness dropping below 70% from 2.5–3.0 Å. Furthermore, the electron density for S55746 in one of the protein chains was more poorly resolved. This copy of S55746 was modelled into the electron density according to the S55746 orientation from the BCL-2 WT: S55746 structure (PDB id 6CL8)32. To avoid phase bias no coordinates from the original WT:S55746 structure were used in refinement and orientations were matched by protein alignments and visual inspection only. The analysis presented here relates to the protein chain without this problem.
BCL-2:venetoclax structures there is a crystal contact between R18 and the acyl oxygen from the venetoclax benzamide (a hydrogen bond) in one of the alternate conformations (conformer A). The conformation of the venetoclax conformer A benzamide is equivalent to the conformation of ABT-263 bound to BCL-2 (PDB id 4LYT) which has no crystal contacts to the bound ligand. The published BCL-2 WT:compound 1 structure (PDB id 4MAN) has a crystal contact between the α6-6 loop and the benzine ring from the benzamide moiety of compound 1 from symmetry related molecules that may influence the conformation of compound 1.

Distances between venetoclax:cytochrome c-ene ring atoms (C1–6), ABT-263 atoms (C30, C9, C29, C26, C25 and C28 ordered according to equivalent positions in ABT-263) and BIMBH3 (4LYT) were determined using the distance command in MacPyMOL version 1.8.0.3. For fair comparison the BCL-2 WT:venetoclax cyclohex-1-eene ring A conformer was used as a reference as it matched the conformers from the ABT-263 and G101V:venetoclax structures. The BCL-2 WT: ABT-263 (PDB id 4LYT), BCL-2 WT:compound 1 (PDB id 4MAN) and BCL-2 WT: G101V:venetoclax structures had two copies of protein and drug in the asymmetric unit. Distances for both copies were calculated and mean values presented in the Supplementary Table 1. RMSD values were calculated for each structure relative to the BCL-2 WT:venetoclax cyclohex-1-eene ring A conformer.

**Surface plasmon resonance.** SPR experiments were performed as previously described. Briefly, experiments were performed in HBS-EP buffer containing 10 mM HEPES pH 7.4, 150 mM sodium chloride, 3.4 mM EDTA, 0.005% tween 20 and optionally 1 mM TCEP for BAXBH3 experiments, at 25 °C. Experiments were performed on either a BIAcore 4000 or BIAcore 2000 using a SA sensor chip (GE healthcare) immobilized with biotinylated BIMBH3 (DRMPEWIA- QELRIGDEAFNAYYARR) or BAXBH3 (DASTKKLSECLKRIGDELDSND- optionally 1 mM TCEP for BAXBH3 experiments, at 25 °C. Experiments were performed using independent protein preparations.

The refitted coordinates and data for all structures were deposited in the PDB: BCL-2:venetoclax PDB id 600K, BCL-2:G101V:venetoclax PDB id 600L, BCL-2:G101A:venetoclax PDB id 600P, BCL-2:FL140:venetoclax PDB id 600M and BCL-2:G101V: SS5746 PDB id 600O. The source data underlying Fig. 3a–h, Fig. 4a and Table 2 are provided as a Source Data File. A reporting summary for this Article is available as a Supplementary Information file. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Author contributions

R.W.B., M.A.A., P.B., I.J.M., A.W.R., D.C.S.H., P.M.C. and P.E.C. made substantial contributions to the conception or design of the work; R.W.B., J.-N.G., C.S.L., D.L., C.A.W., R.T., P.E.C. to the acquisition of data; R.W.B., J.-N.G., P.M.C., P.E.C. to analysis of data; R.W.B., G.L., A.W.R., D.C.S.H., P.M.C., P.E.C. to the interpretation of data; R.W.B., A.W.R., D.C.S.H., P.M.C. and P.E.C. drafted the work or substantially revised it.

Additional information

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Competing interests: R.W.B., J.-N.G., C.S.L., D.L., C.A.W., M.A.A., G.L., I.J.M., R.T., A.W.R., D.C.S.H., P.M.C. and P.E.C. are employees of the Walter and Eliza Hall Institute, which has an agreement with Genentech and AbbVie and receives milestone and royalty payments related to venetoclax. Employees of Walter and Eliza Hall Institute may be eligible for financial benefits related to these payments. J.-N.G., M.A.A., G.L., I.J.M., A.W.R., D.C.S.H., P.M.C. and P.E.C. receive such a financial benefit as a result of previous research related to venetoclax. G.L., D.C.S.H., P.M.C. and P.E.C. have received research funding from Genentech. A.W.R has received research funding from AbbVie. The remaining author declares no competing interests.

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