FTY720 Induces Apoptosis in Multiple Myeloma Cells and Overcomes Drug Resistance

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Abstract
The novel immunomodulator FTY720 down-modulates sphingosine-1-phosphate receptor 1 on lymphocytes at low nanomolar concentrations, thereby inhibiting sphingosine-1-phosphate receptor 1–dependent egress of lymphocytes from lymph nodes into effenter lymphatics and blood. At high micromolar concentration, FTY720 has been shown to induce growth inhibition and/or apoptosis in human cancer cells in vitro. In this study, we investigated the biological effects of FTY720 on multiple myeloma cells. We found that FTY720 induces potent cytotoxicity against drug-sensitive and drug-resistant multiple myeloma cell lines as well as freshly isolated tumor cells from multiple myeloma patients who do not respond to conventional agents. FTY720 triggers activation of caspase-8, -9, and -3, followed by poly(ADP-ribose) polymerase cleavage. Interestingly, FTY720 induces alterations in mitochondrial membrane potential (ΔѰm) and Bax cleavage, followed by translocation of cytochrome c and Smac/Diablo from mitochondria to the cytosol. In combination treatment studies, both dexamethasone and anti-Fas antibodies augment anti–multiple myeloma activity induced by FTY720. Neither interleukin-6 nor insulin-like growth factor-1, which both induce multiple myeloma cell growth and abrogate dexamethasone-induced apoptosis, protect against FTY720-induced growth inhibition. Importantly, growth of multiple myeloma cells adherent to bone marrow stromal cells is also significantly inhibited by FTY720. Finally, it down-regulates interleukin-6–induced phosphorylation of Akt, signal transducers and activators of transcription 3, and p42/44 mitogen-activated protein kinase; insulin-like growth factor-1–triggered Akt phosphorylation; and tumor necrosis factor α–induced IκBα and nuclear factor-κB p65 phosphorylation. These results suggest that FTY720 overcomes drug resistance in multiple myeloma cells and provide the rationale for its clinical evaluation to improve patient outcome in multiple myeloma. (Cancer Res 2005; 65(16): 7478-84)

Introduction
Despite advances in systemic and supportive therapies, multiple myeloma remains an incurable plasma cell malignancy due to both intrinsic and acquired drug resistance (1, 2). High-dose chemotherapy with stem cell rescue has modestly extended event-free and overall survival, but cures few, if any, patients (3). Furthermore, the bone marrow microenvironment also confers drug resistance in multiple myeloma cells via at least two different mechanisms: adhesion of multiple myeloma cells to fibronectin confers cell adhesion–mediated drug resistance, and cytokines [i.e., interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I)] in the bone marrow milieu induce phosphatidylinositol 3-kinase (PI3-K)/Akt and/or Janus-activated kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) signaling, which mediates resistance to conventional and novel therapies (4–6). Biologically based treatments targeting the bone marrow microenvironment, including both bone marrow stromal cells (BMSCs) and bone marrow endothelial cells, as well as multiple myeloma cells, can overcome drug resistance in both preclinical and early clinical studies (7–10).

FTY720, a synthetic sphingosine analogue of myriocine derived from culture filtrates of Isaria sinclairii, has been extensively studied in renal transplantation. It interacts with the sphingosine-1-phosphate–specific G protein–coupled receptors (sphingosine-1-phosphate receptors 1, 3, 4, and 5), formally called EDG receptors, sphingosine-1-phosphate receptors 1, 3, 4, and 5), formally called EDG receptors, which both induce sphingosine-1-phosphate receptor 1 and inhibits the immune response at low nanomolar concentrations, whereas it induces growth inhibition and/or apoptosis in several human cancer cells in vitro at high micromolar levels (15, 16). Previous studies suggested that apoptosis induced by FTY720 might not be related to inactivation of sphingosine-1-phosphate receptor 1 (11, 17); two chiral analogues of FTY720, AAL151 and AAL149, both induce apoptosis in vitro, but only AAL151 inactivates sphingosine-1-phosphate receptor 1 and is active in vivo in transplant and autoimmune models. It is well possible that FTY720, at high concentrations, acts as an intracellular second messenger and mimics sphingosine and ceramide, which both induce apoptosis independent of sphingosine-1-phosphate receptors. To date, however, the molecular mechanisms of its antitumor effects are undefined (18).

In the present study, we show that FTY720 induces apoptosis in multiple myeloma cell lines, as well as patient multiple myeloma...
cells. As with proteasome inhibitor bortezomib (PS-341; ref. 19) and immunomodulatory derivatives of thalidomide (IMiDs; refs. 20, 21), FTY720-induced multiple myeloma cell growth inhibition is enhanced by dexamethasone. Although IL-6 and IGF-1 are major multiple myeloma cell growth factors and confer protection against dexamethasone-induced apoptosis (4), neither exogenous IL-6 nor IGF-1 protects against FTY720-induced cytotoxicity. Adherence of multiple myeloma cells to BMSCs both augments tumor cell growth and protects against dexamethasone-induced apoptosis (22, 23); importantly, FTY720 induces apoptosis even of multiple myeloma cells adherent to BMSCs. Our data therefore show that FTY720 induces cytotoxicity in preclinical models by targeting both multiple myeloma cells and the bone marrow milieu, providing the framework for clinical trials of this novel agent to improve patient outcome in multiple myeloma.

Materials and Methods

Reagents. FTY720 was provided by Novartis Pharma (Basel, Switzerland). It was dissolved in water (10 mmol/L) and stored at −20°C until use. IL-6, IGF-1, and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems (Minneapolis, MN). Pan-caspase inhibitor benzoylcarbonyl-valine-alanine-aspartate fluoromethylketone (Z-VAD-fmk; Calbiochem, San Diego, CA) was dissolved in DMSO, stored at 25°C until use. IL-6, IGF-I, and tumor necrosis factor-α were dissolved in water (10 mmol/L) and stored at −20°C. Pan-caspase inhibitor benzoylcarbonyl-valine-alanine-aspartate fluoromethylketone (Z-VAD-fmk; Calbiochem, San Diego, CA) was dissolved in DMSO, stored at 25°C until use. IL-6, IGF-I, and tumor necrosis factor-α were dissolved in water (10 mmol/L) and stored at −20°C. Pan-caspase inhibitor benzoylcarbonyl-valine-alanine-aspartate fluoromethylketone (Z-VAD-fmk; Calbiochem, San Diego, CA) was dissolved in DMSO, stored at 25°C until use. IL-6, IGF-I, and tumor necrosis factor-α were dissolved in water (10 mmol/L) and stored at −20°C.

Primary multiple myeloma cells, bone marrow mononuclear cells, and bone marrow stromal cells from multiple myeloma patients. Tumor cells (>90% CD138+) were purified from multiple myeloma patient bone marrow using the RosetteSep negative selection system (StemCell Technologies, Vancouver, British Columbia, Canada), as described previously (24). CD138-negative bone marrow mononuclear cells were isolated by depletion of CD138-positive cells using magnetic beads. Bone marrow mononuclear cells were cultured for 3 to 6 weeks to generate BMSCs. Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients in accordance with Declaration of Helsinki protocol.

Cell viability assays. The growth inhibitory effect of FTY720 on multiple myeloma cell lines, bone marrow mononuclear cells, and peripheral blood mononuclear cells using MTT assays. FTY720 induces significant dose- and time-dependent growth inhibition, in MM.1S, U266, and RPMI8226 multiple myeloma cell lines, with IC50 values at 24 hours of 2.85, 5.78, and 5.96 μmol/L, respectively (Fig. 1A), and at 48 hours of 1.70, 4.80, and 3.59 μmol/L, respectively (data not shown). FTY720 also triggers cytotoxicity in dexamethasone-resistant MM.1R, Dox-resistant RPMI-Dox40, and OPM1 cells with IC50 values at 24 hours of 5.97, 5.21, and 7.36 μmol/L, respectively (Fig. 1A), and at 48 hours of 3.57, 6.58, and 9.69 μmol/L, respectively (data not shown). FTY720 also triggers cytotoxicity in dexamethasone-resistant MM.1R, Dox-resistant RPMI-Dox40, and OPM1 cells with IC50 values at 24 hours of 5.97, 5.21, and 7.36 μmol/L, respectively (Fig. 1A), and at 48 hours of 3.57, 6.58, and 9.69 μmol/L, respectively (data not shown). FTY720 also triggers cytotoxicity in dexamethasone-resistant MM.1R, Dox-resistant RPMI-Dox40, and OPM1 cells with IC50 values at 24 hours of 5.97, 5.21, and 7.36 μmol/L, respectively (Fig. 1A), and at 48 hours of 3.57, 6.58, and 9.69 μmol/L, respectively (data not shown).

Cell viability assays. The growth inhibitory effect of FTY720 on multiple myeloma cell lines, bone marrow mononuclear cells, and peripheral blood mononuclear cells separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT; Sigma-Aldrich, Inc., St. Louis, MO) dye absorbance, as previously described (24).

ATP assay. To evaluate the effect of FTY720 on intracellular ATP, MM.1S cells were treated with FTY720 and intracellular ATP was measured using ATP Bioluminescence Assay Kit HS II (Roche Diagnostics, Penzberg, Germany).

Mitochondrial membrane potential. To evaluate the effect of FTY720 on alterations of mitochondrial membrane potential (ΔΨm), MM.1S cells were treated with or without 8 μmol/L FTY720 for 3 or 6 hours, with addition of Mitocupture reagent (Mitocupture Apoptosis Detection Kit, Calbiochem) for the last 20 minutes, followed by flow cytometric analysis (Cytomics FC500, Becton Dickinson, Franklin Lakes, NJ). Viable cells had low fluorescence intensity (FL-1), whereas cells with loss of ΔΨm had high FL-1.

Immunoblotting. Multiple myeloma cells were cultured with FTY720, harvested, washed, and lysed using lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 10 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L Na3VO4, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin], as described previously (25). Cytosolic extracts were obtained using Mitochondria isolation kit (Pierce, Rockford, IL). Total cell lysates were collected, and protein concentration was measured using a protein assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates (40 μg per lane) were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) and immunoblotted with anti-poly(ADP-ribose) polymerase (PARP), caspase-8, caspase-9, Bax, Bcl-XL, nuclear factor-κB (NF-κB) phospho–p65, phospho-IκBα antibodies (Cell Signaling, Beverly, MA); anti-caspase-3 antibody (BD Pharmingen, San Diego, CA); as well as α-tubulin, Bcl-2, Mcl-1, phospho-extracellular signal-regulated kinase (ERK), and phospho-STAT3 antibodies (Santa Cruz Biotech., Santa Cruz, CA). Anti–cytochrome c and Smac/Diablo antibodies were kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX).

Effect of FTY720 on paracrine multiple myeloma cell growth in the bone marrow milieu. To evaluate the effect of drug on growth of multiple myeloma cells adherent to BMSCs, multiple myeloma cells were cultured in BMSC-coated 96-well plates for 48 hours, in the presence or absence of FTY720. Multiple myeloma cells (3 × 104 cells/well) were also incubated in these 96-well culture plates (Costar, Cambridge, MA) in the presence of 104 cells. After 48 hours at 37°C, Cells were pulsed with [3H]thymidine (0.5 μCi/well) during the last 8 hours of 48-hour culture, and DNA synthesis was measured by [3H]thymidine (NEN Life Science Products, Boston, MA) uptake.

Statistical analysis. Statistical significance of differences observed in FTY720-treated compared with control cultures was determined using Student t test. The minimal level of significance was P < 0.01.

Results

Differential cytotoxicity of FTY720 against multiple myeloma cells versus normal cells. We first determined the effect of FTY720 on growth of multiple myeloma cell lines and peripheral blood mononuclear cells using MTT assays. FTY720 induces significant dose- and time-dependent growth inhibition, in MM.1S, U266, and RPMI8226 multiple myeloma cell lines, with IC50 values at 24 hours of 2.85, 5.78, and 5.96 μmol/L, respectively (Fig. 1A), and at 48 hours of 1.70, 4.80, and 3.59 μmol/L, respectively (data not shown). FTY720 also triggers cytotoxicity in dexamethasone-resistant MM.1R, Dox-resistant RPMI-Dox40, and OPM1 cells with IC50 values at 24 hours of 5.97, 5.21, and 7.36 μmol/L, respectively (Fig. 1A). We next assessed viability in MM.1S, U266, and RPMI8226 cells treated with FTY720 for 24 hours using trypan blue exclusion assays (Fig. 1B). FTY720 induces multiple myeloma cell death. FTY720 also induces dose-dependent cytotoxicity in tumor cells from two patients with relapsed multiple myeloma refractory to conventional therapies, with IC50 at 24 hours of 6.10 and 11.36 μmol/L, respectively (Fig. 1C). Importantly, FTY720 is less toxic to peripheral blood mononuclear cells and bone marrow mononuclear cells than tumor cells (Fig. 1D). These data indicate that FTY720 selectively induces cytotoxicity in drug-sensitive and drug-resistant multiple myeloma cells, but less in normal cells.
FTY720 induces caspase-dependent apoptosis. To analyze the mechanism of FTY720-induced cytotoxicity in multiple myeloma cells, we next used immunoblotting to assess activation of caspases in MM.1S cell lines triggered by FTY720. As can be seen in Fig. 2A, FTY720 triggers time-dependent cleavage of caspase-8, -9, and -3, followed by PARP cleavage, a hallmark of apoptosis (Fig. 2A, left). FTY720-triggered caspase/PARP cleavage is observed at ≥4 μmol/L FTY720 (Fig. 2A, right). FTY720 also triggers caspase/PARP cleavage in dexamethasone-resistant MM.1R cells (data not shown), as well as in patient tumor cells (Fig. 2B). The pan-caspase inhibitor Z-VAD-fmk blocks FTY720-induced caspase-8/9 cleavage (Fig. 2C). Moreover, FTY720 decreases intracellular ATP after 8 hours (Fig. 2D; ref. 26). These results indicate that FTY720 induces multiple myeloma cell death via caspase-dependent apoptosis.

FTY720-mediated apoptosis involves mitochondria. We next examined the molecular mechanisms whereby FTY720 activates caspases and triggers apoptosis in multiple myeloma cells. We first examined whether treatment with FTY720 induces a loss in mitochondrial transmembrane potential (∆Ψm) as evidence of mitochondrial outer membrane permeabilization. We cultured cells for 16 hours with FTY720 (8 μmol/L), and then measured changes in ∆Ψm using a tetramethylrhodamine ethyl ester (TMRM) assay. As shown in Fig. 2D, FTY720 decreases ∆Ψm in a dose-dependent manner, indicating that FTY720 induces mitochondrial outer membrane permeabilization.

Figure 1. Differential cytotoxicity of FTY720 against multiple myeloma cells versus normal cells. MM.1S (●), RPMI8226 ( ■ ), U266 ( ▲ ), dexamethasone-resistant MM.1R ( ○ ), doxorubicin-resistant RPMI-Dox40 ( △), and OPM1 ( ▽ ) cells (A); MM.1S ( ■ ), RPMI8226 ( ▲ ), and U266 ( ● ) cells (B); CD138-positive patient multiple myeloma cells [multiple myeloma #1 ( ● ) and multiple myeloma #2 ( ■ )] and MM.1S ( ▲ ) cells (C); as well as peripheral blood mononuclear cells from healthy volunteers ( ●, ■, and ▲; n = 3) and bone marrow mononuclear cells from three different multiple myeloma patients ( ●, ▲, and ▽; n = 3; D) were cultured for 24 hours in the presence of FTY720 (0-32 μmol/L). Cell growth was assessed by MTT assay (A, C, and D); and cell death was assessed by trypan blue exclusion (B). Points and columns, mean of triplicate cultures; bars, SD.

Figure 2. FTY720 induces caspase-dependent apoptosis. A, MM.1S cells were cultured with FTY720 (8 μmol/L) for the indicated times, and with FTY720 for 9 hours at the indicated doses. FL, full-length band; CL, cleavage band. B, CD138-positive patient multiple myeloma cells (multiple myeloma #1) were cultured with FTY720 (8 μmol/L) for 16 hours. C, MM.1S cells were preincubated with Z-VAD-fmk (25 μmol/L) for 30 minutes before treatment with FTY720 (8 μmol/L) for the indicated times. Total cell lysates were subjected to immunoblotting using anti–caspase-3, -8, -9, PARP, and α-tubulin antibodies. D, MM.1S cells were cultured with FTY720 (8 μmol/L) for the indicated times and analyzed by ATP assay. Columns, mean of triplicate cultures; bars, SD.
Further investigated FTY720-induced mitochondrial alterations. Because loss of mitochondrial membrane potential (ΔΨm) is associated with translocation of mitochondrial proteins, such as cytochrome c and Smac/Diablo, to the cytosol, we next examined whether translocation of these proteins to the cytosol is induced by FTY720. As seen in Fig. 3B, treatment with FTY720 induces the release of cytochrome c and Smac/Diablo to the cytosol. These results indicate that cytotoxicity triggered by FTY720, like novel agents bortezomib (29), As2O3 (25), and 2-methoxyestradiol (30), is mediated via activation of caspase-D. Mitochondrial proteins, such as cytochrome c, and 2-methoxyestradiol (30), is mediated via activation of caspase-D, which triggers cleavage of proapoptotic Bax protein.

FTY720-mediated apoptosis involves mitochondria. A, effects of FTY720 on mitochondrial membrane potential (ΔΨm). MM.1S cells were treated with or without FTY720 (8 μmol/L) for the indicated times, with Mitocupture reagent added for the last 20 minutes, followed by analysis by flow cytometry. Values in percent indicate percentage of FL-1 high cells (horizontal bars). B, MM.1S cells were cultured with FTY720 (8 μmol/L) for the indicated times. Cytosolic extracts were obtained using Mitochondria isolation kit and subjected to immunoblotting using anti-Smac/Diablo, cytochrome c, and α-tubulin antibodies. C, MM.1S cells were cultured with FTY720 (8 μmol/L) for the indicated times. Total cell lysates were subjected to immunoblotting using anti–Bcl-XL, Mcl-1, Bcl-2, Bax, and α-tubulin antibodies. Bax is cleaved by calpain to yield p18 Bax, with more apoptotic potential than full-length Bax (31–33). These data suggest that FTY720 triggers cleavage of Bax, followed by mitochondrial alterations and apoptosis.

**FTY720 enhances death signaling via extrinsic and intrinsic pathways.** As FTY720 triggers both extrinsic and intrinsic apoptotic pathways, we next hypothesized that combining it with agents that trigger either extrinsic or intrinsic apoptotic signaling would enhance cytotoxicity. Because we have shown that dexamethasone induces apoptosis via intrinsic apoptotic signaling (27) and augments cytotoxicity of novel chemotherapeutic agents in multiple myeloma cells (19, 20, 25, 30), we next examined whether dexamethasone similarly enhances cytotoxicity of FTY720. MM.1S cells were cultured for 24 hours with dexamethasone, in the presence or absence of FTY720 (2 μmol/L). MTT assays confirmed that FTY720 enhances MM.1S cell death induced by dexamethasone (Fig. 4A). We next examined whether FTY720 enhances anti-Fas antibody–triggered extrinsic apoptotic signaling (34). Whereas the combination with FTY720 and control immunoglobulin M antibody does not alter cell viability, FTY720 augments anti-Fas antibody–induced cytotoxicity (Fig. 4B). These data indicate that combination therapy with FTY720 augments multiple myeloma cell cytotoxicity.

**FTY720 overcomes the protective effects of interleukin-6 and insulin-like growth factor-I on multiple myeloma cell growth.** Because we have shown that IL-6 (35, 36) and IGF-I (37, 38) both mediate growth and prevent apoptosis in multiple myeloma cells in the bone marrow milieu, we next examined whether FTY720 can overcome these protective effects of exogenous IL-6 and IGF-I. Although IL-6 (2 or 10 ng/mL) and IGF-I (10 or 50 ng/mL) overcome dexamethasone-induced MM.1S cytotoxicity and cell growth assessed by MTT assay (data not shown) and thymidine uptake, respectively, IL-6 and IGF-I do not inhibit FTY720-induced cytotoxicity (Fig. 5A and B).

**FTY720 induces growth inhibition in cocultures of multiple myeloma cells with bone marrow stromal cells.** Because we have shown that the bone marrow microenvironment confers growth and drug resistance in multiple myeloma cells (19, 24, 39), we next studied the effect of FTY720 on multiple myeloma cell growth in the bone marrow milieu. We first examined the direct toxicity of FTY720 on patient BMSCs using MTT assay; no toxicity
is induced in BMSCs cultured with FTY720 for 24 hours (Fig. 5C). MM.1S cells were next cultured for 24 hours with or without BMSCs, in the presence or absence of FTY720. Multiple myeloma cell adherence to BMSCs enhanced [3H]thymidine uptake (1.9-fold), which is inhibited in the presence of FTY720. Specifically, 8 μmol/L FTY720 completely blocks this enhancement (Fig. 5D).

**FTY720 inhibits survival signals triggered by cytokines.** Because we (5, 37, 40, 41) and others (42) have shown that STAT3, ERK, and Akt signaling pathways mediate multiple myeloma cell proliferation and survival, we next investigated whether FTY720 inhibits these signaling pathways triggered by IL-6 and IGF-I. Phosphorylation of STAT3, ERK 1/2, and Akt is induced by IL-6 (10 ng/mL) in MM.1S cells; conversely, pretreatment of FTY720 (8 μmol/L for 1 hour) markedly inhibits IL-6–induced STAT3, Akt, and ERK phosphorylation in a dose-dependent manner (Fig. 6A and B). Pretreatment with FTY720 (8 μmol/L for 1 hour) also inhibits IGF-I (50 ng/mL)–induced phosphorylation of Akt in MM.1S cells (Fig. 6C). Because we have shown the importance of NF-κB activation in promoting growth, survival, and drug resistance in multiple myeloma cells in the bone marrow milieu (22, 23, 43), we next examined whether FTY720 inhibits NF-κB activity stimulated by TNF-α. As seen in Fig. 6D, FTY720 inhibits IκBα and NF-κB p65 phosphorylation in MM.1S cells induced by TNF-α. These results suggest that FTY720 blocks IκBα phosphorylation and translocation of NF-κB from cytoplasm to nucleus, thereby overcoming the antipapoptotic effect of activated NF-κB induced by TNF-α.

**Discussion**

FTY720 has initially been used as an immunosuppressant. A phase 2A study of FTY720 in renal transplantation showed that FTY720 at 2.5 mg in combination with cyclosporine and corticosteroids is effective and well tolerated at a steady-state concentration of 22.6 nmol/L (44). Recent studies showed that FTY720 at micromolar levels also induces apoptosis in promyelocytic leukemia (HL-60; ref. 15), T-cell leukemia (Jurkat; ref. 45), bladder cancer (46), glioma (47), prostate (16), and hepatocellular carcinoma (48) cell lines. Moreover, it prevents tumor growth and metastasis in JygMC(A) mouse breast cancer cells both in vitro and in vivo (49). In this respect, high concentrations of FTY720 may mimic effects of the intracellular sphingolipids sphingosine and/or ceramide, which both induce apoptosis independent of sphingosine-1-phosphate receptors (18, 50). The apoptotic processes induced by FTY720 might also be sphingosine-1-phosphate receptor independent (11, 17), because two chiral analogues of FTY720 (AAL151 and AAL149) both induce apoptosis in vitro at identical micromolar concentrations, but only AAL151 targets sphingosine-1-phosphate receptors at low nanomolar concentration and is active in vivo in transplant and autoimmune models. In contrast, AAL149 does not bind to any of the sphingosine-1-phosphate receptors and does not affect lymphocyte migration in vivo (11). It is well possible that FTY720, at high concentrations, acts as an intracellular second messenger and mimics sphingosine and ceramide, which both induce apoptosis independent of sphingosine-1-phosphate receptors (18, 50). To date, however, the molecular mechanisms of its anti–multiple myeloma effects are undefined (18). In this report, we show that FTY720, a novel class of

![](image1.png)

**Figure 5.** FTY720 overcomes the protective effects of IL-6, IGF-I, and BMSCs on multiple myeloma growth. MM.1S cells were cultured for 24 hours in control media (a) and with 3 μmol/L FTY720 (b) and 0.5 μmol/L dexamethasone (c), in the presence or absence of IL-6 (2 or 10 ng/mL; A) or IGF-I (10 or 50 ng/mL; B). C, BMSCs were cultured for 24 hours in the presence of FTY720 (0-8 μmol/L). D, MM.1S cells, BMSCs, and both MM.1S cells and BMSCs were cultured for 24 hours in control media (C), and with 2 μmol/L (D), 4 μmol/L (m), or 8 μmol/L (e) FTY720. Cell growth was assessed by [3H]thymidine uptake (A, B, and D) and MTT assay (C). Columns, mean of triplicate cultures; bars, SD.

![](image2.png)

**Figure 6.** FTY720 inhibits survival signals triggered by cytokines. MM.1S cells were serum starved for 2 hours, and then cultured with or without FTY720 (2-8 μmol/L) for 1 hour. Cells were next stimulated with IL-6 (10 ng/mL; A and B), IGF-I (50 ng/mL; C), or TNF-α (2 or 5 ng/mL; D) for the indicated times. Total cell lysates were subjected to immunoblotting using anti–phospho-STAT3, phospho-Akt-1, phospho-ERK, and α-tubulin antibodies (A-C), as well as with anti-phospho-IκBα, phospho-NF-κB p65, NF-κB p65, and α-tubulin antibodies (D).
immunomodulators, induces apoptosis in drug-sensitive and drug-resistant multiple myeloma cell lines with IC50 at 24 hours of 2.85 to 7.36 μmol/L, as well as in two patient multiple myeloma cells with IC50 of 6.10 and 11.36 μmol/L at 24 hours of culture, respectively; importantly, there is no cytotoxicity in peripheral blood mononuclear cells and bone marrow mononuclear cells.

We have previously shown that apoptosis triggered by conventional and novel anti-multiple myeloma agents is mediated via caspase-8 and/or caspase-9 activation, followed by caspase-3 and PARP cleavage (19, 21, 25). In this study, FTY720 induces activation of caspase-8, -9, and -3, followed by PARP cleavage; conversely, drug-induced PARP cleavage was blocked by Z-VAD-fmk, confirming that FTY720 induces caspase-dependent apoptosis. Mitochondria play an important role in modulating cell death (34); conversely, alterations in expression and/or function of mitochondrial signaling proteins confer drug resistance (29, 51). FTY720 induces caspase-8 and caspase-9 activation, suggesting the potential clinical utility of combining this agent with dexamethasone, which triggers caspase-9 activation, to trigger dual apoptotic signaling, or with IMiD, which also triggers caspase-8 activation, to enhance cytotoxicity. Bax or Bak is essential for initiating the intrinsic apoptotic pathway (52). In this study, FTY720 induces Bax cleavage and decreases mitochondrial membrane potential (ΔΨm), with release of cytochrome c and Smac/Diablo. Although cleavage to p18 Bax is not required to initiate apoptosis, p18 Bax potently accelerates the apoptotic process (31, 32). Because full-length p21Bax is cleaved by calpain at aspartate 33 to yield p18 Bax during apoptosis induced by stress or drugs (53, 54), it is possible that FTY720 also triggers calpain activation, cleavage of Bax, and decrease in mitochondrial membrane potential (ΔΨm) via the intrinsic apoptotic pathway.

As is true for thalidomide/IMiDs (20), bortezomib (19), and lysophosphatidyl acyltransferase-β inhibitor (24), FTY720-induced cytotoxicity in MM.1S cells is enhanced by dexamethasone, suggesting differential apoptotic signaling cascades for these agents versus dexamethasone. For example, dexamethasone induces caspase-9 activation via a cytochrome c-independent and Smac-dependent pathway (27), whereas our study shows that FTY720 triggers caspase-8 activation with both cytochrome c and Smac release from mitochondria. Moreover, we show that anti-Fas antibody, which triggers apoptosis via extrinsic pathway, also enhances FTY720-induced cytotoxicity in MM.1S cells. These results provide a rational framework for clinical use of FTY720 in combination with conventional chemotherapy.


Acknowledgments

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