

1 **Selective BCL-2 Inhibition by ABT-199 Causes On Target Cell Death in Acute Myeloid Leukemia.**

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42 **Conflict of Interest**

43 A. Letai is an advisor for AbbVie and M. Konopleva has a sponsored research agreement from AbbVie.  
44 Dr. Andreeff serves on the Scientific Advisory Board of Eutropics Pharmaceuticals which once had a  
45 license for BH3 profiling.

46

47 **Abstract**

48 B-cell leukemia/lymphoma 2 (BCL-2) prevents commitment to programmed cell death at the  
49 mitochondrion. It remains a challenge to identify those tumors that are best treated by inhibition of BCL-  
50 2. Here we demonstrate that acute myeloid leukemia (AML) cell lines, primary patient samples, and  
51 murine primary xenografts are very sensitive to treatment with the selective BCL-2 antagonist ABT-199.  
52 In primary patient cells, the median IC50 was approximately 10 nM, and cell death occurred within 2 h.  
53 Our *ex vivo* sensitivity results compare favorably with those observed for chronic lymphocytic leukemia  
54 (CLL), a disease for which ABT-199 has demonstrated consistent activity in clinical trials. Moreover,  
55 mitochondrial studies using BH3 profiling demonstrate activity at the mitochondrion that correlates well  
56 with cytotoxicity, supporting an on target mitochondrial mechanism of action. Our protein and BH3  
57 profiling studies provide promising tools that can be tested as predictive biomarkers in any clinical trial of  
58 ABT-199 in AML.

59

60 **Significance**

61 While targeting BCL-2 has largely been investigated in lymphoid cancers, we present preclinical results  
62 of targeting BCL-2 in AML. These results support clinical testing of the small molecule BCL-2  
63 antagonist ABT-199 in AML, accompanied by testing of predictive biomarkers used in this study.

## 64 **Introduction**

65 Acute myelogenous leukemia (AML) is a hematopoietic neoplasia characterized by the rapid, clonal  
66 growth of the myeloid lineage of blood cells. The disease affects approximately 14,000 adults in the  
67 United States each year and unfortunately, despite recent advances in the treatment of AML, 10,400  
68 people die from their disease (1). Most AML patients become resistant to chemotherapy at some point in  
69 their course and succumb to their disease. Therefore, it is necessary to prevent chemo-resistance or  
70 enhance chemosensitivity in a selective fashion to lead to a higher cure rate and a lower toxic burden.

71 A novel strategy to treat cancer cells is to directly stimulate the mitochondrial apoptotic pathway  
72 in them. The mitochondrial apoptotic pathway is regulated by the B-cell leukemia/ lymphoma 2 (BCL-2)  
73 family of proteins. These proteins respond to upstream apoptotic signals that control mitochondrial outer  
74 membrane permeabilization (MOMP) and the release of cytochrome c, a hallmark of mitochondrial  
75 apoptosis (2). The family consists of pro-apoptotic BH3-only proteins, pro-apoptotic multi-domain  
76 effector proteins and anti-apoptotic proteins which together act as a rheostat to control MOMP. The BH3-  
77 only proteins are further subdivided into two groups based on function - the “activators” (BID, BIM and  
78 PUMA) and “sensitizers” (such as BAD, BIK, HRK and NOXA) (3-5). The activators bind to and inhibit  
79 the anti-apoptotics (such as BCL-2, BCL-XL and MCL-1), and also directly interact with the effector  
80 proteins causing the proteins to oligomerize at the mitochondria and cause MOMP (6, 7). However, the  
81 BH3-only sensitizers can only bind to the anti-apoptotics which allows for the release of the activator and  
82 effector proteins to drive MOMP (3).

83 Navitoclax (ABT-263), which functions as a small molecule mimetic of the BH3 domain of the  
84 BH3-only sensitizer protein BAD, efficiently binds to BCL-2, BCL-XL and BCL-W, releasing bound  
85 pro-apoptotic proteins and causing MOMP in BCL-2 dependent cancer cells (8). In early clinical trials,  
86 navitoclax showed potency in the treatment of chronic lymphocytic leukemia (CLL) and small-cell lung  
87 cancer (9, 10). However, treatment with navitoclax causes on-target, dose-limiting thrombocytopenia  
88 because platelets are dependent on the anti-apoptotic protein BCL-XL for their survival (11). The dose-  
89 dependent thrombocytopenia limited navitoclax’s use in many malignancies, particularly leukemias

90 where patients often present with pre-existing thrombocytopenia. This prompted the development of  
91 ABT-199, a modified BH3-mimetic derivative of ABT-263 which maintains specificity for BCL-2, but  
92 lacks affinity for BCL-XL (12). The remodeled drug has shown cancer killing efficacy in CLL *in vivo*,  
93 myc-driven lymphomas in mice and estrogen receptor-positive breast cancer while sparing platelets (12-  
94 14).

95 AML bulk and stem cells are dependent on BCL-2 for survival and BCL-2 inhibition by ABT-  
96 737 (an *in vitro* tool compound with activity very similar to navitoclax) causes cell death in AML cells  
97 (15). Importantly, BCL-2 inhibition relatively spares normal hematopoietic stem cells which are more  
98 dependent on MCL-1 for their survival (16, 17). Thus, the first goal of the present study is to evaluate the  
99 anti-cancer effects of ABT-199 on AML and compare its efficacy with ABT-737/navitoclax, drugs that  
100 have both shown activity in the *ex vivo* treatment of AML cell lines and AML primary patient samples  
101 and in human clinical trials (15). The second goal is to determine if BH3-profiling can be used as a tool to  
102 predict cellular response to ABT-199 treatment.

103 BH3-profiling is a method to determine the mitochondrial priming level of a cell by exposing  
104 cellular mitochondria with standardized amounts of peptides derived from the BH3 domains of BH3-only  
105 proteins and determining the rate of MOMP, as measured by either cytochrome c release or depolarization  
106 across the inner mitochondrial membrane (18). Previously, we have shown that the priming status of the  
107 cell is predictive of the cell's chemo-responsiveness in that the more primed the cell is, the more sensitive  
108 the cell is to various chemotherapeutics (16, 19). Furthermore, BH3-profiling can also identify anti-  
109 apoptotic addictions (16, 19, 20). For instance, the BAD BH3-only peptide binds with high affinity with  
110 BCL-2, BCL-XL and BCL-W, while the HRK BH3 peptide binds with high affinity only to BCL-XL.  
111 Thus, MOMP following BAD peptide incubation suggests an anti-apoptotic dependency on BCL-2, BCL-  
112 XL or BCL-W, while MOMP following HRK peptide incubation indicated dependency on BCL-XL.  
113 Using this tool, we can identify AML cells which depend on BCL-2 for survival and that are more likely  
114 to die following BCL-2 inhibition. Thus, we hypothesize that cells that are addicted to BCL-2 for survival  
115 will be sensitive to ABT-199 and that we can predict this response by BH3 profiling.

116 **Results**

117 **ABT-199 Kills AML Cell Lines Potently and Quickly *in vitro* and *in vivo***

118 As an initial test of the potential utility of ABT-199 in AML, we exposed AML cell lines to increasing  
119 concentrations of ABT-199 for 48 h and then determined the IC<sub>50</sub> values. Comparisons were made with  
120 ABT-737. As shown in Fig. 1A, the IC<sub>50</sub> of ABT-199 ranged from <10 nM to >1000 nM, and sensitivity  
121 to ABT-737 roughly tracked sensitivity to ABT-199. It is notable that in sensitive AML cell lines (IC<sub>50</sub>  
122 < 0.1 μM), ABT-199 is more potent than ABT-737 (Supplemental Figure S1A and S1B), probably due to  
123 ABT-199's 5-fold higher affinity to BCL-2 protein (12). Prior experience with CLL, a disease for which  
124 excellent clinical activity of ABT-199 has been observed, has revealed that CLL cells are killed in an on-  
125 target fashion, and that the killing was evident within 4 h (25). Therefore, we tested whether ABT-199  
126 could rapidly induce apoptosis in a sensitive AML cell line - MOLM-13. In Figure 1B and 1C, it can be  
127 seen that cell growth is inhibited and cell apoptosis is observed within just a few hours of exposure to  
128 ABT-199.

129 To demonstrate that the efficacy seen was consistent with tolerable *in vivo* delivery of ABT-199,  
130 we tested the effect of ABT-199 on an aggressive mouse xenograft model of MOLM-13. NOD SCID  
131 gamma (NSG) mice were injected with luciferase-labeled MOLM-13 cells and monitored by  
132 bioluminescence imaging (BLI) for tumor development. After confirmation of AML engraftment in the  
133 bone marrow (Figure 1D, day 4), the mice were treated with ABT-199 (100 mg/kg) by daily oral gavage  
134 for 2 weeks. Serial BLI images showed that ABT-199 treatment markedly inhibited leukemia progression,  
135 which translated into prolonged overall survival when compared to vehicle-treated mice (p = 0.0004,  
136 Figure 1E). ABT-199 treated mice also carried significantly lower leukemia burden in bone marrow,  
137 spleen and liver as indicated by hematoxylin and eosin staining (H&E, Figure 1F) and  
138 immunohistochemical analysis of human CD45 (Figure 1G).

139

140 **ABT-199 Sensitivity Correlates with BCL-2 Protein Level**

141 Next we tested whether there were correlates of cell line sensitivity to ABT-199 that supported an on-  
142 target action of killing via competition for the BH3 binding site selectively of BCL-2. Relative levels of  
143 several BCL-2 family proteins were measured by Western blot and densitometry (Figure 2A). Spearman  
144 analysis was performed to evaluate the correlation between IC50 values and protein expression. Levels of  
145 BCL-2 correlated with sensitivity to ABT-199, while levels of BCL-XL inversely correlated with ABT-  
146 199 sensitivity (Figure 2B). Levels of MCL-1 demonstrated a trend to anti-correlation with sensitivity to  
147 ABT-199, but the trend was not statistically significant (Figure 2B). These observations supported the on-  
148 target effects of ABT-199.

149 The OCI-AML3 cell line was relatively insensitive to ABT-199 and ABT-737 (Figure 1A). A  
150 quantitative immunoblot showed that OCI-AML3 cells had high expression of BCL-2 and MCL-1 and  
151 relatively low level of BCL-XL (Figure 2A). If ABT-199 is a BH3 mimetic specific for BCL-2, then  
152 MCL-1 knockdown should significantly sensitize OCI-AML3 cells to this compound. To test this, MCL-1  
153 protein level was reduced by 85% in OCI AML3 cells by lentiviral transduction using a previously  
154 validated MCL1-specific shRNA, without affecting BCL-2 protein expression (Figure 2C). Indeed, MCL-  
155 1 knockdown greatly increased sensitivity to ABT-199 (Figure 2D) as well as to ABT-737 (Supplemental  
156 Figure S2). HL-60 cells with high levels of BCL-2 protein and relatively low BCL-XL and MCL-1  
157 expression are very sensitive to ABT-199 (Figure 1A). BCL-XL overexpression conferred resistance to  
158 ABT-199 in HL-60 cells, while BCL-2 overexpression made HL-60 cells moderately resistant to ABT-  
159 199 (Figure 2E-F). All these results are consistent with a killing mechanism operating via selective  
160 targeting of BCL-2 in AML cells.

161

### 162 **ABT-199-resistant MOLM-13 cells express lower BCL-2 levels compared to the parental cells**

163 Although ABT-199 effectively induced apoptosis in MOLM-13 cells, a fraction of MOLM-13 cells  
164 remained alive after 24 h treatment with 50 nM ABT-199 (Figure 2B). To determine whether surviving  
165 cells represent a sub-clone with low target (Bcl-2) expression levels, we selected ABT-199-resistant

166 MOLM-13 cells by culturing parental cells in medium containing 50nM ABT-199. Intracellular flow  
167 cytometry was performed to measure cellular BCL-2 protein in the parental and ABT199-resistant  
168 MOLM-13 cells. Although no distinct sub-populations were observed, the BCL-2 associated MFI of the  
169 parental MOLM-13 cells distributed between ~1 to ~20 (Supplemental Figure S3A), indicating a  
170 heterogeneity of BCL-2 expression in the parental cells. In sum, resistant cells expressed lower levels of  
171 BCL-2 protein compared to the parental cells (resistant cells: MFI = 3.48; parental cells: MFI = 5.51;  $P <$   
172 0.0001, Supplemental Figure S3B).

173

#### 174 **ABT-199 Operates Selectively on BCL-2 Dependent Mitochondria**

175 If ABT-199 is killing cancer cells via displacement of pro-apoptotic proteins from BCL-2, it should be  
176 operating on mitochondria. As one would expect if this were the case, we observed a correlation between  
177 direct mitochondrial toxicity and cellular toxicity for ABT-199 and ABT-737 in the 12 cell lines studied  
178 in Figure 1A (Figure 3A, B). We also tested whether detection of BCL-2 dependence using  
179 mitochondrial exposure to the BAD BH3 peptide correlated with cellular sensitivity to these agents. We  
180 found that while there was a good correlation between mitochondrial sensitivity to the BAD peptide and  
181 cellular drug sensitivity for the most sensitive cell lines, there was a group of relatively drug -resistant cell  
182 lines that still demonstrated mitochondrial sensitivity to the BAD BH3 peptide (Figure 3 C, D). A clue to  
183 the reason for this was revealed by the tendency of these cell lines to have mitochondria that were also  
184 quite sensitive to the HRK peptide, an indicator of BCL-XL dependence. To ensure that we were  
185 studying BCL-2 dependence specifically, especially in these less drug-sensitive cells, we made a  
186 correction, by subtracting the HRK signal from the BAD signal. In Figure 3E and F, we used this  
187 modified metric to observe a good correlation between mitochondrial BCL-2 dependence and cellular  
188 sensitivity.

189

#### 190 **ABT-199 Efficiently Kills Primary AML Myeloblasts**



191 We observed ABT-199 selectively kills BCL-2 dependent cell lines; therefore, we next wanted to test the  
192 sensitivity of primary patient AML samples to treatment with ABT-199. AML myeloblasts from patient  
193 bone marrow or peripheral blood were exposed to ABT-199 for 48 h in minimum essential medium alpha  
194 supplemented with cytokines. It is notable that the primary cells were quite sensitive, with median IC50  
195 values less than 10 nM (Figure 4A). Note that this is significantly lower than the IC50 observed in the  
196 AML cell lines (Figure 1A). Note also that this is comparable to the sensitivity observed for *ex vivo*  
197 exposure of CLL cells, a disease in which ABT-199 has shown clinical activity in most patients treated  
198 (25).

199 Because prolonged *ex vivo* culture by itself can promote sensitivity to apoptosis of AML  
200 myeloblasts, we wanted to test whether we would see sensitivity at shorter time points as well. Another  
201 potential confounding element is that the ABT class of compounds tend to be highly bound by serum  
202 proteins (26). We found that culturing AML cell lines or primary cells in the absence of serum for 8 h did  
203 not alter the mitochondrial priming or BCL-2 dependence, compared to culture in the presence of serum  
204 (Supplemental Figure S4). Therefore, we tested sensitivity of AML myeloblasts to ABT-199 at 8 h in the  
205 absence of serum (Figure 4B). Clinical and genetic data for both sets of AML samples is available in  
206 Supplemental Table 1. Again, AML myeloblasts proved to be sensitive to ABT-199 with a median IC50  
207 of 20 nm. Indeed, when we reduced exposure times further, to 2 hours, we could still see induction of  
208 apoptosis by ABT-199 in AML myeloblasts (Figure 4C). Similarly rapid induction of cell death has  
209 been observed for the clinically sensitive CLL, consistent with a direct action of ABT-199 on AML  
210 myeloblast mitochondria, promoting apoptosis in the absence of a requirement for additional cell  
211 signaling extrinsic to the mitochondria.

212 Upon testing of additional cryopreserved AML patient samples, including AML cells with diploid  
213 cytogenetics and mutations in *FLT3*, *NRAS*, and *NPM1* genes, 20 out of 25 (80%) were sensitive to ABT-  
214 199 (100 nM), while 5 samples were resistant to both ABT-199 and ABT-737 (Table S2). However,  
215 samples from patients with complex cytogenetics and *JAK2* mutation (n = 9) were largely insensitive to  
216 ABT-199 (1 of the 9, or 11.1% response rate, p = 0.0005 by two-tailed Fisher exact test). Further we

217 found no correlation between ABT-199 sensitivity and FAB classification (Supplemental Figure S5A) or  
218 NPM1 (Supplemental Figure S5B) or FLT-3 mutational status (Supplemental Figure S5C). There was no  
219 difference in ABT-199 sensitivity between samples sensitive or resistant to conventional induction  
220 chemotherapy (Supplemental Figure S5D), consistent with prior findings with ABT-737 (16).

221 We next tested whether sensitivity to ABT-199 correlated with protein expression for primary  
222 AML myeloblasts. As we found with AML cell lines, sensitivity to ABT-199 correlated directly with  
223 BCL-2 expression and inversely with BCL-XL expression as measured by quantitative Western blot  
224 (Figure 4D-E). No significant relationship was observed between MCL-1 level and sensitivity to ABT-  
225 199 in primary AML myeloblasts (Supplemental Figure S6). AML myeloblasts also demonstrate higher  
226 BCL-2 mRNA expression than normal bone marrow (Figure 4F). Higher expression of mRNA for  
227 MCL-1, BCL-XL, and BIM in AML myeloblasts was not observed (Supplemental Figure S7).

228

### 229 **ABT-199 Induces Apoptosis in AML Stem/Progenitor Cells (LSPCs)**

230 We next tested whether ABT-199 is capable of inducing cell death not only in AML blasts, but also in the  
231 phenotypically defined AML stem/progenitor cells characterized by CD34<sup>+</sup>CD38<sup>-</sup>CD123<sup>+</sup>  
232 immunophenotype (27). Samples from six ABT-199-sensitive AML patients with high blast counts were  
233 incubated with ABT-199 or ABT-737 for 24 h, and apoptosis induction was determined by Annexin V  
234 flow cytometry in electronically gated AML blasts (CD45<sup>dim</sup>SSC<sup>low</sup>) and AML stem/progenitor cells  
235 (CD45<sup>dim</sup>SSC<sup>low</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD123<sup>+</sup>). ABT-199 (Figure 4G) and ABT-737 (Supplemental Figure S8)  
236 induced apoptotic cell death in both bulk AML blasts and AML stem/progenitor cells.

237

### 238 **BH3 Profiling Predicts AML Myeloblast Killing by ABT-199**

239 We next tested whether killing of primary AML myeloblasts by ABT-199 acted as a true BH3 mimetic in  
240 an on-target fashion on BCL-2 dependent mitochondria. If this is the case, we would expect that  
241 mitochondria sensitive to the BAD BH3 peptide should also be sensitive to the ABT-199 peptide. Indeed,  
242 we found an extremely tight correlation between mitochondrial sensitivity to BAD BH3 and ABT-199

243 across 30 independent patient samples (Figure 5A). Supporting the on-target effect of this class of drugs,  
244 a similar correlation was found for ABT-263 (Supplemental Figure S9). No such correlation was  
245 observed for the comparison of the BCL-XL selective peptide HRK BH3 and the IC50 of ABT-199,  
246 supporting BCL-2 selective action of ABT-199 (Figure 5B). We observed a weak anti-correlation  
247 between cellular sensitivity to ABT-199 and sensitivity to the MCL-1 selective peptide NOXA BH3  
248 (Figure 5C). This suggests that there is a minor tendency for MCL-1 dependent mitochondria to be less  
249 sensitive to ABT-199.

250 In other diseases, BH3 profiling has proven a useful tool for predicting the cytotoxic effect of  
251 BH3 mimetic small molecules (28, 29). Here we tested whether BH3 profiling using the BAD BH3  
252 peptide predicted cytotoxicity from ABT-199, and found that the correlation was very good (Figure 5D).  
253 In addition, the mitochondrial effect of ABT-199 correlated well with the cytotoxic effect (Figure 5E),  
254 again supporting a direct mitochondrial effect of ABT-199, consistent with a mechanism of action of  
255 direct competition for the BH3 binding site of BCL-2 on mitochondria.

256

### 257 **BH3 Profiling Predicts Response to ABT-199 in an AML Xenograft Model**

258 Tumor xenograft models established by inoculation of cancer cell lines into immunodeficient mice have  
259 been used widely for testing novel therapies. However, cultured tumor cells can undergo changes in their  
260 gene expression patterns after prolonged passage in *in vitro* culture. Therefore, the preclinical results  
261 obtained from patient-tumor derived xenograft (PDX) models may offer superior modeling of the human  
262 disease, especially for testing target-oriented therapies. We have shown that ABT-199 was very effective  
263 in a murine AML cell line xenograft model (Figure 1E). As a more clinically relevant test of ABT-199's  
264 anti-leukemic efficacy *in vivo*, NSG mice were injected with primary AML cells from two different  
265 patients (R and S) and monitored for leukemia engraftment by measurements of human CD45<sup>+</sup> cells in  
266 peripheral blood. After confirmation of AML engraftment, the mice were randomly divided into vehicle  
267 and treatment groups. Treated mice received ABT-199 for 2 weeks, after which all the mice were  
268 sacrificed, and bone marrows were examined for AML tumor burden by human CD45 flow cytometry.

269 FACS analysis showed that 2-wks of ABT-199 treatment significantly reduced leukemia burden in  
270 murine bone marrows in mice injected with cells from patient S (mean,  $70 \pm 16\%$  human CD45<sup>+</sup> cells in  
271 bone marrow of control mice ( $n = 9$ ) and  $32.7 \pm 12\%$  in ABT-199 treated mice ( $n = 11$ ,  $p = 0.0004$ ,  
272 Figure 6A). We did not observe a decrease in tumor burden in mice injected with cells from patient R  
273 (mean  $70.3 \pm 8.1\%$  human CD45<sup>+</sup> cells in bone marrow of control mice ( $n = 8$ ) and  $74.3 \pm 6.4\%$  in ABT-  
274 199 treated mice ( $n = 8$ ,  $p = 0.1930$ , Figure 6B).

275         Since we observed a difference in response in the xenograft model following ABT-199 treatment,  
276 we asked whether the response to ABT-199 could be predicted by BH3 profiling. In blinded fashion, pre-  
277 treatment AML myeloblasts from each model were subjected to BH3 profiling in which mitochondria  
278 were exposed to the BAD BH3 peptide as well as ABT-199 itself. We found that mitochondria from  
279 AML myeloblasts from patient S released more cytochrome c following incubation with the BAD peptide  
280 or ABT-199 compared to patient R (Figure 6C). These results provide evidence that ABT-199 kills AML  
281 myeloblasts by the expected mechanism of inhibition of mitochondrial BCL-2. Furthermore, these results  
282 suggest that BH3 profiling might predict the response of AML primary cells to ABT-199 *in vivo*.

283

284

285 **Discussion**  
286

287 Relapsed AML is a difficult cancer to treat effectively; therefore, there is need for improved treatment  
288 options for refractory AML. Here, we show that selective inhibition of BCL-2 by ABT-199 kills AML  
289 cell lines and primary patient cells both *ex vivo* and in *in vivo* mouse xenografts as a single agent in the  
290 low nano-molar range. The concentrations used in our studies here are in the 0.001-1  $\mu\text{g/ml}$  range, a  
291 range readily achievable in clinical trials where serum concentrations of 3-4  $\mu\text{g/ml}$  have been observed  
292 (25). Moreover, the drug acts very quickly *in vitro*, killing cells within 2 hours of drug exposure. We also  
293 show that as in CLL, ABT-199 functions on-target at the mitochondria. This is consistent with the  
294 observation that AML myeloblasts from chemorefractory patients showed no difference in their BCL-2  
295 dependence, as measured by BH3 profiling, or sensitivity to ABT-199 compared to chemosensitive cells  
296 (16).

297 Our *in vitro* results suggest there will be heterogeneity in clinical response (IC50s ranged from  
298 0.43 to >1000 nm), so that a predictive biomarker would be of great utility. Here we present four methods  
299 that may be predictive of clinical response to ABT-199. The first method is cytogenetics. The cellular  
300 death response to ABT-199 appears to be largely independent of cytogenetic and genetic mutation status,  
301 except perhaps for complex karyotype and *JAK2* mutant patients, suggesting that treatment with ABT-199  
302 could be useful for patients who have poor prognostic factors. The utility of cytogenetics as a more  
303 general predictive biomarker for response to ABT-199 needs to be examined across many more samples.

304 A second method is *ex vivo* short term culture of the primary patient samples with ABT-199. The  
305 disadvantage of this method is that it is difficult to reliably culture primary AML cells for the requisite  
306 time frame to observe cell death. We observed that even after a short 8 h culture there could be upwards  
307 of 60% spontaneous apoptotic death in the control un-treated primary AML cells. Therefore, it would not  
308 be ideal to rely on an *in vitro* cell death assay where many samples could be potentially lost due to  
309 spontaneous cell death during culture.

310           The third predictive biomarker method is to measure BCL-2 levels by Western blot. We show  
311 that increased expression of BCL-2 is associated with increased sensitivity to ABT-199. However, given  
312 the complex interactions of the BCL-2 family members, individual measurements of the various anti-  
313 apoptotics alone may not provide accurate data on the *in vivo* biology of the anti-apoptotic dependencies  
314 in AML. Many of the BCL-2 family members are regulated by post-translational modifications and  
315 interactions with other proteins. These types of interactions are difficult to capture in static Western blot  
316 measurements.

317           The fourth method, BH3 profiling, may prove useful as a predictive biomarker. BH3 profiling is a  
318 functional assay which accounts for the relative amounts and interactions of all of the BCL-2 family  
319 members. We show here that the mitochondrial response to the BAD peptide as well as mitochondrial  
320 ABT-199 correlates with the *ex vivo* drug treatment. Most significantly, BH3 profiling could discriminate  
321 *in vivo* sensitivity of human AML cells to ABT-199 (Figure 6). Thus, we may be able to use BH3  
322 profiling of pre-treatment AML samples to direct ABT-199 treatment to AML cases that are most BCL-2  
323 dependent. While the assay is less familiar to many, it is a straightforward protocol using reagents and  
324 equipment available in most clinical and research laboratories. Moreover, results are available the same  
325 day the sample is acquired. As for the other putative biomarkers, empiric testing in the clinical setting is  
326 the only way to truly validate BH3 profiling as a useful predictive biomarker.

327           Although we focused largely on the expression of BCL-2 and BCL-XL in AML, we also detected  
328 the expression of MCL-1. It has been recently reported that the anti-apoptotic MCL-1 is necessary for the  
329 development and sustained growth of AML (30). Since ABT-199 does not inhibit MCL-1, increased  
330 expression of MCL-1 could be a potential source of upfront resistance to BCL-2 inhibition by ABT-199.  
331 However, we show that the majority of AML patient samples tested did not show MCL-1 dependence (as  
332 indicated by the NOXA response). This suggests that although MCL-1 may be necessary for the  
333 development of AML, most cases of AML may not depend on MCL-1 for survival as much as on BCL-2.  
334 Indeed, in a pertinent direct comparison, we have found that most AML myeloblasts are more BCL-2

335 dependent and less MCL-1 dependent than HSC, though there are about 20% exceptions (16). It may well  
336 be that dependence on individual anti-apoptotic proteins varies with myeloid differentiation state.

337 BCL-2 was discovered in lymphoid cancer cells, and much of the research on this protein has  
338 been conducted in lymphoid cells, where it is highly expressed (31). It is therefore understandable that  
339 clinical testing of ABT-199 has so far focused on lymphomas and CLL. Here we demonstrate that  
340 selective, on-target BCL-2 inhibition using a clinically active drug is a promising avenue for clinical  
341 investigation in the myeloid malignancy AML. It is particularly important to recognize that even AML  
342 myeloblasts that are resistant to conventional therapies appear to be quite sensitive to BCL-2 inhibition.  
343 Thus, BCL-2 inhibition by ABT-199 offers hope to those AML cases that most need novel therapeutic  
344 intervention. Our results strongly support the testing of ABT-199 for treatment of AML patients as the  
345 majority of patient samples were sensitive to the drug in *ex vivo* culture. Furthermore, our results support  
346 the testing of BH3-profiling as a predictive biomarker for ABT-199 response in the clinic.

347

348 **Methods**

349 **Cell lines**

350 The AML cell lines were purchased from the American Type Culture Collection (Manassas, VA) or  
351 Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or were kindly  
352 provided by Dr. James Griffin (Dana-Farber Cancer Institute, Boston, MA). HL-60, MOLM-13, OCI-  
353 AML2, OCI-AML3, KG-1, U937 and Kasumi-1 were validated in September 2010 by short tandem  
354 repeat DNA fingerprinting using the AmpFISTR Identifiler kit according to manufacturer's instructions  
355 (Applied Biosystems). HL-60, MOLM-13, OCI-AML2, OCI-AML3, KG-1, U937 and Kasumi-1 were  
356 validated in September 2010 by short tandem repeat DNA fingerprinting using the AmpFISTR Identifiler  
357 kit according to manufacturer's instructions (Applied Biosystems). HL-60 cell lines with stable  
358 overexpression of BCL-2 or BCL-XL, and the control cell line with empty vector, were kindly provided  
359 by Dr. Kapil N. Bhalla (The Methodist Hospital Research Institute, Houston, TX). AML cell lines were  
360 cultured in RPMI 1640 medium supplemented with 10% or 20% fetal bovine serum, 10mM L-glutamine,  
361 100 U/ml penicillin and 10 mg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of  
362 5% CO<sub>2</sub>.

363 **Treatment of AML Cell Lines with ABT-737 and ABT-199**

364 AML cells were incubated for appropriate time in RPMI 1640- medium supplemented with 10% FBS and  
365 titrated concentrations of ABT-199 or ABT-737. Viable AML cells were enumerated by flow cytometry  
366 using counting beads with concurrent Annexin-V and propidium iodide (PI) staining. IC50 values were  
367 calculated using Calcsyn software (Biosoft, Cambridge, MA) based on the number of live cells  
368 (Annexin-V/PI). For specific details, see Supplemental Methods.

369 **Quantitative Western Blot**

370 Cell lysates were prepared and immunoblotted as previously described (22). Antibodies used for  
371 quantitative Western blot were: BCL-2 (#M0887, Dako, Carpinteria, CA), BCL-XL (# 2764, Cell  
372 Signaling Technology, Danvers, MA), MCL-1 (#559027, BD Biosciences, San Diego, CA),  $\alpha$ -Tubulin  
373 and  $\beta$ -Actin (loading controls, #T6199 and #A5441, Sigma-Aldrich, St. Louis, MO). Blots were scanned



374 with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The band intensity was  
375 quantified using Odyssey software v2.0. The ratio of band intensity of BCL-2 proteins relative to that of  
376 loading control was normalized to the ratio in untreated OCI-AML3 cells.

### 377 **Gene Knockdown by shRNA**

378 MCL1 was knocked down by lentiviral transduction using a MCL1-specific shRNA transfer vector  
379 targeting residues 2421-2440 on RefSeq NM\_021960.4. Lentivirus was prepared by co-transfection of  
380 HEK293T cells (ATCC) with an equal molar mix of transfer vector and packaging plasmid (psPAX2 and  
381 pMD2.G, Addgene, Cambridge, MA) using JetPrime transfection reagent as directed by the manufacturer  
382 (Polyplus, Illkirch, France). Fresh lentiviral supernatants were passed through 0.45  $\mu$ m surfactant-free  
383 cellulose acetate membranes; polybrene was added to 8  $\mu$ g/mL, and the virus stock was used at once to  
384 spinoculate OCI-AML3 cells as described before (23). Infected cells were selected with 0.5  $\mu$ g/mL  
385 puromycin. In parallel, control cells were transduced using lentivirus delivering a hairpin targeting GFP in  
386 pLKO.1 (Addgene). Knockdown was verified by Western blot analysis.

### 387 **Selection of Resistant MOLM-13 Cells and Measurements of BCL-2 Protein by Intracellular Flow** 388 **Cytometry**

389 Resistant MOLM-13 cells were selected in RPMI 1640 medium supplemented with 10% FBS and 50 nM  
390 of ABT-199. Every two days, the cells were pelleted by centrifugation and resuspended in fresh medium  
391 with 50 nM ABT-199. Cell viability was monitored by Vi-CELL viability analyzer (Beckman Coulter,  
392 Irving, TX) until MOLM-13 cells reached a viability higher than 90%. The cellular content of BCL-2  
393 protein was measured in both parental and resistant MOLM-13 cells by flow cytometry. Briefly, 1 million  
394 highly viable cells were washed twice with PBS and fixed in 1 mL of 4% paraformaldehyde for 15  
395 minutes on ice, followed by washing with PBS and permeabilization with 1 mL of 0.1% Triton X-100 in  
396 PBS-buffered bovine serum albumin (BSA, 1% w/v). After incubation on ice for 10 minutes, cells were  
397 then washed with washing buffer (1% BSA in 1 x PBS), resuspended in 90  $\mu$ L of washing buffer and  
398 stained with 10  $\mu$ L of FITC-conjugated BCL-2 antibody or FITC-conjugated IgG1 isotype control

399 (#F7053 and #X0927, DAKO, Carpinteria, CA). After incubation in dark at room temperature for 30  
400 minutes, the cells were washed again with washing buffer and analyzed by flow cytometry using  
401 Gallios™ flow cytometer (Beckman Coulter). The intensity of BCL-2-associated fluorescence was  
402 measured on a logarithmic scale. For each sample, 20,000 cells were analyzed for Median Fluorescence  
403 Intensity (MFI) using Kaluza flow analysis software (Beckman Coulter).

#### 404 **BH3 Profiling of Cell Lines**

405 AML cell lines were seeded at a density of  $4 \times 10^5$  cells/mL in 10% FBS RPMI media supplemented with  
406 10 mM L-glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin 24 h before BH3 profiling. Two  
407 million cells of each cell line were pelleted at 400 xg for 5 minutes at RT and resuspended in 2 mL DTEB  
408 (135 mM Trehalose, 10 mM HEPES-KOH, 0.1% w/v BSA, 20 uM EDTA, 20 uM EGTA, 50 mM KCl, 5  
409 mM succinate, final pH 7.4). Cell lines were profiled by using the plate-based JC-1 BH3 profiling assay  
410 previously described (16). Cells were permeabilized with digitonin, exposed to BH3 peptides, and  
411 mitochondrial transmembrane potential loss was monitored using the ratiometric dye JC-1.

#### 412 **Isolation and Treatment of Primary AML Cells with ABT-263, ABT-737 and ABT-199**

413 Primary AML cells were obtained by informed consent from the Dana-Farber Cancer Institute, Leukemia  
414 Group, the Pasquarello Tissue Bank at the Dana-Farber Cancer Institute, the University of Texas MD  
415 Anderson Cancer Center, Leukemia Tissue Bank Shared Resource from the Ohio State University  
416 Comprehensive Cancer Center and the Germany-Austrian Study Group according to protocols approved  
417 by the Institute's Institutional Review Board. Samples were Ficoll purified, used immediately or viably  
418 frozen in 90% FBS/10%DMSO.

419 Fresh (Figures 4A, 4D and 4E) or thawed (Figures 4B, 5) mononuclear cells were resuspended in  
420 culture medium supplemented with cytokines. Cells were treated with ABT-199, ABT-263 or ABT-737  
421 for appropriate time. Cells were then washed with PBS and resuspended in Annexin binding buffer. Cell  
422 viability was assessed by FACS analysis following concurrent Annexin V and PI (or 7-AAD) staining.  
423 For more detailed methods, see Supplemental Methods.

#### 424 **Apoptosis of AML Stem/Progenitor Cells**

425 AML mononuclear cells were isolated by Ficoll density centrifugation and cultured with 100 nM ABT-  
426 199 or ABT-737 as described above. After 24 h, AML cells were washed twice in Annexin binding buffer  
427 (ABB) and resuspended in 100  $\mu$ L ABB containing 1:100 dilution of Annexin-V-APC (#550475), 1:50  
428 dilution of CD45-APC-Cy7 (#557833), CD34-FITC (#555821), CD38-PE-Cy7 (#335790) and CD123-  
429 PercP-Cy5.5 (#58714) (all from BD Biosciences) for 20 minutes at room temperature in dark. Following  
430 staining, cells were washed with ABB and resuspended in 95  $\mu$ L ABB containing 5  $\mu$ L DAPI. Cells were  
431 analyzed by Gallios Flow Cytometer (Beckman Coulter). Results were expressed as percentage of  
432 specific apoptosis calculated as: (% AnnV+ cells sample-%AnnV+ cells control)/(100-%AnnV+ cells  
433 control).

434

#### 435 ***In vivo* Study of ABT-199 Efficacy in AML Mouse Models**

436 All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal  
437 Care and Use Committees at the University of Texas MD Anderson Cancer Center. Twenty female NOD  
438 SCID gamma (NSG) mice (6-wk old, Jackson Laboratory, Bar Harbor, MA) were intravenously injected  
439 with luciferase-labeled MOLM-13 cells ( $0.7 \times 10^6$  cells/100  $\mu$ L) and randomly divided into two groups.  
440 Four days post injection, the mice were treated with vehicle or ABT-199 (100 mg/kg body weight) daily  
441 by oral gavage for 2 weeks. For oral dosing, ABT-199 (10 mg/mL) was formulated in 60% phosal 50  
442 propylene glycol, 30% polyethyleneglycol-400, and 10% ethanol. Bioluminescence imaging (BLI) was  
443 used to monitor tumor burden on different time points. Briefly, mice were anaesthetized and injected  
444 intraperitoneally with firefly luciferase substrate D-luciferin and then imaged noninvasively using IVIS-  
445 200 *in vivo* imaging system (PerkinElmer, Waltham, MA). Three mice from each group were sacrificed  
446 by CO<sub>2</sub> asphyxiation after 15 d. Bone marrow, spleen, and liver were collected for H&E and  
447 immunohistochemical staining. The remaining seven mice in each group were followed for survival.

448 For primary AML derived xenograft models, NSG mice were sub-lethally irradiated (250 cGy)  
449 the day prior to intravenous injection of  $10^5$  PDX21 patient-derived AML cells. Three weeks following  
450 injection and after confirmation of AML engraftment, the mice were randomly divided into two groups  
451 and treated with 100 mg/kg ABT-199 or vehicle via gavage daily for 2 weeks. All mice were then  
452 sacrificed and femur bone marrows were analyzed for leukemia burden by CD45 flow cytometry (using  
453 anti-human CD45-PE antibody #555483, BD Biosciences, San Jose, CA).

#### 454 **Immunohistochemistry Analysis**

455 Immunohistochemistry was performed as described previously (24). Briefly, the tissue was formalin-  
456 fixed, paraffin-embedded, sectioned into 5- $\mu$ m thickness and mounted onto microscope slides. Tissue  
457 sections were then deparaffinized and rehydrated using xylene and ethanol in decreasing concentration.  
458 Samples were stained with hematoxylin and eosin (H&E) for histopathological evaluation. For  
459 immunohistochemical staining, the tissue sections were incubated with primary antibody against human  
460 CD45 (#555480, BD Biosciences, San Jose, CA ), followed by sequential incubation with biotinylated  
461 secondary antibody, peroxidase labeled streptavidin and 3,3' diaminobenzidine tetrahydrochloride/H<sub>2</sub>O<sub>2</sub>  
462 (Dako), which resulted in a brown precipitate at the antigen site. Images were taken using an optical  
463 microscope under the same magnification.

#### 464 **Microarray-Based Gene Expression Profiling in AML**

465 The expression of BCL-2 family genes was determined using oligonucleotide microarrays (HG-U133  
466 Plus 2.0, Affymetrix) in 288 AML samples comprising all cytogenetic groups, and in 103 normal samples  
467 (healthy BM and non-leukemia conditions) as described in Haferlach et al (21). All samples in this study  
468 were obtained from untreated patients at the time of diagnosis. Cells used for microarray analysis were  
469 collected from the purified fraction of mononuclear cells after Ficoll density centrifugation. The study  
470 design adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committees of  
471 the participating institutions before its initiation. The analysis is conducted at logarithm-2 transformed  
472 gene expression intensities. Correlation analysis based on Pearson correlation coefficient and Spearman's  
473 rank correlation coefficient was performed to identify probe sets that have consistent expression pattern

474 corresponding to a common gene. Two-sample t-test was performed for each two-group comparison, and  
475 the *P* value threshold of 0.005 was used to moderately control for multiple testing.

#### 476 **iBH3 of Primary AML Patient Cells**

477 Thawed cells were washed 1x with PBS and stained with 1:100 Invitrogen Live/Dead – near IR stain  
478 (#10119, Life Technologies, Grand Island, NY) in FACS Buffer (2% FBS PBS, 1:100) for 20 min on ice,  
479 washed with FACS buffer and subsequently stained with CD45-V450 (#642275; BD Biosciences; San  
480 Jose, CA) 1:100 FACS buffer on ice for 20 min. Cells were pelleted at 400 x g for 5 min at RT and  
481 resuspended in DTEB. 100 uL of cells in DTEB was added to each tube containing twice the final  
482 concentration of each peptide treatment in 100 uL of DTEB with 0.002% w/v digitonin. Mitochondria in  
483 the permeabilized cells were exposed to peptides for 60 minutes at RT before the addition of 200 uL 4%  
484 v/v formaldehyde at RT for 15 minutes, quenched with 50 uL of 100 mM Tris / 2.5 M glycine pH 8.2 for  
485 5 minutes at RT, and pelleted at 1500 xg for 5 minutes at RT. Cells were stained with anti-cytochrome c-  
486 Alexa488 (#560263, BD Bioscience) 1:100 in 0.1% Saponin/1%BSA/PBS overnight at 4°C and diluted  
487 1:5 in PBS an hour before FACS on a LSR Fortessa flow cytometer (BD Bioscience) to quantify  
488 cytochrome c loss calculated from the median fluorescence intensity (MFI) as

$$489 \quad \% \text{ cyto c loss} = 100 \times \left( 1 - \frac{[\text{MFI}_{\text{sample}} - \text{MFI}_{\text{isotype}}]}{[\text{MFI}_{\text{DMSO}} - \text{MDI}_{\text{isotype}}]} \right)$$

490

491 AML blasts were identified by low-mid CD45/low SSC-A.

#### 492 **Statistical analysis**

493 Statistical analyses were performed using GraphPad Prism software v6.0 (GraphPad, La Jolla, CA).

494 Unless otherwise indicated, the results are expressed as the mean ± standard error of the mean (SEM)  
495 from at least three independent experiments. Differences with *P* values ≤ 0.05 were considered  
496 statistically significant.

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### Figure legends

**Figure 1. Selective inhibition of BCL-2 by ABT-199 kills AML cell lines quickly and effectively.**

A). AML cell lines were treated with ABT-199 or ABT-737 for 48 h. Calcsyn software was used to calculate the IC50 values based on the number of viable cells (i.e., Annexin V/PI) determined by FACS analysis. B). MOLM-13 AML cells were treated with indicated concentrations of ABT-199. Apoptosis induction was determined by Annexin V/PI flow cytometry. C). Viable (i.e., Annexin V/PI) cell counts were quantified by FACS analysis using CountBright counting beads. D). Serial bioluminescence images of mice bearing MOLM-13 tumors treated with the vehicle or ABT-199 (treatment started on day 4, administered by oral gavage at dose of 100 mg/kg). E). Kaplan-Meier survival curves for mice treated as described in E (n = 7 per arm). Statistical significance was calculated using Log-rank (Mantel-Cox) test (p < 0.0004 ). F). H&E staining of histological sections of liver, spleen, and bone marrow 15 d post leukemia cell injection. Age- and sex-matched mice without tumor were used as controls. Representative MOLM-13 cells are indicated by arrows. Representative engraftment areas are circled in green. All pictures were taken under the same magnification; scale bar equals 50  $\mu$ m. G). Immunohistochemical staining of histological sections of liver, spleen, and bone marrow with human CD45 antibody 15 d post leukemia cell injection. Scale bar equals 50  $\mu$ m.

**Figure 2. Sensitivity to ABT-199 positively correlates with endogenous BCL-2 protein level and negatively correlates with BCL-XL protein level in AML cell lines.**

A) Western blot analysis of BCL-2 family proteins in untreated AML cells. The band intensity was quantified using Odyssey v2.0 software, and displayed numerically as a ratio of the band intensity detected in the OCI-AML3 cells. B) Significant correlations were observed between ABT-199 IC50 values and BCL-2/BCL-XL protein levels. The non-parametric one-tailed Spearman test was used to determine the correlation coefficient. The p values provided are nominal p values not corrected for



615 multiple comparisons. C). MCL-1 knockdown by 85% was achieved by lentiviral shRNA. D). MCL-1  
616 knockdown significantly sensitized OCI-AML3 cells to ABT-199. E). Western blot analysis showing HL-  
617 60 AML cells transfected to stably overexpress BCL-XL or BCL-2. F). Overexpression of BCL-XL or  
618 BCL-2 in HL-60 cells confers complete resistance to ABT-199-induced apoptosis.

619

620 **Figure 3. ABT-199 functions selectively on BCL-2 dependent mitochondria in AML cell lines.**

621 A). The IC<sub>50</sub> values of AML cell lines treated with ABT-737 from Figure 1A were correlated with the  
622 mitochondrial response of ABT-737 (1 $\mu$ M). Mitochondrial response was measured by JC1 based BH3  
623 profiling. B). IC<sub>50</sub> values of cell lines treated with ABT-199 from Figure 1A were correlated with the  
624 mitochondrial response of mitochondrial ABT-199 (0.1 $\mu$ M). C). IC<sub>50</sub> values of AML cells treated with  
625 ABT-737 were correlated with the response to the BAD BH3 (80 $\mu$ M). The mitochondrial responses to the  
626 BAD BH3 peptide were measured by JC1 based BH3 profiling. D). IC<sub>50</sub> values of AML cell treated with  
627 ABT-199 from Figure 1A were correlated with the mitochondrial response of the BAD BH3 (80 $\mu$ M)  
628 peptide. E). IC<sub>50</sub> values of AML cells treated with ABT-737 were correlated with the response to the  
629 BAD BH3 (80 $\mu$ M) – HRK BH3 (80 $\mu$ M). The mitochondrial responses to the BAD and HRK BH3  
630 peptides were measured by JC1 based BH3 profiling. F). IC<sub>50</sub> values of AML cells treated with ABT-199  
631 from Figure 1A were correlated with the mitochondrial response of the BAD BH3 (80 $\mu$ M) – HRK BH3  
632 (80 $\mu$ M) peptide. Statistical correlation was performed using a one-tailed Spearman r using GraphPad  
633 Prism 6.

634

635 **Figure 4. ABT-199 efficiently kills primary AML myeloblasts as a single agent.**

636 A). IC<sub>50</sub> determination for ABT-199 and ABT-737 treatment of primary AML samples. Fresh  
637 mononuclear cells from AML patients were isolated from bone marrow or peripheral blood and treated  
638 with ABT-199 and ABT-737 for 48 h. The IC<sub>50</sub> values were calculated based on viable (i.e., Annexin V<sup>-</sup>  
639 /PI<sup>-</sup>) cell numbers determined by FACS analysis. Samples with ABT-199 IC<sub>50</sub> < 0.1  $\mu$ M were defined as

640 “sensitive”, while those with ABT-199 IC<sub>50</sub> > 1 μM were defined as “resistant”. B). Frozen primary  
641 AML myeloblasts were thawed treated with ABT-199 and ABT-263 for 8 h in the absence of fetal bovine  
642 serum. Viability was assessed by Annexin-/PI- via FACS analysis and IC<sub>50</sub> values were calculated using  
643 GraphPad Prism software. C). Thawed primary AML samples were treated for 2 h with 1-1000 nM of  
644 ABT-199 and viability was assessed by Annexin V-PI- by FACS analysis D). Nonparametric Spearman  
645 correlation analysis shows a significant (p = 0.017) negative correlation between ABT-199 IC<sub>50</sub> values  
646 and BCL-2 protein levels. E) A non-significant (p = 0.069) positive correlation was observed between  
647 ABT-199 IC<sub>50</sub> values and BCL-XL protein levels. F). Boxplots represent the quartiles and range of log<sub>2</sub>  
648 values of mRNA expression for BCL-2 genes in different subgroups of AML and normal bone marrows.  
649 The median is indicated by the black line in each box. Numbers on top indicate number of patients in each  
650 specified subgroup. Differences in gene expression with P values ≤ 0.005 were considered statistically  
651 significant, as denoted by \*. G). Patient AML samples treated with 100 nM ABT-199 for 24 hours were  
652 subjected to FACS analysis of specific apoptosis based on Annexin V staining in the bulk AML  
653 myleoblast and CD34+/CD38-/CD123+ LSC-containing population. P value determined via paired t-test.

654

### 655 **Figure 5. BH3 profiling predicts AML myeloblast killing by ABT-199**

656 A). Intracellular BH3 (iBH3) profiling was performed on thawed primary AML cells using the BAD BH3  
657 (80 μM) and ABT-199 (1μM). The mitochondrial sensitivity to BAD BH3 and ABT-199 were positively  
658 correlated. B). There is no correlation between the IC<sub>50</sub> of primary AML samples from Figure 4B with  
659 the BCL-XL specific BH3 peptide HRK (80 μM). C). The IC<sub>50</sub> of primary AML samples from Figure 4B  
660 were correlated with the NOXA (80μM), a MCL-1 specific NOXA BH3 peptide. D). The ABT-199 IC<sub>50</sub>  
661 of primary AML samples from Figure 4B were correlated with the BAD BH3 peptide (80uM). E). The  
662 ABT-199 IC<sub>50</sub> from Figure 4B was correlated with the ABT-199 mitochondrial response (1μM). All  
663 correlations were tested using a one-tailed Spearman r correlation using GraphPad Prism software.

664

### 665 **Figure 6. BH3 profiling predicts AML progression in a primary AML xenograft model**

666 A-B). NSG mice were injected with primary AML cells as described under Methods. Mice were treated  
667 with ABT-199 100 mg/kg oral daily dose starting 3 weeks after AML cell injection, for two weeks. The  
668 graph represents % of human CD45<sup>+</sup> leukemic cells in the murine bone marrow in mice sacrificed upon  
669 completion of the therapy. A non-parametric, unpaired, two-tailed *t*-test was used to evaluate the  
670 significance of mean difference. C). Intracellular BH3 profiling using the BAD BH3 (80 μM) and ABT-  
671 199 (10 μM) was performed on pre-treatment patient samples.

672

Figure 1

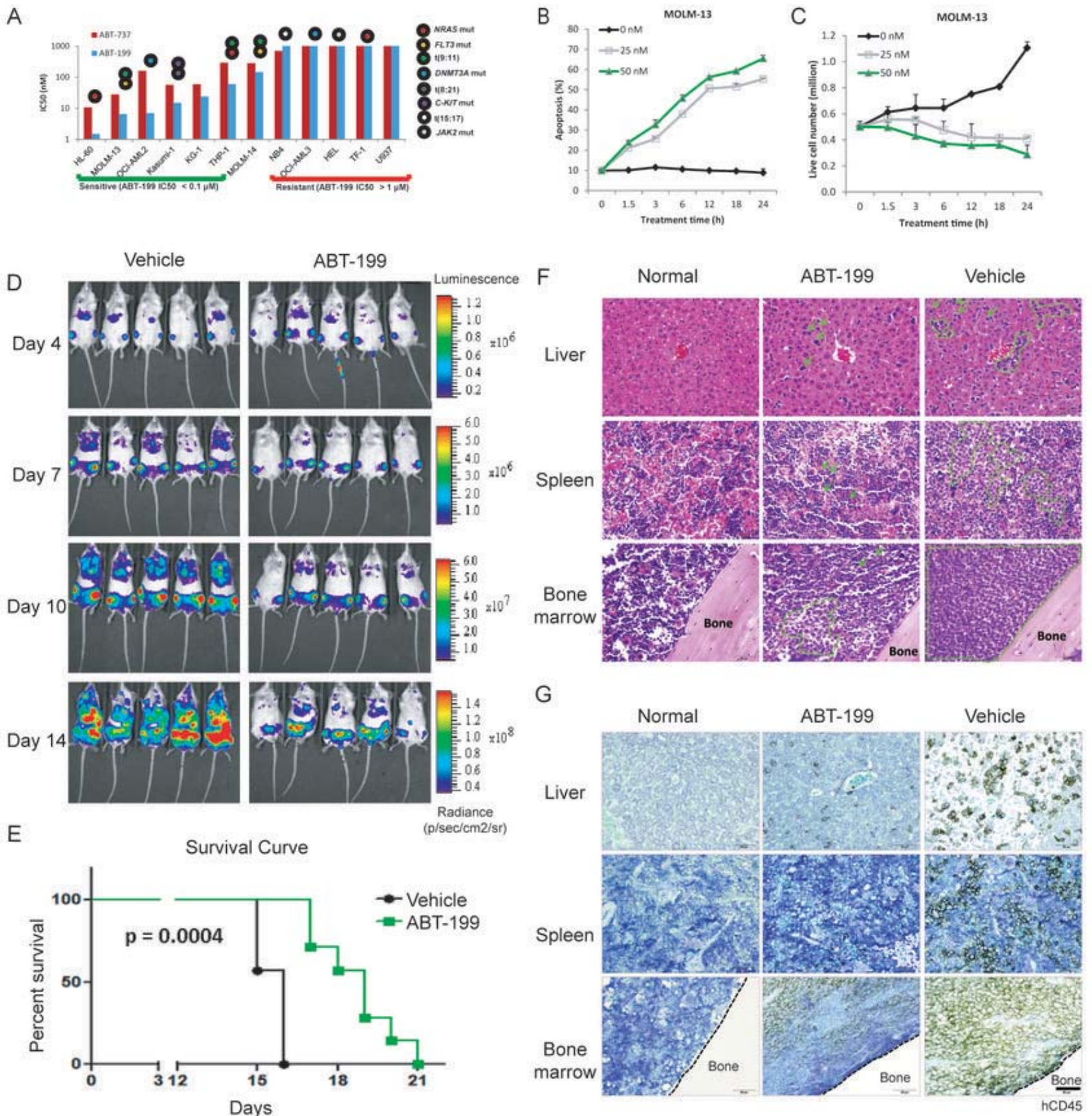
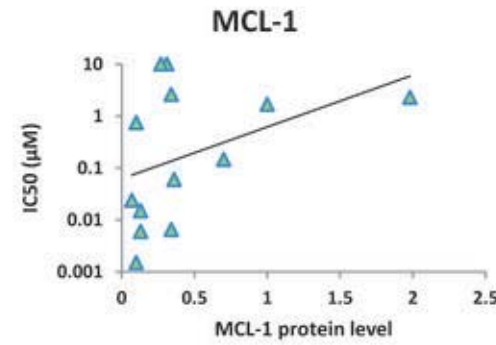
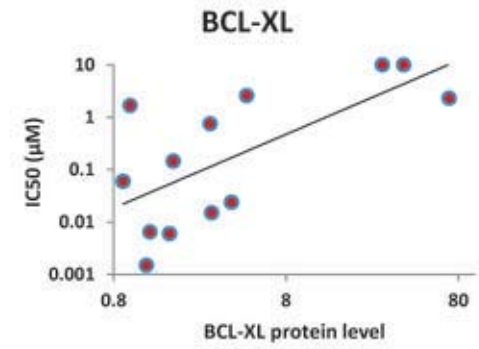
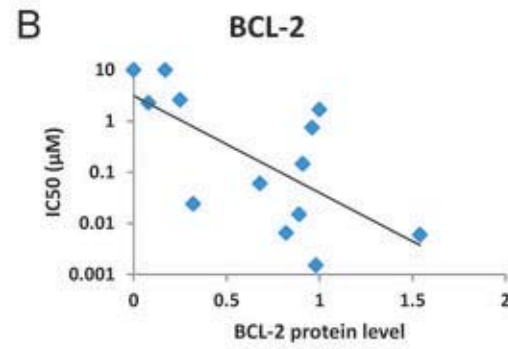
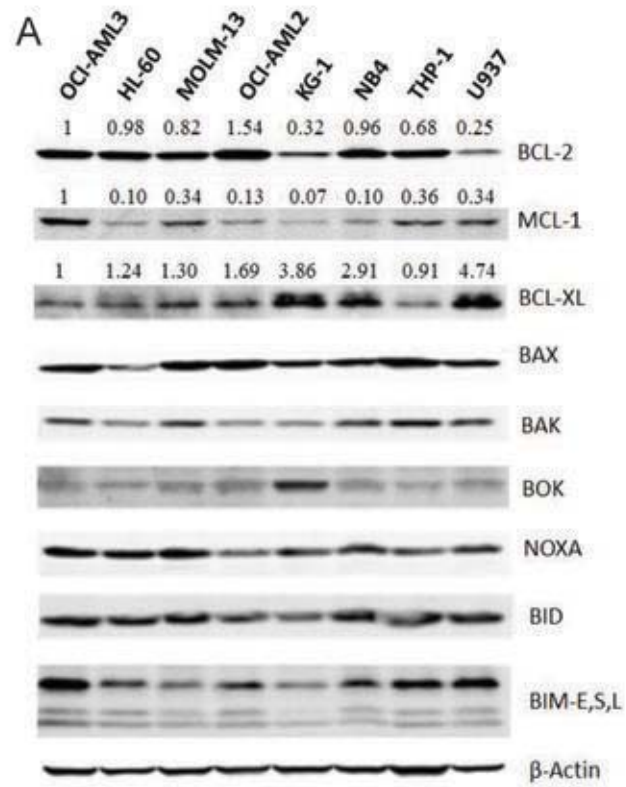


Figure 2



	ABT-199 IC50 v.s. protein level	
	Spearman r	P value
BCL-2	-0.666	0.015
BCL-XL	0.630	0.024
MCL-1	0.409	0.165

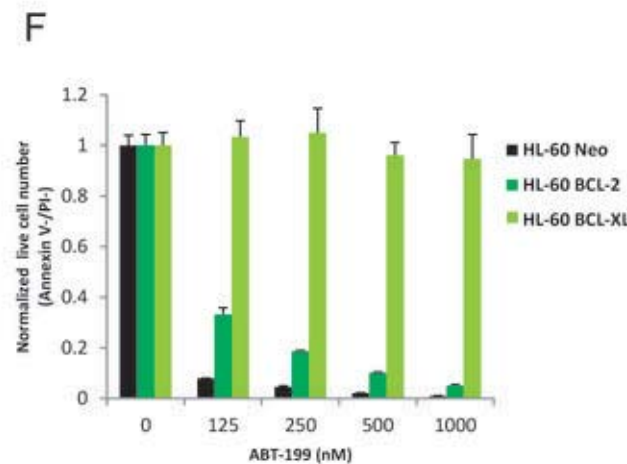
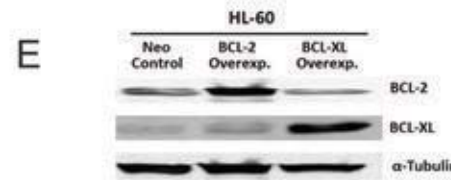
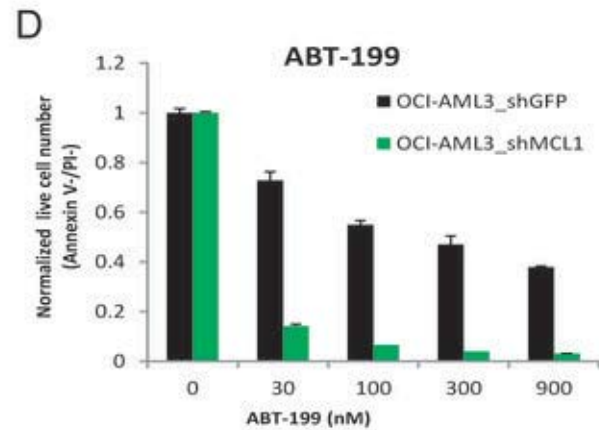
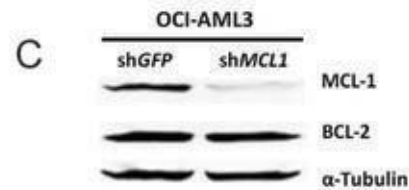
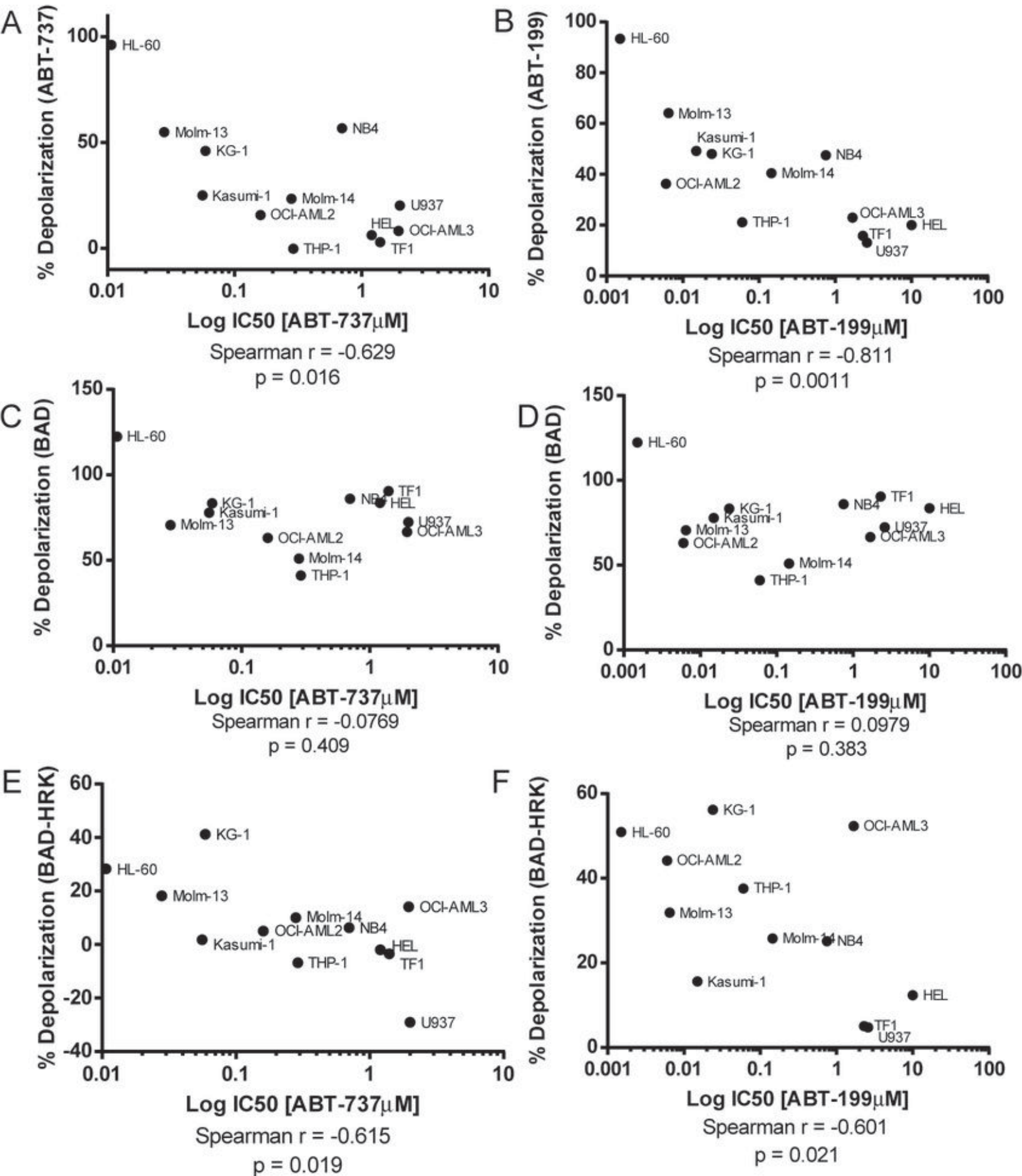


Figure 3



**Figure 4**

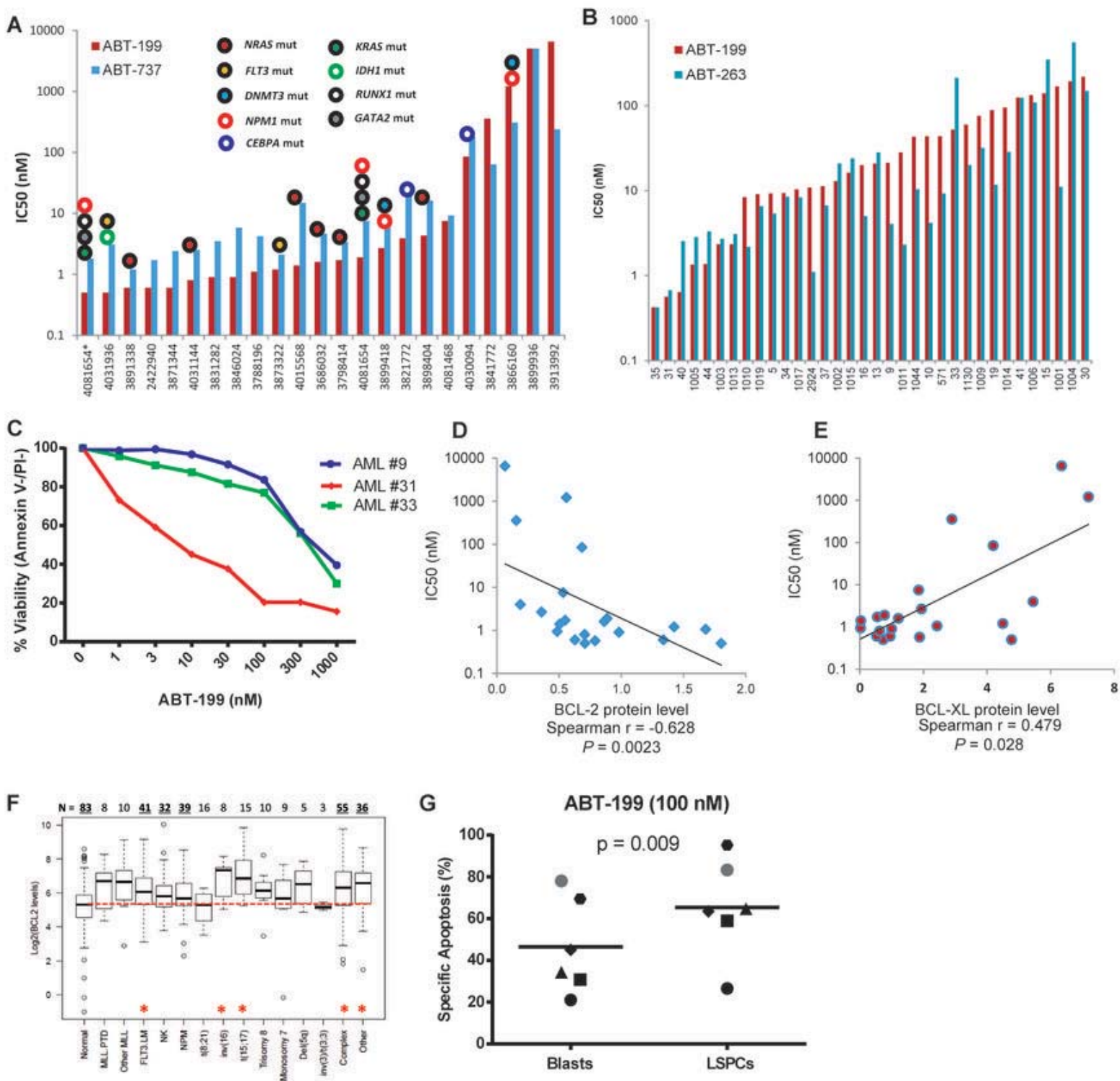


Figure 5

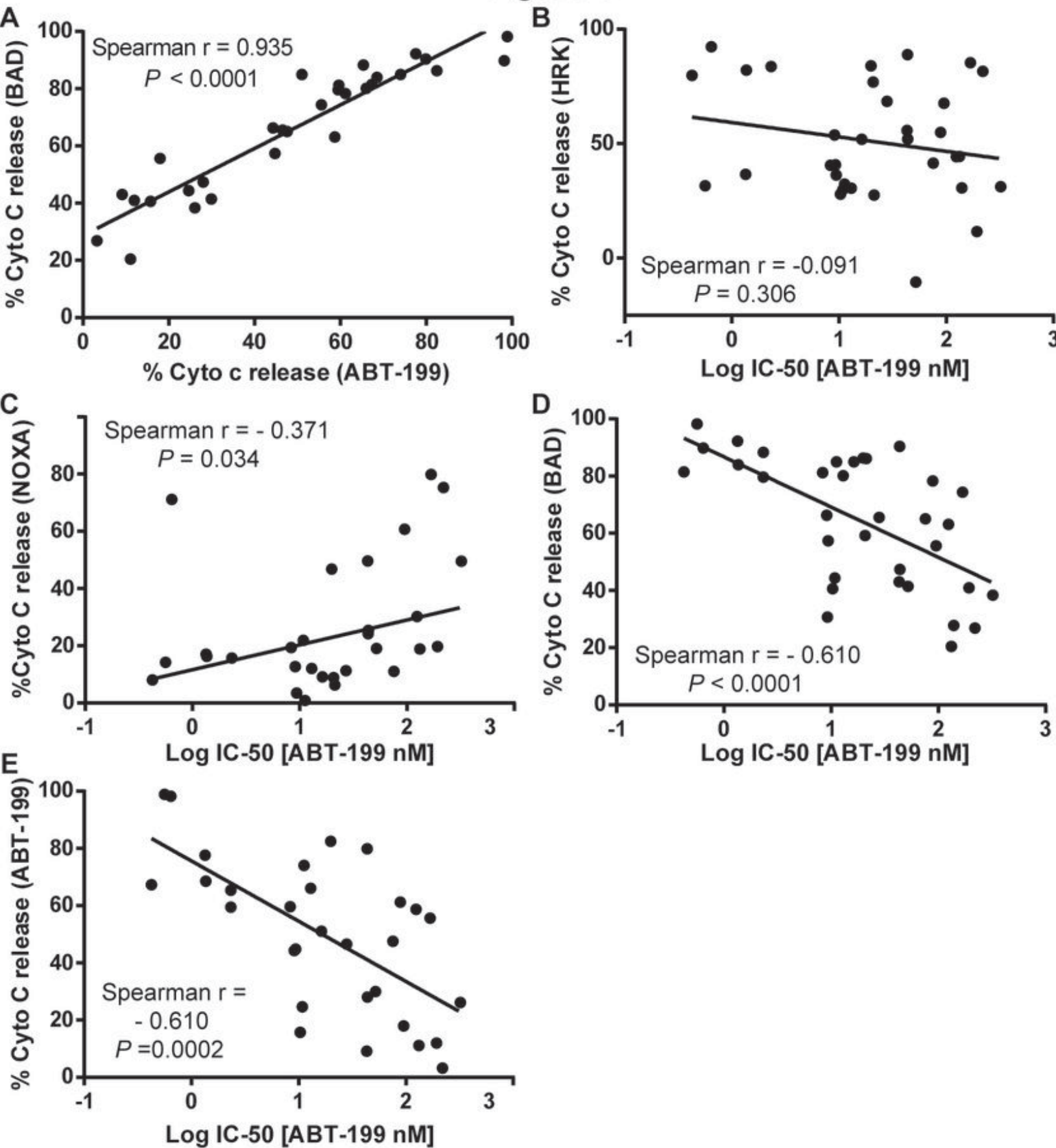
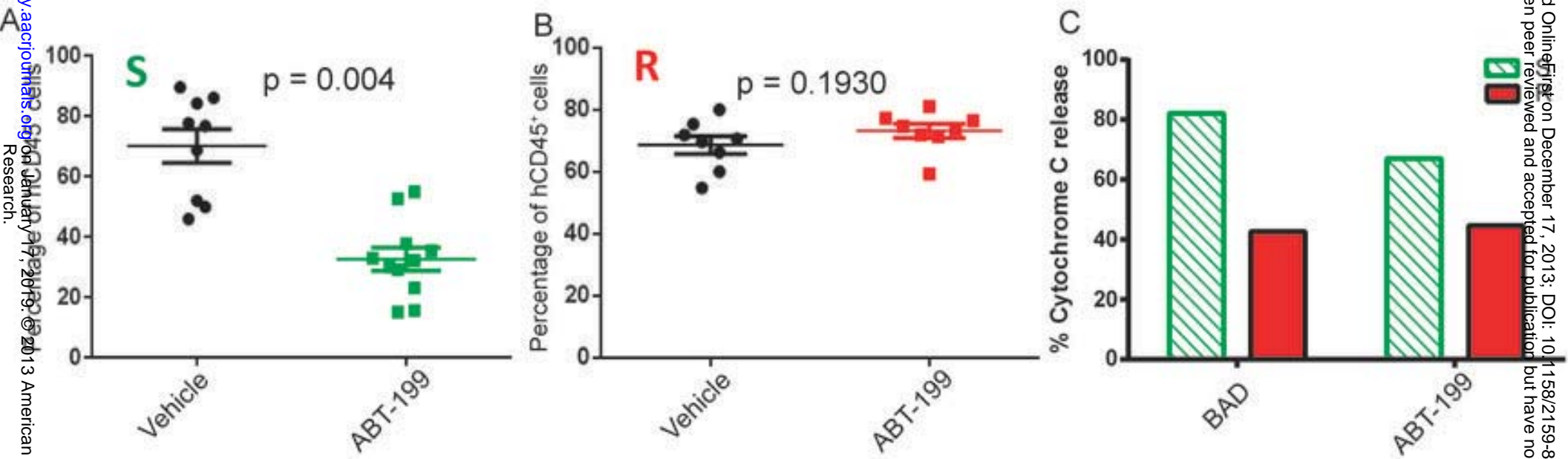




Figure 6



# CANCER DISCOVERY

## Selective BCL-2 Inhibition by ABT-199 Causes On Target Cell Death in Acute Myeloid Leukemia

Rongqing Pan, Leah J Hogdal, Juliana Maria Benito, et al.

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