Both leukaemic and normal peripheral B lymphoid cells are highly sensitive to the selective pharmacological inhibition of pro-survival Bcl-2 with ABT-199

Bcl-2 inhibition selectively kills mature B cells

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Conflict of Interest Statement

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Abstract

Overexpression of the pro-survival protein Bcl-2 marks many B lymphoid malignancies and contributes to resistance to many commonly used chemotherapeutic agents. The first effective BH3 mimetic inhibitors of Bcl-2, ABT-737 and navitoclax, also target Bcl-x_L causing dose-limiting thrombocytopenia. This prompted the development of the Bcl-2 selective antagonist, ABT-199. Here, we show that in lymphoid cells, ABT-199 specifically causes Bax/Bak-mediated apoptosis that is triggered principally by the initiator BH3-only protein Bim. As expected, malignant cells isolated from patients with chronic lymphocytic leukaemia (CLL) are highly sensitive to ABT-199. However, we found that normal, untransformed mature B cells are also highly sensitive to ABT-199, both *in vitro* and *in vivo*. By contrast, the B cell precursors are largely spared, as are cells of myeloid origin. These results pinpoint the likely impact of the pharmacological inhibition of Bcl-2 by ABT-199 on the normal mature haemopoietic cell lineages in patients and have implications for monitoring during ABT-199 therapy, as well as for the clinical utility of this very promising targeted agent.

Keywords

ABT-199, ABT-737, BCL2, Chronic lymphocytic leukaemia, Apoptosis, Lymphocyte subsets

Introduction

Bcl-2 family proteins are key regulators of the intrinsic apoptotic pathway. They are generally subdivided into three groups based on their structure and function. Bax and Bak are critical for unleashing the effector phase of apoptosis by mediating mitochondrial outer membrane permeabilisation (MOMP). This key step triggers the release into the cytosol of pro-apoptogenic factors, such as cytochrome *c*, to activate caspases, proteolytic enzymes that drive cellular demolition [1]. Under conditions which favour cell survival, Bax and Bak are kept in check by the pro-survival Bcl-2 family members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bfl-1/A1) [2]. Stress-induced signalling pathways promote apoptosis by mobilising members of the third group of the family, the BH3-only proteins. These proteins (e.g. Bim) trigger the activation of the essential apoptosis mediators Bax/Bak either through direct binding or indirectly, by binding to the pro-survival Bcl-2 proteins thereby unleashing Bax/Bak.

Evasion of apoptosis is generally regarded as necessary for malignant transformation by permitting the acquisition and tolerance of otherwise deleterious oncogenic capabilities such as uncontrolled proliferation [3]. Moreover, as most anti-cancer agents trigger cellular stress pathways (e.g. DNA damage), defects in the intrinsic apoptotic pathway (e.g. Bcl-2 overexpression) confer relative protection against many cytotoxic agents. Importantly, this frequently correlates with inferior patient outcomes. For example, the adverse prognostic significance of loss-of-function mutations in p53 across a diverse range of cancers [4] is largely attributable to the consequent loss of the apoptotic response to DNA damaging agents [5].

BH3 mimetics were developed to directly tackle or bypass such blocks to promote cell

death by binding to and inactivating the pro-survival Bcl-2 proteins [6]. The first validated small molecule to kill tumour cells solely by mimicking the action of the BH3-only proteins is ABT-737. Both ABT-737 and the orally bioavailable navitoclax (ABT-263) bind with high affinity to Bcl-2, Bcl-x_L and Bcl-w, but poorly to Mcl-1 and A1 [7]. In Phase I/II clinical trials, navitoclax demonstrated substantial anti-tumour efficacy, particularly in chronic lymphocytic leukaemia (CLL) and small lymphocytic lymphoma (SLL) [7-9]. However, navitoclax has demonstrated only limited clinical activity in other cancers (such as non-CLL/SLL lymphoid neoplasms and small cell lung cancer) that frequently overexpress the pro-survival Bcl-2 proteins that it targets and were demonstrably susceptible to navitoclax in preclinical studies [8, 10]. A plausible explanation for this finding is that inadequate drug exposures were achieved in patients due to the dose limiting toxicity of navitoclax which causes thrombocytopenia through inhibition of Bcl-x_L in platelets [9, 11-13].

We [14] and others [15, 16] have demonstrated previously that at least in lymphoid cells, ABT-737 and navitoclax antagonise Bcl-2 much more effectively than Bcl-x_L or Bcl-w despite comparable affinities for these proteins in biochemical studies. The possibility of separating the anti-tumour efficacy of navitoclax from thrombocytopenia, its major toxicity, provided the basis for the development of the Bcl-2-selective BH3 mimetic compound ABT-199 (GDC-199/RG7601) [17]. Preclinical studies indicated that relative to navitoclax, ABT-199 had superior potency across a range of non-Hodgkin lymphoma (NHL) cell lines and primary CLL cells *in vitro*, with markedly reduced anti-platelet effects *in vitro* and *in vivo*. This has been borne out to date in an ongoing Phase 1 clinical trial, where ABT-199 has demonstrated potent anti-tumour activity in patients with relapsed/refractory CLL (notably with Grade 3 tumour lysis syndrome

92 observed in all patients in the initial dose cohort) and NHL, without significant treatment-
93 induced thrombocytopenia [17-20].

94 Changing the profile of BH3 mimetic activity can be predicted to alter the collateral
95 impact on normal cell lineages that depend on Bcl-2 or Bcl-x_L at various stages of their
96 development, such as lymphocytes. For example, using murine models of genetic deletion, the
97 survival of early B-cell progenitors has been demonstrated to be Bcl-x_L-dependent [21], with
98 maturation associated with a gradual switch to Bcl-2 in this tissue compartment [13, 22, 23]. In
99 contrast, the earliest thymic progenitors ($CD4^-CD8^-$ double negative (DN) thymocytes) are Bcl-
100 2-dependent, as are mature single positive (SP) thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$) [24-29].
101 However, Bcl-x_L is the primary pro-survival protein required for maintaining the viability of
102 immature double positive (DP) thymocytes ($CD4^+CD8^+$) [21, 30].

103 In order to better identify parameters that will guide the optimal deployment of the Bcl-2
104 selective antagonist ABT-199 in the clinic, we have explored its effect on normal lymphocyte
105 subsets, both *in vitro* and *in vivo*, and characterised the mechanism by which cells are killed.
106 Importantly, we address the recent suggestion that the greater cytotoxicity of ABT-199 compared
107 to navitoclax may reflect a novel capability to directly activate Bax [31]. In addition, we
108 demonstrate that contrary to a widespread belief that cancerous but not healthy cells are primed
109 for killing by BH3-mimetic therapy [32], we find that normal human B cells are as sensitive to
110 ABT-199 *in vitro* as their malignant counterparts.

111

112

Materials and Methods

Mice

The generation of *Bim*$^{-/-}$ [33], *Puma*$^{-/-}$, *Noxa*$^{-/-}$ [34], *Bmf*$^{-/-}$, *Bax*$^{-/-}$ [35], *Bak*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$[1], Eμ-Myc [36], Mcl-1$^{fl/fl}$ [37] and Rosa26-CreERT2 mice has been described. All animal experiments were performed in accordance with the guidelines of the institutional Animal Ethics Committee.

Cell lines, FACS analysis and *in vitro* cell survival assays

Single cell suspensions were prepared from thymus, spleen or BM, and cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% foetal calf serum (FCS) with graded concentrations of ABT-737 or ABT-199 (1nM-4μM). Cell viability was quantified by flow cytometric analysis of cells that excluded propidium iodide (5 μg/mL; Sigma-Aldrich, St Louis, MO, USA). Results were normalized to the viability of cells that had been left untreated for 24 h. Leukocyte sub-populations were identified by staining with mAbs coupled to FITC, R-PE, APC, followed by flow cytometric analysis: anti-B220 (clone RA3-6B2), anti-IgD (clone 11-26C), anti-IgM (clone 333.12 or 5.1), anti-CD8 (clone YTS169), anti-CD4 (clone H129 or YTA321), anti-CD21 (clone 7G6) and anti-CD23 (clone B3B4). AML cells retrovirally transduced with the MLL-ENL expression vector were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Thermo Scientific HyClone, Logan, UT, USA), 2 mM L-glutamine, 6 ng/mL IL-3 (Peprotech, Rocky Hill, NJ, USA). Cre-mediated recombination *in vitro* was induced by treatment with 10^{-7} M 4-hydroxy tamoxifen (Sigma-Aldrich).

Haemopoietic reconstitution

Fetal liver cells from were harvested, cultured for 24 h (in medium containing 100 ng/ml of SCF,

10 ng/ml of IL-6, 10 ng/ml of Flt-3L and 50 ng/ml of TPO) and infected with retroviruses encoding Bcl-x_L, Bcl-2, Mcl-1 (human) or Bcl-w (mouse). Transduced cells were injected into lethally irradiated (2x550 Rads) recipient mice. Hematopoietic and lymphoid organs were harvested from reconstituted mice 8 weeks later.

In vivo drug treatment

Mice were administered daily doses of ABT-737 (100mg/kg) by intraperitoneal injection or ABT-199 (100mg/kg) by oral gavage for one week, then sacrificed. The haemopoietic organs were harvested and processed into single cell suspension, stained with the above antibodies and analysed on a FACSCalibur™ analyser (BD Biosciences, San Jose, CA, USA). Lymphocyte subsets were enumerated based on manual cells counts and proportional representation on flow cytometry.

Western blot

Protein lysates were separated by SDS-PAGE (NuPAGE Novex Bis-Tris pre-cast gels, Invitrogen) and immunoblotted as previously described[38] with primary antibody (mouse anti-Bcl2 clone Bcl2-100 ; rat anti-Bim clone 3C5 (Enzo Life Sciences, Farmingdale, NY, USA); rat anti-Bcl-w clone 16H12 [39]; mouse anti-actin clone AC-40 (Sigma-Aldrich); rat anti-Mcl1 clone 19C4; rat anti-Bcl-x_L clone 9C9, then secondary goat anti-mouse or –rat antibody (conjugated with IRDye™ 800 (Rockland Immunochemicals, Gilbertsville, PA, USA) or Alexa Fluor 680 (Molecular Probes, Life Technologies, Carlsbad, CA, USA) fluorochromes), then scanned and analysed using the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA).

Human samples

Samples were collected from normal volunteers and patients with CLL who had provided written informed consent. These studies were approved and monitored by the human research ethics committees (HREC) of participating centres.

In vitro cytotoxicity assays with human samples

Mononuclear cells were isolated as previously described [38]. Unsorted mononuclear cells were cultured for 24 h at 37°C, in a humidified atmosphere containing ambient O_2 and 10% CO_2, in IMDM plus 10% FCS with titrated drug concentrations. Stock solutions were made up in DMSO (Sigma-Aldrich).

Viable CLL and normal cells were enumerated by flow cytometry on a FACSCalibur™ analyser (BD Biosciences), using BD CaliBRITE™ beads (BD Biosciences) with concurrent PI exclusion and surface immunophenotyping with anti-CD5-FITC (clone BL1a), anti-CD8-FITC (clone B9.11), anti-CD19-PE (clone J3.119) and anti-CD4-APC (clone 13B8.2) antibodies (Beckman Coulter, Villepinte, France). Viability was determined by normalising to the number of CLL cells in drug-free wells. Concentration response curves were fitted using Graphpad Prism to determine LC50.

Statistics

Analysis was performed using Graphpad Prism software. Paired groups compared using Wilcoxon matched pairs signed rank test. Multiple t tests corrected for multiple comparisons using Holm-Sidak method (α=0.05).

Results

Normal and malignant human peripheral blood B cells are highly sensitive to ABT-199, unlike T cells and myeloid cells

In Phase 1 clinical trials, navitoclax induced moderate depletion of human $CD3^+$ cells after just 14 days of therapy [8]. Along the same lines, identification of human lymphocyte subsets sensitive to selective Bcl-2 inhibition would provide the opportunity to anticipate any possible excess risk of opportunistic infections with ABT-199.

We firstly determined the *in vitro* sensitivity to ABT-199 of normal human B cells, as well as $CD4^+$ and $CD8^+$ T cells in peripheral blood sampled from healthy donors (n = 9). Significantly, normal peripheral B cells were intrinsically more sensitive (~1,000-fold) to ABT-199 than either T cell subset (Fig 1; mean ABT-199 LC50 ± SEM for B cells, CD4 T cells and CD8 T cells were 3.0 ± 0.9 nM, 2.5 ± 0.6 μM and 1.3 ± 0.7 μM respectively; B versus CD4 T cells p=0.008; B versus CD8 T cells p=0.004). This difference was also observed when the cells were treated with ABT-737 (Fig 1; mean ABT-737 LC50 ± SEM for B cells, CD4 T cells and CD8 T cells were 6.8 ± 2.2 nM, 1.0 ± 0.1 μM and 0.2 ± 0.1 μM respectively; B versus CD4 T cells p=0.008; B versus CD8 T cells p=0.004). There was no significant difference between the sensitivity of B, $CD4^+$ T or $CD8^+$ T cells to ABT-737 compared to ABT-199 (correcting for multiple comparisons using the Holm-Sidak method (α=0.05).

The overexpression of Bcl-2 observed in some human B cell malignancies, such as CLL, contributes to chemoresistance, but is also believed to prime such malignancies for killing by BH3 mimetics such as ABT-199 [40]. Surprisingly, we found that normal B cells have sensitivities

196 *in vitro* to ABT-737 and ABT-199 comparable to that observed in CLL cells freshly isolated
197 from patients (Fig 1).

198 We were unable to compare the sensitivity of normal and malignant B cells within
199 individual samples from CLL patients as almost all lack sufficient circulating normal B cells
200 reflecting previous anti-leukaemic therapy. We were, however, able to determine the relative *in*
201 *vitro* sensitivity to both ABT-199 and ABT-737 of CLL cells and normal T cells and
202 granulocytes in the same patient (Fig 1). Consistent with our findings in the samples from
203 healthy donors, circulating T cells and granulocytes were significantly less sensitive than CLL to
204 both BH3 mimetics (mean ABT-199 LC50 ± SEM for CLL cells, T cells and granulocytes were
205 10.0 ± 3.2 nM, 2.5 ± 2.8 μM and > 4 μM respectively; CLL versus T cells $p<0.0001$; CLL versus
206 granulocytes $p<0.0001$; mean ABT-737/navitoclax LC50 ± SEM for CLL cells, T cells and
207 granulocytes were 20.8 ± 3.7 nM, 1.6 ± 0.3 μM and > 4 μM respectively; CLL versus T cells
208 $p<0.0001$; CLL versus granulocytes $p<0.0001$).

209

210 **ABT-199 and ABT-737 have overlapping activities in many lymphoid subsets**

211 In order to better understand the differences in the sensitivity of lymphoid cells to BH3 mimetics
212 and to explore such differences in tissues not readily available from human donors, we next
213 examined the effect of ABT-199 on murine lymphocyte subpopulations isolated from primary
214 and secondary lymphoid organs. By comparing the *in vitro* activity of ABT-737 and ABT-199,
215 we also sought to delineate the specific contribution of Bcl-2 inhibition to that described for
216 ABT-737 on these cells [14].

B cells As observed for normal human blood, circulating murine B cells were highly susceptible to ABT-199 (Fig 2A, B), with other mature B cells (lymph node B cells, recirculating B cells in the bone marrow and splenic follicular B cells) and the precursor splenic T2 cells also being highly susceptible (EC50s < 1μM). Other than nodal B cells, for each of these subsets, ABT-199 was more potent than ABT-737, consistent with its higher affinity for Bcl-2 [17]. In contrast, B cell precursor subsets from BM and splenic T1 cells were modestly sensitive to ABT-737, but resistant to ABT-199.

T cells For the T cell lineage, DN thymocytes and the CD4 and CD8 SP T cells (Fig 2C, D), were at least as susceptible to ABT-199 as to ABT-737. Circulating and splenic T cells were more susceptible than thymic SP cells. DP thymocytes were resistant to ABT-199, but moderately sensitive to ABT-737.

The observed pattern of *in vitro* sensitivity to ABT-199 is consistent with genetic evidence indicating reliance on Bcl-2 for survival of murine mature B cells, DN thymocytes and mature peripheral T cells [22-29]. Resistance to ABT-199 and moderate sensitivity to ABT-737 were observed in those lymphoid subsets considered to be Bcl-x_L-dependent (such as bone marrow pro/pre-B cells or DP thymocytes) as defined genetically. Further, the data indicate differences in intrinsic susceptibility to ABT-199 and ABT-737 according to the organ of origin for mature B and T cells.

ABT-199 activates the intrinsic apoptotic pathway and kills lymphocytes by Bax/Bak-mediated apoptosis

238 Interpretation of the data presented above depends on knowledge of the mechanism-of-action of
239 ABT-199 in the cells of interest. We next focussed on identifying the key Bcl-2 family members
240 that mediate killing of lymphoid cells by ABT-199. Firstly, we established that ABT-199-
241 induced cell killing is absolutely dependent on the presence of Bax and Bak, with its cytotoxicity
242 being completely abolished in their absence, in all lymphoid subsets studied (Fig 3A). This
243 confirms that the cytotoxicity of ABT-199 is entirely mediated by the intrinsic apoptotic
244 pathway. Once activated, Bax and Bak are generally regarded as functionally interchangeable [2].
245 However, there are key differences in the mechanisms by which they are restrained in healthy
246 cells. In particular, Bcl-x_L has been shown to keep both Bax and Bak in check, whereas Bcl-2
247 appears to restrain only Bax [41]. Lymphoid subsets sensitive to ABT-199 (recirculating B cells,
248 DN thymocytes, CD4 SP and CD8 SP thymocytes) isolated from mice which lack Bax (and thus
249 rely on Bak alone to mediate MOMP) were as sensitive to ABT-199 *in vitro* as their wild-type
250 counterparts (Fig 3A). This may indicate that the direct de-repression of Bax in complex with
251 Bcl-2 is not the only mechanism of ABT-199-induced apoptosis in lymphocytes. Surprisingly,
252 recirculating B cells expressing only Bax were significantly less sensitive to ABT-199 compared
253 to those expressing both Bax and Bak (LC50 280 nM and 23 nM respectively; $p<0.0001$).
254 However, this was not observed in other lymphocyte subsets.

255 We next examined the role of BH3-only proteins known to be important in initiating
256 lymphocyte apoptosis [33, 42-44]. Complete absence of Bim, but not loss of Puma, Bmf or Noxa
257 markedly reduced the intrinsic sensitivity of recirculating B cells, DN thymocytes, as well as
258 CD4 and CD8 SP thymocytes to ABT-199 (Fig 3B). This is consistent with the notion that the
259 cytotoxicity of ABT-199 is primarily mediated (at least in lymphoid cells) by the liberation of

Bim from sequestration by Bcl-2 [17], as we and others have demonstrated previously with ABT-737 and navitoclax [14, 15]. The fact that Bim is so critical for killing by ABT-199 suggests that ABT-199 is unlikely to activate Bax or Bak directly [31]. It is notable however, that the absence of Bim conferred varying levels of resistance to ABT-199 in different lymphoid subsets. In particular, DN thymocytes remained moderately sensitive to ABT-199 (LC50 3.0 μM), in contrast to other subsets where the LC50 exceeded the highest tested concentration (4 μM).

Overexpression of Bcl-2 sensitises lymphoid cells to ABT-199 whereas elevated levels of other pro-survival Bcl-2 proteins confer resistance

We have previously demonstrated that Bim protein is stabilised when in complex with the pro-survival Bcl-2 family members: consequently the steady state levels of Bim are substantially elevated in cells overexpressing these proteins [14]. Consistent with the increased level of Bim available for displacement in these settings, the lymphoid subsets that are normally resistant to ABT-199 are sensitised to this drug by the overexpression of Bcl-2 (Fig 4A; Supplementary Fig 1). As expected, for intrinsically sensitive recirculating BM B cells, no additional sensitisation to ABT-199 was observed with enforced overexpression of Bcl-2.

The narrow target specificity of ABT-199 offers the theoretical advantage of reduced toxicity, but also widens the spectrum of potential resistance mechanisms. As we anticipated, overexpression of pro-survival proteins that are not directly targeted by ABT-199, such as Bcl-x_L, Bcl-w and Mcl-1, confers high-level resistance to ABT-199 in normally sensitive cells, such as recirculating B cells (Fig 4A; Supplementary Fig 1).

We next examined the effect of Bcl-2 overexpression in two mouse leukaemia/lymphoma models that express high levels of Mcl-1: Eμ-Myc B lymphoma cells and MLL-ENL driven acute myeloid leukaemia (AML). As previously shown in Eμ-Myc lymphoma cells [45], we found that Bcl-2 overexpression significantly increased ABT-199 sensitivity (Fig 4B-C; Supplementary Fig 2). However, these cells became highly sensitive to ABT-199 after deletion of floxed *Mcl-1* alleles by tamoxifen-induced activation of Cre-ERT2 recombinase (Fig 4B-C; Supplementary Fig 2). These results are reminiscent of findings with ABT-737 [14] and suggest that ABT-199 might be efficient in the treatment of other leukaemias, when combined with drugs that directly (or indirectly) target Mcl-1.

In vivo sensitivity of the normal lymphoid compartments to ABT-737 and ABT-199

As well as intrinsic factors, microenvironmental cues can significantly modulate sensitivity to BH3 mimetics [46, 47]. We therefore examined the effect of short-term treatment with ABT-199 or ABT-737 (both used at 100mg/kg) on the lymphoid subpopulations *in vivo* to assess this and to model likely changes during therapy of patients. ABT-199 was administered orally, whereas ABT-737 was administered by intraperitoneal injection because of its lack of oral bioavailability.

Consistent with our *in vitro* observations with murine and human cells, both drugs substantially reduced peripheral B cells to a similar extent. ABT-199 spared the B cell precursor subsets which were sensitive to ABT-737 *in vivo* (Fig 2A), as in the *in vitro* studies. Intriguingly, BM recirculating B cells were reduced to a greater extent in mice injected with ABT-737, despite the fact that they were more sensitive to ABT-199 than ABT-737 *in vitro*. This paradox suggests

either partial resistance to Bcl-2 inhibition by recirculating B cells while within the BM environment, or that there is greater compensatory production of new recirculating B cells from their precursors during ongoing ABT-199 treatment than is possible with ABT-737 therapy. In ABT-199-treated mice, the depletion of mature peripheral B cells is associated with a relative increase in numbers of bone marrow B cell precursors, compared to vehicle-treated control animals. This contrasts strongly with the reduction in precursors induced by dual inhibition of Bcl-2 and Bcl-x_L with ABT-737.

Within the T cell compartment, ABT-199 reduced the most primitive thymic T cell precursors to the same extent as ABT-737. However, a compensatory increase in double positive thymocytes was observed in the same ABT-199 treated mice, which contrasted with a clear reduction of this population in ABT-737-treated mice (Fig 5B). Mature T cell depletion was much less marked in mice treated with ABT-199 than in mice treated with ABT-737.

Taken together, these data suggest that because of intrinsic insensitivity to selective Bcl-2 inhibition of key B and T precursor cells, longer term administration of ABT-199 may impact normal lymphopoiesis to a lesser degree than ABT-737 treatment.

Discussion

The overexpression of Bcl-2 plays a key role in the pathogenesis of many cancers, particularly lymphoid malignancies such as chronic lymphocytic leukaemia and follicular lymphoma. Importantly, by rendering cells resistant to diverse apoptotic stimuli, elevated Bcl-2 levels not only facilitate the tolerance of deleterious oncogenic lesions, thereby overcoming many physiological safeguards against malignancy, but also confers intrinsic chemoresistance which translates to markedly inferior clinical outcomes. For example, mature B-cell lymphomas which harbour concomitant chromosomal rearrangements of both *MYC*/8q24 and *BCL-2*/18q21 ('double hit' lymphomas) are recognised to be associated with markedly poorer long term survival rates following contemporary immune-chemotherapy compared to those lymphomas bearing only *MYC* gene rearrangements (<40% versus ~80% 5-year overall survival, respectively) [48, 49].

We have previously demonstrated that Bcl-2 is the critical target required for ABT-737 and navitoclax cytotoxicity in CLL and normal mature B cells [9, 13, 14]. Our new findings reported here reveal that ABT-199 recapitulates the cytotoxicity of ABT-737 and navitoclax in cells that rely on Bcl-2 for their survival. Cell killing by these drugs is mediated through an identical Bim-dependent mechanism of action (Fig 3B), where elevated Bcl-2 markedly potentiates its activity (Fig 4A). It is interesting that Bim-deficient DN thymocytes remain moderately sensitive to ABT-199. It has been demonstrated that Bcl-2-Puma complexes can also be disrupted by ABT-199 [50]. While the absence of Puma alone did not reduce sensitivity to ABT-199 in this cell type, these results do not exclude the possibility that Bim may cooperate with Puma, or another BH3-only protein in this setting.

In Phase 1 clinical trials, navitoclax demonstrated potent clinical activity (with objective response rates >30%) in patients with CLL, small lymphocytic lymphoma and NK/T cell lymphoma [8, 9] refractory to multiple frontline chemotherapeutic regimens. Notably however, none of the patients treated in these trials achieved a complete response and achievable clinical exposures were significantly constrained by the thrombocytopenia caused by Bcl-x_L inhibition [9, 11, 12]. This left open the possibility that the clinical potential of targeting Bcl-2 could only be fully realised in the absence of this dose limiting toxicity.

We have demonstrated here that selective targeting of Bcl-2 with ABT-199 kills non-transformed Bcl-2-dependent murine lymphoid subsets (mature B and T cells) *in vitro* with comparable potency to ABT-737, paralleling our recent findings that ABT-199 completely recapitulates the anti-tumour efficacy of navitoclax against CLL [17]. Notably however, Bcl-x_L dependent murine lymphoid subsets such as pro/pre-B cells and DP thymocytes are spared following treatment with ABT-199 *in vitro* and *in vivo*. In contrast, ABT-737 is moderately cytotoxic in these cell types. Indeed, the observed increase in numbers of B progenitors following ABT-199 treatment appears to reflect compensatory increased B lymphopoiesis driven by depletion of peripheral B cells, a response not seen with ABT-737 treatment because of its direct toxicity to these precursors (Fig 3). It should be noted that our *in vivo* observations are unlikely to be an artefact of the different routes of administration as the published pharmacokinetic data indicate comparable bioavailability with exposures of 82.0μM*h and 90.9μM*h following 100mg/kg dosing [7, 17] of ABT-737 and ABT-199 respectively.

Our results suggest that, compared with navitoclax, ABT-199 may induce less haematological toxicity, with a correspondingly lower relative risk of opportunistic infections

and thus a more favourable therapeutic window. This may mean that not only could ABT-199 be a viable option in patients unable to tolerate more toxic treatments but it could also translate to a safer therapeutic window when ABT-199 is used in combination with other agents. It will be important to determine whether these trends translate to a genuinely superior safety profile in the context of clinical trials.

Interestingly, we have also demonstrated that in human samples, ABT-199 is highly potent in killing both normal and malignant circulating B cells. Indeed, the virtually identical degree of sensitivity in normal and malignant cells was somewhat surprising in view of the prevailing model that Bcl-2 overexpression in malignant cells selectively primes them for killing by BH3 mimetics. Notably however, this was in keeping with our observation that overexpression of Bcl-2 in normal lymphocytes highly sensitive to ABT-199 (such as recirculating B cells) does not result in further sensitisation. This raises the possibility of a clinical niche for ABT-199 in non-malignant B cell disorders, such as humoral autoimmune diseases, and suggests that B lymphopaenia could serve as a biomarker of ABT-199 exposure. In contrast, human T cells and circulating granulocytes appear relatively intrinsically resistant to ABT-199, highlighting the potential clinical advantage of this drug over more immunosuppressive anti-cancer therapies.

While the narrow specificity of ABT-199 for Bcl-2 alone may offer significant clinical advantages in terms of tolerability, the converse may apply in terms of efficacy. For example, while we have demonstrated that selective Bcl-2 inhibition recapitulates the efficacy of navitoclax in CLL [17], it remains to be seen whether ABT-199 will retain the potent activity previously demonstrated by ABT-737 and navitoclax in malignancies of more primitive origin, such as acute lymphoblastic leukaemia [51], particularly if such malignant cells also rely on Bcl-x_L

385 for their survival.

386 In conclusion, we undertook detailed studies to examine the mechanism of action of the Bcl-
387 2-selective BH3 mimetic ABT-199. We have delineated the effect of selective Bcl-2 inhibition
388 on non-transformed lymphoid subsets, and demonstrated a potential safety advantage of this new
389 drug over its more promiscuous predecessors, ABT-737 and navitoclax, which also target Bcl-x_L
390 and Bcl-w. However, much work remains to define its optimal clinical niche, including
391 identification of optimal combination regimens.

392

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Conflict of Interest Disclosures

SLK, DM, PB, AWR and DCSH, are employees of Walter and Eliza Hall Institute, which receives commercial income and has received research funding from Genentech and Abbott Laboratories (now Abbvie).

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620

621

Figure legends

Fig 1: Sensitivity of normal and malignant human blood cells to ABT-199, ABT-737 or navitoclax *in vitro*

Samples collected from healthy donors or patients with CLL were treated *in vitro* for 24h with various concentrations of ABT-199, or ABT-737 and/or navitoclax (data pooled for these two drugs as they have similar activity), and analysed by FACS for viability using PI exclusion. The left panel represents the LC50s for ABT-199 or ABT-737 for normal B cells, $CD4^+$ T cells, $CD8^+$ T cells and granulocytes from 9 individual healthy donors. The right panel represents the LC50s for CLL cells, T cells and granulocytes from 42 samples tested against ABT-199 and 53 samples tested against ABT-737 or navitoclax (data pooled for these two drugs as they have similar activity [52]).

Fig 2: Comparing the *in vitro* sensitivity of murine lymphoid subsets to ABT-199 or ABT-737 reveals sub-populations that are Bcl-2 dependent

(**A**) Response of diverse freshly isolated normal mouse B cell lymphoid subsets (presented in approximate ontological order) to the Bcl-2-selective BH3 mimetic ABT-199 (red symbols) and to ABT-737 (black symbols) after 24 h culture *in vitro*. Data shown represent means + 1 SEM from at least three (and up to 14 for the bone marrow subsets) independent experiments, each involving organs from an individual mouse. Differences in sensitivity of each cell type to ABT-199 compared to ABT-737 were analysed by t-test, with correction for multiple comparisons using the Holm-Sidak method (α=0.05). Statistically significant differences are indicated by asterisks (**: p value < 0.01; ***: p value < 0.001).

(**B**) Dose response curves demonstrating the *in vitro* sensitivity of bone marrow B lymphoid subsets to ABT-199 (red symbols) and ABT-737 (black symbols) .

(**C** and **D**) Response of T lymphoid subsets to ABT-199and ABT-737 . Data shown are means $\pm$ 1 SEM from at least three (up to 17 for the thymic subsets) independent experiments.

Note: The *in* vitro sensitivity of the bone marrow and thymic lymphoid subsets to ABT-737 has previously been published [14]. The data is included in this manuscript to allow for direct comparison between ABT-199 and ABT-737, as well as to contrast the responses of peripheral (spleen, blood and lymph node) lymphoid subsets.

Fig 3: ABT-199 triggers the BH3-only protein Bim to initiate Bax/Bak-mediated killing of lymphoid cells

(**A**) ABT-199 triggers Bax/Bak-mediated apoptosis. Lymphoid subsets isolated from WT (n=12), *Bax*$^{-/-}$ (n=3), *Bak*$^{-/-}$ (n=9) or *Bax*$^{-/-}$*Bak*$^{-/-}$ (n=1) mice treated with varying concentrations of ABT-199 *in vitro* for 24 hours and analysed by FACS for cell viability using PI exclusion. Data shown represent means ± 1 SEM from n=1-12 independent experiments. Dose-response curves are shown only for lymphoid subsets (recirculating B cells, CD4^{-}CD8^{-} DN thymocytes, CD4^{+} and CD8^{+} single positive thymocytes) that are most sensitive to ABT-199.

(**B**) Bim is the principal trigger for killing by ABT-199. Primary lymphocytes from *WT* (n=12), *Bim*$^{-/-}$ (n=5), *Noxa*$^{-/-}$ (n=4), *Puma*$^{-/-}$ (n=3), *Bmf*$^{-/-}$ (n=4) mice were treated *in vitro* with ABT-199 and analysed as described above. Data shown represent means ± 1 SEM from n=3-12 independent experiments.

Fig 4: Overexpressing Bcl-2 markedly sensitises normal lymphoid cells and leukaemia cells to ABT-199

(**A**) Mouse foetal liver cells were transduced with GFP expressing retroviral vectors that encode for FLAG-tagged Bcl-2, Bcl-x_L, Bcl-w or Mcl-1, and transplanted into lethally irradiated recipient mice. Bone marrows were harvested following hematopoietic reconstitution eight weeks later and responses of donor (GFP^{+ve}) lymphoid subsets to ABT-199 *in vitro* assessed following 24 h of exposure to the drug.

(**B**, **C**) Eμ-Myc pre-B/B lymphoma and MLL-ENL driven acute myeloid leukaemia (AML) cells bearing *Mcl-1* alleles flanked by *LoxP* sites and oestrogen-receptor regulated Cre were transduced with vectors bearing Bcl-2, Bcl-x_L or no insert and treated with ABT-199 for 24 h, with or without prior tamoxifen to induce deletion of *Mcl-1*.

Fig 5: *In vivo* response of normal B and T cell subsets to ABT-737 or ABT-199

Wild-type C57/BL6 mice were treated for one week with ABT-199 (100mg/kg) daily delivered by oral gavage (n=9), ABT-737 (100mg/kg) by daily intraperitoneal (IP) injections (n=6), or the respective carrier vehicles as controls (n=6 and 9 respectively; normalised to 100%) and then sacrificed. The lymphoid organs and peripheral blood were analysed for cellular composition and cell number. Relative numbers of B (**A**) and T (**B)** cells after 1 week of treatment with ABT-199 or ABT-737 are summarised. Data shown represent means + 1 SEM. Differences between groups were analysed by t-test, with correction for multiple comparisons using the Holm-Sidak method (α=0.05). Statistically significant differences are indicated by asterisks (**: p value < 0.01; ***: p value < 0.001).

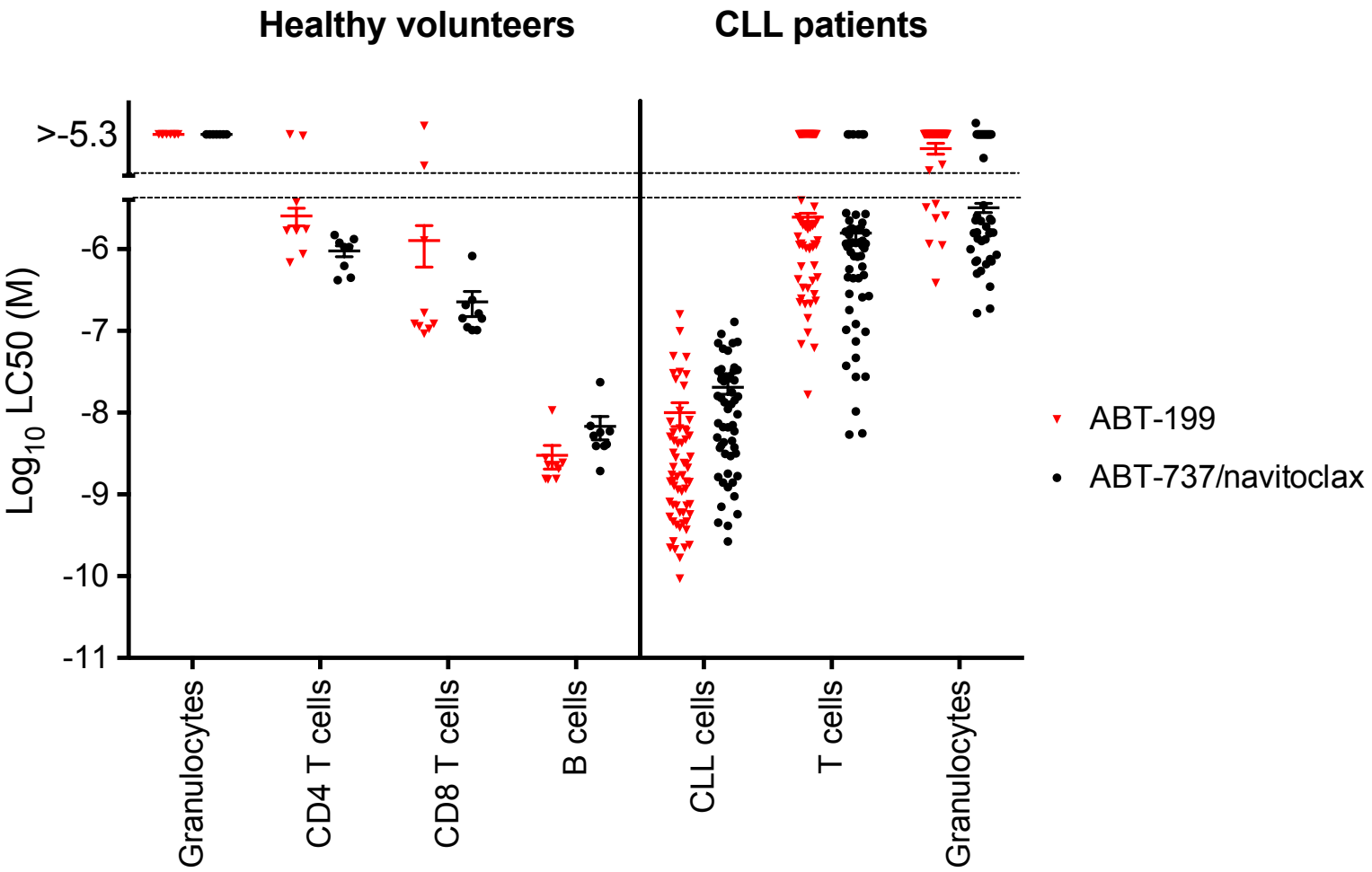

Healthy volunteers
CLL patients
>-5.3
-6
-7
-8
-9
-10
-11
Log10 LC50 (M)
Granulocytes
CD4 T cells
CD8 T cells
B cells
CLL cells
T cells
Granulocytes
ABT-199
ABT-737/navitoclax

Fig. 1

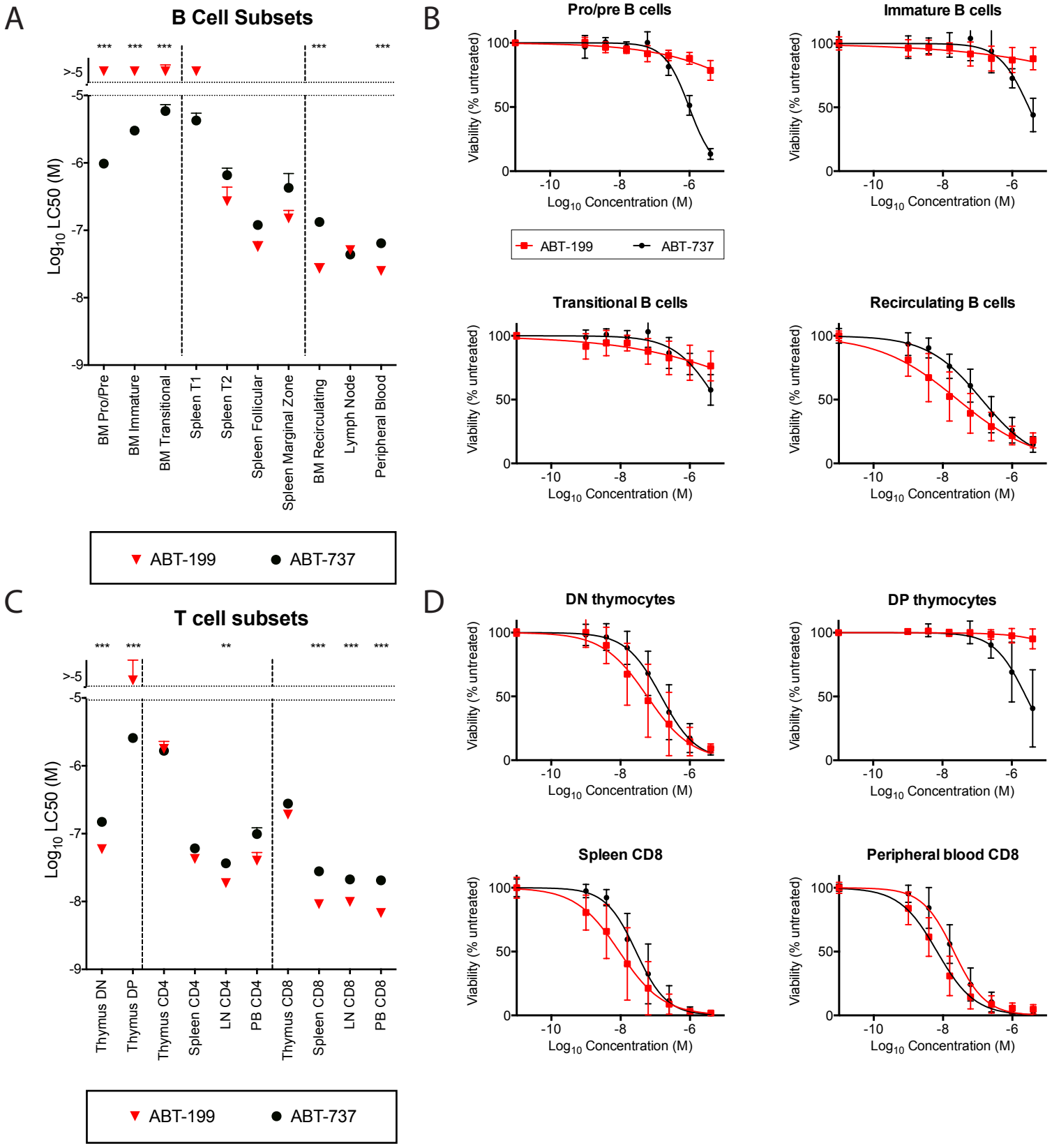

A
B Cell Subsets
Log10 LC50 (M)
BM Pro/Pre
BM Immature
BM Transitional
Spleen T1
Spleen T2
Spleen Follicular
Spleen Marginal Zone
BM Recirculating
Lymph Node
Peripheral Blood
ABT-199
ABT-737
B
Pro/pre B cells
Immature B cells
Transitional B cells
Recirculating B cells
Viability (% untreated)
Log10 Concentration (M)
ABT-199
ABT-737
C
T cell subsets
Thymus DN
Thymus DP
Thymus CD4
Spleen CD4
LN CD4
PB CD4
Thymus CD8
Spleen CD8
LN CD8
PB CD8
D
DN thymocytes
DP thymocytes
Spleen CD8
Peripheral blood CD8

Fig. 2

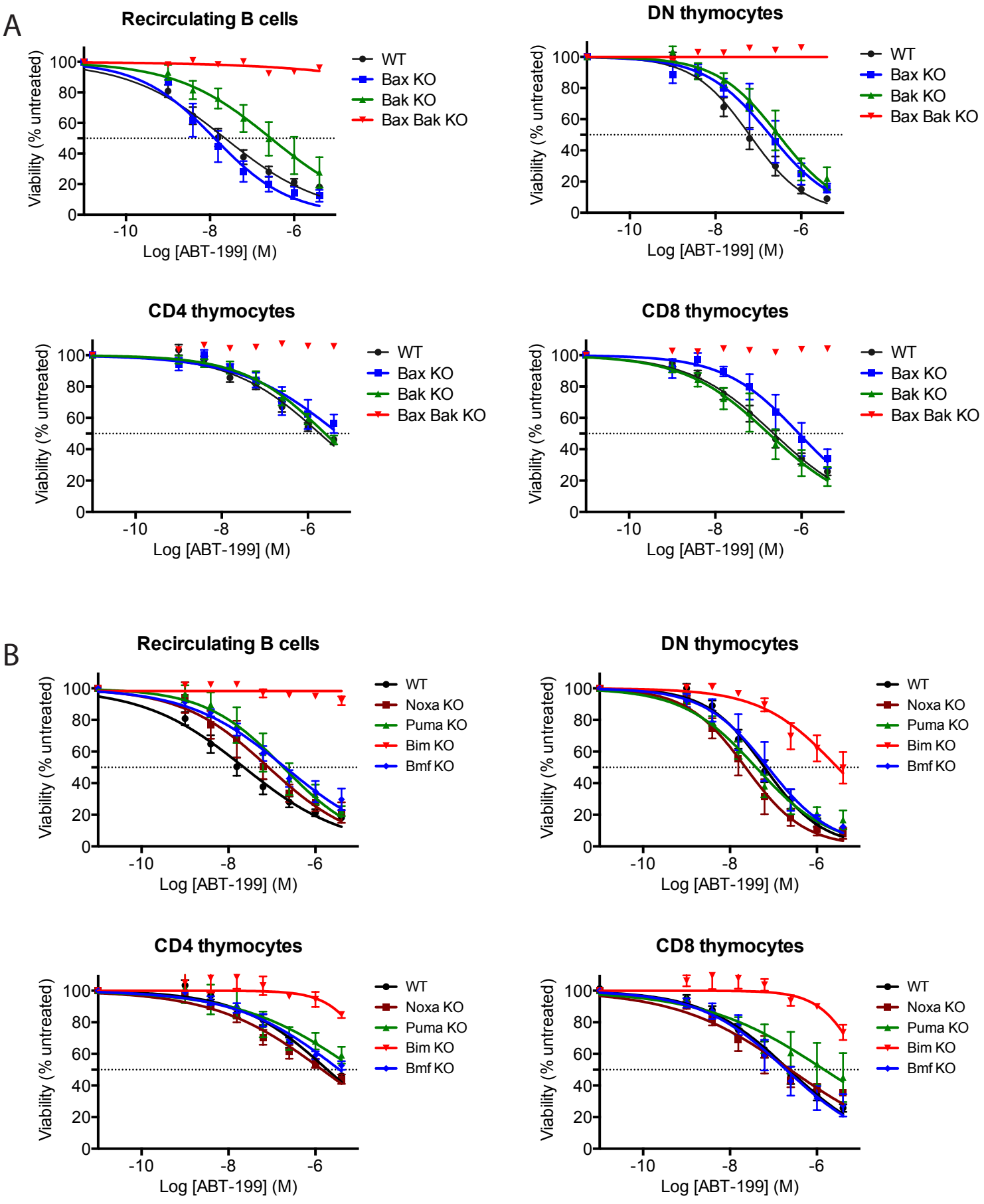
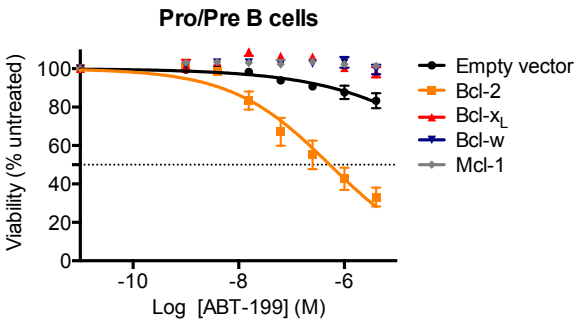
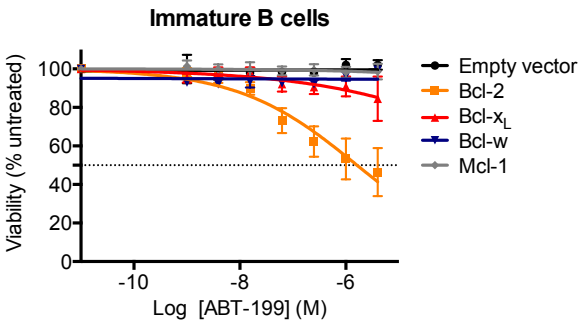
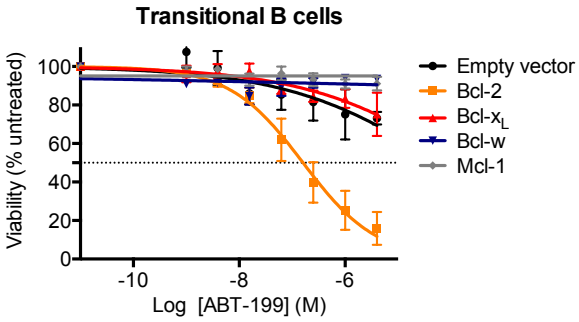

A
Recirculating B cells
DN thymocytes
CD4 thymocytes
CD8 thymocytes
Viability (% untreated)
Log [ABT-199] (M)
WT
Bax KO
Bak KO
Bax Bak KO
B
Recirculating B cells
DN thymocytes
CD4 thymocytes
CD8 thymocytes
Viability (% untreated)
Log [ABT-199] (M)
WT
Noxa KO
Puma KO
Bim KO
Bmf KO

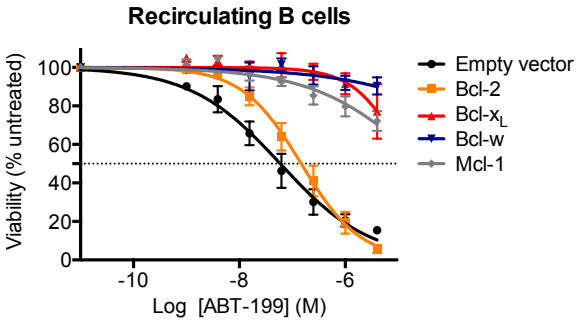
Fig. 3

A

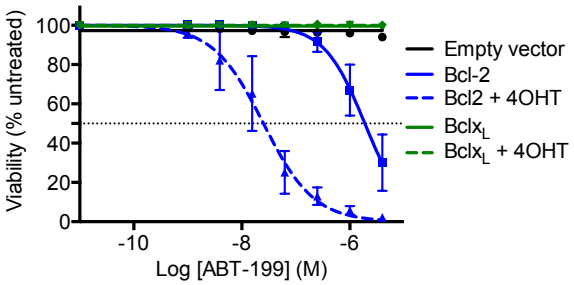

Pro/Pre B cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl-2
Bcl-xL
Bcl-w
Mcl-1


Immature B cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl-2
Bcl-xL
Bcl-w
Mcl-1


Transitional B cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl-2
Bcl-xL
Bcl-w
Mcl-1


Recirculating B cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl-2
Bcl-xL
Bcl-w
Mcl-1

B


Mcl-1fl/fl / Eμ-Myc cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl-2
Bcl2 + 4OHT
BclxL
BclxL + 4OHT

C

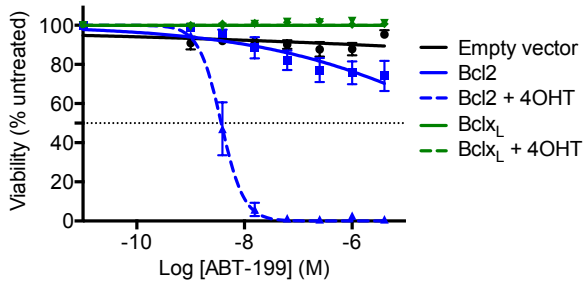

Mcl-1fl/fl / MLL cells
Viability (% untreated)
Log [ABT-199] (M)
Empty vector
Bcl2
Bcl2 + 4OHT
BclxL
BclxL + 4OHT

Fig. 4

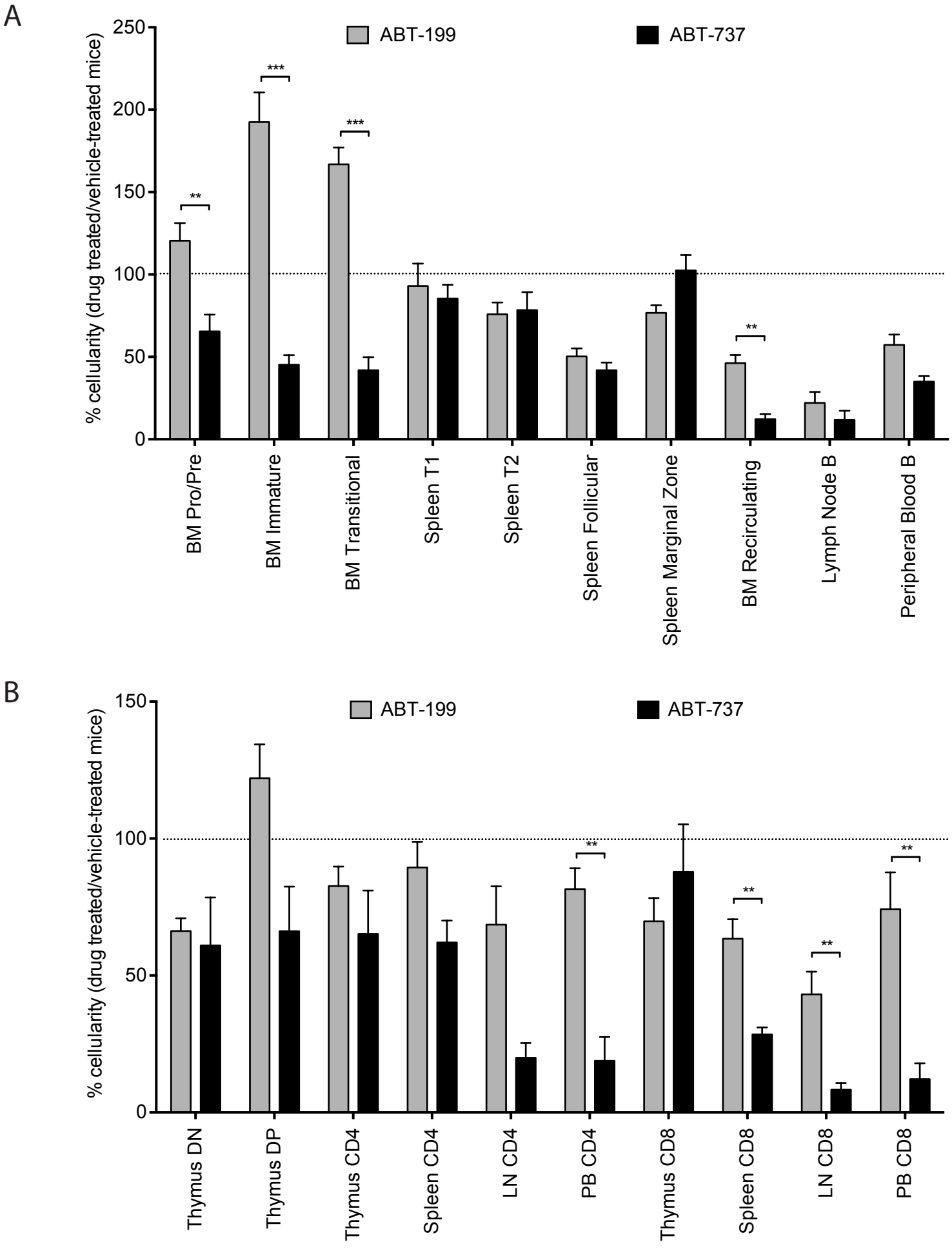
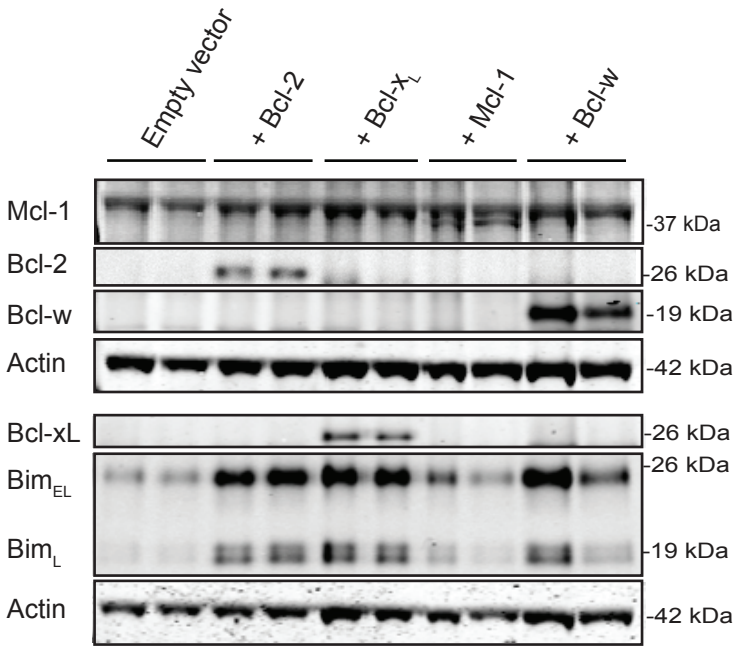
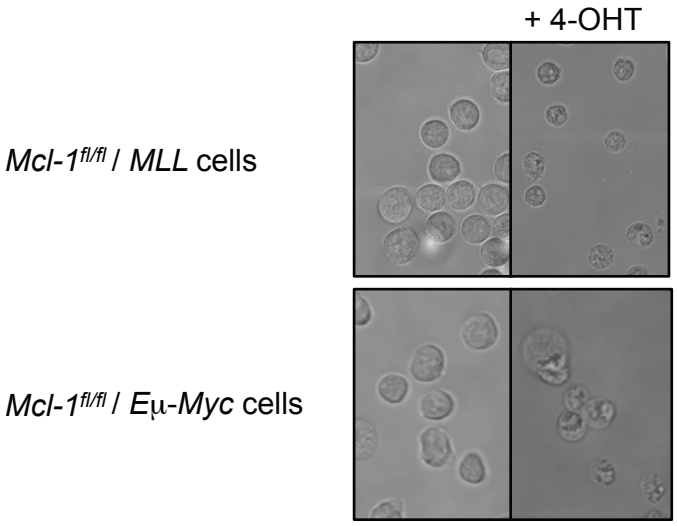

A
ABT-199
ABT-737
% cellularity (drug treated/vehicle-treated mice)
250
200
150
100
50
0
**
***
***
**
BM Pro/Pre
BM Immature
BM Transitional
Spleen T1
Spleen T2
Spleen Follicular
Spleen Marginal Zone
BM Recirculating
Lymph Node B
Peripheral Blood B
B
ABT-199
ABT-737
% cellularity (drug treated/vehicle-treated mice)
150
100
50
0
**
**
**
**
Thymus DN
Thymus DP
Thymus CD4
Spleen CD4
LN CD4
PB CD4
Thymus CD8
Spleen CD8
LN CD8
PB CD8

Fig. 5

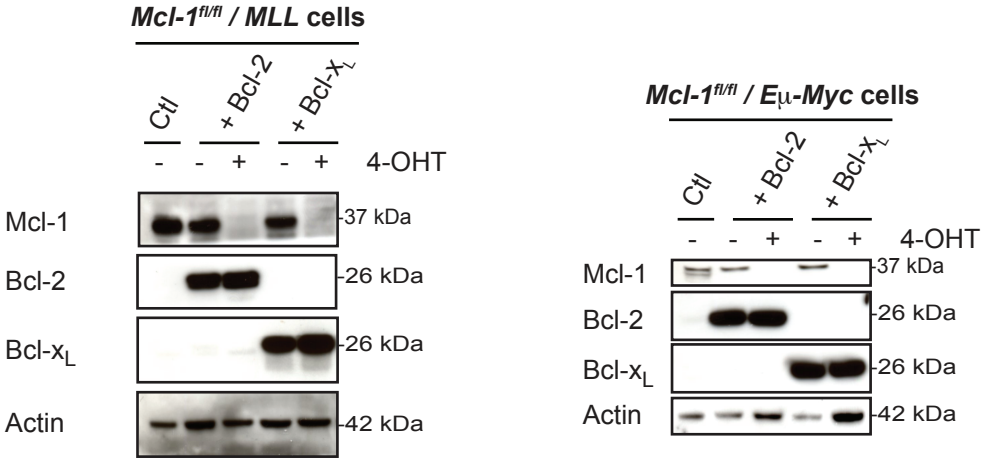


Supplementary Fig. 1

A



B



Supplementary Fig. 2

Supplementary Figure 1:

Western blots of whole spleen lysates prepared from lethally irradiated mice reconstituted with E13.5 foetal liver cells transduced with vectors bearing no insert, Bcl-2, Bcl-x_L, Mcl-1 or Bcl-w were immunoblotted to confirm overexpression of transduced proteins. As demonstrated previously [1], overexpression of each protein was associated with elevated steady state levels of Bim.

Supplementary Figure 2:

(**A**) Light microscope images demonstrating death of Mcl-$1^{fl/fl}$/MLL-ENL/Cre-ER and Mcl-$1^{fl/fl}$/Eμ-Myc/Cre-ER cells following deletion of Mcl-1 induced by addition of 4-hydroxytamoxifen (4-OHT).

(**B**) Western blots demonstrating efficient deletion of *Mcl-1* induced by addition of 4-OHT in Mcl-$1^{fl/fl}$/MLL-ENL/Cre-ER and Mcl-$1^{fl/fl}$/Eμ-Myc/Cre-ER cells retrovirally transduced with Bcl-2 or Bcl-x_L. No cells transduced with the control vector survived deletion of Mcl-1.

Reference

1. Merino D, Khaw SL, Glaser SP, et al. Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood*. 2012;119(24):5807-5816.