Aminopeptidase Expression in Multiple Myeloma Associates with Disease Progression and Sensitivity to Melflufen

Poster
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Juho J. Miettinen[1], Romika Kumari [1], Gunnhildur Á. Traustadóttir [2], Maiju-Emilia Huppunen [1], Muntasir M. Majumder [1], Alexander Schepsky [2], Juha Lievonen [3], Pekka Anttila [3],Ana Slipicevic [4], Raija Silvennoinen [3], Thorarinn Gudjónsson [2], Nina N. Nupponen [4], Fredrik Lehmann [4], Caroline A. Heckman [1]

1 Institute for Molecular Medicine Finland - FIMM, HiLIFE – Helsinki Institute of Life Science, iCAN Digital Precision Medicine Flagship, University of Helsinki, Helsinki, Finland, 2 Stem Cell Research Unit, Biomedical Center, Department of Anatomy, Faculty of Medicine, School of Health Sciences, University of Iceland, Reykjavik, Iceland, 3 Department of Hematology, Helsinki University Hospital and Comprehensive Cancer Center, University of Helsinki, Helsinki, Finland, 4 Oncopeptides AB, Stockholm, Sweden

BACKGROUND

Multiple myeloma (MM) is characterized by extensive immunoglobulin production leading to an excessive load on protein homeostasis in tumor cells. The function of the aminopeptidase (AP) gene family is to catalyze the hydrolysis of amino acids from proteins or peptides leading to proteolysis. They are a substantial group of enzymes implicated in many cellular functions such as cell cycle, DNA repair, and apoptosis. APs function downstream of the ubiquitin-proteasome pathway, and enzymatic activity of these proteases can be utilized in the delivery of peptide conjugated drugs, such as melphalan flufenamide (melflufen), a peptidase-drug conjugated to an alkylating payload. Melflufen is currently investigated in clinical trials. The role of APs in MM has not yet been characterized.

OBJECTIVES

- To characterize aminopeptidase expression in CD138+ cells enriched from bone marrow aspirates of patients with MM.
- To study the role of aminopeptidases in MM progression and sensitivity to melflufen.
- To confirm melflufen as a substrate for aminopeptidases.

METHODS

- **Gene expression analysis:** In total, 123 bone marrow aspirates from Finnish patients with MM (41 newly diagnosed (NDMM), 82 relapsed/refractory (RRMM) samples) were obtained after written informed consent and following approved protocols in compliance with the Declaration of Helsinki. CD138+ cells were enriched by immuno-magnetic bead selection (StemCell Technologies) from MM patient bone marrow aspirates and used for RNA isolation. Illumina compatible RNA sequencing libraries were prepared and further sequenced. The CoMMpass database was utilized for gene expression validation (n= 892).
- **Survival analysis:** The impact of AP gene expression on survival outcome was estimated by Kaplan-Meier analysis. Significance for survival curves between two groups (high vs. low expression) were calculated using Mantel-Cox log rank test.
- ***Ex vivo* drug sensitivity testing:** Viably cryopreserved bone marrow mononuclear cell samples (n=15) from 14 different patients, including 6 NDMM and 9 RRMM samples, were used for *ex vivo* drug sensitivity assessment of melflufen, melphalan, bortezomib, selinexor, and 4-hydroperoxycyclophosphamide. The IntelliCyt multicolor high throughput flow cytometer iQue Screener Plus (Sartorius) was used for sample analysis. CD138+CD38+ cell population response was measured by counting the numbers of live cells in seven different drug concentrations in duplicate after 72h of incubation. Annexin V and 7-AAD double negative cells were considered as live cells.
- **Testing of AP inhibitory effects on melflufen:** MM cell lines RPMI-8226 and MM.1S were used to test the inhibitory effect of AP inhibitors tosedostat and bestatin against melflufen activity. The cells were pretreated with DMSO (control), bestatin (10 µM) and tosedostat (10 µM) for 1h and then treated with 3 different concentations of melflufen/melphalan for 15 min, whereafter the medium was replaced. The cells were incubated for 48h and cell viability was measured using PrestoBlue cell viability reagent.
- **Hydrolysis assay:** The enzymatic hydrolysis of melflufen by AP LAP3 was measured using liquid chromatography tandem-mass spectrometry (LC-MS/MS). The release of para-fluoro-L-phenylalanine (4-F-Phe-OEt) from melflufen was measured after melflufen incubation with LAP3 for 2h.

RESULTS

Based on AP gene expression levels, there where 20/36 AP genes that had a median expression of 0>log2(RPKM). *LAP3*, *ERAP2*, *METAP2*, *TTP2*, *ERAP1* and *DPP7* were the most highly expressed APs in the samples from Finnish patients with MM (Fig 1.) and further validated in the CoMMpass dataset. In total, 9 APs (*LAP3*, *ERAP2*, *METAP2*, *TPP2*, *NPEPPS*, *BLMH*, *RNPEP*, *PEPD* and *DNPEP*) were found to be more highly expressed in RRMM than NDMM samples (P-value<0.05), indicating potential roles in disease progression.

RESULTS

Figure 1. Aminopeptidase gene expression in CD138+ cells enriched from myeloma patient bone marrow aspirates.

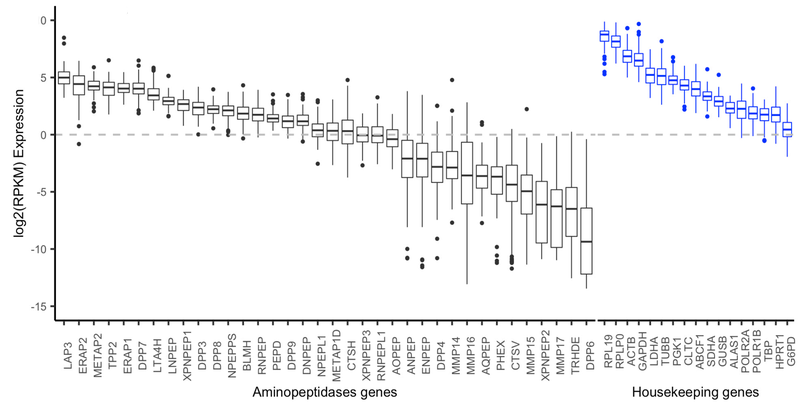
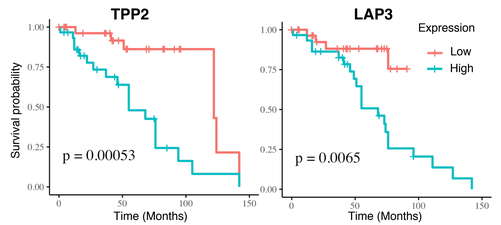
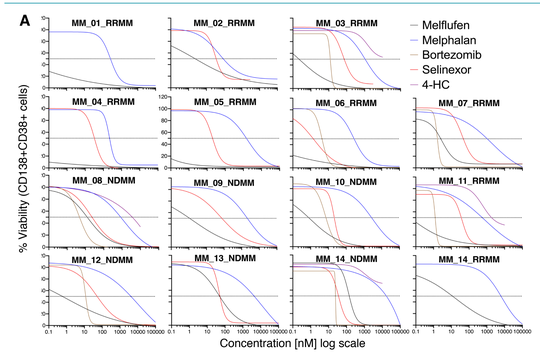


Figure 2. Impact of *TPP2* & *LAP3* gene expression on the survival of patients with MM as estimated by Kaplan-Meier analysis. Gene expression was measured from CD138+ cells enriched from MM patient bone marrow aspirates



Survival analysis revealed higher expression of many AP genes associated with disease progression including *TPP2*, *LAP3*, *DPP3*, *BLMH*, *RNPEP*, *NPEPL1* (P-value<0.05). MM patients having higher *TPP2* or *LAP3* expression in malignant cells had poorer prognosis with a median survival of 55 and 68 months, (*TPP2*; p=0.0005, *LAP3*; p=0.0065) respectively (Fig 2.).

Figure 3. A) Dose response curves and B) EC50 values from 15 MM patient sample CD138+CD38+ cells. Drug sensitivity was measured with melflufen, melphalan, selinexor, bortezomib and 4-hydroperoxycyclophosphamide (4-HC) after 72h incubation. Cell viability was measured by multicolor high throughput flow cytometry.



B

	EC50 [nM]				
	Melflufen	Melphalan	Selinexor	Bortezomib	4-HC
MM_01_RRMM	0,001	326	NA	NA	NA
MM_02_RRMM	2,2	52	31,0	NA	NA
MM_03_RRMM	0,2	1059	61,4	12,3	>1E+4
MM_04_RRMM	2E-23	191	32,3	NA	NA
MM_05_RRMM	0,003	1671	19,7	NA	NA
MM_06_RRMM	0,0002	303	1,6	4,2	NA
MM_07_RRMM	3,0	1935	35,2	0,9	NA
MM_08_NDMM	6,8	1170	20,9	3,4	3800
MM_09_NDMM	1,6	1831	63,3	NA	NA
MM_10_NDMM	0,9	3052	21,4	6,8	NA
MM_11_RRMM	0,04	526	39,1	1,2	1900
MM_12_NDMM	0,4	10154	48,1	11,5	NA
MM_13_NDMM	44	344	46,2	NA	NA
MM_14_NDMM	134	17000	35,4	13,8	>1E+4
MM_14_RRMM	14	7207	NA	NA	NA

EC50 values from samples considered as responsive to the particular drug are marked in red.

Melflufen *ex vivo* response was detected in 73% (11/15), melphalan in 40% (6/15), selinexor in 69% (9/13), bortezomib in 63% (5/8) and 4-hydroperoxycyclophosphamide in 0% (0/4) of tested MM patient samples (Fig 3.). RRMM samples (median EC50=0.04 nM) were more sensitive to melflufen than NDMM samples (median EC50=4.2 nM) (Fig 4.).

Figure 4. Comparison CD138+CD38+ cell EC50 values between NDMM and RRMM patient samples in the five tested drugs.

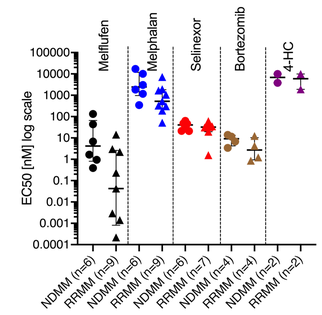
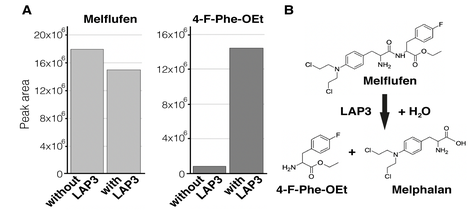


Figure 5. Enzymatic hydrolysis of melflufen by LAP3.

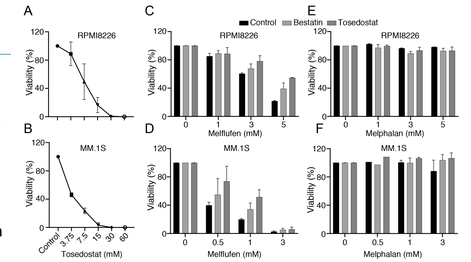
A) Melflufen and para-fluoro-L-phenylalanine ethyl ester (4-FP-Phe-Oet) amounts were measured after 2h incubation of melflufen with LAP3. The measurements were performed using LC-MS/MS and compound amounts are indicated as peak areas. B) Reaction pathway of the hydrolysis of melflufen peptide bond by LAP3 aminopeptidase.



The hydrolysis assay demonstrated that melflufen is a substrate for LAP3 by confirming the decrease in melflufen and increase in 4-FP-Phe-OEt after melflufen incubation with LAP3 for 2h (Fig 5.).

Figure 6. Aminopeptidase (AP) inhibitors reduce sensitivity to melflufen in MM cell lines.

MM cell lines A) RPMI-8226 and B) MM.1S were treated with different concentrations of AP inhibitor tosedostat and cell viability was measured using PrestoBlue cell viability reagent. C) RPMI-8226 and D) MM.1S were pretreated with DMSO (control), bestatin or tosedostat and then either were left without additional treatment or were treated with 3 different concentrations of melflufen and after 48h cell viability was measured. E) RPMI-8226 and F) MM.1S were pretreated with DMSO, bestatin or tosedostat and then either were left without additional treatment or were treated with 3 different concentrations of melphalan and after 48h cell viability was measured.



AP inhibitors tosedostat and bestatin decreased the viability of MM cell lines RPMI-8226 and MM.1S in a dose-dependent manner and reduced their sensitivity towards melflufen (Fig 6.).

CONCLUSION

- **Multiple aminopeptidase genes were found to be differentially expressed in MM samples and associated with disease progression and reduced survival.**
- **Very good *ex vivo* sensitivity for melflufen was observed especially in samples from patients with RRMM.**
- **Melflufen activity is dependent on aminopeptidase activity.**
- **Melflufen is a substrate for LAP3 aminopeptidase.**

DISCLOSURES

JL: Amgen, Bristol-Myers Squibb, Celgene, Takeda, Janssen, Janssen-Cilag, Novartis, Pfizer, and Roche: Personal fees. **RS**: Amgen, BMS, Celgene, and Takeda: Funding and personal fees; Sanofi: Personal fees. **NNN**: Oncopeptides: Consultant AB. **AS and FL:** Oncopeptides AB: Employment. **TG:** Oncopeptides AB: Research funding. **CAH**: Oncopeptides AB, Novartis, Kronos Bio, Celgene, Pfizer, Orion Pharma, and the IMI2 consortium project HARMONY: Research funding. **JM, RK, GAT, MH, MMM, AS, and PA**: Nothing to disclose.

CONTACT INFORMATION

Juho Miettinen, Postdoctoral researcher
juho.miettinen@helsinki.fi

Caroline Heckman, Principal investigator
caroline.heckman@helsinki.fi

Institute for Molecular Medicine Finland
Nordic EMBL Partnership for Molecular Medicine

HiLIFE UNIT
UNIVERSITY OF HELSINKI

UNIVERSITY OF ICELAND

Melflufen is an abbreviated form of the international non-proprietary name (INN) melphalan flufenamide, an investigational product not yet approved for commercial use in any market globally

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