HemaSphere

Article
Open Access

The Peptide–Drug Conjugate Melflufen Modulates the Unfolded Protein Response of Multiple Myeloma and Amyloidogenic Plasma Cells and Induces Cell Death

Ken Flanagan[1], Romika Kumari[2], Juho J. Miettinen[2], Staci L. Haney[3], Michelle L. Varney[3], Jacob T. Williams[3], Muntasir M. Majumder[2], Minna Suvela[2], Ana Slipicevic[1], Fredrik Lehmann[1], Nina N. Nupponen[1], Sarah A. Holstein,[3] Caroline A. Heckman[2]

Correspondence: Caroline A. Heckman (caroline.heckman@helsinki.fi).

ABSTRACT

Immunoglobulin light-chain (AL) amyloidosis is a rare disease caused by clonal plasma cell secretion of misfolded light chains that assemble as toxic amyloid fibrils, depositing in vital organs including the heart and kidneys, causing organ dysfunction. Plasma cell–directed therapeutics are expected to reduce production of toxic light chain by eliminating amyloidogenic cells in bone marrow, thereby diminishing amyloid fibril deposition and providing the potential for organ recovery. Melphalan flufenamide (melflufen) is a first-in-class peptide–drug conjugate that targets aminopeptidases and rapidly releases alkylating agents inside tumor cells. Melflufen is highly lipophilic, permitting rapid uptake by cells, where it is enzymatically hydrolyzed by aminopeptidases, resulting in intracellular accumulation of the alkylating agents, including melphalan. Previous data demonstrating sensitivity of myeloma cells to melflufen suggest that the drug might be useful in AL amyloidosis. We describe the effects of melflufen on amyloidogenic plasma cells in vitro and ex vivo, demonstrating enhanced cytotoxic effects in comparison to melphalan, as well as novel mechanisms of action through the unfolded protein response (UPR) pathway. These findings provide evidence that melflufen-mediated cytotoxicity extends to amyloidogenic plasma cells, and support the rationale for the evaluation of melflufen in patients with AL amyloidosis.

INTRODUCTION

Immunoglobulin light-chain (AL) amyloidosis, though rare, is the most common form of systemic amyloidosis and is caused by plasma cell production of misfolded light chain which deposits as amyloid fibrils in organs, causing damage and organ dysfunction.[1] Currently, treatment of AL amyloidosis relies on plasma cell-directed therapy, with agents used in the treatment of multiple myeloma (MM) applied to AL amyloidosis, though evidence of safety and efficacy in MM does not always translate directly to AL amyloidosis.[2] By targeting the amyloid-producing plasma cells, new amyloid fibril deposition is diminished,

potentially enabling organ recovery. Melphalan is currently one of the standard treatment options available for patients with AL amyloidosis, often in combination with bortezomib or dexamethasone, or as part of an autologous stem cell transplant.[3–5]

Despite decades of progress for patients with MM and AL amyloidosis, there exists a substantial unmet medical need for novel therapeutics. In AL amyloidosis, patients would benefit from additional effective therapies with better tolerability, considering the increased risk of adverse events due to organ involvement resulting from amyloid deposition,[6] while in MM, there remains a population of patients with high-risk disease who have a poor prognosis and limited remaining therapeutic options.[7,8] These high-risk MM patients are defined by clinical, cytogenetic, and biological features resulting in poor patient outcomes. Such poor patient prognosis is often correlated with the outgrowth of drug-resistant clones which expand in response to inhibition of specific targeted pathways.[9,10] Targeting upstream processes with multiple mechanisms of action that are exploited by pathogenic plasma cells compared to healthy cells are expected to provide therapeutic potential to both MM and AL amyloidosis patients, particularly where clonal evolution has led to the development of these treatment resistant clones.[11]

Melflufen (melphalan flufenamide) is a novel peptide–drug conjugate that rapidly induces cytotoxicity in tumor cells.[12–17] Melflufen is highly lipophilic which results in rapid cellular uptake and hydrolysis by aminopeptidases within cells, culminating in intracellular accumulation of the melphalan alkylating agent.[18] The reduced systemic toxicity of melflufen observed in

[1]Oncopeptides AB, Stockholm, Sweden

[2]Institute for Molecular Medicine Finland - FIMM, HiLIFE – Helsinki Institute of Life Science, iCAN Digital Precision Cancer Medicine Flagship, University of Helsinki, Finland

[3]Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska, USA

SAH and CAH contributed equally to this study.

MM patients in comparison to other alkylators may be particularly relevant in the more fragile amyloidosis patient population.[19] Melflufen is currently being investigated for the treatment of MM.[20–25] Previous data demonstrating MM plasma cell sensitivity for melflufen suggests that the drug might be useful to directly eliminate amyloidogenic plasma cells, thereby reducing the amyloid load in patients.[26,27]

Perhaps as a consequence of increased intracellular concentrations or differential subcellular localization resulting from increased lipophilicity, the enhanced sensitivity to melflufen in targeted tumor cells suggests that the drug is functioning via related, yet potentially distinct, mechanisms from melphalan. As such, it is of interest to dissect and differentiate the cellular mechanism(s) of melflufen activity to better understand the patient populations within MM, as well as additional MM-related indications, including AL amyloidosis, where the drug could provide benefit to patients.

The UPR is a pathway of particular interest in MM and AL amyloidosis, where antibody-producing plasma cells possess an amplified requirement for mechanisms to cope with the increased load of unfolded protein and associated endoplasmic recticulum (ER) stress.[28] Activation of the UPR in plasma cells augments pathways allowing the cell to survive, potentially leading to drug resistance.[29] Drugs with mechanisms of action targeting the UPR, including proteasome inhibitors, function by disrupting the UPR and result in accumulation of unfolded proteins; this leads to apoptotic death of pathogenic plasma cells, and has potential applicability to AL amyloidosis.[30,31] UPR activation is also linked to initiation of immunogenic cell death, which has been implicated in additional mechanisms of antitumor immune responses.[32]

We hypothesized that melflufen may possess enhanced cytotoxicity via effects on the UPR, potentially explaining the increased toxicity on plasma cells. The purpose of this study is to explore the effects of melflufen and melphalan in pathogenic plasma cells, with a specific focus on the UPR pathway.

MATERIALS AND METHODS

Patient material

The Finnish Hematology Registry and Biobank (Helsinki, Finland) provided viably frozen bone marrow mononuclear cells (BM-MNCs) from 6 amyloidosis patients. The samples were collected at diagnosis as bone marrow aspirates after informed consent and following protocols approved by an ethical committee of the Helsinki University Hospital Comprehensive Cancer Center, and in compliance with the Declaration of Helsinki. The BM-MNCs were enriched from the aspirates by gradient centrifugation before cryopreservation. Clinical features are listed in Suppl. Table S1.

Single-cell RNA sequencing

The BM-MNCs were thawed and sorted based on cell viability (7AAD, BD Biosciences, Santa Jose, CA) and CD138 cell surface expression (APC, clone MI15, BD Biosciences) using fluorescence-activated cell sorting (BD Influx Cell Sorter, BD Biosciences). CD138+ and CD138− sorted cell fractions were mixed at a 1:1 ratio with a maximum of 800,000 cells per sample. If there were fewer than 400,000 CD138+ cells, then all CD138+ cells were mixed with 400,000 CD138− cells. Single cell RNA-sequencing library prepration was performed on the mixed CD138+ and CD138+ cell samples using the Chromium Single Cell 3′ Gene Expression v3 reagent kit (10x Genomics, Pleasanton, CA). The prepared libraries were then sequenced on a NovaSeq instrument (Illumina, San Diego, CA).

To assign cell type identity to the clusters, the specific cell type markers for the immune cells were obtained from ScType database.[33] To predict interaction networks in the genes upregulated in plasma cells the "search tool for the retrieval of interacting genes/proteins (STRING)" was used.[34]

Flow cytometry–based drug sensitivity testing

BM-MNCs from 6 amyloidosis patients were tested for melflufen and melphalan sensitivity. The cells were thawed, DNase I (Promega, Madison, WI) treated for 60 minutes, and incubated in conditioned medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L glutamine, penicillin [100 U/mL], streptomycin [100 μg/mL] and 25% conditioned medium from the HS-5 human BM stromal cell line)[35] overnight to exclude apoptotic cells. The cells were plated in conditioned medium (100,000 cells in 100 μL/well) in a 96-well plate in the presence of either melflufen or melphalan at 6 different concentrations between 1 and 100,000 nM. Following incubation for 72 hours at 37°C and 5% CO2, the cells were stained with cell surface marker antibodies for CD138 (BV510, clone MI15) and CD38 (BV421, clone HIT2), followed by staining with apoptotic (Annexin-V) and dead (7-AAD) cell markers (BD Biosciences). Flow cytometry analysis was performed using the IntelliCyt iQue Screener PLUS instrument (Sartorius, Goettingen, Germany). Plasma cells were identified from the live cells based on cell surface expression of both CD138 and CD38 markers. Live cell counts for the CD138+CD38+ cell population were used to analyze effects of different doses on cell viability. The surviving fraction for melflufen- and melphalan-treated cells was computed by normalizing with viability of untreated (0.2% dimethyl sulfoxide) cells.

Cell culture

MM.1S, RPMI-8226, and U266 cells were obtained from American Type Culture. JJN3 cells were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures. ALMC-1 and ALMC-2 cells were obtained from Dr. Diane Jelinek, Mayo Clinic (Rochester, MN).[36] Mycoplasma testing was performed using MycoAlert mycoplasma detection kit (Lonza, Rockland, ME).

Detection of apoptosis by flow cytometry

Cells were grown in the presence or absence of melphalan or melflufen for 24 hours. Cells were washed and stained with APC-conjugated Annexin V antibody and propidium iodide (PI) according to the manufacturer's instruction (eBioscience, San Diego, CA, USA). FlowJo software was used for all data analysis and generation of flow diagrams. We define early apoptotic cells as being AnnexinV+/PI− and late apoptotic cells as AnnexinV+/PI+.

Immunoblotting

Myeloma cells were incubated in the presence or absence of melphalan, melflufen, or brefeldin A (BFA; positive control). Cells were washed with phosphate-buffered saline solution and lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (1% sodium deoxycholate, 0.15 M NaCl, 0.1% SDS, 1% [v/v] Triton X-100, 0.05 M Tris HCl, pH 7.4). The bicinchoninic assay (BCA) method was used for protein quantification. Equal amounts of total protein were run on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes before being probed with primary and secondary antibodies. Images were obtained using an ECL chemiluminescence detection kit and a Bio-Rad ChemiDoc MP imaging system.

Quantitative reverse transcriptase-polymerase chain reaction

Cells were incubated in the presence or absence of drugs before RNA was isolated using an Omega E.Z.N.A. total RNA isolation kit (Omega Bio-tek, Norcross, GA). cDNA was generated from 1 μg of RNA using the i-Script cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA, gene-specific primers, and i-Taq Sybr green super mix (Bio-Rad) were mixed according to manufacturer's instruction. Quantitative reverse transcriptase-polymerase

chain reaction (q-RT-PCR) assays were performed in triplicate in a Bio-Rad CFX96 real time machine and data were analyzed using the Bio-Rad CFX manager 3.1 software. The housekeeping gene β-ACTIN was used for normalization.

XBP-1 splicing

RNA isolation and cDNA synthesis were performed as described above. PCR was performed using XBP-1-specific primers as described by Yan et al.[37] PCR products were separated on a 2% agarose gel, stained with SybrSafe dye (Invitrogen, Carlsbad, CA) and imaged using a Bio-Rad ChemiDoc MP imaging system. The upper band denotes unspliced XBP-1 and the lower band denotes spliced XBP-1.

Light chain enzyme-linked immunosorbent assay

Cells were incubated in the presence of melphalan, melflufen, or BFA (positive control) for 12 hours before being lysed in RIPA buffer containing protease and phosphatase inhibitors (1% sodium deoxycholate, 0.15 M NaCl, 0.1% SDS, 1% [v/v] Triton X-100, 0.05 M Tris HCl, pH 7.4). Protein was quantified using the BCA method. Where indicated, cell culture supernatants were collected for analysis of secreted light chain. The human lambda or kappa light chain kit (Bethyl Laboratories, Montgomery, TX) was used to quantify intracellular monoclonal protein levels by enzyme-linked immunosorbent assayper the manufacturer's instruction.

RESULTS

Melflufen demonstrates superior ex vivo toxicity in plasma cells from patients with AL amyloidosis

Previous data demonstrating superior efficacy of melflufen compared to melphalan in MM cells suggested that there might be similar increased toxicity in the related plasma cell dyscrasia, AL amyloidosis.[12,38] To test this, the cytotoxic effects of melflufen and melphalan were assessed in CD138+CD38+ plasma cells from patients with confirmed AL amyloidosis (Suppl. Table S1) within the BM-MNC fractions. The age range of the patient cohort was 53–81 years, and 4 of 6 samples had light chain of lambda type. Nearly all (5 of 6) of the samples demonstrated significant levels of toxicity (greater than 50%) in response to melflufen (Figure 1A, Suppl. Table S2) below concentrations of 1 µM within 72 hours (half maximal effective concentration [EC_{50}] 0.1833–22.55 nM), while none of the samples responded significantly to melphalan at these concentrations. When these 5 melflufen-sensitive samples were compared for their melphalan responses, it was evident across all patient samples, including those with high-risk cytogenetics, that primary plasma cells from AL amyloidosis patients were significantly more sensitive to melflufen than melphalan (Suppl. Table S2).

These samples were further analyzed by single cell RNA sequencing, revealing clusters of cells with identifiable plasma cell populations (Suppl. Figure S1). As peptidase-mediated hydrolysis of melflufen is required to convert the lipophilic melflufen molecule to an intracellular hydrophilic metabolite with high alkylating potential, we examined expression of aminopeptidase genes across the patient samples. The analysis identified increased expression of DPP7 (log2 fold-change 0.38 to 0.62; P value <0.05), a peptidase gene previously identified as having high capacity for melflufen hydrolysis in cells[39] in plasma cell clusters of 4 of the 6 samples potentially providing the pathogenic cells with the necessary cellular pathways to process melflufen into an active alkylator (Suppl. Figure S2). One patient sample was refractory to both melflufen and melphalan (AL_01), and notably did not demonstrate elevated expression of DPP7 in plasma cells compared to other cell clusters. Future analyses with additional patient samples may be able to identify genes and transcriptional pathways responsible

for resistance to both melphalan and melflufen, as multiple aminopeptidases are capable of hydrolyzing melflufen.[38]

Furthermore, scRNAseq data identified 30 genes with a role in ER stress and UPR that demonstrated increased expression in plasma cell clusters, including DERL3,[40] XBP1,[41] HERPUD1,[42] UBE2J1,[43] RRBP1,[44] SEL1L,[45] PD1A4,[46] LMAN1,[47] and CRELD2[48] (Figure 1B).

Over half (16/30) of these genes were predicted to be located in the ER. Protein–protein interaction (PPI) analysis revealed that many of these genes have significant functional associations with each other (PPI enrichment P value = 1.0e-16), with genes HSP90B1 (n = 12), PDIA4 (n = 10), XBP1 (n = 9), DERL3 (n = 9), and MANF (n = 8) showing the interactions with the maximum number of genes (Suppl. Figure S3). These pathways could contribute to the increased efficacy of melflufen in ex vivo patient samples (Figure 1). We therefore sought to further explore the mechanism of action of melflufen in relationship to ER stress, apoptosis pathways, and UPR.

Melflufen induces superior cytotoxic effects by apoptosis in comparison to melphalan in relevant light chain secreting plasma cell lines

To develop an in vitro cell system to analyze the melflufen mechanism of action relevant to AL amyloidosis patients, the efficacy of melflufen was tested for in vitro cytotoxic activity in one human MM cell line with kappa light chain secretion (JJN3), as well as 2 cell lines derived from an AL amyloidosis patient with lambda light chain secretion (ALMC-1 and ALMC-2) to determine the level of potency in head-to-head comparison with melphalan (Figure 2A) following 72 hours of exposure.

Similar to the results in ex vivo patient samples, melflufen demonstrated increased potency in comparison to melphalan in JJN3 cells, with an approximately 5-fold decrease in EC_{50} values (Suppl. Table S3). In ALMC-1 and ALMC-2 cell lines, melphalan demonstrated minimal toxicity within 72 hours, with undeterminable EC_{50} values at the concentrations tested, while melflufen demonstrated toxicity at low micromolar concentrations. This decrease in cell viability in response to melflufen corresponded with a decrease in kappa light chain secretion into the supernatant in JJN3 cells (with no detectable lambda light chain), and lambda light chain secretion into the supernatant in ALMC-1 and ALMC-2 cells (with no detectable kappa light chain), likely directly due to the decreased cell viability, however leaving open the possibility of decreased light chain production in viable cells as well (Figure 2B). Thus, while melflufen demonstrated potent effects on viability and light chain secretion in all 3 cell lines, melphalan had minimal effects. Notably, DPP7 expression, previously detected in primary amyloidogenic plasma cells, was detectable by qRT-PCR in all 3 cell lines (data not shown).

Melflufen induces cellular events associated with apoptosis

To further explore the mechanism of cell toxicity, a panel of myeloma and light chain secreting cell lines (ALMC-2, JJN3, MM.1S, RPMI-8226, and U266) was exposed to either melflufen or melphalan for 24 hours, and apoptosis was assessed using flow cytometric analysis of Annexin V/propidium iodine staining. These studies revealed enhanced induction of apoptosis pathways mediated by melflufen compared to melphalan across all tested cell lines (Figure 3, Suppl. Table S4).

To more directly assess the capacity of melflufen to induce apoptosis, caspase and poly ADP-ribose polymerase cleavage were analyzed via immunoblotting. Melflufen treatment resulted in caspase cleavage within 12 hours in all tested cell lines at doses at or above 1 µM, while melphalan did not induce caspase cleavage at any of the concentrations tested up to 10 µM (Figure 4). Melflufen induced consistent cleavage of caspases 3, 8, and 9 at doses as low as 1 µM as early as 6 hours after treatment (Suppl. Figure S4), with similar lack of effect on melphalan treatment.
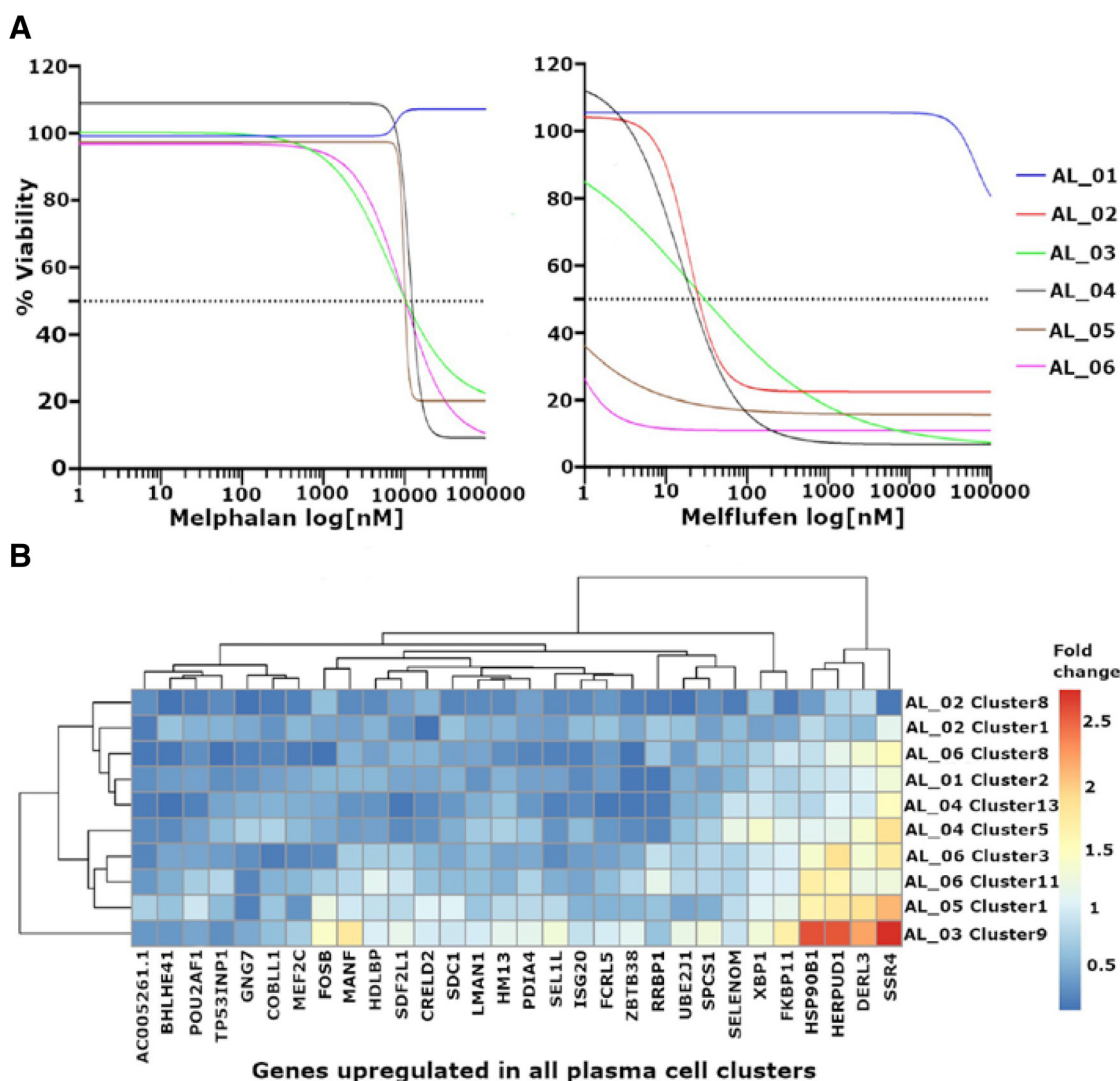
Figure 1. Melflufen demonstrates enhanced efficacy compared to melphalan in ex vivo samples from AL amyloidosis patients. (A) Sensitivity to melphalan (left) or melflufen (right) within CD138+ cells in primary bone marrow mononuclear cell samples from amyloidosis patients (n = 6) following 72 h of treatment was determined by flow cytometry and normalized to vehicle control (100% viability) as described in the Materials and Methods. Different samples showed varying response to melflufen with sample AL_01 being resistant, samples AL_02, AL_03, and AL_04 showing intermediate response and samples AL_05 and AL_06 being highly sensitive to melflufen compared to melphalan. (B) Heatmap showing hierarchical clustering of 30 genes, which were commonly over expressed in plasma cell clusters from 6 amyloidosis samples.

The lipophilicity of the melflufen molecule is expected to increase intracellular concentrations of the alkylator, and may function to redistribute drug within cells, which could lead to an altered, and increased pattern of efficacy. The distinct apoptosis induction mediated by melflufen compared to melphalan suggested a potential separate mechanism of action for the compound.

Melflufen induces markers of ER stress and the UPR, but does not directly affect the production of immunoglobulin light chain

Immunoblot analysis of UPR markers revealed that treatment with melflufen results in an increase of ATF4 and phosphorylated eukaryotic translation initiation factor 2 α (eIF2α) (Figure 5A), but no clear effect on IRE1 expression within 3 hours of treatment. Furthermore, there was a notable increase in spliced XBP-1 in response to 10 µM melflufen treatment within 6 hours in all cells except ALMC-2 cells (Figure 5B). However, XBP-1 cleavage was detected in ALMC-2 cells in response to 10 µM melflufen, but not melphalan, within 12 hours (data not shown). These responses were absent or greatly reduced in

response to equal concentrations of melphalan across cell lines and time points analyzed. In these studies, BFA was used a positive control as an agent which induces the UPR via disruption of ER-golgi trafficking.

Ultimately, the protein kinase RNA-like endoplasmic reticulum kinase (PERK)-mediated pathway of the UPR results in increased expression of C/EBP homologous protein (CHOP), resulting in apoptosis.[49] As ATF4 and phosphorylated eIF2α implicate the PERK branch of the UPR, we examined *CHOP* gene expression by qRT-PCR in response to melphalan or melflufen. Higher doses of melflufen resulted in increased expression of *CHOP* in all cell lines tested within 3 hours, while there was no effect on *CHOP* in response to melphalan (Figure 6).

Because of the potential for effects within the ER, as well as the decrease in immunoglobulin light chain in the supernatant of melflufen-treated cells described earlier, we hypothesized that melflufen might directly affect plasma cell production of light chain separately from the effects on cell toxicity. However, cell lysates of melflufen-treated cells did not demonstrate significant
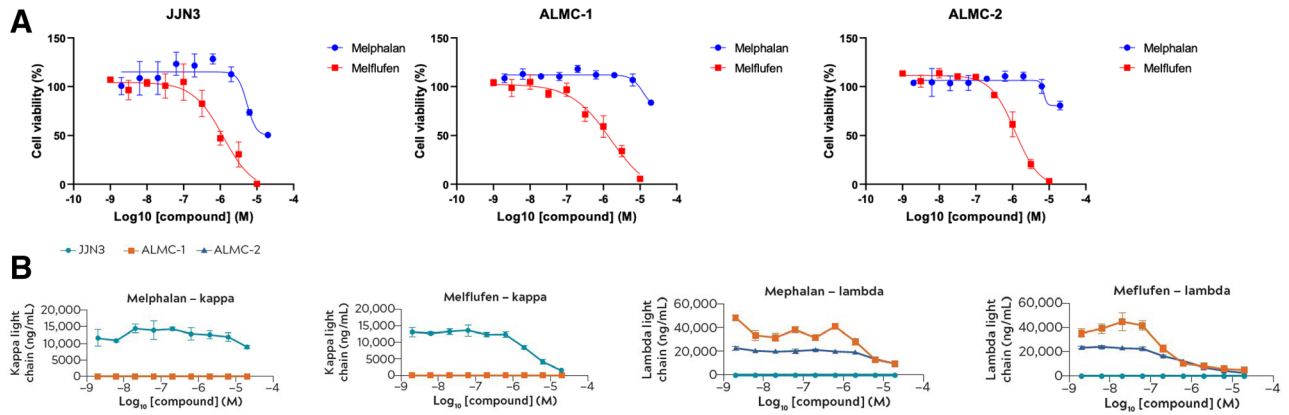
Figure 2. Melflufen demonstrates increased efficacy compared to melphalan in immunoglobulin secreting plasma cell lines. (A) The indicated cell line (JJN3, ALMC-1, or ALMC-2) was incubated for 72 h in the presence of either melphalan (blue) or melflufen (red) at increasing concentrations and cell viability was determined by CellTiter-Blue assay with normalization to vehicle treated cells (100% viability). (B) Kappa light chain secretion was determined in response to increasing concentrations of melphalan or melflufen for the kappa light chain secreting JJN3 cell line, with no detection in the lambda light chain secreting ALMC-1 cell line as a control. Similarly, lambda light chain secretion was determined for the lambda light chain secreting ALMC-1 and ALMC-2 lines, with no detection in the kappa light chain secreting JJN3 cell line as a control.

effects on accumulation of intracellular light chain (Figure 7) in the remaining live cells, indicating that effects on secreted light chains mediated by melflufen are likely the direct result of decreased viability of light chain producing plasma cells and not effects on light chain production or trafficking. It remains possible that the lack of changes in detected intracellular light chain is due to light chain accumulated within cells before melflufen treatment, which remains within cells and further experiments

will be performed to confirm whether melflufen has affects on light chain production separate from the indirect effects mediated by MM cell toxicity.

DISCUSSION

We and others have previously demonstrated toxicity of melflufen in MM cells, with increased tumor cell death by
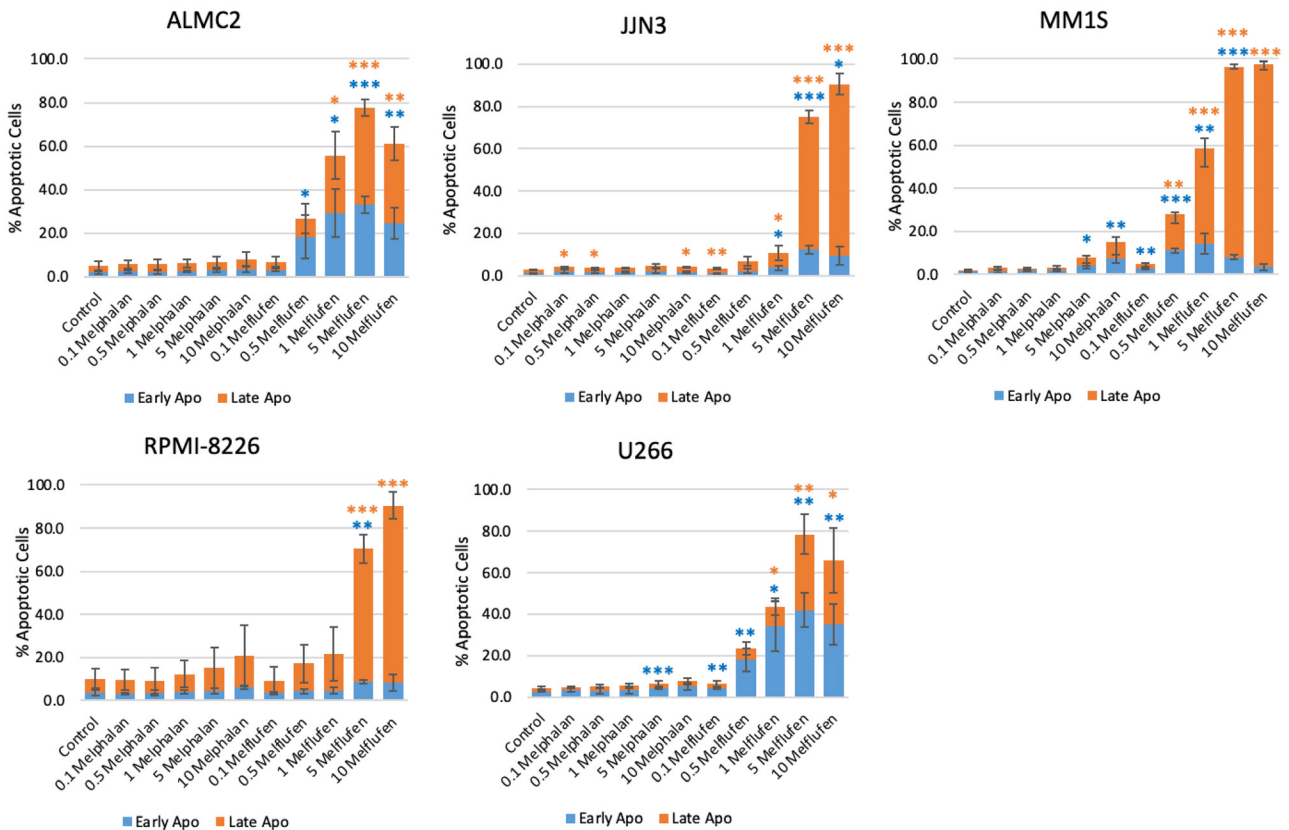

Figure 3. Enhanced induction of apoptosis by melflufen compared to melphalan across all tested MM and AL amyloidosis cell lines. The indicated cell line was incubated for 24 h in the presence of either melphalan or melflufen at increasing concentrations, as indicated, and apoptosis/necrosis was determined by Annexin V/PI staining and flow cytometry. Early apoptosis was defined by Annexin V staining, while late apoptosis was defined by staining both annexin V and PI. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
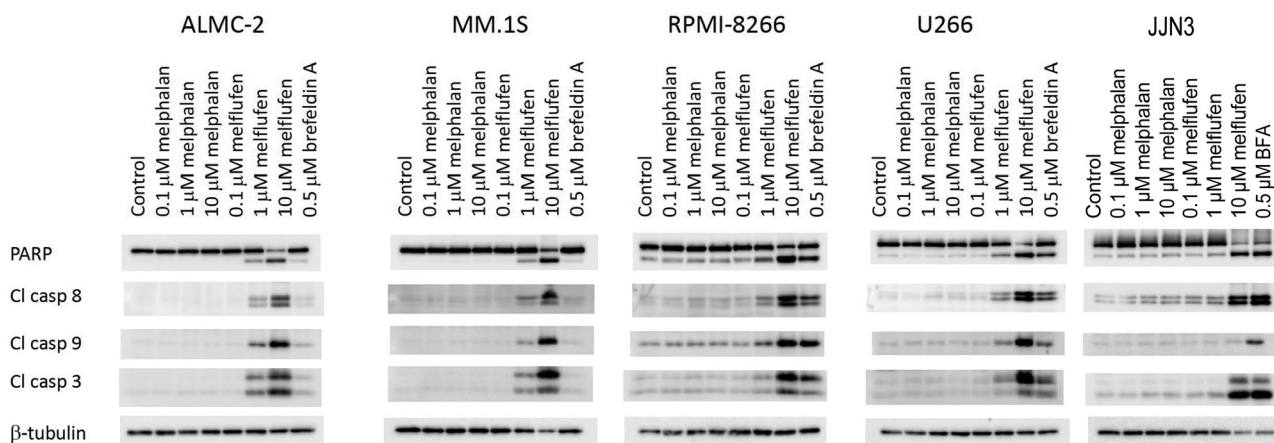
Figure 4. Melflufen mediates cellular events associated with apoptosis within 12 h. The indicated cells were incubated in the presence of either melphalan or melflufen (with brefeldin A as a positive control) for 12 h and cells were harvested and analyzed cleavage of PARP, or caspase 3, 8, and 9 as indicated, by immunoblot analysis, with β-tubulin as a loading control. PARP = poly ADP-ribose polymerase.

apoptosis, resulting in enhanced preclinical efficacy in mouse models compared to melphalan, which is currently used for MM patients.[12] The related pathogenesis between MM and AL amyloidosis has led to an exploration of multiple drugs designed to target MM plasma cells for patients with AL amyloidosis. We therefore sought to extend the melflufen data into amyloidogenic plasma cell models, and to further explore the potentials mechanism(s) of action mediated by melflufen.

Herein, we describe that consistent with data in MM cell lines, melflufen demonstrates superior efficacy to melphalan in amyloidogenic cells, including primary plasma cells from amyloidosis patients. Furthermore, the data have revealed a unique mechanism mediated by melflufen, but not melphalan, via induction of the UPR resulting in rapid induction of apoptosis at clinically relevant drug concentrations. Due to their high rate of protein synthesis, pathogenic plasma cells in MM and amyloidosis are particularly susceptible to therapeutic strategies that target the UPR, and this is an active area of therapeutic intervention.[50] Furthermore, the clonal evolution of plasma cells in both MM and amyloidosis necessitates strategies that target
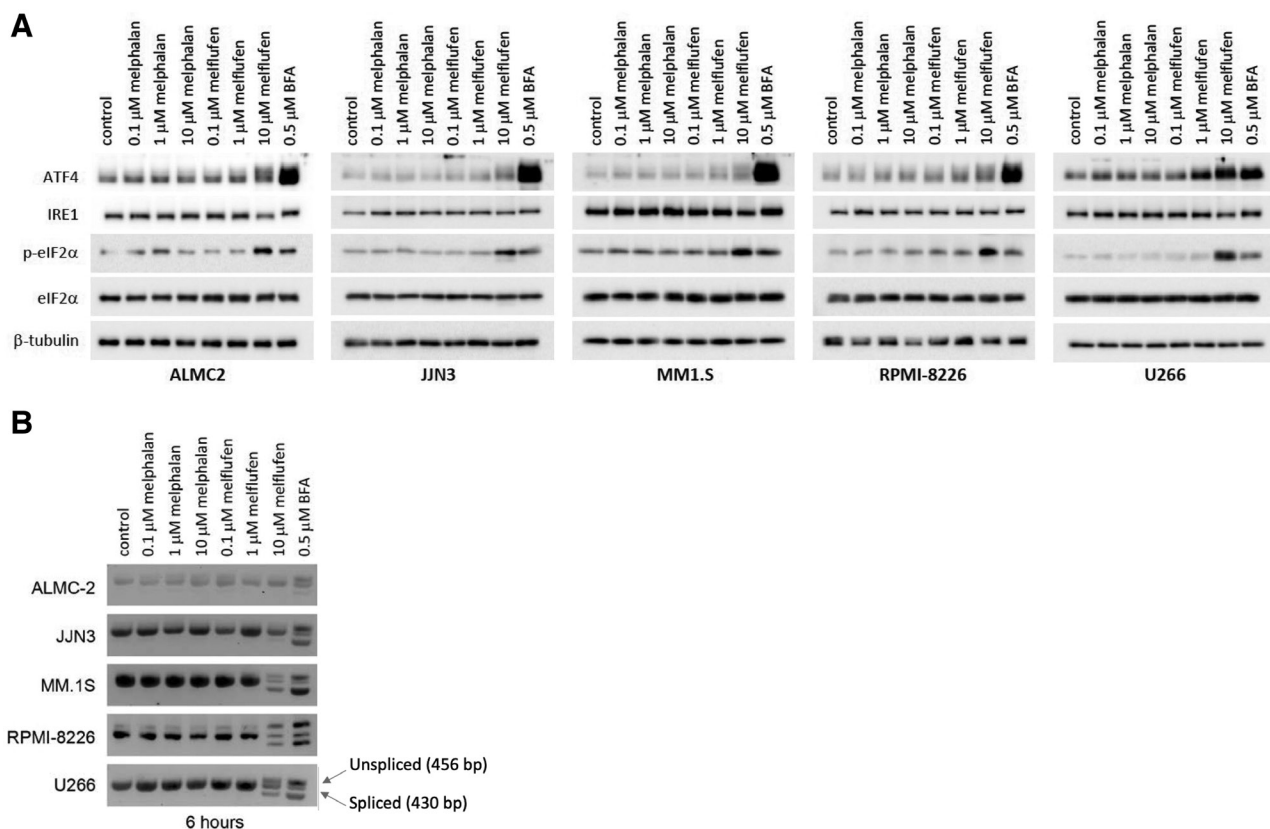

Figure 5. Melflufen induces expression of markers of endoplasmic recticulum stress and the UPR. (A) Immunoblot analysis of UPR markers (ATF4, IRE1, and p-eIF2α) from cells incubated with vehicle control, or 0.1–10 μM melphalan or melflufen for 3 h. β-tubulin and total eIF2α were used as protein controls, while BFA is included as a positive control for UPR induction. (B) PCR analysis of XBP-1 cleavage from cells incubated with 0.1–10 μM melphalan or melflufen for 6 h. BFA is included as a positive control for UPR induction. BFA = brefeldin A; PCR = polymerase chain reaction; UPR = unfolded protein response.
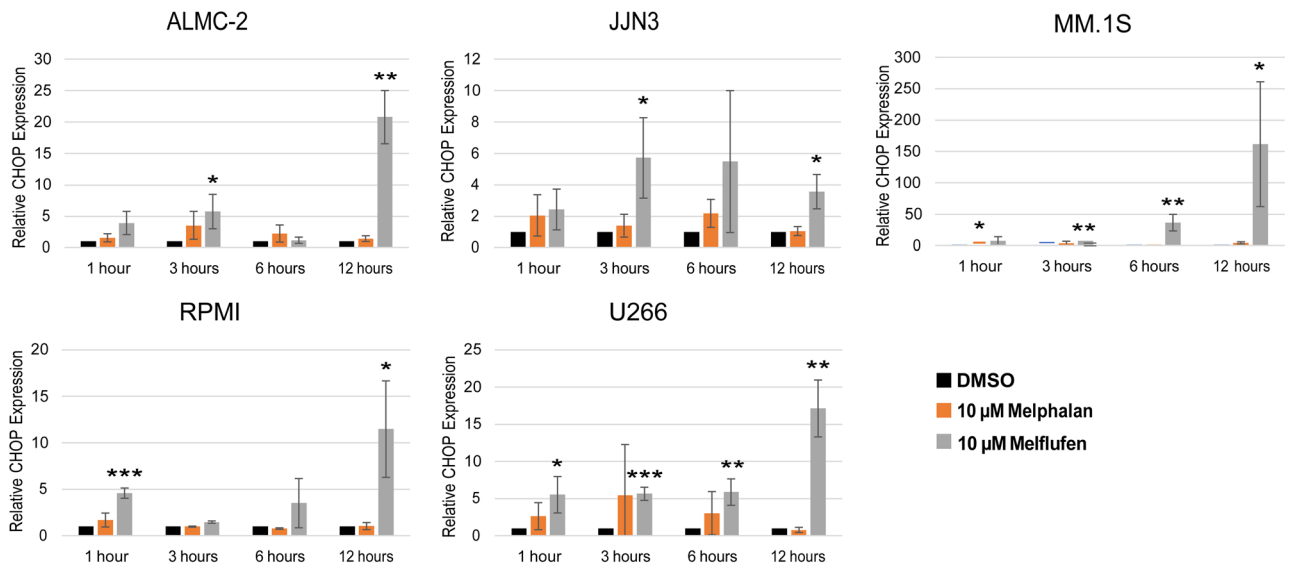
Figure 6. Melflufen induces upregulation of CHOP. The indicated cell lines were incubated with either melphalan or melflufen (10 μM) for 1, 3, 6, or 12 h before analysis for CHOP expression by qRT-PCR (Suppl. Table S5). DMSO was used as the vehicle control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. CHOP = C/EBP homologous protein; DMSO = dimethyl sulfoxide.

multiple mechanisms of action that obviate resistance mechanisms of pathogenic plasma cells.[51] The study is limited in scope to in vitro analysis due to an unfortunate lack of appropriate animal models of amyloidosis.[52] However, previous studies of xenografts of MM.1S cells demonstrated in vivo activity of melflufen consistent with our in vitro results.[52]

We compared the toxicity of melflufen to the clinically used drug melphalan, and demonstrated that plasma cells producing amyloidogenic light chains were considerably more sensitive to melflufen than melphalan, with particular sensitivity in ALMC-1 and ALMC-2 lines.[36] In these two lines, melflufen demonstrated low micromolar EC_{50} values, while melphalan did not induce demonstrable toxicity. The toxicity mediated by melflufen correlated with an overall decrease in secreted light

chain, likely a directly result of cell death, as we did not detect significant diminishment of light chain production within live cells at subtoxic doses of melflufen. The reduction of light chain by targeting the pathogenic plasma cells could provide a benefit to AL amyloidosis patients where accumulation of light chain fibrils is believed to be the cause of organ toxicity in the disease.

As the mechanism of action mediated by melflufen appears linked to plasma cell death, rather than a direct decrease in light chain production, we further explored the disparate effects mediated by melflufen compared to melphalan. Across 5 cell lines tested, melflufen demonstrated toxicity by apoptosis at doses at or below 5 μM, while melphalan demonstrated limited, if any, induction of apoptosis. Whether enhanced induction of
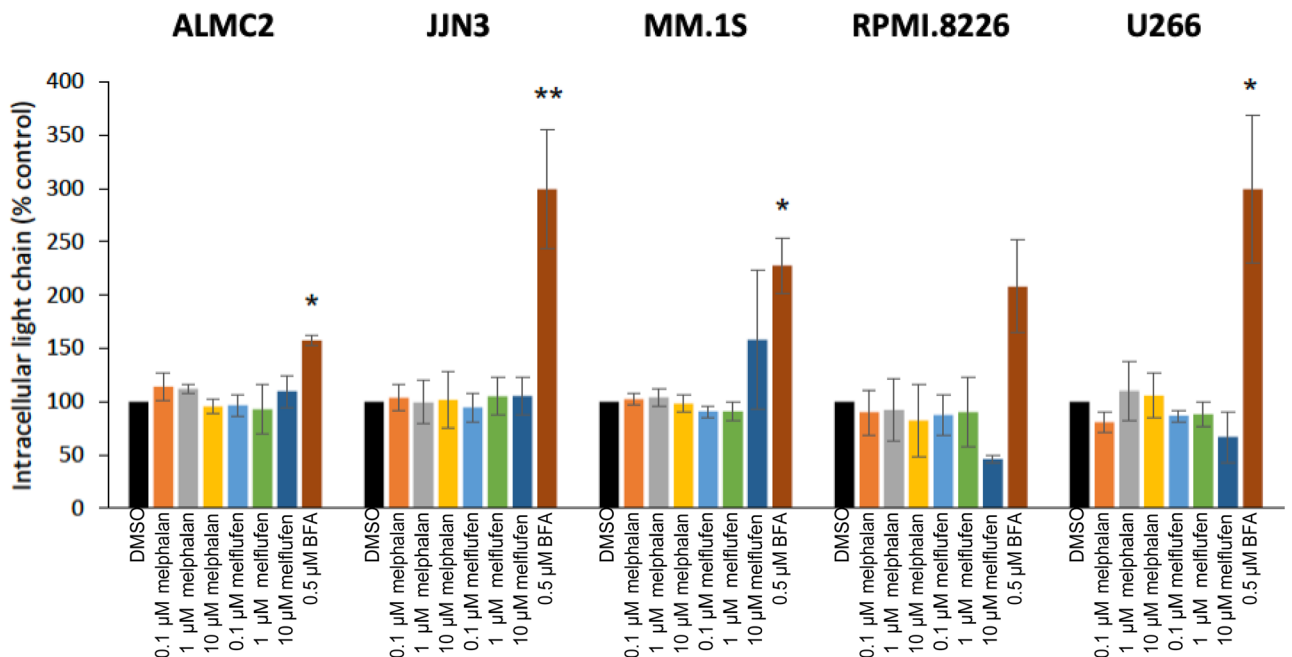

Figure 7. Melflufen does not directly affect the intracellular light chain levels in viable cells after 12 h of treatment. Whole cell lysate subjected to kappa (JJN3) or lambda (ALMC-2, MM.1S, RPMI-8226, U266) light chain ELISA. Brefeldin A was included as a positive control. *$P < 0.05$, **$P < 0.01$. ELISA = enzyme-linked immunosorbent assay.

apoptosis mediated by melflufen is the result of increased cellular uptake, altered intracellular distribution or other unique mechanisms mediated by melflufen is an area of active study.

Evidence for induction of the UPR pathway by melphalan was limited to cells treated at higher concentrations and was only seen in sensitive cell lines at later time points, while melflufen induction of the UPR was rapid and included both sensitive and resistant cell lines. Induction of the UPR is believed to occur through 3 distinct branches, mediated by ATF6, PERK, or IRE1, respectively.[53] While the observed XBP-1 cleavage mediated by melflufen (Figure 5B) is shared between the IRE1 and PERK pathway, analysis of components of the ATF6 and IRE1 pathway in response to melflufen did not demonstrate consistent evidence of activation (data not shown). However, induction of phosphorylated eIF2α and ATF4 in multiple plasma cell lines (Figure 5A) implicates the PERK pathway, which is of particular interest with regard to plasma cells.[54] Furthermore, melflufen-mediated upregulation of CHOP (Figure 6) is a late event in the PERK UPR pathway connecting these cellular pathways to the observed tumor cell apoptosis (Figure 3). While induction of the PERK pathway did not appear to directly affect protein trafficking in viable cells (Figure 7), apoptosis mediated by melflufen, subsequent to PERK activation, was sufficient to limit production of light chain from amyloidogenic plasma cells (Figure 2).

This additional mechanism of action mediated by melflufen through UPR activation suggests a potential synergy with its established role as an alkylating agent. Multiple mechanisms of action could allow melflufen to circumvent standard cellular mechanisms of drug resistance which inevitably develop in response to current therapeutics.[55] Therapeutic options targeting additional mechanisms of action in MM and AL amyloidosis are necessary to overcome these significant challenges in both MM and AL amyloidosis.[56] Bortezomib is currently used as a standard treatment option for patients with AL amyloidosis in combination with melphalan to effectively combine 2 distinct mechanisms of action[57]: modulation of ER stress[58] and DNA alkylation.[59] It is tempting to speculate that melflufen, by virtue of combining both mechanisms in a single moiety could further synergize with proteasome inhibitors, and comparisons of melflufen to bortezomib, both alone and in combination, are an area of active research.

Furthermore, effective plasma cell directed therapeutics have advantages compared with immunotherapeutic targeting of toxic light chain species which not only require identification of the specific toxic amyloid species but are not expected to affect the continuous plasma cell production of toxic light chain from amyloidogenic plasma cells. This provides a challenge to generating the level of suppression of toxic light chain production required for clinical efficacy.[60]

Melflufen provides a potential opportunity to achieve maximum therapeutic benefit by combining plasma cell–directed therapeutics with antifibril approaches in clinical development.[61]

It is interesting to note that a significant number of the genes identified with increased expression in plasma cells of AL amyloidosis patients compared with other cell populations have a role in ER stress and UPR, underscoring the importance of these pathways in the biology of myeloma and amyloidogenic plasma cells. The significance of this pathway opens the possibility that the enhanced lipophilicity of melflufen leads not only to increased uptake by cells, but perhaps directs redistribution within cells into cellular compartments, including the ER and/or Golgi apparatus, otherwise inaccessible to melphalan.

In particular, higher expression of DPP7, a peptidase with the potential to hydrolyze melflufen, was detected in ex vivo patient samples of plasma cells with melflufen sensitivity. While the small sample size precludes a conclusive determination of a causative role of *DPP7* in the sensitivity of amyloidogenic plasma cells to melflufen, these data are consistent with a role for peptidase expression in general, and *DPP7* in particular.[39] Previous data indicated a role for DPP7 in hydrolysis of melflufen required for

toxicity in breast cancer cells.[39] Increased expression of peptidases responsible for melflufen cleavage within targeted pathogenic plasma cells could provide an additional layer of specificity for the drug, with potential implications for increased efficacy without increasing toxicity. Additional single cell sequencing with a larger data set may permit a better understanding of the mechanism of both melphalan and melflufen resistance in amyloidogenic cells, to tailor treatments and identify patient populations more likely to respond to treatment.

In summary, these data extend previous findings regarding the efficacy of melflufen into amyloidogenic plasma cells, and further expand the potential mechanism of action to include effects on the UPR. While the kinetics and sensitivity of cell lines varied somewhat, the totality of the data indicates that melflufen induction of apoptosis and ER stress response is superior to melphalan in plasma cells. Although additional clinical aspects of the melflufen therapy in MM are currently investigated, results presented in this study focus on the preclinical aspect in AL-amyloidosis with interesting scientific results adding valuable knowledge on the biology and the potential of alkylating agents and the concept of peptide–drug conjugate drugs like melflufen in this disease. The increased efficacy with melflufen compared with melphalan suggests clinical efficacy in amyloidosis for melflufen above what has already been demonstrated for the clinically used drug, melphalan, with the potential for lower efficacious doses and consequently attenuated systemic toxicity.

REFERENCES

1. Merlini G, Dispenzieri A, Sanchorawala V, et al. Systemic immunoglobulin light chain amyloidosis. *Nat Rev Dis Primers*. 2018;4:38.
2. Koh Y. AL amyloidosis: advances in diagnosis and management. *Blood Res*. 2020;55(suppl 1):S54–S57.
3. Aimo A, Buda G, Fontana M, et al. Therapies for cardiac light chain amyloidosis: an update. *Int J Cardiol*. 2018;271:152–160.
4. Milani P, Palladini G. Conventional therapy for amyloid light-chain amyloidosis. *Acta Haematol*. 2020;143:365–372.
5. Roccatello D, Fenoglio R, Sciascia S, et al. CD38 and Anti-CD38 monoclonal antibodies in AL amyloidosis: targeting plasma cells and beyond. *Int J Mol Sci*. 2020;21:E4129.
6. Basset M, Nuvolone M, Palladini G, et al. Novel challenges in the management of immunoglobulin light chain amyloidosis: from the bench to the bedside. *Expert Rev Hematol*. 2020;13:1003–1015.
7. Muchtar E, Magen H, Gertz MA. High-risk multiple myeloma: a multifaceted entity, multiple therapeutic challenges. *Leuk Lymphoma*. 2017;58:1283–1296.
8. Kastritis E, Dimopoulos MA. Recent advances in the management of AL Amyloidosis. *Br J Haematol*. 2016;172:170–186.

9. Furukawa Y, Kikuchi J. Molecular basis of clonal evolution in multiple myeloma. *Int J Hematol*. 2020;111:496–511.

10. Mishima Y, Mishima Y, Shirouchi Y, et al. The clonal evolution during long-term clinical course of multiple myeloma. *Int J Hematol*. 2020;113:279–284.

11. Anderson KC. Progress and paradigms in multiple myeloma. *Clin Cancer Res*. 2016;22:5419–5427.

12. Wickström M, Nygren P, Larsson R, et al. Melflufen - a peptidase-potentiated alkylating agent in clinical trials. *Oncotarget*. 2017;8:66641–66655.

13. Byrgazov K, Anderson C, Salzer B, et al. Targeting aggressive osteosarcoma with a peptidase-enhanced cytotoxic melphalan flufenamide. *Ther Adv Med Oncol*. 2020;12:1758835920937891.

14. Strese S, Hassan SB, Velander E, et al. In vitro and in vivo anti-leukemic activity of the peptidase-potentiated alkylator melflufen in acute myeloid leukemia. *Oncotarget*. 2017;8:6341–6352.

15. Carlier C, Strese S, Viktorsson K, et al. Preclinical activity of melflufen (J1) in ovarian cancer. *Oncotarget*. 2016;7:59322–59335.

16. Delforoush M, Strese S, Wickström M, et al. In vitro and in vivo activity of melflufen (J1)in lymphoma. *BMC Cancer*. 2016;16:263.

17. Holstein SA, Hillengass J, McCarthy PL. Melflufen: a next-generation nitrogen mustard. *J Clin Oncol*. 2021;39:836–839.

18. Berglund A, Ullén A, Lisyanskaya A, et al. First-in-human, phase I/IIa clinical study of the peptidase potentiated alkylator melflufen administered every three weeks to patients with advanced solid tumor malignancies. *Invest New Drugs*. 2015;33:1232–1241.

19. Merlini G. AL amyloidosis: from molecular mechanisms to targeted therapies. *Hematology Am Soc Hematol Educ Program*. 2017;2017:1–12.

20. Richardson PG, Bringhen S, Voorhees P, et al. Melflufen plus dexamethasone in relapsed and refractory multiple myeloma (O-12-M1): a multicentre, international, open-label, phase 1-2 study. *Lancet Haematol*. 2020;7:e395–e407.

21. Oriol A, Larocca A, Leleu X, et al. Melflufen for relapsed and refractory multiple myeloma. *Expert Opin Investig Drugs*. 2020;29:1069–1078.

22. Schjesvold F, Robak P, Pour L, et al. OCEAN: a randomized Phase III study of melflufen + dexamethasone to treat relapsed refractory multiple myeloma. *Future Oncol*. 2020;16:631–641.

23. Terpos E, Ntanasis-Stathopoulos I. Multiple myeloma: clinical updates From the American Society of Hematology Annual Meeting 2018. *Clin Lymphoma Myeloma Leuk*. 2019;19:e324–e336.

24. Bringhen S, Voorhees PM, Plesner T, et al. Melflufen plus dexamethasone in relapsed/refractory multiple myeloma: long-term survival follow-up from the Phase II study O-12-M1. *Br J Haematol*. 2021;193:1105–1109.

25. Dhillon S. Melphalan flufenamide (Melflufen): first approval. *Drugs*. 2021;81:963–969.

26. Ray A, Ravillah D, Das DS, et al. A novel alkylating agent Melflufen induces irreversible DNA damage and cytotoxicity in multiple myeloma cells. *Br J Haematol*. 2016;174:397–409.

27. Chakraborty R, Lentzsch S. Emerging drugs for the treatment of light chain amyloidosis. *Expert Opin Emerg Drugs*. 2020;25:299–317.

28. Hetz C, Papa FR. The unfolded protein response and cell fate control. *Mol Cell*. 2018;69:169–181.

29. Nikesitch N, Lee JM, Ling S, et al. Endoplasmic reticulum stress in the development of multiple myeloma and drug resistance. *Clin Transl Immunology*. 2018;7:e1007.

30. Nikesitch N, Ling SC. Molecular mechanisms in multiple myeloma drug resistance. *J Clin Pathol*. 2016;69:97–101.

31. Cooley CB, Ryno LM, Plate L, et al. Unfolded protein response activation reduces secretion and extracellular aggregation of amyloidogenic immunoglobulin light chain. *Proc Natl Acad Sci U S A*. 2014;111:13046–13051.

32. Rufo N, Garg AD, Agostinis P. The unfolded protein response in immunogenic cell death and cancer immunotherapy. *Trends Cancer*. 2017;3:643–658.

33. Ianevski A, Giri AK, Aittokallio T. Fully-automated cell-type identification with specific markers extracted from single-cell transcriptomic data. bioRxiv preprint 2021. doi: 10.1101/812131.

34. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607–D613.

35. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood*. 1995;85:997–1005.

36. Arendt BK, Ramirez-Alvarado M, Sikkink LA, et al. Biologic and genetic characterization of the novel amyloidogenic lambda light chain-secreting human cell lines, ALMC-1 and ALMC-2. *Blood*. 2008;112:1931–1941.

37. Yan Y, Gao YY, Liu BQ, et al. Resveratrol-induced cytotoxicity in human Burkitt's lymphoma cells is coupled to the unfolded protein response. *BMC Cancer*. 2010;10:445.

38. Miettinen JJ, Kumari R, Traustadottir GA, et al. Aminopeptidase expression in multiple myeloma associates with disease progression and sensitivity to melflufen. *Cancers (Basel)*. 2021;13:1527.

39. Schepsky A, Traustadottir GA, Joelsson JP, et al. Melflufen, a peptide-conjugated alkylator, is an efficient anti-neo-plastic drug in breast cancer cell lines. *Cancer Med*. 2020;9:6726–6738.

40. Oda Y, Okada T, Yoshida H, et al. Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J Cell Biol*. 2006;172:383–393.

41. Yoshida H, Matsui T, Yamamoto A, et al. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*. 2001;107:881–891.

42. Ma Y, Hendershot LM. Herp is dually regulated by both the endoplasmic reticulum stress-specific branch of the unfolded protein response and a branch that is shared with other cellular stress pathways. *J Biol Chem*. 2004;279:13792–13799.

43. Elangovan M, Chong HK, Park JH, et al. The role of ubiquitin-conjugating enzyme Ube2j1 phosphorylation and its degradation by proteasome during endoplasmic stress recovery. *J Cell Commun Signal*. 2017;11:265–273.

44. Tsai HY, Yang YF, Wu AT, et al. Endoplasmic reticulum ribosome-binding protein 1 (RRBP1) overexpression is frequently found in lung cancer patients and alleviates intracellular stress-induced apoptosis through the enhancement of GRP78. *Oncogene*. 2013;32:4921–4931.

45. Sopha P, Ren HY, Grove DE, et al. Endoplasmic reticulum stress-induced degradation of DNAJB12 stimulates BOK accumulation and primes cancer cells for apoptosis. *J Biol Chem*. 2017;292:11792–11803.

46. Tufo G, Jones AW, Wang Z, et al. The protein disulfide isomerases PDIA4 and PDIA6 mediate resistance to cisplatin-induced cell death in lung adenocarcinoma. *Cell Death Differ*. 2014;21:685–695.

47. Nyfeler B, Nufer O, Matsui T, et al. The cargo receptor ERGIC-53 is a target of the unfolded protein response. *Biochem Biophys Res Commun*. 2003;304:599–604.

48. Oh-hashi K, Koga H, Ikeda S, et al. CRELD2 is a novel endoplasmic reticulum stress-inducible gene. *Biochem Biophys Res Commun*. 2009;387:504–510.

49. Hu H, Tian M, Ding C, et al. The C/EBP Homologous Protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. *Front Immunol*. 2018;9:3083.

50. Vangala JR, Potluri A, Radhakrishnan SK. BET inhibitors synergize with carfilzomib to induce cell death in cancer cells via impairing Nrf1 transcriptional activity and exacerbating the unfolded protein response. *Biomolecules*. 2020;10:E501.

51. Kumar SK. Targeted management strategies in multiple myeloma. *Cancer J*. 2019;25:59–64.

52. Chauhan D, Ray A, Viktorsson K, et al. In vitro and in vivo antitumor activity of a novel alkylating agent, melphalan-flufenamide, against multiple myeloma cells. *Clin Cancer Res*. 2013;19:3019–3031.

53. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011;334:1081–1086.

54. Obeng EA, Carlson LM, Gutman DM, et al. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood*. 2006;107:4907–4916.

55. Saltarella I, Desantis V, Melaccio A, et al. Mechanisms of resistance to anti-CD38 daratumumab in multiple myeloma. *Cells*. 2020;9:E167.

56. García-Guerrero E, Sierro-Martínez B, Pérez-Simón JA. Overcoming chimeric antigen receptor (CAR) modified T-cell therapy limitations in multiple myeloma. *Front Immunol*. 2020;11:1128.

57. Kastritis E, Leleu X, Arnulf B, et al. Bortezomib, melphalan, and dexamethasone for light-chain amyloidosis. *J Clin Oncol*. 2020;38:3252–3260.

58. Ri M. Endoplasmic-reticulum stress pathway-associated mechanisms of action of proteasome inhibitors in multiple myeloma. *Int J Hematol*. 2016;104:273–280.

59. Esma F, Salvini M, Troia R, et al. Melphalan hydrochloride for the treatment of multiple myeloma. *Expert Opin Pharmacother*. 2017;18:1127–1136.

60. Parrondo RD, Majeed U, Sher T. Antibody-based immunotherapy for treatment of immunoglobulin light-chain amyloidosis. *Br J Haematol*. 2020;191:673–681.

61. Popkova T, Hajek R, Jelinek T. Monoclonal antibodies in the treatment of AL amyloidosis: co-targeting the plasma cell clone and amyloid deposits. *Br J Haematol*. 2020;189:228–238.