Poster 3100

In Vitro and *In Vivo* Activity of Melflufen in Amyloidosis

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BACKGROUND

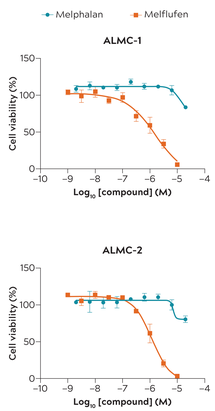
- Immunoglobulin light chain (AL) amyloidosis is a rare disease caused by plasma cell secretion of misfolded light chains that assemble as amyloid fibrils and deposit on vital organs, including the heart and kidneys, causing organ dysfunction
- Plasma cell directed therapeutics, aimed at preferentially eliminating the clonal population of amyloidogenic cells in bone marrow, are expected to reduce production of toxic light chains and alleviate deposition of amyloid, thereby restoring healthy organ function
- Melphalan flufenamide ethyl ester, melflufen, is a peptide-drug conjugate with potent toxicity in myeloma cells
- Melflufen is highly lipophilic, permitting rapid cellular uptake, and is subsequently enzymatically cleaved by aminopeptidases within cells, resulting in augmented intracellular concentrations of toxic molecules, providing a more targeted and localized treatment
- Previous data demonstrating multiple myeloma plasma cell sensitivity for melflufen suggest that the drug might be useful for directly eliminating amyloidogenic plasma cells, thereby reducing the amyloid load in patients
- Increased intracellular concentrations of melflufen in myeloma cells indicates a potential reduction in systemic toxicity in patients, an important factor in the fragile amyloidosis patient population

METHODS

- Cellular toxicity and apoptosis were measured in response to either melflufen or melphalan in multiple malignant human plasma cell lines, including the amyloidosis patient-derived light chain secreting ALMC-1 and ALMC-2 cells, as well as primary bone marrow cells from AL amyloidosis patients, using annexin V and live/dead cell staining by multicolor flow cytometry and measurement of cleaved caspases
- Lambda and kappa light chain secretion was measured in the supernatant of cells by ELISA
- Bone marrow aspirates were taken from amyloidosis patients at diagnosis stage (n=10) after informed consent and following approved protocols in accordance with the Declaration of Helsinki. CD138+ enriched mononuclear cells were loaded to the 10x Genomics Chromium Single Cell 3′RNAseq platform for studying single-cell gene expression profiles. The Chromium Single Cell 3′RNAseq run and library preparation were done using Chromium™ Single Cell 3′ Reagent v3 chemistry. 10x Genomics Cell Ranger v3.0.1 pipelines were used for the initial data processing, including alignments against human genome GRCh38. Furthermore, the clustering and differential gene expression of the data was performed using Seurat 3.0
- MM.1S, RPMI-8826, U266 or ALMC-2 cells were incubated in the presence or absence of compound at the indicated concentrations; cells were harvested and analyzed for annexin V/propidium iodide by flow cytometry, and cell lysates or DNA were purified and analyzed by either PCR or western blot analysis for established markers of apoptosis and/or UPR
- In a pilot study to develop a model to assess the efficacy of melflufen, the light chain secreting human myeloma cell line, JJN3, was transduced with luciferase and adoptively transferred into NSG mice. Cell death in response to melflufen or melphalan will be measured by *in vivo* bioluminescence, and serum light chain in subsequent studies

RESULTS

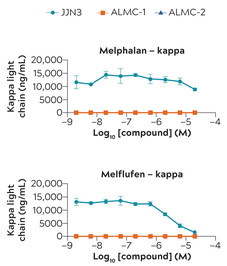
Figure 1: Melflufen-mediated sensitivity of light chain producing plasma cell lines



	ALMC-1 IC_{50} (μM)	ALMC-2 IC_{50} (μM)	JJN3 IC_{50} (μM)
Melphalan	–	–	5.4
Melflufen	1.6	1.2	1.2

- **Methods:** Cells were plated in a 96-well format in the presence of the indicated compound in doses ranging from 0.014 to 10 mM for 72 hours at 37°C. Cell viability was determined by CellTiter-blue with the manufacturer's standard protocol
- **Results:** All three light chain secreting cell lines (JJN3, ALMC-1 and ALMC-2) tested demonstrated increased sensitivity to melflufen compared with melphalan

Figure 2: Melflufen-mediated reduction in light chain secretion in plasma cell lines



Kappa ELISA

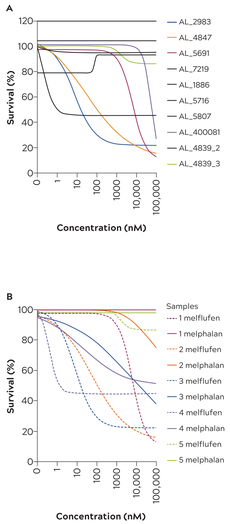
	AMCL-1 IC_{50} (μM)	AMCL-2 IC_{50} (μM)	JJN3 IC_{50} (μM)
Melphalan	NA	NA	ND
Melflufen	NA	NA	2.9

Lambda ELISA

	AMCL-1 IC_{50} (μM)	AMCL-2 IC_{50} (μM)	JJN3 IC_{50} (μM)
Melphalan	2.5	5.1	NA
Melflufen	0.20	0.45	NA

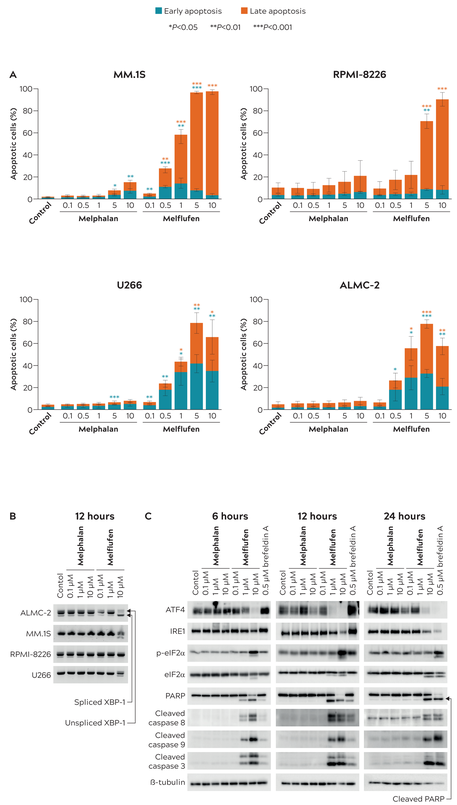
- **Methods:** Cells were plated in a 96-well format in the presence of the indicated compound in doses ranging from 0.014 to 10 mM for 72 hours at 37°C in three cell lines with established lambda (ALMC-1 and ALMC-2) or kappa (JJN3) light chain secretion. Human kappa and lambda light chain secretion was determined in the supernatant by ELISA using commercial kits and the manufacturer's standard protocols
- **Results:** Melflufen demonstrated superior inhibition of light chain secretion to melphalan in all three amyloidogenic plasma cell lines

Figure 3: Sensitivity to melflufen within CD138+ cells in primary bone marrow samples from amyloidosis patients



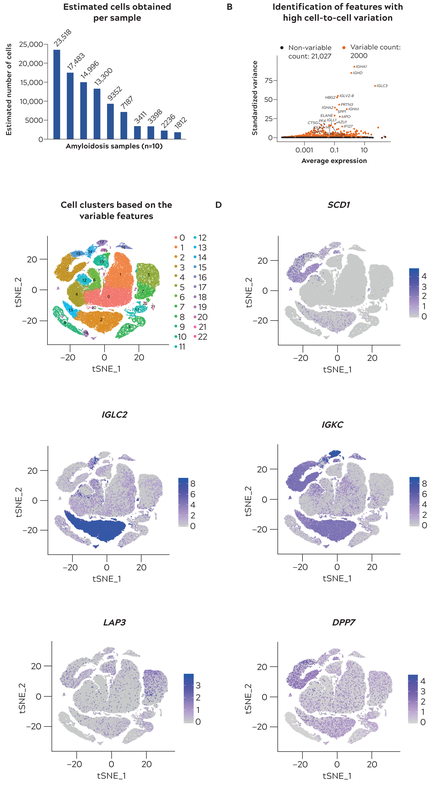
- **Methods:** Viably frozen bone marrow mononuclear cells from 10 amyloidosis patients were selected based on optimal viability. Viably frozen cells were thawed and incubated overnight to exclude apoptotic cells. Cells were plated in a 96-well format in the presence of either melflufen or melphalan at concentrations between 1 and 100,000 nM. Plasma cells were identified by multiplexed flow cytometry based on expression of CD138, and cell survival was determined by 7-AAD and expression of annexin V surface antigens. Live cell counts for each cell population were used to analyze effect of different doses on cell viability. Surviving fraction for melflufen dilutions were computed by normalizing with viability of untreated cells for each cell subset
- **Results:** A) 50% (5 out of 10) of samples from amyloidosis patients (n=9) demonstrated responsiveness to melflufen at concentrations below 10,000 nM. B) CD138+ plasma cells from amyloidosis patients with demonstrated responsiveness to melflufen exhibited significantly increased sensitivity compared with melphalan

Figure 4: Effects of melflufen on apoptosis and markers of UPR



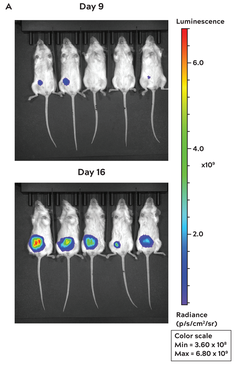
- **Methods:** The indicated cells were incubated in the presence or absence of compound at the indicated concentrations. Following 24 hours of incubation, cells were harvested and analyzed for annexin V/propidium iodide by flow cytometry (A), XBP-1 splicing by PCR (B), or the indicated UPR and apoptosis marker by western blot (C), which was analyzed at multiple time points
- **Results:** All cell lines tested were significantly more sensitive to melflufen than melphalan with regard to inducing apoptosis, stimulating endoplasmic reticulum stress as indicated by XBP-1 splicing, as well as stimulation of multiple markers of UPR and apoptosis

Figure 5: Single-cell sequencing data in CD138+ cells in primary bone marrow samples from amyloidosis patients



- **Methods:** Bone marrow aspirates from 10 individual subjects with amyloidosis were analyzed by single-cell sequencing.
- **Results:** A) A minimum of 1800 individual cells were analyzed for each patient sample. B) Analysis of heterogeneous bone marrow samples identified genes with high cell-to-cell variability, including transcripts consistent with amyloidosis pathogenesis such as immunoglobulin light chains. C) The heterogeneous bone marrow samples were clustered based on variable gene expression identifying discrete populations of cells that can be used for additional analyses, including expression of genes associated with amyloidogenic plasma cells, as well as genes associated with the melflufen mechanism of action, such as *LAP3* and *DPP7* (*D*).

Figure 6: Pilot xenograft mouse study to test the efficacy of melflufen in a model of amyloidosis



BCMA (pg/mL)	855	2303	686	0	0
Kappa light chain (pg/mL)	742	2431	987	0	0
Tumor burden (BLI)	6.02 × 10^6	7.63 × 10^6	5.51 × 10^6	1.87 × 10^6	1.01 × 10^6

- **Methods:** The human myeloma cell line, JJN3, was transduced with luciferase for *in vivo* monitoring of tumor burden (A) by *in vivo* bioluminescent imaging following luciferin administration on days 6, 9, 13 and 16 after tumor inoculation into female NSG mice. B) Serum levels of BCMA and kappa and lambda light chains were monitored by ELISA on days 9 and 16 after tumor inoculation using the manufacturer's protocols for each
- **Results:** JJN3 tumors were detectable in all inoculated mice, although tumor burden was variable. BCMA and kappa light chains were detectable in the serum of 3/5 mice, which corresponded to the mice with the greatest detected tumor burden

CONCLUSIONS

- Melflufen demonstrated superior efficacy in amyloidogenic plasma cell lines compared with melphalan, with increased plasma cell death and decreased secretion of light chains
- *Ex vivo* patient samples demonstrated an increased probability of response to melflufen compared with melphalan treatment
- IC_{50} values associated with light chain secretion were superior to those associated with cellular viability, suggesting efficacy in affecting amyloidogenic potential of the cells at doses below those considered toxic to plasma cells and further opening the possibility of improving the light chain load in patients without overt amyloidogenic plasma cell toxicity in response to melflufen
- Single-cell sequencing with heterogeneous bone marrow samples from amyloidosis patients has identified discrete populations of cells, including plasma cells with high expression of immunoglobulin genes. Further analysis of these data will explore these populations of cells for differential expression patterns, identifying patients whose amyloidogenic cells are sensitive to melflufen
- Melflufen demonstrated significant effects on apoptosis, with increased induction of apoptotic and UPR markers in comparison with melphalan
- An animal model using transfer of amyloidogenic plasma cells that are sensitive to melflufen *ex vivo* has been established and will be used to analyze the *in vivo* effects of melflufen on both amyloidogenic plasma cell toxicity and serum levels of BCMA and light chains

DISCLOSURES

Flanagan: *Oncopeptides AB*: Employment. **Slipicevic**: *Oncopeptides AB*: Employment. **Holstein**: *Genentech*: Membership of an entity's Board of Directors or advisory committees; *GSK*: Consultancy; *Celgene*: Consultancy; *Takeda*: Membership of an entity's Board of Directors or advisory committees; *Adaptive Biotechnologies*: Membership of an entity's Board of Directors or advisory committees; *Sorrento Consultancy*; *Oncopeptides*: Membership of an entity's Board of Directors or advisory committees, research funding. **Varney**: None. **Suvela**: None. **Lehmann**: *Oncopeptides AB*: Employment. **Nupponen**: *Oncopeptides AB*: Consultancy. **Heckman**: *Celgene*: Research funding; *Novartis*: Research funding; *Oncopeptides*: Research funding; *Orion Pharma*: Research funding.

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