Caspase Activation Is Required for Gemcitabine Activity in Multiple Myeloma Cell Lines

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Abstract
The objective of this study was to determine potential mechanisms of apoptotic activity of gemcitabine, a pyrimidine nucleoside analogue, in the MM1.S multiple myeloma (MM) cell line. A MM cell line that is sensitive to glucocorticoids (MM1.S) was used for this study. Immunoblotting analysis, cell cycle assays, and annexin V staining were performed to determine whether gemcitabine induced apoptosis in this model. Furthermore, we attempted to delineate the apoptotic pathway by measuring caspase-8 and -9 activity using fluorometric assays. Loss of mitochondrial membrane potential was measured by flow cytometry. Gemcitabine treatment caused apoptosis in MM cell lines as measured by an increase in DNA cleavage, an increase in annexin V binding, a decrease in the mitochondrial membrane potential, and activation of caspase activity. Furthermore, cleavage of the caspase substrate poly(ADP-ribose) polymerase and caspase-3 activation were documented as early as 8 h after treatment with gemcitabine. Caspase-8 and -9 were activated by gemcitabine treatment in this cell line, suggesting several mechanisms of action including death receptor pathway and mitochondrial damage. The addition of interleukin 6 to MM1.S cells treated with gemcitabine offered no protection against gemcitabine-induced cell death. Gemcitabine induced apoptosis in the MM1.S cell line, and its activity required caspase activation. There is a suggestion that mitochondrial integrity being affected with gemcitabine in this system. Gemcitabine acts independently of interleukin 6, suggesting potential important therapeutic implications in MM patients.

Introduction
MM accounts for 1% of all cancers and 10% of hematological malignancies, with 13,700 new cases diagnosed and >11,000 deaths in 1999–2000 (1). It is a clonal B-cell disorder in which malignant plasma cells accumulate in the bone marrow and produce an immunoglobulin, usually monoclonal IgG or IgA (2). Treatment of MM with conventional chemotherapy is not curative, with a median survival of about 3 years (3). Although high-dose chemotherapy with hematopoietic stem cell support increases the rate of complete remission and event-free survival (4), almost every patient relapses, mandating the crucial need for salvage therapy options (5).

Gemcitabine (2',2'-difluorodeoxycytidine) is a nucleoside analogue with significant activity in solid tumors (6–9) and some efficacy in hematological malignancies (10). The major effect of gemcitabine is directed against DNA synthesis (11) by causing masked chain termination and ribonucleotide reductase inhibition (12–14). Several investigations, including our recent report, have suggested activity of gemcitabine against MM cell lines (15, 16). Comparison of several purine and pyrimidine nucleoside analogues clearly demonstrated that gemcitabine was the most active in MM cell lines, including those resistant to glucocorticoids (16). Although the activity of gemcitabine is established in a variety of MM cell lines, and some aspects of its cytotoxicity have been demonstrated, the apoptotic pathway is not clearly defined. In the present report, we elucidate the role of caspase activation in gemcitabine-induced cytocytotoxicity in MM cell lines. Our data demonstrate that gemcitabine-mediated cell death is due to apoptosis, is independent of IL-6, a known growth factor in this disease (17), and requires caspase activation.

Materials and Methods

Chemicals. Gemcitabine was obtained from Eli Lilly and Co. (Indianapolis, IN). ZVAD-fmk was purchased from Enzyme Systems Products (Livermore, CA). Staurosporine was obtained from Alexis Chemical (San Diego, CA), CMX-Ros was from Molecular Probes (Eugene, OR), whereas caspase-8 and -9 fluorometric kits were purchased from R&D Systems (Minneapolis, MN). IL-6 was purchased from R&D Systems. All other chemicals were obtained from Sigma (St. Louis, MO).

Cell Culture. MM1.S is a glucocorticoid-sensitive cell line established from the peripheral blood of a MM patient (18),
and it was used in our current investigation. Cells were routinely cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were maintained at 37°C in an incubator with 5% CO2.

**Gemcitabine Treatment.** The MM cells were seeded into culture at concentrations that would ensure logarithmic growth over the duration of the experiment. These concentrations are detailed in the individual experiments. The standard treatment of MM cells consists of 100 nM gemcitabine; however, in some experiments, 100 μM gemcitabine was used as specified. The time of treatment was specified in the individual experiments, and the cells were maintained in standard culture conditions over the course of the experiment. To block apoptosis, ZVAD-fmk, a pan-specific caspase inhibitor, was used at 40 μM. As a positive control for apoptosis, cells were treated with staurosporine at 100 nM. Cells were maintained at 37°C in an incubator with 5% CO2.

**Cell Proliferation Assay.** MM cells were cultured into 96-well plates at a concentration of 25,000 cells/well and incubated with the indicated drugs for 72 h. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Cell Titer aqueous assay (Promega, Madison, WI), which measured the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in live cells. The amount of formazan is proportional to the number of living cells present in the assay mixture. Each data point was the average of four independent determinations, and the error bars represented the SD. The data were expressed as the percentage of formazan produced by the cells treated with the control medium in the same assay.

**Immunoblotting Analysis.** MM1.S cells were cultured and grown at a concentration of 10 × 10^6 in 30 ml of tissue culture media in 75-cm² flasks. Cells were treated according to the specific experiment and harvested at the desired time points. Anti-FAS antibody (obtained from Dr. Kenneth Anderson, Boston, MA) was used as a positive control for activation of the death receptor pathway and activation of caspase-8 and -3. Cell pellets were homogenized in lysis buffer [10 mM KPO4 (pH 7.0), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM B-glycerol phosphate, 1 mM sodium orthovanadate, 2 mM DTT, 1 mM phenylmethylsulfonil fluoride, 0.5% NP40, and 0.1% deoxycholate]. Homogenates were centrifuged at 4°C for 10 min at 16,100 × g, and the supernatants were collected and stored at −20°C until time of use. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein, at a concentration of 40 μg, was mixed with sample buffer [125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mM DTT, and 0.05% bromophenol blue] and fractionated on a precast 8–16% Tris-glycine gel (Invitrogen/Novex, Carlsbad, CA). Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). After protein transfer, membranes were blocked with block buffer (5% nonfat milk in PBS) for at least 1 h and subsequently incubated with the appropriate primary antibody at a 1:1000 dilution overnight at 4°C. To detect PARP, a mouse monoclonal antibody was used (PharMingen, San Diego, CA). A polyclonal rabbit antibody (PharMingen) was used to detect caspase-3. The following day, blots were washed with PBS-T (PBS with 0.1% Tween 20) and incubated for 1 h at room temperature with a 1:1000 dilution of horseradish peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL). After washing the blots with PBS-T, they were developed using enhanced chemiluminescence (ECL; Amersham), and the signal was visualized with X-ray film (Hyperfilm; Amersham).

**Cell Cycle Analysis.** Cells were grown in 25-cm² flasks at a concentration of 5 × 10^6 cells/ml and treated with gemcitabine at the concentration indicated in the figure legends. After incubation for 48 h, cells were collected, centrifuged at 500 × g, washed with cold PBS, and fixed with 40% ethanol at 4°C overnight. Cells were then washed with cold PBS, and the pellet was resuspended in 50 μg/ml RNase A (diluted in PBS) and incubated for 30 min at 37°C. The samples were then resuspended in 25 μg/ml PI in 38 mM sodium citrate buffer. Flow cytometry was performed on a Coulter EPICS XL instrument, and data were analyzed using the System II software package.

**Annexin V FITC Assay.** As cells undergo apoptosis, the integrity of the cell membrane is disrupted, and PS is exposed. Using a fluorescent conjugate of annexin V, the presence of PS is detected by annexin V binding, which has a high selective affinity for PS. MM1.S cells were grown in 25-cm² flasks at a concentration of 2.5 × 10^6 cells in 5 ml of media. Cells were treated with gemcitabine at 100 nM, and under some conditions, the cells received 40 μM ZVAD before the addition of gemcitabine. Cells were then harvested, washed twice with cold PBS, and centrifuged. Samples were then resuspended in the diluted binding buffer provided with the kit, and a 100-μl portion of each sample was mixed with 1 μl of annexin solution and 5 μl of PI staining solution. The tubes were then placed on ice and incubated for 10 min in the dark. Samples were analyzed by flow cytometry using Coulter EPICS XL instrument, and data were analyzed using the System II software package.

**Caspase-8 and -9 Fluorometric Assay.** Cells were cultured in 25-cm² flasks with 5 × 10^6 cells/5 ml media. Cells were treated with various drugs at the indicated concentration. After harvesting, cells were washed twice with cold PBS and resuspended with cell lysis buffer following the manufacturer’s protocol. Protein concentrations in all cell lysates were determined using Bio-Rad protein assay (Bio-Rad Laboratories), and 200 μg of total protein were used for each examined sample. Samples were added to the reaction buffer with the appropriate caspase fluorogenic substrate. Reactions were incubated for 2 h at 37°C, and fluorescence was determined using the Packard Fluorocyte fluorometer plate reader that allowed for light excitation at 400 nm wavelength and collected emitted light at a wavelength of 505 nm.

**Mitochondrial Membrane Potential Assay.** Cells were cultured in 25-cm² flasks with 5 × 10^6 cells in 5 ml of media. ZVAD-fmk was added to the appropriate flasks 1 h before drug therapy. Cells were then treated with gemcitabine and
harvested at the indicated time points. Cells were washed with cold PBS, and samples were transferred into two tubes with 1 ml PBS/tube. Samples were incubated with 10 nM CMX-Ros fluorescent dye for 15 min at 37°C and analyzed by flow cytometry as described in “Materials and Methods.” Data are representative of three similar experiments.

Results

Gemcitabine Induces Apoptosis in a Caspase-dependent Manner. MM1.S cells were treated with 100 nM gemcitabine alone or in combination with ZVAD for 48 h, and cell cycle was determined as described in “Materials and Methods.” As shown in Fig. 1, ZVAD alone did not increase the population in the sub-G1 cell cycle. Treatment with 100 nM gemcitabine resulted in an almost 3-fold increase (28.7 ± 1.67%) in cells at G2-M, compared with untreated controls (9.1 ± 0.97%). The combination of gemcitabine and ZVAD resulted in an almost 50% reduction in cells at the sub-G1 phase, suggesting that caspase activation is involved in the mechanism of gemcitabine-induced apoptosis in this cell line.

The distribution of cells in different phases of cell cycle was most perturbed in S and G2-M phase after gemcitabine treatment. Untreated cells and the ZVAD-treated cell population had about 18–20% cells in S and G2-M phases. Cells treated with gemcitabine had a significant reduction in these two phases of the population. Again, this reduction was affected by ZVAD treatment. These results indicate that gemcitabine treatment reduces the cell population in S phase and concomitantly increases the sub-G1 population, indicating that cells are undergoing apoptosis. This process was inhibited by the pan-specific caspase inhibitor ZVAD, suggesting the requirement for caspase activation.

Gemcitabine Promotes PS on Cell Surface. Cells treated with 100 nM gemcitabine for 48 h showed a >6-fold increase in annexin binding compared with untreated controls (41.5 ± 3.25% compared with 6.8 ± 0.28%). Pretreatment with ZVAD did not affect the pattern of annexin binding, suggesting that gemcitabine-induced PS exposure occurs upstream of caspase activation. Annexin uptake was also apparent after 24 h of gemcitabine treatment, albeit with less intensity (data not shown).

Gemcitabine Promotes Activation of Caspase-3. PARP is a 116,000 protein that is a substrate for activated caspase-3 (19). The cleaved form of PARP is detected at M, 85,000 by immunoblotting. Cells were treated with gemcitabine for the indicated times and harvested, and whole cell extracts were prepared, fractionated, and immunoblotted as described. Anti-Fas antibody was used as a positive control because activation of the death receptor pathway results in cleavage of the caspase cascade and subsequent PARP cleavage. As shown in Fig. 2A, immunoblots of whole cell lysates of MM1.S cells treated with gemcitabine for 0, 4, 8, 12, 18, or 24 h showed PARP cleavage as early as 4 h of incubation. The presence of cleaved PARP appears to increase over time, with a substantial amount of cleavage by 18 and 24 h of incubation. This was accompanied by a decrease in the detection of the uncleaved form of PARP.

The activation of caspase-3 from its pro-form (M, 32,000) to its active form (M, 19,000) was also measured by immunoblotting (Fig. 2B). Although PARP cleavage implies activation of effector caspase-3, these studies validate that caspase-3 was activated. Cells were treated with 100 nM gemcitabine for the indicated times, and anti-Fas antibody (at 6 h) treatment was used as a positive control for the induction of caspase-3. The cleaved form of caspase-3 was evident by 4 h of incubation with gemcitabine, indicating increased caspase-3 activity. Together, these results suggest that gemcitabine is activating apoptosis as evidenced by an increase in PS on the cell surface, activation of caspase-3, and cleavage of PARP and an increase in cells with a sub-G1 content of DNA resulting from nucleosomal...
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Mitochondrial membrane potential, a phenomenon associated with activation of apoptotic pathways. Gemcitabine is able to dissipate the inner mitochondrial membrane potential compared with 15.4 ± 3.96% of cells, which was used as a positive control.

Gemcitabine Promotes Mitochondrial Damage. In apoptosis, there are two classical pathways that lead to activation of effector caspases such as caspase-3. The first pathway involves activation of the death receptor, leading to cleavage and activation of caspase-8. The other pathway is initiated by mitochondrial injury, leading to cytochrome c release that interacts with pro-caspase-9 and APAF-1 (an apoptotic protease-activating factor) forming the apoptosis signaling complex and causing activation of caspase-9 (20). We first examined whether gemcitabine was causing changes to the mitochondria because many chemotherapeutic drugs appear to initiate apoptosis through the mitochondria.

The loss of the mitochondrial membrane potential (ΔΨₘ) occurs in cells undergoing apoptosis. CMX-Ros is a cationic fluorescent dye that associates with the inner membrane of active mitochondria by charge. This is detected as a high fluorescence signal by flow cytometry. When the mitochondria are injured, the charge across the membrane dissipates, and this decrease in the ΔΨₘ is measured by the inability of the dye to associate with the membrane and a shift in the fluorescence to a lower peak. After treatment with the maximally effective concentration of gemcitabine, MM.1S cells were analyzed by flow cytometry to determine the percentage of cells exhibiting either high or low fluorescence with CMX-Ros staining, and these data are represented in Fig. 3. Staurosporine treatment (100 nM) was included as a positive control as described in “Materials and Methods.”

Gemcitabine administration resulted in an increase in cells with decreased ΔΨₘ, as seen by an increase in the number of cells with a low CMX-Ros signal. The results were accentuated after 48 h of treatment, when 59.3 ± 3.96% of cells exhibited a loss in the inner mitochondrial membrane potential compared with 15.4 ± 9.06% in untreated controls. This indicated that gemcitabine is able to dissipate the inner mitochondrial membrane potential, a phenomenon associated with activation of apoptotic pathways.

Fig. 3. Gemcitabine causes disruption of the inner mitochondrial membrane at 48 h. The disruption of the mitochondrial membrane causes less CMX-Ros uptake. Data are representative of three similar experiments and are expressed as mean ± SD. The dark bars represent cells with high CMX-Ros uptake, and the light gray bars represent cells with low uptake. STS (100 nm) refers to staurosporine, which was used as a positive control.

Fig. 4. Time-dependent activation of caspases by gemcitabine. Increase in fluorometric activity of caspase-8 (A) and caspase-9 (B) was measured after gemcitabine treatment. Cells were treated with 100 nm gemcitabine for 4, 8, 12, 18, 24, and 48 h. Activity of caspase-8 and -9 was measured as described in “Materials and Methods.” Data are representative of three independent experiments. Error bars, SD.

Gemcitabine Promotes Activation of Both Caspase-8 and -9. We next measured which initiator caspases are activated to determine whether the death receptor pathway (caspase-8 activation) or the mitochondrial injury pathway (caspase-9 activation) is activated by gemcitabine. Fluorometric assays were used to detect the enzymatic activity of caspase-8 and -9. This assay uses a caspase-specific peptide substrate that is conjugated to 7-amino-4-trifluoromethyl coumarin; a fluorescent reporter molecule. When the peptide binds to active caspases, the fluorescent molecule is released by cleavage, and the amount of fluorescence is quantified. In Fig. 4, cells were treated with gemcitabine at 100 nm for the indicated time points and then harvested and assayed as described in “Materials and Methods.” Treatment of MM1.S cells with gemcitabine resulted in activation of both caspase-8 and -9. Cells showed increased activity of caspase-8, which was substantial at 48 h of incubation. This activity was more than 10-fold that of untreated controls (66,205 ± 5,366 fluorescent units in treated cells versus 6,599 ± 1,086 fluorescent units in untreated controls; Fig. 4A). Caspase-9 was also activated by the administration of gemcitabine to these cells. It appears that the activity increases with time of incubation. The maximum is seen at 48 h (40,000 ± 3,033 fluorescent units in treated cells versus 3,951 ± 898.73 fluorescent units in untreated cells; Fig. 4B).

To confirm these results and ensure that we were detecting caspase activity, ZVAD was added to select samples as
hematological malignancies (29–28). Several studies have suggested activity for this agent in MM cell lines, including those that are resistant to traditional therapies (16). Here, we have investigated the pathway by which gemcitabine caused apoptosis in the MM.1S cell line, showing that caspase activation is required for this activity.

The increase in sub-G1 cell population documented in our cell cycle analysis suggested that apoptosis is induced by this agent. Adding a caspase inhibitor to the cell culture partially prevented the gemcitabine-induced apoptotic process, suggesting that caspase activation is required. Caspases are cysteine proteases that cleave substrates on the carboxyl side of the aspartate residue (35). To be activated, caspases undergo proteolytic cleavage that removes a pro-domain that differs in length between different caspases (36, 37). Once activated, initiator caspases can activate effector caspases, eventually causing cleavage of PARP and cell death. Immunoblotting revealed PARP cleavage as early as 4 h after gemcitabine treatment of the MM.1S cell line. Consistent with PARP cleavage, caspase-3 activation was witnessed in the same time-dependent manner. Additionally, annexin V binding to PS on cells treated with gemcitabine confirmed that these cells are undergoing programmed cell death. Together, these results demonstrate that gemcitabine induces apoptosis in the MM1.S cell line.

Apoptotic pathways can be divided into those that involve death receptor signaling and caspase-8 activation and those that involve mitochondrial damage and caspase-9 activation (19). In this report we investigated both pathways using the ability of each caspase to cleave a specific substrate bound to a fluorescent compound. Increased caspase activity is detected by increased fluorescence as the fluorescent tag is released by cleavage. Our results show that both caspase-8 and -9 are activated. Adding a caspase inhibitor abrogated this activity, suggesting that gemcitabine affects both pathways.

Mitochondria are involved in the apoptotic process by releasing cytochrome c that binds to APAF-1 and pro-caspase-9 forming the apoptosome (38). Because we have shown that caspase-9 is activated in gemcitabine-treated cells, we studied potential mitochondrial injury in our system. We have demonstrated that gemcitabine causes disruption in $\Delta \Psi_m$ as reflected by diminished binding of a cationic dye (CMX-Ros). The maximal effect occurs at 48 h after drug treatment. Studies to investigate and delineate the exact mitochondrial damage with gemcitabine are being initiated in our laboratory.

Because mitochondrial damage is noted at 48 h after gemcitabine treatment, and caspase-3, -8, and -9 cleavage is demonstrated as early as 8 h, it is possible that cell death rather than gemcitabine itself is causing the loss in mitochondrial membrane potential. Other time points were tested to detect whether mitochondrial damage occurs early in the course of the signaling pathway, but the most activity was demonstrated at 48 h as shown in Fig. 3. It is likely that gemcitabine causes caspase activation and subsequently causes cell death, resulting in mitochondrial injury.

The efficacy of gemcitabine in the MM1.S cell line makes this agent an attractive choice for patients who failed prior therapies. In our investigation, we show that the actions of gemcitabine are independent of IL-6 in vitro, suggesting that

**Fig. 5.** Cell proliferation assay showing that IL-6 does not prevent gemcitabine-induced cell death in the MM.1S cell line. The light gray bars indicate cells treated with no drug, 100 nm gemcitabine, or 100 μM gemcitabine as indicated on the X axis. The dark bars indicate cells with the addition of IL-6 (500 pg/ml). The response in untreated control cells is set at 100%, and the response of the gemcitabine-treated cells was compared with that of the untreated cells. Data are representative of two similar experiments and are expressed as mean ± SD.

Discussion

Gemcitabine is a pyrimidine nucleoside analogue that has shown significant activity in many solid organ cancers (25–28). Several studies have suggested activity for this agent in hematological malignancies (29–31). Nucleoside analogues such as cytarabine, cladribine, and fludarabine were studied in MM patients but showed no activity (32–34). Our investigation suggests a potential effect for gemcitabine in the MM.1S cell line. A previous report by our group showed that gemcitabine is more potent than other nucleoside analogues in MM cell lines and that its activity is manifested in various ways.

**Gemmicitabine Acts Independently of IL-6.** IL-6 is a cytokine that has pleiotropic effects on hematopoietic and non-hematopoietic cells (21, 22). IL-6 induces purified B cells to differentiate into Ig-secreting plasma cells. It acts as a growth factor for MM, mediating the expansion of plasma-blastic cells. IL-6 supports the survival and/or expansion of MM cells not only by stimulating cell division but also by preventing apoptosis (23). Because IL-6 is an important growth factor for MM cells, it is essential to investigate whether the addition of IL-6 would affect gemcitabine-induced apoptosis in MM1.S cell line.

Cells were treated with gemcitabine (either 100 nm or 100 μM), with and without IL-6 at a total concentration of 500 pg/ml. Gemcitabine-induced cell death, which was detected using a MTS assay, was not affected by the addition of IL-6 (Fig. 5). We have shown previously that IL-6 partially inhibits dexamethasone-induced cell death in the MM1.S cell line (24). The data here suggest that gemcitabine, contrary to dexamethasone, acts independently of IL-6, making this agent an important therapeutic option for patients with MM.
its pathway bypasses IL-6 and therefore might have important therapeutic implications. There have been two small trials evaluating gemcitabine as a single agent in MM (39, 40) and one study with gemcitabine in combination with paclitaxel (41). In the latter study, reported by Gazitt et al. (41), nine patients were enrolled, seven of whom were evaluable. Four patients had a partial response, two patients had a minimal response, and one patient was without response. It is difficult to speculate how active gemcitabine was in this regimen because it was studied in combination therapy, but our data suggest that this agent should be active and warrant evaluation in the context of a well-designed clinical trial as a single agent.

In a recently published clinical trial with gemcitabine as a single agent for the treatment of MM (39), 29 heavily pretreated, elderly patients with MM received gemcitabine at 1000 mg/m² over a 30-min infusion. Of the 23 evaluable patients, 16 (70%) had stable disease, demonstrating that gemcitabine is active in this disease. In addition, the hematological toxicity reported in this study resulted in many patients not receiving all of the courses, suggesting that growth factors or other supports to control the hematological toxicity may improve the quality or quantity of the response rates. In a clinical investigation reported by Offidani et al. (40), 16 heavily pretreated MM patients received gemcitabine at 1250 mg/m² over 30 min. After three courses of gemcitabine as a single agent, the response rate was 31% (one complete remission, one partial remission, and three minimal responses). In addition to these responses, an additional eight patients (50%) had stable disease. Overall, in this clinical trial, 81% of heavily pretreated patients with MM responded to single agent gemcitabine. Taken together, these two clinical studies provide sufficient evidence for the efficacy of gemcitabine in MM patients to warrant further investigation into the actions of this drug.

Both these trials used gemcitabine on a 30-min infusion schedule. However, it has previously been demonstrated that the rate of gemcitabine triphosphate accumulation is saturated at a dose-rate of 10 mg/m²/min (42). Hence, consideration should be given to administering gemcitabine at this pharmacologically guided fixed dose-rate schedule. The toxicity could also have been controlled by infusing gemcitabine at a different dose-rate to more favorable patients with MM to fully evaluate the efficacy of this drug. Based on these considerations, our present observations, and previous clinical trials, a Phase II study has been initiated to test fixed dose-rate gemcitabine as a single agent for treatment of MM.

References


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