Caloric restriction modulates Mcl-1 expression and sensitizes lymphomas to BH3 mimetic in mice

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Key Points

- Caloric restriction reduces Mcl-1 expression and sensitizes lymphoma cells to ABT-737 in vivo.
- Caloric restriction mimetics can sensitize lymphomas to ABT-737–induced death independently of p53 and of the main BH3-only proteins.

Introduction

In addition to cell-autonomous changes that drive a cancer cell to proliferate and contribute to tumorigenesis, alterations in whole-organism metabolism have been observed to be associated with heightened risks for a variety of cancers. Intriguingly, although excess calories are associated with human cancer and shortened lifespan, caloric restriction (CR) is known to prolong lifespan and decrease tumorigenesis. However, the mechanism by which CR limits tumors and how it can affect responses to chemotherapy are still poorly understood.

Most DNA-damaging chemotherapeutic drugs kill tumor cells through the so-called intrinsic (or mitochondrial) apoptotic pathway. Upon cytotoxic insult, the mitochondrial outer membrane will be permeabilized (MOMP) by the proapoptotic members of the Bcl-2 family, which leads to the release of apoptotic proteins from the intermembrane space, resulting in the activation of caspases. Bcl-2 family members have classically been grouped into three classes. The prosurvival class consists of Bax, Bak, and Bok, with Bax and Bak being critical for the induction of MOMP and the subsequent release of apoptogenic molecules. The third class includes the proapoptotic BH3-only proteins (Bim, Puma, Noxa, Bad, Bmf, Bid, Bik, and Hrk), which promote apoptosis by regulating the antiapoptotic Bcl-2 proteins.

Caloric restriction (CR) is proposed to decrease tumorigenesis through a variety of mechanisms including effects on glycolysis. However, the understanding of how CR affects the response to cancer therapy is still rudimentary. Here, using the Eμ-Myc transgenic mouse model of B-cell lymphoma, we report that by reducing protein translation, CR can reduce expression of the prosurvival Bcl-2 family member Mcl-1 and sensitize lymphomas to ABT-737–induced death in vivo. By using Eμ-Myc lymphoma cells lacking p53, we showed that CR mimetics such as 2-deoxyglucose led to a decrease in Mcl-1 expression and sensitized lymphoma cells to ABT-737–induced death independently of p53. In keeping with this, Eμ-Myc lymphoma cells lacking the BH3-only proapoptotic members Noxa, Puma, or Bim were also sensitized by CR mimetics to ABT-737–induced death. Remarkably, neither the loss of both Puma and Noxa, the loss of both Puma and Bim, nor the loss of all three BH3-only proteins prevented sensitization to ABT-737 induced by CR mimetics. Thus, CR can influence Mcl-1 expression and sensitize cells to BH3 mimetic–induced apoptosis, independently of the main BH3-only proteins and of p53. Exploiting this may improve the efficacy of, or prevent resistance to, cancer therapy. (Blood. 2013;122(14):2402-2411)
The prominent tumor suppressor p53 is found to be eliminated or mutated in approximately 50% of sporadic human cancers. In addition to its role in cell cycle control, the loss of p53 tends to favor glycolysis. Importantly, the loss of p53 function also increases the resistance of tumor cells to a broad range of cancer therapeutics. Therefore, the development of new strategies that can kill tumor cells in a p53-independent manner are a priority.

The link between metabolism and the expression of Bcl-2 family members is poorly understood. By using the Eμ-Myc transgenic mouse model, in which all animals spontaneously develop clonal pre-B or B-cell lymphomas, we have demonstrated that restriction of caloric intake by 25% can significantly reduce the translation of Mcl-1 through the AMPK/mTOR pathway and restore the sensitivity of lymphomas to ABT-737 in vivo. By using genetic approaches to define the impact of p53 and key BH3-only proteins, we established that Eμ-Myc lymphoma cells lacking p53 or Noxa, Puma, or Bim; or lacking both Puma and Noxa or both Puma and Bim; or lacking all three BH3-only proteins (Noxa, Puma, and Bim) were also sensitized by CR mimetics to ABT-737–induced death.

Unexpectedly, our results suggested that CR and CR mimetics (2-deoxyglucose [2DG] and lomidamine [LND]) can sensitize lymphomas in vivo or lymphoma cells in vitro, respectively, to ABT-737–induced apoptosis, regardless of the presence of p53 or the absence of the BH3-only proteins. These data support the metabolic control of Mcl-1 expression as a key event in this setting.

### Methods

#### CR experiment

All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee and of the regional ethics committee (approval reference NCE/2011-35). Eμ-Myc/wild-type (WT) mice were obtained from the Jackson Laboratory. We observed that WT C57BL/6 mice age 6 to 8 weeks eat an average of 3 g of food per day. WT syngenic C57BL/6 were intravenously injected with 0.2 × 10^6 Eμ-Myc cells and were then fed normally or in CR mode (75% of normal dose is 2.25 g per day per mouse). Upon the appearance of lymphoma, mice were intraperitoneally injected daily for 10 days with vehicle or ABT-737 (75 mg/kg). At the end of the ABT-737 treatment, all mice were fed normally. Animals were euthanized as soon as they showed signs of illness. After 9 days of the dietary study, glycemia was measured by using a freestyle Optium blood glucose monitoring device. The number of white blood cells (WBCs) was counted 2 days after the end of the ABT-737 treatment by using a Hemavet 950FS (Drew Scientific, Inc., Le Rheu, France).

#### Cell culture and in vitro treatments

MEF Mcl-1<sup>−/−</sup> cells (and controls) were a kind gift of Dr. Opferman. MEF cells from Bcl-2<sub>−/−</sub> double-knockout mice (and controls) were a kind gift of Dr. Korsmeyer. Eμ-Myc/WT lymphoma cells were harvested from enlarged lymph nodes of C57BL/6 transgenic Eμ-Myc mice isolated and cultured as described. Eμ-Myc lymphomas generated to lack specific BH3-only genes or p53 have been previously described<sup>12</sup>-<sup>25</sup>; mice were intercrossed with Eμ-Myc mice to produce single and compound-deficient lymphomas, as described in Happo et al.<sup>26</sup> Eμ-Myc/Noxa<sup>−/−</sup> and Eμ-Myc/Puma<sup>−/−</sup> Bim<sup>−/−</sup> triple-knockout lymphoma cell lines have been previously described.<sup>26</sup> Next, 0.8 × 10<sup>5</sup> cells per milliliter were treated for 20 hours with various doses of ABT-737 in the presence or absence of 2DG or LND.

#### Statistics

The data are expressed as mean ± standard deviation. For in vitro experiments, differences in the calculated mean values (Gaussian law) between the groups were assessed by one-way analysis of variance, followed by a Fisher test, and in cases in which significant differences were detected, a Tukey honestly significant difference test was used. Kaplan-Meier survival analyses were performed, and survival curves were compared by using log-rank tests.

### Results

**CR reduces Mcl-1 expression through inhibition of protein translation and sensitizes Eμ-Myc mice to ABT-737 treatment**

We sought to determine the impact of CR on the expression of key Bcl-2 family members. To that end, we reduced the dietary intake of the mice by 25%, which had no significant effect on the body weights of C57BL/6 mice over the duration of the experiment (Figure 1A), but which significantly reduced the glycemia of these mice compared with the control group (fed ad libitum; Figure 1B). Upon CR, syngeneic C57BL/6 mice were intravenously injected with Eμ-Myc/WT primary cells; 24 days later, mice from control and CR groups were euthanized in order to analyze the level of expression of several Bcl-2 members in lymphoma tissue by immunoblots (Figure 1C). Mcl-1 levels were reduced (by 39% ± 10%; P < .05; Figure 1D), but no other antiapoptotic Bcl-2 family members tested (supplemental Figure 1). In contrast, Bim S seemed to be enhanced in response to CR (P < .05; Figure 1D).

We and others previously established that inhibition of protein translation via the AMPK/mTOR pathway was an efficient way to decrease Mcl-1 protein levels. It is well established that when nutrient availability is compromised, mTORC1 is inactivated, in turn leading to the inactivation of eEF2. The translation efficiency of the most energy-demanding process in the cell is reduced. As presented in Figure 1E, CR induced the expression and activation of AMPK (and the phosphorylation of ACC, one of its targets). We also observed a decrease in raptor expression, which is in line with mTORC1 destabilization and inhibition. eEF2 is a key regulator of protein translation that is inhibited upon CR via AMPK activation and mTOR inhibition. We confirmed that eEF2 was inactivated (phosphorylated) in lymphoma cells upon CR. Finally, translation inhibition upon CR was further supported by ATF4 overexpression, since it is one of the rare proteins induced upon general inhibition of the translation in cells.

Altogether, our data indicate that upon CR, there is an AMPK activation and reduction of mTORC1, resulting in a decrease in protein translation. Since the Mcl-1 protein has a short half-life, a block in translation results in the reduction of its expression. CR is well established that Eμ-Myc lymphomas are resistant to ABT-737 treatment, at least in part because they express high levels of Mcl-1.<sup>12,21</sup> Therefore, we evaluated whether CR could sensitize Eμ-Myc lymphomas to this BH3 mimetic. We intravenously injected Eμ-Myc lymphoma cells into syngeneic mice and fed the mice either ad libitum (control) or according to CR conditions. One week later, mice were treated with 75 mg/kg ABT-737 or vehicle for 10 days; mice were euthanized when they became unwell due to progressive lymphoma (Figure 2A). Although neither treatment with ABT-737 alone nor CR increased survival of mice over that of control mice, the combination of ABT-737 and CR did so. Indeed, median survival was 30 days in the control group, 33 days in the ABT-737 group, 30 days in the CR group, and 41 days in the CR + ABT-737 group (P < .001 for ABT-737 [n = 8] vs CR + ABT-737 [n = 10]).
We confirmed that the ABT-737 treatment was active because it induced thrombocytopenia (Figure 2B), as previously described.32 The degree of thrombocytopenia was not different between the ABT-737 and CR + ABT-737 groups (Figure 2B), consistent with Mcl-1 levels not having an impact on platelet survival. Finally, while neither ABT-737 nor CR alone modulated the number of circulating...
WBCs (Figure 2C), the combination of ABT-737 and CR did so, suggesting that the combination could sensitize both circulating lymphoma cells and normal WBCs to apoptosis. This reduction in WBCs is consistent with the protective effects seen following combination therapy of mice bearing lymphomas (Figure 2A). These results imply that CR can modulate Mcl-1 levels and sensitize lymphomas in mice to treatment with ABT-737.

CR mimetics sensitize cells to ABT-737, even in the absence of p53

Because p53 is frequently mutated in tumors and because it is a key regulator of glycolysis, which can control Mcl-1 levels, we tested whether the benefits of co-treatment were dependent on p53 status. We performed ex vivo experiments using Eμ-Myc lymphoma cells isolated from Eμ-Myc mice that were either WT or heterozygous for p53 (during lymphomagenesis, Eμ-Myc/p53<sub>WT</sub>/p53<sub>Het</sub> lymphoma cells undergo obligatory loss of the WT allele; supplemental Figure 2). Unlike MEF cells isolated from Mcl-1 knockout pups, Eμ-Myc/p53<sub>WT</sub>/p53<sub>Het</sub> cells were not sensitive to ABT-737–induced death, indicating that BH3-mimetic treatment of Eμ-Myc lymphoma was ineffective, regardless of p53 status (Figure 3A), in accordance with a previous study.

To analyze ex vivo the role of CR in ABT-737–induced cell death, we used 2DG and LND, two classic CR mimetics (see Kang and Wang for a review). First, we verified that 2DG and LND sensitized lymphoma cells to ABT-737–induced death through a caspase-dependent mechanism (ie, apoptosis; supplemental Figure 3). Next, we established that 2DG and LND led to reduced Mcl-1 expression in both Eμ-Myc/WT and Eμ-Myc/p53<sub>WT</sub>/p53<sub>Het</sub> cells (Figure 3B). In addition, by using m<sub>7</sub>GTP (guanosine 5′-triphosphate) Sepharose beads that mimic the messenger RNA cap structure, we verified that 2DG or LND led to a block in protein translation in lymphoma cells (supplemental Figure 4A) and to a decrease in cyclin D1, a protein with a short half-life (supplemental Figure 4B).

It appeared that both Eμ-Myc/WT and Eμ-Myc/p53<sub>WT</sub>/p53<sub>Het</sub> lymphoma cells were resistant to ABT-737 (Figure 3A) and were similarly sensitized to ABT-737–induced death in the presence of 2DG or LND (Figure 3C-D). Results were reproduced in independent Eμ-Myc/p53<sub>WT</sub>/p53<sub>Het</sub> lymphoma cells (supplemental Figure 6A). Thus, ex vivo studies support the sensitization by CR mimetics of lymphoma cells to ABT-737–induced death, independently of p53 expression status.

Figure 2. CR increases the lifespan of mice that receive ABT-737 treatment. (A) Syngeneic C57BL/6 mice were intravenously injected with lymphoma cells that were isolated from Eμ-Myc/WT mice and fed ad libitum or under CR for 17 days. Seven days after intravenous injection, mice were treated or not for 10 days with 75 mg/kg ABT-737. Next, all mice were fed ad libitum until the time of ethical euthanasia (see the experimental procedure scheme, left panel). Lifespans of the mice from the beginning of each treatment regimen are indicated for each group (control vs CR + ABT-737: median survival, 30 and 41 days, respectively; n = 10; P = .00005; CR vs CR + ABT-737: median survival, 30 and 41 days, respectively; n = 9 and n = 10, respectively; P = .0061; ABT-737 vs CR + ABT-737: median survival, 33 and 41 days, respectively; n = 8 and n = 10, respectively; P = .00049). (B) Numbers of platelets were measured in each group at 14 days after the intravenous injection of Eμ-Myc cells (control, n = 5; CR and CR + ABT-737, n = 4 for ABT-737). (C) Numbers of WBCs were analyzed in each group 21 days after the intravenous injection of Eμ-Myc cells (control, n = 7; ABT-737 and CR + ABT-737, n = 8; CR, n = 9). In vivo experiments were conducted twice with similar results. **P < .01; ***P < .001. i.v., intravenous.; ns, nonsignificant.
Results were reproduced in independent E\textsubscript{2}tization by CR mimetics to ABT-737 Mcl-1 protein expression in E\textsubscript{2}t cells from mice bearing E\textsubscript{2}t cells (supplemental Figure 5B-C). We isolated lymphoma cells from mice bearing either E\mu-Myc/Puma\textsuperscript{−/−} or E\mu-Myc/Noxa\textsuperscript{−/−} cells (supplemental Figure 5B-C).

As expected, we verified that the presence or absence of Puma or Noxa did not affect the response of E\mu-Myc cells to ABT-737−induced cell death (Figure 5A). As seen with E\mu-Myc lymphoma cells lacking Bim, neither the loss of Puma nor the loss of Noxa prevented the ability of CR mimetics to reduce Mcl-1 expression (Figure 5B) or to sensitize cells to ABT-737 death (Figure 5C-D).

Next, we reasoned that the removal of individual proapoptotic binding partners of Mcl-1 might not be sufficient to prevent sensitization to ABT-737 induced by CR mimetics, since the other BH3-only proteins are still present and could be sufficient to elicit apoptosis. We therefore examined the combinatorial roles of Bim, Puma, and Noxa in our models. We used E\mu-Myc/Puma\textsuperscript{−/−}/Noxa\textsuperscript{−/−} lymphoma cells. Because it was not feasible to generate E\mu-Myc lymphomas lacking Puma and Bim or lacking Puma, Noxa, and Bim by conventional breeding due to breeding complications and early lethality of intermediate genotypes, fetal liver cells from Puma\textsuperscript{−/−}/Bim\textsuperscript{−/−} double-knockout mice and from Puma\textsuperscript{−/−}/Bim\textsuperscript{−/−}/Noxa\textsuperscript{−/−} triple-knockout mice were infected with a c-MYC retrovirus, injected into lethally irradiated recipient mice, and lymphomas were harvested for subsequent analysis.

Lymphoma genotypes were confirmed (supplemental Figure 5D-E). We verified that sensitization to ABT-737 was not affected in those
cells (Figure 6A) and that CR mimetics led to a decrease in Mcl-1 protein expression regardless of the genotype (Figure 6B). Perhaps surprisingly, neither Eμ-Myc/Puma−/− Noxa−/− lymphoma cells nor rv-MYC/Puma−/− Bim−/− nor rv-MYC/Puma−/− Bim−/− Noxa−/− knockout lymphoma cell lines (TKO cells) were protected from sensitization to 10 μM ABT-737–induced death observed in the presence of CR mimetics, although some protection was observed at 5 μM compared with death seen in Eμ-Myc/WT lymphoma cells (Figure 6C). Results shown are mean ± SD from 3 independent experiments. Each experiment was performed on cells from 2 independently derived lymphomas. ***P < .001.

Discussion

Over the last few years, many attempts have been made to develop more specific cancer therapies that target specific properties of tumor cells, such as their avidity for glucose (Warburg effect) or their dependence on the Bcl-2 protein family (using BH3 mimetics). By using the Eμ-Myc model, we have shown that CR can modulate the expression of the important prosurvival member of the Bcl-2 family, Mcl-1, and can promote the sensitization of lymphomas to ABT-737 treatment in mice (Figures 1C and 2A). We have extended this observation by using lymphoma cells that were originally isolated from knockout mice to show that this sensitization was independent of Bim, Puma, or Noxa, the three BH3-only proteins known to bind Mcl-1 and Puma. Thus, the relative reduction in availability of Mcl-1, together with the neutralization of Bcl-2, Bcl-xL and possibly Bcl-w by ABT-737, prevents sequestration of Bax and Bak, freeing them to initiate the apoptotic cascade required for therapeutic efficacy.
to Mcl-1 (Figures 4-6). Notably, this sensitization was also independent of p53 status (Figure 3), thereby introducing an innovative strategy with the potential to enhance the efficiency of killing tumor cells.

To address whether CR could modulate the expression of Bcl-2 family members, we restricted the food intake of mice by 25% for 24 days (Figure 1C) because this approach has been shown to have no significant impact on the weights of the animals, but will lead to a significant decrease in glycemia (Figure 1A-B). We used a lymphoma transplantation model because our goal was to analyze the impact of CR on established tumors and not to study the lymphoma transplantation model, because our goal was to analyze that in the E/Myc mice, CR mimetics could affect the translation of Mcl-1 protein. It has been demonstrated extensively that CR can modulate messenger RNA translation regulation (mainly via the mTOR pathway) and that the reduction of glucose metabolism (using CR mimetics) affects the translation of Mcl-1 protein.

It was worth noting that the number of WBCs that were measured in circulating blood and the overall survival rates of the mice in the CR group (fed ad libitum), ABT-737, or CR groups were not significantly different (Figure 1A-B). We used a preliminary data from freshly isolated human lymphoma cells from patients suffering from Burkitt or diffuse large B-cell lymphoma to test the efficiency of our cotreatment. However, preliminary data from freshly isolated human lymphoma cells from patients with follicular B-cell lymphoma (n = 1) or Hodgkin lymphoma (n = 1) showed sensitization to killing with ABT-737 by the...
addition of either 2DG or LND (P < .05; supplemental Figure 8), underlying the potential benefit of such cotreatment in patients. Primary tumor cells were isolated from the remaining part of patients’ lymph node biopsies collected for diagnosis after patients’ agreement and directly analyzed in our laboratory. Informed consent was obtained in accordance with the Declaration of Helsinki.

Figure 6. There is no compensatory effect of Bim, Puma, or Noxa on the CR mimetics–mediated sensitization to ABT-737–induced death. (A) The indicated cells were treated for 20 hours with increasing doses of ABT-737. Cell death (percentage of PI-positive cells) was determined by FACS. The insert shows Mcl-1 expression in the indicated cell types. (B) The indicated cells were incubated with 2DG 100 μg/mL or LND 100 μM for 20 hours, and the levels of Mcl-1 were analyzed by immunoblot. HSP60 was used as a loading control. Quantifications of Mcl-1 over HSP60 levels are indicated. (C-D) The indicated cells were treated for 20 hours with 1, 5, or 10 μM ABT-737 with or without 2DG 100 μg/mL or LND 100 μM. Dead cells were counted as in Figure 3A. The results represent the mean ± SD of 3 independent experiments. Each experiment was performed on cells from 2 independently derived lymphomas for each genotype. ***P < .001.
Several reports have suggested that DNA-damaging agents\textsuperscript{31,39} could sensitize tumor cells to ABT-737–induced death. However, since p53 is mutated or deleted in approximately 50% of human tumors, this approach may reduce the potential impact of such observations. In striking comparison, we have demonstrated that CR could sensitize lymphomas to ABT-737–induced apoptosis independently of p53 (Figure 3), as suggested in other models,\textsuperscript{13} thereby introducing new strategies to kill such cancer cells.

A question remains concerning our work: Which proapoptotic member(s) are involved in sensitization to ABT-737 induced by CR mimetics? The Bcl-2 family comprises at least 11 proapoptotic members that share some redundant functions.\textsuperscript{4} It is now generally accepted that the BH3-only members play an important role in Bax and Bak activation, even though the mechanisms for this process are still not well understood.\textsuperscript{4}

We investigated the roles of some of the preferential binding partners of Mcl-1 (Bim, Puma, Noxa) by using single, double, or triple gene deletion models, since these were recently found to be required for the induction of maximal killing of lymphoma cells in response to DNA-damaging agents.\textsuperscript{26} In addition, Noxa has been described to play a role in glucose metabolism by promoting glucose uptake.\textsuperscript{40} It has further been suggested that the CPT-11 or bortezomib–described to play a role in glucose metabolism by promoting glucose deprivation expression. Among these proteins, Bad has been linked extensively activated or de-repressed upon the CR-dependent decrease in Mcl-1 and thus a dependence on Noxa-induced killing.

In fact, we repeatedly established that our combination therapy was efficient, regardless of the genotype of the myc–driven lymphoma cells (Figures 4–6 and supplemental Figure 6). Therefore, it is possible that one (or several) other proapoptotic members are activated or de-repressed upon the CR-dependent decrease in Mcl-1 expression. Among these proteins, Bad has been linked extensively to glucose metabolism and glucose deprivation–induced cell death.\textsuperscript{43} Recent studies report that upon glycolysis inhibition, Bak dissociation from Mcl-1 or BMF was induced, thereby sensitizing the cells to BH3 mimetic–induced death.\textsuperscript{13,14} According to the roles of these proteins in cell death and the impact of metabolism on their activation, it is possible that Bad, BMF, and/or Bak are playing roles in myc–driven lymphoma. Nevertheless, we cannot rule out the possibility that Bid, Bax, HRK, or Bik could also play central roles in CR-mediated sensitization to ABT-737–induced death.

It has been shown recently that specific oncogenes, such as activated PI3K, render tumors unresponsive to CR, suggesting that the efficacy of reduced food intake may be limited to certain molecular subsets of cancers.\textsuperscript{44} Additionally, the side effects of long-term CR may impose significant risks to cancer patients who are receiving chemotherapy.\textsuperscript{45} However, our work establishes that short-term CR may represent a safe and efficient way to sensitize tumor cells to BH3 mimetics, independently of the status of p53. This study suggests that one challenge for future investigations is the identification of the therapeutic window and the necessary conditions to sensitize the patient to this innovative treatment.

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Authorship

Contribution: O.M. and B.Z. performed most experiments; B.Z. and M.C. performed statistical analysis; J.C., M.A.J., and L.M. performed experiments; L.A.P., L.H., J.-F.T., C.L.S., and E.M.M. contributed reagents and scientific input; B.T., G.G., J.R.-M., N.M., and J.-F.M. collected patient samples; and J.-E.R. designed research, interpreted data, and wrote the manuscript.

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