BCL-2 inhibition with ABT-737 prolongs survival in an NRAS/BCL-2 mouse model of AML by targeting primitive LSK and progenitor cells

Stephanie Beurlet,1,2 Nader Omidvar,3 Petra Grombei,1,2 Patricia Krief,1,2 Carole Le Pogam,1,2 Niclas Setterblad,2 Pierre de la Grange,4 Christophe Leboeuf,2,5,6 Anne Janin,2,5,6 Maria-Elena Noguera,6 Florence Hervatin,7 Laure Sarda-Mantel,2,8 Marina Konopleva,9 Michael Andreff,9 Andrea W. Tu,10 Alice C. Fan,11 Dean W. Felsher,11 Anthony Whetton,12 Marika Pla,1,2 Robert West,13 Pierre Fenaux,1,2,6 Christine Chomienne,1,2,6 and Rose Ann Padua1,2,6

1Institut National de la Santé et de la Recherche Médicale U940, Paris, France; 2Université Paris-Diderot, Sorbonne Paris Cité, Institut Universitaire d’Hématologie, Unité Mixe de Recherche de Santé 940, Unité Mixe de Recherche de Santé 728, Paris, France; 3Haematology Department, Cardiff University School of Medicine, Cardiff, United Kingdom; 4GenoSplice Technology, Institut Universitaire d’Hématologie, Paris, France; 5Institut National de la Santé et de la Recherche Médicale U728, Paris, France; 6Assistance Publique-Hôpitaux de Paris, Hôpital Saint-Louis, Paris, France; 7Commissariat à l’Energie Atomique, Service Hospitalier Frédéric Joliot, Orsay, France; 8Institut National de la Santé et de la Recherche Médicale U773, Université Paris-Diderot, Sorbonne Paris Cité, Assistance Publique-Hôpitaux de Paris Hôpital Bichat, Paris, France; 9The University of Texas, MD Anderson Cancer Center, Houston, TX; 10ProteinSimple, Santa Clara, CA; 11Stanford University Cancer Center, Stanford, CA; 12School of Cancer and Enabling Science, University of Manchester, Manchester, United Kingdom; and 13Public Health, Cardiff University School of Medicine, Cardiff, United Kingdom

Myelodysplastic syndrome (MDS) transforms into an acute myelogenous leukemia (AML) with associated increased bone marrow (BM) blast infiltration. Using a transgenic mouse model, MRP8[NRASD12/hBCL-2], in which the NRAS:BCL-2 complex at the mitochondria induces MDS progressing to AML with dysplastic features, we studied the therapeutic potential of a BCL-2 homology domain 3 mimetic inhibitor, ABT-737. Treatment significantly extended lifespan, increased survival of lethally irradiated secondary recipients transplanted with cells from treated mice compared with cells from untreated mice, with a reduction of BM blasts, Lin-/Sca-1+/c-Kit+, and progenitor populations by increased apoptosis of infiltrating blasts of diseased mice assessed in vivo by techniciun-labeled annexin V single photon emission computed tomography and ex vivo by annexin V/7-amino actinomycin D flow cytometry, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, caspase 3 cleavage, and re-localization of the NRAS:BCL-2 complex from mitochondria to plasma membrane.

Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell hematologic disorders evolving to acute myelogenous leukemia (AML) and models multi-step leukemogenesis. The prognosis of patients who transform is poor and there are no efficient therapeutic strategies available for this largely elderly population. Activating neuroblastoma–Rasheed sarcoma virus cellular homologue (N-RAS) mutations have been associated with poor prognosis in MDS patients and increased risk of progression to AML.1 We used an NRASD12 mouse2,3 and over-expression of human B cell lymphoma 2 (hBCL-2)4 to study MDS–AML progression.

The BCL-2 family of apoptotic regulators contains proteins that have at least 1 BCL-2 homology domain 3.5 Apoptosis plays a key role in the physiopathology of MDS. Apoptosis of bone marrow (BM) hematopoietic cells has been shown in MDS and correlates with activation of apoptosis-related proteins.6 Decrease of apoptosis may also explain the clinical disease progression and accumulation of immature progenitor cells, correlating with a decrease of the BCL-2 associated protein X (BAX):BCL-2 ratio.7 Fifty percent of AML have increased BCL-2 expression, which correlates with poor prognosis.8,9

Key Points

- BCL-2 homology domain 3 mimetic inhibitor ABT-737 targets leukemia initiating cells and progenitors.
- Dephosphorylates RAS signaling proteins and regulates proliferation and differentiation genes detected by gene expression profiling.

Phosphoprotein analysis showed restoration of wild-type (WT) AKT or protein kinase B, extracellular signal-regulated kinase 1/2 and mitogen-activated protein kinase patterns in spleen cells after treatment, which showed reduced mitochondrial membrane potential. Exon specific gene expression profiling corroborates the reduction of leukemic cells, with an increase in expression of genes coding for stem cell development and maintenance, myeloid differentiation, and apoptosis. Myelodysplastic features persist underscoring targeting of BCL-2-mediated effects on MDS–AML transformation and survival of leukemic cells. (Blood. 2013; 122(16):2864-2876)
Using mutant-NRAS<sup>1</sup> and hBCL-2<sup>4</sup> transgenes, we created a mouse model of AML.<sup>3</sup> The BM, spleen, and liver of NRASD12/hBCL-2 mice are infiltrated by myeloblasts associated with dysplastic features. The liver infiltration evaluated by temporal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) is anti-apoptotic, as expected by the overexpression of hBCL-2 in these cells. The Lin-/Sca-1<sup>+</sup>/c-Kit<sup>+</sup> (LSK) primitive compartment of marrow cells is expanded, correlating with an increase in myeloid progenitors. An activated RAS-guanosine triphosphate (GTP) and BCL-2 complex was noted in both Mac1<sup>+</sup> and Sca1-<sup>+</sup> cells of the double transgenic mice and was absent in the Sca1<sup>+</sup> compartment of the single NRASD12 and BCL-2 transgenic mice.<sup>5</sup> In the Mac1<sup>+</sup> compartment, single BCL-2 recruits endogenous RAS to activate it.<sup>3</sup> This complex is also observed in MDS patients correlating with the World Health Organization classification and percentage of BM blasts.<sup>10</sup>

ABT-737 is a small molecule able to bind the hydrophobic pocket of BCL-2, B cell lymphoma X protein (BCL-X), and BCL-w with a very high affinity, but with a low affinity for myeloid cell leukemia (MCL-1) and BCL-2 related fetal liver homologous murine A1. This binding prevents BCL-2 from blocking the formation of the BAX/BCL-2 homologous antagonist killer complex at the mitochondria and has been shown to effectively alter mitochondrial membrane potential (MMP).<sup>11</sup> The efficacy of ABT-737 has already shown in vitro inducing apoptosis of carcinoma and AML cell lines,<sup>12</sup> ex vivo on patients’ blasts,<sup>13</sup> in vivo on a mouse model of lymphoma<sup>14</sup> and on a xenograft model of small cell lung cancer.<sup>15</sup> The combination of ABT-737 with chemotherapeutic agents<sup>15-17</sup> or other targeted therapy molecules<sup>18,19</sup> has synergistic efficacy. A related orally bioavailable derivative, ABT-263 (Navioclax) has been tested in a phase I clinical trial on patients with both small cell lung cancer and other solid tumors.<sup>20,21</sup> and chronic lymphocytic leukemia<sup>22,23</sup> in which acceptable toxicity and promising efficacy has been reported. Here we report on a preclinical study with ABT-737 in an AML mouse model.

**Methods**

**Transgenic mice**

The MRP8[NRASD12/hBCL-2] mice were generated from crosses of MRP8/hBCL-2 with MRP8[NRASD12 hemizygote mice in Friend leukemia Virus B strain from the National Institutes of Health (FVB/N), as previously described.<sup>2,4</sup> Mice were classified according to the Bethesda method<sup>24</sup> as myeloid leukemia without maturation and will be referred to here as AML. Mice were maintained in the barrier facilities of the Institut Universitaire d’Hématologie, Paris, Hôpital Saint Louis, under the appropriate animal project licenses. All procedures complied with national regulations on the use of animals for experimentation. Mice were sacrificed based on veterinary advice or when they were moribund. The mice were bred and genotyped using standard husbandry and polymerase chain reaction techniques as described.<sup>3</sup> These mice have some myeloid dysplasia in the BM, 60% to 90% of blasts, pancytopenia, and weight loss as we previously described<sup>3</sup> (Figures 1A and 2B; supplemental Figure 1). Two-week-old pups were euthanized to identify a pre-leukemic phase of the disease.

**ABT-737**

ABT-737 was available either as a stock solution at 200 mg/mL in dimethylsulfoxide or as a powder (kindly provided and synthesized by MD Anderson Cancer Center or as a generous gift by Abbott Laboratories, Green Oaks, IL); both preparations were equally active. The final formulation was 30% propylene glycol, 5% Tween 80, 65% double distilled water, pH 4.5, 75 mg/kg and was administered 3 times per week (15 injections for 33 days) unless otherwise indicated (Figure 1B).

**Tissue and cell preparation**

The BM and spleen were prepared as previously described and detailed in the supplemental Methods.<sup>3</sup>

**Secondary transplantation**

Spleen cells (10<sup>7</sup>) from untreated and treated mice were injected intravenously into lethally irradiated (2 × 5 gray) syngeneic FVB/N mice. Three weeks after transplant hBCL-2 expression was measured by flow cytometry.

**Progenitor colony assay**

 Colony assays were performed using the Methocult media, as recommended by the manufacturer (Stem Cell Technologies, Vancouver, BC, Canada) detailed in the supplemental Methods.

**Immunofluorescence and confocal microscopy**

Immunofluorescence and microscopy was undertaken, as we had previously described<sup>25</sup>, detailed in the supplemental Data.

**Flow cytometric apoptosis assessment: annexinV- 7AAD**

Apoptosis was assessed using annexinV/7-amino actinomycin D (7AAD) staining and flow cytometry, as detailed in the supplemental Data.<sup>25</sup>

**MMP**

MMP was measured using DiOC<sub>3</sub>(3) (Invitrogen, Carlsbad, CA), as detailed in the supplemental data. Background corrected mean fluorescence intensity using unstained cells was used to calculate the FL3/FL1 ratios of DiOC<sub>3</sub>(3) test samples, as previously described.<sup>26</sup>

**Single photon emission computed tomography (SPECT)**

**ANX-labeling.** Technicium (99mTc)-Annexin-V (ANX), which allows non-invasive in vivo detection of apoptosis, was prepared by injecting sodium pertechnetate (400 ± 20 mega becquerel drawn-up from a 99mTc generator, freshly eluted) with stannous Tricine in a sterile vial containing annexin V (Thescus Imaging Corp., Cambridge, United Kingdom). After shaking, the preparation was left to stand 15 minutes at room temperature. The quality control was performed with instant thin-layer chromatography, using citric acid/dextrose solution as eluant. The radiolabeling yield was always greater than 88%. ANX-scintigraphy imaging is detailed in the supplemental Data.

**TUNEL**

Apoptosis was assessed by in situ detection of fragmented DNA using the TUNEL assay on 5-μm paraffin embedded liver sections as detailed in the supplemental Data.<sup>3</sup>

**RAS activation assays and western blot analysis**

RAS pull down assays and western blots were undertaken as detailed in the supplemental Data. For in vitro assays, AML spleen cells were isolated and cultured in the presence of 10, 100, or 500 nM of ABT-737 dissolved in dimethylsulfoxide and diluted in culture media for 24 hours. Western blot analysis was undertaken to determine the phosphorylation states of RAS signaling proteins extracellular signal-regulated kinase (ERK) and murine thymoma viral oncogene homologue 1 isolated from the ak mouse strain (AKT) as detailed in the supplemental Data.

**NanoPro assay**

Splenocyte cells (10<sup>7</sup>) isolated from transgenic mice were lysed in radio immunoprecipitation assay buffer and subjected to a nanofluidic proteomic
immunoassay (NIA) run on the NanoPro1000\textsuperscript{27} (ProteinSimple, Santa Clara, CA) mixing 0.1 mg/mL of lysate in a final volume of 15 µL loaded in a 384-well plate with 5 to 8 gradient ampholyte mix. Antibodies were diluted in ProteinSimple antibody diluent: mitogen-activated protein kinase (MEK)\textsuperscript{1} (Upstate 07-641; Millipore, Billerica, MA) 1:100, MEK2 (Cell Signaling Technology, MA). Samples were run in triplicate.
Figure 2. ABT-737 rescues clinical features of AML mice. (A) Kaplan-Meier survival curves showing prolonged survival of AML mice who completed treatment with ABT-737 (n = 26, red line), mice who failed to complete treatment (n = 10, blue line), and untreated mice (n = 63, black line) plotted from date of birth (P < .0001). (B) White blood cell counts (WBC) (pretreatment vs posttreatment, P < .0001), neutrophil counts (pretreatment vs posttreatment, P < .0001), lymphocytes (pretreatment vs posttreatment, P < .0001), and platelet counts (PLT) (pretreatment vs posttreatment, P < .0001) are shown (mean ± standard deviation, n = 30 mice in the pretreatment and posttreatment groups; n = 31 in the untreated group). There were no significant differences between untreated and pretreatment AML groups. (C) Percentage AML BM blasts pretreatment and posttreatment after day 33 from start of treatment (n = 6 mice). Giemsa stained BM smears showing decreased marrow blasts after treatment (shown in low power [LP] × 50 magnification) and hematoxylin and eosin stained liver sections with infiltration in the untreated mice and clearance in the treated mice postday 33. Representatives of 3 mice: (D) differential survival of mice transplanted each with 10^7 spleen cells from AML untreated (black) or treated (red) mice into lethally irradiated FVB/N recipients (n = 5 in each group) (log-rank X^2 = 9.496; P < .005). (E) Significant reduction in the Lin-/Sca-1^-/c-Kit^- (LSK) population of untreated (n = 12) and treated (n = 8) (P < .01); untreated mice analyzed compared with WT FVB/N mice (P = .71); treated compared with WT mice (n = 6) (P = .1). (F) ABT-737 targets myeloid progenitors. Dot plots showing numbers of day 7 colony-forming unit–granulocyte macrophage per 3 × 10^4 cells plated per dish from BMs of diseased untreated and normal mice (P < .001); untreated compared with treated mice were statistically different (P < .01) (mean ± standard deviation of n = 4 mice analyzed postday 33). There was no statistical difference between the WT and post-ABT-737 treatment groups (P = .3).
Table 1. Target genes regulated after ABT-737 treatment of AML mice

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Regulation</th>
<th>Fold change</th>
<th>P value</th>
<th>Entrez gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc</td>
<td>Down</td>
<td>2.08</td>
<td>3.89E-02</td>
<td>17869</td>
</tr>
<tr>
<td>Mcm5</td>
<td>Down</td>
<td>1.64</td>
<td>2.31E-02</td>
<td>17218</td>
</tr>
<tr>
<td>Mcm7</td>
<td>Down</td>
<td>1.53</td>
<td>4.95E-02</td>
<td>17220</td>
</tr>
<tr>
<td>Ez11</td>
<td>Down</td>
<td>1.52</td>
<td>3.06E-02</td>
<td>13555</td>
</tr>
<tr>
<td>Pole4</td>
<td>Down</td>
<td>1.65</td>
<td>1.36E-02</td>
<td>66979</td>
</tr>
<tr>
<td>Myeloid differentiation genes upregulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Csf1</td>
<td>Up</td>
<td>1.80</td>
<td>2.24E-02</td>
<td>12977</td>
</tr>
<tr>
<td>Csf4</td>
<td>Up</td>
<td>2.08</td>
<td>2.81E-02</td>
<td>12475</td>
</tr>
<tr>
<td>Csf3</td>
<td>Up</td>
<td>3.36</td>
<td>2.40E-02</td>
<td>12986</td>
</tr>
<tr>
<td>Itgb2</td>
<td>Up</td>
<td>1.89</td>
<td>2.33E-02</td>
<td>16414</td>
</tr>
<tr>
<td>Itgb2l</td>
<td>Up</td>
<td>3.51</td>
<td>3.55E-02</td>
<td>16415</td>
</tr>
<tr>
<td>Rara</td>
<td>Up</td>
<td>1.56</td>
<td>9.60E-03</td>
<td>19401</td>
</tr>
<tr>
<td>Apoptosis related genes upregulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trtnsf23</td>
<td>Up</td>
<td>5.22</td>
<td>2.20E-03</td>
<td>79201</td>
</tr>
<tr>
<td>Mapt13</td>
<td>Up</td>
<td>3.10</td>
<td>3.00E-02</td>
<td>26415</td>
</tr>
<tr>
<td>Trtnsf17</td>
<td>Up</td>
<td>2.17</td>
<td>3.66E-02</td>
<td>21935</td>
</tr>
<tr>
<td>Card10</td>
<td>Up</td>
<td>1.67</td>
<td>3.17E-02</td>
<td>105844</td>
</tr>
<tr>
<td>Ctds</td>
<td>Up</td>
<td>1.72</td>
<td>2.16E-02</td>
<td>13033</td>
</tr>
<tr>
<td>Dapk2</td>
<td>Up</td>
<td>1.63</td>
<td>3.07E-02</td>
<td>13143</td>
</tr>
<tr>
<td>Capn2</td>
<td>Up</td>
<td>1.62</td>
<td>4.03E-02</td>
<td>12334</td>
</tr>
<tr>
<td>Dapk1</td>
<td>Up</td>
<td>1.54</td>
<td>3.06E-02</td>
<td>69635</td>
</tr>
<tr>
<td>Nkbiz</td>
<td>Up</td>
<td>1.53</td>
<td>6.20E-03</td>
<td>80859</td>
</tr>
<tr>
<td>Cox42</td>
<td>Up</td>
<td>1.53</td>
<td>2.12E-02</td>
<td>84682</td>
</tr>
<tr>
<td>Parp4</td>
<td>Up</td>
<td>1.51</td>
<td>1.78E-03</td>
<td>328417</td>
</tr>
<tr>
<td>Stem cells related upregulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcf72</td>
<td>Up</td>
<td>2.13</td>
<td>3.38E-02</td>
<td>21416</td>
</tr>
<tr>
<td>Ptw2</td>
<td>Up</td>
<td>2.20</td>
<td>3.06E-02</td>
<td>57746</td>
</tr>
<tr>
<td>Bmpr1a</td>
<td>Up</td>
<td>2.31</td>
<td>4.63E-02</td>
<td>12166</td>
</tr>
<tr>
<td>Spp1</td>
<td>Up</td>
<td>4.32</td>
<td>1.38E-02</td>
<td>20750</td>
</tr>
</tbody>
</table>

Fold change of treated compared with untreated mice was considered significant when \( P \leq 0.15 \) and \( P \) value \( \leq 0.05 \). The arrays were normalized to FVB/N, and then the treated was compared with the untreated group (n=3) (GEO accession no. GSE48601).

Results

Identification of preleukemia

The MRP5[NRASD12/hBCL-2] mice developed AML with dysplastic features in approximately 21 to 27 days (Figure 1A). Some degree of dysplasia was noted and some mice had pancytopenia at about day 27 correlating with expression of BCL2 in 40% cells. Two-week-old double transgenic NRASD12/hBCL-2 mice showed reduced BM blasts (5% to 8% [n = 3]) with dysplasia compared with 60% to 90% in the 4-week-old AML mice with absence of tissue invasion, whereas the adult mice have extensive tissue invasion and evidence of extramedullary hematopoiesis (Figure 1A). Late apoptosis measured by annexinV- and TUNEL+ in the BM shows increased apoptosis in these 2-week-old mice compared with wild-type (WT) and BCL-2 mice (data not shown). These young mice may be considered as the preleukemic or MDS stage.

Statistical

Survival of treated mice was compared with untreated controls by using the Kaplan Meier method with log-rank test of significance. Some AML mice were euthanized according to veterinary advice (at the end of treatment) due to disease; all of these mice were shown to have advanced disease at necropsy. The combined end point (died naturally or euthanized due to advanced disease) of untreated and treated mice was used for comparison. Further analysis compared only those that received the full course of treatment with controls to test “biological effectiveness.”

Blood counts, LSK, progenitor, apoptosis, and MMP assays were presented as the mean ± standard deviation, and between group differences as the mean difference (95% confidence interval difference). The unpaired Student t test was also used.

Results

Identification of preleukemia

The MRP5[NRASD12/hBCL-2] mice developed AML with dysplastic features in approximately 21 to 27 days (Figure 1A). Some degree of dysplasia was noted and some mice had pancytopenia at about day 27 correlating with expression of BCL2 in 40% cells. Two-week-old double transgenic NRASD12/hBCL-2 mice showed reduced BM blasts (5% to 8% [n = 3]) with dysplasia compared with 60% to 90% in the 4-week-old AML mice with absence of tissue invasion, whereas the adult mice have extensive tissue invasion and evidence of extramedullary hematopoiesis (Figure 1A). Late apoptosis measured by annexinV- and TUNEL+ in the BM shows increased apoptosis in these 2-week-old mice compared with wild-type (WT) and BCL-2 mice (data not shown). These young mice may be considered as the preleukemic or MDS stage.
similar (Figure 2B), whereas lymphopenia and thrombocytopenia persisted (Figure 2B); toxicity of ABT-737 on platelets in mice and humans is well known.30 A significant reduction in BM blast counts (19% ± 7% vs 60% ± 6% in treated vs untreated mice), with a reduced to complete clearance of blast invasion of the spleen and liver was observed (Figure 2C). Secondary transplants of lethally
irradiated syngeneic mice showed that recipients with untreated AML cells died in 4.5 weeks, whereas those transplanted with treated cells remained alive for 11 to 11.5 weeks after which they were euthanized based on veterinary advice, as they were ill (Figure 2D) \( (P < .005) \). All the engrafted mice demonstrated hBCL-2 expression measured by flow cytometry (data not shown). As we had previously reported, AML mice have an increased myeloid immature LSK cell population in the BM. After 33 days of ABT-737 treatment, the proportion of BM LSK cell population decreased to nearly normal (12.9 ± 1.4% untreated vs 6.4 ± 1.1% treated \( (P < .01) \) and treated vs 1.8 ± 0.3% WT \( (P < .1) \) (Figure 2E), with a complete restoration of colony growth to normal range (79.6 ± 7.0 untreated vs 48.6 ± 13.5 treated colony-forming unit–granulocyte macrophage progenitors per dish \( (P < .01) \), in which expected normal values are 40.8 ± 2.9 \( (P < .3) \) compared with treated group) (Figure 2F).
ABT-737 treatment induces apoptosis and inhibition of cell proliferation

Transformation of MDS to AML in patients is linked to a reduction of apoptosis and increased expression of BCL-2. Likewise, in the AML transgenic mouse overexpression of BCL-2 results in decreased apoptosis in the liver and in early apoptosis in the spleen. To be able to document the mechanism of blast clearance during ABT-737 treatment, the apoptosis status of the infiltrated tissues was measured. Spleen cells of ABT-737–treated mice showed a decrease in annexinV from 40.0 ± 7.7% in the WT mice, 35.9 ± 10.3% in the untreated AML to 4.6 ± 2.4% cells being annexinV/7AAD after 23 to 25 days of treatment (ie, after 10 to 11 injections) and an increased trend toward late apoptosis at treatment (49.2 ± 4.9% untreated vs 59.5 ± 14.4% treated) (Figure 3A). No significant differences in apoptosis was observed between the WT untreated and treated mice (early P ~ .08; late apoptosis P ~ .21). Liver and spleen were studied by single photon emission computed tomography (SPECT); at days 23 to 25, analyses of paired pretreated and posttreated WT and AML mice were imaged (Figure 3B; supplemental Figure 3). Untreated and treated mice show that in AML mice, ABT-737 treatment induces a more than twofold increase of the radioactive 99mTc annexinV uptake in the liver and spleen area (Figure 3B). Imaging of WT mice showed no increase in uptake of radioactivity post treatment compared with pretreatment profiles (Figure 3B; supplemental Figure 3). This result was corroborated by an increased apoptosis in the treated AML mice assayed by the TUNEL of liver sections (Figure 3C); the nuclei of the hepatocytes are stained clearly in blue, and the brown staining apoptotic invading hematopoietic cells are in the sinusoid spaces. An increased caspase 3-dependent cleavage was detected (Figure 3D). BCL-X, a known target of ABT-737 is downregulated in spleen cells of ABT-737 AML mice while MCL-1, a protein known to be linked to ABT-737 resistance, is unaffected by the treatment (Figure 3E).

Then the control on cell growth by ABT-737 treatment was analyzed. Cyclin D1, which is maximally expressed in mid-to-late G1 phase, was found to be markedly decreased with treatment in both the Mac-1 and Sca-1 compartments. This data are consistent with the proliferative capacity of AML leukemic cells and that of mutant RAS-mediated myelopoiesis, as well as the previously reported anti-proliferative property of ABT-737. The increased myelopoiesis is not reflected in the expression of the alternative target of ABT-737, BCL-xL, which is only marginally downregulated with treatment in the Mac-1 and Sca-1 compartments, consistent with the previously reported high expression levels of MCL-1–mediating resistance. Phosphorylated p38-MAPK was significantly upregulated after treatment. p21 expression is stable, as previously observed in the MRP8NRASD12 mouse models, through
a cyclin D1 imposed block. Likewise, cyclin D3 and cyclin-dependent kinase inhibitors (p15^INK4B and p27^Kip1) were not significantly modified (Figure 3E). The marginal decrease in the level of p27^Kip1 in the Mac-1 and Sca-1 compartments of treated animals is indicative of a release of the G1-arrest, whereas no such change was observed in the S-phase entry p15^INK4B protein or the pRb tumor suppressor (data not shown).

Localization of the RAS:BCL-2 complex to the plasma membrane and dephosphorylation of RAS signaling proteins in AML mice by reducing the MMP

BCL-2 expression was increased with disease progression from single to double transgenic mice (Figure 4A); we have previously reported that this may be due to the expansion of the Sca-1^+ compartment. ABT-737 treatment increased the percentage of cells expressing BCL-2 in the peripheral blood (Figure 4A). Similarly, RAS and BCL-2 expression measured by western blot analysis also revealed an increase of both transgenes with disease progression, as we previously described (Figure 4B). We compared RAS activity and co-localization of hBCL-2 in the NRAS:BCL-2 complex in ABT-737-treated mice compared with untreated mice using RAS-GTP pull down assays and confocal microscopy. Normalized levels of activated RAS-GTP are increased after treatment (Figure 4C). Likewise, normalized hBCL-2 level remains in the RAS complex after treatment (Figure 4C), with no significant change in its phosphorylated status whether on serine 70 or threonine 56 (Figure 4D). It has been previously shown that ABT-737 alters the MMP in this present study, there is a significant increase in MMP in AML compared with WT mice (P < 0.005). Treatment with the inhibitor significantly reduced the MMP of the WT and diseased mice (Figure 4E) (P < 0.005 and P < 0.001, respectively). No difference in total mitochondria numbers were observed between the groups measured by the cell-permeant MitoTracker probe (Invitrogen, Carlsbad, CA) (data not shown). AML transgenic mice are characterized by an NRAS:BCL-2 complex localized at the mitochondria, where BCL-2 is normally found (Figure 4F). However, after ABT-737 treatment, the localization of the NRAS:BCL-2 complex shifts to the plasma membrane (Figure 4F). These results indicate that despite the clinical and apoptotic effect, ABT-737 is not capable of disrupting the NRAS:BCL-2 complex (Figures 4C,F).

As RAS is found in its activated RAS-GTP form after ABT-737 treatment, we next examined whether differential patterns of RAS downstream protein phosphorylations could be identified after treatment by immunoblot and NIA. After treatment in vivo, phosphorylated ERK (pERK)1/2 and pAKT levels were highly reduced in comparison with untreated mice (Figure 5A-B). Culturing the AML spleen cells ex vivo for 24 hours showed that ABT-737 is acting directly on the leukemic cells to reduce pERK and pAKT (Figure 5C). Using the NIA, we estimated the level of different phosphorylated MEK (pMEK) isoforms; the traces of the 2 phospho-forms of the MEK1 and MEK2 isoforms are modulated during disease progression and restored to near normal levels by treatment in vivo (Figure 5D). Total MEK1 levels are found very much reduced in 2 AML mice compared with WT controls with decreased pMEK1-5 and pMEK1-6 peaks (Figure 5Di). ABT-737 treatment induced a significant trend to restore WT MEK1 levels and phosphorylation patterns (Figure 5Di). Similar results are observed with the MEK2 isoforms with the restoration of peaks MEK2-3 and MEK2 which were lost in the AML mice upon treatment. (Figure 5Dii).

Discussion

ABT-737 shows biological efficacy in this mouse model of AML; the difference in survival between the untreated and treated groups is highly significant in mice that had the treatment completed. The mice clearly gain weight with treatment. The blood counts show that the platelets and lymphocytes are not rescued due to the reported toxicity of the drug as it targets BCL-XL. However, the other blood parameters, such as neutrophil count and white blood cell counts are rescued. BM blasts are significantly reduced, and the organ invasion is reduced, consistent with the increase of differentiated cells illustrated by the increase in Mac-1^hi/Gr-1^hi populations (data not shown) and the diminution of the expanded LSK compartment and the late (colony-forming unit) progenitors, thereby indicative of targeting the primitive leukemic cells. The finding that genes important for stem cell development, maintenance, and differentiation are upregulated implies that the drug treatment may reflect the partial restoration of normal stem cell function, as evidenced by the increased survival of mice transplanted with cells from the AML-treated group. Interestingly, the upregulation of Spp1 is compatible with previous reports of its effect on dampening hematopoietic stem cell/progenitor proliferative capacity within the BM microenvironment, as well as the raised levels of its gene product (osteopontin) in the serum of leukemia patients as compared with normal controls, with its transcriptional regulation via a RAS-mediated...
In this context, Spp1 may be influencing the proliferation of leukemia initiating cells by reducing their proliferative capacity and restoring the stem cells to their normal phenotype.

Treatment with ABT-737 induces apoptosis of myeloid blasts that invade organs, as measured by annexinV/7AAD flow cytometry, SPECT, and TUNEL, consistent with clearance of the disease, as observed by the reduction in BM blasts. The hepatocytes are clearly morphologically intact and therefore ABT-737 does not appear to be toxic to these cells, and the apoptosis induced appear to be targeting the infiltrating myeloid cells. The apoptosis profiles by flow and SPECT confirm the specificity of the ABT-737 for BCL-2-expressing cells and cell death is not due to liver toxicity. This shows there is differential targeting of diseased BCL-2-expressing cells sparing the normal cells. The upregulation of pro-apoptotic signaling pathway. In this context, Spp1 may be influencing the proliferation of leukemia initiating cells by reducing their proliferative capacity and restoring the stem cells to their normal phenotype.

Figure 5. Modification of signaling proteins after treatment. Western blot analysis showing dephosphorylation of proteins from spleen cells of (A) ERK1/2 and (B) AKT on day 0 and after ABT-737 treatment (postday 33), and normalized protein loading confirmed by probing for β-actin (n = 2 mice). (C) Representative western blot analysis showing dephosphorylation of proteins of ppERK and pAKT of spleen cells after culturing in vitro with vehicle (V), 10, 100, or 500 nM ABT-737 for 24 hours (n = 2 mice). (D) Nanoimmunoassay using the NanoPro (ProteinSimple) isoelectric focusing for detection of MEK signatures. Representative trace of spleen cells of WT FVB/N and AML untreated mice (day 0) and after ABT-737 treatment (postday 33) showing the dephosphorylated isoform pMEK2-3 and restoration of MEK2 expression (arrowed); histogram showing restoration of normal FVB/N signatures of the different isoforms after treatment. Each reaction was done in triplicate. Representative of at least 2 mice (n = 2) in each group.
genes were observed in gene expression profiles. Interestingly, among the downregulated genes, we found the anti-apoptotic Bcl-2a1b gene that encodes the protein A1b implicated in the resistance to ABT-737.39 These data are consistent with the reduced phosphorylation of anti-apoptotic AKT, which normally inhibits pro-apoptotic BCL-2 associated death promoter protein BAD, which in turn inhibits anti-apoptotic BCL-2; dephosphorylation of AKT results in the dominance of pro-apoptotic features. AKT also induces mammalian target of rapamycin, which in turn activates p70S6K, which is anti-apoptotic. Therefore, the downregulation of this pathway by dephosphorylation of AKT results in increased apoptosis, as we have observed; nevertheless, this efficacy does not lead to complete remission, although hematopoiesis returns to an MDS phenotype with reduced BM blasts and increased dysplasia. The relatively increased MMP of AML as compared with WT splenocytes is consistent with increased levels of reactive oxygen species, as previously reported in these mice.40

The RAS:BCL-2 complex is not disrupted by treatment, which is to be expected, as the binding site of RAS to BCL-2 is the BH4 domain,41 whereas ABT-737 binds the BCL-2 homology domain 3 domain. The localization of the RAS:BCL-2 complex shifts from the mitochondria where BCL-2 is localized3 to the plasma membrane reminiscent of the localization of the single NRASD12 transgenic mice and consistent with increased apoptosis.3,10 Thus, presumably due to the reduction in MMP induced by ABT-737, downstream of RAS, dephosphorylation of AKT, ERK, and MEK are more likely to reflect the return to a more normalized hematopoiesis, albeit with remaining MDS features. Although high levels of MCL-1 are critical for survival of AML cells,42 increased expression of MCL-1 is also thought to confer resistance to ABT-737, as the drug binds poorly to this protein.3,1,12,43 ERK promotes MCL-1 expression by ELK144 and stabilizes the protein by phosphorylation within the proline/glutamic acid/serine/threonine site45; therefore, its dephosphorylation in our mouse model post-ABT-737 treatment is consistent with the absence of increased expression of MCL-1 and the sensitivity of the AML mice to this reagent. It has been shown that MEK inhibition enhances ABT-737–induced leukemia cell apoptosis by preventing ERK-mediated MCL-1 induction46; in our mouse model, the MEK signatures are restored to nearly WT levels observed in normal mice, which again is consistent with sensitivity of the mice to this treatment. These patterns are likely to be a consequence of the restoration of normal hematopoiesis, although our ex vivo data indicates a direct effect of ABT-737 on the abnormal cells with reduced phosphorylation of RAS signaling proteins, ERK and AKT. The cells after the course of treatment in vivo are most likely a mixture of abnormal and normal cells, as evidenced by the reconstitution of longer-lived mice in the secondary transplants of treated cells and the subsequent delayed development of disease.

The rescue from lethality with ABT-737 was associated with a concomitant suppression of cyclin D1/p27kip S-phase–associated proteins. These findings are wholly consistent with the reduced clonogenic capacity of treated cells and with the G1/S checkpoint commonly compromised in many tumor cells. These mechanisms allow the repair of DNA damage due to genetic instability, as reported in AML patient cells37 and in this AML model.40 Furthermore, ABT-737 was found to profoundly induce stress signals as illustrated by upregulation of phospho-p38MAPK in both Sca-1 and Mac-1 compartments; hence, concomitant signals emanating via this pathway may represent a viable target in AML. These data indicate that NRASD12 and BCL-2 cooperate in vivo via tightly regulated cell cycle cyclins and inhibitors consistent with the proliferative capacity...
of hematopoietic cells during AML disease expansion, which is in turn is modulated by ABT-737.

As with other reported studies, with ABT-737, the other targets of the molecule are not completely elucidated in vitro. The increase of BCL-2 expression at the end of treatment may be due to the increased proliferation of the mature myeloid cells that harbor the BCL-2 transgene; this hypothesis is supported by the upregulation of the myeloid differentiation genes observed in the arrays. The short duration of treatment may not have allowed complete terminal differentiation and recovery of normal hematopoiesis, and because this is a transgenic model, complete and durable normal hematopoiesis can never be restored, and the mice inevitably relapse and die of disease. Furthermore, careful cytological analysis of the BM and peripheral blood documented the persistence of granulocytes with dysplasia.

In summary ABT-737 was found to be of benefit in this mouse model of AML, targeting leukemia initiating cells, LSK cells, and progenitors, and inducing upregulation of differentiation, and downregulation of cell cycle and proliferation genes, characteristic of the NRASD12 MDS mouse with a shift in NRAS:BCL-2 localization and protein modification signatures toward a normal phenotype. These measured changes translate to a significantly prolonged lifespan that supports the notion that clinical trials may be warranted in AML patients with this class of inhibitor or its BCL-2–specific derivative, ABT-199, which has recently been developed.48,49

Acknowledgments

The authors thank Annie Soulie and Murielle Reboul for their technical assistance with confocal microscopy and tissue preparation in the early stages of the project, members of the Institut Universitaire d’Hématologie specifically the Département d’Expérimentation Animale, Bernard Boursin and Elisabeth Savariau from the photography laboratory, the Imagery Department for confocal microscopy (who are supported by grants from the Conseil Régional d’Île-de-France and the Ministère de la Recherche), and Michael Bishop and Scott Kogan (University of California, San Francisco) for the MRP8NRA5D12, Irving Weissman and Eric Lagasse (Stanford University) for the MRP8BCL-2 mice and William G. Bornmann (MD Anderson Cancer Center) for generously providing ABT-737 in the early part of the study and Abbott for generously providing ABT-737 to enable us to complete this study.

This work was supported by grants from Fondation de France, Fondation ARC pour la Recherche sur le Cancer, Association Laurette Fugain, Ligue Contre le Cancer, Institut National de la Santé et de la Recherche Médicale, Leukaemia & Lymphoma Research (United Kingdom), and the National Institutes of Health: National Cancer Institute (K23CA140722) (A.C.F. and D.W.F.). R.A.P. and C.C. are members of the European Cooperation in Science and Technology Action BM0801 and the European Leukemia Network. S.B. and C.L.P. are recipients of the European Cooperation in Science and Technology Action BM0801 short-term scientific mission awards.

Authorship


Conflict-of-interest disclosure: P.d.I.G. is a salaried employee of Genosplice. A.T. is a salaried employee of Protein Simple. D.W.F. and A.C.F. have patents pending through Stanford University relating to the nanoinmunoassay technology. The remaining authors declare no competing financial interests.

Correspondence: Rose Ann Padua, UMRS-940, InstitutUniversitaire d’Hématologie, F-75475 Paris, France; e-mail: rose-ann.padua@inserm.fr.

References

17. Zhang C, Cai TY, Zhu H, et al. Synergistic Antitumor Activity of Gemcitabine and ABT-737 In Vitro and In Vivo through Disrupting the


BCL-2 inhibition with ABT-737 prolongs survival in an NRAS/BCL-2 mouse model of AML by targeting primitive LSK and progenitor cells

Stephanie Beurlet, Nader Omidvar, Petra Gorombei, Patricia Krief, Carole Le Pogam, Niclas Setterblad, Pierre de la Grange, Christophe Leboeuf, Anne Janin, Maria-Elena Noguera, Florence Hervatin, Laure Sarda-Mantel, Marina Konopleva, Michael Andreeff, Andrea W. Tu, Alice C. Fan, Dean W. Felsher, Anthony Whetton, Marika Pla, Robert West, Pierre Fenaux, Christine Chomienne and Rose Ann Padua

Updated information and services can be found at:
http://www.bloodjournal.org/content/122/16/2864.full.html

Articles on similar topics can be found in the following Blood collections
- Myeloid Neoplasia (1588 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml