Promising Preclinical Activity of 2-Methoxyestradiol in Multiple Myeloma[1]

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ABSTRACT

Purpose: **2-Methoxyestradiol (2ME2), a natural endogenous product of estradiol metabolism, has demonstrated activity against tumor cell lines and can inhibit angiogenesis. There are limited treatment options for patients with multiple myeloma (MM) who relapse after high-dose therapy and stem cell transplantation. We studied the preclinical activity of 2ME2 as a therapeutic agent for myeloma.**

Experimental Design: **Five established myeloma cell lines as well as primary plasma cells from patients with MM were exposed to 2ME2 at various concentrations. We evaluated the activity of the drug to inhibit cell replication and induction of apoptosis *in vitro* as well as the ability of the drug to inhibit myeloma tumor xenograft growth in severe combined immunodeficient mice.**

Results: **2ME2 inhibited tritiated thymidine uptake in all myeloma cell lines tested in a dose-dependent fashion and induced G_2-M phase cell cycle arrest. The drug induced apoptosis in all cell lines tested and in half of the primary plasma cells evaluated in a dose-response manner. Forty-eight h after drug exposure, a large proportion of the cells were dead by propidium iodide staining. Injection of the drug i.p. suppressed myeloma tumor xenograft growth, and the effect was sustained after cessation of therapy.**

Conclusions: **2ME2 has significant activity against myeloma cell lines and primary myeloma cells both *in vitro* and in an animal model. Clinical trials are required to evaluate its activity in patients with MM.**

INTRODUCTION

MM[3] is a malignant neoplasm of terminally differentiated plasma cells residing in the bone marrow. In 2002, approximately 14,600 new cases of myeloma will be diagnosed in the United States, and over 10,800 patients will die of the disease (1). Current treatment strategies including alkylating agents, corticosteroids, stem cell transplantation, and thalidomide are not curative, and the median survival is approximately 3–4 years (2–4); therefore, there is a need for new agents to combat this disease.

2ME2 is a natural metabolite of the endogenous estrogens 17β-estradiol and 2-hydroxyestradiol (5). It has shown promising antitumor effects in preclinical studies (6, 7). 2ME2 directly induces tumor cell apoptosis, in addition to its potent antiangiogenic properties (6–9). Because angiogenesis is increased in myeloma (10, 11), and given the unique antitumor and antiangiogenic properties of 2ME2, we hypothesized that the agent may be of therapeutic benefit in this disease. In this study, we report the results of our laboratory studies with 2ME2 against a variety of myeloma cell lines and primary cells *in vitro*. In addition, we also studied its effects on myeloma xenograft growth in a mouse model.

MATERIALS AND METHODS

2ME2 and Controls. 2ME2 (a generous gift of Entremed, Rockville, MD) was dissolved in DMSO as a 30 mM stock solution and diluted in S-10 (RPMI 1640, 10% FCS, penicillin/streptomycin, and L-glutamine) for the *in vitro* experiments. DMSO control was used for the *in vitro* studies. The solubility of 2ME2 in water is very low. To ensure uniform absorption of the drug, a liposomal preparation of 2ME2 dissolved in 1,2-dioleoyl-SN-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL), a gift from Glenn Swartz (Entremed), was used for the *in vivo* xenograft experiments. Empty liposomes and untreated animals were used as controls for the xenograft studies. In cell culture media, the drug is not free but is complexed with serum proteins that enhance its solubility, and hence a liposomal preparation was not needed.

Cell Lines. The MM cell lines KAS-6/1, ANBL6 and ARH9 were a generous gift of Diane F Jelinek (Mayo Clinic, Rochester, MN). RMPI 8226 and My-5 were purchased from the American Type Culture Collection. RMPI 8226, My-5, and ARH9 were maintained in S-10 medium. KAS-6/1 and ANBL6 were maintained in S-10 supplemented with interleukin 6 (R&D, Minneapolis, MN) at a concentration of 1 ng/ml.

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[3] The abbreviations used are: MM, multiple myeloma; 2ME2, 2-methoxyestradiol; SCID, severe combined immunodeficient; 7-AAD, 7-amino actinomycin D; PI, propidium iodide.

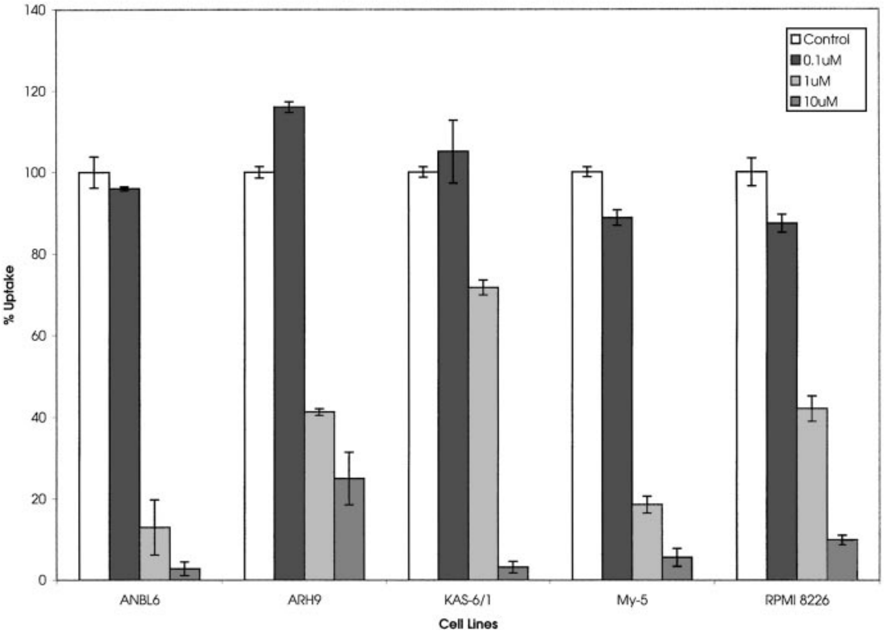

Fig. 1 Relative *in vitro* tritiated thymidine uptake of myeloma cell lines in the presence of various concentrations of 2ME2, expressed as a percentage of untreated control. Cell lines were incubated in S-10 without and with 2ME2 at 0.1, 1, and 10 μM. There was a dose-dependent decrease in thymidine uptake in all cell lines tested. The absolute values of incorporated thymidine (radioactivity cpm) in the control cells were as follows: ANBL6, 66,400; ARH9, 61,800; KAS-6/1, 81,000; My-5, 53,000; and RPMI 8226, 97,600.

Measurement of Apoptosis on Patient Marrow Plasma Cells. Apoptosis was detected as described previously (12). In brief, bone marrow samples were obtained from waste bone marrow aspirates taken from patients with myeloma. All patients provided signed informed consent for the research use of the waste marrow, and this study was approved by the Institutional Review Board of the Mayo Clinic/Foundation. RBCs in the bone marrow were lysed with ACK (0.155 M NH_4Cl and 0.1 M $KHCO_3$), and the nucleated cells were washed twice in PBS and cultured in S-10 with and without 2ME2 at 37°C. After 48 h, cells were washed with PBS and stained with the monoclonal antibodies CD45-FITC (Becton Dickinson) and CD38 phycoerythrin (CD38-PE; Becton Dickinson, San Jose, CA). To differentiate viable, apoptotic, and dead cells, the DNA dye 7-AAD (Calbiochem) was also used as the third color. Samples were analyzed on a FACScan (Becton Dickinson) within 30 min of staining.

Cell Line Viability and Apoptosis Assays. A two-color flow cytometry assay using annexin V-FITC (Caltag, Burlingame, CA) and PI (Sigma) was used to differentiate viable, apoptotic, and dead cells after 48 h of incubation with 2ME2. Briefly, 1×10^6 cells were taken from each well, washed twice in PBS, and resuspended in 100 μl of annexin V binding buffer (Ca^{2+}). Five μl of annexin V were added and incubated at 4°C for 15 min. Samples were then washed in 3 ml of binding buffer, resuspended in 500 μl of binding buffer with 5 μg/ml PI, and then analyzed on a flow cytometer within 30 min. Cells that take up PI are dead, whereas cells that are positive for annexin V but negative for PI have undergone apoptosis. The flow cytometry data were analyzed using the Cell Quest software package (Becton Dickinson).

Cell Proliferation Assays. Cell proliferation assays were performed using tritiated thymidine uptake. Cells (50,000) were seeded in triplicate in 96-well plates, and 2.5 μCi of tritiated thymidine were added followed by incubation at 37°C for 4 h. The cells were then washed, and retained activity was measured using a beta counter (Beckman, Fullerton, CA).

Cell Cycle Analysis. Cells (1×10^6) were washed twice in PBS, resuspended in 2 ml of lysolecithin, and incubated on ice for 30 min. The cells were washed again twice in PBS and resuspended in 0.5 ml of PBS with RNase (30 units/ml). After a 30-min incubation at 37°C, 10 μl of PI (1 mg/ml) were added, and the cells were stored at 4°C and run on a flow cytometer (Becton Dickinson).

***In Vivo* Studies.** Four-week-old female SCID mice (CB17) were purchased from Harlan Sprague Dawley (Madison, WI). The mice were maintained in a pathogen-free environment at the institutional animal care facility and cared for using accepted standards. The mice were given 250 cGy of total body irradiation, and 24 h later, the mice received injection of 1×10^7 KAS-6/1 cells in the right flank. The cells were counted and assessed for viability by trypan blue exclusion and washed three times in PBS (Life Technologies, Inc.) before injection. Two weeks after implantation of the cells, therapy was initiated. One group of mice did not receive any therapy (control group), the second group was injected i.p. with empty liposomes, and the third group was treated i.p. with liposomal 2ME2 at a dose of 150 mg/kg. This dose was based on studies performed at Entremed and represented a well-tolerated dose that showed efficacy in other tumor models; there are no reported maximum tolerated dose or LD_{10} for this drug.[4] The mice were treated with the drug daily for 2 weeks and observed for another 3 weeks after cessation of therapy. Tumor xenografts were measured in two dimensions, three times a week, using calipers. Tumor volumes were calculated using the following formula: vol-

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[4] V. Pribluda and G. Swartz, personal communication.

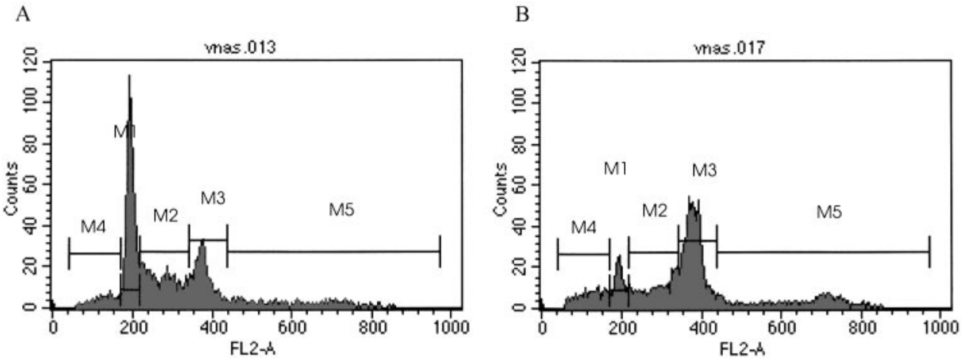

Fig. 2 Cell cycle analysis of ANBL6 cells in the absence (*A*) and presence (*B*) of 2ME2. The results shown are for ANBL6 cells after exposure to 10 μM 2ME2 for 24 h. The cells were stained with PI as described in "Materials and Methods." The *M1* peak represents cells in G_0-G_1, the *M3* peak represents cells in G_2-M, and *M4* represents cells in the sub-G_1 phase of the cell cycle. The number of cells in M4 doubled in the presence of 2ME2.

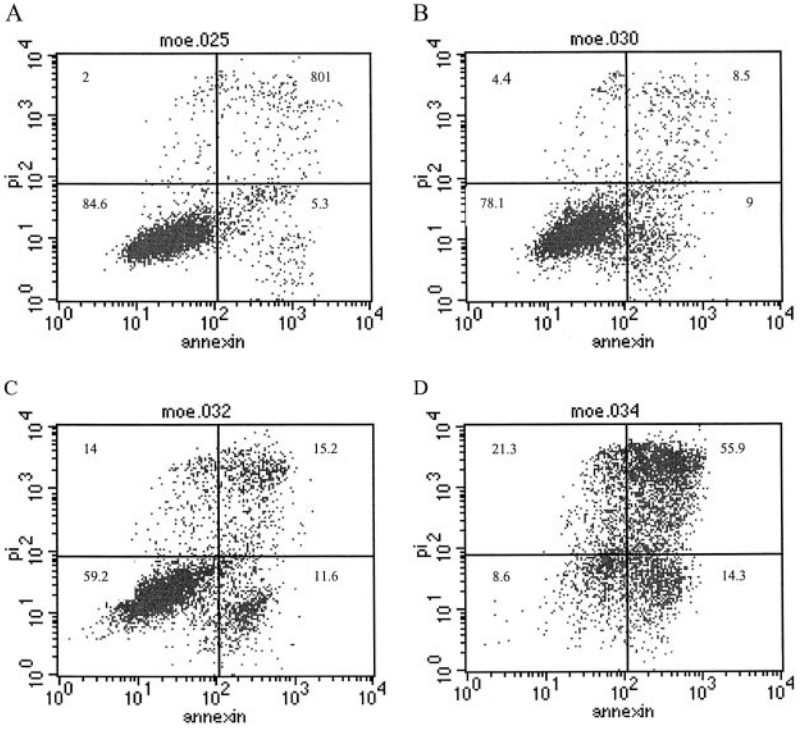

Fig. 3 Flow cytometry analysis of KAS-6/1 cells after 48 h of exposure to 2ME2 (*A,* no drug; *B,* 0.1 μM; *C,* 1 μM; and *D,* 10 μM). Annexin V-positive cells are undergoing apoptosis, whereas PI-positive cells are dead. There is an increase in the number of cells undergoing apoptosis and death with increasing 2ME2 concentrations. *Numbers* represent the percentage of cells in each quadrant.

ume = $(a^2b)/2$, where a is the smaller diameter. The protocol was approved by the Institutional Animal Care and Use Committee at Mayo Foundation in accordance with federal regulations.

RESULTS

2ME2 Inhibits Myeloma Cell Proliferation and Induces G_2-M Arrest. Five different myeloma cell lines (ANBL6, ARH9, KAS-6/1, My-5, and RPMI 8226) were incubated in growth medium with the addition of 2ME2 at three different concentrations (0.1, 1, and 10 μM). Tritiated thymidine uptake was measured as a marker of DNA replication and cell proliferation. 2ME2 inhibited myeloma cell proliferation in a dose-dependent manner in all cell lines tested (Fig. 1). Exposure of plasma cell lines to 2ME2 resulted in cell cycle arrest at the G_2-M phase (Fig. 2, *M3*; 18–38.6%) with a concomitant decrease in the number of cells in G_0-G_1 phase (Fig. 2, *M1*; 36–9.4%). As expected, the number of cells in the sub-G_1 phase increased from 5.6% to 10% (Fig. 2, *M4*).

2ME2 Induces Myeloma Cell Apoptosis. 2ME2 induced a dose-dependent increase in the number of cells undergoing apoptosis in three (ANBL6, KAS-6/1, and My-5) of the five cell lines tested (Figs. 3 and 4). Accompanying this effect on apoptosis (annexin V positive, PI negative), there was an increase in the number of dead cells (PI positive) in all cell lines tested. The proportion of dead cells increased with higher concentrations of 2ME2, suggesting a drug-induced effect (Fig. 5).

2ME2 Induces Apoptosis in Primary Myeloma Cells. Plasma cells are identified by their strong CD38 expression and dim to absent CD45 expression (Fig. 6; Ref. 12). This method identifies cells that are in excess of 95% pure myeloma cells. These cells were selected and analyzed for 7-AAD positivity as a measure of apoptosis (12). Apoptotic cells are permeable to

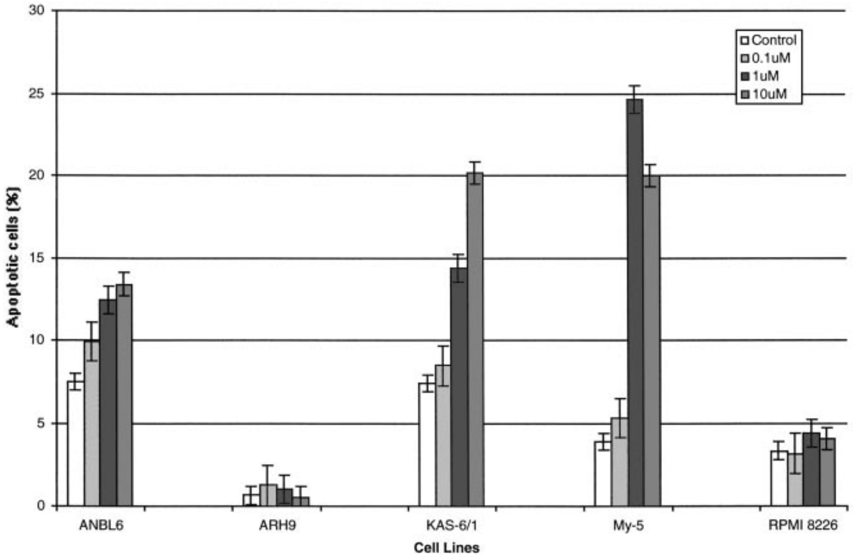

Fig. 4 Fraction of cells undergoing apoptosis after exposure to 2ME2. The cell lines were incubated with 2ME2 at the indicated concentrations for 48 h and analyzed for annexin V as described in "Materials and Methods.: The drug induced apoptosis in three of the cell lines in a dose-dependent manner.

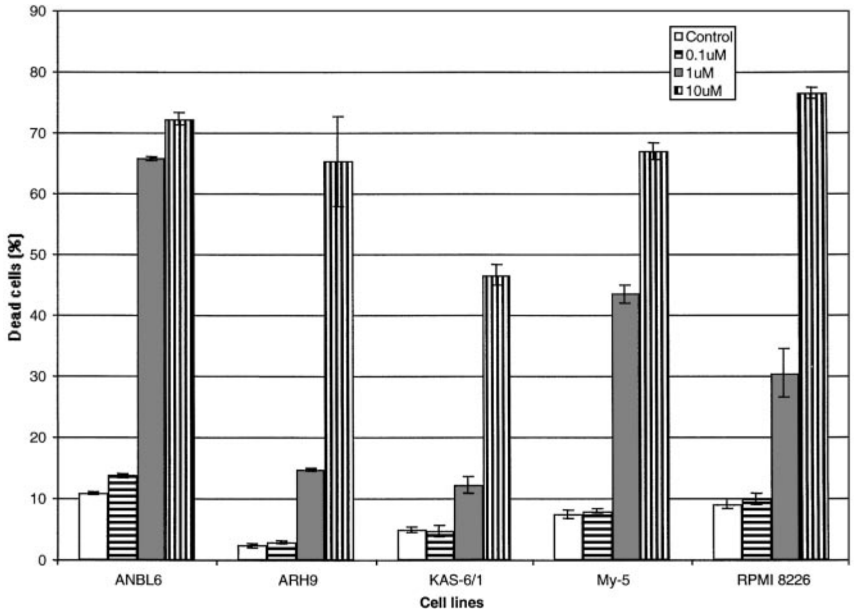

Fig. 5 2ME2 induced cell death in all cell lines tested. The fraction of cells dead by PI (including annexin V-positive cells) staining increased with higher concentrations of 2ME2.

7-AAD, allowing it to bind to DNA. In two of the patient samples (patients 1 and 4), there was a dose-dependent increase in the number of cells undergoing apoptosis compared with controls (Fig. 7). However, in two of the primary myeloma cell samples tested, 2ME2 inhibited apoptosis. Thus, it appears that, similar to other drugs used for myeloma, only a fraction of the samples may be sensitive to the drug.

2ME2 Inhibits Growth of Myeloma Tumor Xenografts in SCID Mice. Measurable tumor xenografts developed in all mice (30 mice; 10 mice/group). All tumors were measured in two dimensions three times a week, and tumor volumes were estimated as described in "Materials and Methods." 2ME2 induced a rapid and complete regression of tumor growth, whereas the tumors continued to grow in both the control and placebo-treated mice (Fig. 8). All of the mice had a postmortem looking for small foci of disease, and there were none in the treated group. Mice treated with 2ME2 developed weight loss after 2 weeks of therapy; therefore, 2ME2 was discontinued, and all mice were observed. The mice regained the weight without the reappearance of the tumors during the remaining observation period.

DISCUSSION

The mainstay of therapy for MM for many years has been a combination of melphalan and prednisone, which produces a response rate of 50% (13). However, complete responses are

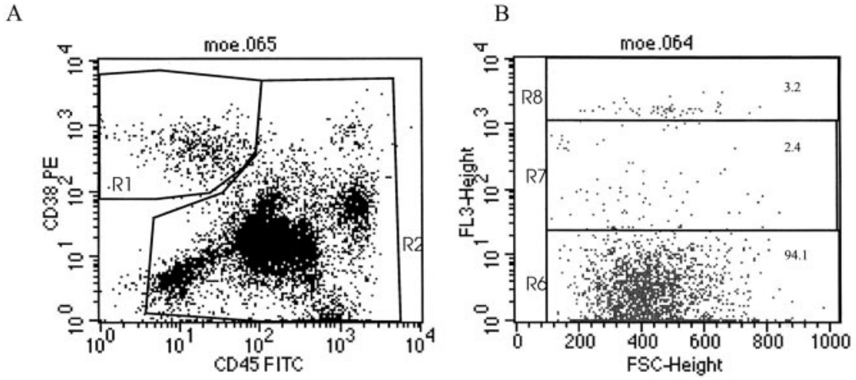

Fig. 6 Flow cytometry of bone marrow samples after RBC lysis. Plasma cells are identified by their bright CD38 expression and dim to absent CD45 expression (*A*). The gated cells are then analyzed for apoptosis by 7-AAD staining and appear in region 7 (*B*). Data shown are from patient 1 at 48 h, with no drug. *Numbers* represent the percentage of cells in each quadrant.

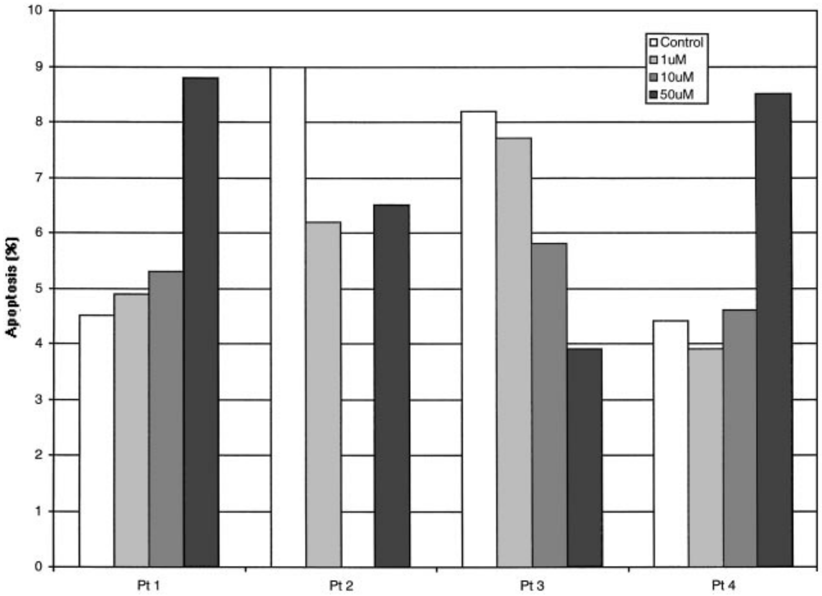

Fig. 7 Drug-induced apoptosis in primary plasma cells from patients with MM. Bone marrow samples were lysed with ACK (0.155 M NH_4Cl and 0.1 M $KHCO_3$) and cultured in S-10 with or without 2ME2. After 48 h, samples were analyzed as described in the Fig. 6 legend, and 2ME2 induced apoptosis in two of the patient samples.

uncommon (<10%), and only one-fourth of patients treated with this combination survive up to 5 years (13). Recently, many centers have been offering high-dose therapy with autologous stem cell transplantation to patients with MM. High-dose therapy produces response rates of 75–90% (14, 15) with complete responses in 20–40% and a 5-year survival of 52% in one series (16). However, transplantation does not cure the disease, and the survival curves do not achieve a plateau. Patients who relapse after high-dose therapy can be treated with thalidomide, and approximately 25–35% of patients will respond (17).

2ME2, a natural endogenous product of estradiol metabolism, has been shown to have activity against tumor cell lines in preclinical models (6). In this study, 2ME2 induced apoptosis and cell death in all myeloma cell lines tested and had significant activity against primary plasma cells in two of the four patient samples studied. This compares favorably with other agents currently used for therapy of myeloma. Other studies have shown that 2ME2 induced apoptosis both by stabilizing wild-type p53 and by inducing phosphorylation of Bcl-2 leading to its inactivation (18, 19). However, these mechanisms are not universal in all cells assessed (6).

In our *in vitro* studies, the percentage of myeloma cells undergoing apoptosis after a 48-h incubation with 2ME2 was relatively small (<25%) compared with what has been observed with pancreatic cancer cell lines (30–90%; Ref. 20). This difference may be due to differences in sensitivity to 2ME2-induced apoptosis between the cell lines. However, myeloma cell viability was low at 48 h. Our studies show that 2ME2 also inhibits myeloma cell proliferation by inducing cell cycle arrest at the G_2-M phase. This supports similar observations with the drug against pancreatic carcinoma cell lines, prostate cancer cells, and lymphoblastic cell lines (20–22). In addition, 2ME2 has been shown to arrest cells in mitosis without inhibiting tubulin depolymerization (23). Our data are in keeping with other reports that 2ME2 blocks the cell cycle and induces apoptosis (22–26). The concentrations of 2ME2 that are required to effect apoptosis are much higher in patient samples compared with myeloma cell lines, probably because the latter

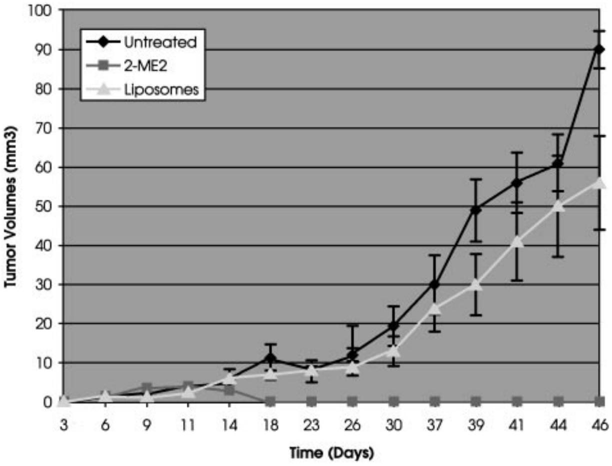

Fig. 8 Plot of tumor volumes in three groups of mice implanted with the KAS-6/1 cell line and treated with liposomal 2ME2 or empty liposomes or controls. The mice were sacrificed at day 46.

proliferate slowly and are typically resistant to most forms of therapy compared with cell lines. However, given the anticipated low toxicity of 2ME2, we fully expect to achieve adequate tissue concentrations in clinical trials.

Finally, our *in vivo* studies show that 2ME2 can suppress myeloma xenograft growth in SCID mice. 2ME2 was reasonably tolerated by the mice. Transient weight loss was observed, but this resolved rapidly with cessation of therapy. However, the tumors did not reappear, suggesting that the effect of the drug may be prolonged even after therapy is stopped.

Angiogenesis is essential for tumor growth because tumor cells require a constant supply of nutrients and growth factors to sustain their growth. Indeed, without new vessel formation, tumors cannot reach diameters beyond 1 mm because diffusion will not be able to keep up with their demands for nutrients (11, 24). Angiogenesis is increased in myeloma, and bone marrow microvessel density correlates with prognosis. Patients with lower levels of new vessel formation have an improved survival compared with those with higher levels of new vessel formation (25). Treatment of patients with MM using high-dose therapy does not lead to a significant change in marrow microvessel density, and this may possibly be a factor in relapse of the disease (26). A number of studies have shown that 2ME2 can inhibit angiogenesis (8, 27–29). The postulated mechanisms include inhibition of endothelial cell proliferation and migration (8), induction of endothelial cell apoptosis (28), and down-regulation of vascular endothelial growth factor expression (29). Vascular endothelial growth factor is a survival factor for myeloma cells; therefore, its down-regulation could add to the effects of the drug against myeloma cells (30). Targeting blood vessels in myeloma may lead to improved responses or decreased risk of relapse after high-dose therapy and stem cell transplantation. The antiangiogenic properties of 2ME2 and the importance of angiogenesis in myeloma provide additional rationale for the study of 2ME2 in myeloma. However, we have no data indicating that 2ME2 inhibits myeloma angiogenesis at present, and we are planning such studies in the context of clinical trials.

Recently, Phase I clinical trials with 2ME2 have been conducted in breast and prostate cancer. Based on the preclinical activity seen in this study, the antiangiogenic properties of the drug, and the limited options available to patients with advanced myeloma, we believe translational clinical trials to test the efficacy of 2ME2 are warranted in the disease. We have thus initiated a multi-institutional Phase II clinical trial with 2ME2 for patients with relapsed and plateau-phase myeloma, and accrual to this study is ongoing.

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