Cell Death and Differentiation (2012) 19, 623–632

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Pharmacological blockade of Bcl-2, Bcl-x_L and Bcl-w by the BH3 mimetic ABT-737 has only minor impact on tumour development in *p53*-deficient mice

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The tumour suppressor *p53* transcriptionally regulates a range of target genes that control cell growth and survival. Mutations of *p53* have been implicated in the development of ~50% of human cancers, including those instigated by exposure to mutagens. Although numerically rare, cancers can arise as a consequence of inherited mutations, such as in the Li–Fraumeni syndrome, which is caused by mutation of one *p53* allele. Gene-targeted mice deficient for p53 have been generated to study this familial cancer syndrome. On a C57BL/6 background, *p53*-deficient mice develop primarily thymic lymphoma and more rarely sarcoma. Evasion of apoptosis is considered to be essential for neoplastic transformation. As proteins of the Bcl-2 family are the critical regulators of apoptosis, we investigated the role of the pro-survival members Bcl-2, Bcl-x_L and Bcl-w in cancer development in $p53^{+/-}$ and $p53^{-/-}$ mice by testing whether ABT-737, a pharmacological inhibitor of these proteins, could prevent or delay tumourigenesis. Our studies showed that ABT-737 prophylaxis only caused a minor delay and reduction in γ-radiation-induced thymic lymphoma development in $p53^{-/-}$ mice, but this was accompanied by a concomitant increase in sarcoma. These data show that, collectively, Bcl-2, Bcl-x_L and Bcl-w have only minor roles in thymic lymphoma development elicited by defects in p53, and this may indicate that Mcl-1 and/or A1 may feature more prominently in this process.
Cell Death and Differentiation (2012) **19,** 623–632; doi:10.1038/cdd.2011.133; published online 14 October 2011

Li–Fraumeni syndrome (LFS) is a rare inherited autosomal disorder characterised by development of a diverse range of cancer types, including sarcomas, leukaemias/lymphomas, colon, pancreatic or breast cancers and brain or adrenocorticoid tumours before the age of 45. Srivastava *et al.*[1] and Malkin *et al.*[2] have linked LFS to heterozygous germline mutations within the tumour-suppressor gene *p53*. Obligatory loss or mutation of the wild-type (wt) *p53* allele plus additional lesions in oncogenes and/or tumour-suppressor genes drives the development of cancers in these individuals. To establish a mouse model of LFS, several groups have performed gene targeting in ES cells to generate mice that either lack *p53*[3,4] or carry point mutations in *p53* that are commonly found in human cancers.[5] *p53* mutant mice are highly prone to develop a similar range of cancers as those found in LFS patients,[3–5] although lymphomas and sarcomas predominate with their relative frequencies affected by the genetic background.[4]

The p53 tumour-suppressor gene encodes a transcription factor that can be activated by a broad range of stress stimuli, including DNA damage, hypoxia or activation of certain oncogenes (e.g., *c-myc*).[6] Upon activation, p53 transcriptionally regulates various target genes that control a broad range of effector pathways, including cell-cycle arrest, DNA repair, cellular senescence and apoptosis.[6] Somatically acquired mutation or loss of *p53* have been implicated in the development of ~50% of sporadic human cancers. Importantly, loss of p53 function also increases resistance of tumour cells to a broad range of anticancer therapeutics, particularly those that elicit DNA damage.[6] Therefore, development of new strategies that can kill tumour cells in a p53-independent manner is an important area of research.

Apoptosis is a form of programmed cell death that is essential for normal development and tissue homeostasis in multicellular organisms.[7] Evasion of apoptosis is a hallmark of cancer cells that promotes neoplastic transformation in concert with other tumourigenic processes, such as self-sufficiency for cell growth and evasion of cellular senescence.[8] Apoptosis is regulated by interactions between the pro-survival and pro-apoptotic members of the Bcl-2 family and, accordingly, many cancer types show abnormalities in the balance between these two factions.[9] The pro-survival Bcl-2-family members share four distinct Bcl-2 Homology domains (BH1–BH4), and experiments using gene-targeted mice have shown that these proteins have tissue-specific as well as overlapping critical roles in cell survival.[9] The pro-apoptotic proteins Bax and Bak also contain four BH domains and share substantial structural similarity to their Bcl-2 pro-survival relatives, but serve critical functions in the

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Keywords: p53; ABT-737; Bcl-2 family; lymphoma; cancer
Abbreviations: qRT-PCR, quantitative PCR; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; DMSO, dimethyl sulphoxide; BSS, balanced salt solution; CL, cell line; wt, wild type; Ctrl, control value; WBC, white blood cell count; *Eμ-myc*/rv-*bcl-2*, *Eμ-myc* B lymphoma cells that had been retrovirally transduced with a Bcl-2 expression construct
Received 25.5.11; revised 06.9.11; accepted 07.9.11; Edited by S Kaufmann; published online 14.10.11

activation of the effector stages of apoptosis by mediating mitochondrial outer membrane permeabilisation (MOMP) and consequent activation of the caspase cascade.[9,10] The BH3-only members among the Bcl-2-family members (Bim, Puma, Noxa, Bid, Bad, Bmf, Bik and Hrk) share with each other and the wider family only the 16- to 24-amino-acid BH3 domain. Studies using gene-targeted mice have shown that BH3-only proteins are essential for initiation of apoptosis signalling, functioning in a cell death stimulus-specific as well as a cell type-specific manner.[9,11]

New anticancer therapeutics are being developed to specifically target the pro-survival members of the Bcl-2 family using small-molecule mimetics of BH3-only proteins.[12] ABT-737 and (the orally available) ABT-263, two BH3-mimetics that bind with high affinity to Bcl-2, Bcl-x_L and Bcl-w, but not to Mcl-1 or A1, have proven to be highly effective as single agents in inhibiting the growth of small-cell lung carcinoma cells in xenograft mouse models.[13,14] In addition, ABT-737 synergised potently with many conventional chemotherapeutic drugs (e.g., cyclophosphamide and taxol) and inhibitors of oncogenic kinases (e.g., Gleevec for blockade of BCR-ABL in CML) in the killing of a broad range of cancers.[15,16]

Given that evasion of apoptosis, often due to deregulated expression of pro-survival Bcl-2-family members, is thought to be critical for tumour development, it is theoretically possible that BH3 mimetics may find utility as a prophylactic strategy to prevent or delay development of malignant disease in genetically predisposed individuals, such as Li–Fraumeni patients. To test this hypothesis experimentally, we explored whether blockade of Bcl-2, Bcl-x_L and Bcl-w by the BH3-mimetic ABT-737 could inhibit or delay tumourigenesis in *p53*-deficient mice. ABT-737 prophylactic treatment had only a minor impact, although it did cause a minor delay in onset and reduction in the incidence of γ-radiation-induced thymic lymphoma in *p53*-deficient mice, which was accompanied by a compensatory increase in sarcoma. These results indicate that Bcl-2, Bcl-x_L and Bcl-w must only have a minor role in sustaining the survival of cells undergoing neoplastic transformation owing to defects in p53.

Results

Prophylactic treatment with the BH3 mimetic ABT-737 does not prevent tumour development in $p53^{+/-}$ and $p53^{-/-}$ mice. Expression of endogenous pro-survival Bcl-2-family members is thought to be critical for the development of many cancers.[17] We wanted to determine which pro-survival Bcl-2-family members are critical to sustain survival of cells undergoing neoplastic transformation and hence are essential for tumourigenesis in cancer-prone *p53*-deficient mice. To block all of Bcl-2, Bcl-x_L and Bcl-w, we prophylactically treated $p53^{+/-}$ and $p53^{-/-}$ mice from the age of 4 weeks with ABT-737 (twice per week 50 mg/kg body weight, i.p.) or vehicle for 20 consecutive weeks and monitored the mice for tumour development. This dose of ABT-737 caused a significant decrease in platelets ($P^{**}=0.0036$) as well as leukocytes ($P^{***}=0.0009$) in the blood, and also resulted in a decrease in thymocytes ($P^{**}=0.0024$) and splenocytes ($P^{*}=0.0321$) of C57BL/6 (wt) mice (Supplementary Figure 1), indicating that this treatment regime was efficacious. Surprisingly, ABT-737 had no significant impact on the incidence or rate of tumour development in the $p53^{+/-}$ ($P=0.0623$) or $p53^{-/-}$ mice ($P=0.5477$; Figure 1a). Although vehicle-treated $p53^{+/-}$ mice showed a trend towards increased survival compared with ABT-737-treated $p53^{+/-}$ animals (median survival: vehicle = 469 days *versus* ABT-737 = 312 days), this difference did not reach statistical significance ($P=0.0623$).

Sick ABT-737 or vehicle-treated $p53^{+/-}$ mice succumbed mostly to either sarcoma or thymic lymphoma, and a small number developed carcinoma, such as prostate cancer (Figure 1b). There was no significant difference in lymphoma or sarcoma incidence between both treatment groups. The ABT-737-treated $p53^{+/-}$ mice presented with lower spleen and lymph node weights, and lower WBCs, compared with the vehicle-treated animals, but none of these differences were statistically significant (Figures 2a–c). Thymus weights were similar in both cohorts (Figure 2a). PCR genotyping of thymus samples collected from $p53^{+/-}$ mice that had to be killed either because of thymic lymphoma or sarcoma revealed loss of the wt p53 allele only in samples from thymic lymphoma-burdened mice but not from those that had developed sarcoma (Supplementary Figure 2). This result is consistent with previous reports that loss of heterozygosity is essential for thymic lymphoma development in $p53^{+/-}$ mice.[3,4] Flow-cytometric analysis using surface marker-specific antibodies (CD3, CD4 and CD8 for T cells and B220 for B cells) revealed no significant difference in T- and B-lymphoid-cell subset composition in thymi and spleens between both treatment groups.

Sick $p53^{-/-}$ mice mostly succumbed to thymic lymphoma, although in some cases this was also associated with sarcoma development and/or liver/kidney failure (Figure 1c). Mice that developed thymic lymphoma also commonly presented with infiltration of lymphoma cells into the lungs (which frequently led to breathing difficulties in the affected animals), spleen and, to a lesser extent, the liver and kidney (Figure 3c). No consistent differences in thymic lymphoma incidence (Figure 1c) and tumour dissemination (Figure 3) were observed between the two treatment groups at killing. Our analysis revealed a trend towards a decrease in thymus, spleen and lymph node weights as well as blood leukocyte counts in sick, tumour-burdened $p53^{-/-}$ mice that had been prophylactically treated with ABT-737 in comparison with mice that had been treated with vehicle, but none of these differences reached statistical significance (Figures 3a and b). The majority of thymi from sick animals (regardless of treatment cohort: ABT-737 or vehicle analysed by flow cytometry) revealed the presence of lymphoma cells, characterised by abnormal increase in cell size and an unusual distribution of T-cell surface markers, such as $CD4^{Low}CD8^{High}$. Collectively, these data show that prophylactic treatment with ABT-737 is unable to delay spontaneous tumour development or reduce the severity of malignant disease in $p53^{+/-}$ and $p53^{-/-}$ mice.

Prophylactic treatment with the BH3 mimetic ABT-737 has a significant, albeit minor, impact on low-dose γ-irradiation-induced thymic lymphoma development in $p53^{-/-}$ mice. In order to mimic the effects of environmental

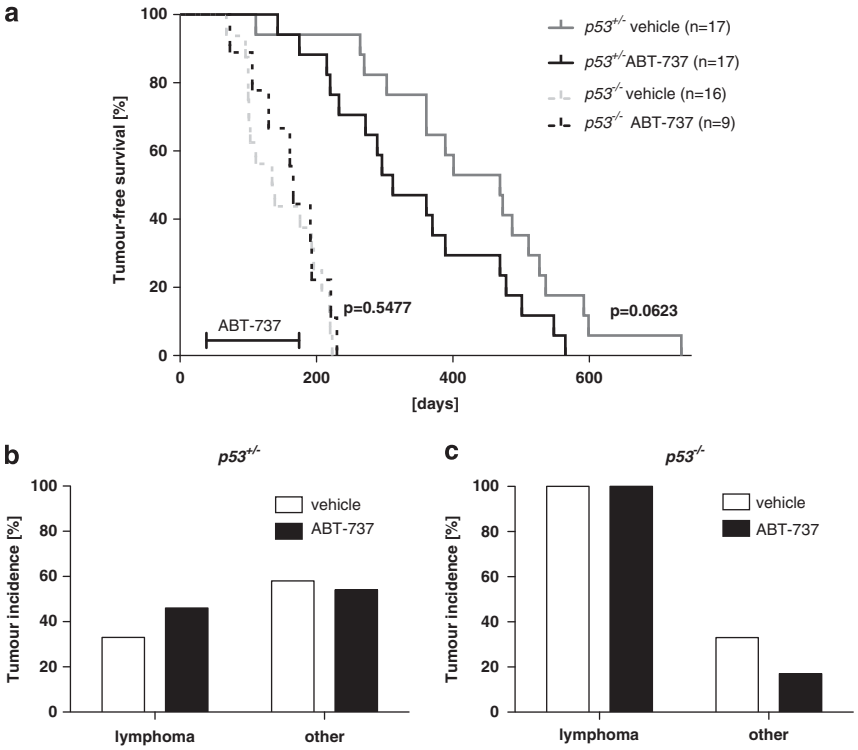

Figure 1 Prophylactic treatment of pre-malignant $p53^{+/-}$ and $p53^{-/-}$ mice with the BH3 mimetic ABT-737 does not prevent or even delay tumourigenesis. (**a**) Kaplan–Meier analysis of tumour-free survival comparing two cohorts of $p53^{+/-}$ mice as well as two cohorts of $p53^{-/-}$ mice, one prophylactically treated with the BH3 mimetic ABT-737 the other with vehicle between 4 and 24 weeks after birth (treatment period is indicated with a line). Median tumour onset of $p53^{+/-}$ mice prophylactically treated with ABT-737 was 312 days *versus* 469 days for $p53^{+/-}$ mice that had been treated with vehicle. This difference is not statistically significant ($n_{ABT-737} = 17$, $n_{Vehicle} = 17$; Mantel–Cox log–rank test: $P = 0.0623$). Median tumour onset for $p53^{-/-}$ mice prophylactically treated with ABT-737 was 166 days *versus* 137 days for mice that had been treated with vehicle. This difference was also not statistically significant ($n_{ABT-737} = 9$, $n_{Vehicle} = 16$; Mantel–Cox log-rank test: $P = 0.5477$). (**b**) Percentages of $p53^{+/-}$ mice within each cohort (ABT-737 prophylaxis or vehicle treatment) that developed the different types of malignancies ($n_{ABT-737} = 17$, $n_{Vehicle} = 17$). (**c**) Percentages of $p53^{-/-}$ mice within each cohort (ABT-737 prophylaxis or vehicle treatment) that developed the different types of malignancies ($n_{ABT-737} = 6$, $n_{Vehicle} = 6$)

mutagenic factors on tumourigenesis, we examined the impact of prophylactic treatment with ABT-737 on low-dose γ-radiation-induced thymic lymphoma development in $p53^{+/-}$ and $p53^{-/-}$ mice. Repeated exposure to low-dose γ-radiation elicits thymic lymphoma development in certain strains of mice, including C57BL/6, owing to activation of the endogenous radiation-induced leukemia virus (RadLV) and generation of oncogenic mutations resulting from DNA-strand breaks.[18] Loss of one or both alleles of *p53* significantly accelerates γ-radiation thymic lymphoma development,[19] most likely because of loss of p53's ability to coordinate cell-cycle arrest and DNA-repair responses. As expected, when compared with non-irradiated $p53^{+/-}$ mice, both treatment cohorts (ABT-737 prophylactic treatment or vehicle) of γ-irradiated $p53^{+/-}$ mice showed a considerably accelerated tumour onset (compare Figures 1a and 4a). The median survival and tumour incidence of γ-irradiated $p53^{+/-}$ mice did not significantly differ between animals that had been treated prophylactically with ABT-737 and vehicle-treated mice (172 *versus* 190 days, respectively, $P = 0.8992$; Figures 4a and b). The majority of mice in both treatment groups presented with thymic lymphoma and these tumours routinely showed, as expected,[19] loss of the wt *p53* allele (Supplementary Figure 2). Analysis of thymus, spleen and lymph node weights (Figure 5a), as well as blood leukocyte counts (Figure 5b), of sick, tumour-burdened, γ-irradiated $p53^{+/-}$ mice revealed no significant differences between the treatment groups despite a trend towards a decrease in lymph node weights ($P = 0.0691$; Figure 5a). Flow-cytometric analysis revealed that most lymphomas were $CD8^{+}CD4^{-}$ or $CD4^{Low}CD8^{High}$ in both the ABT-737 and vehicle-treated mice. Histological analysis revealed some dissemination of lymphoma cells into the lungs and occasionally the liver and kidney (Figure 5c), but the extent of these pathological features was not different between the ABT-737 and vehicle-treated animals.

Remarkably, prophylactic treatment with ABT-737 significantly, albeit to a relatively minor extent, prolonged the tumour-free survival of γ-irradiated $p53^{-/-}$ mice (median survival: 134 days *versus* 121 days, respectively, $P^{**} = 0.0070$; Figure 4a). Autopsy of sick animals from both treatment groups showed that the majority developed thymic lymphoma that was routinely associated with lymphoma cell infiltration into the lungs, and in some cases also sarcoma or splenomegaly and lymphadenopathy (Figure 6c). In addition to delaying overall tumour development, ABT-737 prophylactic treatment also caused a pronounced reduction in the incidence of thymic lymphoma and a significant reduction in thymus weight and a marked but not significant decrease in leukocytes in the peripheral blood (Figures 4c, and 6a and b).

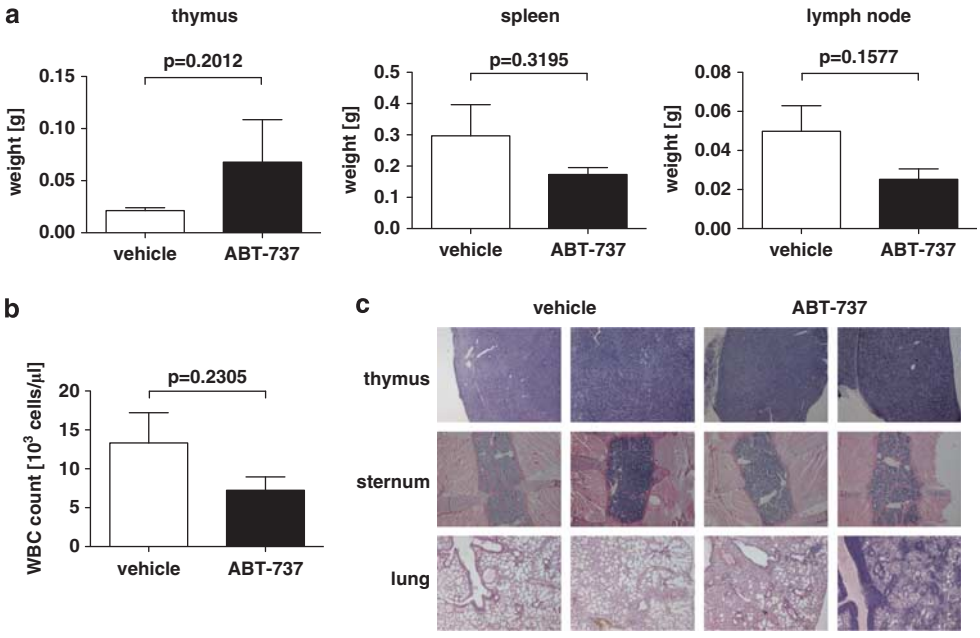

Figure 2 Prophylactic treatment with ABT-737 does not alter the severity and manifestation of thymic lymphoma in $p53^{+/-}$ mice. (**a**) Thymus ($P=0.2012$, ± S.E.M.), spleen ($P=0.3195$, ± S.E.M.) and lymph node ($P=0.1577$, ± S.E.M.) weights from $p53^{+/-}$ mice that had been prophylactically treated with ABT-737 as compared with their vehicle-treated counterparts. (**b**) WBCs in tumour-burdened sick $p53^{+/-}$ mice that had been prophylactically treated with ABT-737 or vehicle ($P=0.2305$, ± S.E.M.). (**c**) Histological analysis of representative thymus, sternum and lung tissue samples of $p53^{+/-}$ mice treated with ABT-737 or vehicle ($n_{ABT-737}=2$, $n_{Vehicle}=2$). Tissue sections were stained with haematoxylin and eosin

This was, however, accompanied by a compensatory increase in sarcoma in γ-irradiated $p53^{-/-}$ mice that had been exposed to ABT-737 (Figure 4c). No marked differences in lymphoma burden or lymphoma cell dissemination into lungs and other tissues were detected between the two cohorts (Figures 6a–c). Most of these tumours were CD8 SP or $CD4^{Low}CD8^{High}$ (Supplementary Figure 3), a phenotype frequently observed in radiation-induced thymic lymphomas.[20,21] These results demonstrate that prophylactic treatment with ABT-737 can inhibit γ-radiation-induced thymic lymphoma development in $p53^{-/-}$ mice, albeit only to a minor extent.

Thymic lymphoma cells from *p53*-deficient mice, regardless of ABT-737 prophylaxis or vehicle treatment, are relatively resistant to *in vitro* treatment with ABT-737. To explore whether thymic lymphoma cells that develop under the pressure of ABT-737 prophylactic treatment might be selected for resistance to apoptotic stimuli, we generated cell lines (CLs) from three different primary thymic lymphomas of untreated $p53^{-/-}$ mice as well as from γ-irradiated $p53^{-/-}$ that had either been treated prophylactically with ABT-737 or vehicle. These CLs and as control, the ABT-737-sensitive pre-B lymphoma-derived CL Eμ-*myc*/rv-*bcl-2*,[15] were exposed to 5 μM ABT-737, 30 nM dexamethasone or vehicle (dimethyl sulphoxide (DMSO)) in culture, and their viability was monitored over 24 h (Figure 7). We observed no significant differences in the response to ABT-737 or dexamethasone between the thymic lymphoma-derived CLs from ABT-737 prophylactically treated mice and those from vehicle control-exposed animals. In comparison with the Eμ-*myc*/rv-*bcl*-2 lymphoma cells, all thymic lymphoma cells from *p53*-deficient mice were relatively resistant to ABT-737 (Figure 7). In agreement with a previous study,[22] dexamethasone efficiently induced apoptosis of *p53*-deficient thymic lymphoma cells, whereas, as expected,[23] the Bcl-2-overexpressing Eμ-*myc*/rv-*bcl*-2 lymphoma cells were highly resistant to this glucocorticoid. Taken together, these data show that ABT-737 prophylaxis does not alter the response of thymic lymphomas originating in *p53*-deficient mice to ABT-737 or dexamethasone.

Thymic lymphomas arising in γ-irradiated *p53*−/− mice prophylactically treated with ABT-737, display no differences in RNA and protein expression of Bcl-2 family members when compared to those from vehicle treated control mice. Quantitative RT-PCR and western blot analysis revealed that the levels of Bcl-2 ($P=5379$), Bcl-x_L ($P=0.3121$), Mcl-1 ($P=0.6667$), Bcl-w ($P=0.5119$) and A1 ($P=0.7927$) were not markedly different between thymic lymphomas from the two treatment groups (Figures 8a and b). In certain tumours, however, we occasionally observed a minor increase in the protein levels of Mcl-1 and/or Bcl-x_L of ABT-737- or vehicle-treated animals as compared with extracts from C57BL/6 thymi, which were used as control. In addition, there was no consistent difference between the various thymic lymphomas in the levels of the pro-apoptotic BH3-only proteins Bim, Bid or Puma. We did, however, observe a downregulation of Bim and Bid protein levels when comparing the primary tumour cells and their derivative CLs, which might explain the decreased sensitivity to ABT-737 treatment in our *in vitro* experiment (Figure 7). Collectively,

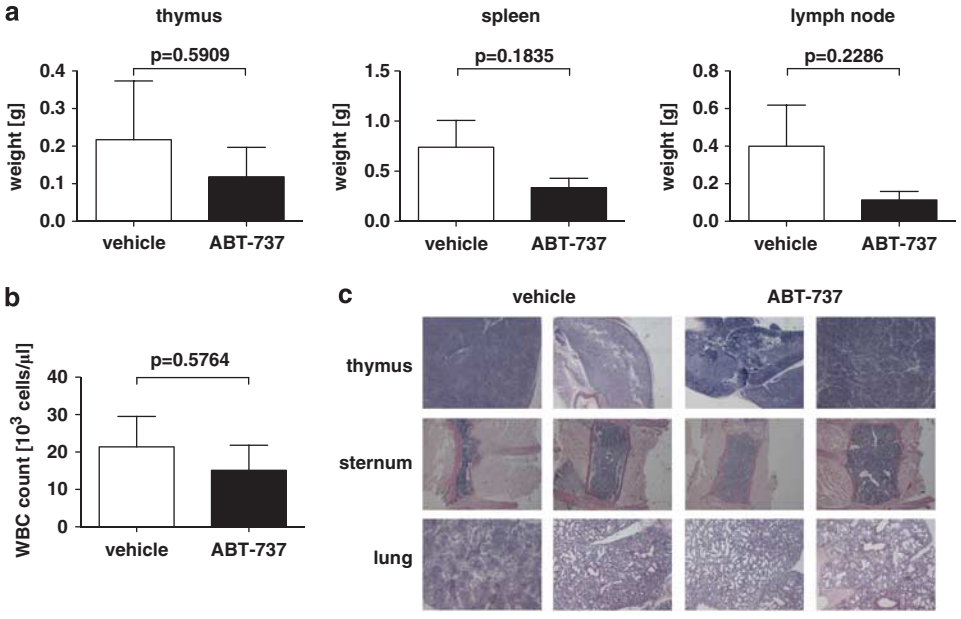

Figure 3 Prophylactic treatment with ABT-737 does not alter the severity and manifestation of thymic lymphoma in $p53^{-/-}$ mice. (**a**) Thymus ($P = 0.5909$, ± S.E.M.), spleen ($P = 0.1835$, ± S.E.M.) and lymph node ($P = 0.2286$, ± S.E.M.) weights from $p53^{-/-}$ mice that had been treated with either ABT-737 or vehicle. (**b**) WBCs of sick $p53^{-/-}$ mice that had been prophylactically treated with ABT-737 or vehicle ($P = 0.5764$, ± S.E.M.). (**c**) Histological analysis of representative thymus, sternum and lung tissue samples of $p53^{-/-}$ mice treated with ABT-737 or vehicle ($n_{ABT-737} = 2$, $n_{Vehicle} = 2$). Tissue sections were stained with haematoxylin and eosin

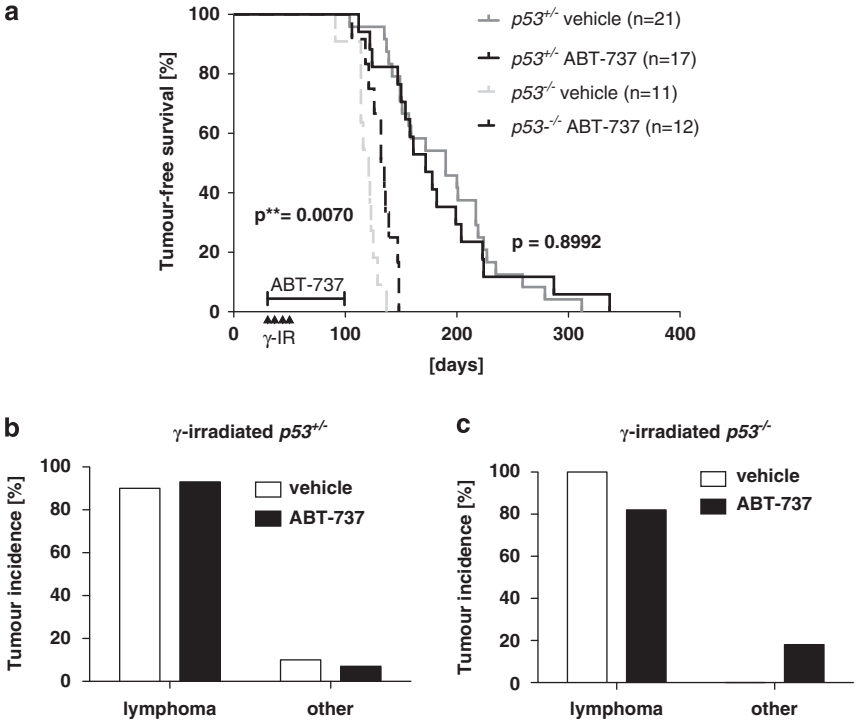

Figure 4 Prophylactic treatment with ABT-737 slows the development of thymic lymphoma in γ-irradiated $p53^{-/-}$ but not $p53^{+/-}$ mice. (**a**) Kaplan–Meier analysis of tumour-free survival comparing two cohorts of γ-irradiated $p53^{+/-}$ as well as two cohorts of γ-irradiated $p53^{-/-}$ mice, one prophylactically treated between 4 and 14 weeks after birth with the BH3 mimetic ABT-737 and the other with vehicle. The arrowheads indicate the days when mice had been γ-irradiated. The time of treatment with ABT-737 or vehicle is indicated by a line. Median tumour onset for γ-irradiated $p53^{+/-}$ mice prophylactically treated with ABT-737 was 172 days *versus* 190 days for γ-irradiated $p53^{+/-}$ mice that had been treated with vehicle ($n_{ABT-737} = 17$, $n_{Vehicle} = 21$). This difference is not statistically significant (Mantel–Cox log-rank test: $P = 0.8992$). Median tumour onset for γ-irradiated $p53^{-/-}$ mice prophylactically treated with ABT-737 was 134 days *versus* 121 days for γ-irradiated $p53^{-/-}$ mice treated with vehicle. This difference is statistically significant ($n_{ABT-737} = 12$, $n_{Vehicle} = 11$; Mantel–Cox log-rank test: $P^{**} = 0.0070$). (**b**) Percentages of γ-irradiated $p53^{+/-}$ mice within each cohort (ABT-737 prophylaxis or vehicle treatment) with the indicated type of malignancy ($n_{ABT-737} = 17$, $n_{Vehicle} = 21$). (**c**) Percentages of γ-irradiated $p53^{-/-}$ mice within each cohort (ABT-737 prophylaxis or vehicle treatment) with the indicated type of malignancy ($n_{ABT-737} = 12$, $n_{Vehicle} = 11$)

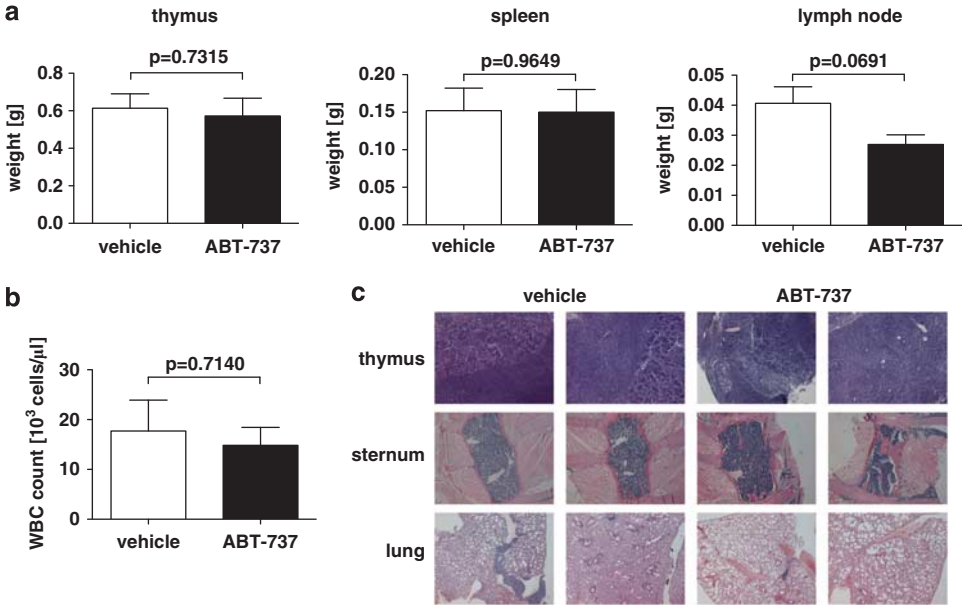

Figure 5 Prophylactic treatment with ABT-737 does not alter the severity and manifestation of thymic lymphoma in γ-irradiated $p53^{+/-}$ mice. (**a**) No significant changes were observed in thymus ($P=0.7315$, $\pm$S.E.M.), spleen ($P=0.9649$, $\pm$S.E.M.) and lymph node ($P=0.0691$, $\pm$S.E.M.) weights in tumour-burdened sick $p53^{+/-}$ mice that had been γ-irradiated and prophylactically treated with ABT-737 as compared with vehicle-treated controls. (**b**) WBCs in sick $p53^{+/-}$ mice that had been γ-irradiated and prophylactically treated with ABT-737 or vehicle ($P=0.7140$, $\pm$S.E.M.). (**c**) Histological analysis of representative thymus, sternum and lung tissue samples from γ-irradiated $p53^{+/-}$ ($n_{ABT-737}=2$, $n_{Vehicle}=2$). Tissue sections were stained with haematoxylin and eosin

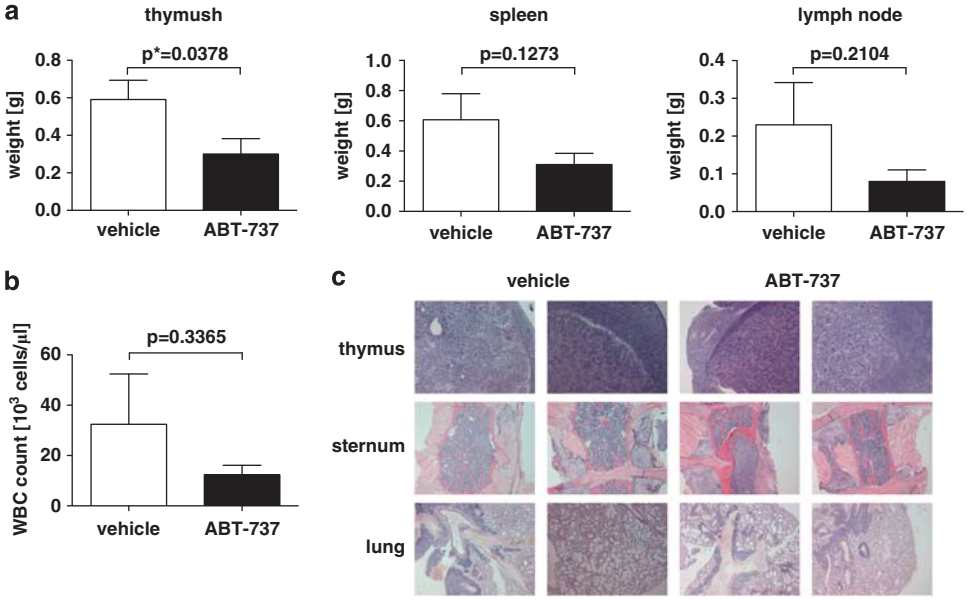

Figure 6 Prophylactic treatment with ABT-737 has a minor impact on the severity and manifestation of thymic lymphoma in γ-irradiated $p53^{-/-}$ mice. (**a**) Significantly decreased thymus ($P^*=0.0378$, $\pm$S.E.M.), but not spleen ($P=0.1273$, $\pm$S.E.M.) and lymph node ($P=0.2104$, $\pm$S.E.M.), weights in tumour-burdened sick $p53^{-/-}$ mice that had been γ-irradiated and prophylactically treated with ABT-737 as compared with vehicle-treated controls. (**b**) WBCs of sick $p53^{-/-}$ mice that had been γ-irradiated and prophylactically treated with ABT-737 or vehicle ($P=0.3365$, $\pm$S.E.M.). (**c**) Histological analysis of representative thymus, sternum and lung tissue samples of sick γ-irradiated $p53^{-/-}$ mice ($n_{ABT-737}=2$, $n_{Vehicle}=2$). Tissue sections were stained with haematoxylin and eosin

our results show that prophylactic treatment with ABT-737 during thymic lymphoma development does not select for aberrations in the expression of the pro-survival or pro-apoptotic Bcl-2-family members or other general defects in the apoptotic machinery.

Discussion

Small molecules that can directly activate the programmed cell death machinery are of great clinical interest for anti-cancer therapy.[12] Much effort has been invested to develop

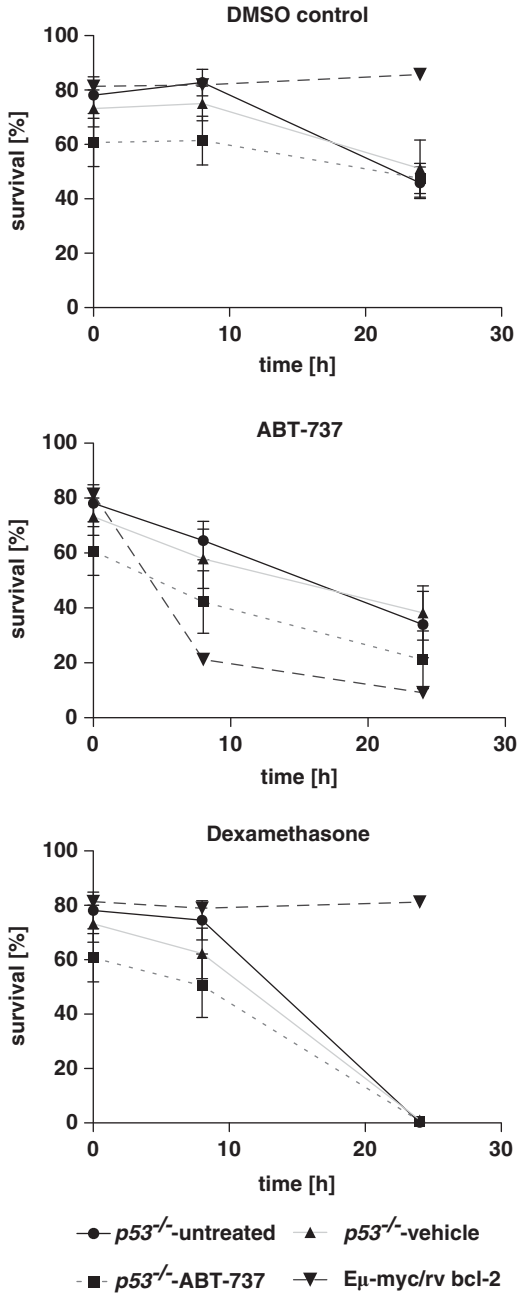

Figure 7 Response of thymic lymphoma derived CLs from $p53^{-/-}$ mice that had been prophylactically treated with ABT-737 or vehicle to apoptotic stimuli *in vitro*. Thymic lymphoma-derived CLs from γ-irradiated $p53^{-/-}$ mice that had either been left untreated ($n=2$) or had been γ-irradiated and prophylactically treated with ABT-737 ($n=3$) or vehicle ($n=3$) and an ABT-737-sensitive control CL, Eμ-*myc*/rv-*bcl*-2 (triplicate), were treated in culture with either 5 μM ABT-737, 30 nM dexamethasone or DMSO (vehicle control) for 0, 8 or 24 h and cell viability was monitored by fluorescence-activated cell sorting (FACS) analysis

small-molecule mimetics of the pro-apoptotic BH3-only proteins that can bind pro-survival Bcl-2-family members to elicit apoptotic death in cancer cells.[13,14] Because of their ability to directly induce apoptosis, Bcl-2-family inhibitors offer great potential for treatment of cancer, especially in treating malignancies with a dependency on Bcl-2 or Bcl-x_L for their survival.[12] Here, we investigated the role of Bcl-2, Bcl-x_L and Bcl-w inhibition not as a modality for treatment of established malignant cancers, but as a potential prophylactic strategy to prevent tumour development in the first place.

To address this question, we used gene-targeted mice deficient for the tumour suppressor p53, a suitable model of the human LFS, although these animals are more predisposed to thymic lymphoma rather than sarcoma as seen in the human condition.[3,4] We examined whether pharmacological blockade of Bcl-2, Bcl-x_L and Bcl-w using the BH3 mimetic ABT-737 in pre-malignant $p53^{+/-}$ and $p53^{-/-}$ mice could prevent or delay tumour development. In particular, given that Bcl-x_L[24] and Bcl-2[25] are both critical for cell survival during normal T lymphopoiesis, it appeared promising that their inhibition could interfere with thymic lymphoma development. Moreover, although Bcl-w is not readily detectable in thymic lymphomas elicited by p53 deficiency (Figure 8b) and is not essential for T-cell development,[26] it is expressed in T lymphoid cells (including progenitors)[27] and hence might contribute to the survival of nascent thymic lymphoma cells. Our results showed, however, that prophylactic treatment with ABT-737 had no significant impact on spontaneous tumour development in *p53*-deficient mice (Figure 1a). This indicates that endogenous expression of pro-survival Bcl-2-family members other than or in addition to Bcl-2, Bcl-x_L or Bcl-w may be critical for survival of cells undergoing neoplastic transformation in the development of both thymic lymphoma and sarcoma. Mcl-1 appears to be a good candidate for this function as it is critical for survival of T-cell progenitors[28] as well as stem/progenitor cells in many other lineages.[29] Moreover, it is also possible that repression of pro-apoptotic Bcl-2-family members, such as Bim[30] or Puma,[31] maintains survival of *p53*-deficient cells undergoing neoplastic transformation. Finally, it remains of course possible that prophylaxis with higher doses of ABT-737, such as the ($\sim$4–7 times higher) conventional dose of 75–100 mg/kg body weight/day commonly used for short-term studies (e.g., see Cragg *et al*.[32]), could significantly delay tumour development in *p53*-deficient mice. The treatment regime applied in this study was chosen because of the intent to administer ABT-737 and vehicle for a prolonged rather than short period of time; notably this dose caused a significant reduction in platelets and certain lymphocyte populations (Supplementary Figure 1), demonstrating that it was efficacious.

We also investigated whether prophylactic treatment with ABT-737 could delay tumourigenesis in $p53^{+/-}$ and $p53^{-/-}$ mice that had been exposed to repeated low-dose γ-irradiation to mimic environmental mutagenic factors that can promote neoplastic transformation, as a model for tumourigenesis provoked by environmental insults that cause DNA damage. Consistent with previous studies,[19] the γ-irradiated $p53^{-/-}$ and $p53^{+/-}$ mice succumbed to tumours, almost exclusively thymic lymphomas, considerably faster than their non-irradiated counterparts. Interestingly, the γ-irradiated $p53^{-/-}$ mice prophylactically treated with ABT-737 survived significantly, albeit only marginally, longer ($P^{**}=0.0070$) than the corresponding control mice injected with vehicle (Figure 4a). Although ABT-737 prophylaxis caused a 20% reduction in lymphoma incidence, this was accompanied by a compensatory increase in sarcoma

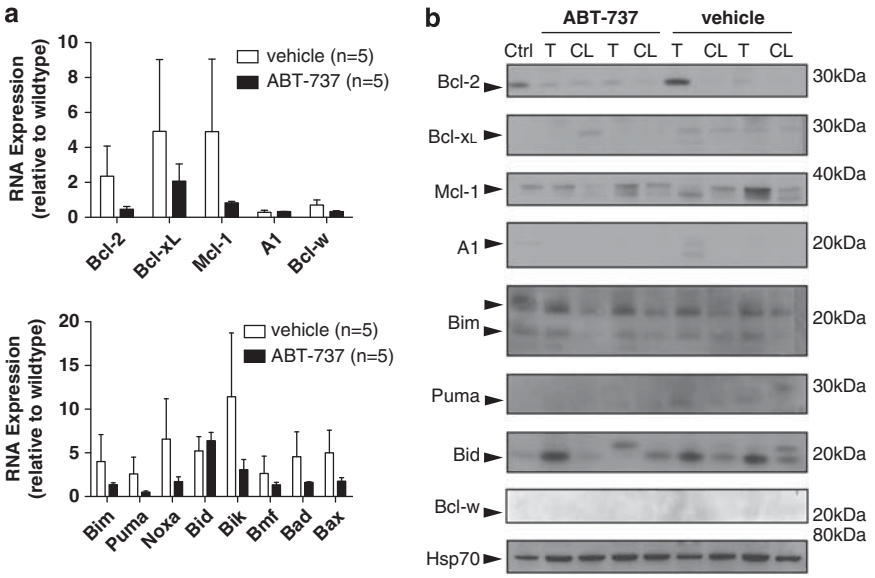

Figure 8 Prophylactic treatment with ABT-737 does not alter the expression of mRNA or the protein levels of Bcl-2-family members in thymic lymphomas arising in γ-irradiated $p53^{-/-}$ mice. (**a**) Analysis of the mRNA levels of the genes indicated using quantitative RT-PCR on cDNAs generated from total RNA from thymic lymphomas arising in γ-irradiated $p53^{-/-}$ mice that had been prophylactically treated with either ABT-737 ($n=5$) or vehicle ($n=5$). Levels of mRNAs were normalised to the mRNA levels of these genes found in thymi of healthy, untreated (i.e., non-irradiated, no ABT-737 prophylaxis) C57BL/6 (wt) mice. (**b**) Western blot analysis using antibodies against the indicated Bcl-2-family members and Hsp-70 (loading control) of primary thymic lymphoma cells (T) and CLs derived from these tumours that arose in γ-irradiated $p53^{-/-}$ mice that had been prophylactically treated with ABT-737 ($n=2$) or vehicle ($n=2$). As control we used a protein extract from thymocytes of a healthy, untreated (i.e., non-irradiated, no ABT-737 prophylactic treatment) C57BL/6 (wt, control (Ctrl)) mouse

(Figure 4c). These observations indicate that Bcl-2, Bcl-x_L and Bcl-w may have a minor role in maintaining the survival of 'cancer-initiating cells' that give rise to γ-radiation-induced thymic lymphoma, but appear dispensable for the survival of 'cancer-initiating cells' giving rise to sarcomas.

Thymic lymphoma burden was frequently observed to be somewhat lower in ABT-737 prophylactically treated mice as compared to vehicle-treated animals (Figures 3, 5 and 6). This reduction may have been caused by a minor cytotoxic effect on lymphoma cells by the BH3 mimetic, particularly in mice where the final injection of ABT-737 and killing coincided because of the advanced progress of the malignant disease. Alternatively, ABT-737 might affect lymphoma growth indirectly, for example, by impairing the survival of stromal cells that are critical for lymphoma dissemination and/or survival. This latter explanation may account for the reduction in overall lymphoma burden seen in moribund mice that had received their final injection of ABT-737 a longer period of time before they were killed.

In conclusion, our studies showed that Bcl-2, Bcl-x_L and Bcl-w have only a minor role in the development of thymic lymphoma and sarcoma in *p53*-deficient mice. We therefore reason that Mcl-1 and/or A1, the other two anti-apoptotic Bcl-2-family members, may have a more critical role in sustaining the survival of cells undergoing neoplastic transformation in this hematopoietic malignancy. This would be consistent with the finding that both Mcl-1[28] and A1[33] are critical in sustaining the survival of non-transformed T lymphoid cells at several stages of differentiation. Our observations may indicate that blockade of Mcl-1 and/or A1, rather than inhibition of Bcl-2, Bcl-x_L and/or Bcl-w, is required to impede tumourigenesis in patients with germline mutations in *p53*. Although blockade of Bcl-2, Bcl-x_L and Bcl-w by ABT-737 had only a minor impact on tumour development in *p53*-deficient mice, such treatment may still be efficacious in inhibiting tumourigenesis in other inherited cancer syndromes, most likely those that are associated with high levels of Bcl-2 and/or Bcl-x_L expression.

Materials and Methods

Experimental animals. All experiments were conducted according to the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. *p53*-deficient mice on a mixed C57BL/6/129SV genetic background[4] were provided by Professor Tyler Jacks from the Massachusetts Institute of Technology (Cambridge, MA, USA) and were backcrossed onto a C57BL/6 background for >20 generations before commencement of these studies.

Treatment of mice with γ-radiation and/or ABT-737. Mice ($p53^{+/-}$ and $p53^{-/-}$) at the age of 4 weeks were either left untreated or exposed to 1.5 Gy of γ-radiation once a week for four consecutive weeks using a ^{60}Co source to elicit thymic lymphoma development.[18] Between the ages of 4 and 24 weeks, nine mice of the non-irradiated cohort were injected intra-peritoneally twice a week with ABT-737 (50 mg/kg body weight) and 16 mice with vehicle. The γ-irradiated cohort was treated for the first time with ABT-737 or vehicle on the day when the first dose of γ-radiation commenced. Subsequently, ABT-737 or vehicle was applied twice a week for 10 consecutive weeks. Mice were examined daily and killed when declared unwell by the animal technicians. ABT-737 was formulated in 30% propylene glycol, 5% Tween-80 and 65% D5W (5% dextrose in water, pH 4.2).

Blood analysis. Blood was taken at the time of killing by cardiac puncture and analysed using an Advia blood analyser (Siemens, Deerfield, IL, USA). Blood parameters were plotted by using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Flow-cytometric analysis. Lymphoid organs were harvested and single-cell suspensions were prepared. Red cells were depleted using red cell lysis buffer. Cells (5×10^4) were stained for surface markers using fluorochrome-conjugated monoclonal antibodies to mouse CD4, CD8, CD3 and B220 for 30 min in balanced

salt solution (BSS) supplemented with 2% fetal calf serum (FCS) and 10% normal rat serum.

Histological analysis. Soft tissues, sternum and spine were harvested and fixed in 80% Histochoice/20% ethanol and embedded in paraffin, and stained with haematoxylin and eosin and analysed by a qualified pathologist (PW). Representative images of the thymus, sternum and lung were taken at a magnification of $\times 25$.

Genotyping. DNA samples from tail biopsies of mice or thymic lymphoma cells were analysed by PCR using primer sets that can detect wt and deleted *p53* alleles. Samples were compared to tail-derived DNA from 3-week-old $p53^{+/-}$ and $p53^{-/-}$ mice. *Mcl*-1 primers were used as control for quality of extracted DNA.

Cell culture and cell survival assays. Thymic lymphoma cells were cultured at 37 °C in a humidified 10% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Bovogen Biologicals PTY, East Keilor, Victoria, Australia), 50 μM β-mercaptoethanol (Sigma Aldrich, Castle Hill, NSW, Australia) and 100 μM asparagine (Sigma Aldrich). Cells were grown for 2 weeks in culture before being subjected to cell survival assays. For cell survival assays, 5×10^4 cells/well in a 96-well flat bottom plate were treated with either 5 μM ABT-737, 30 nM dexamethasone or DMSO (Sigma Aldrich). Cell viability was determined by flow-cytometric analysis, considering cells that are not stained with either FITC-conjugated Annexin-V (1 μg/ml) or propidium iodide (2 μg/ml) as live cells.

qRT-PCR analysis. Quantitative PCR (qRT-PCR) analysis was performed by using TaqMan probes according to the manufacturer's instructions and analysed by ABI-7900 (Applied Biosystems, Mulgrave, Victoria, Australia). Details of TaqMan gene expression assays will be provided on request.

Western blot analysis. Western blot analysis was conducted following standard protocols using extracts from primary thymic lymphomas or CLs derived from these tumours. Western blots were probed using monoclonal hamster anti-mouse Bcl-2, monoclonal mouse anti-mouse Bcl-x_L (BD Pharmingen, San Diego, CA, USA), monoclonal rat anti-mouse Mcl-1, monoclonal rat anti-mouse Bcl-w, monoclonal rat anti-mouse A1, monoclonal rat anti-mouse Bid[34] (last four, all gifts from Professor David Huang, The Walter and Eliza Hall Institute), polyclonal rabbit anti-mouse Bim (Stressgen, Sapphire Bioscience PTY.LTD, Waterloo, NSW, Australia) and polyclonal rabbit anti-mouse Puma (Abcam, Sapphire Bioscience PTY.LTD; Ab-27669) antibodies. Probing using monoclonal anti-mouse Hsp-70 antibody (gift from Robin Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia) was used as a loading control. Monoclonal goat anti-mouse, anti-rat and anti-rabbit IgG antibodies coupled to HRP were purchased from Southern Biotech (*In Vitro* Technologies PTY.LTD, Noble Park, Victoria, Australia).

Statistical analysis. Kaplan–Meier mouse survival curves were generated and analysed using GraphPad Prism (GraphPad Software Inc). Mouse cohorts were compared by log-rank Mantel–Cox test. *P*-values less than 0.05 were considered significant.

In vitro cell survival, blood cell counts, organ weights and RNA levels were plotted and analysed using GraphPad Prism, using two-tailed Student's *t*-test for comparing two groups.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Abbott Laboratories and Genentech Inc. for providing us with ABT-737; Dr. T Jacks for *p53*-deficient mice; Drs. LA O'Reilly and DC Huang, and Mr. ARD Delbridge, for advice and reagents; K McKenzie, G Siciliano, N Iannarella, L Reid and K Vella for expert animal care; B Helbert and C Young for genotyping; J Corbin for automated blood analysis; E Tsui, V Babo, K Weston, Y Hoang and S Hasanein for histology, and D Quilici, T Nikolaou and G Thomas for γ-irradiation. This work was supported by grants and fellowships from the Cancer Council of Victoria (to PNK), the National Health and Medical Research Council (Program Grant no. 461221; NHMRC Australia Fellowship), the NIH (CA43540) and the Leukemia and Lymphoma Society (SCOR Grant no. 7413), University of Melbourne International Research Scholarship, University of Melbourne International Fee Remission Scholarship, Cancer Therapeutics CRC Top-up Scholarship (last three to SG) and operational infrastructure grants through the Australian Government IRISS and the Victorian State Government OIS.

1. Srivastava S, Zou ZQ, Pirollo K, Plattner W, Chang EH. Germline transmission of a mutated p53 gene in a cancer-prone family with Li–Fraumeni syndrome. *Nature* 1990; **348**: 747–749.
2. Malkin D, Li FP, Strong LC, Fraumeni JFJ, Nelson CE, Kim DH *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 1990; **250**: 1233–1238.
3. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CAJ, Butel JS *et al.* Mice deficient for p53 are developmentally normal but are susceptible to spontaneous tumours. *Nature* 1992; **356**: 215–221.
4. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT *et al.* Tumor spectrum analysis in *p53*-mutant mice. *Curr Biol* 1994; **4**: 1–7.
5. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT *et al.* Mutant p53 gain of function in two mouse models of Li–Fraumeni syndrome. *Cell* 2004; **119**: 847–860.
6. Vousden KH, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol* 2007; **8**: 275–283.
7. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. *N Engl J Med* 2009; **361**: 1570–1583.
8. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
9. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; **9**: 47–59.
10. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; **305**: 626–629.
11. Huang DCS, Strasser A. BH3-only proteins – essential initiators of apoptotic cell death. *Cell* 2000; **103**: 839–842.
12. Lessene G, Czabotar PE, Colman PM. BCL-2 family antagonists for cancer therapy. *Nat Rev Drug Discov* 2008; **7**: 989–1000.
13. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; **435**: 677–681.
14. Park CM, Bruncko M, Adickes J, Bauch J, Ding H, Kunzer A *et al.* Discovery of an orally bioavailable small molecule inhibitor of prosurvival B-cell lymphoma 2 proteins. *J Med Chem* 2008; **51**: 6902–6915.
15. Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC *et al. In vivo* efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *Proc Natl Acad Sci USA* 2008; **105**: 17961–17966.
16. Cragg MS, Harris C, Strasser A, Scott CL. Unleashing the power of inhibitors of oncogenic kinases through BH3 mimetics. *Nat Rev Cancer* 2009; **9**: 321–326.
17. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003; **22**: 8590–8607.
18. Kaplan HS, Brown MB. Further observations on inhibition of lymphoid tumor development by shielding and partial-body irradiation of mice. *J Natl Cancer Inst* 1951; **12**: 427–436.
19. Kemp CJ, Wheldon T, Balmain A. *p53*-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nat Genet* 1994; **8**: 66–69.
20. Michalak EM, Vandenberg CJ, Delbridge ARD, Wu L, Scott CL, Adams JM *et al.* Apoptosis-promoted tumorigenesis: gamma-irradiation-induced thymic lymphomagenesis requires Puma-driven leukocyte death. *Genes Dev* 2010; **24**: 1608–1613.
21. Labi V, Erlacher M, Krumschnabel G, Manzl C, Tzankov A, Pinon J *et al.* Apoptosis of leukocytes triggered by acute DNA damage promotes lymphoma formation. *Genes Dev* 2010; **24**: 1602–1607.
22. Strasser A, Harris AW, Jacks T, Cory S. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell* 1994; **79**: 329–339.
23. Strasser A, Harris AW, Cory S. *Bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 1991; **67**: 889–899.
24. Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T. *bcl-x* prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. *J Exp Med* 1999; **189**: 1691–1698.
25. Bouillet P, Cory S, Zhang L-C, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency are prevented by loss of its BH3-only antagonist Bim. *Dev Cell* 2001; **1**: 645–653.
26. Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N *et al.* Apoptosis regulator Bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci USA* 1998; **95**: 12424–12431.
27. O'Reilly LA, Print C, Hausmann G, Moriishi K, Cory S, Huang DCS *et al.* Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. *Cell Death Differ* 2001; **8**: 486–494.
28. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature* 2003; **426**: 671–676.

29. Opferman J, Iwasaki H, Ong CC, Suh H, Mizuno S, Akashi K *et al.* Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 2005; **307**: 1101–1104.
30. Bouillet P, Metcalf D, Huang DCS, Tarlinton DM, Kay TWH, Köntgen F *et al.* Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; **286**: 1735–1738.
31. Villunger A, Michalak EM, Coultas L, Müllauer F, Böck G, Ausserlechner MJ *et al.* p53- and drug-induced apoptotic responses mediated by BH3-only proteins Puma and Noxa. *Science* 2003; **302**: 1036–1038.
32. Cragg MS, Jansen ES, Cook M, Strasser A, Scott CL. Treatment of B-RAF mutant human tumor cells with a MEK inhibitor requires Bim and is enhanced by a BH3 mimetic. *J Clin Invest* 2008; **118**: 3651–3659.
33. Mandal M, Borowski C, Palomero T, Ferrando AA, Oberdoerffer P, Meng F *et al.* The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J Exp Med* 2005; **201**: 603–614.
34. Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK *et al.* The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell* 2007; **129**: 423–433.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)