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 chain and alleviate deposition of amyloid thereby restoring healthy organ function.

Melphalan flufenamide ethyl ester, melflufen, is a peptidase potentiated alkylating agent 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 suggests that the drug might be useful to directly eliminate amyloidogenic plasma cells, thereby reducing the amyloid load in patients. Furthermore, the 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.

To assess potential efficacy in amyloidosis patients and to explore the mechanism of action, we examined effects of melflufen on amyloidogenic plasma cells *in vitro* and *in vivo*.

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 light chain was measured in supernatant by ELISA, and intracellular levels were detected by flow cytometry. To assess efficacy of melflufen in vivo, 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 was measured by *in vivo* bioluminescence, and serum light chain was monitored.

Results: Melflufen demonstrated increased potency against multiple myeloma cell lines compared to melphalan, inducing malignant plasma cell death at lower doses on established light chain secreting plasma cell lines. While ALMC-1 cells were sensitive to both melphalan and melflufen, the IC50 for melphalan at 960 nM was approximately 3-fold higher than melflufen (334 nM). However, ALMC-2 cells were relatively insensitive to melphalan (12600 nM), but maintained a 100-fold increase in sensitivity to melflufen (121 nM). Furthermore, while 40% of primary CD138+ plasma cells from patients with diagnosed AL amyloidosis responded to

melflufen treatment in vitro, only 20% responded to melphalan with consistently superior IC50 values for melflufen (Figure 1). Light chain secreting cell lines and AL amyloidosis patient samples were further analyzed by single cell sequencing.

We further examined differential effects on apoptosis and the unfolded protein response in vitro in response to either melflufen or melphalan. This is of particular interest in amyloidosis, where malignant antibody producing plasma cells possess an increased requirement for mechanisms to cope with the amplified load of unfolded protein and associated ER stress.

As AL amyloidosis is ultimately a disease mediated by secretion of toxic immunoglobulin, we assessed the effects of melflufen on the production of light chain *in vitro*, measuring a decrease in production of light chain in response to melflufen treatment. Finally, we took advantage of a recently described adoptive transfer mouse model of amyloidosis to assess the efficacy of melflufen and melphalan in eliminating amyloidogenic clones and reducing the levels of toxic serum light chain *in vivo*.

Conclusions: These findings provide evidence that melflufen mediated toxicity, previously described in myeloma cells, extends to amyloidogenic plasma cells and further affects the ability of these cells to produce and secrete toxic light chain. This data supports the rationale for the evaluation of melflufen in patients with AL amyloidosis.

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