ABT-199, a new Bcl-2-specific BH3 mimetic, has *in vivo* efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia

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Key Points
ABT-199, a new Bcl-2-specific BH3 mimetic, is efficacious in vivo against mouse lymphomas without provoking thrombocytopenia.

Treating mouse lymphomas with BH3 mimic ABT-737 combined with bortezomib or purvalanol achieved long-term remission.

Abstract
BH3-only proteins trigger the stress apoptosis pathway and chemical mimetics have great potential for cancer therapy. BH3-only proteins inhibit anti-apoptotic members of the Bcl-2 family. Promising BH3 mimetic ABT-737 and the related orally available compound ABT-263 (navitoclax) bind avidly to anti-apoptotic Bcl-2, Bcl-xL and Bcl-w. However, their interaction with Bcl-xL provokes thrombocytopenia, which has proven to be the dose-limiting toxicity. We have tested the efficacy of ABT-199, a new Bcl-2-specific BH3 mimetic, against aggressive progenitor cell lymphomas derived from bi-transgenic myc/bcl-2 mice. As a single agent, ABT-199 was as effective as ABT-737 in prolonging survival of immunocompetent tumor-bearing mice, without causing thrombocytopenia. Both drugs acted rapidly but, contrary to prevailing models, their apoptotic activity did not rely upon the BH3-only protein Bim. When ABT-737 was combined with the proteosome inhibitor bortezomib or cdk inhibitor purvalanol, many treated animals achieved long-term remission.

Introduction
Whether cells live or die by apoptosis when confronted with diverse stresses, including radiation and chemotherapy, is determined by interactions between opposing factions of the Bcl-2 family of proteins. Bcl-2 prevents apoptosis, as do Bcl-xL, Bcl-w, Mcl-1 and A1, but other close relatives Bax and Bak instead provoke apoptosis. Stress signals induce distant relatives known as BH3-only proteins, which bind avidly to a hydrophobic groove on pro-survival proteins, preventing them from restraining any activated Bax or Bak molecules. Certain BH3-only proteins (Bim, cleaved Bid and perhaps Puma, Noxa) can also bind weakly and transiently to Bax and/or Bak, triggering their conformational change and homo-oligomerization on the
outer mitochondrial membrane. As a consequence, cytochrome c is released into the cytoplasm and provokes activation of the proteases (caspases) that demolish the cell.

Chemical mimetics of BH3-only proteins represent an exciting new class of cancer therapeutic. The most promising thus far have been ABT-737 and the related orally available compound ABT-263 (navitoclax), which bind avidly to Bcl-2, Bcl-xl and Bcl-w but not to Mcl-1 or A1. Both have significant efficacy against human tumor cell lines having high levels of Bcl-2 or Bcl-xL but little Mcl-1, particularly lymphoid malignancies and small cell lung carcinoma (SCLC). However, because Bcl-xl is critical for the survival of platelets, ABT-737 and ABT-263 induced transient thrombocytopenia in pre-clinical trials, and the dose limiting toxicity for navitoclax proved to be thrombocytopenia.

Abbott Laboratories have recently developed a high affinity Bcl-2-selective BH3 mimetic, ABT-199, which spared human platelets in vitro and dog platelets in vivo. Tumor regression was achieved for xenografts of human lymphoma cell lines and, excitingly, the first clinical tests for refractory chronic lymphocytic leukemia (CLL) resulted in rapid tumor lysis in 3/3 patients.

We have investigated the efficacy of ABT-199 in a preclinical model responsive to ABT-737: immunocompetent mice transplanted with lymphomyeloid progenitor cell tumors derived from Eμ-myc/Eμ-bcl-2 (hereafter myc/bcl-2) transgenic mice. These lymphomas have high levels of Bim, which is thought to be critical for responsiveness. To test the requirement for Bim, we also generated and treated bim-/- myc/bcl-2 tumors. In addition, we trialled combination therapy with ABT-737 and either the proteosome inhibitor bortezomib or the cyclin dependent kinase (cdk) inhibitor purvalanol.

Methods

Treatment with BH3 mimetics. Non-irradiated C57BL/6 mice were injected (i.v.) with 3x10^6 lymphoma cells (3 mice/treatment arm/tumor) and monitored by tail bleed. Starting on d11, when the white blood cell (WBC) count had become elevated, the BH3 mimetic or relevant vehicle was administered daily for 10d (ABT-737, Abbott...
Lectroes, 75\text{mg/kg}, i.p. in 30\% propylene glycol, 5\% Tween 80, 10\% DMSO, 3.3\% dextrose in water pH4; ABT-199, Abbott Laboratories, 100\text{mg/kg}, oral gavage in 60\% phosal 50PG, 30\% PEG 400, 10\% ethanol).

**Results and Discussion**

All procedures involving animals were approved by the Walter and Eliza Hall Institute Animal Ethics Committee. Mice were transplanted with \textit{bim}\textsuperscript{++} or \textit{bim}\textsuperscript{+-} \textit{myc/bcl-2} lymphoma cells and treatment with ABT-737 or ABT-199 was initiated once WBC counts were elevated (d11). The impact of ABT-737 and ABT-199 on tumor-bearing mice was rapid, with the WBC count dropping within 3 h (Figure 1A,B). By d6, the WBC count had dropped further in most mice, except for one \textit{bim}\textsuperscript{+-} lymphoma (#324), which had a mixed phenotype (see Figure 1 legend; supplemental Figure S1A).

Platelet counts were low prior to treatment, presumably due to the tumor burden (Figure 1C, D). Following the first dose of ABT-737, platelet counts dropped significantly within 3h but had rebounded by d6, as previously reported\textsuperscript{7}. In mice treated with ABT-199, however, there was no drop in platelets at 3 h and by d6 they had increased to \textasciitilde normal levels, most likely due to the reduction in tumor cells. Thus, as anticipated for a Bcl-2-selective inhibitor, ABT-199 treatment did not cause acute thrombocytopenia.

Mice treated with the BH3 mimetics lived significantly longer than controls (Figure 1E, F). Those transplanted with \textit{bim}\textsuperscript{++} lymphomas responded equally well to ABT-737 and ABT-199, their median survival being extended by approximately 10d (Figure 1B). Indeed, had the treatment been continued, the mice may well have survived longer.

ABT-737 has been thought to act by displacing Bim from Bcl-2, thereby making it available to neutralise Mcl-1 or A1, which would otherwise contribute to resistance\textsuperscript{13}. Surprisingly, however, the \textit{bim}\textsuperscript{+-} tumors responded well to treatment. With ABT-199, survival was only modestly less than for those with \textit{bim}\textsuperscript{++} tumors (P = 0.039) and with ABT-737, the response was comparable (Figure 1E, F). These results indicate
that Bim is not essential for apoptosis induced by these BH3 mimetics, at least in this tumor type.

We hypothesized that bim\(^{-/-}\) tumors might have had a compensatory increase in another BH3-only protein. However, the level of Bid, Noxa and Bad did not vary between tumors and, while some had higher Puma or Bmf than others, there was no consistent difference between the bim\(^{-/-}\) and bim\(^{+/-}\) tumors (supplemental Figure S1B). Presumably a combination of other BH3-only proteins had sufficed to replace Bim. It will be important to determine if these findings extend to other Bcl-2-dependent tumor types, particularly in a clinical setting.

Long-term exposure to ABT-737 in vitro can result in the emergence of resistant clones through up-regulation of anti-apoptotic Bcl-2-related proteins\(^{14}\). To test whether selection was occurring during treatment in vivo, we compared 5 tumors from sick mice that had been treated earlier with a BH3 mimic or vehicle (supplemental Figure S2). No increase in endogenous Bcl-x\(_L\), Mcl-1 or Bcl-2 was observed but transgenic Bcl-2 was higher in certain treated tumors (bim\(^{+/-}\) #16 and, to a lesser extent, bim\(^{+/-}\) #9). Whether treatment selects those cells having higher Bcl-2 or induces higher expression is unclear. Of note, the duration of treatment used here (10d) is comparatively short relative to expected clinical regimens.

Despite their encouraging response, all mice treated solely with a BH3 mimetic eventually died from their tumors (within 30d following cessation of treatment). We previously showed striking synergy between ABT-737 and low dose cyclophosphamide, achieving many complete remissions for mice transplanted with myc/bcl-2 lymphomas, and attributed the synergy to neutralisation of Mcl-1 by BH3-only proteins Noxa and Puma induced by cyclophosphamide (via p53)\(^{12}\). We have now tested ABT-737 in combination with two agents expected to lower Mcl-1 levels: the proteasome inhibitor bortezomib and cdk (1,2,4) inhibitor purvalanol A (Figure 2; supplemental Tables S1, S2). While proteasome inhibition can lead to accumulation of Mcl-1\(^{15}\), it also results in an increase in Noxa (eg\(^{16}\)). CDK inhibitors down-regulate Mcl-1 transcription\(^{17}\) and purvalanol A has been shown to induce apoptosis of Myc-overexpressing cells and extend survival of mice transplanted with E\(\mu\)-myc tumors\(^{18}\).
For direct comparison with the earlier study, treatment was started on d4 rather than on d11. None of the lymphomas responded to bortezomib or purvalanol A as single agents. Tumor #9 responded better to ABT-737 plus bortezomib than ABT-737 alone (P = 0.02), the response being comparable (P=0.19) to that achieved with ABT-737 and low dose cyclophosphamide, but ABT-737/purvalanol was not significantly better than ABT-737 alone (P = 0.12). For tumor #12, there was no significant benefit with ABT-737 plus bortezomib versus ABT-737 alone (P=0.52), but the outcome with ABT-737/purvalanol was comparable to that achieved with ABT-737/cyclophosphamide (P=0.47). For tumor #16, ABT-737 produced complete remission as a single agent, all mice remaining healthy until the experiment was terminated at 150d (supplemental Table S2); the greater sensitivity may be due to higher levels of Puma and Noxa in this tumor (supplemental Figure S1B). When treated with ABT-737 from d11 rather than d4, however, all mice transplanted with #16 had died by d50 (Figure 1E). This dramatic difference in outcome most likely reflects the relative tumour burden at the outset of treatment and longer treatment may have been more effective.

Early clinical trials for treating CLL with navitoclax and now ABT-199 show promising potential. In view of our results, combination therapy with cyclophosphamide, bortezomib (velcade) and clinically approved cdk inhibitors would be worthwhile considering. Others have also reported encouraging *in vivo* synergies with ABT-737 and, very recently, with ABT-199.

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Authorship Contributions
CJV and SC conceived the studies, planned experiments, analysed data and wrote the manuscript. CJV performed the experiments.

Disclosure of Conflicts of Interest
The authors declare no competing financial interests. The Walter and Eliza Hall Institute had a tripartite research collaboration with Genentech Inc and Abbott Laboratories at the time of these studies.
References


Figure 1. Comparison of responsiveness to ABT-737 and ABT-199

(A, B) White blood cell counts, (C, D) platelet counts and (E, F) Kaplan-Meier survival curves of mice transplanted with 4 bim^{+/+} (#9, #12, #16 and #47) and 3 bim^{-/-} (#58, #96 and #324) myc/bcl-2 lymphomas and treated with ABT-737, ABT-199 or the respective vehicles (3 mice per treatment arm for each independent tumor). Blood analyses were performed at 0h, 3h and 6d after starting treatment (d11 after transplantation), using an ADVIA hematology analyser (Bayer). Bars represent mean + SEM; significant changes observed at 3h are indicated *<0.05, ** P<0.01, ***P<0.001, Student’s t-test. The x-axis in E, F indicates days elapsed since start of treatment and the bar indicates the duration of treatment (10d). Significance for Kaplan-Meier survival curves was determined using the log-rank (Mantel-Cox) test. The median survival of bim^{+/+} lymphomas treated with ABT-737 was 21.5d versus 14d for vehicle, P=0.0088; and 23.5d with ABT-199 vs. 13d for vehicle, P = 0.0003. The median survival of bim^{-/-} lymphomas treated with ABT-737 was 20.5d vs. 14d for vehicle, P < 0.0001; and 19.5d with ABT-199 vs 13d for vehicle, P < 0.0001. One bim^{-/-} lymphoma (# 324) rebounded early following treatment; unusually, it comprised 50% progenitor (B220^{+}CD4^{+}) and 50% B lymphoid (B220^{+}CD4^{+}) cells, the latter having higher levels of Mcl-1 (see supplemental Figure S1A), which would confer greater resistance.

Figure 2. Combination therapy with ABT-737 extends survival.

Safe regimens were established from previously published data^{12,18,19} and our own dose-finding studies (data not shown). Non-irradiated C57BL/6 recipients were transplanted with three independent bim^{+/+} myc/bcl-2 lymphomas (#9, #12, #16) (3x10^6 tumor cells/mouse) 4d prior to commencement of treatment. ABT-737 was administered i.p. at 75 mg/kg/d for 10d (bar), starting on d1; cyclophosphamide (CTX; 50 mg/kg) i.p. on d3, 8; bortezomib (0.5 mg/kg) ip on d1, 3, 5, 8, 10; purvalanol A (20 mg/kg) ip on d1, 3, 5, 8, 10, alone or in combination with ABT-737, with at least 6 mice per indicated treatment arm (supplemental Tables S1, S2). With tumor #16, all mice treated with ABT-737 alone remained healthy until the experiment was terminated at d150 (supplemental Table S2 and data not shown; controls treated with vehicle were all dead by d35).
**Figure 1**

**A**

*bim\(^{+/+}\)myc/bcl-2*

- WBC (x10\(^3/\)μL)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199

**B**

*bim\(^{-/-}\)myc/bcl-2*

- WBC (x10\(^3/\)μL)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199

**C**

*bim\(^{+/+}\)myc/bcl-2*

- platelets (x10\(^9/\)L)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199

**D**

*bim\(^{-/-}\)myc/bcl-2*

- platelets (x10\(^9/\)L)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199

**E**

*bim\(^{+/+}\)myc/bcl-2*

- percent survival
- time (d)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199

**F**

*bim\(^{-/-}\)myc/bcl-2*

- percent survival
- time (d)
- vehicle 737
- ABT-737
- vehicle 199
- ABT-199
Figure 2

*myc/bcl-2 #9*

*myc/bcl-2 #12*

- **percent survival**
- **time (d)**

- **vehicle**
- **ABT-737**
- **ABT-737/CTX**
- **bortezomib**
- **ABT-737/bortezomib**
- **purvalanol**
- **ABT-737/purvalanol**
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