Safety and efficacy of vorinostat, bortezomib, doxorubicin and dexamethasone in a phase I/II study for relapsed or refractory multiple myeloma (VERUMM study: vorinostat in elderly, relapsed and unfit multiple myeloma)

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Supplementary methods

Study

The primary objective of this prospective, phase I/II, open-label trial was the determination of the maximum tolerable dose (MTD) of vorinostat, given in combination with BDD (Fig. S1A). The trial included a phase I part with a standard dose escalation design to determine the MTD (Fig.S1B) and a phase II part to further evaluate safety, tolerability and potential biomarkers of response. In the phase I part of the study, consecutively recruited patients received oral vorinostat at three doses (100, 200, 300mg on day [d] 1-4, d8-11, d15-18) according to a standard 3+3 design (Suppl. Fig. S1B). The 28d schedule incorporated fixed doses of subcutaneous (sc) bortezomib (1·3mg/m² d1, 8, 15), intravenous (iv) doxorubicin (9mg/m² d1, 8), and oral dexamethasone (cycle 1: 40mg, cycle 2-6: 20mg weekly). Before initiating the VBDD trial, possible vorinostat schedules had been discussed with renowned experts in the field: our aim being to use a VBDD combination that was both well-tolerable and efficacious. Since prior HDACi trials had reported of prevailing side effects, precluding RRMM patients to remain on triplet or quadruplet medication, we chose a potentially well-tolerable continuous vorinostat use of '4-days on and 4-days off', and repeating this throughout 28d cycles (Fig. S1B). This was adapted from the '4d-on and -off' dexamethasone treatment as compared to HDACi regimens applied for 8, 14 or even 21 days.

Our cumulative vorinostat doses of 1200mg, 2400mg and 3600mg per cycle were comparable to previous trials (Table 1A). Our study aim was not to limit the total HDACi dose per cycle, rather than to test a new combination schedule with best tolerability. Enrollment to the next dose level was permitted, if fewer than one of three, or two of six patients experienced a dose-limiting toxicity (DLT) at a given dose level. Having reached the maximum dose of vorinostat, additional patients were treated within the phase II study part. Patients continued VBDD for a total of six cycles. The study protocol was approved by the institutional review board of University of Freiburg Medical Center and compiled with governmental regulatory requirements. It was performed in accordance with the Declaration of Helsinki and the International Conference on Harmonization guidelines for Good Clinical Practice. All patients provided written informed consent before treatment was started. The trial was registered at ClinicalTrials.gov (NCT01394354, https://clinicaltrials.gov/ct2/show/NCT01394354?term=vbdd&rank=1).

Participants

Patients with RRMM were eligible after at least first-line therapy (including bortezomib and/or IMiDs). Patients were required to be ≥18 years, with a Karnofsky performance status (KPS) ≥60% and adequate hematologic (ANC ≥1x10e9/L, platelet count ≥50x10e9/L and hemoglobin >7g/dl, unless myelosuppression was secondary to bone marrow [BM] plasmacytosis with infiltration rates >70%), hepatic and renal function (AST, ALT ≤2·5 x upper limit of normal (ULN), Bilirubin ≤1·5 x ULN, eGFR >20ml/min). Patients were ineligible if they suffered from severe organ disease (including neuropathy NCI-CTC ≥grade 3, hepatic impairment, pulmonary or pericardial disease), myeloma-related CNS involvement or if they had received prior HDACis.

Outcomes

The primary endpoint of the study was the MTD of vorinostat, given in combination with fixed doses of BDD (Fig.1B). The MTD is defined as the highest dose at which six patients have been treated and less than two patients experienced DLT within the first cycle of treatment. DLTs were defined as any possibly drug-related adverse event (AE) ≥grade 3 (CTCAE) or abnormal laboratory value assessed as clinically relevant that occurred within the first VBDD cycle (d1-28). AEs were documented according to CTCAE Version 3·0 and reported until three months after the last application. The secondary endpoints included overall response rate (ORR) according to EBMT and IMWG criteria. Disease assessments incorporated measurements of serum M-protein, serum-free light chains, serum immunofixation and PC infiltration of BM-aspirates and -histology sections. PFS, OS, and quality of life (QoL) were

assessed before and at the end of study treatment (EoT). These tests included fitness ratings, functional comorbidity tests and comorbidity indices as described by us previously. Additional endpoints were HDAC expression in BM samples and pan-HDAC activity in peripheral blood mononuclear cells (PBMCs). Response and disease progression were assessed by clinicians blinded for the investigator-reported responses; next line treatment is summarized in Fig. S2.

Pharmacodynamics

HDAC6 expression in BM specimens and pan-HDAC activity in PBMCs served as correlative endpoints and were assessed throughout the trial (screening and cycle 2, d8). For the measurement of pan-HDAC activity, PBMCs were isolated from whole blood using Ficoll density gradient centrifugation. The interphase was separated, incubated, washed and the cells were counted in RPMI 1640 without phenol red by adding Türk's solution. 49µL cell suspension containing $8x10^5$ PBMCs were incubated with 0·5µL 30mM MAL (Boc(Ac)Lys-AMC) substrate and 0·5µL Igepal CA-630 solution for 20min at 37°C. The enzymatic conversion of MAL into the intermediate product ML (BocLys-AMC) was stopped with Trichostatin A. Addition of trypsin led to proteolytic release of the fluorescent product AMC (7-Amino-4-methylcumarine), which was detected at λ_{Ex}=390 and λ_{Em}=460nm, correlating with uninhibited HDAC activity. The pan-HDAC activity was quantified against positive and negative controls containing no enzyme and either AMC or MAL, respectively (Fig.S3). Immunohistochemistry (IHC) was performed before, in cycle 2 and EoT assessing the extent of BMPCs and HDAC6 expression. IHC was performed as follows: 2µm BM tissue sections were deparaffinized using Xylol, heat-induced antigen retrieval was done by cooking at 95°C for 20 min in antigen retrieval buffer. Incubation with the primary antibody (Abcam, anti-HDAC6 antibody, ab1440, 1:200) was performed for 1h, followed by staining with Dako Real Detection System and counterstained with hematoxylin and eosin reagent. The HDAC6 IHC was interpreted based on the intensity (0=negative; 1=mild; 2=moderate; 3=strong) and the proportion of positive cells, evaluated in 100-200 PCs/case. HDAC IHC scores were calculated through multiplication of staining intensity, differentiating low (0-1), moderate (2), and high expression (3). Because HDACi trials had shown pharmacokinetics these were not repeated.

Statistical analysis

Sample size considerations were based on the primary objective of the trial: it was anticipated that at most 15-18 patients would be sufficient to determine the MTD. The decision to enroll a total of 33 patients was based on feasibility considerations and to obtain more convincing data on safety and efficacy of our schedule. Statistical analyses were performed with SAS 9·2 (SAS Institute Inc., Cary, NC, USA). Data are presented as of December 3, 2016. For the primary endpoint, MTD was estimated as the highest dose at which less than two DLTs in six patients were observed in the first cycle. MTD estimation was based on the phase I part of the trial. The comparison of QoL pre- and post-treatment was evaluated with the Wilcoxon's signed rank test in patients where both assessments were available. OS and PFS were calculated as time from start of treatment until death/death or first observation of progression. Patients without an event of interest were considered as censored observation at the time last seen alive/without observation of disease progression. OS and PFS were estimated using the Kaplan Meier method.

Supplementary Table S1. Patient variables in PBMCs' HDAC activity: responders vs. non-responders in 8 patients each with PBMC HDAC measurements (see details also in Fig. 1)

Patient variables	PBMC HDAC-responders	PBMC HDAC-SD / Non-responders
Sex: male (m) / female (f)	4 / 4	5 / 3
Median age [years]	64	65
ISS at ID	stage III: 50%	stage III: 50%
Pretreatment		
Prior ASCT [n, %]	8 (100%)	7 (87.5%)
Prior proteasome inhibitors (PI; n, %)	7 (87.5%)	7 (87.5%)
Prior IMiDs (n, %)	5 (62.5%)	3 (37.5%)
Thalidomide (n, %)	1 (12.5%)	0
Lenalidomide (n, %)	1 (12.5%)	1 (12.5%)
Both thalidomide and lenalidomide (n, %)	3 (37.5%)	2 (25%)
eGFR (MDRD) [ml/min]	67 (34-111)	50 (28-114)
BM PC infiltration [%] (pathology)	45 (10-80)	65 (25-70)
BM PC infiltration [%] (cytology)	33 (9-90)	66 (25-85)
Unfavorable cytogenetics [%]	57	66
Best ORR [%]	50	38
EoT ORR [%]	25	38

Abbreviations and explanations:
SD: stable disease, ISS: International Staging System; ID: initial diagnosis; n: number of patients; eGFR - ß2-MG score; MDRD: Modification of Diet in Renal Disease; BM: bone marrow; PC: plasma cell; ASCT: autologous stem cell transplantation; IMiD: immunomodulatory drugs (thalidomide, lenalidomide, pomalidomide); EoT: end of treatment; ORR: overall response rate; CBR: clinical benefit rate (≥SD)
In 16 patients, HDAC measurements in PBMC samples were performed (as also depicted in Fig. 1). In total, we observed 14 serologic responders and only two non-responders. In those two non-responders, only minor reductions in HDAC activity were observed, whereas the 14 responders presented with a mixed pattern of HDAC activity reduction, the 8 patients with most prominent HDAC PBMC reductions being depicted above 'responders'. Comparing HDAC decreases between baseline and cycle 2 day 8 for these patient groups via Wilcoxon's 2sample test did not yield a statistically significant result (p=0.94).

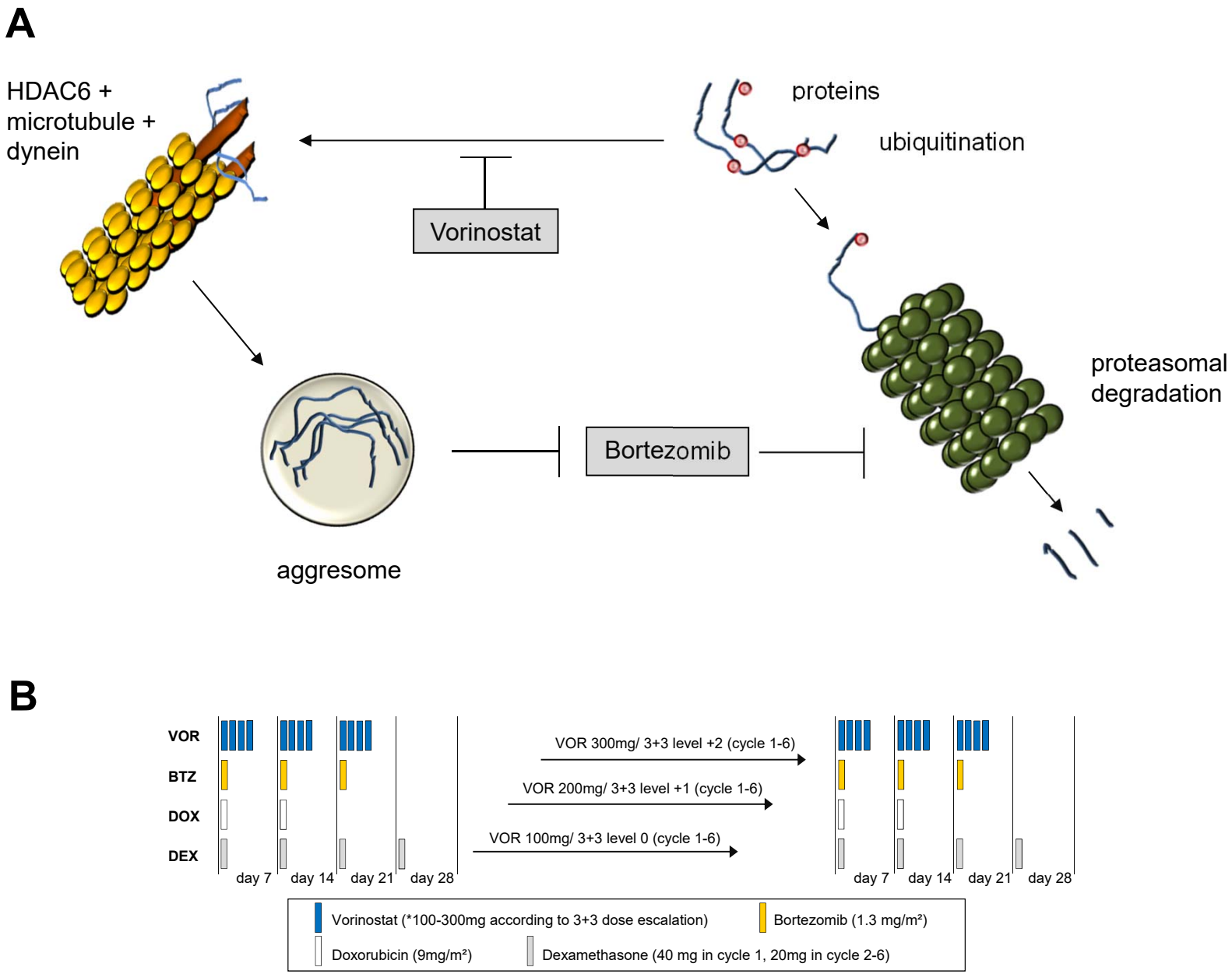
Figure legends

Supplementary Figure S1A. Molecular synergism of vorinostat and bortezomib. HDACi are a group of deacetylating agents with multiple effects on intracellular metabolism, including modifying impact on transcription factors, molecular chaperones, and signal transduction. Synergistic activity has been observed in combination with bortezomib. HDACi antagonize escape mechanisms secondary to proteasome inhibitor treatment by facilitating the aggregation of misfolded protein and its removal along microtubules by dynein motor proteins. The combination of proteasome inhibitors and vorinostat thus amplifies cellular stress and adds up to a substantial increase in apoptosis. Apart from the latter main impact on the aggresome pathway, vorinostat has been described to exert further mechanisms which account for a potential synergism with bortezomib, including enhanced cytochrome-c release, caspase and PARP cleavage and inactivation of NF-κB.

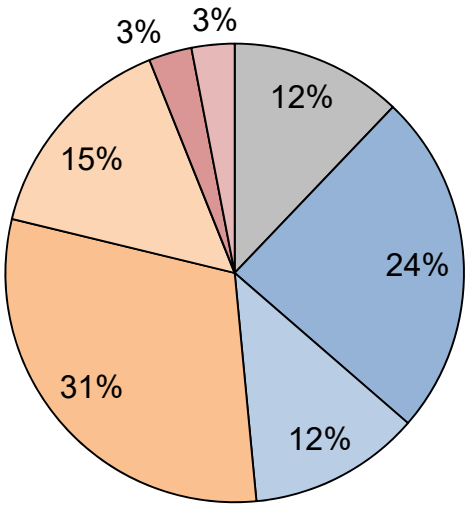
Suppl. Fig. S1B. Dosing schedule: boxes represent individual days with shaded boxes indicating days on which medication was administered. Patients were enrolled onto three dose levels and treated on 28-day cycles with vorinostat (VOR) 100-300mg daily (depending on dose escalation cohort) d1-4, d8-11 and d15-18. Subcutaneous bortezomib (BTZ) was administered once weekly on d1, 8 and 15 at a dose of 1·3mg/m², doxorubicin (DOX) once weekly on d1 and 8 at a dose of 9mg/m² (i.v.) and dexamethasone (DEX) on d1, 8 and 15 (40mg within cycle 1, reduced to 20mg in cycle 2-6), repeated for six cycles.

Suppl. Fig. S2. Pie chart and table illustrating next line treatment after VBDD.
Abbreviations: VD: bortezomib-dexamethasone; VTD: bortezomib-thalidomide-dexamethasone; PBSCT: peripheral blood stem cell transplantation; allo-SCT: allogeneic stem cell transplantation; Pom/Dex: pomalidomide + dexamethasone; MOR03087: anti-CD38-antibody treatment/dexamethasone.

Suppl. Fig. S3. Pharmacodynamic assessment of pan-HDAC activity. Pan-HDAC activity was measured in PBMCs at screening and cycle 2, d8. For this purpose, PBMCs were isolated from PB samples of patients at baseline and cycle 2, d8. Lysis of PBMCs led to HDAC release. Next, Boc(Ac)Lys-AMC (MAL), a fluorescent substrate incorporating a monitoring acetyl group, was added to the samples. In samples with low vorinostat activity, HDACs deacytelated MAL. Decetylated MAL (ML) is prone to trypsin cleavage leading to fluorophor 7-amino-4-methylcoumarin (AMC) release. AMC release emits a fluorecent signal correlating with high pan-HDAC activity in the respective sample. Contrarily, acetylated Boc(Ac)Lys-AMC (MAL) may not be cleaved by trypsin and gives no signal.



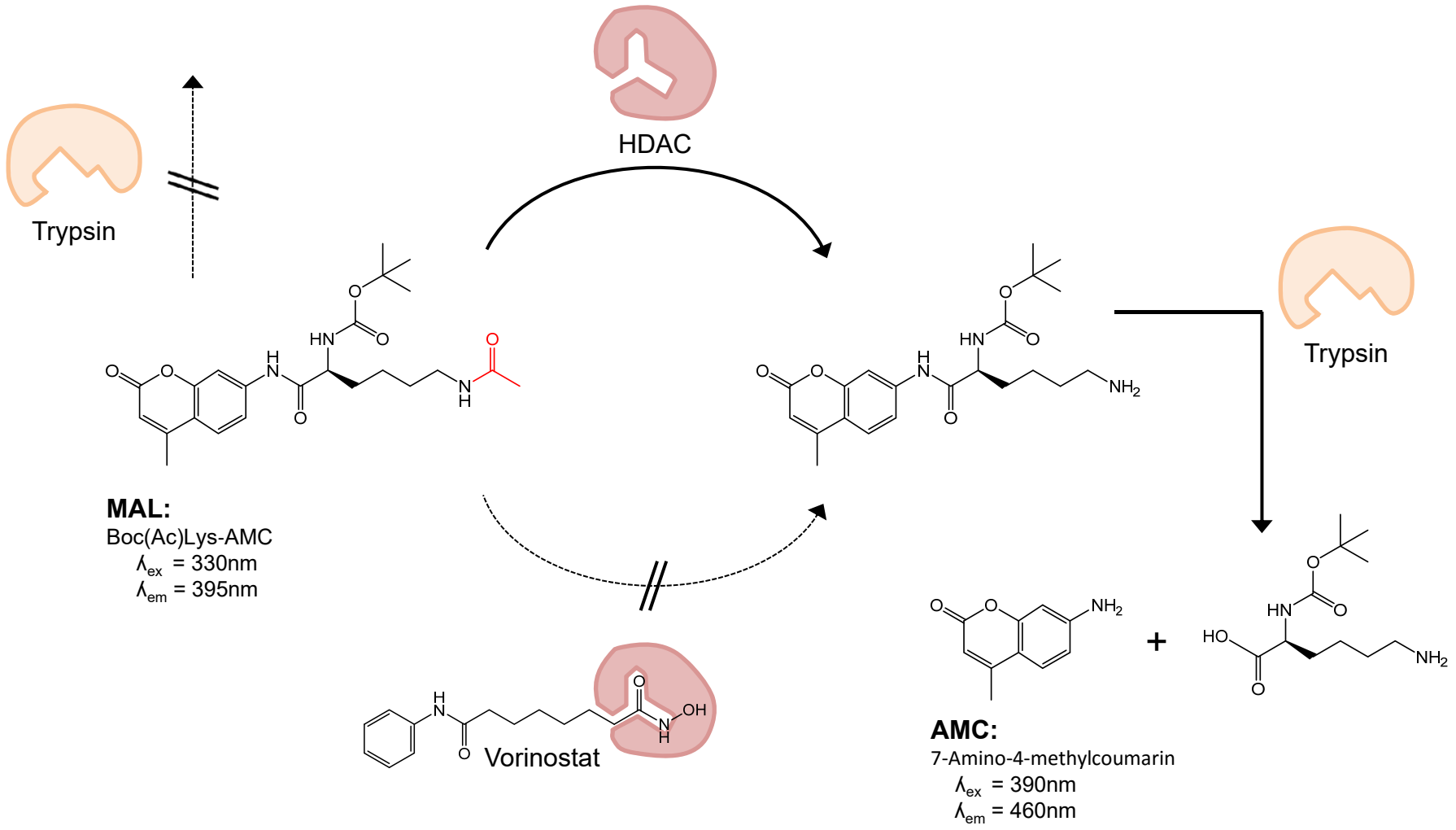
Suppl. Figure S1



No maint. VD VTD ASCT allo-SCT Pom/Dex Mor03087

Subsequent treatment after VBDD		
n=33	**# patients**	**Therapeutic intention + reason**
No maintenance	4	no AEs desired and treatment free interval
VD	8	maintenance
VTD	4	maintenance
ASCT	10	consolidation
Allo-SCT	5	consolidation
Poma + Dex	1	next line
MOR03087	1	next line

Suppl. Figure S2



Suppl. Figure S3