IMP dehydrogenase inhibitor mycophenolate mofetil induces caspase-dependent apoptosis and cell cycle inhibition in multiple myeloma cells

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Abstract

Multiple myeloma is an incurable disease for the majority of patients, therefore requiring new biological targeted therapies. In primary myeloma cells, IMP dehydrogenase (IMPDH) was shown to be consistently overexpressed. We therefore tested the IMPDH inhibitor mycophenolate mofetil (MMF) currently available as a clinical therapeutic agent for its antmyeloma activity in vitro. MMF depleted intracellular guanosine 5’-triphosphate (GTP) levels in myeloma cells. We showed apoptosis induction in myeloma cell lines and primary myeloma cells between 1 and 5 μmol/L MMF. MMF was also cytotoxic at this concentration in dexamethasone-resistant and Mcl-1-overexpressed myeloma cell lines shown by the tetrazolium salt XTT assay along with cell survival measured by a modified flow cytometric assay. Apoptosis was not inhibited by the presence of an antioxidant, suggesting that MMF-induced apoptosis is less likely to be associated with reactive oxygen species. However, apoptosis was abrogated by exogenously added guanosine, which activates an alternative pathway for GTP formation, implicating that this effect is directly mediated by IMPDH inhibition. MMF-induced G1-S phase cell cycle arrest and its apoptosis induction mechanism were associated with a caspase-dependent pathway as shown by alteration of mitochondrial membrane potential and cytochrome c release followed by activation of the caspases. MMF-induced apoptosis was also inhibited by a pan-caspase inhibitor Z-VAD-fmk. MMF-treated myeloma cells showed an up-regulation of Bak, which most likely together with Bax resulted in the release of cytochrome c. In summary, MMF attenuates G1-S phase cell cycle progression and activates the pathway of mitochondrial dysfunction, leading to cytochrome c release followed by activation of caspases. [Mol Cancer Ther 2006;5(2): 457–66]

Introduction

Multiple myeloma is a largely incurable B-cell neoplasia characterized by accumulation of malignant cells due to deregulated apoptosis of incompletely differentiated plasma cells. Proliferation and survival of myeloma cells occur through overexpression of antiapoptotic and cell cycle regulatory proteins, which are mainly activated by stroma-derived factors (1). Deregulation of various signal transduction pathways, including nuclear factor-kB, phosphoinositide 3 kinase/Akt, signal transducers and activators of transcription 3 (STAT3), and Ras/mitogen-activated protein kinases, are associated with chemoresistance (2, 3). These newly gained insights prompted the search for new compounds with specific targets.

Mycophenolate mofetil (MMF, Cellcept), an immunosuppressive drug, is approved for clinical use in the prevention of acute allograft rejection following organ transplantation and hematopoietic stem cell transplantation and has shown efficacy in autoimmune diseases (4, 5). Mycophenolic acid (MPA), the active metabolite of MMF, is a selective inhibitor of IMP dehydrogenase (IMPDH). MMF was developed as a prodrug to improve its bioavailability (6) and is rapidly completely converted to the active metabolite MPA by plasma esterases (7). IMPDH catalyzes the rate-limiting reaction of de novo guanosine 5’-triphosphate (GTP) biosynthesis at the IMP metabolic branch point (8). Inhibition of IMPDH enzyme leads to the reduction of xanthine monophosphate (XMP), GTP, and dGTP resulting in inhibition of cell proliferation.

Previous studies have shown that MPA inhibits DNA synthesis in T and B lymphocytes by blocking de novo guanosine synthesis, which is critical for their proliferation. IMPDH activity is increased significantly in cancer cells, and it was considered a sensitive target for chemotherapy (8). IMPDH inhibitors were used to induce differentiation in cancer cell lines, implicating alterations in the activity of IMPDH and the levels of guanine nucleotides in the regulation of cell growth and differentiation (9). Patient-derived nonlymphocytic leukemia cells were used to study induction of differentiation by IMPDH inhibitors. Phase I/II
clinical trials using tiazofurin (2-b-D-ribofuranosylthiazole-4-carboxamide), a selective IMPDH inhibitor, have been conducted (10–13). The result showed a good correlation between biochemical variables (decline in IMPDH activity and GTP concentrations in blast cells) and clinical response. This compound was not tested further clinically due to its toxicity profile.

MPA has also been shown to induce apoptosis in human T lymphocytic and monocyte cell lines (4). Recently, we reported the result of cDNA microarray analysis done on purified primary myeloma cells and revealed a consistent elevation of IMPDH2 gene expression compared with the normal control plasma cells (14). We also did a phase I study using MMF in advanced myeloma patients to test whether MMF can be tolerated in such patients and to perform a laboratory correlative study measuring intracellular nucleotide levels from patient bone marrow and peripheral blood mononuclear cells (14). IMPDH inhibition and its effect on cell proliferation and apoptosis by MMF in myeloma cells have not been reported to our knowledge. Most recently, a new IMPDH inhibitor VX-944 was reported as a novel apoptosis inducer in myeloma cells by a non–caspase-dependent pathway (15). These data provide a strong rationale for testing IMPDH targeting drugs, such as MMF. The present study is based on our hypothesis that MMF may inhibit tumor growth of myeloma cells and may induce apoptosis by IMPDH inhibition.

Materials and Methods

Reagents

MPA (Sigma Chemical Co., St. Louis, MO) and MMF (Roche Laboratories, Inc., Nutley, NJ) were dissolved in 95% ethanol and used at indicated concentrations. Dexamethasone (Sigma) and interleukin-6 (IL-6; R&D Systems, Minneapolis, MN) were added to cultures at indicated concentrations. N-acetyl-l-cysteine (L-NAC; Sigma) was prepared in sterile water immediately before use. Z-VAD-fmk (Calbiochem, La Jolla, CA) and tumor necrosis factor–related apoptosis-inducing ligand (Biomerieux, Plymouth Meeting, PA) were used for caspase inhibition study.

Antibodies for Western Blotting

The following antibodies were used: anti-cytochrome c (Biosource, Camarillo, CA); anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-caspase-9 and anti-caspase-3 (BioVision, Mountain View, CA); anti-poly(ADP-ribose) polymerase cleavage site specific (Biosource); anti-Bcl-xL, anti-Bcl-2, anti-Bak, anti-Bax, anti-p21, anti-cyclin D1, anti-cyclin A, and anti-cyclin E (Santa Cruz Biotechnology); anti-STAT3 and phospho-STAT3 (Ser727) monoclonal antibody (Cell Signaling Technology, Beverly, MA); anti-Mcl-1 (BD Pharmingen, San Jose, CA).

Myeloma Cell Lines, Purified Myeloma Cells, and Bone Marrow Stromal Cells

The myeloma cell lines RPMI8226 and RPMI8226-HAmcl1 were obtained from Dr. R.G. Fenton (The University of Maryland Greenebaum Cancer Center) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1-glutamine (2 mmol/L), and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin; all from Life Technologies, Inc., Gaithersburg, MD) at 37°C in a humidified 5% CO2 as has been previously described (16). The IL-6-dependent ARP1 and ARP1-bcl2 cell lines were obtained as has been previously described (2, 16). Both were maintained in the same media as other myeloma cell lines except for adding 1 ng/mL IL-6. Heparinized bone marrow samples were obtained from patients with relapsed myeloma after obtaining informed consent under Institutional Review Board–approved protocol at the University of Maryland, School of Medicine. Mononuclear cells separated by LSM Lymphocyte Separation Medium (ICN Biomedicals, Inc., Aurora, OH) were saved and frozen until their use. The stored mononuclear cells were thawed and subjected to CD138 immunomagnetic purification according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA) before use. The CD138 enriched fraction contained >95% plasma cells as determined by Wright-Giemsa staining and morphology. A bone marrow sample from one patient was used to obtain bone marrow stromal cells as described previously (17).

Western Blot Analysis

Western blot analysis was done as described previously (18). In brief, myeloma cells were cultured with MMF, harvested, washed, and lysed. Equal amount of protein loading was confirmed by Ponceau staining. Immunoreactivity was detected by sequential incubation with secondary antibody and enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Subcellular Fractionation

Myeloma cells were harvested by centrifugation at 1,000 × g for 10 minutes at 4°C as reported previously (19). In brief, cells were homogenized, and the nuclei were pelleted by centrifugation at 750 × g for 10 minutes at 4°C. The supernatant was centrifuged at 10,000 × g for 25 minutes. The supernatant (cytosolic fractions) was saved for further analysis, and the protein concentrations were determined by Bradford method (Bio-Rad, Hercules, CA).

Measurement of Mitochondrial Membrane Potential $\Delta \Psi_m$

Mitochondrial energization was determined by retention of JC-1 dye (Calbiochem) as described previously (18). Briefly, 1 × 106 cells were loaded with JC-1 dye (1 μg/mL) during the last 30 minutes of incubation. Cells were washed in PBS twice. Fluorescence was monitored in a fluorometer using 570-nm excitation/595-nm emission for the J-aggregate of JC-1.

Measurement of Apoptosis and Growth Inhibition Assay

Apoptosis was determined by DNA staining with Annexin V-FITC apoptosis Detection Kit per manufacturer’s instructions (BD Biosciences, Palo Alto, CA). Controls included unstained cells, cells stained with Annexin V-FITC alone, and cells stained with propidium iodide alone to subtract the percentage of apoptotic cells in...
the untreated from the treated population to determine the actual number of cells that underwent apoptosis.

Cell survival was measured using either a modified method of flow cytometry-based cell viability assessment previously published (20) or a colorimetric assay using XTT sodium salt [2,3-bis(2-Methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide], as described previously (21). In brief, a flow cytometry-based cell viability assay was measured using Annexin V-FITC apoptosis kit to stain myeloma cells treated with various concentrations of MMF for 4 days. Then, the cells were submitted for flow cytometric analysis to measure the total cell number and cells in apoptosis from each treatment after calibration of the instrument (FACScan, Becton Dickinson, San Jose, CA) as described previously (20).

**Caspase Assays**

Cell lysates were prepared as reported previously (16). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Caspase assays were done in 96-well plates using Ac-DEVD-AMC fluorogenic substrate (PharMingen, San Diego, CA), as described previously (16). Measurement of the AMC cleavage product was done using a CytoFluor II Microplate Fluorescence Reader (PerSeptive Biosystems, Inc., Bedford, MA) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

**Cell Cycle Analysis**

Synchronized ARPI cells were treated with 1 μmol/L MMF for 8, 16, and 24 hours and then fixed in 70% ethanol for 30 minutes at 4°C. Cells were treated with 1,000 units/mL of DNase-free RNase (Roche Molecular Biochemicals, Mannheim, Germany) for 30 minutes at 37°C. The DNA was stained with 40 mg/mL of propidium iodide for 30 minutes at room temperature and then analyzed by FACScan flow cytometry. Data were analyzed using Modfit LT modeling software (Verity Software House, Inc., Topsham, ME).

**Bone Marrow Stromal Cell Coculture Experiment**

We previously cultured patient-derived plasma cells in media without bone marrow stromal cells for >2 days in the presence of IL-6. However, there was extensive apoptosis induction after 2 days of *in vitro* culture, and we found it difficult to maintain primary plasma cells in a conventional suspension culture for longer than 2 days. Thus, we tested the effect of MMF on isolated CD138+ plasma cells from myeloma patients using a bone marrow stromal cell coculture system for 3 days and submitted it to apoptosis analyses. Bone marrow stromal cell culture for 3 days without RPMI8226 cells showed no cell detachment or suspension cells in the media, suggesting that suspension cells in the coculture system were 100% RPMI8226 cells.

Bone marrow–derived human stromal cells were thawed and grown in DMEM-HG with 10% fetal bovine serum with L-glutamine and antibiotics. The origin of bone marrow stromal cells used in experiments was derived from one patient but different from the origin of primary myeloma cells used for the coculture experiment. The cells were plated onto a six-well plate to become 80% confluent. Patient-derived myeloma cells after CD138 enrichment were seeded at 1 × 10^6 per well in a six-well plate coated with human stromal cells. The cells were grown for 3 days in Iscove’s modified Dulbecco’s medium, 10% fetal bovine serum, and 2 mmol/L L-glutamine with 1 ng/mL IL-6 followed by immediate addition of MMF.

**Determination of Intracellular Nucleotide Triphosphates**

We optimized the high-performance liquid chromatography method by others (12, 22) for the simultaneous determination of intracellular ATP, GTP, dGTP, and XMP as described previously (14).

**Statistical Analysis**

The mean and SD were calculated for each data point. Differences between groups were analyzed by one-way or two-way ANOVA. Significant differences among groups were calculated at $P < 0.05$.
MMF Inhibits Dexamethasone-Resistant ARP1-bcl2 Cell Growth

To test whether MMF can induce apoptosis in cells expressing a drug resistance phenotype, we treated the ARP1-bcl2 cells with increasing doses of MMF. ARP1-bcl2 cells constitutively overexpress Bcl-2 and are resistant to dexamethasone (2). We first confirmed that ARP1-bcl2 cells were indeed resistant to dexamethasone, whereas ARP1 cells were relatively sensitive by the XTT assay (data not shown). These results were consistent with previous reports (2). Next, we treated both cell lines with MMF for 4 days. As shown in Fig. 1C, MMF was equally cytotoxic to ARP1 and ARP1-bcl2 cells and reversed the ARP1-bcl2 drug-resistant phenotype. We also tested MMF on RPMI8226 and RPMI8226-HAmcl1, which is a RPMI8226 line overexpressing myeloma survival factor Mcl-1 (16). MMF was equally cytotoxic to both cell lines at the higher concentrations (Fig. 1D).

MMF-Induced Apoptosis Is Salvaged by Guanosine

To test whether MMF blocks the IMPDH pathway and causes depletion of intracellular GTP levels, we added 100 nmol/L guanosine (11) simultaneously with MMF to investigate whether restoration of GTP pools via a salvage pathway protects against apoptosis induction in myeloma cells. Figure 2A shows the result of reversing apoptosis induction by the addition of guanosine to MMF in RPMI8226 cells. This result suggests that MMF causes depletion of intracellular GTP pool by blocking the IMPDH pathway, which can be reversed with the addition of exogenous guanosine.

MMF Induced Apoptosis in Myeloma Cells via Caspase-Dependent Pathway

Apoptosis induction by dexamethasone in myeloma cells is associated with caspase-9 activation, triggered by the second mitochondrial-derived activities of caspases (Smac) but not cytochrome c (24). To test whether MMF-induced

Figure 1. A, MMF-induced apoptosis in ARP1 cells. The ARP1 cells were incubated with MMF for 3 d to determine the apoptosis effect of MMF. Cells were stained with Annexin V/propidium iodide to detect early apoptosis. Apoptotic cells were determined as the percentage of cells stained with either propidium iodide, Annexin V, or Annexin V[propidium iodide at each MMF concentration analyzed by flow cytometry. Columns, mean % apoptosis of three separate experiments; bars, SD. B, MMF is cytotoxic to myeloma cell lines. The RPMI8226 and the ARP1 cells were incubated with various doses of MMF for 4 d to determine the cell survival. The nonapoptotic cell number was calculated using the analysis described in the Materials and Methods using a flow cytometry – based Annexin V[propidium iodide staining method as the total number of untreated cells as 100%. The RPMI8226 is designated by a black circle line, and the ARP1 is designated by a white circle line. Points, mean % cell survival of three separate experiments; bars, SD. C, MMF was cytotoxic to the myeloma cell line overexpressing bcl-2, which confers dexamethasone resistance. The ARP1 and ARP1-bcl2 cell lines were treated with increasing concentration of MMF for 4 d. Surviving cells were analyzed by the tetrazolium salt XTT assay as described in Materials and Methods. Points, mean % cell survival of three separate experiments; bars, SD. ARP1 (open circle) and ARP1-bcl2 (closed circle) cells were shown as percentage cell survival. D, MMF was cytotoxic to the myeloma cell line overexpressing Mcl-1. The RPMI8226 and RPMI8226HA-Mcl1 cell lines were treated with MMF for 4 d and submitted to XTT analysis. The RPMI8226 (open circle) and RPMI8226HA-Mcl1 (closed circle) cells are presented as percent cell survival. Points, mean % cell survival of three separate experiments; bars, SD.
apoptosis was associated with mitochondria-derived activators, we measured caspase activity after MMF treatment, using fluorogenic substrate on four myeloma cell lines, RPMI8226, RPMI8226-HAMcl1, ARP1, and ARP1-bcl2, treated with 1 μmol/L MMF. An aliquot of cells was harvested every 24 hours for up to 3 days. Interestingly, the caspase activation in RPMI8226-HAMcl1 cells was delayed on days 1 and 2 compared with RPMI8226 (Fig. 2B). ARP1-bcl2 showed a moderate effect in delaying caspase-3 activity on days 1 and 2 but not as significant as the delay with RPMI8226-HAMcl1 (Fig. 2C). Although, baseline (day 0) caspase activity was different in ARP1 and ARP1-bcl2 cells (and this phenomenon was reproducible in multiple experiments), there was a delayed caspase-3 activity in ARP1-bcl2 cells. These data suggested that MMF activates caspase-3, which causes myeloma cells to undergo apoptosis. The delayed activation of caspase-3 in myeloma cells over-expressing Bcl-2 or Mcl-1 suggests that apoptosis induced by MMF was associated with mitochondria-derived activators (2, 16, 25).

We next examined the protein expression of both caspase-3 and its upstream signal caspase-9, which is activated in response to mitochondrial disturbance (26). We did Western blotting using anti-caspase-9, anti-caspase-3 antibodies, and a PARP cleavage site-specific antibody in ARP1 cells treated with 1 μmol/L MMF for the indicated time points and then lysed as described in Materials and Methods. Western blot analysis using caspase-9, caspase-3 antibodies, and a PARP cleavage site-specific antibody was performed. Antibodies stained both procaspase-9 and caspase-3 as well as cleaved caspase-9 and caspase-3. Cleaved PARP (bottom). Cell extracts were analyzed for the presence of caspase-9, caspase-3, and PARP before and after MMF addition for 12, 24, 36, and 60 h. The densitometry result of the bands for cleaved caspase-9, caspase-3, and PARP were shown under each band at different time points.

Figure 2. A, MMF-induced apoptosis is salvaged by guanosine. RPMI8226 cells were cultured in the presence of 5 μmol/L MMF with and without 100 μmol/L guanosine (Gua) for 4 d. The cells were harvested and analyzed for Annexin V/propidium iodide–based flow cytometric analysis to determine the percentage apoptotic cells. RPMI8226 cells incubated with culture media for 4 d were used as a control and calculated as 100%. Columns, mean; bars, SD. *, P < 0.05, significantly different from respective controls. B, caspase-3 activity in RPMI8226 cell lines treated with MMF. Caspase-3 activity was measured using Ac-DEVD-AMC fluorogenic substrate as described in Materials and Methods at days 0, 1, 2, and 3 in RPMI8226 and RPMI8226-HAMcl1; cells were incubated with MMF, and the caspase-3 activity was measured at days 0, 1, 2, and 3. C, caspase-3 activity in ARP1 cell lines treated with MMF. Caspase-3 activity was measured as the method used in B. The ARP1 and ARP1-bcl2 cells were incubated and the activity was measured at days 0, 1, 2, and 3. D, MMF-induced activity of caspase-3 and caspase-9 in ARP1 cells. The time course of recruitment and cleavage of caspase-9 and caspase-3 and cleavage of PARP by MMF was measured by immunoblotting. ARP1 cells were treated with 1 μmol/L MMF for the indicated time points and then lysed as described in Materials and Methods. Western blot analysis using caspase-9, caspase-3 antibodies, and a PARP cleavage site-specific antibody was performed. Antibodies stained both procaspase-9 and caspase-3 as well as cleaved caspase-9 and caspase-3. Cleaved PARP (bottom). Cell extracts were analyzed for the presence of caspase-9, caspase-3, and PARP before and after MMF addition for 12, 24, 36, and 60 h. The densitometry result of the bands for cleaved caspase-9, caspase-3, and PARP were shown under each band at different time points.
we measured the cytochrome c levels in MMF-treated ARP1 cells. As shown in Fig. 3A, cytochrome c release was detected 24 hours after MMF incubation. The onset of cytochrome c release is consistent with the caspase-3 and caspase-9 activation shown by the Western blot as shown in Fig. 2D. It is well known that mitochondrial membrane potential \( \Delta \psi_m \) plays an important role in the exchange of ions and various molecules, which may have roles in apoptosis (27). Loss of membrane potential leads to opening of the permeability transition pore leaking the inner components into the cytosol (28, 29). As shown in Fig. 2B, we observed a significant drop in \( \Delta \psi_m \) in MMF-treated ARP1 cells.

Recently, reactive oxygen species have been shown to be implicated in the mediation of apoptosis by anti-myeloma agents (30). To rule out the possibility of a reactive oxygen species–dependent mechanism as a cause of MMF-induced apoptosis, we incubated myeloma cells with 1 \( \mu \)mol/L MMF and the free radical scavenger L-NAC at 15 mmol/L. If reactive oxygen species–dependent apoptosis is a major cause for MMF-induced apoptosis, then we would expect to see a reduction of apoptosis through the addition of L-NAC. After 3 days of incubation, the apoptosis induction was not significantly blocked by L-NAC (data not shown).

**MMF Effect on Bcl-2 Family Proteins in Myeloma Cells**

To address the issue of whether any proteins of the Bcl-2 family were implicated in MMF-induced apoptosis, the expression of Bcl-2, Bcl-xL, Bax, and Bak protein levels were measured in ARP1 cells. As shown in Fig. 4, Bax and Bcl-2 protein expression levels did not show any significant changes through each time point. In contrast, a remarkable increased expression of a proapoptotic protein Bak and a decrease expression of a prosurvival protein Bcl-xL were shown.

**Decreased Phosphorylation of STAT3 (Ser^{727}) after MMF Treatment in Myeloma Cells**

STAT3 is constitutively activated in some myeloma cell lines and primary myeloma cells (31). IL-6 receptor–mediated signaling pathways in the myeloma cell lines RPMI8226 and ARP1 were determined previously (32, 33). On IL-6 stimulation, STAT is activated through tyrosine phosphorylation by Janus kinase 2 or Src-kinase. Tyrosine-phosphorylated STAT then dimerizes and translocates to the nucleus. To test whether MMF treatment affects STAT3 phosphorylation, myeloma cells were treated with MMF in the presence of IL-6 for various times. As shown in Fig. 5, MMF treatment caused the down-regulation of phosphorylation at STAT3 (Ser^{727}) in ARP1 and RPMI8226 cells. The cells cultured with IL-6 (1 ng/mL) were prepared for protein extracts at the different time points after 1 \( \mu \)mol/L MMF was added. The total STAT3 protein level did not change.

**Effects of MMF on Cell Cycle Distribution and Expression of p21 Protein and Cyclins in ARP1 Cells**

The synchronized ARP1 cells were treated with 1 \( \mu \)mol/L MMF for 16 hours and then were harvested for cell cycle analysis. The control group and MMF-treated group showed the following percent cell distribution in each cell cycle phase: G1, 49 \( \pm 0.1/72 \pm 0.3 \) (control/MMF); S, 36 \( \pm 0.3/18 \pm 0.6 \); G2-M, 15 \( \pm 0.4/10 \pm 0.6 \), respectively. MMF-treated ARP1 cells clearly showed a G1-S phase cell cycle block compared with the control group without MMF treatment. The effects of MMF on the distribution of cell cycle phases in the myeloma cell line prompted us to investigate the MMF effect on the expression of cell cycle regulatory proteins. ARP1 cells were treated with 1 \( \mu \)mol/L MMF and harvested at different time points to obtain total lysates for p27 and p21 immunoblotting. Cell cycle inhibitors, such as p21 protein expression level, increased at 24 hours (Fig. 6). p27 protein expression was decreased at 6 and 12 hours but up-regulated at 24 hours. The Western blot for cyclins showed cyclin D1 was consistently up-regulated, whereas cyclin E was down-regulated at 12 hours and beyond. By contrast, cyclin A was up-regulated at 12 hours and maintained this status through each time point. These pattern changes in cyclins were consistent with the cell cycle block at G1-S phase.

**MMF Induced Apoptosis in Primary Myeloma Cells**

Primary myeloma cells in the control group (no MMF) cocultured with the bone marrow stroma showed 70% cell survival after 3 days of in vitro culture. MMF (1 \( \mu \)mol/L) treated primary myeloma cells showed 15% to 40% more apoptotic cells compared with the control group (\( P < 0.05 \); Fig. 7). In addition to this experiment, we tested a higher concentration of MMF at 5 \( \mu \)mol/L on patient samples.
This concentration was able to inhibit growth of primary myeloma cells at up to 70% growth inhibition compared with the non-MMF-treated control group (data not shown).

**Discussion**

IMPDH inhibitors were initially found to be antineoplastic more than two decades ago (34). Clinical studies involving tiazofurine were conducted in acute and chronic myelogenous leukemia and revealed remarkable clinical and laboratory correlative responses (11, 12, 35). However, the toxicity of the drug prevented its clinical use. MMF has been previously reported to induce leukemia differentiation but not apoptosis. Our study shows for the first time that a clinically available IMPDH inhibitor MMF also has a broad antimyeloma effect, including apoptosis induction and cell cycle arrest in myeloma cell lines and primary myeloma cells.

A significant antimyeloma activity was observed with MMF, showing up to 80% growth inhibition at an *in vitro* drug concentration between 1 and 5 μmol/L. This concentration is a clinically achievable trough level after administration of the drug between 2 and 5 g/d (36, 37). Myeloma cell samples from patients with refractory myeloma cocultured with bone marrow stroma showed a modest response at the 1 μmol/L range, but it increased to 70% with 5 μmol/L MMF. Thus, we hypothesize that higher concentrations of MMF may be active in myeloma cells. Although these primary myeloma cells were derived from heavily treated patients and despite there might be possibly some dose-response relationship, the limited number of patient samples used in this study only can provide preliminary results on the MMF effect on primary myeloma cells.

MMF blocked the pathway involving the IMPDH enzyme in myeloma cells because MMF-induced apoptosis is inhibited with guanosine, which causes the repletion of the GTP pools through a salvage pathway. In addition, IMPDH inhibition by MMF was further confirmed by depletion of the intracellular GTP, dGTP, and XMP levels in myeloma cells treated with MMF. These results indicate that inhibition of *de novo* guanine nucleotide biosynthesis is a crucial step for apoptosis induction in myeloma cells after treatment with MMF.

The time to achieve the maximum apoptosis effect by MMF required 3 to 4 days, which was longer than that of other antimyeloma agents. We hypothesize that the difference was due to the mechanism of the DNA-damaging process as drugs, such as doxorubicin, are a direct DNA-damaging agent. By contrast, nucleotide biosynthesis inhibitors, such as MMF, act more indirectly requiring intracellular depletion of the GTP pool for apoptosis induction. Our experiment using a high-performance liquid chromatography–based assay for intracellular GTP measurement showed that it took 3 to 4 days to have an intracellular GTP drop after MMF treatment. The result was consistent with that done by others using HL60 cells treated with MPA (11), which caused a significant reduction of intracellular pools of GTP after 3 to 4 days of MPA incubation. The delay in apoptosis induction is thought to be strongly associated with the time to deplete intracellular nucleotide levels.

Due to Ishitsuka et al. showing a non–caspase-dependent mechanism of apoptosis in VX-944-treated myeloma cells, we did further experiments to confirm the evidence of a caspase-dependent apoptosis pathway by MMF. In addition to a series of experiments, such as demonstration of mitochondria membrane potential disruption, cytochrome c release, caspase-9, caspase-3, and poly(ADP-ribose) polymerase activation in Results, we treated the cells with pan-caspase inhibitor Z-VAD-fmk at a low dose (10 μmol/L) to block the MMF-induced apoptosis effects.
apoptosis effect. As expected, there was >50% apoptosis inhibition by the caspase inhibitor use compared with the control (data not shown). Furthermore, apoptosis induction caused by MMF was not reversed by adding L-NAC, thus showing that the mechanism of apoptosis is less likely to be associated with mitochondria-derived reactive oxygen species.

We did not fully investigate the MMF effect on IL-6-induced signal transduction cascade in myeloma cells. Previous investigators have shown that IL-6 protects against dexamethasone-induced apoptosis and also showed an up-regulation of Mcl-1 through IL-6 signaling pathways (33, 38–40). However, as we showed in this article, MMF caused decreased phosphorylation of STAT3 (Ser727) in myeloma cell lines. Similarly, down-regulation of phosphorylation of STAT3 (Ser727) was shown in IL-3-dependent murine myeloid and lymphoid cell lines after treatment with MPA (41). The novel IMPDH inhibitor VX-944 did not down-regulate this pathway (15). Phosphorylation of STAT3 (Ser727) is regulated by two major pathways, the mitogen-activated protein kinase pathway and mammalian target of rapamycin pathway (42, 43). In the experiment using IL-3-dependent murine myeloid and lymphoid cell lines after treatment with MPA, the authors found that both mitogen-activated protein kinase pathway and mammalian target of rapamycin pathway inhibition was required to down-regulate phosphorylation of STAT3 (Ser727; ref. 41). We hypothesize that MMF-induced intracellular GTP depletion in myeloma cells may inhibit both upstream pathways, such as mitogen-activated protein kinase and mammalian target of rapamycin pathways, and our future experiments include demonstration of inhibition of these pathways. Another piece of evidence that supports the possibility of MMF to be involved in attenuation of this pathway is that Bcl-xL, which is the known STAT3-regulated gene, was repressed in protein expression levels after MMF treatment. We also did reverse transcription-PCR to measure Bcl-xL mRNA expression and found that it decreased 18 to 24 hours after MMF treatment (data not shown). Bcl-xL is known to form heterodimers with proapoptotic members of the Bcl-2 family and inhibits their apoptotic action (44).

MMF-induced cytotoxicity effect was not affected by overexpression of Bcl-2 or Mcl-1. Bak is known to have functions to promote cell death by neutralizing the antiapoptotic effect of Bcl-2 (45, 46). The present study revealed that MMF caused a marked increased in the level of Bak protein. The attenuation of Bak protein by MMF may be one of the major reasons to induce apoptosis in dexamethasone-resistant Bcl-2 overexpressing myeloma cell line.

Mcl-1 has been shown to be an important survival factor in myeloma cells, and our result with a significant growth inhibition of Mcl-1 overexpressing cell line by MMF suggests that IL-6-induced Mcl-1 up-regulation may be overcome by MMF. Of note, Mcl-1 up-regulation is only

Figure 6. MMF induced p21 protein and caused attenuation of cyclins. Time-dependent effects of MMF on expression of p27 and p21 protein in ARP1 cells. Total lysates from ARP1 cells were prepared before (time 0) and after 6, 12, 24 h of culture with 1 μmol/L MMF. Western blotting was done using anti-p21 and anti-p27 monoclonal antibody as described in Materials and Methods. An equal protein loading was confirmed by Ponceau staining. The effect on cyclin D1, cyclin E, and cyclin A protein by MMF was also confirmed. Total lysates from ARP1 cells were prepared before (time 0) and after 12, 24, 36, and 60 h of culture, and Western blotting was done using anti-cyclin D1, anti-cyclin E, and anti-cyclin A monoclonal antibodies as described in Materials and Methods. Actin was used as a loading control. The band intensity measured by densitometry was shown under each time point.

Figure 7. MMF induced apoptosis in primary myeloma cells. CD138⁺ enriched plasma cells from four patients were treated with 1 μmol/L MMF for 3 d in a human bone marrow stromal cell coculture system as described in Materials and Methods. Cells were harvested after 3 d of culture and analyzed for Annexin V/propidium iodide–based flow cytometry analysis. CD138⁺-enriched cells without MMF treatment but cultured for 3 d were analyzed and calculated as 100% survival (white columns). The viable plasma cells after MMF treatment (black columns) was calculated as % nonapoptotic cells based on the control cell survival as 100% (P < 0.05).
one of several mechanisms by which IL-6 affects myeloma cells. Our data on Mcl-1 protein expression level after MMF treatment did not remarkably depress their levels, but antiapoptotic protein Bcl-xL expression levels decreased significantly. These data raise the possibility that MMF may overcome Mcl-1 overexpression by suppressing antiapoptotic protein levels. Future experiments include measuring Bcl-2 family proteins in Mcl-1 overexpressing cells treated with MMF and to further delineate the association between IL-6 signaling and MMF.

In agreement with these results, MMF resulted in down-regulation of Bcl-xL, up-regulation of Bak, and disruption of mitochondrial membrane potential in myeloma cells, which triggered release of cytochrome c and activation of caspase-3. MMF-induced cell death may at least in part be due to a decrease in Bcl-xL level, especially because Bcl-xL down-regulation preceded caspase-3 cleavage. Thus, it seems reasonable to postulate that Bak and Bax proteins play an important role in regulation of MMF-induced apoptosis.

MMF also induced G1-S phase block in myeloma cells with corresponding cell cycle regulatory protein expression changes consistent with this cell cycle phase block. This result is consistent with the cell cycle analysis in human T-lymphocytic and monocytic cell lines (4) after treatment with MMF. The importance of cell cycle inhibition by MMF is also highlighted by the fact that cell cycle deregulation, in particular of cyclin D1, is thought to be associated with myeloma pathogenesis (47).

We did not test any apoptosis effect of MMF on human T or B cells in vitro, because these findings were published by others (4, 7). MMF effect on T-cell lineage may raise concerns in myeloma patients by causing possible immune suppression or reduction of peripheral blood count. However, we found that these toxicities were rare in myeloma patients treated with MMF in the range of 1 to 5 g/d shown by our phase I dose escalation study (14).

Although we did not test any MMF effect in the setting of combination with other antimonyeloma agents in vitro, to combine different apoptosis inducers, which share the downstream apoptosis cascade with MMF but different upstream cascades at the initiation of apoptosis would be worth testing for activity against myeloma cells. For example, combinations of MMF with tumor necrosis factor–related apoptosis-inducing ligand, Revlimid, Velcade, or suberylanilide hydroxamic acid, which are mainly caspase-8 activators (47), would be interesting to test for an additive or synergistic apoptosis induction.

In summary, we have shown the apoptosis effect of MMF on myeloma cells is caspase dependent and provided the basis for future drug development involving IMPDH inhibition. Although MMF and VX-944 are both IMPDH inhibitors, their chemical structure and the mechanism of apoptosis induction seem to be different. Studies using IMPDH inhibitors in myeloma cells may hold promise as to develop novel antimonyeloma agents.

References
IMP dehydrogenase inhibitor mycophenolate mofetil induces caspase-dependent apoptosis and cell cycle inhibition in multiple myeloma cells

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