Cancer Therapy: Preclinical

In Vitro and In Vivo Antitumor Activity of a Novel Alkylating Agent, Melphalan-Flufenamide, against Multiple Myeloma Cells

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Abstract

Purpose: The alkylating agent melphalan prolongs survival in patients with multiple myeloma; however, it is associated with toxicities and development of drug-resistance. Here, we evaluated the efficacy of melphalan-flufenamide (mel-flufen), a novel dipeptide prodrug of melphalan in multiple myeloma.

Experimental Design: Multiple myeloma cell lines, primary patient cells, and the human multiple myeloma xenograft animal model were used to study the antitumor activity of mel-flufen.

Results: Low doses of mel-flufen trigger more rapid and higher intracellular concentrations of melphalan in multiple myeloma cells than are achievable by free melphalan. Cytotoxicity analysis showed significantly lower IC50 of mel-flufen than melphalan in multiple myeloma cells. Importantly, mel-flufen induces apoptosis even in melphanal- and bortezomib-resistant multiple myeloma cells. Mechanistic studies show that siRNA knockdown of aminopeptidase N, a key enzyme mediating intracellular conversion of mel-flufen to melphalan, attenuates anti–multiple myeloma activity of mel-flufen. Furthermore, mel-flufen–induced apoptosis was associated with: (i) activation of caspases and PARP cleavage; (ii) reactive oxygen species generation; (iii) mitochondrial dysfunction and release of cytochrome c; and (iv) induction of DNA damage. Moreover, mel-flufen inhibits multiple myeloma cell migration and tumor-associated angiogenesis. Human multiple myeloma xenograft studies showed a more potent inhibition of tumor growth in mice treated with mel-flufen than mice receiving equimolar doses of melphalan. Finally, combining mel-flufen with lenalidomide, bortezomib, or dexamethasone triggers synergistic anti–multiple myeloma activity.

Conclusion: Our preclinical study supports clinical evaluation of mel-flufen to enhance therapeutic potential of melphalan, overcome drug-resistance, and improve multiple myeloma patient outcome.

Introduction

Multiple myeloma remains incurable due to the development of a drug-resistant phenotype after prolonged therapy (1, 2). For many years, combined melphalan (mustard-phenylalanine) and prednisone has been a mainstay of multiple myeloma treatment in the nontransplant candidates. In transplant candidates, a treatment regimen comprising a high-dose melphalan (HDM) in conjunction with autologous stem cell transplantation (ASCT) has improved progression-free and overall survival in patients with multiple myeloma (3–5). More recent studies have combined melphalan and steroids with several novel agents such as bortezomib, thalidomide, or lenalidomide, as initial therapy of elderly newly diagnosed patients and have improved response extent and frequency, as well as prolonged progression free an overall survival (6–12). In a parallel fashion, integration of these novel therapies into the transplant paradigm as induction, consolidation, and maintenance has further improved outcome in this setting (13, 14). These studies exemplify the use of melphalan in the current multiple myeloma therapy, and provided impetus for the development of melphalan prodrug to increase tumor specificity, reduce toxicity, and prevent drug-resistance.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance
The alkylating agent melphalan is actively used in multiple myeloma therapy; however, dose-limiting toxicities and development of resistance limits its use. Recent studies have focused on developing a prodrug to enhance the therapeutic potential of melphalan. Melphalan-flufenamide (mel-flufen) is an enzyme-activated prodrug of melphalan which allows for a more rapid and higher intracellular accumulation of melphalan in tumor cells than is achievable by direct exposure to equimolar doses of melphalan. Mel-flufen is undergoing evaluation in phase I/IIa clinical trials in solid tumors. Here, we used both in vitro and in vivo multiple myeloma xenograft models to show that mel-flufen is a more potent anti–multiple myeloma agent than melphalan, which can overcome conventional drug resistance. Moreover, the combination of mel-flufen with bortezomib, lenalidomide, or dexamethasone induces synergistic anti–multiple myeloma activity. Our preclinical data therefore provide the framework for clinical evaluation of mel-flufen, either alone or in combination, to improve patient outcome in multiple myeloma.

Materials and Methods

Pharmacologic screening of alkylating oligopeptides led to the identification of a novel melphalan-containing prodrug melphalan-flufenamide (mel-flufen; \(\text{L-melphalanyl-p-L-fluoro phenylalanine ethyl ester}\)), a molecular entity with a more potent antitumor activity than parental drug melphalan despite identical alkylating capacity (Fig. 1A; refs. 15–17). Mel-flufen is rapidly incorporated into the tumor cells, followed by intracellular hydrolysis, which in part is mediated by aminopeptidase N (ANPEP), an enzyme overexpressed in several tumor cell malignancies (18). Studies using solid tumor cell models showed that treatment with mel-flufen causes at least a 10-fold higher loading of melphalan, which explains its higher tumor cell cytotoxicity (15–17, 19). To date, the mel-flufen activity against multiple myeloma cells is undefined. In the present study, we examined the antitumor activity of mel-flufen in multiple myeloma cells using both in vitro and in vivo model systems. Our studies show that mel-flufen is more potent than melphalan and can overcome resistance not only to melphalan, but also to novel agents, providing the rationale for its clinical evaluation to improve patient outcome in multiple myeloma.

Measurement of intracellular concentrations of mel-flufen and melphalan
The intracellular concentration of melphalan in RPMI-8226 cells was assessed at various time points after treatment with freshly made solutions of mel-flufen or melphalan. For each treatment series, RPMI-8226 cells were resuspended at a concentration of 2.5 × 10^6 cells/mL in a total volume of 6 mL of complete prewarmed RPMI media, and 1 mL sample was removed at 0, 5, 15, 30, 60, or 120 minutes after addition of each drug. The 1 mL sample was added to 4 mL of precooled PBS and centrifuged for 5 minutes at 1,000 rpm; the resulting cell pellet was washed in 5 mL of precooled PBS and solubilized by adding 50 μL of ethanol/acetonitrile (1:1, v/v). The resulting precipitated cell debris was cleared by centrifugation at 10,000 rpm for 5 minutes and the supernatant was collected and frozen at −80°C until further analyses. The intracellular amount of mel-flufen or melphalan was measured in a aliquot of 25 μL of the sample, which was mixed with 75 μL of an internal standard solution consisting of 1 μg/mL of fluorescein diluted in 1:1 (acetonitrile:ethanol), centrifuged for 4 minutes at 3,700 rpm (Heraeus Biofuge 13). Supernatant was transferred to 200 μL high-performance liquid chromatography (HPLC) vials and then analyzed for mel-flufen or melphalan content by liquid chromatography/mass spectrometry (LC/MS; SIM of 498 Da for mel-flufen, 305 Da for melphalan, and 333 Da for the fluorescein standard). The above analyses were conducted at OncoTargeting AB. The mean ± SEM of peak area of mel-flufen and melphalan at each time point was calculated. The area under the curve for 0 to 120 minutes of melphalan [area under curve (AUC) 0–120 minutes] was determined for each treatment and is shown relative to AUC 0 to 120 minutes of 0.5 μmol/L of mel-flufen.

Cell viability, proliferation, and apoptosis assays
Cell viability was assessed by using colorimetric assay with MTT (Calbiochem), and cell proliferation analysis in coculture experiments with patient-derived bone marrow stromal cells (BMSC) was conducted using thymidine.
incorporation, as described previously (20). Apoptosis was quantified using Annexin V–FITC/propidium (PI) iodide apoptosis detection kit, as per manufacturer’s instructions (BD Biosciences), followed by an analysis on FACS Calibur (BD Biosciences).

Aminopeptidase N activity assay
Enzymatic activity of ANPEP was measured with the substrate L-alanine-4-nitro-anilide (Sigma–Aldrich) as previously described (21).

Immunoblotting
Western blot analysis was conducted as previously described (22) using antibodies recognizing full length and cleaved forms of caspase-3, -7, -8, -9, and PARP (Cell signaling), as well as p53, γ-H2AX, ANPEP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam).

Transient transfection assays
MM.1S cells were transiently transfected with genome control (scrambled) siRNA or siRNA against ANPEP (Smart pool siRNA; Dharmacon, Inc.) using Nucleofector Kit V, according to manufacturer’s instructions (Amaxa Biosystems).

Human plasmacytoma xenograft model
All animal studies were approved by the Dana-Farber Cancer Institute (DFCI) Institutional Animal Care and Use Committee. The xenograft tumor model was conducted as previously described (23, 24). CB-17 severe combined immunodeficient (SCID) mice were subcutaneously inoculated with $6.0 \times 10^6$ MM.1S cells in 100 μL of serum-free RPMI-1640 medium, and then randomized to treatment groups when tumors reached approximately 100 mm$^3$. Mice were treated intravenously with vehicle, mel-flufen, and mel-flufen as myeloma therapy. Figure 1. A, chemical structures of the melphalan-containing dipeptide mel-flufen and melphalan. B, RPMI-8226 cells were treated with indicated concentrations of either mel-flufen or melphalan; samples were harvested at 0, 5, 15, 30, 60, and 120 minutes, followed by analysis for intracellular accumulation of mel-flufen using LC/MS. The peak area of mel-flufen and melphalan was analyzed at each time point after normalization of signal intensity in each sample using the internal fluorescein standard. AUC 0 to 120 minutes was calculated from the individual samples. The values are given relative to AUC for Mel-flufen 0.5 μmol/L mean value. Data presented are mean ± SEM (n = 3). C, multiple myeloma cell lines were treated with indicated doses of mel-flufen for 24 hours, and cytotoxicity was assessed using MTT assay (n = 3; mean ± SD; P < 0.005 for all cell lines). D, MM.1R and RPMI-8226 cells were treated with mel-flufen (2 μmol/L) for 24 hours and analyzed for apoptosis by AnnexinV/PI staining assay (n = 2; mean ± SD; P < 0.001); E, MM.1R and RPMI-8226 multiple myeloma cells were treated with mel-flufen (1 and 3 μmol/L, respectively) for 24 hours; protein lysates were subjected to immunoblotting using indicated antibodies. F, MM.1S cells were treated with mel-flufen in the presence or absence of BMSCs for 24 hours, and DNA synthesis was measured by $^3$H-TdR uptake (mean ± SD of triplicate cultures; P < 0.002 for all samples).
or melphalan with indicated concentrations and treatment duration times. Animals were euthanized when their tumors reached 2 cm³.

**In vitro** migration and capillary-like tube structure formation assays

Transwell Insert Assays (Chemicon) were used to measure migration, and *in vitro* angiogenesis was assessed by Matrigel capillary-like tube formation assay, as previously described (25). For endothelial tube formation assay, human vascular endothelial cells (HUVEC) were obtained from Clonetics and maintained in endothelial cell growth medium-2 (EGM2 MV SingleQuots; Clonetics) containing 5% FBS.

**Statistical analysis**

Statistical significance of differences observed in drug-treated versus control cultures was determined by using the Student t test. The minimal level of significance was P < 0.05. Tumor volume and survival in mice was measured using the GraphPad PRISM (GraphPad Software/version 5). Isobologram analysis (26) was conducted using “Calcusyn” software program (Biosoft). Combination index (CI) values of less than 1.0 indicate synergism and values more than 1.0 indicate antagonism.

**Results and Discussion**

**Mel-flufen delivers high intracellular concentration of melphalan**

RPMI-8226 multiple myeloma cells were treated with either mel-flufen (0.5, 1, or 5 µmol/L) or melphalan (10 or 100 µmol/L) and the intracellular content of mel-flufen or melphalan was measured using HPLC/MS. AUC 0 to 120 minutes was calculated from melphalan peak area curves in each sample and relative AUC is shown in Fig. 1B. Treatment of RPMI-8226 cells with mel-flufen led to high intracellular concentration of free melphalan as compared with cells exposed directly to melphalan (Fig. 1B). A high concentration was reached as early as after 15 minutes (data not shown). Importantly, treatment of RPMI-8226 cells with 5 µmol/L of mel-flufen load the cells with much more melphalan than can be achieved using even 100 µmol/L of melphalan (Fig. 1B). These data suggest that mel-flufen allows for a more rapid and higher intracellular accumulation of melphalan in multiple myeloma cells than is achievable by direct exposure to equimolar doses of melphalan. Our results using multiple myeloma cells are consistent with similar observation in other cancer cell lines (19). Previous studies showed that lipophilicity and an early intracellular hydrolysis of melphalan by peptidases inside the cells to release melphalan contributes to achieving rapid and high intracellular concentrations of melphalan (19, 27, 28). Earlier findings also showed that in tumor cells a limited exposure time, which simulate short half-life in *vivo*, proved more favorable for mel-flufen than for melphalan indicating a trapping mechanism through the enzymatic activation (29). Together, these results suggest that mel-flufen administration in patients with multiple myeloma is a more efficient therapeutic strategy for delivering higher concentrations of intracellular melphalan than by directly exposing cells to free melphalan.

**Anti–multiple myeloma activity of mel-flufen in vitro**

Human multiple myeloma cell lines (MM.1S, INA-6, RPMI-8226, MM.1R, Dox-40, ARP-1, and ANBL-6) were treated with various concentrations of mel-flufen for 24 hours, followed by assessment of cell viability using MTT assays. A significant concentration-dependent decrease in viability of all cell lines was observed in response to mel-flufen treatment (Fig. 1C). The cytotoxicity of mel-flufen was observed in multiple myeloma cell lines sensitive and resistant to conventional and novel therapies, as well as representing distinct cytogenetic profiles. For example, we examined isogenic cell lines dexamethasone-sensitive MM.1S and dexamethasone-resistant MM.1R with t(14;16) translocation and c-maf overexpression; RPMI-8266 with TP53, K-Ras, and EGF receptor (EGFR) mutations; and INA-6, an IL-6-dependent cell line with N-Ras activating mutation. The variable IC₅₀ of mel-flufen observed against multiple myeloma cell lines may be attributed to their distinct genetic background and/or drug-resistance characteristics (30–33).

We next examined whether anti–multiple myeloma activity of mel-flufen is due to induction of apoptosis. Treatment of MM.1R or RPMI-8226 cells with mel-flufen triggered accumulation of cells in early (Annexin V⁺/PI½) and late-stage (Annexin V⁺/PI⁺) apoptosis (Fig. 1D). Moreover, mel-flufen–induced apoptosis was associated with: (i) activation of caspase-3, -7, -8, and -9, as well as PARP cleavage; (ii) reactive oxygen species (ROS) generation; and (iii) decrease in mitochondrial transmembrane potential (Δψm) accompanied by release of cytochrome c (Fig. 1E and Supplementary Fig. S1A–S1D). Studies using pan-caspase inhibitor (Z-VAD-FMK) showed attenuation of mel-flufen–induced cytotoxicity in 3 multiple myeloma cell lines (Supplementary Fig. S1E). These findings suggest that mel-flufen triggers both mitochondria-dependent and—independent apoptotic signaling pathways.

Interaction and adhesion of multiple myeloma cells with BMSCs triggers cytokine secretion, which mediates paracrine growth of multiple myeloma cells, as well as confers cell adhesion–mediated drug resistance (CAM-DR; refs. 34, 35). To determine whether mel-flufen can overcome these protective effects, MM.1S cells were cultured with or without BMSCs in the presence or absence of various concentrations of mel-flufen. A significant inhibition of BMSCs-induced proliferation of MM.1S cells was observed in response to mel-flufen treatment (Fig. 1F). These data suggest that (i) mel-flufen not only directly targets multiple myeloma cells, but also can overcome the cytoprotective effects of the multiple myeloma-host bone marrow microenvironment.

The mechanism(s) of melphalan-resistance include decreased melphalan uptake, reduced induction of DNA cross-links, and/or increased repair of such DNA lesions, which otherwise are converted to DNA single
or double-strand breaks. Because mel-flufen exhibit a higher potency and rapid kinetics of action in tumor cells as compared with melphalan, we examined whether these attributes impart mel-flufen the ability to overcome melphalan-resistance. We used previously characterized (36) melphalan-sensitive RPMI-8226 and its melphalan-resistant derivative of RPMI-8226 (LR5) multiple myeloma cell lines. As seen in Fig. 2A, mel-flufen induces cytotoxicity even in melphalan-resistant LR5 cells, whereas melphalan alone does not significantly affect the viability of LR5 cells at tested concentrations. Higher concentrations of melphalan (100 \( \mu \text{mol/L} \)) showed similar resistance to melphalan in LR5 cells (data not shown). The mechanism(s) whereby mel-flufen overcomes melphalan-resistance remains to be examined; however, it is likely that mel-flufen induces a more potent and cumulative DNA damage and thereby can overcome the DNA cross-link repair capacity of multiple myeloma cells. Besides reduced DNA damage, melphalan resistance is also linked to other factors, such as increased glutathione-S-transferase activity, CAM-DR, mitochondrial alterations, or impaired caspase activation (36–39); and many of these mechanism(s) may likely be circumvented by achieving a sustained and higher intracellular concentrations of melphalan with mel-flufen.

We next examined the effects of mel-flufen in dexamethasone- or proteasome inhibitor-resistant multiple myeloma cells. We observed a significantly more potent anti–multiple myeloma activity of mel-flufen than melphalan when tested against dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) cells (Fig. 2B). To determine whether mel-flufen can overcome bortezomib-resistance, we used bortezomib-sensitive (ANBL6.WT) and bortezomib-resistant ANBL6 (ANBL6.BR) multiple myeloma (MM) cells were treated with indicated concentrations of mel-flufen or melphalan, and cytotoxicity was measured using MTT assay (\( n = 3 \); mean ± SD; \( P < 0.005 \)).

Figure 2. A, melphalan-sensitive RPMI-8226 [wild-type (WT)] and melphalan-resistant LR-5 cells were treated with indicated concentrations of mel-flufen for 24 hours, and cytotoxicity was assessed using MTT assay (\( n = 3 \); mean ± SD; \( P < 0.005 \)). B, MM.1S and MM.1R cells were treated with mel-flufen or melphalan at the indicated concentrations for 24 hours, and cytotoxicity was measured by MTT assay (\( n = 3 \); mean ± SD; \( P < 0.005 \)). C, bortezomib-sensitive ANBL6 (ANBL6.WT) and bortezomib-resistant ANBL6 (ANBL6.BR) multiple myeloma (MM) cells were treated with indicated concentrations of mel-flufen or melphalan, and cytotoxicity was measured using MTT assay (\( n = 3 \); mean ± SD; \( P < 0.003 \)). D, purified patient multiple myeloma (CD138 \(^{+} \)) cells were treated with mel-flufen at indicated doses for 24 hours, and cell viability was measured using Trypan blue assay (mean ± SD of triplicate cultures; \( P < 0.001 \) for all patient samples).
ANBL6.BR cells are needed to delineate the mechanism(s) whereby mel-flufen overcomes bortezomib-resistance, as well as mechanism(s) conferring cross-resistance to melphalan and bortezomib. Nevertheless, our data show that mel-flufen, but not melphalan, overcomes bortezomib-resistance. Figure 3. A, total protein lysates from MM.1S, MM.1R, RPMI-8226, LR-5, and INA6 cells were subjected to immunoblot analysis using anti-ANPEP, or anti-GAPDH antibodies (Ab). B, bar graph shows the baseline ANPEP activity in various multiple myeloma (MM) cell lines. C, MM.1S cells were transfected with scr-siRNA or ANPEP-siRNA; 24 hours after transfection, cells were treated with indicated concentrations of melphalan or mel-flufen for 24 hours, followed by analysis of viability (n = 3; mean ± SD; P < 0.001 in scr- versus ANPEP siRNA-transfected cells in response to mel-flufen). Percentage cell viability was normalized (as 100%) for scr- or ANPEP-siRNA controls, respectively. D, MM.1R and RPMI-8226 multiple myeloma cells were treated with mel-flufen (2 μmol/L for MM.1R and 5 μmol/L for RPMI-8226 cells) for 24 hours; protein lysates were prepared and subjected to immunoblotting using indicated antibodies. E, p53-null ARP-1 multiple myeloma cells were treated with indicated concentrations of either mel-flufen or melphalan for 24 hours, and cytotoxicity was assessed using MTT assay (n = 3; mean ± SD; P < 0.001). F, MM.1S cells were treated with mel-flufen (1 μmol/L) or melphalan (1 μmol/L) for 1, 2, 6, and 24 hours; cells were then washed and stained with Alexa Fluor 647-conjugated anti-H2AX antibody, followed by quantification of γ-H2AX-positive cells using flow cytometry (n = 3; mean ± SD; P < 0.005). MFI, mean fluorescence intensity. G, MM.1S cells were treated with melphalan (3 μmol/L) or mel-flufen (0.5 μmol/L) for 4 hours, harvested, and subjected to alkaline comet assay. For each slide, images were randomly captured by fluorescence microscopy and representative images from 3 independent experiments are shown.
We next determined whether mel-flufen similarly affects purified patient multiple myeloma cells. Tumor cells from 5 patients with multiple myeloma, including those relapsing after multiple prior therapies including bortezomib, lenalidomide, and dexamethasone, were treated with mel-flufen. Patients were considered refractory to specific therapy when disease progressed on therapy or relapsed within 2 months of stopping therapy. A significant dose-dependent decrease in viability of all patient multiple myeloma cells was noted after mel-flufen treatment (Fig. 2D). These data show that mel-flufen induces cytotoxicity in tumor cells obtained from patients whose multiple myeloma is resistant to bortezomib, dexamethasone, or lenalidomide therapies. Importantly, mel-flufen at the IC50 for multiple myeloma cells does not significantly affect the viability of normal PBMCs (data not shown), suggesting specific anti–multiple myeloma activity and a favorable therapeutic index for mel-flufen.

Mechanism(s) mediating mel-flufen activity

Earlier studies established that (i) ANPEP (also known as CD13) plays a key role in catalyzing the release of free melphalan from mel-flufen, and (ii) hydrolysis of the peptide bond in mel-flufen by ANPEPs is a prerequisite for mel-flufen–induced cytotoxicity (19). Of note, the activity and/or the expression of ANPEP is elevated in many cancer types, and is associated with various characteristics of malignant phenotype including cell proliferation, cytokine secretion, tumor invasion, and angiogenesis (18). These studies suggest ANPEP as a viable therapeutic target in cancer (18). As seen in Fig. 3A and B, both ANPEP expression and activity are constitutively elevated in multiple myeloma cells. Importantly, transfection of ANPEP siRNA, but not negative-control (scrambled) siRNA, significantly inhibited mel-flufen–induced apoptosis in MM.1S cells, whereas no marked difference in melphalan-induced apoptosis was noted (Fig. 3C). These data suggest ANPEP as a viable therapeutic target in cancer (18). As seen in Fig. 3A and B, both ANPEP expression and activity are constitutively elevated in multiple myeloma cells. Importantly, transfection of ANPEP siRNA, but not negative-control (scrambled) siRNA, significantly inhibited mel-flufen–induced apoptosis in MM.1S cells, whereas no marked difference in melphalan-induced cytotoxicity was evident (Fig. 3C). The residual mel-flufen cytotoxic activity in ANPEP-siRNA–transfected cells may be attributed to limitations of RNA interference strategy, which usually results in incomplete gene loss. Another possibility is that mel-flufen or its intermediate metabolite in multiple myeloma cells is substrate of other aminopeptidases as reported in solid tumor cells (19). Nevertheless, our data provide the evidence that mel-flufen–triggered apoptosis...
in multiple myeloma cells is facilitated, at least in part, u ANPEP.

Previous studies have shown that melphalan triggers DNA-damage response/repair signaling, associated with activation of p53 (41). We therefore next examined whether mel-flufen–induced apoptosis in multiple myeloma cells correlates with induction of similar signaling cascades. Examination of mel-flufen–treated multiple myeloma cells showed a robust increase in p53 (Fig. 6, A, B, C). As expected, we found that mel-flufen trigger cytotoxicity even in p53-null ARP-1 cells. As expected, we found that mel-flufen trigger cytotoxicity even in p53-null ARP-1 cells.

Figure 6. Combination of low doses of mel-flufen and lenalidomide, bortezomib, or dexamethasone trigger synergistic antiproliferative effect of mel-flufen and lenalidomide, and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of mel-flufen and lenalidomide. The graph (right) is derived from the values given in the table (left). The numbers 1 to 9 in graph represent combinations shown in the table. CI < 1 indicates synergy. B, MM.1S cells were treated for 24 hours with mel-flufen, bortezomib, or mel-flufen plus bortezomib, and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of mel-flufen and bortezomib. The graph (right) is derived from the values given in the table (left). C, MM.1S cells were treated for 24 hours with mel-flufen, dexamethasone, or mel-flufen plus dexamethasone, and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of mel-flufen and dexamethasone. The graph (right) is derived from the values given in the table (left). D, melphalan-resistant LR-5 cells were treated for 24 hours with mel-flufen, bortezomib, or mel-flufen plus bortezomib, and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of mel-flufen and bortezomib. The graph (right) is derived from the values given in the table (left).
that high concentrations of melphanal (5–10 μmol/L) are required to achieve 30% to 40% cell death; importantly, mel-flufen (0.5 μmol/L) at the concentrations 10-fold lower than melphanal (5 μmol/L) is able to trigger significant cytotoxicity in p53-null ARF-1 multiple myeloma cells (Fig. 3E). These data suggest that although mel-flufen increases p53 levels (Fig. 3D), its cytotoxic activity in multiple myeloma cells is not dependent on p53. Our findings have important clinical implications, as 10% to 15% of patients with multiple myeloma have p53 mutations/deletions at presentation, which confer drug resistance, and majority of patients acquire this abnormality with disease progression; a therapeutic approach using mel-flufen would allow for potent anti–multiple myeloma activity even in this patient population.

An early event in the response of mammalian cells to DNA double-strand breaks is the phosphorylation of histone H2AX (γ-H2AX) at the sites in proximity to DNA breaks (42). A robust induction of γ-H2AX was observed in mel-flufen–treated MM.1R and RPMI-8226 cells (Fig. 3D, middle), suggesting that mel-flufen–induced DNA cross-links were indeed converted to DNA double-strand breaks. We next compared the potency of mel-flufen and melphanal in inducing DNA double-strand breaks. MM.1S cells were treated with mel-flufen (1 μmol/L) or melphanal (1 μmol/L) for 2, 6, and 24 hours; cells were then washed and stained with Alexa Fluor 647–conjugated anti-H2AX (pS139) antibody, followed by quantification of γ-H2AX–positive cells using flow cytometry. Results show an early and potent induction of DNA double-strand breaks in mel-flufen– versus melphanal–treated MM.1S cells (Fig. 3F).

To confirm the differential induction of DNA damage by mel-flufen and melphanal, we next conducted alkaline comet assay. The comet assay is a single cell gel electrophoresis assay and uses the principle that damaged DNA migrates, forming a ‘tail,’ whereas undamaged DNA with intact supercoiled structure, does not migrate, forming the head of the comet. The intensity and length of the comet tail is proportional to extent of DNA damage. Results showed that even low concentrations of mel-flufen (0.5 μmol/L) are able to trigger more potent and greater DNA damage than is observed in cells treated with higher concentrations (3 μmol/L) of melphanal (Fig. 3G and Supplementary Fig. S2). Together, these results suggest that mel-flufen is more efficient inducer of DNA damage than melphanal.

Overall, our mechanistic studies shows that (i) mel-flufen–induced cytotoxicity is facilitated via ANPEP; (ii) mel-flufen triggers DNA damage associated with induction of γ-H2AX and p53; (iii), although p53 is upregulated in response to mel-flufen treatment, the cytotoxic activity of mel-flufen is not dependent on p53, suggesting that there may be a p53-independent component to mel-flufen–induced cytotoxicity; and (iv) mel-flufen–induced apoptosis is associated with activation of caspases and PARP cleavage. Of note, DNA damage response signaling is linked to activation of p53/caspases signaling axis (43), suggesting a potential cross-talk between these pathways during mel-flufen–induced apoptosis. It is possible that mel-flufen, such as melphanal, triggers pleiotropic signaling pathways; however, due to the rapid intracellular accumulation characteristics of mel-flufen compared with melphanal, the kinetics of alterations in apoptotic response signaling may vary and this issue remains to be defined.

**Effect of mel-flufen on migration of multiple myeloma cells and angiogenesis**

Multiple myeloma cell growth is associated with angiogenesis (44, 45). As noted earlier, ANPEP expression/activity is associated with malignant phenotype, including angiogenesis (46); and importantly, mel-flufen is ANPEP-activated prodrug of melphanal. We examined the effect of mel-flufen on multiple myeloma cell migration and angiogenesis using Transwell insert systems and in vitro tube formation assays. VEGF is elevated in the multiple myeloma bone marrow microenvironment, and prior studies showed that VEGF triggers growth, migration, and angiogenesis in multiple myeloma (44, 45, 47). We first examined whether mel-flufen affects VEGF–induced multiple myeloma cell migration. VEGF alone markedly increases MM.1S cell migration; conversely, mel-flufen inhibits VEGF-dependent multiple myeloma cell migration, evidenced by a decrease in the number of crystal violet–stained cells (Fig. 4A and B). These cells were more than 95% viable before and after conducting the migration assay, excluding the possibility that drug-induced inhibition of migration is due to cell death. Additional experiments show that melphanal also inhibits tumor-associated angiogenesis and multiple myeloma cell migration, albeit at much higher concentrations than mel-flufen (data not shown). These findings are consistent with more potent and robust accumulation of intracellular melphanal upon mel-flufen treatment. These results suggest that mel-flufen may negatively regulate homing of multiple myeloma cells to the bone marrow.

We next used in vitro capillary-like tube structure formation assays to examine the antiangiogenic activity of mel-flufen. Angiogenesis was measured in vitro using Matrigel capillary-like tube structure formation assays in which HUVECs plated onto Matrigel differentiate and form capillary-like tube structures similar to in vivo neovascularization. This assay therefore provides evidence for antiangiogenic effects of drugs. HUVECs were seeded in 96-well culture plates precoated with Matrigel; treated with vehicle [dimethyl sulfoxide (DMSO)], mel-flufen for 16 hours; and then examined for tube formation using an inverted microscope. As seen in Fig. 4C and D, tube formation was markedly decreased in the mel-flufen–treated cells versus vehicle control. HUVEC cell viability was assessed using Trypan blue exclusion assay, and less than 5% cell death was observed with mel-flufen treatment. Our results are consistent with earlier in vivo data showing that mel-flufen decreases the number of blood vessel formation in SH-SY5Y xenograft model (17). Taken together, our findings suggest that mel-flufen...
blocks multiple myeloma cell migration and inhibit tumor-associated angiogenesis.

**Anti–multiple myeloma activity of mel-flufen in xenograft mouse model**

Having shown that mel-flufen induces apoptosis in multiple myeloma cells in vitro, we next examined the in vivo efficacy of mel-flufen using a human plasmacytoma MM.1S xenograft mouse model (23). Treatment of tumor-bearing mice with mel-flufen intravenously significantly inhibited multiple myeloma tumor growth (P = 0.001) and prolonged survival (P < 0.001) of these mice (Fig. 5A and B, respectively). Equimolar doses of melphalan also reduced tumor progression (Fig. 5C), albeit to a lesser extent than mel-flufen. Moreover, mel-flufen–treated mice survived for a longer time than mice receiving equimolar doses of melphalan (P < 0.01; CI, 95%; Fig. 5D). These in vivo data confirm our in vitro findings showing more potent anti–multiple myeloma activity and tumor cell selectivity of mel-fluten versus melphalan.

**Combined treatment with mel-flufen and lenalidomide, bortezomib, or dexamethasone induces synergistic anti–multiple myeloma activity**

We next examined whether mel-flufen can be combined with other drugs to enhance cytotoxicity and overcome melphalan-resistance. MM.1S cells were first treated with both mel-flufen and lenalidomide simultaneously across a range of concentrations for 24 hours, and then analyzed for viability using MTT assay. An analysis of synergistic anti–multiple myeloma activity using the Chou and Talalay method (26) showed that the combination of low concentrations of mel-flufen and lenalidomide triggered synergistic anti–multiple myeloma activity, with a CI < 1.0 (Fig. 6A, graph and table). We next examined whether mel-flufen adds to the anti–multiple myeloma activity of proteasome inhibitor bortezomib and conventional anti–multiple myeloma agent dexamethasone. As with lenalidomide, the combination of mel-flufen with bortezomib or dexmethasone triggered synergistic anti–multiple myeloma activity, evidenced by a significant decrease in viability of MM.1S cells (Fig. 6B and C, graphs and tables). Importantly, a similar synergism was observed between mel-flufen and lenalidomide, bortezomib, or dexamethasone in melphalan-resistant L55 multiple myeloma cells (Fig. 6D–F, graphs and tables). Although definitive evidence of decreased toxicity of combination therapy awaits results of clinical trials, the synergy observed in vitro may allow for use of lower doses and decreased toxicity.

Collectively, our preclinical studies therefore show potent in vitro and in vivo anti–multiple myeloma activity of mel-flufen at doses that are well tolerated in human multiple myeloma xenograft mouse models. These findings provide the framework for clinical trials of mel-flufen both as a single agent and together with lenalidomide, bortezomib, or dexamethasone to increase response, overcome drug resistance, reduce side effects, and improve patient outcome in multiple myeloma.

**Disclosure of Potential Conflicts of Interest**

D. Chauhan is a consultant to Oncopeptide AB. K. Viktorsson is employed as Head of Preclinical Research at Oncopeptides AB. R. Lewensohn has ownership interest (including patents) in Oncopeptides AB. No potential conflicts of interest were disclosed by the other authors.

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