

Cancer Therapy: Preclinical

FTY720 Shows Promising *In vitro* and *In vivo* Preclinical Activity by Downmodulating Cyclin D1 and Phospho-Akt in Mantle Cell Lymphoma

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Abstract

Purpose: Despite the progress that has been made in the treatment of mantle cell lymphoma (MCL), all patients invariably relapse with the currently available therapies. Because of the absence of curative therapy for MCL, we explored FTY720 as a novel agent against MCL.

Experimental Design: The cytotoxic effect of FTY720 in primary MCL tumor cells and cell lines were evaluated *in vitro*. The effects of FTY720 on caspase activation, generation of reactive oxygen species, and modulation of Cyclin D1 and Akt, which are implied in the pathogenesis of MCL, were investigated. The *in vivo* efficacy of FTY720 was evaluated in a Jeko-severe combined immunodeficient xenograft model of human MCL.

Results: FTY720 mediated time- and dose-dependent cytotoxicity in primary MCL tumor cells and MCL cell lines *in vitro*. FTY720-induced cytotoxicity occurred independent of caspase activation but dependent on the generation of ROS in MCL. In addition, FTY720 treatment resulted in the time-dependent downmodulation of Cyclin D1 and accumulation of cells in G₀-G₁ and G₂-M phases of the cell cycle with concomitant decrease in S-phase entry. Furthermore, concentrations of FTY720 that induced cytotoxicity led to decreased phospho-Akt in primary MCL cells and cell lines. Most importantly, the *in vivo* therapeutic activity of FTY720 was shown in severe combined immunodeficient mice engrafted with the Jeko MCL cell line.

Conclusions: These results provide the first evidence for a potential use of FTY720 in targeting key pathways that are operable in the pathogenesis of MCL and warrant further investigation of FTY720 in clinical trials to treat patients with MCL. *Clin Cancer Res*; 16(12); 3182-92. ©2010 AACR.

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy characterized by the abnormal accumulation of CD20⁺/CD5⁺ B cells in lymph nodes, spleen, bone marrow, and blood (1). Although treatment with combination chemotherapeutic regimens can be effective, virtually all patients relapse and the outcome of patients with MCL remains poor, with a median survival of only

3 years (2). Currently available therapies including combination chemotherapy, high-dose chemotherapy followed by stem cell transplant, and monoclonal antibody therapy have shown limited success (3). The pathogenesis of MCL is, in part, attributed to the constitutively active Ser/Thr kinase, Akt (4), a survival pathway associated with defective phosphatase activity, and overexpression of Cyclin D1 driven by the chromosomal translocation t(11;14)(q13; q32) between the *IgH* and *Bcl-1* genes (4, 5). Dysregulation of antiapoptotic and proapoptotic proteins also have been implicated in this disease (4, 5). Given the absence of curative therapy for MCL, it is essential to explore new treatment options.

FTY720 (fingolimod) is a synthetic compound produced by the modification of ISP-1 (myriocin), a naturally occurring substance with immunosuppressive properties (6-9). FTY720 is phosphorylated *in vivo* by sphingosine kinase 2 and binds to all four of the currently known sphingosine 1 phosphate (S1P) receptors (S1PR₁, S1PR₃, S1PR₄, and S1PR₅) with high affinity. Upon binding of p-FTY720, the S1PRs are internalized from the cell membrane and degraded, resulting in the sequestration of

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Translational Relevance

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy with a median survival of 3 years. The pathogenesis of MCL is, in part, attributed to the overexpression of Cyclin D1 and constitutively active Akt survival pathway. FTY720, a synthetic compound produced by the modification myriocin, is a naturally occurring substance. Herein, we report that FTY720 promotes the death of MCL cell lines and primary human MCL tumor cells concurrent with the downmodulation of Cyclin D1 and phospho-Akt, two critical targets implicated in the pathogenesis of MCL. On going clinical trials using FTY720 to prevent renal transplant rejection has proven this drug to be safe for human use. More importantly, demonstration of novel mechanisms, including caspase-independent and reactive oxygen species-dependent cytotoxicity and *in vivo* therapeutic efficacy of single-agent FTY720 in an aggressive Jeko-severe combined immunodeficient xenograft mouse model, provides clear rationale for further development and use of FTY720 in clinical trials to treat patients with MCL.

lymphocytes in secondary lymphoid organs (6). FTY720 produces lymphopenia and is being developed as an immunosuppressive therapeutic agent (7). A phase III clinical trial using FTY720 as an immunosuppressant to prevent renal transplant rejection has been completed (7). In addition, FTY720 was recently shown to be therapeutically active against several solid tumors, multiple myeloma, and chronic lymphocytic leukemia (CLL; refs. 8, 9). Herein, we report that FTY720 promotes the death of MCL cell lines and primary human MCL tumor cells concurrent with the downmodulation of Cyclin D1 and phospho-Akt (p-Akt), two critical targets implicated in the pathogenesis of MCL. We therefore provide *in vitro* and *in vivo* evidence to support the use of FTY720 as a promising therapeutic agent for the treatment of patients with MCL.

Materials and Methods

Patient samples and cell lines

All patient samples were obtained following informed consent detailed in a protocol approved by the Ohio State University Institutional Review Board. Primary tumor cell were isolated from the peripheral blood of patients diagnosed with MCL, according to the WHO classification (10). The purity of primary tumor cell exceeded 90%. MCL cell lines (Mino, Jeko, and SP53, generous contribution from Dr. Raymond Lai, University of Alberta, Edmonton, Alberta, Canada) have been previously described (11). All cells were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L

L-glutamine, and penicillin (100 U/mL)/streptomycin (100 g/mL; Invitrogen), at 37°C in a humidified atmosphere of 5% CO₂.

Analyses of apoptosis by flow cytometry

FTY720 was synthesized as previously described (9). An Annexin V-FITC- and propidium iodide (PI)-binding assay was used to detect cell apoptosis as described by us previously (9).

Cytofluorometric analysis of reactive oxygen species

Reactive oxygen species (ROS) was measured as previously described (12). Briefly, Jeko and Mino cells (1×10^6 /mL) treated with FTY720 at indicated concentrations and time periods were washed and incubated in 10 μmol/L dihydroethidine (Molecular Probes) at 37°C for 30 minutes. The cells were then washed and analyzed by flow cytometry. Dihydroethidine enters the cell and is oxidized by ROS, particularly superoxide, to yield fluorescent ethidium that binds to DNA further amplifying its fluorescence. Thus, increases in ethidium fluorescence are suggestive of superoxide generation. For rescue experiments, Jeko and Mino cells were then incubated with the nonspecific ROS scavenger N-acetyl cysteine (NAC; at 1, 5, and 10 mmol/L; Sigma-Aldrich) for 24 hours in the presence or absence of FTY720. NAC was added 15 minutes before the addition of FTY720. Experiments were done in triplicate.

Immunoblot analyses

Whole-cell lysates were prepared using the radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate, containing protease and phosphatase inhibitors]. Proteins were analyzed by immunoblot following standard procedures (9) using indicated antibodies. Anti-Caspase-3, Cyclin D2, and Cyclin D3 were from Cell Signaling Technology). Cyclin D1, Akt, Actin, Bax, Mcl-1, phospho-extracellular signal-regulated kinase (p-Erk), Erk, and Bcl-2-specific antibodies were from Santa Cruz Biotech. Digital quantification was done using a ChemiDoc instrument (Bio-Rad). P-Akt and Cyclin D1 expression was calculated relative to expression in the control-treated cells, arbitrarily set to 1.0.

Development of a preclinical MCL xenograft model

Six- to eight-week-old female severe combined immunodeficient (SCID) mice (cb17 scid/scid; Taconic Farms) were depleted of murine natural killer cells with intraperitoneal injections of 0.2 mg of rat anti-mouse interleukin-2 receptor β monoclonal antibody (TMβ1), 1 day before engraftment with human tumor cell lines and then every week, as previously described (13). Cell-dose titration trials with three separate MCL cell lines (SP53, Jeko, and Mino) were done to determine the optimal dose of cells that would lead to consistent engraftment and development of tumor burden in all mice. This dose was found to be 4×10^7 cells (injected i.v. through tail vein) for Mino and Jeko cell lines.

Evaluation of FTY720 *in vivo* therapeutic activity

Freshly cultured Jeko cells (4×10^7 cells; >90% viability) were injected i.v. in sterile PBS through the tail vein. Two days after injection, 20 mice were randomly distributed into four cages. The first control group (ten mice) received saline i.p. every day. The second group (10 mice) was treated with FTY720 (5 mg/kg) every day for 2 weeks i.p., starting at day 18 after engraftment. Animals were monitored daily for signs of tumor burden, hind limb paralysis, respiratory distress, weight loss, ruffled coat, and distended abdomen. Body weight was measured weekly for the first 3 weeks and then every day. Animals were sacrificed if they exhibited either hind limb paralysis, 30% reduction in body mass, or 10% reduction in body mass together with respiratory distress and/or ruffled coat or lethargic behavior. Tissues (including spleen, liver, ovaries, brain, and lungs) obtained from sacrificed mice were subjected to histopathologic evaluation to confirm the presence of MCL. The immunohistochemical analysis of the tissues was done using monoclonal mouse anti-human CD45 (BD Pharmingen) to stain human neoplastic lymphocytes. The primary end point of the study was survival defined as the time to develop defined clinical criteria leading to removal from the study. The dosing schedule was based on our reported studies in SCID mice (9) and *in vivo* pharmacokinetic (PK) study of FTY720 in rats (14, 15). Rat PK study showed that FTY720 achieved a 3.5 $\mu\text{mol/L}$ maximum plasma concentration at a 4 mg/kg single i.v. dose. Therefore, a regimen of 5 mg/kg daily dose for 2 weeks was predicted to achieve a steady-state concentration. The Jeko cell line represents a blastoid variant of aggressive MCL. Because this line showed a resistant phenotype to several immunotherapeutic agents, we chose this cell line for our preclinical model. This model in three independent experiments has resulted in the development of lethal tumor burden by 26 to 30 days (95% confidence interval). All mice died with lethal tumor burden as verified by histologic examination. The animal research was reviewed and approved by the University Laboratory Animal Resources (ULAR)-Institutional Animal Care and Use Committee at the Ohio State University.

Statistical analysis of data

Linear mixed models were used for the analysis of *in vitro* studies. Holm's procedure was used to correct for multiple comparisons. *P* value of <0.05 was claimed as significant for single comparisons or after correction for multiple comparisons. Log-rank test was used for the animal survival study and Kaplan-Meier survival curves were generated from these tests.

Results

FTY720 induces cell death in MCL cell lines and primary MCL tumor cells

To analyze the *in vitro* effect of FTY720 on the viability of MCL cell lines and primary MCL cells, a dose-response

study was first performed. Incubation of primary MCL tumor cells (from six separate patients), and MCL cell lines (Mino and Jeko) in the presence of increasing concentrations of FTY720 ranging from 0 to 15 $\mu\text{mol/L}$, resulted in significant dose-dependent cell death as determined by Annexin V-FITC/PI staining at 24 hours. Figure 1 shows the percent viable cells as determined by Annexin V⁻ PI⁻ cells normalized to nontreated controls ($P < 0.0001$). The Jeko cell line, derived from the peripheral blood of a patient with blastoid variant of MCL, showed the most resistance to FTY720-induced apoptosis.

FTY720-induced apoptosis is not associated with caspase activation or downmodulation of antiapoptotic Bcl-2 or Mcl-1 protein levels

FTY720 has been shown to mediate cytotoxicity by caspase-3 activation-dependent and caspase-3 activation-independent mechanisms in different cell types (14, 15). We have recently shown FTY720 to mediate cytotoxicity in CLL B cells through a caspase-independent mechanism (9). Consistent with this observation, continuous incubation of Jeko and Mino cells with increasing doses of FTY720 failed to mediate caspase-3 activation (Fig. 2A and B). To determine if the FTY720-mediated cytotoxicity was due to the downregulation of antiapoptotic proteins, we tested the effect of FTY720 exposure on the expression of Bcl-2 and Mcl-1, two critical antiapoptotic proteins in MCL. As shown in Fig. 2, the expression of Bcl-2 or Mcl-1 was not altered in response to FTY720 in Jeko (Fig. 2C) and Mino cells (Fig. 2D). Similar results were observed in primary MCL tumor cells (data not shown).

FTY720-induced cytotoxicity is dependent on the generation of ROS

Caspase-independent mechanisms of apoptosis are mediated by the generation of ROS (16). To directly determine if FTY720-induced apoptosis is mediated by the generation of ROS, Jeko cells were treated with FTY720 for 2, 4, and 6 hours, and were examined for changes in ROS generation using the specific fluorescence probe, dihydroethidine (16). FTY720 induced an increase of dihydroethidine fluorescence (right shift of the dihydroethidine curves), indicating an increase in ROS generation as early as 4 hours in both Jeko (Fig. 3A) and Mino (Fig. 3C) cell lines that is further increased at 6 hours. The levels of ROS in nontreated control cells remained unchanged over all incubation time periods. To further explore the implication of ROS in FTY720-induced apoptosis, Jeko cells ($1 \times 10^6/\text{mL}$) were incubated with NAC (1, 5, and 10 mmol/L), a nonspecific ROS scavenger, for 8 and 24 hours, in the absence or presence of FTY720. NAC was added 15 minutes before the addition of FTY720 and cell viability was determined by Annexin V/PI staining and flow cytometry. As shown in Fig. 3B, NAC, at each concentration except at 1 mmol/L ($P = 0.0425$) led to a significant increase in the viability of Jeko cells when compared with FTY720 only-treated cells averaged across 8 and 24 hours of incubation ($P < 0.0001$). Similar FTY720-induced ROS production

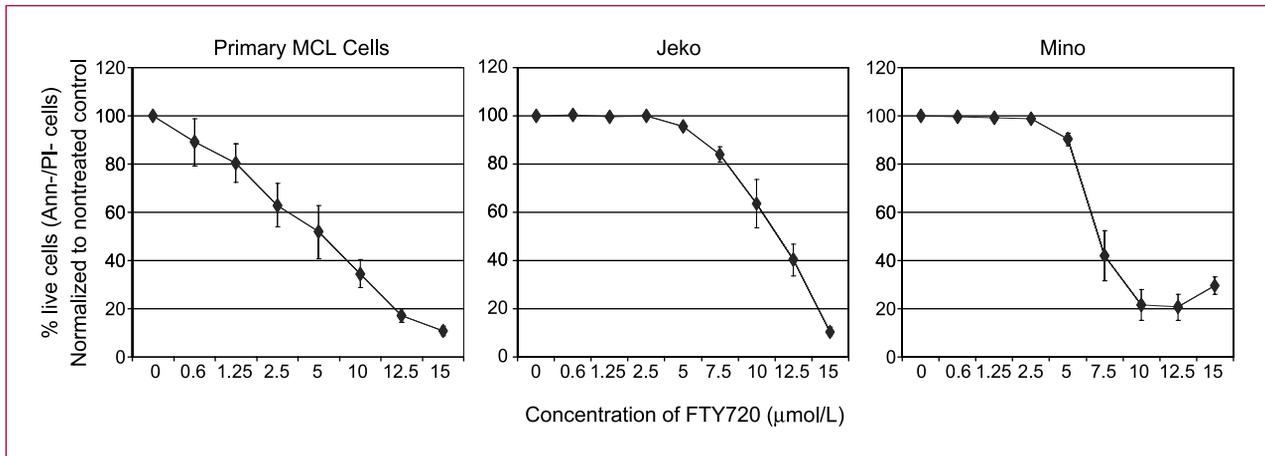


Fig. 1. FTY720 induced apoptosis in MCL cells. Six primary MCL tumor cells (2×10^6 /mL), Jeko, and Mino cell lines (5×10^5) were treated with indicated concentrations of FTY720 for 24 h and viability was analyzed using Annexin V (Ann)-FITC and PI staining as described in Materials and Methods. The Annexin V⁺/PI⁻ cells normalized to vehicle controls are represented as % live cells. The results showed that FTY720 treatment averaged across doses induced significantly higher apoptosis compared with nontreated control ($P < 0.0001$).

and NAC-mediated rescue of FTY720-induced cytotoxicity was also observed in Mino cells (Fig. 3C and D). Furthermore, a dose-dependent effect of NAC on both cell lines was observed. ROS production has been shown to initiate apoptotic signals through upregulation of the proapoptotic protein Bax (16). Consistent with this, FTY720 treatment resulted in the upregulation of Bax in the Jeko and Mino cell lines as early as 8 hours posttreatment (Fig. 3E). A sustained 3- to 4-fold increase in the Bax levels were observed in both Jeko and Mino cells tested as late as 24 hours (Fig. 3E). FTY720 also induced Bax upmodulation in primary MCL tumor cells (data not shown).

FTY720-induced apoptosis is associated with the downmodulation of Cyclin D1 and cell cycle arrest in MCL cells

Cyclin D1 overexpression has been implicated in the pathogenesis of MCL (4, 5). To determine if FTY720-induced apoptosis was mediated through the modulation of this target, we evaluated the effect of FTY720 on Cyclin D1 levels by immunoblot analysis. Treatment of primary MCL cells with FTY720 resulted in the time-dependent downregulation of Cyclin D1 protein (Fig. 4A, left), resulting in 65% reduction by 8 hours after (normalized to 100% of DMSO treatment) and progressively decreased to 30% by 24 hours (Fig. 4A, left). A reduction in Cyclin D1 protein expression was also observed in Mino and to a lesser extent in Jeko cells (Fig. 4A, right and middle). We observed similar results in Jeko and Mino cell lines showing Cyclin D3 downmodulation following treatment with FTY720 (data not shown). No changes in Cyclin D2 were observed (data not shown).

To determine if the FTY720-induced Cyclin D1 downmodulation resulted in defects in cell cycle progression, Jeko and Mino cells were treated with FTY720 for 12

and 24 hours and subjected to cell cycle analysis by flow cytometry (Fig. 4B). FTY720 treatment resulted in dose- and time-dependent decrease in the S-phase entry of the cell cycle. This decrease in the S-phase entry is further reflected by the accumulation of cells in the G₁ phase of the cell cycle (Fig. 4B). Interestingly, compared with Mino cells, FTY720 induced relatively lesser Jeko cells to enter into S phase (48% reduction in Mino cells compared with 28% reduction Jeko cells; Fig. 4B). This is consistent with the observation that FTY720 induced lesser Cyclin D1 downmodulation in Jeko cells compared with Mino cells (Fig. 4A).

FTY720-induced apoptosis is associated with the downmodulation of p-Akt

The Akt pathway has been implicated in MCL survival and pathogenesis as evidenced by constitutive phosphorylation of Akt in primary MCL tumor cells (4). To determine if FTY720 induced the modulation of p-Akt levels in MCL cell lines, we evaluated the effect of FTY720 exposure on Jeko and Mino cells. As shown in Fig. 5A and C, FTY720 induced p-Akt downmodulation in Jeko and Mino cells. Because of the established link between the Akt pathway and the mitogen-activated protein kinase (MAPK) cascade in solid tumors (17, 18), we next sought to determine if the FTY720-mediated p-Akt downmodulation induced Erk phosphorylation. The Erk1/2 phosphorylation status was analyzed by immunoblot using pErk1/2 and Erk1/2 antibodies. Interestingly, in contrast to Akt, FTY720 induced phosphorylation of Erk1/2 in Jeko and Mino cells as early as 2 hours posttreatment (Fig. 5B and D). Maximal phosphorylation of Erk1/2 occurred at 4 hours and remained elevated as late as 10 hours posttreatment. Similar results were observed in primary MCL tumor cells (data not shown).

Development of a preclinical MCL xenograft model and *in vivo* therapeutic evaluation of FTY720

To determine the *in vivo* therapeutic activity of FTY720, in treating established MCL tumor burden, we developed an *in vivo* preclinical mouse model for human MCL using previously described MCL cell lines (11). SCID mice treated with the monoclonal antibody TM β 1 to deplete murine natural killer cells were engrafted with 4×10^7 MCL cells (Jeko, Mino, and SP53) *i.v.* and observed daily for signs of tumor burden. Animals engrafted with Jeko cells developed cachexia/wasting syndrome and respiratory distress between days 18 and 20 following engraftment. All mice engrafted with Jeko cells showed central nervous system and diffuse organ tumor burden and splenomegaly (Fig. 6A, left). Evaluation of brain tissue showed an impressive meningeal infiltrate and perivascular invasion (Fig. 6B, left). Mice engrafted with Mino cells developed cachexia/wasting syndrome and respiratory distress from 30 to 50 days. Mice engrafted with Mino cells developed large bilateral neck masses, mediastinal tumor, and splenomegaly (Fig. 6A, middle). Histopathologic examination of neck masses showed tumor effacement of parotid glandular tissue (Fig. 6B, middle). Mice engrafted with SP53 cells survived longest, developing respiratory distress from 40 to 60 days. All mice engrafted with SP53 cells developed ret-

roperitoneal and abdominal tumor burden (Fig. 6A, right). Histopathologic examination of retroperitoneal lymph nodes showed marked effacement of normal lymph node architecture (Fig. 6B, right). Tumor burden was verified by immunohistochemistry staining for the human CD19 and CD45 antigen (data not shown). Figure 6C shows a Kaplan-Meier survival plot reflecting time to lethal tumor burden, at which time animals were sacrificed, examined for tumor location, and the organs were collected for histology and flow cytometry. Engraftment of each cell line resulted in a characteristic pattern of tumor burden and highly reproducible time to develop advanced disease.

To determine the *in vivo* therapeutic activity of FTY720 against MCL, we engrafted 20 SCID mice with 4×10^7 Jeko cells and randomized mice to two treatment groups. The dose used in this study (5 mg/kg/d) was determined based on *in vivo* PK studies of FTY720 in a rat model as previously reported (9, 19, 20). This regimen, in prior studies using a murine Raji xenograft model (9), did not lead to significant toxicity, which further justified its implementation for our *in vivo* studies using the MCL preclinical model. As shown in Fig. 6D, mice treated with 5 mg/kg FTY720 ($n = 10$) have a median survival of 38 days (95% confidence interval, 30-39), which is significantly longer than the median survival of 26.5 days (95% confidence interval, 26-27)

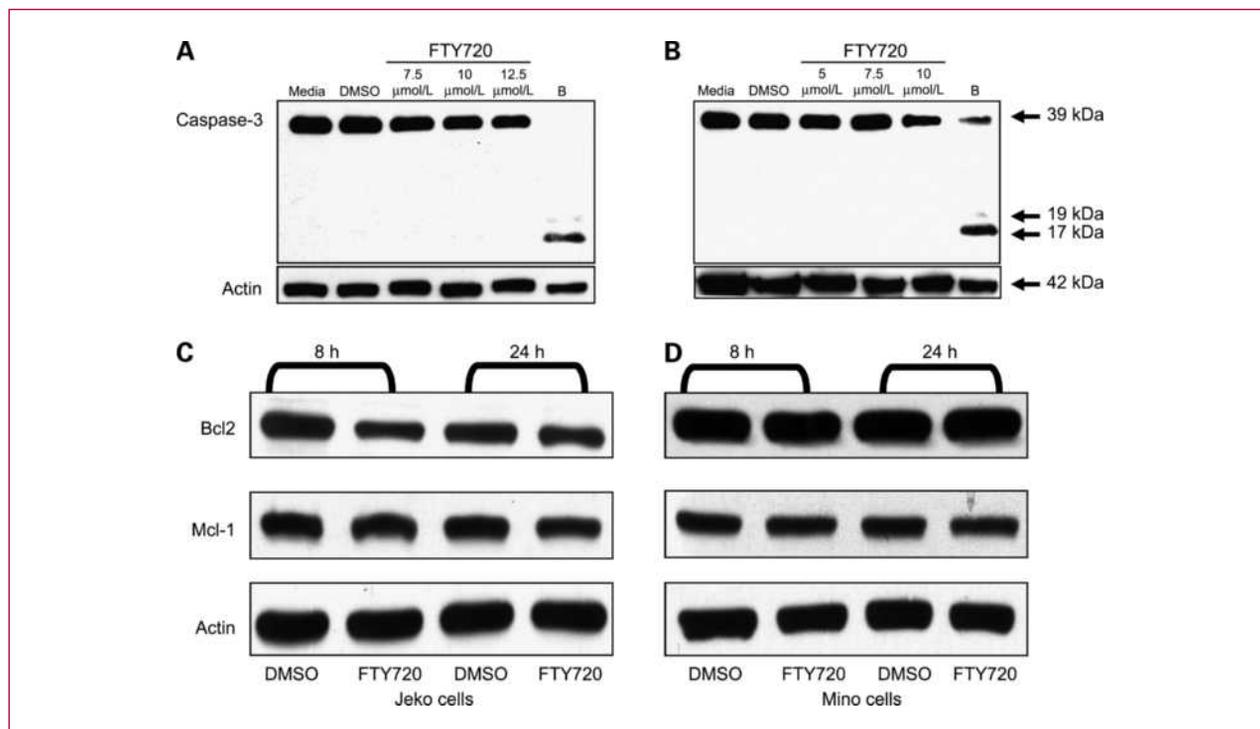


Fig. 2. FTY720-induced apoptosis is not associated with caspase activation or downmodulation of antiapoptotic Bcl-2 or Mcl-1 protein levels. A and B, FTY720-induced apoptosis is independent of caspase activation. Jeko (A) and Mino (B) cells (1×10^6 /mL) were treated with indicated concentrations of FTY720. Immunoblots of 24-h lysates using anti-Caspase-3 antibody is shown. Lysates from bortezomib-treated (B; 10 nmol/L) cells were used as positive control. Actin was used as loading control. C and D, failure of FTY720 to modulate Bcl-2 and Mcl-1 expression. Jeko (C) and Mino (D) cells (1×10^6 /mL) were treated with 7.5 and 5 μ mol/L FTY720, respectively. Immunoblots of 8- and 24-h lysates using indicated antibodies are shown. Actin was used as loading control.

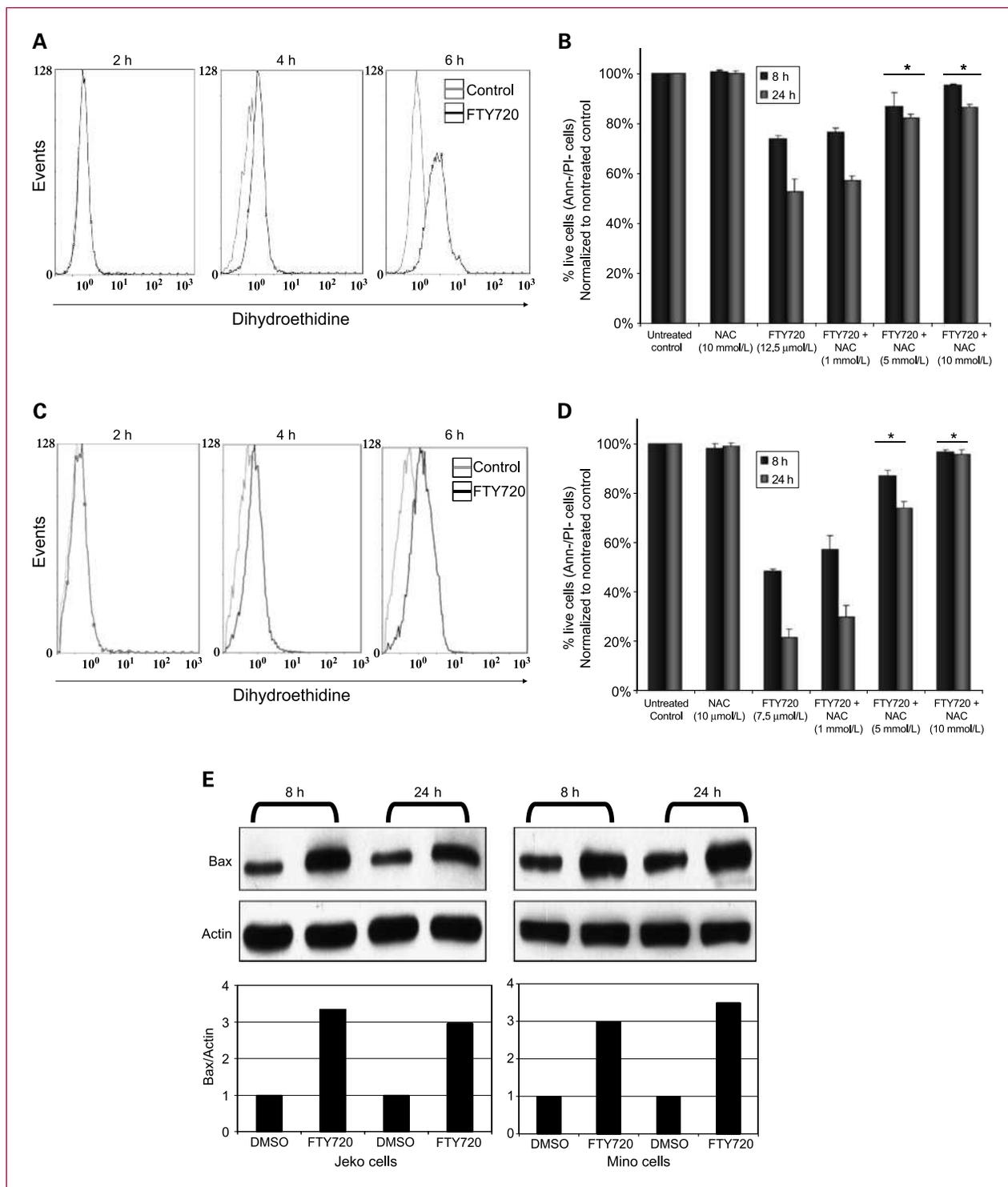


Fig. 3. FTY720-induced cytotoxicity is dependent on the generation of ROS. A to D, FTY720 induced ROS generation in MCL Jeko (A) and Mino (C) cells (1×10^6 /mL) treated with FTY720 (12.5 and 7.5 μ mol/L, respectively) or DMSO control and the ROS generation determined using 10 μ mol/L dihydroethidine as described in Materials and Methods. For rescue experiments, Jeko (B) and Mino (D) cells (1×10^6 /mL) were incubated with NAC for 8 and 24 h in the absence or presence of FTY720. NAC was added 15 min before the addition of FTY720 and cell viability determined by Annexin V/PI staining and flow cytometry. Preincubation with NAC led to a significant enhancement in cell survival at 8 and 24 h in Jeko and Mino cells [1 mmol/L NAC: $P = 0.0425$ and $P = 0.005$ versus FTY720 alone, respectively; (*) 5 mmol/L NAC and (*) 10 mmol/L NAC: $P < 0.0005$ versus FTY720 alone, respectively]. E, FTY720 induced Bax upregulation in MCL Jeko and Mino (1×10^6 /mL) cells were treated with 7.5 and 5 μ mol/L FTY720, respectively. Immunoblots of 8- and 24-h lysates using indicated antibodies are shown. Bottom, levels of Bax normalized with actin.

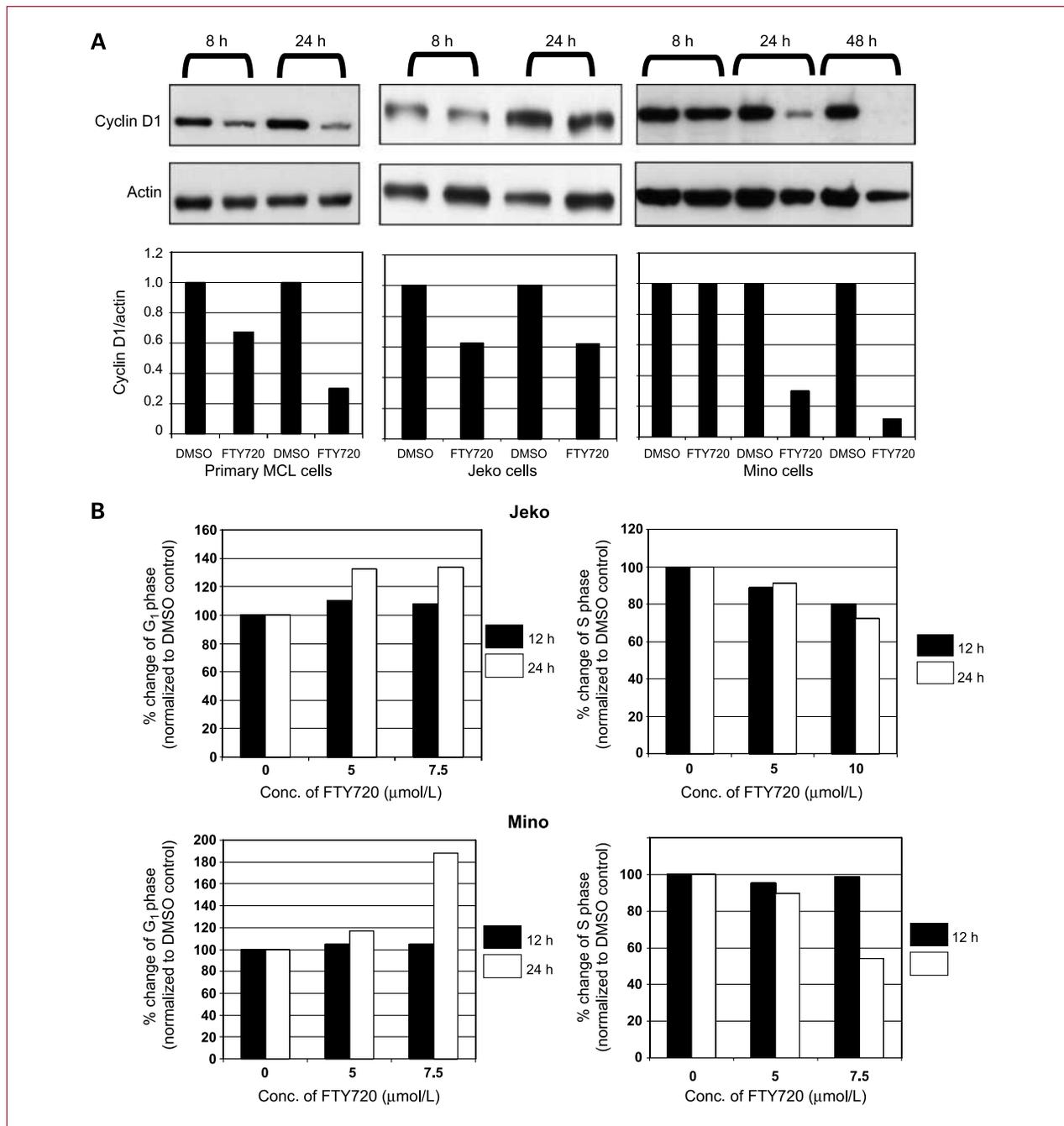


Fig. 4. FTY720 induced the downregulation of Cyclin D1 and cell cycle arrest in MCL cells. A, FTY720 induced the downmodulation of Cyclin D1 in MCL. Primary MCL cells (2×10^6 /mL; left) were treated with $15 \mu\text{mol/L}$ FTY720 (representative of two separate experiments). Jeko (middle) and Mino cells (1×10^6 /mL) were treated with 12.5 and $7.5 \mu\text{mol/L}$ FTY720, respectively. The different doses were chosen based on the IC_{50} of FTY720 with each of the cell types. Cell lysates made at indicated time points were subjected to immunoblotting using anti-Cyclin D1 antibody; actin was used as loading control. Bottom, Cyclin D1 normalized with actin. B, FTY720 induced cell cycle dysregulation in Jeko and Mino cells. Jeko and Mino cells (1×10^6 /mL) were treated with indicated concentrations of FTY720 or vehicle DMSO for 12 and 24 h. Cells were processed and cell cycle was analyzed by flow cytometry as described in Materials and Methods. Results shown are representative of three independent experiments. Percent change in G₁- or S-phase cells was normalized to DMSO vehicle control.

for PBS-treated mice ($n = 10$; $P = 0.001$). The histopathologic analysis of the spleens from both FTY720- and PBS-treated mice showed diffuse infiltration of human CD19+/CD45+ cells, with neoplastic lymphocytes completely

effacing the normal splenic parenchyma, consistent with our observation that FTY720 increases the median survival of MCL-engrafted mice but, as a single agent, does not result in complete elimination of disease.

Discussion

The studies described in this report have identified FTY720 as a potential therapeutic agent targeting Cyclin D1 and p-Akt, two critical signaling molecules implicated in pathogenesis of MCL. Overexpression of Cyclin D1

contributes to the aggressive nature of this disease (21, 22). Several lines of evidence indicate that the downmodulation of Cyclin D1 and associated cell cycle regulation may have potential therapeutic relevance in MCL. Inhibition of HSP90 with the small-molecule 17-allylamino-17-demethoxy-geldanamycin induced G₀-G₁ cell cycle arrest

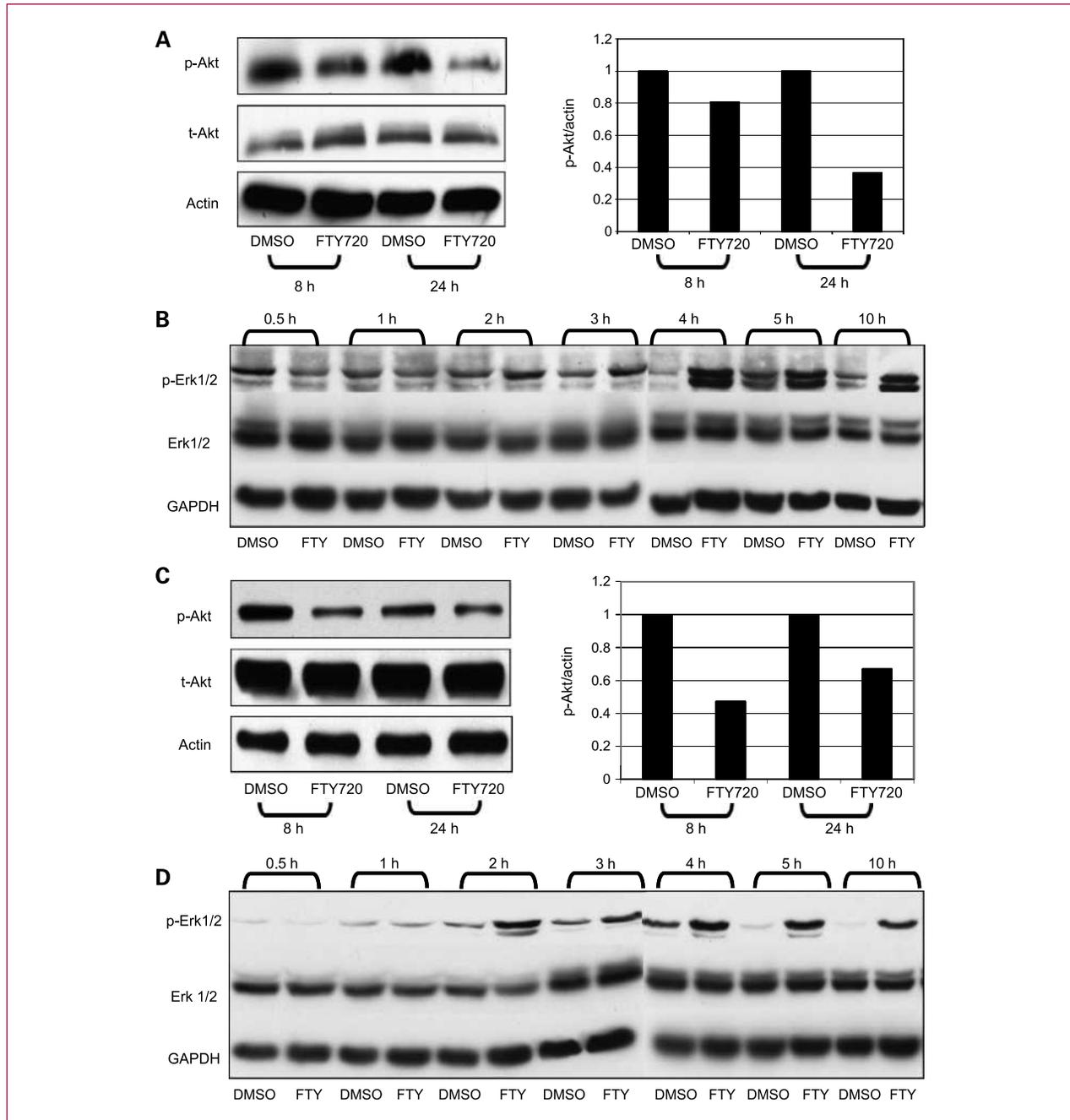


Fig. 5. FTY720-induced differential modulation of p-Akt and p-ERK proteins in MCL. A and C, FTY720-induced changes of p-Akt status in MCL cell lines. Jeko (A) and Mino cells (C; 1×10^6 /mL) were treated with 7.5 and 5 μ mol/L FTY720, respectively. Immunoblots of 8- and 24-h lysates using indicated antibodies are shown. Levels of p-Akt normalized with actin are shown. B and D, FTY720-induced changes of ERK phosphorylation status in MCL cell lines. Jeko (B) and Mino cells (D; 1×10^6 /mL) were treated with FTY720. Cell lysates were collected at indicated posttreatment time intervals (0.5-10 h) and subjected to immunoblotting using indicated antibodies.

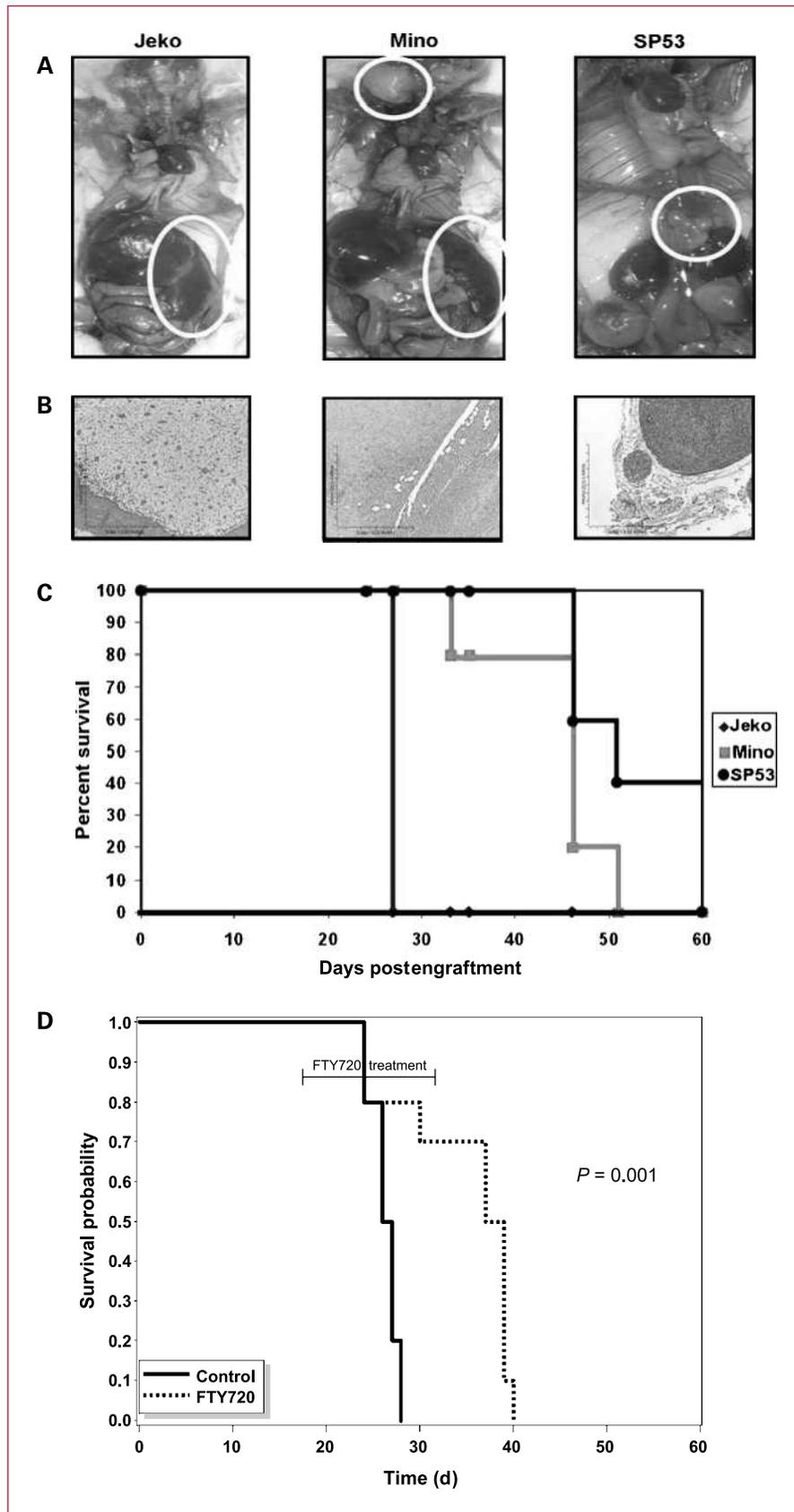


Fig. 6. FTY720 prolonged survival of Jeko-SCID xenografted mice. A and B, development of a preclinical *in vivo* MCL model. SCID mice were engrafted with 4×10^7 cells i.v., as described in Materials and Methods, and observed daily for signs of tumor burden.

Tumor-bearing mice were sacrificed and examined for tumor location and organs were collected for histology and flow cytometry. A, left, Jeko, mice engrafted with Jeko cells all showed wasting/cachexia, central nervous system tumor burden, and splenomegaly; middle, Mino, mice engrafted with Mino cells developed large bilateral neck masses, mediastinal tumor, and splenomegaly; right, SP53, mice engrafted with SP53 cells developed retroperitoneal and abdominal tumor burden. B, left, Jeko, evaluation of brain tissue from mice engrafted with Jeko cells showed meningeal spread and perivascular invasion in the central nervous system; middle, Mino, histopathologic examination of neck mass from a mouse engrafted with Mino cells shows tumor effacement of parotid glandular tissue; right, SP53, retroperitoneal lymph nodes showed marked effacement of normal lymph node architecture. C, Kaplan-Meier survival plot reflecting time to lethal tumor burden using three different MCL cell lines (Jeko, Mino, and SP53; five animals per group). D, evaluation of *in vivo* therapeutic activity of FTY720 in the preclinical MCL model. SCID mice injected i.v with 4×10^7 JeKo cells described in the Materials and Methods section were observed daily for signs of tumor burden. The mean survival for FTY720-treated mice ($n = 10$) was 38 d (range, 30-39). This is significantly prolonged compared with vehicle control group ($n = 10$; 26.5 d; range, 26-27 d). The results from the log-rank test indicated an overall statistically significant difference in survival functions between the FTY720 treatment and the control group ($P = 0.001$).

and cell death associated with the downregulation of Cyclin D1 and activation of the intrinsic/mitochondrial caspase pathway in MCL cell lines (23). FTY720-induced downregulation of Cyclin D1 and associated cell cycle block observed in primary MCL tumor cells and MCL cell lines indicates a potential application of FTY720 as an agent for both nodal and blastic variant MCL. Of note, the blastic variant Jeko cell line exhibited relatively less Cyclin D1 downmodulation in response to FTY720, suggesting possible contribution to the relatively increased resistance of Jeko cells compared with primary MCL tumor cells and Mino cell line to FTY720-mediated apoptosis. Recent data have indicated a role for Cyclin D2 and Cyclin D3 in the pathogenesis of MCL tumors that show the downmodulation of Cyclin D1 (24, 25). Our work with Jeko and Mino cell lines showed no changes in Cyclin D2 and downmodulation of Cyclin D3 following brief exposure to FTY 720. These data indicate that a potential resistance mechanism allowing for the induction of other Cyclin proteins following Cyclin D1 downmodulation does not become operable following treatment of MCL cells with FTY-720. Consistent with its effect on cell cycle regulation, FTY720-treated MCL cells exhibited defective S-phase entry associated with the accumulation of cells in the G₀-G₁ phase of the cell cycle. FTY720 has been shown to induce G₀-G₁ cell cycle arrest in HL-60RG and Jurkat cells (9, 26).

The FTY720-mediated downmodulation of p-Akt also has implications relevant to the pathogenesis of MCL. The Ser/Thr kinase, Akt, is a major mediator of survival signals that protect MCL cells from apoptosis. Akt phosphorylation is seen in 100% of aggressive blastoid MCL variants and cell lines tested, and in 30% of classic MCL cases. Because constitutive activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway contributes to the pathogenesis and survival of MCL, particularly in blastoid variants (4), agents that target Akt activity have great potential in MCL therapy. Inhibition of the PI3K/Akt pathway in MCL cell lines have been shown to reduce or abrogate the phosphorylation of Akt, p27 (kip1), FRKHL-1, MDM2, Bad, mammalian target of rapamycin, Gsk-3 β , and I κ B, and lead to cell cycle arrest and apoptosis (4, 27, 28). The precise mechanism by which FTY720 mediated reduction in Akt phosphorylation in MCL is not known and is presently being explored in our laboratory.

Akt inhibits apoptosis by inactivating proapoptotic proteins such as Bad, forkhead, and Nur77, and by activating antiapoptotic proteins such as NF- κ B (4, 27, 28). Suppression of Bax translocation from the cytosol to mitochondria by Akt pathway has been reported (29). Here, we show Bax upregulation in Jeko cells starting as early as 8 hours after the incubation with FTY720. It is also important to note that Bax upregulation takes place with a p53-independent mechanism because Jeko cells have no detectable p53 (data not shown). Although with a delayed kinetic, we also show p-Akt downmodulation that might contribute to Bax upregulation, suggesting a link between the Akt pathway and the expression level of Bax.

Bax has been reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel, which leads to the loss in membrane potential and the release of cytochrome *c* (29). It has previously been shown that Bax upregulation can further enhance the generation of ROS (30, 31). Here, together with Bax upregulation following FTY720 treatment, we show that FTY720 induces the generation of ROS starting as early as 4 hours. Interestingly, the apoptosis can be rescued by incubation with NAC, a nonspecific ROS scavenger, indicating that the elevation of ROS is essential for the FTY720-induced apoptosis. These results also suggest a link between ROS generation and Bax upregulation in which Bax upregulation could represent a positive feedback loop in the apoptosis cascade. It is also interesting to note that the FTY720-induced apoptosis is a caspase-independent process that is distinct from several currently used chemotherapeutic agents such as fludarabine, bortezomib, and chlorambucil.

Several studies suggest a link between the PTEN/PI3K/Akt cascade and the MAPK (Ras/Raf/MAP/Erk) cascade, two essential pathways frequently dysregulated in tumorigenesis (17, 18, 32). Our studies showing FTY720-induced inhibition of p-Akt and induction of p-ERK1/2 is consistent with the recent report showing that the inhibition of the PTEN/PI3K/Akt leads to MAPK pathway activation through a PI3K-dependent feedback loop in breast cancer (18).

FTY720 has been shown to induce dephosphorylation of Erk protein in multiple myeloma and CLL (8, 9). Whereas early PP2A-dependent Erk dephosphorylation by FTY720 has been observed in CLL B cells, PP2A-independent delayed phosphorylation of Erk1/2 was also noticed at later time points (9).⁷ Signaling through S1PRs, phosphorylated FTY720 provided cell survival signals and function through phosphorylation of downstream effectors, such as Akt and Erk1/2. The role of Sphingosine Kinase 2 in phosphorylation of FTY720 and its subsequent engagement and activation of high-affinity S1PRs in MCL cells remains to be tested. Erk1/2 phosphorylation is a survival signal in a variety of cancers. Thus, FTY720-induced phosphorylation of Erk1/2 may impart resistance of tumor cells to apoptosis *in vivo*. In such cases, pharmacologic agents that inhibit Sphingosine Kinase 2 or agents that inhibit S1PR-mediated G protein-coupled receptor signaling may overcome the effects of activated Erk1/2. Taken together, these findings underscore the rationale of a combined therapeutic approach with FTY720 and MAPK pathway inhibitors in MCL.

It is interesting to note that although there is minimal interpatient variability in the sensitivity of the primary MCL cells tested, differences in sensitivity to FTY720 between MCL primary tumor cells and cell lines were observed *in vitro* (Fig. 1). This is very likely attributed to the differences associated with adaptation and selection pressures of actively proliferating cell lines under *in vitro* culture conditions. In addition, Fig. 1 suggested that MCL tumor cells treated with the FTY720 may become more sensitive at higher

⁷ Q. Lin and N. Muthusamy, unpublished data.

concentrations of the drug. This in fact is consistent with what is suggested by the survival studies (Fig. 6D) in which survival was extended but disease was not eliminated. Given these observed data, clinical application of FTY720 in MCL patients warrants detailed PK modeling as factors such as disease stage and aggressiveness may influence the therapy outcome *in vivo*.

In summary, downregulation of cyclin D1 and Akt, two critically relevant targets, use of novel caspase-independent and ROS-dependent cytotoxic mechanisms and *in vivo* therapeutic efficacy in a reproducible, aggressive Jeko-SCID xenograft mouse model provide potential basis for further development of FTY720 for clinical applications in MCL therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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