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Novel Peptide-drug Conjugate Melflufen Efficiently Eradicates Bortezomib-resistant Multiple Myeloma Cells Including Tumor-initiating Myeloma Progenitor Cells

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Abstract

Introduction of the proteasome inhibitor bortezomib has dramatically improved clinical outcomes in multiple myeloma. However, most patients become refractory to bortezomib-based therapies. On the molecular level, development of resistance to bortezomib in myeloma cells is accompanied by complex metabolic changes resulting in increased protein folding capacity, and less dependency on the proteasome. In this study, we show that aminopeptidase B, encoded by the *RNPEP* gene, is upregulated in bortezomib-resistant myeloma cell lines, and in a murine in vivo model. Moreover, increased *RNPEP* expression is associated with shorter survival in multiple myeloma patients previously treated with bortezomib-containing regimens. Additionally, expression is increased in plasma cell precursors, a B-lymphoid compartment previously associated with myeloma stem cells. We hypothesized that increased aminopeptidase B expression in aggressive myeloma clones may be used therapeutically toward elimination of the cells via the use of a novel peptide-drug conjugate, melphalan flufenamide (melflufen). Melflufen, a substrate of aminopeptidase B, efficiently eliminates bortezomib-resistant myeloma cells in vitro and in vivo, and completely suppresses clonogenic myeloma growth in vitro at subphysiological concentrations. Thus, melflufen represents a novel treatment option that is able to eradicate drug-resistant myeloma clones characterized by elevated aminopeptidase B expression.

Introduction

Proteasome inhibitors (PI) are one of the backbones of multiple myeloma (MM) therapy.[1] The first-in-class PI, bortezomib (BTZ), is a boronate-based inhibitor approved for the treatment of MM that significantly improved patient outcomes. BTZ is a backbone of several therapy regimens currently approved for the treatment of newly diagnosed MM (NDMM) as well as relapsed/refractory multiple myeloma (RRMM) disease. In the NDMM setting, BTZ is used in dexamethasone-containing triplet combinations with an alkylating agent cyclophosphamide or immunomodulatory drug lenalidomide (LEN). In the treatment of RRMM, BTZ is combined with dexamethasone or prednisone and various novel agents used in the advanced disease, such as immunomodulatory drug pomalidomide (POM), anti-CD38 monoclonal antibody daratumumab, and other agents, according to the latest review.[2] However, despite the initial response, MM patients often relapse after BTZ treatment, or become BTZ-refractory,[3] a condition with a very poor prognosis.[4] The development of BTZ resistance, under continuing selective pressure with BTZ-based therapy, is an important clinical problem.

The activity of the β5 proteasome subunit has initially been considered the rate-limiting protease of the proteasome, and consequently all currently approved PI were designed to preferentially target the β5 subunit.[5] Based on initial findings in PI-resistant cell lines generated in vitro, point mutations in the β5-encoding gene *PSMB5* have been proposed as a mechanism of resistance to PI.[6] However, *PSMB5* mutations are rarely found in MM patient samples, despite a common development of resistance to BTZ.[7] Recent studies unraveled a complex molecular mechanism of BTZ resistance, which includes concerted changes in cell metabolism, conferring more effective protein folding and suggesting less dependence on proteasome β5 activity in BTZ-resistant myeloma cells.[8,9] Such complex changes in the biology of BTZ-resistant cells imply an increased peptidase activity that may be involved in protein turnover. Intriguingly, elevated aminopeptidase B (ApB) was found during global proteome profiling by mass-spectroscopy in BTZ-resistant cells.[8] ApB is encoded by the *RNPEP* gene located on chromosome 1q32, a region often gained or amplified in high risk MM.[10,11] ApB belongs to the M1 family of aminopeptidases.[12–14] The enzyme recognizes N-terminal basic amino acids lysine and arginine, and hydrolyzes the following peptide bond removing the N-terminal basic amino acid from the oligopeptide chain.[15,16]

Elevated levels of aminopeptidases, including proalgetic enzymes, are observed in different cancer types[17–21] and can

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be used therapeutically by a peptide-conjugate, melphalan flufenamide, hereafter referred to as melflufen.[22–24] Melflufen is a lipophilic peptide-drug conjugate that rapidly delivers an alkylating payload, melphalan (MPH), into tumor cells by utilizing both the high lipophilic molecular properties and high intracellular peptidase expression. Melflufen is passively transported across cell and organelle membranes where it is hydrolyzed by aminopeptidases, resulting in high entrapment of hydrophilic alkylating payload within the cancer cells.[25] Melflufen has shown efficacy in PI-naive myeloma cell lines,[24,26] as well as in RRMM patients in clinical trials.[27–29] Intriguingly, RRMM patients, refractory to a prior PI-containing therapy, showed better response to melflufen (overall response rate [ORR] = 41.7%, 95% confidence interval [CI], 15.2-72.3) in comparison to the all-patient population (ORR = 31.1%, 95% CI, 18.2-46.6).[30] Therefore, we have decided to investigate the functional role of elevated levels of ApB in BTZ-refractory MM patients, in the in vitro model of BTZ resistance, and its implication in the susceptibility of high risk myeloma to melflufen.

Methods

Ethical aspects

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Tumor sampling and data collection were performed following informed consent; the study was approved by a regional ethical committee (2007/237). In vivo experiments were performed using chick embryos (INOVOTION, Inc). This model is recognized as an alternative to mouse xenografts for in vivo experiments by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R). Experiments with chick embryos do not require administrative procedures for obtaining ethics committee approval for animal experimentation (European Directive 2010/63/EU). It has been confirmed by the regional Ethical Committee in Grenoble (Inovotion-JV-01).

Cell lines and media

Cell lines RPMI-8226, AMO1, and ARH77 were obtained from commercial sources (American Type Culture Collection, ATCC, Wesel, Germany; Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany) and were maintained under standard conditions in RPMI-1640 medium (Sigma-Aldrich, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin (Sigma-Aldrich). Cells were adapted to BTZ (AMO-BTZ, RPMI-BTZ, and ARH-BTZ) by continuous exposure to increasing drug concentrations as previously described[8] and were cultured in a presence of 90 nM BTZ. Cell lines were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Switzerland) and STR-typed to confirm the authenticity of the derived cell line with parental cell lines (at DSMZ, Braunschweig, Germany).

Drugs

Melflufen was obtained from Oncopeptides AB. Bestatin was obtained from MedChem Express (HY-B0134); BTZ was obtained from MedChem Express (HY-10227). The other drugs used throughout the study were obtained from SelleckChem. All chemicals were dissolved in dimethyl sulfoxide (DMSO) as 10 or 100 mM (where solubility is appropriate) stock solutions.

Cytotoxicity assays

Serial drug dilutions of melflufen were prepared in phenol red-free RPMI-1640 culture medium. Cells were seeded in 96-well plates as 1×10^4 cells/well and incubated with the drugs for 48 hours at 37°C and 5% CO_2. Cell viability was assessed using CCK-8 reagent (GLPBio, Montclair, CA) according to a protocol provided by the manufacturer. The read out was done on Tecan Sunrise plate reader. To estimate the half maximal inhibitory concentration (IC_{50}), smoothed dose-response curves were fitted using GraphPad Prism v8 (GraphPad Software). The IC_{50} values were calculated from the mean of 3 independent experiments. Synergy scoring was calculated using SynergyFinder platform.[31]

Apoptosis assay

1×10^6 cells were seeded for the experiment and treated with respective concentration of the drug for 24 h. Apoptosis was determined by staining the cells with the Annexin V/FITC Detection Kit (Biotool, Switzerland) according to the manufacturer's instructions. Afterward, cells were analyzed on FACS Canto II (BD Biosciences), data were analyzed using FlowJo v10 Software (FlowJo Company, Ashland, OR).

Cell cycle distribution assay

1×10^6 cells were seeded for the experiment and treated with a respective concentration of the drug for 24 h. Subsequently, the cells were washed and fixed with ice-cold 70% methanol. Next, the cells were incubated with RNAse A (Sigma-Aldrich) for 30 min/37°C and propidium iodide for 10 min/RT (Sigma-Aldrich). Cells were analyzed on FACS Canto II (BD Biosciences, CA) and cell cycle distribution was evaluated using FACS Diva Software (BD Biosciences).

Analysis of gene expression and statistical methods

Gene expression data matrices GSE2658, GSE9782, GSE24080, GSE111921, GSE136400, GSE141005 were downloaded from GEO Omnibus. The values for gene expression measured by microarray were extracted using GEO2R software. The survival was assessed by Cox regression analysis. The statistical significance between different samples was evaluated using the Mann Whitney *U* test for unpaired samples and the Wilcoxon test for the paired samples. Spearman correlation analysis was employed for the correlation analysis. All statistical analyses were performed using GraphPad Prism.

SDS-PAGE and Western blot

SDS-PAGE was performed using bis-tris 10% gels (ThermoFisher Scientific, MA) with 30 µg of protein per well. Western blot was performed as described previously[32] with following antibodies: RNPEP (Aminopeptidase B, ANB ApB; A305-611A-M, Bethyl laboratories, Montgomery, TX) and GAPDH (#G8795, Sigma-Aldrich) as a protein loading control.

RNA isolation and quantitative PCR

Total RNA was extracted from the cells by Trizol (ThermoFisher Scientific) and Direct-zol RNA MiniPrep (Zymo Research, CA). 1 µg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) according to manufacturer's recommendations. Subsequently, 10 ng of cDNA was used into quantitative PCR

reactions with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and primers for RNPEP and GAPDH as a housekeeping gene (all from Thermo Fisher Scientific). The reactions were performed in technical duplicates and the amount of specific RNA was quantified using QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific).

Hydrolysis of melflufen by ApB

In vitro hydrolysis was performed in the incubation buffer (50 mM Tris, 100 mM KCl, 1 mM DTT, pH 7.5). ApB (R&D Systems, MN) was thawed and diluted with incubation buffer to desired concentration. Stock solution (1000 µM) of the test substrate melflufen was prepared in DMSO and further diluted in incubation buffer to desired concentration shortly before the experiment to minimize autohydrolysis of melflufen. Incubation buffer, substrates were mixed with ApB for 2 h at a substrate to enzyme ratio of 50:1. Control incubations were performed without ApB. All incubations were performed in duplicate at 37°C. The incubation was stopped by adding ice cold acetonitrile (Th. Geyer, Germany). The samples were centrifuged and subjected to liquid chromatography-high resolution mass spectrometry analysis. Results were evaluated using LCquan (Thermo Fisher Scientific).

Colony forming cell outgrowth assay

Clonogenic progenitors of MM were assessed in a semisolid methylcellulose-based media formulation containing 10% phytohemagglutinin (PHA)-stimulated leukocyte conditioned medium (ReachBio, WA). Human bone marrow mononuclear cells were obtained from 7 distinct RRMM patients on signing the informed consent and stored at −152°C until required for the assay. The patients were diagnosed with Durie-Salmon stages III-A (N = 6) and III-B (N = 1). All patients were relapsed (within 6 months) or refractory to cyclophosphamide/BTZ/dexamethasone therapy. On the day of the experiment, the cells were rapidly thawed; the content of each vial was diluted in 10 mL of Iscove's modified Dulbecco's medium containing 10% FBS (Thermo Fisher Scientific) and cleared from cellular debris by centrifugation. The supernatant was discarded, cell pellets were diluted to the appropriate concentration for the CFC assay and added to tubes containing the methylcellulose-based medium formulation ColonyGEL 1150 optimized for MM-CFC assays (ReachBio, WA) mixed with 10% PHA-stimulated leukocyte conditioned medium and the test compounds (final concentration as follows: melflufen at 10 and 50 nM, BTZ at 5 and 10 nM, carfilzomib [CFZ] at 10 nM, LEN at 2000 nM, POM at 100 nM, iberdomide [IBER] at 5 and 50 nM, CC-92480 at 5 and 50 nM, selinexor [SXR] at 100 nM, bendamustine [BDM] at 5000 nM, MPH at 500, 1000, 2500, and 5000 nM) or solvent control (DMSO). Each tube was vortexed to ensure equal distribution of the cells throughout the media. A minimum of 3 replicates were set-up for each control and each concentration of the compound. Typically, 2×10^4 cells were plated for CFC assays for each condition. The replicate dishes were placed at 37°C, 5% CO_2 for a total of 14 to 16 days. After 14 to 16 days, the colonies were evaluated, enumerated based on morphology, and documented by photography. For each patient, representative MM-CFC in the presence of the solvent control as well as in the presence of the test compounds were documented.

Chick chorioallantoic membrane assay

Fertilized White Leghorn eggs were incubated at 37.5°C with 50% relative humidity for 9 days (Inovotion INC). On day E9, the upper chorioallantoic membrane (CAM) was dropped down by drilling a small hole through the eggshell into the air sac, and a 1 cm^2 window was cut in the eggshell above the CAM. Myeloma cell lines AMO1 and AMOaBTZ were washed with complete medium and suspended in graft medium. An inoculum of 1×10^5 cells was added onto the CAM of each egg. Eggs were then randomized into 8 groups, with at least 20 eggs per group. On day E10, tumors became detectable, and were treated with either vehicle (0.325% DMSO in 1x PBS), BTZ at 3.2 µg/kg, or melflufen at 178.3 µg/kg. For all conditions, the injection volume of 100 µL/egg was dropped onto the tumor. On day E18, the upper portion of the CAM containing tumor was removed, washed in 1× PBS and then directly transferred in paraformaldehyde, fixed for 48 hours, and put in embedding cassettes. The tumor was then washed, carefully cut away from normal CAM tissue and weighed. To estimate toxicity, eggs were checked at least every 2 days, for the viability and visible macroscopic abnormalities. The number of dead embryos counted on day E18, combined with reported abnormalities was used to evaluate total toxicity.

Histology analysis

Paraffin blocks were sectioned at approximately 4 µm thickness. The sections were put on glass slides and stained with hematoxylin & eosin (H&E). Pictures were taken using Olympus microscope (BX60, serial NO. 7D04032) at objective magnifications of ×110, and microscope camera (Olympus DP73, serial NO. OH05504). H&E-stained sections were examined. Microscopically, 10 nonoverlapping fields (HPF) from each section of the tumor were evaluated and graded by a semiquantitative scoring system for the presence of pathological changes.

Results

Elevated ApB expression in MM is associated with resistance to BTZ and worse clinical outcomes in BTZ-treated patients

In our previously published proteomics analysis, ApB showed significant upregulation on acquisition of resistance to BTZ.[8] This has been confirmed in the current study using quantitative reverse transcription PCR and densitometric western blot analysis (Figure 1A; Supplemental Digital Content, Figure S1, http://links.lww.com/HS/A168). Increased ApB expression in BTZ-resistant MM cell lines can be confirmed in an independent murine model of Vk*MYC-driven high-risk MM,[33] where murine *Rnpep* mRNA expression is significantly increased in BTZ-resistant tumors compared with BTZ-naive tumors (Figure 1B). Next, the association of elevated *RNPEP* mRNA expression with poor survival in MM patients was tested. Analysis of gene expression profiling datasets GSE24080 and GSE136400 has shown elevated *RNPEP* mRNA expression to be attributed to patients with significantly shorter progression-free survival (62 versus 70.9 months, hazard ratio = 0.87, 95% CI, 0.73-1.05, Figure 1C) and overall survival (118 versus 144 months, hazard ratio = 0.82, 95% CI, 0.66-1.01, Figure 1D). These data confirm inferior survival of MM patients with higher *RNPEP* gene expression, observed in an independent cohort of patients published elsewhere.[34] Together, these data suggest association of ApB with BTZ-resistance and poor prognosis in MM.

Acquisition of BTZ resistance sensitizes myeloma cells to melflufen in vitro and in vivo

As the cytotoxic activity of melflufen is directly linked to increased levels of aminopeptidases, we next examined the

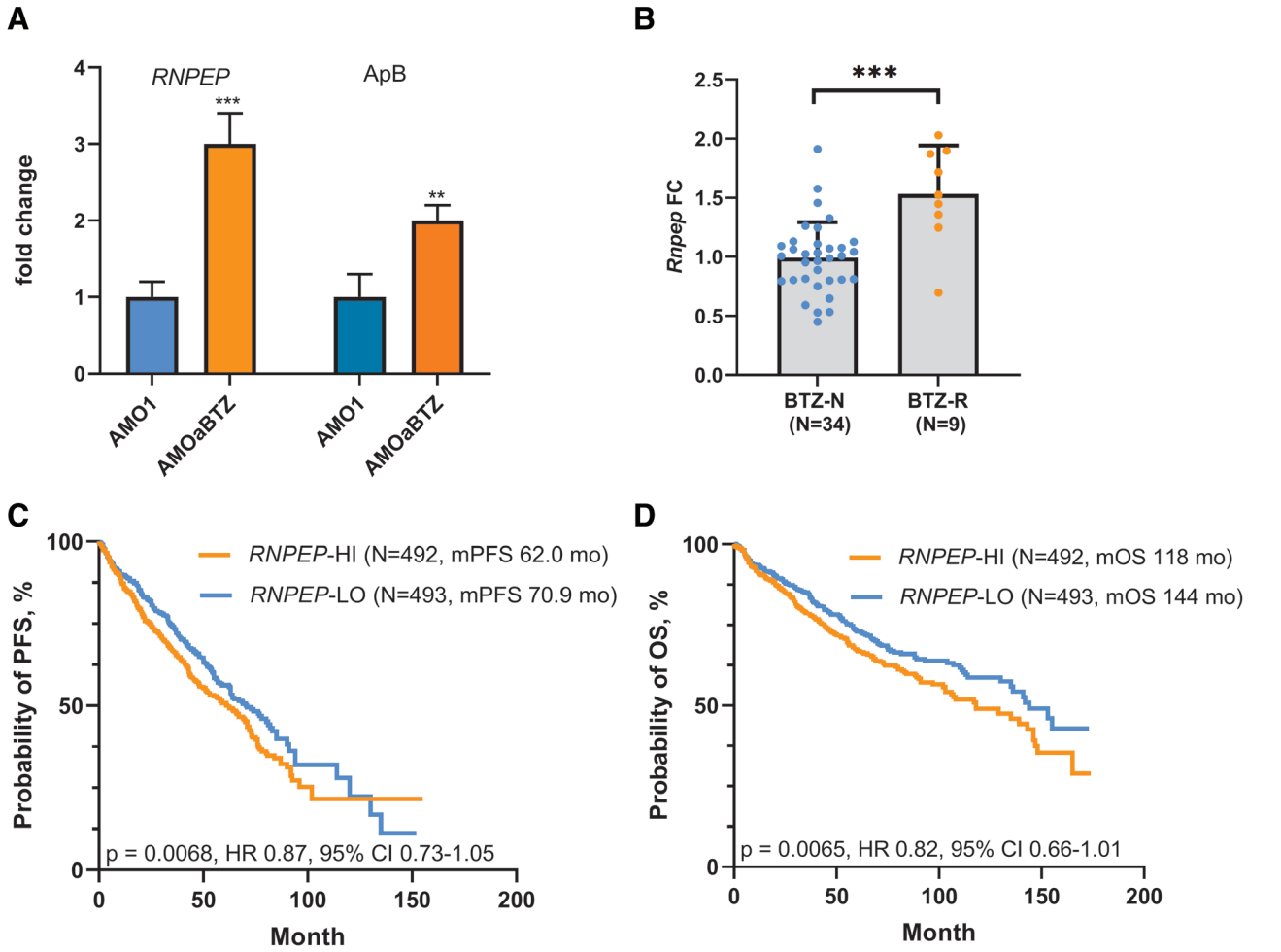

Figure 1. Aminopeptidase B expression in multiple myeloma and its association with resistance to bortezomib. (A), Expression of ApB on a protein level and corresponding RNPEP mRNA in AMOaBTZ cells assessed by densitometric analysis of western blot (presented in Supplemental Digital Content, Figure S1, http://links.lww.com/HS/A168) and RT-qPCR analyses, respectively. In both methods, GAPDH was used as a housekeeping control. The data represent mean ± SD of 3 independent experiments evaluated by paired *t* test; $^{**}P < 0.01$; $^{***}P < 0.001$. (B), Expression of murine Rnpep mRNA in BTZ-naive and BTZ-resistant myeloma cells isolated from a high-risk MYC-driven murine model of multiple myeloma, available at GEO Omnibus as GSE111921. The data represent median ± SD, $^{***}P < 0.001$, analyzed by Mann-Whitney *U* test. (C, D), Regression analysis of PFS (C) and OS (D) of MM patients from cohorts with GSE24080 and GSE136400 with available censored survival and gene expression data. The patients are divided according to the gene expression of *RNPEP* in their samples into a *RNPEP*-LO (low expression, below the median within the cohort) and *RNPEP*-HI (high expression, above the median within the cohort). Number of patients (N), mPFS, mOS, *P* value (*P*), HR, 95% CI of HR are presented on the corresponding figures. AMOaBTZ = AMO1 bortezomib adapted cells; ApB = aminopeptidase B; CI = confidence interval; HR = hazard ratio; MM = multiple myeloma; mOS = median OS; mPFS = median PFS; OS = overall survival; PFS = progression-free survival.

cytotoxic effect of melflufen in BTZ-resistant myeloma cell lines and their BTZ-naive parental cell line. Intriguingly, melflufen demonstrated enhanced cytotoxicity in all tested BTZ-resistant myeloma cells (Figure 2A) that have been previously generated by us.[35] In addition, melflufen treatment induced significantly higher levels of apoptosis in BTZ-adapted AMO1 MM cells (AMOaBTZ) (Figure 2B). Likewise, melflufen induced cell cycle arrest in both AMO1 and AMOaBTZ cells; however, AMOaBTZ cells treated with melflufen displayed higher levels of cells in sub-G0/G1 phase, indicating increased level of DNA damage and cell death (Figure 2C). Moreover, melflufen showed higher cytotoxicity than equimolar amount of MPH in BTZ-naive and BTZ-resistant MM cells (Figure 2D) and this difference was the most pronounced in BTZ-resistant cells.

To investigate the in vivo effect of melflufen on myeloma, a chick embryo CAM assay with AMO1 and AMOaBTZ MM cells was established. Both cell lines are capable of robust tumor growth in this model (Figure 3), allowing for further investigation of the effect of drug treatment on tumor growth. Histopathology examination of the MM cell–derived tumors showed complete tumors with minimal inflammation, high mitotic figures index, almost no necrosis and tumor matrix, and a high grade of pleomorphic nuclear shapes (Figure 4). The dose of melflufen used in this model was previously established as 178.3 µg/kg, which corresponds to a 440 nM concentration,[36,37] closely resembling the achievable plasma levels in melflufen-treated cancer patients.[22] The dose of BTZ was established here as the maximal tolerated dose of 3.2 µg/kg. Both melflufen and BTZ were able to significantly reduce the growth of AMO1-derived tumors (Figure 3A), although histopathology has revealed some remaining surviving MM cells in BTZ-treated tumors (Figure 4). On the contrary, in the BTZ-resistant AMOaBTZ-established tumors, only melflufen could significantly prevent tumor growth. BTZ treatment resulted in the increase of tumor volume in some CAM (Figure 3C), although this increase was not significant on average (Figure 3A). Interestingly, treatment of the xenografted tumors with melflufen was associated with increased lymphocyte infiltration in both BTZ-sensitive and BTZ-resistant models (Figure 3B). Thus, melflufen is a very effective drug at overcoming BTZ resistance in vitro and in vivo.

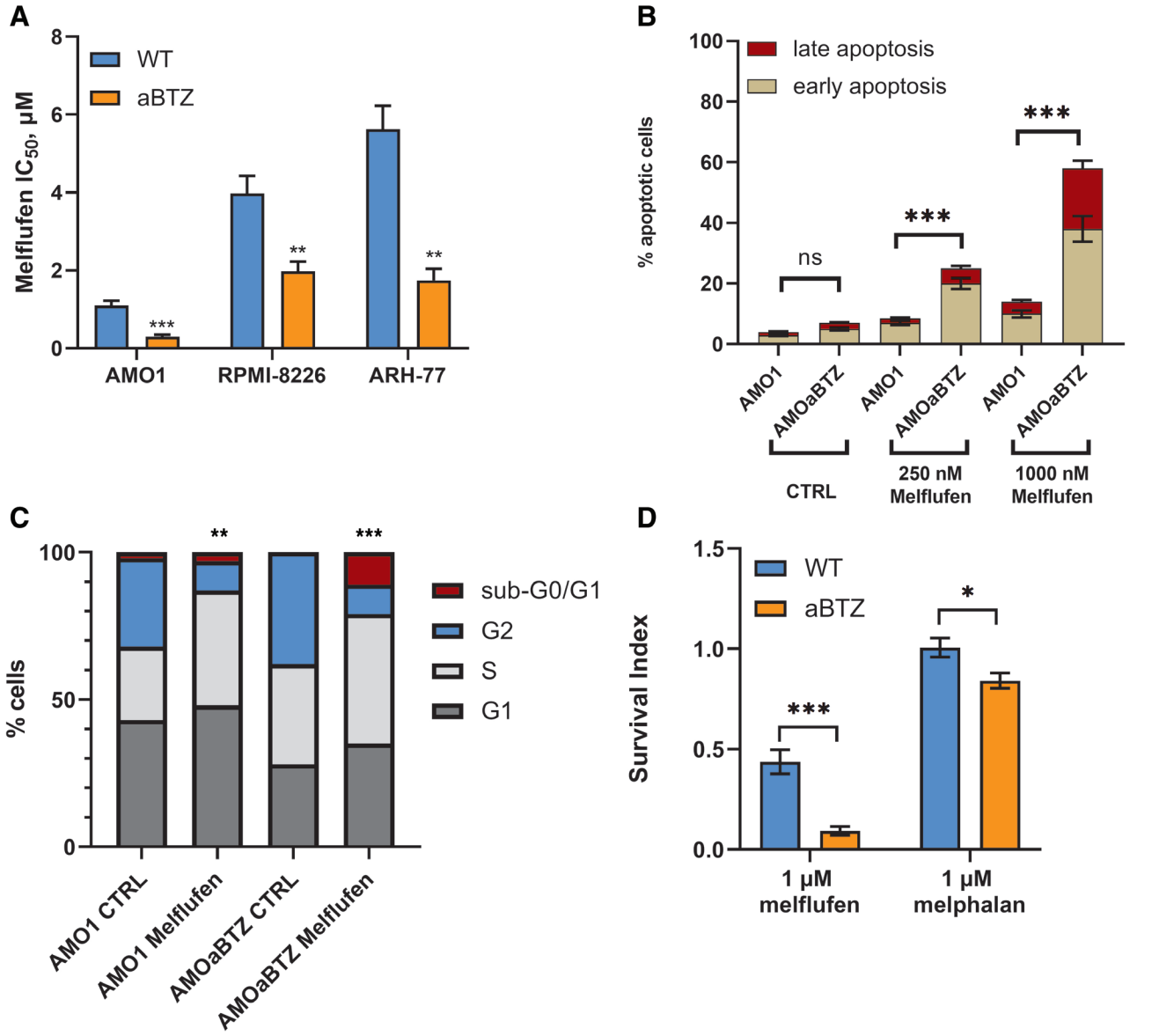

Figure 2. Effect of melflufen in bortezomib-resistant cells in vitro. (A), Cytotoxicity of melflufen in myeloma cell lines AMO1, RPMI-8226, and ARH-77 and their bortezomib-resistant derivatives (aBTZ). Data represent mean ± SD of 3 independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$, evaluated by paired *t* test (B), Apoptosis assessment in AMO1 and BTZ-adapted AMOaBTZ cell lines treated by melflufen. Data represent mean ± SD of 3 independent experiments; $^{***}P < 0.001$, evaluated by paired *t* test. (C), Cell cycle analysis in AMO1 and AMOaBTZ cell lines on treatment with melflufen. Data represent mean of the distribution of cell cycle calculated from 3 independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$, evaluated by paired *t* test. (D), Effect of equimolar amounts (1.0 µM) of melflufen and melphalan on survival of AMO1 and AMOaBTZ cells after 48 h of continuous treatment, presented as survival index. Data represent mean ± SD of 3 independent experiments; $^{*}P < 0.05$; $^{***}P < 0.001$, evaluated by *t* test. aBTZ = bortezomib-adapted cells ; AMOaBTZ = AMO1 bortezomib adapted cells; CTRL = control, untreated cells; IC_{50} = half maximal inhibitory concentration; ns = not significant; WT = wild type, PI-naive cells.

Melflufen is a substrate of ApB

As a next step, we aimed to establish a link between melflufen's cytotoxicity in BTZ-resistant MM and elevated expression of ApB. Aminopeptidase inhibitor bestatin[16] significantly impaired melflufen's cytotoxicity in AMOaBTZ cells (Figure 5A). Further calculation of synergy indices clearly demonstrated antagonism between bestatin and melflufen (CI > 1.2, HSA and BLISS δ < −15), further strengthening the hypothesis of aminopeptidase-mediated potentiation of melflufen's efficacy. Purified ApB promoted hydrolysis of melflufen in vitro, as demonstrated by colorimetric assay (Figure 5B). ApB recognizes N-terminal arginine and lysine, which both have basic nitrogen atoms in their side chains. Interestingly, melflufen has an N-terminal MPH, with a side chain bearing basic nitrogen located at a similar distance from the peptide bond as in arginine and lysine (Figure 5C).Overall, the data indicate that ApB is involved in melflufen's cytotoxicity.

Melflufen prevents clonogenic outgrowth of myeloma colonies derived from bone marrow mononuclear cells of RRMM patients

Resistance to BTZ has previously been described in MM progenitor cells (MMPC).[38,39] These are a drug-resistant minor population of myeloma "stem" cells, with self-renewal potential and the ability to support clonogenic outgrowth of myeloma bulk tumors in a colony forming assay.[40,41] Intriguingly, gene expression analysis of B-lymphoid subsets (GSE141005) revealed

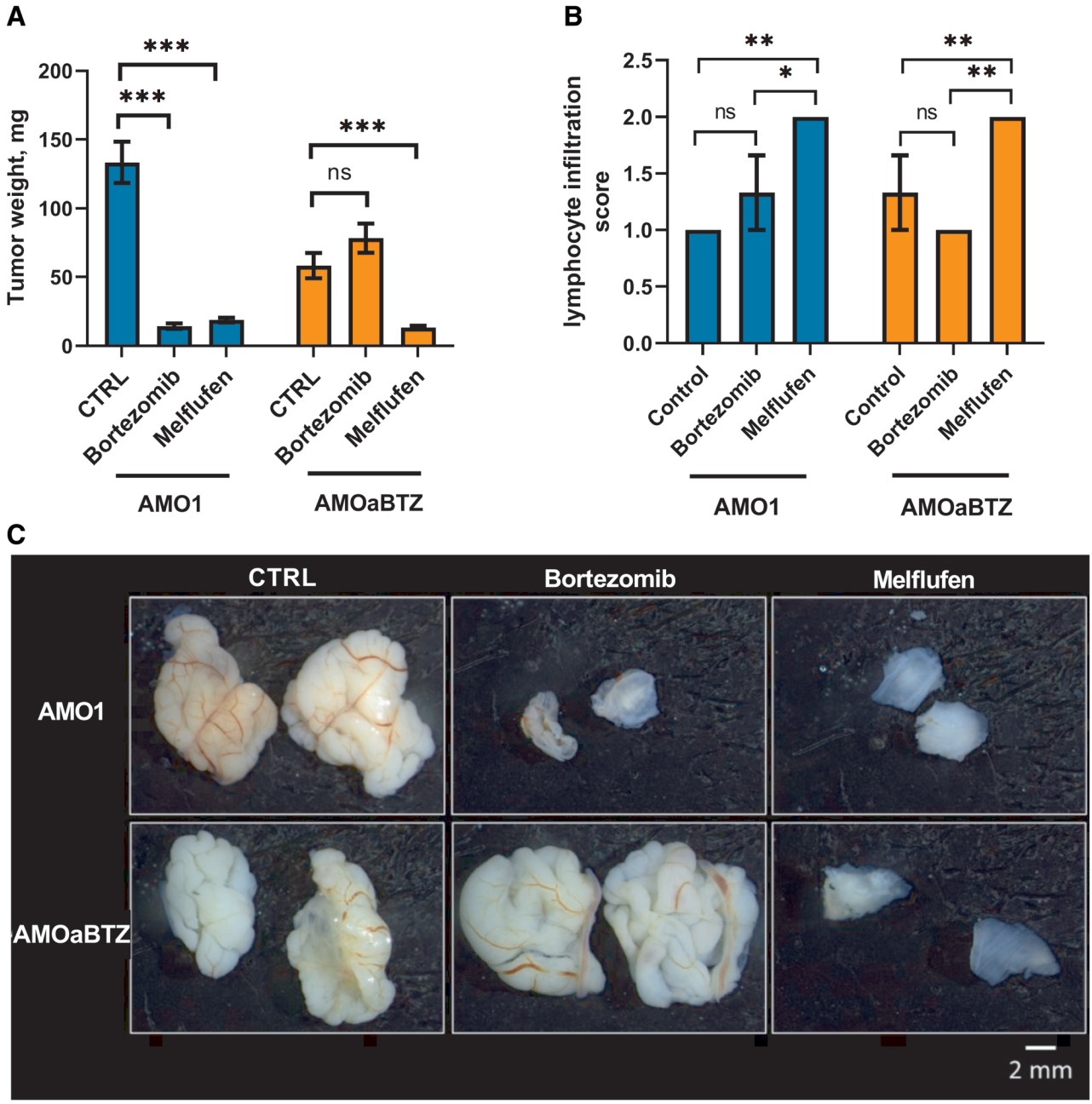

Figure 3. Effect of melflufen in bortezomib-resistant cells in the CAM assay. (A), Tumor weight analysis in chick embryos xenografted with AMO1 and AMOaBTZ cells and treated with solvent control, bortezomib, or melflufen at concentrations indicated in the Materials and Methods. Data represent mean ± SEM of 3 independent experiments; ***$P < 0.001$, evaluated by paired t test. (B), Lymphocyte infiltration score calculated based on evaluation of microscopically 10 nonoverlapping fields (HPF) from each section of the tumor by a semiquantitative scoring system for the presence of pathological changes. Scoring for lymphocytes infiltration within the tumor: 0 (absence of lymphocytes in the tumor); 1 (below 5 lymphocytes per HPF); 2 (between 5 and 20 lymphocytes per HPF); 3 (above 20 lymphocytes per HPF). Two sections from 3 tumors from each conditions were analyzed. Data represent mean ± SEM of 3 independent experiments; *$P < 0.05$; **$P < 0.01$, evaluated by t test. (C), Photographs of representative tumors removed from the CAM at the end of the experiment, 2 from each condition. AMOaBTZ = AMO1 bortezomib adapted cells; CAM = chick chorioallantoic membrane; CTRL = control conditions; HPF = high-power field; ns = not significant.

elevated *RNPEP* gene expression in plasma cell progenitors, which decreases on plasma cell differentiation (Figure 6A). Therefore, melflufen and other myeloma drugs (BTZ, CFZ, BDM, MPH, LEN, POM, IBER, CC-92480, and SXR) were tested for the ability to suppress the clonal outgrowth of colonies derived from mononuclear cell fractions obtained from the bone marrow of RRMM patients, who relapsed within 6 months or became refractory to a triplet therapy consisting of cyclophosphamide, BTZ, and dexamethasone. The drugs were used at clinically reachable concentrations, based on available pharmacokinetic data and previous studies.[42–46] BTZ could not suppress the clonal MM outgrowth, indicating a weak anti-MMPC activity, which is in line with previous results.[39] Of the other antimyeloma drugs tested, only melflufen and MPH showed a 100% suppressive effect on clonogenic recovery of RRMM cells, whereas the other drugs demonstrated little (BDM, LEN, POM) to moderate (CFZ, IBER, CC-92480, SXR) anti-MMPC effect (Figure 6B). The data further show that melflufen is almost 100-fold more active in colony forming suppression than MPH (which also fully suppressed the clonal

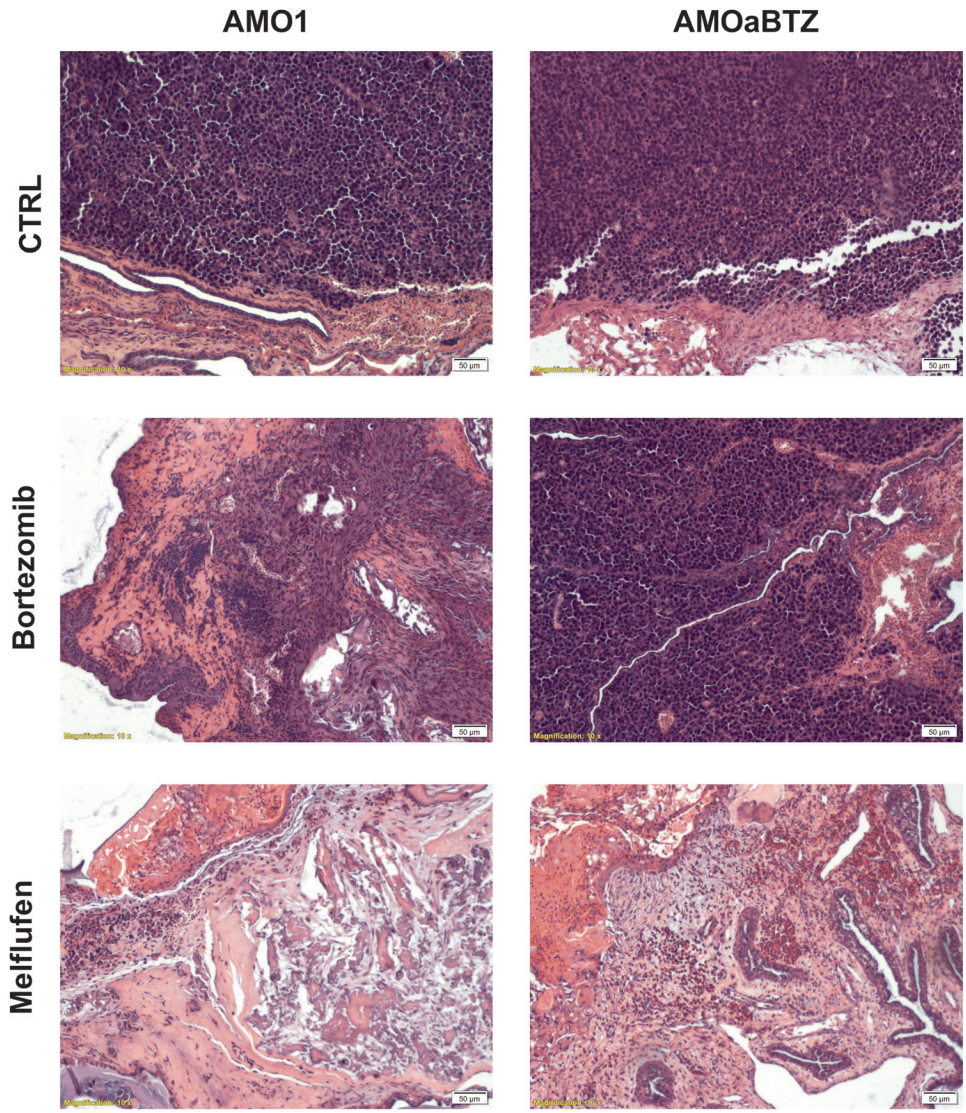

Figure 4. Histopathology analysis of AMO1 and AMOaBTZ-derived tumors. Hematoxylin & eosin staining of AMO1 and AMOaBTZ-derived tumors treated with a solvent control, bortezomib, and melflufen. AMOaBTZ = AMO1 bortezomib adapted cells; CTRL = control condition.

outgrowth at higher doses), despite carrying equimolar amount of alkylating moiety.

Discussion

BTZ is one of the common backbones in the treatment of NDMM patients. Nevertheless, patients with MM ultimately develop resistance to BTZ and relapse. Nowadays, almost all RRMM patients become BTZ-resistant and require novel agents for further treatment. Acquisition of PI resistance is a complex process associated with metabolic changes allowing malignant myeloma cells to become less dependent on the proteasome, a state where inhibition of proteasome activity does not result in cell death.[8,9,47]

Elevated levels of aminopeptidases are observed in different cancer types[17–21] and are associated with aggressive disease and tumor dissemination.[36,37] There are at least 2 possible strategies to target aggressive neoplastic cells, based on their elevated aminopeptidase expression. One is to apply aminopeptidase inhibitors, such as bestatin or tosedostat. However, bestatin is only used as an adjunct to chemotherapy of acute leukemia and tosedostat is yet to reach the clinic.[21,48] In addition, there is a risk that aminopeptidase activity may not be essential for a cancer cell survival, thus their inhibition may not bring the desirable cytotoxic effect. The other approach is to employ the enzymatic activity of aminopeptidases against the neoplastic cells by using a lipophilic peptide-drug conjugate, such as melflufen.[22,49] Lipophilic melflufen is passively transported through cellular membranes and rapidly hydrolyzed by cellular aminopeptidases into MPH, which becomes trapped within the cellular compartment of the cancer cell due to its hydrophilic nature.[25] However, in order for this scenario to take place, the peptide-drug conjugate must serve as a substrate for the aminopeptidase that is upregulated within the aggressive neoplastic cell.

In this study, ApB encoded by *RNPEP* gene was found to be upregulated on acquisition of resistance to BTZ in human MM cell lines and in a murine model of high-risk MM. Moreover, elevated *RNPEP* gene expression is associated with a shorter progression-free and overall survival of MM patients, thus confirming higher *RNPEP* expression as a risk factor previously described in other patient cohorts.[34] Likewise, elevated *RNPEP* gene expression is observed in plasma cell precursors, a B-lymphoid cell compartment, where the tumor-initiating MMPC or "stem" cells are residing.[41] Hence, elevated ApB

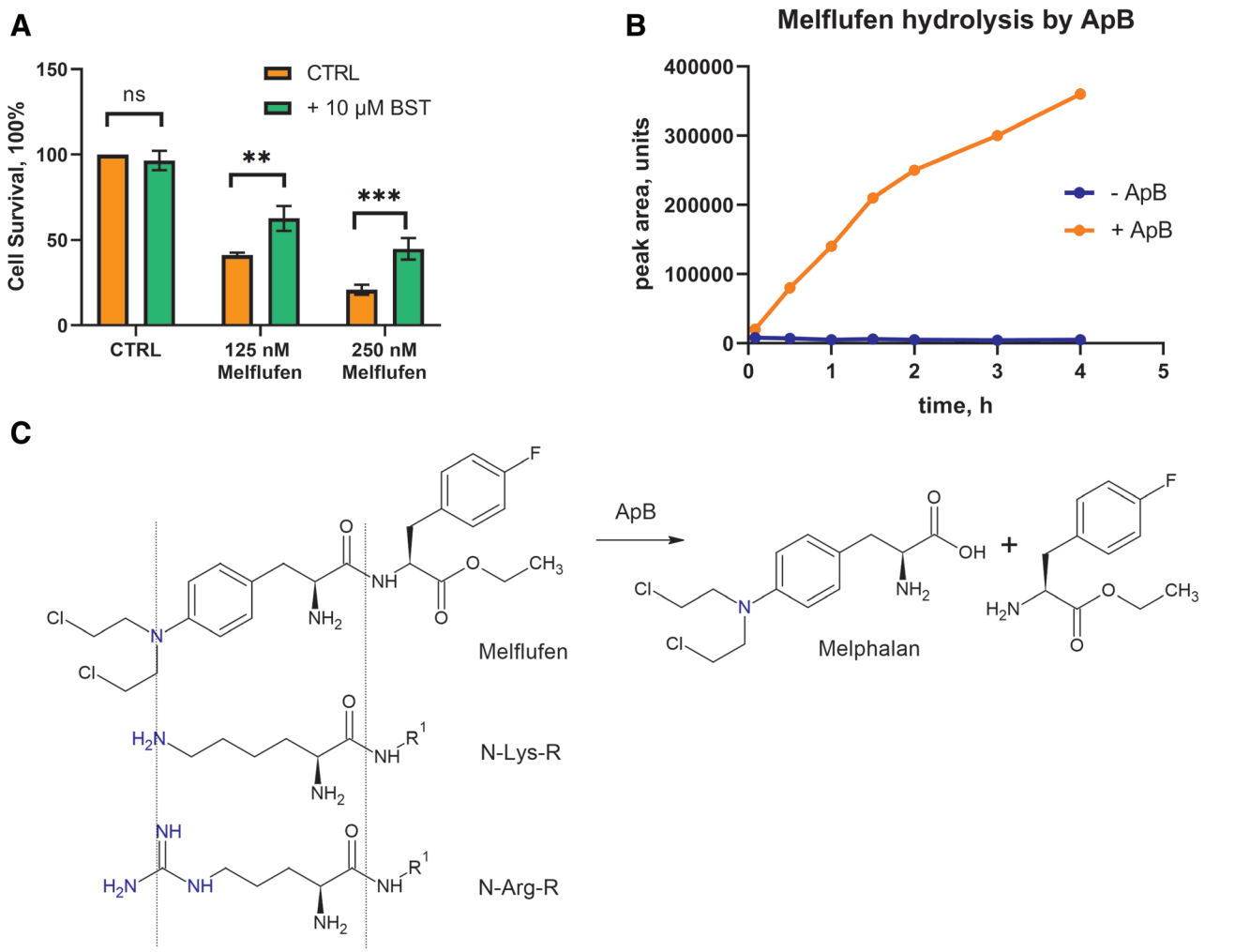

Figure 5. Analysis of melflufen as a substrate of aminopeptidase B. (A), Cytotoxicity of 125 and 250 nM melflufen in AMOaBTZ cells in the presence of 10 μM BST. Data represent mean ± SD of 3 independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$, evaluated by paired *t* test. (B), In vitro hydrolysis of melflufen by aminopeptidase. The data represent 2 independent experiments. (C), Structural comparison of side chains in melflufen and aminopeptidase B substrates, N-terminal Lys and Arg. Basic nitrogen atoms with the side chains are depicted in blue.

levels are associated with aggressive myeloma clones conferring tumor initiation and therapy resistance in MM.

ApB recognizes N-terminal basic amino acids arginine and lysine. The side chains of these amino acids carry additional nitrogen atoms. MPH moiety within melflufen is often seen as a phenylalanine analog. However, the nitrogen atom within the alkylating mustard moiety of MPH is located approximately the same distance from the peptide bond as in lysine and arginine. Thus, ApB may utilize a peptide like melflufen, which carries N-terminal MPH residue as a substrate. Indeed, in this study, ApB was shown to promote hydrolysis of the peptide bond in melflufen, resulting in the release of hydrophilic MPH, and its entrapment within aggressive myeloma cells, resulting in cell death. Melflufen-mediated cell death could be attenuated by aminopeptidase inhibitor bestatin, confirming the aminopeptidase-mediated antineoplastic activity of melflufen. Thus, melflufen has a potential to eradicate aggressive myeloma clones characterized by elevated ApB expression, such as BTZ-resistant clones and MMPC.

MMPC can give rise to differentiated progeny generating the bulk of myeloma tumor in a patient's bone marrow, and are proposed to be one of the reasons of incurability of MM.[50] It has been previously shown that dexamethasone, LEN, BTZ, and 4-hydroxy-cyclophosphamide had only little effect on MMPC clonogenic recovery.[41] There are different opinions on the exact phenotype of MMPC; however, they all agree that MMPC are capable of clonogenic outgrowth.[40,41] In this study, other antimyeloma drugs, such as BDM and BTZ, had little effect on the clonogenic recovery of MM-colony forming cells (MM-CFC) from mononuclear cell fractions obtained from the bone marrow of RRMM patients. More advanced drugs, such CFZ, IBER, novel immunomodulatory drug CC-92480, and XPO1 inhibitor SXR, were active in suppressing the MM clonal outgrowth, but could not prevent it with 100% efficiency at physiological concentrations. On the contrary, melflufen and MPH showed total suppression of MM clonal outgrowth. As for MPH, it may reflect its bone marrow ablation when provided in high doses. However, melflufen has shown the same effect at concentrations 100-fold lower, probably reflecting its potentiation within MMPC by elevated ApB levels observed in plasma cell precursors. It is tempting to speculate that a superior anti-MMPC activity of melflufen in the clonogenic assay might explain a prolonged overall survival in RRMM patients treated with melflufen/dexamethasone combination.[25] However, further studies identifying exact phenotype of MMPC and their vulnerabilities need to be conducted, since MMPC are likely a heterogeneous cell population reflecting the complexity of the disease.

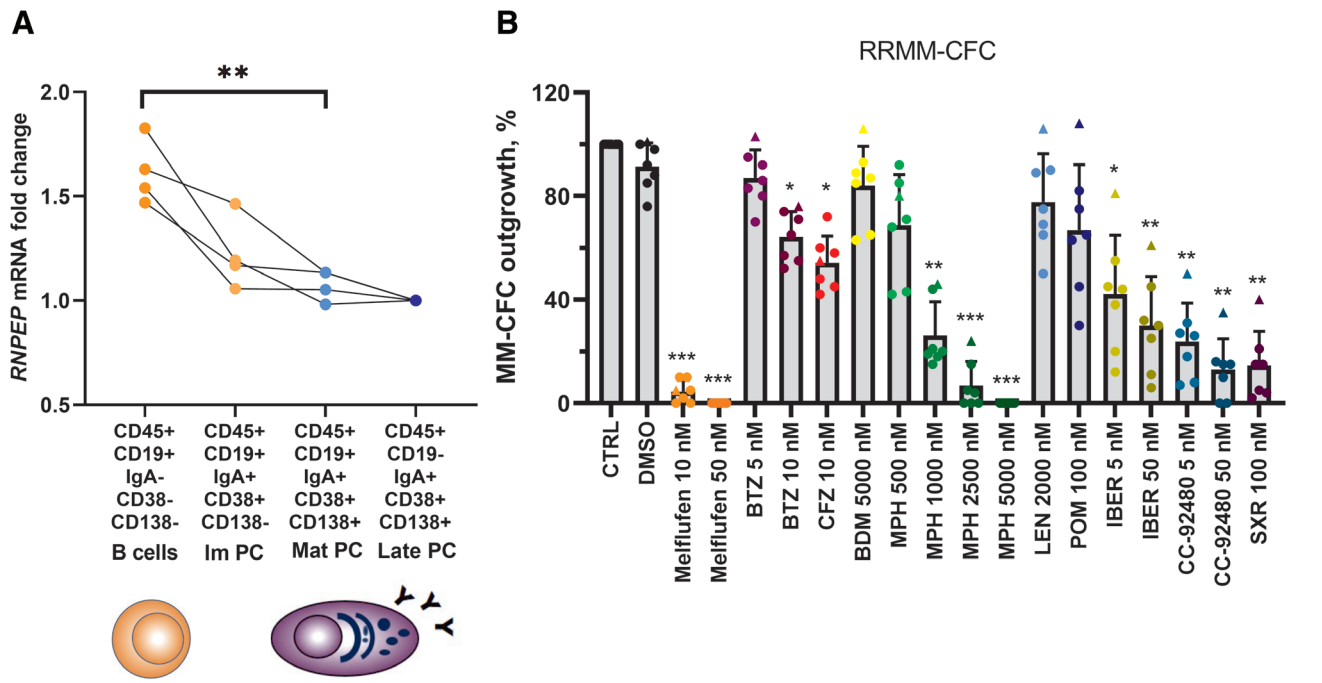

Figure 6. Clonogenic outgrowth of MM progenitor cells. (A), Aminopeptidase B expression in B-lymphoid subsets including plasma cell precursors and mature plasma cells as determined by depicted immunophenotype. $^{**}P<0.01$. (B), Quantification of colony-forming cells from mononuclear cell fractions of RRMM patients' bone marrows, untreated or treated with solvent control (DMSO), MFL, BTZ, CFZ, BDM, MPH, LEN, POM, IBER, CC-92840, and SXR at given concentrations. Data represent 3 independent experiments for each patient sample at each concentration; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; evaluated by Mann-Whitney *U* test. BDM = bendamustine; BTZ = bortezomib; CFC = colony forming cell; CFZ = carfilzomib; CTRL = control condition; DMSO = dimethyl sulfoxide, solvent; IBER = iberdomide; LEN = lenalidomide; MFL = melflufen; MM = multiple myeloma; MPH = melphalan; PC = plasma cells; POM = pomalidomide; RRMM = relapsed/refractory multiple myeloma; SXR = selinexor.

Although the exact role of ApB in MM progression and drug resistance is still not fully elucidated, its elevated expression in aggressive myeloma subclones may already be utilized therapeutically, as shown by promising results of melflufen/dexamethasone combination in patients with RRMM, including those with high risk cytogenetics, as well as those suffering from triple-class refractory and extramedullary diseases.[25,29,30,51,52] As for the future development, myeloma treatment paradigm is currently moving toward the use of dexamethasone-based triplet and even quadruplet combinations. In line with this treatment paradigm shift, 2 triplet combinations based on BTZ or daratumumab and melflufen/dexamethasone were investigated in a phase I/II clinical trial ANCHOR (OP-104).[25,53] Monoclonal anti-CD38 antibody daratumumab works through promoting antimyeloma immune responses by various mechanism involving lymphocytes.[54] Thus, by stimulating lymphocyte infiltration into the tumor, as observed in this study, melflufen may build an effective combination with daratumumab. Indeed, RRMM patients recruited to the daratumumab/melflufen/dexamethasone arm of the ANCHOR study have shown ORR of 76% and clinical benefit rate of 79%, with 2 patients (6%) having achieved complete response.[53] Therefore, future studies of melflufen combination with therapeutic modalities that promote antimyeloma immune response are warranted.

Disclosures

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