Investigation of antitumor effects of synthetic epothilone analogs in human myeloma models in vitro and in vivo

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26-Trifluoro-(E)-9,10-dehydro-12,13-desoxyepothilone B [Fludelone (Flu)] has shown broad antitumor activity in solid tumor models. In the present study, we showed, in vitro, that Flu significantly inhibited multiple myeloma (MM) cell proliferation (with 1–15 nM IC50), whereas normal human bone marrow stromal cells significantly inhibited multiple myeloma (MM) cell proliferation (with Flu) (12). In mice, Flu appears to have a particularly broad therapeutic index in vitro. It seems to be curative over a range of tumor types, without tumor relapse upon suspension of treatment. Moreover, Flu is orally available and is completely curative against human tumor xenografts by parenteral and oral therapy. In addition, treatment with Flu gave complete tumor remission against Taxol-resistant tumors (10, 12). These very promising antitumor data were obtained in s.c. solid tumor models; however, drug efficacy in orthotopic models has not been investigated. In the present study, we focused on the evaluation of the anti-multiple-myeloma (MM) effects of the second generation analog Flu, in comparison with dEpoB, in a disseminated MM model. Our data demonstrated that Flu had a profound antitumor activity in human MM both in vitro and in vivo, indicating that this compound might be a promising agent against MM, particularly in late-stage refractory disease.

Materials and Methods

Cell Lines and Primary Specimens. For information about cell lines and primary specimens, see Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Reagents. Flu and dEpoB, were synthesized in-house (the BioOrganic Laboratory at Memorial Sloan–Kettering Cancer Center). Paclitaxel was purchased from Sigma. For in vivo mouse-model experiments, Flu and dEpoB were dissolved in 50% DMSO/50% ethanol as a stock solution (30 mg/ml) and diluted with normal saline at the time of injection.

Cell-Viability Assay. Cell-viability measurement was assessed by a colorimetric assay using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma) and phenazine methosulfate (PMS, Sigma) according to the manufacturer’s protocol.

Cell-Cycle Analysis. Cells were fixed with 66.6% ethanol at −20°C overnight, resuspended in 50 µg/ml RNase A (diluted in PBS), and incubated for 30 min at 37°C. The samples were resuspended in 25 µg/ml propidium iodide and 38 mM sodium citrate buffer, pH 7.2. Flow cytometry was performed on a FACSCalibur automated system (Becton Dickinson), and data were analyzed by using the program FLOWJO (Tree Star, Ashland, OR).

Abbreviations: BLI, bioluminescence imaging; dEpoB, 12,13-desoxyepothilone B; Dex, dexamethasone; Dox, doxorubicin; Flu, Fludelone; JNK, c-Jun N-terminal kinase; MM, multiple myeloma; MTD, maximum tolerated dose; NOD, nonobese diabetic; PARP, poly(adenosine diphosphate-ribose) polymerase; SCID, severe combined immunodeficient; SPAK, stress-protein-activated kinase; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

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Annexin-V-Binding and DNA-Fragmentation Assays. Based on cell-cycle analysis, the optimal concentration of dEpoB and Flu causing complete cell-cycle arrest was 50 nM; therefore, this concentration was used in the following in vitro study. MM cells were treated with or without 40 μM pancaspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk, Calbiochem) for 1 h before adding the compounds. Cells were stained with annexin V phycocrythrin and 7-aminoactinomycin D (7-AAD, Sigma), following standard protocol. A DNA-fragmentation assay was performed by using a Suicide-Track DNA ladder isolation kit (Oncogene Research Products, San Diego).

Caspase-8 and -9 Fluorometric Assays. Caspase-8 and -9 activities were measured with assay kits, following the manufacturer’s instructions (Calbiochem). MM cells treated with tumor-necrosis-factor-related apoptosis-inducing ligand (50 ng/ml) were used as a positive control. The result was expressed as the relative fluorescent units in three independent experiments.

Nonradioactive Stress-Protein-Activated Kinase (SPAK)/c-jun N-Terminal Kinase (JNK) Assay. The SPAK/JNK activity was determined by using a commercially available kit (Cell Signaling Technology, Beverly, MA). In brief, cells were lysed, and equal amounts of protein were incubated overnight with glutathione S-transferase (GST)-c-jun fusion (amino acids 1–89) protein beads. The immune body and rabbit polyclonal cytochrome of protein were incubated overnight with glutathione technology, Beverly, MA). In brief, cells were lysed, and equal amounts of protein were incubated overnight with glutathione S-transferase (GST)-c-jun fusion (amino acids 1–89) protein beads. The immune body and rabbit polyclonal cytochrome of protein were incubated overnight with glutathione technology, Beverly, MA). In brief, cells were lysed, and equal amounts of protein were incubated overnight with glutathione S-transferase (GST)-c-jun fusion (amino acids 1–89) protein beads. The immune body and rabbit polyclonal cytochrome of protein were incubated overnight with glutathione

Preparation of Cytosolic and Mitochondrial Extracts from CAG Cells. A mitochondrial fractionation kit (Active Motif, Carlsbad, CA) was used for the isolation of mitochondrial or cytosolic fractions, following the manufacturer’s instructions.

Immunoblotting Analysis. Protein lysates were prepared by using Laemmli buffer, and protein concentration was determined by Lowry protein assay (Bio-Rad). An equal amount of protein (50 μg) was fractionated on a precast 4–20% Tris-glycine gel (Bio-Rad). After transfer, membranes were processed for standard Western blotting assay. The primary antibodies used were as follows: poly(adenosine diphosphate-ribose) polymerase (PARP, BD PharMingen); caspase-3 (BD PharMingen); anti-phospho-SPAK/JNK and anti-SPAK/JNK (Cell Signaling Technology); rabbit polyclonal Ab against actin (Sigma); and monoclonal second antibody and rabbit polyclonal cytochrome c antibody (Cell Signaling Technology).

Establishment of Subcutaneous and Disseminated MM Xenografts and Therapy. Nonobese diabetic (NOD) severe combined immunodeficient (SCID) mice (The Jackson Laboratory) were housed and maintained in facilities under an institute-approved animal protocol. For the s.c. xenograft MM RPMI 8226 mouse model, 10- to 12-wk-old female mice were sublethally irradiated with 3 Gy from a cesium 60-6 radiation source and inoculated with 10 × 10^6 tumor cells in the right flank. When tumor volumes approached 100 mm^3, the mice were divided into experimental cohorts of five to eight mice each. Injections (i.p.) of Flu or dEpoB (20 mg/kg of body weight, five doses (one dose every 2 d) then three doses (one dose every 3 d), were administered. Control mice were treated with the same amount of vehicle under the same protocol. Tumor volume was calculated by using the formula mm^3 = 0.5 × width^2, where r = (length + width)/4, measuring the two largest perpendicular axes of the tumor.

For establishing a disseminated xenograft NOD/SCID mouse model, 10 × 10^6 CAG cells, stably expressing the HSV-Tk-eGFP-luciferase fusion protein (13), were injected intravenously via the tail vein. The tumor distribution was followed by serial whole-body noninvasive imaging of visible light emitted by luciferase-expressing MM cells, upon injection of mice with luciferin. At 7–10 d after tumor injection, a group of NOD/SCID mice with established disseminated MM was divided into four cohorts, with a statistically equivalent tumor burden in each of the cohorts. The cohorts were treated with either Flu or dEpoB alone (20 mg/kg of body weight, five doses, with one dose every 2 d followed by 6 d of rest and then five doses, with one dose every 3 d).

Bioluminescence Imaging (BLI). Mice were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL), and t-luciferin (Xenogen, Alameda, CA) in PBS was administered, at a dose of 75 mg/kg of body weight, by retroorbital injection. The BLI with a charge-coupled device camera (IVIS, Xenogen) was initiated 2 min after the injection of luciferin. Dorsal and ventral images were acquired from each animal at each time point to better determine the origin of photon emission. The data were expressed as photon emission (photons per second per cm^2 per steradian).

Statistical Analysis. All analyses were done by using the program STATA 7.0 (StataCorp, College Station, TX), with P < 0.05 considered to be significant. For comparing tumor-associated parameters, the nonpaired Student’s t test was used. Log-rank tests were used to calculate the statistical significance of the difference in Kaplan-Meier survival curves.

Results

Epothilone Inhibits the in Vitro Growth of MM Cell Lines and Patient CD138+ Cells. We first evaluated the direct effect of Flu and dEpoB on proliferation and survival of MM cell lines. Both drugs decreased the survival of RPMI 8226, CAG, H929, MOLP-5, and D dex-sensitive MM.1S lines in a dose-dependent manner. As shown in Table 1, the IC50 in MM cell lines after 72-h incubation was between 6 and 14.4 nM for Flu, and between 37 and 68.6 nM for dEpoB. MOLP-5 is a stroma-dependent MM line that was sensitive to Flu (IC50, 14.4 nM) but relatively resistant to dEpoB (IC50, 68.6 nM). In addition, three of four MM lines tested in vitro were sensitive to paclitaxel, with IC50 values between 9 and 11.5 nM, whereas one line, H929, was relatively resistant (IC50, 75 ± 10 nM). We also evaluated the drug sensitivity of Dex-resistant MM.1R cells and DEX-resistant RPMI 8226/Dox40 cell lines. Flu still showed an IC50 comparable to that obtained on the Dex- or Dox-sensitive parent lines; however, paclitaxel was not as effective as Flu in killing these MM cell lines, showing much higher IC50 values. In contrast to MM lines, two lymphoma lines showed 5- to 10-fold higher IC50 after Flu or dEpoB treatment. Two human marrow stroma lines, HS-27A and HS-5, were relatively resistant to both dEpoB and Flu (IC50 ~ 100 nM).

All MM lines exposed to Flu (10 × IC50) for 24 h exhibited typical morphological changes, characterized by chromatin condensation, development of ring-like structures, a reduction of cell volume, membrane blebbing, and appearance of apoptotic bodies (Fig. L4A). The DEX-sensitive and -resistant RPMI 8226 lines showed similar morphology after Flu treatment.

It has been reported that epothilone B was only minimally effective against MM and colon cancer cells after 4-h drug exposure in vitro, possibly because of the kinetics of its intracellular accumulation and retention (14, 15). To investigate the time course of efficacy of Flu and dEpoB on MM cells, RPMI 8226 cells were pulse-exposed to 125 nM drugs for 1, 2, 4, 8, and 24 h, followed by drug wash-out and continued incubation in drug-free medium for up to 48 h. As shown in Fig. 1B, both Flu (Right) and dEpoB (Left) were active, even with short-term drug exposure. However, the effect was most dramatic in cells treated by Flu, because the cell proliferation was completely inhibited at 24 h with only 1-h pulse exposure, whereas the same degree of inhibition with dEpoB
Table 1. Cell growth inhibition IC50, nM by epothilones and paclitaxel against a panel of human tumors and normal human cell populations as determined by XTT assay

<table>
<thead>
<tr>
<th>Histology</th>
<th>Cell line</th>
<th>dEpoB</th>
<th>Flu</th>
<th>Paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloma</td>
<td>RPMI 8226/S</td>
<td>37 ± 2</td>
<td>7.6 ± 1.2</td>
<td>11.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>RPMI 8226/Dox40</td>
<td>38 ± 3</td>
<td>31 ± 2</td>
<td>6,000 ± 100</td>
</tr>
<tr>
<td>CAG</td>
<td>61.3 ± 4.2</td>
<td>12 ± 1.8</td>
<td>11 ± 2</td>
<td></td>
</tr>
<tr>
<td>NCI-H929</td>
<td>43 ± 5</td>
<td>9 ± 2</td>
<td>75 ± 10</td>
<td></td>
</tr>
<tr>
<td>MOLP-5</td>
<td>68.6 ± 6</td>
<td>14.4 ± 3</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MM.1S</td>
<td>52.3 ± 7</td>
<td>6 ± 1</td>
<td>9 ± 2</td>
<td></td>
</tr>
<tr>
<td>MM.1R</td>
<td>74 ± 4.3</td>
<td>14.2 ± 1.6</td>
<td>68 ± 8</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>RL</td>
<td>90 ± 11</td>
<td>80 ± 11</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SKI-DLBCL1</td>
<td>72 ± 9.8</td>
<td>60 ± 4.2</td>
<td>NA</td>
</tr>
<tr>
<td>BM stroma</td>
<td>HS-27A</td>
<td>100 ± 10</td>
<td>102 ± 8</td>
<td>89 ± 8</td>
</tr>
<tr>
<td></td>
<td>HS-5</td>
<td>100 ± 8</td>
<td>96 ± 7</td>
<td>NA</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SD from three independent experiments. NA, no data available; BM, bone marrow.

required 8 h of pulse exposure. By 48 h, all cells had died after 4- to 8-h exposure to Flu, but 24-h exposure to dEpoB was needed for comparable toxicity (Fig. 1B).

We evaluated the drug sensitivity of MM cells from three patients and normal plasma cells from three healthy donors. The MM CD138⁺ cells were incubated in a 24-well-plate with a preestablished confluent monolayer of marrow stromal cells in the presence of IL-6, sIL-6R, and IGF-I for up to 72 h to facilitate viability and proliferation. Flu, dEpoB, paclitaxel (50 nM), or control vehicle was added to the culture. The viability of MM cells was significantly decreased after 48-h incubation with Flu and even more so after 72-h incubation, relative to controls (Fig. 1C; P < 0.01); however, incubation with the same concentration of dEpoB or paclitaxel had no significant effect on MM cell viability (P > 0.05). Flu had no significant inhibitory effects on normal plasma cells, indicating that these cells are resistant to Flu treatment, presumably because of their G0 state. Whereas untreated MM cells appeared as typically round or elliptical, treated cells showed apoptotic features, such as chromatin condensation, ring-like nuclear structure, cell shrinkage, and apoptotic bodies (Fig. 1D).

Flu Treatment Blocked MM Cells at G2/M Phase, Resulting in Apoptosis. To confirm that synthetic epothilone analogs and paclitaxel shared a similar mechanism of induction of cell-cycle arrest (16), we examined the cell-cycle profile of Flu-treated MM cell lines. As shown in Fig. 2A, the addition of 125 nM Flu induced a shift of OPM-2 cells from G1 to G2/M as early as 6 h. The cell cycle was completely blocked at G2/M by 24 h, followed by a sharp increase in sub-G1 cells (data not shown). To determine the minimal concentration of Flu sufficient to induce cell-cycle arrest, serial concentrations (7.8–125 nM) were incubated with cells for 24 h. Complete cell-cycle arrest of RPMI 8226 cells in the G2/M phase was seen at a concentration of 31.3 nM Flu (Fig. 2B).

To determine the consequence of Flu-induced cell-cycle arrest at the G2/M phase, we evaluated annexin V staining of treated cells. Cells treated with Flu for only 12 h showed a >10-fold increase in annexin binding, compared with untreated controls (data not shown). After 24 h, Flu-treated cells were 100% annexin-V-positive vs. control cells (<10% positive). Pretreatment with a pancaspase...
inhibitor, z-VAD-fmk, reduced the number of annexin-binding cells by ∼60%, indicating that the apoptotic process requires caspase activation (Fig. 2C). We also performed DNA-fragmentation assays on RPMI 8226 and CAG cells treated by either Flu or dEpoB. Typical DNA laddering was detected within 24 h (Fig. 2D).

To evaluate the dynamic changes of microtubules in 50 nM Flu-treated RPMI 8226 cells, we showed that Flu and dEpoB shared with paclitaxel the capacity to enhance microtubule-bundle formation in tumor cells, without appreciably changing the total mass of microtubules in the cell shortly after exposure (6–12 h). At later stages (∼24 h), microtubules were disrupted, and cell apoptosis occurred (refs. 16 and 17; and see Supporting Materials and Methods and Fig. 5, which is published as supporting information on the PNAS web site).

**Epothilones Induce Apoptosis by Activation of Caspase-3, -8, and -9.** To determine whether caspase is involved in epothilone-mediated apoptosis, we first examined the activation of caspase-3 and PARP by standard Western blotting assay. Immunoblots of whole-cell lysates showed typical 17-kDa products of caspase-3 or 85-kDa products of PARP cleavage, accompanied by a decrease in the detection of the uncleaved form of caspase-3 or PARP (Fig. 3A and B). The activation of caspase-3 was further confirmed by immunohistochemistry, in which only the cleaved caspase-3 was stained (see Supporting Materials and Methods and Fig. 6, which is published as supporting information on the PNAS web site).

We next explored what initiator caspases are activated, to determine whether the death-receptor pathway (caspase-8 activation) or the mitochondrial-injury pathway (caspase-9 activation) was activated by Flu. As illustrated in Fig. 3, the activity of caspase-8 showed a 3- to 4-fold increase (Fig. 3C), whereas caspase-9 increased 8- to 10-fold, relative to untreated controls (Fig. 3D).

**Epothilones Promote the Release of Mitochondrial Proteins Cytochrome c and Second Mitochondrial-Derived Activator of Caspase (Smac) and Activation of Phosphorylated JNK.** Activation of caspase-9 is closely associated with mitochondrial injury in the apoptotic pathway. We therefore examined the role of mitochondria in triggering apoptosis in MM cells after treatment with Flu. Treatment of CAG cells with Flu or dEpoB induced the release of both cytochrome c and Smac detected at 12 h and, particularly, at 24 h of incubation (Fig. 3E).

We next looked at an upstream activator of this pathway. Several reports have linked JNK to apoptosis (18, 19), in which stress stimuli activate JNK (20, 21). We demonstrated significant increases (7- to 10-fold) in GST-Jun phosphorylation, indicating activation of JNK (Fig. 3F Top and Middle). Moreover, cotreatment of CAG cells with SP600125 (BioSource International, Camarillo, CA), a specific inhibitor of JNK (22), blocked Flu-induced JNK activity (Fig. 3F Top). Activation of JNK in response to Flu was not associated with changes in total JNK protein levels (Fig. 3F Bottom).

**Flu Is a Potent Inhibitor of s.c. Xenografts in NOD/SCID MM Model.** To assess the activity of Flu and dEpoB on MM tumor growth in vivo, we first evaluated its effect in an s.c. RPMI 8226 xenograft MM model in NOD/SCID mice. Based on a previous study on solid tumor xenografts at our institute (12, 23), we established a maximum tolerated dose (MTD) for dEpoB of 15–25 mg/kg of body weight, based on i.p. administration every other day and, for Flu, an MTD of 15–30 mg/kg of body weight, based on 6-h i.v. infusion. For the present study, we chose to administer both agents by an i.p. route at 20 mg/kg of body weight, every second day. Animals treated with Flu alone showed a dramatic inhibition of RPMI 8226 tumor growth (Fig. 4A). After three doses of Flu, there was significant tumor shrinkage, and after five doses, the tumor was undetectable. We have followed the mice for >100 d, and there has
been no recurrence of tumor. In contrast to the remarkable effect of Flu, dEpoB showed no significant effect on tumor growth, compared with vehicle controls (Fig. 4A). Quantification of tumor burden by BLI at day 50 (just before killing moribund mice in the control and dEpoB groups) showed an inhibition comparable to that obtained by tumor-diameter measurement (Fig. 4 B and C). There was a significant treatment-related body-weight loss (≈13%) noted in comparison with the vehicle-treated control animals (see Fig. 7, which is published as supporting information on the PNAS web site). However, after treatment was suspended, the body weight recovered to control values within 1 wk.

Flu Treatment Significantly Prolonged Overall Survival in a Disseminated Xenograft MM Model. We used luciferase-based, noninvasive BLI in a disseminated MM model in NOD/SCID mice by using CAG cells stably transfected with a triple-modality fusion reporter gene expressing herpes simplex virus 1 thymidine kinase, eGFP, and firefly luciferase (13). Anatomical distribution and pathophysiological manifestations in this model were consistent with the clinical course of MM in human patients, i.e., hallmarked by major involvement of the axial skeleton, osteolytic bone lesions captured by both pathology and x-ray examinations (e.g., spine, skull, and pelvis), and frequent development of hind-limb paralysis secondary to spinal lesions, without significant tumor spread to lungs, liver, spleen, or kidney. Most importantly, the tumor burden in individual mice can be quantified by real-time photon emission that correlates with tumor-cell number (Fig. 4D).

We used this disseminated CAG MM xenograft model to test Flu and dEpoB therapy and Flu followed by bortezomib. At the end of treatment (day 40), in mice treated with Flu alone, the tumor burden decreased ≈50-fold, compared with the control; however, there was no difference in tumor burden between dEpoB- and vehicle-treated mice (Fig. 4E). We also compared the tumor burden before and after bortezomib treatment. After five doses of bortezomib (0.25 mg/kg of body weight), the median tumor burden (dorsal and ventral) further decreased 10.1- and 12.3-fold, respectively, relative to tumor burden before bortezomib treatment (data not shown). Overall survival was significantly prolonged in the Flu-treated group (Fig. 4F, P < 0.005). There was a significant body-weight loss (≈17%) in the treatment groups compared with vehicle controls (P < 0.01), and, as in the s.c. study, body weight was recovered ≈1 wk after the cessation of treatment. There was no significant difference in hemoglobin levels and white blood cell count between treated and control mice at the end of drug administration. However, the platelet count was significantly lower in vehicle control mice than in mice treated by Flu, probably because of the extensive tumor infiltration in their bone marrow that resulted in suppression of hematopoiesis (see Fig. 8, which is published as supporting information on the PNAS web site).

Discussion

MM accounts for 1% of all cancers and 10% of hematological malignancies. Treatment of MM with conventional chemotherapy is not curative, with a median survival of ≈3 yr (24). Although high-dose chemotherapy with hematopoietic stem-cell support increases the rate of complete remission and event-free survival, almost every patient relapses, mandating viable salvage therapy options.

Drugs that target microtubules are among the most commonly prescribed anticancer therapies in recent years. The great success of paclitaxel in the market is attributable, in part, to the efficacy of these drugs in solid tumors (17). Paclitaxel has also been tried in the treatment of MM but failed in clinical trials (25, 26). The application of paclitaxel in MM is limited, not only because of the drug’s high toxicity but also because of the development of multidrug resistance, because paclitaxel serves as a substrate for the MDRI/P-glycoprotein drug-efflux pump. Although patients with MM at presentation have a low percentage of tumor cells that express MDRI protein, this percentage increases up to 50% in patients after chemotherapy (27, 28). These findings suggest that efforts should focus on overcoming some of the problems associated with paclitaxel-based therapy, including issues with formulation, administration, and susceptibility to resistance conferred by
the drug-efflux protein P-glycoprotein. Epothilones have emerged from these efforts as a class of microtubule-targeting drugs that should be evaluated in MM.

In preclinical models, we have evaluated Flu and dEpoB against a panel of human MM and non-Hodgkin lymphoma (NHL) lines. Flu inhibits MM and lymphoma cell proliferation significantly. However, MM cell lines are more sensitive to Flu, with extremely low IC50, whereas two NHL lines were inhibited at doses of Flu that were 5- to 10-fold higher than those effective in MM (Table 1). Compared with dEpoB, Flu has ≈5-fold greater potency on MM cell lines. Importantly, we showed that Flu is effective against paclitaxel-resistant MM cells (8226/Dox40) line that overexpress the MDR1/Pgp drug-efflux pump (15), as well as the DEX-resistant MM.1R line. In addition, we evaluated the duration of drug exposure necessary to cause apoptosis in the MM cells, showing that a much shorter duration of exposure to Flu than to dEpoB was needed to produce extensive tumor-cell kill. The viability of primary MM cells (29). Furthermore, we showed that Flu significantly decreased the proliferation potential than the bulk of the tumor mass (35, 36), might be responsive to Flu. Failure to eradicate these surviving tumor stem cells has proven to be the obstacle to achieving a curative therapy in this disease.

The data presented above indicate the superior performance of the fully synthetic epothilone analog Flu, in which metabolic stability and bioavailability were incorporated into the molecule through chemical synthesis. Flu was superior to the earlier generation epothilone analog dEpoB in our MM xenograft models. Flu caused complete tumor regression in s.c. tumors, with no subsequent recurrence, and significantly prolonged the overall survival of mice with disseminated tumor, with tolerable side effect profile. We anticipate that Flu may prove to be a promising agent in MM therapy.

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