Published in final edited form as:

Clin Cancer Res. 2021 April 01; 27(7): 1974–1986. doi:10.1158/1078-0432.CCR-20-2839.

VLA4-nanoparticles hijack cell adhesion mediated drug resistance (CAM-DR) to target refractory myeloma cells and prolong survival

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Abstract

Background.—In multiple myeloma (MM), drug resistant cells underlie relapse or progression following chemotherapy. Cell adhesion mediated-drug resistance (CAM-DR) is an established mechanism used by MMC to survive chemotherapy and its markers are upregulated in residual disease. The integrin VLA4 ($\alpha_4\beta_1$) is a key mediator of CAM-DR and its expression affects drug sensitivity of MMC. Rather than trying to inhibit its function, we here hypothesized that up-regulation of VLA4 by resistant MMC could be exploited for targeted delivery of drugs, which would improve safety and efficacy of treatments.

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Contributions:

FF led the design, execution and analysis of the research. MJS, SA, FF conducted experiments. DP and GM co-invented CPT-PD. SF aided in the scale up of chemical synthesis. GC synthesized and characterized CPT-PD and VLA4 ligand. XY, MJS and KS prepared nanomicelles and optimized nanoformulation synthesis procedures. NY performed the histology experiments. JR managed animals and assisted in sampling. MR and JO participated in the experimental design and execution of flow cytometry and SPEP. MF and RV provided the myeloma patient tumor data and BM samples. RV and JDP provided clinical insight and editing of the paper. JDP and GML provided funding. FF and GML wrote the manuscript. GML provided nanomedicine expertise, experimental direction, training, infrastructure and funding.

Conflicts of Interest

Washington University has intellectual property (co-inventors: GML, DP) regarding the phospholipid prodrug technology and the targeted micellar nanoparticle.

Methods: We synthesized 20 nm VLA4-targeted micellar nanoparticles (V-NP) carrying DiI for tracing or a novel camptothecin prodrug (V-CP). Human or murine MMC, alone or with stroma, and immunocompetent mice with orthotopic MM were used to track delivery of NP and response to treatments.

Results: V-NP selectively delivered their payload to MMC in vitro and in vivo, and chemotherapy increased their uptake by surviving MMC. V-CP, alone or in combination with melphalan, were well tolerated and prolonged survival in myeloma-bearing mice. V-CP also reduced the dose requirement for melphalan, reducing tumor burden in association to sub-optimal dosing without increasing overall toxicity.

Conclusions: V-CP may be a safe and effective strategy to prevent or treat relapsing or refractory myeloma. V-NP targeting of resistant cells may suggest a new approach to environment-induced resistance in cancer.

Introduction

Multiple myeloma (MM) is a plasma cell neoplasia characterized by a recurring clinical course (1). Newer treatments have significantly improved survival. However, all patients relapse, requiring multiple lines of treatment (2). As MM affects older patients and induces multi-organ damage, therapeutic options are limited by side-effects, and preventing and treating chemoresistance without aggravating toxicity remains an unmet need.

The relationship between myeloma cells (MMC) and the bone microenvironment (BME) evolves through progression: in active lesions, most cells are found in homogeneous areas with reduced stromal contact (3). Chemotherapy can select for dormant MMC in tight contact with the bone surface (4,5), from which relapse can initiate (6,7). In “Cell Adhesion Drug Resistance” (CAM-DR), signaling through adhesion molecules, engaged with the bone matrix and stromal cells (8), protects MMC from chemotherapy (4,9). MMC resistant to anti-myeloma drugs such as doxorubicin have higher levels of integrin $\alpha_4\beta_1$, (Very Late Antigen 4; VLA4; CD49d/CD29), and adherence to fibronectin promotes survival to drug treatment (4,8). In patient samples, VLA4 expression is increased in relapsing disease or minimal residual disease (5,10). Pharmacological interventions to inactivate VLA4-mediated adherence to the BME (11,12) and sensitize myeloma cells to chemotherapy have been tested with variable success (13). This research considers an alternative approach and hypothesizes that VLA4 up-regulation by resistant MMC (R-MMC) could be exploited as a target for nanotherapy (Suppl Fig 1A).

Camptothecin (CPT) and its derivatives topotecan and irinotecan (Suppl Fig. 1B) are potent anti-cancer agents that inhibit DNA topoisomerase I (TOP1)(14,15). CPT analogues have been considered for combination regimens in MM (16-18), but serious off-target side effects and limitations in stability and bioavailability (14) have limited their use.

We hypothesized that coupling PAz-PC (1-palmitoyl-2-azelaoyl PC) to camptothecin through the E ring (Suppl. Fig 1B) would stabilize (14) and inactivate CPT (Fig. 1A), and allow its protected incorporation within the hydrophobic aspect of the nanoparticle

phospholipid surfactant, which also presents the high affinity peptidomimetic ligand for VLA4 (16) (V-CP, Suppl fig 1C).

Upon binding to their target, nanomicelles fuse and the prodrug transfers into the cell membrane (20) (Fig 1B), a mechanism referred to as “contact-facilitated drug delivery” (CFDD) (11,20). After fusion, cellular membrane trafficking carries prodrugs until lipases release them into the cytosol (11,20,21).

In lieu of attempting to dislodge cancer cells from the protective microenvironment by adhesion receptor antagonists (12,22), we leveraged selective pressure from free chemotherapy to upregulate VLA4 expression in surviving cells for therapeutic advantage (Suppl Fig 1A). This work demonstrates increased MM uptake of VLA4 targeted NP in R-MMC. Consistent with that, treatment of MM bearing mice with V-CP in combination with melphalan improved survival and reduced tumor burden without increasing toxicity.

Materials and methods:

Synthesis and targeting of Sn2-labile CPT pro-drug and micellar nanoparticles

Preparation of CPT-PD was achieved via a one-step coupling of oxidized lipid PAzPC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine) with CPT (Supplementary methods). A VLA-4 antagonist based on the highly selective peptidomimetic, LLP2A (19), was synthesized and coupled to a polyethylene glycol–phosphatidylethanolamine anchor as detailed in Supplementary methods (Fig 1A, Suppl 1D-E). Therapeutic mixed micelles were prepared by microfluidization. The surfactant co-mixture of nanoparticles included phosphatidylcholine (Lipoid, Ludwigshafen, Germany) and 0.18 mol% of VLA4-PEG-PE. The peptidomimetic $\alpha_v\beta_3$ -integrin homing ligand was a gift from Kereos, Inc. (St. Louis, MO) and incorporated into control micellar nanoparticles as previously described (21). For particle uptake assays, DiI (1 mol%, Thermo Fisher Scientific Inc, Waltham, MA) was incorporated. V-CP included 4 mol% of CPT-PD, and was replaced in “No-Drug” (ND) nanoparticles by phosphatidylcholine. Dynamic light scattering was performed with ZetaPlus (Brookhaven Instruments Corporation) on every batch: particle size (average 18.2 ± 3.0), polydispersity (0.24), and electrophoretic zeta potential (-3.1 mV). Data from several batches V-NP illustrated high batch-to-batch reproducibility and long-term shelf-life stability (~ 1 year at 4°C) (Suppl Fig 1F).

RNAseq and Datamining

Patient RNAseq data were obtained from the MMRF/CoMMpass study (23) and expressed as transcripts per million (TPM). Data for myeloma cell lines were obtained from the Gene Expression Atlas (<https://www.ebi.ac.uk/gxa>) and RNAseq data were obtained for MM and healthy tissues from ArchS4 (<http://amp.pharm.mssm.edu/archs4/index.html>) (24).

Cell culture and in vitro assays

Human myeloma cell lines (HMCL) U266, MM.1S, OPM2, RPMI8226, mouse bone marrow stromal cells (BMSC) and macrophages were obtained as previously described (25,26). DexR-8226 cells were selected by culturing RPMI8226 cells in increasing doses

followed by maintenance in 20 μ M dexamethasone. Myeloma cell identity was confirmed by flow cytometry (CD138, CD38, CD184, CD319).

BM and PBMC of MM patients were sorted for CD138, cryopreserved and thawed in complete media one day prior to treatment. MMC media was further supplemented with IL6 4ng/mL. To obtain MM stroma, CD138-negative cells from the bone marrow of newly diagnosed or relapsing patients were selected by culturing them in adhesion as described (26) and then assigned to four experiments: viability by flow cytometry, staining with DiI prior to co-culture, co-culture staining with live/dead, pre-treatment then co-culture.”.

Ex-vivo melphalan-resistant and osteotropic 5TGM1 were obtain by culturing whole bone marrow from a 5TMM mouse and two KaLwRij mice with subcutaneous 5TGM1 (25), and identified by GFP expression by microscopy and fluorimetry.

MTT assay and EdU staining (Abcam) were performed as described. Live/dead staining was performed by incubating with Calcein AM (2 μ g/mL, BD) and propidium iodide (PI, 2 μ g/ml), then imaging with an inverted Nikon Diaphot 300 microscope (Nikon Instruments Inc., Melville, NY). Viability was evaluated as live/(live+dead) cells by ImageJ in 4-12 microscope fields per treatment. Fluorimetry for DiI, Calcein, PI, or GFP was performed with a SpectraMax (Molecular Devices,CA) and normalized to the average of untreated samples.

Flow cytometry

Antibodies against CD45, CD138, CD184, CD38, CD49d, CD29, CD139, CD269 and viability stains were purchased from BD Biosciences (BD; San Jose, CA), Miltenyi Biotec (Auburn, CA) or Invitrogen (Carlsbad, CA) (Supplemental materials). Samples were analyzed on a FACS Calibur (BD) or ZE5 flow cytometer (Bio-Rad, Hercules, CA) and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Animal studies

The Washington University School of Medicine (WUMS) Animal Research Committee approved all experiments. C57Bl/KaLwRij mice were a kind gift from Dr. G Mundy, bred in-house and inoculated as previously described (25,27). Mice were monitored for behavioral change and weight and euthanized for paralysis or weight loss over 20% in accordance with the AVMA Guidelines. Melphalan was prepared and administered as described (25,28). Nanoparticle emulsions (2ml/kg) were infused i.v. Serum protein electrophoresis (SPEP) utilized a Helena QuickScan 2000 workstation(25); complete blood counts (CBC) and serum analyses were performed with a Liasys 330 analyzer (AMS Diagnostics) by the WUMS Department of Comparative Medicine (21).

Optical imaging

Optical imaging was performed on IVIS50 (PerkinElmer, Waltham, MA). Epifluorescence was acquired for λ 535/580nm for DiI and 465/520nm for GFP. Average radiant efficiency (aRE) was measured from regions of interest (ROIs) using Living Image 2.6 (PerkinElmer, Walthman, MA).

Histology and immunohistochemistry

Sections (5 μ m) of paraffin-embedded spleen and liver were stained with hematoxylin and eosin. Bones were fixed in 4% paraformaldehyde and decalcified with 10% EDTA prior to embedding (21). Antigen retrieval for immunostaining was performed with proteinase K (#V302A Promega), direct incubation with HRP-conjugated rabbit anti-mouse IgG (Abcam, #ab97046), developed by DAB substrate (#SK-4100 VECTOR) and counter-staining with hematoxylin (#H-3401 VECTOR) or methyl green (#H-3402 VECTOR). ApopTag Peroxidase In Situ Apoptosis Detection Kit (#S7100, Millipore) was used for TUNEL staining. Slides were imaged using a Nanozoomer scanner and analyzed with NDPview (Hamamatsu, Japan).

Statistical analysis

Two-tailed t-test, ANOVA, Kruskal-Wallis test, and post-hoc tests were used to assess group differences. Kaplan-Meier survival curves were analyzed with the Log-rank (Mantel-Cox) test. Tests were considered significant at $p < 0.05$. Analyses were performed with Prism 6 (GraphPad).

Results

Targeting topoisomerase 1 (TOP1) in multiple myeloma cells

RNAseq from the CoMMpass study (23) showed expression of the *TOP1* gene in 887/887 MMC samples from patients (Fig 1C). Previously treated MM had higher expression of *TOP1* relative to naïve ($p = 0.0101$, Fig 1C). Patient-matched samples showed a trend for increased expression in subsequent sampling ($N = 73$, $p < 0.01$ by two-way ANOVA, Fig 1D). Datamining revealed high levels of *TOP1* in human myeloma cell lines (HMCL) compared to established targets, such as the glucocorticoid receptor (*NR3C1*) or cereblon (*CRBN*) (Suppl. Fig 1G, Gene Expression Atlas <https://www.ebi.ac.uk/gxa>) (29), and over-expression in HMCL relative to normal plasma cells (Suppl. Fig 1H), bone or spleen cells ($p < 0.0001$) (Suppl Fig 1I, ArchS4 <http://amp.pharm.mssm.edu/archs4/index.html>) (24)).

Effects of free CPT were tested in vitro on HMCL. Low micromolar concentrations induced apoptosis in RPMI8226 (Fig 1E) and nanomolar CPT reduced proliferation of MM.1S (EdU incorporation) by over 90% (Fig 1F). To model acquired drug resistance, a sub-line of dexamethasone-resistant RPMI8226 cells (DexR-8226) was developed ($IC_{50} > 300 \mu M$ vs 250 nM) (Suppl. Fig 1J). DexR-8226 cells exposed to CPT had a similar IC_{50} to parental RPMI8226 cells (WT, Fig 1G). Further, combinations of melphalan and CPT enhanced toxicity in WT RPMI8226 (Fig 1H), DexR-8226 (Suppl Fig 1K), U266 (Suppl Fig 1L), MM.1S (Suppl Fig 1M), and 5TGM1 (Suppl Fig 1N). Similar results were also noted combining low-dose dexamethasone and CPT (Suppl Fig 1O). These results suggest that CPT, alone or in association with chemotherapy, can exert anti-cancer effects on MMC or R-MMC.

Effect of VLA4-CPT-PD-NP (V-CP) on myeloma cells *in vitro*

V-CP of 20 nm diameter were synthesized and characterized for stability (Suppl Fig 1B-F).

Datamining on HMCL for target receptor and intracellular phospholipases, capable of releasing active CPT from the CPT-PD, showed high expression of both cytosolic *PLA2G4A* and membrane-bound phosphocholine-preferring *PLA2G6* phospholipases (Suppl Fig 1P). Expression of VLA4 subunits *ITGA4* and *ITGB1* was compared to another osteotropic integrin, $\alpha_v\beta_3$, showing significantly higher, although variable, levels (Suppl Fig 1Q). Further investigating the rationale for pairing a VLA4 homing ligand to a TOP1 inhibitor, patient RNAseq data were analyzed for association between the two targets. In MMC samples that had high levels of both *ITGB1* and *ITGA4* RNA (Fig 1I), hence greater probability of having VLA4 heterodimer expression, TOP1 was significantly higher than patients with intermediate ($p<0.001$) or low levels ($p<0.001$) of VLA4 subunits ($p<0.001$, Fig. 1I).

Binding and fusion of V-NP was tested with fluorescently-labeled NP targeting VLA4 (V-DiI) or $\alpha_v\beta_3$ ($\alpha_v\beta_3$ -DiI), as a control for nonspecific binding (Suppl. Fig Q) in HMCL. Fluorescence was significantly higher after exposure to V-DiI than after $\alpha_v\beta_3$ -DiI ($p<0.001$, Fig 1J), showing target-dependent payload delivery from V-NP to MMC.

Relative V-NP carrying no treatment (V-ND), V-CP reduced proliferation in RPMI8226 cells (Fig 1K). By MTT, V-CP reduced viability (5TGM1, OPM2 Suppl Fig 1R-S) and potentiated anti-myeloma effects of low-dose melphalan or dexamethasone (Fig 1L-M, Suppl Fig 1T-U). In primary human MMC V-CP induced cell death alone and enhanced the effects of 10 μ M melphalan (Fig 1O, Suppl fig 1V). By contrast, in PBMC from MM patients 10 μ M melphalan had toxic effects, while V-CP did not (Fig. 1P).

V-CP overcome stroma-mediated drug resistance *in vitro* and *ex-vivo*

Co-culture with Human Vascular Endothelial Cells (HUVEC) (30) or macrophages (31) has been reported to increase resistance to anti-myeloma agents. Both in HUVEC-U266 (Fig 2A) and 5TGM1-macrophages (Fig 2B) V-CP reduced MMC viability.

BMSC from MM patients (MM-SC) are particularly chemoprotective for MMC (32). HMCL were co-cultured with BMSC from patients and treated with V-ND or V-CP. Flow cytometry showed a 90% reduction in viable MMC, but no changes in stroma alone (Fig 2C). Live/dead staining showed extensive MMC cell death with V-CP, and normal BMSC (Fig 2D, Suppl Fig 4C). Unlabeled MM.1S, U266 (Fig 2E-F), or RPMI8226 (Suppl Fig 4D) were parachuted on MM-BMSC, pre-stained with DiI for tracking, and treated with V-CP or V-ND. Colony formation monitoring showed that BMSC were unaffected, maintaining their adhesions, shape and ability to replicate (Fig 2E-F, Suppl Fig 4D). Melphalan, initial reduction, allowed the growth of tumor clones. Upon co-treatment with V-CP, no MMC clusters emerged (Fig 2E-F, Suppl Fig 4D).

To model bone-adhering MMC, BM cultures were established from mice (33) bearing subcutaneous plasmacytomas (25) (Fig 2G). In re-expanded GFP+ cells, V-CP reduced viability relative to melphalan alone (Fig 2H). GFP+ colonies appeared within a month in 100% of wells treated with saline or V-ND, 10-20% of single-agent treated, and no cultures exposed to V-CP plus melphalan (Fig 2I-J). These results indicate that V-CP anti-myeloma effects overcome stromal protection and blunt the re-emergence of tumor cells.

In vivo biodistribution of NP

KaLwRij mice injected i.v. with 5TGM1 develop aggressive orthotopic myeloma (33-35) with average survival of one month (5TMM). Epifluorescence imaging of V-DiI injected 5TMM mice ex-vivo on whole organs and flow cytometry were used to trace V-NP in vivo. Three hours after injection, DiI co-localized with GFP+ tumor sites (Fig 3A), while non-tumor controls showed low and diffuse signal. V-DiI and non-targeted DiI-NP accumulated over time in the liver and gut, and were traced to fecal matter (Fig 3A-D, Suppl Fig. 3A-C) (36). 5TMM mice had higher DiI fluorescence intensity in the bones and spleen and lower in gut and liver (Fig 3A, C-D). Flow cytometry of hepatic cells of non-tumor-bearing mice showed similar fluorescence intensity in mice injected with V-DiI or saline (Fig 3E, Suppl Fig 3D), suggesting that NP transited into the biliary system without major hepatocyte retention. Accordingly, 48h after injection, mice receiving V-ND or V-CP had similar serum ALT relative to controls (Suppl Fig 3C), despite reported hepatotoxicity of TOP1 inhibitors. In 5TMM mice, MMC infiltrating the liver were DiI+, while most hepatocytes did not show sign of dye retention (Suppl Fig 3D). A small population of resident spleen and BM cells (3-5% and 1-3%) accumulated V-DiI in both 5TMM and controls (Fig 3F). The majority of dye uptake, however, occurred in GFP+ MMC (8-40% of total, Fig 3F-G, Suppl Fig 3F). Dedicated and extensive experiments across multiple species would be needed to thoroughly understand biodistribution, particularly in the gut and liver. These data align with previous observations in rodents demonstrating that NP are preferentially eliminated via hepatobiliary excretion (36,37) regardless of targeting. Here, the transit of micellar VLA4-NP through the liver did not result in significant payload delivery to resident cells. Notably, in 5TMM mice, VLA4-NP in vivo delivered DiI into MMC, demonstrating specific targeting.

Treatment with dexamethasone or melphalan increases VLA4 expression and VLA4-NP uptake

Drug-resistant myeloma cells have been reported to increase membrane expression of VLA4(4,5,9). Relative to WT RPMI8226, DexR-8226 cells had a 2.5x-fold (1.2x-2.7x) increase in fluorescence when exposed to V-DiI (Fig 4A), but not $\alpha_v\beta_3$ -DiI. Flow-cytometry confirmed that DexR-8226 cells had higher membrane expression of both α_4 (CD49d) and β_1 (CD29) (Fig 4B, Suppl Fig 4A-B). By contrast, expression of CD138 (Fig 4B) and CD269 (BCMA, Suppl Fig 6A) was unchanged and CD38 was downregulated (Fig 4B). DexR-8226 cells had twice the level of β_1 (CD29) (Suppl Fig 4B) and a higher percentage of cells were positive for both α_4 and β_1 . DexR-8226 were selectively more sensitive to V-CP (Fig 4D). These data suggest that acquired drug resistance correlated with increased VLA4 expression on their membrane, which sensitized DexR-8226 cells to the anti-myeloma effects of V-CP.

To establish whether short-term treatments would induce selection or up-regulation of VLA4, RPMI8226 cells were treated with melphalan. Live cells showed no change in CD38, CD138 or BCMA, but had higher levels of CD29 and CD49d (Suppl Fig 4D), and 21-26% more VLA4+ cells (Fig 4E).

To further evaluate the effects of prior drug exposure in the context of CAM-DR, patient stroma-MMC co-cultures were exposed to dexamethasone for 24h, washed to remove the

free drug and non-adherent cells, then treated with V-ND or V-CP. Live/dead staining at 72h showed that V-CP induced maximal cell death on pre-treated co-cultures (Fig 4F) with no toxicity on BMSC.

Melphalan treatment increases MM uptake of fluorescent V-Dil in vivo

The effect of chemotherapy on V-Dil uptake in vivo was evaluated in the 5TMM model, in which high-dose melphalan (HD-Mel, 5mg/kg i.p/ twice/week) has been shown to reduce but not eradicate MM (6,7). 5TMM mice were treated from day 21 with HD-Mel and three injections of V-Dil i.v. (Fig 4G). Ex-vivo organ imaging showed that red fluorescence from V-Dil accumulation relative to GFP signal was significantly increased at the sites affected by tumor lesions in the melphalan relative to saline-treated 5TMM mice (Fig. 5I, $p<0.05$). Flow cytometry of BM cells showed that HD-Mel had reduced the % of GFP+ cells (Fig 4J, Suppl Fig 4E), but the fluorescence intensity for DiI was higher in those MMC (GFP+) that had survived melphalan treatment relative to MMC from saline/V-Dil mice (Fig 4J-K). Finally, cells isolated from the BM of melphalan-treated 5TMM mice showed high sensitivity to V-CP (Fig 4L). Together, these studies demonstrate melphalan treatment in vivo favors delivery of VLA4-NP to R-MMC.

Anti-myeloma treatment with V-CP alone in 5TMM mice shows no toxicity and mild efficacy

In vivo efficacy and toxicity of V-ND and V-CP were assessed in male and female 5TMM mice. Saline, V-ND, or V-CP i.v. were administered three times a week for a cumulative dose equivalent of 0.0167 mg/kg of CPT in 7 doses (Fig 5A). Despite hepatobiliary clearance, V-CP did not induce significant weight loss relative to controls (Fig 5B). Clinical pathology performed one week after the last treatment showed no anemia (Fig 5C), normal leukocyte and erythrocyte counts, and mild thrombocytopenia in all tumor-bearing mice regardless of treatment (Suppl Fig 5A-C). ALT levels were within normal limits in V-CP mice (Fig 5D). AST, less specific, was relatively elevated in all tumor-bearing mice: notably, no treatment-related differences were noted (Suppl Fig 5D). Serum albumin was normal in mice given V-CP treatment (Fig 5E), suggesting preserved liver function. Unlike larger (>200nm) integrin-targeted perfluorocarbon nanoparticles, previously associated with hepatic swelling and transaminase release (38), neither V-ND nor V-CP had impact on liver weights (Suppl Fig 5E). Spleen weight was increased in all tumor-bearing mice, but no treatment-related differences were noted (Suppl Fig 5F).

No treatment reduced paraproteinemia (Fig 5F), but V-CP improved overall animal survival by 3 and 4 days, approximately 10%, relative to saline (Fig 5G; $p=0.0052$) and V-ND (Fig 5G; $p=0.0008$). Since saline and V-ND treatments showed no difference, V-ND was selected as a vehicle control in further experiments.

V-CP with melphalan prolong survival in 5TMM mice

Because uptake of V-NP was highest in R-MMC, we hypothesized that free chemotherapy alternated with V-CP would enhance anti-myeloma effectiveness in vivo. To establish the impact of this combination treatment on survival, skeletally mature male 5TMM mice were treated with V-ND, V-ND plus HD-Mel or V-CP plus HD-Mel for 2 weeks (7 NP doses) (Fig 5H). HD-Mel plus V-ND or V-CP lowered paraproteinemia by serum protein electrophoresis

(SPEP) on day 28 relative to V-ND control ($p<0.001$, Fig 5I). Consistent with slower disease progression in mature male 5TMM, control mice had a median overall survival of 35 days and a disease specific survival (DSS) of 38.5 days (Fig 5J). Treatment with V-ND plus HD-Mel relative to V-ND yielded a survival increase (OS, median 42 days, $p=0.0257$, Suppl Fig 5I) but a non-significant change on DSS ($p=0.743$, Fig 5J). V-CP plus HD-Mel prolonged survival to 46 days, 30% longer than V-ND ($p=0.0015$) and 9.5% than V-ND plus HD-Mel ($p=0.0139$) (Suppl Fig 5I). DSS also improved ($p=0.0057$ and $p=0.0139$; Fig 5I), and 30% of mice survived for a month after treatment.

Transitory anemia (Fig 5K) and a decrease in blood cell counts (Suppl Fig 5G-H), was noted in both V-CP/HD-Mel and V-ND/HD-Mel, with no differences at nadir or in the efficiency of recovery after melphalan discontinuation. With similar kinetics, body weight decreased during melphalan treatment with subsequent recovery (Fig 5L). Necropsy and tissue analysis revealed no evidence of macroscopic organ damage or liver swelling (Fig 5M), and no increase in TUNEL staining of the liver relative to controls (suppl fig 5J-K). Collectively, these data show that V-CP alternated with melphalan delayed relapse and prolonged survival without further systemic toxicity.

V-CP with low-dose melphalan (Mel-LD) reduce MM tumor burden *in vivo*

The effects of V-CP in combination with low-dose melphalan (Mel-LD) was studied in 6 week-old female 5TMM mice, in which MM is more aggressive (39,40). To unmask combination effects, melphalan dose was reduced by 40% (3mg/kg x2/week) (7,28), starting on day eight (Fig 6A). At the end of treatment 20% of mice treated with V-ND/saline had hindlimb paralysis due to osteolytic spine lesions. Also 16% of mice given V-ND/Mel-LD were paralyzed, but none treated with V-CP/Mel-LD. Whole-body epifluorescence, showed no reduction of tumor burden in V-ND/Mel-LD treatment, but significant decrease in mice given V-CP/Mel-LD (Suppl Fig 6A $p<0.05$). At end-of treatment sacrifice, paraproteinemia was lower in V-CP/Mel-LD compared to both V-ND/Mel-LD and controls (Fig 6B; $p<0.05$). Ex-vivo imaging showed fewer hotspots and reduced epifluorescence intensity in the spleen, pelvic girdle, spine, and hindlimb bones of mice given V-CP/Mel-LD (Fig 6D-E; $p<0.05$, Suppl Fig 6B). By contrast, Mel-LD with V-ND did not lower epifluorescence (Fig 6D-E; $p>0.05$).

Clinical pathology at the end of treatment showed some impact of Mel-LD, but no animals developed severe anemia or thrombocytopenia (Fig 6F, Suppl Fig 6C-F). One mouse in the V-CP/Mel-LD and two animals in the V-ND/Mel-LD had numerically mild granulopenia, but overall counts did not differ from the controls (Suppl Fig 6E). Serum creatinine, BUN and ALT were within the normal range across treatment groups (Fig 6H-J). Mild elevations of AST were similar to the V-ND group (Suppl Fig 6G), and albumin levels were normal (Fig 6K). Mice treated with V-CP Mel-LD showed no weight loss during treatment, and had higher relative body weight at the end of the study ($p<0.05$, Suppl Fig 6H). Further, liver and gut histopathology showed normal morphology and no increase in TUNEL staining (41)(Fig 6L, Suppl Fig 6K-L).

Histological examination of the spleen suggested diffuse plasmablast/plasma cell accumulations with partial loss of follicular structure in V-ND and, to a lesser extent, in V-

ND/Mel-LD mice (Fig 6M). Spleens from V-CP/Mel-LD mice showed a conserved structure with some evidence of plasma cell infiltration. Immunohistochemistry (IHC) for the tumor isotype confirmed a widespread prevalence of IgG-positive cells in the spleens of tumor-bearing mice (Fig 6L-M). However, IgG staining was significantly reduced in V-CP/Mel-LD mice relative to V-ND/saline and V-ND/Mel-LD (Fig 6M).

H&E of the hindlimb bones revealed extensive bone marrow infiltration and osteolytic lesions consistent with plasma cell neoplasm in VLA4-ND/saline and VLA4-ND/Mel-LD treated mice. The size and prevalence of these lesions were reduced in mice given V-CP/Mel-LD (Fig 6N). Bone IHC showed intense staining for IgG in the V-ND/saline and VLA4-ND/Mel-LD groups that was significantly reduced in V-CP/Mel-LD mice (Fig 6O).

In all, Mel-LD alone was ineffective against MM yet still induced hematological toxicity. V-CP in combination with Mel-LD were effective in reducing tumor burden in 5TMM mice, with no added toxicity. Therefore, V-CP co-treatment lowered the free chemotherapy requirement to achieve anti-tumor effects without aggravating toxicity.

Discussion:

Therapeutics emerging during the last two decades have made MM a treatable disease, extending survival for responding patients. However, all patients relapse or develop chemoresistant progression. Novel therapeutics with low toxicity capable of treating the chemoresistant cells are needed, to delay, treat, or prevent relapse.

A key determinant of chemoresistance in MM is the protective effect of the BME, where contact with endothelial (30), myeloid (31), or mesenchymal cells (42), or with matrix proteins (43) induce pro-survival signaling (4,9,13). VLA-4 is upregulated by MMC in response to environmental stimuli (e.g. CXCL12) (42,44), and favors survival and retention within the bone niche of tumor cells. VLA4 engagement in adhesive interactions with fibronectin or VCAM1 increase MMC chemoresistance (4,8,9,12).

Recognizing its chemoprotective function, several investigators have attempted to neutralize or inhibit VLA4 (12,22) in order to dislodge tumor cells from their protective niche and sensitize them to chemotherapy. Others have used VLA4 as a biomarker to target therapeutics in MM (27,37,45-47). Unfortunately, VLA4 expression is variable across patients and heterogeneous even within a single tumor lesion. To overcome these barriers, other investigators proposed multiplexing ligands to increase carrier functionality (46,47) at the expense of higher synthesis complexity and larger hydrodynamic size.

To the best of our knowledge, this report reflects the first attempt to obtain a therapeutic advantage by addressing the equilibrium between VLA4^{low} and VLA4^{high} MMC, combining free chemotherapy with VLA4-targeted nanotherapy. In vitro or in vivo, dexamethasone or melphalan exposure increased VLA4 surface expression and V-NP uptake in MMC. Delivery of CPT-PD by incorporation into the lipid surfactant of V-NP, (V-CP), had antitumor effects on MMC in vitro or co-culture with BMSC, and enhanced response to chemotherapy. V-CP prolonged survival of immunocompetent mice bearing orthotopic diffuse MM, alone or in combination with melphalan. V-CP co-treatment reduced dosing

requirement of chemotherapy to achieve anti-myeloma effects with Mel-LD. These anti-myeloma effects were concordantly verified through SPEP, in vivo and ex-vivo epifluorescence, and tissue analysis at extra- and intra-medullary sites.

A limitation of this study is the extensive use of MM cell lines, which often represent more aggressive MM; future studies to address the effects of V-CP on slower-growing and more heterogeneous MMC populations are warranted.

Relapsing MMC often harbor mutations altering the targets of previous treatments, or downregulating them (48). Therefore, the present research explored a potent chemotherapeutic uncommonly used in MM. RNAseq showed that camptothecin's target, *TOP1*, was highly expressed in MMC and increased in patients with relapsing or refractory disease. Kazmi et al (17) showed that a cyclophosphamide and topotecan combination reduced dosing requirements of melphalan. However, in other trials, free CPT analogues or untargeted nanoformulations of SN-38 (49) showed remarkable toxicity (17,18,50), with varying results on efficacy (16-18,49), including no activity (49).

The V-NP utilized in the present study have a stable diameter of ~20 nm, comparable to the size of an antibody (14~40 nm), high shelf-life stability, and incorporate an inactivated, stabilized camptothecin lipase-labile prodrug (CPT-PD). These nanoparticles reach deeply into disseminated bone cancer lesions (21) or solid tumors (37). V-NP were preferentially taken up and effective in R-MMC. Conversely, *TOP1* was increased in *VLA4^{high}* MMC from patients. In all, a relatively low dose-equivalent of inhibitor reduced lowered dose-requirements and extended survival in combination therapies without increasing toxicity.

Pegylated particles targeting doxorubicin to VLA4 were previously shown to reduce tumor size in subcutaneous myeloma xenografts (37), and to accumulate in the liver and gut of rodents. Here, hepatic accumulation is confirmed, but ex-vivo flow cytometry shows that only negligible levels of DiI were delivered to non-tumor liver cells. Moreover, while CPT derivatives are associated with high liver and gut toxicity (41), treatment with V-CP did not cause transaminase release, steatosis, increase in TUNEL staining in the liver or gut, or weight loss. Therefore, CPT-PD inclusion in V-NP prevented toxic effects even when the micelles accumulated into the target organs of toxicity. More to the point, V-NP delivered payloads into MMC in vivo, and with a preference for R-MMC.

In conclusion, this study suggests that V-CP in combination with free chemotherapy provides a novel strategy to treat chemoresistant MMC, addressing the initiator of progression and relapse. More broadly, this study provides proof of principle that cancer cell adhesion to the microenvironment can be exploited, rather than circumvented, with targeted therapeutic nanoparticles to improve outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Funding was provided by: NIH R01 CA216840-01A1 (GML), U54 CA199092 (GML, JFD), R50 CA211466 (MR). The CoMMpass study was organized and funded by the MMRF.

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Statement of significance

In multiple myeloma (MM), relapse or drug resistance (RR) eventually ensue in all patients. Aggressive treatments need to be balanced for toxicity, and many compounds have had limited applicability in this frail patient population. Cell adhesion-mediated drug resistance (CAM-DR) promotes survival of myeloma cells (MMC), and alterations of VLA4 expression affect MMC chemosensitivity. Overexpression of VLA4 by chemoresistant MMC was here exploited to deliver nanomicelles targeted through a high-affinity ligand. A camptothecin prodrug was designed and included into 20 nm micellar particles (V-CP), which showed anti-myeloma effects in vitro and in vivo. In combination with melphalan, V-CP further reduced tumor burden and prolonged survival in mice, without adding to toxicity. Hence, a CAM-DR mechanism was co-opted to target and effectively treat refractory MM cells. These results suggest that V-CP may be a safe and effective strategy to treat or prevent relapsing or refractory myeloma.

Fig 1. Targeting of topoisomerase 1 in MMC with free CPT or VLA4-CPT-PD

A) Synthesis and structure of CPT-PD by conjugation of camptothecin (CPT) and phosphocholine (PC) B) V-CP are mixed-micelles (20nm) including in the single membrane layer Sn2-labile PD (red stars) and the homing ligand (green arrowhead), which bind to dimeric activated $\alpha_4\beta_1$. Binding allows the PC of the NP to fuse to the plasma membrane, transferring the PC-conjugated PD. C-D) *TOP1* expression by RNAseq in MMC samples from patients. C) Patients (Naïve, N=769) vs with previous treatments (Post-Tx, N=118). D) in the same patients (N=73) over consecutive samples (ANOVA $p < 0.01$). E) RPMI8226 sensitivity to CPT (AnnexinV-PI flow cytometry) F) MM.1S proliferation as ratio of EdU +/PI+ (all) nuclei (6 wells/treatment) G) RPMI8226-WT vs. DexR-8226 sub-line sensitivity to CPT H) RPMI8226 response to CPT +/- melphalan 25 μ M (MTT). I-J) Analysis of RNAseq data from MMRF CoMMpass study for association between VLA4 and *TOP1*. Samples (N) were classified as high, med, and low relative to 1 SD from the mean for ITGA4 (horizontal) and ITGB1 (vertical). Samples were grouped based on four categories likely to associate with $\alpha_4\beta_1$ (VLA4) heterodimer: Low-Low (blue N=14), high-high (pink N=35), one high and one at medium levels as Med-High (yellow N=159), and combinations of low to medium expression as “others” (dark green N=683). *TOP1* expression across

groups (in J) shows that Low-Low MM have lower *TOP1* mRNA levels than any other group ($p<0.01$ - $p<0.001$), and High-High have the highest *TOP1* levels ($p<0.01$ - $p<0.001$). Medium-High VLA4 are higher than Others (Medium-Medium/Low-Medium/High-Low) and lower than High-High ($p<0.01$), further suggesting a correlation between expression of dimer subunits and TOP1 levels K) Uptake by HMCL of V-DiI vs. $\alpha_v\beta_3$ -DiI by mean 520/600 nm fluorescence (ratio of control) L) effect of V-CP vs -ND on proliferation of RPMI8226: green EdU, red propidium iodide. M-N) MTT viability 48h after treatment with V-CP vs -ND -/+ free drugs: M) MM.1S -/+ melphalan 2.5 μ M; N) RPMI8226 -/+ dexamethasone 10 μ M; O) live/dead staining of CD138 positive MMC from a patient with relapsing MM 48h after treatment with V-ND or V-CP -/+ melphalan 10 10 μ M; O) viability of PBMC from a newly diagnosed MM patient based on live/dead staining (P, ratio of calcein/(calcein+PI cells), one of three concordant experiments; # $p<0.001$ relative to control, ** $p<0.01$, *** $p<0.001$.

Fig 2. Anti-myeloma effects of V-CP in the presence of stroma

A) Viability of U266-HUVEC co-culture by MTS after treatment with V-CP vs -ND; B) Growth of 5TGM1 in co-culture with BM macrophages (BM Mφ) as GFP MFI 5 days after treatment; C) Viability by of MM.1S or RPMI8226 cells in co-culture with MM-BMSC from a newly diagnosed patient, versus MM-BMSC alone, assessed by propidium iodide exclusion with flow cytometry 72h after treatment with V-ND or V-CP, normalized to no drug; D) live/dead staining (green live, red dead) three days after treatment of co-cultures of RPMI8226 (round) and stromal cells (large, adherent) from a relapsing MM patient (VRD plus Velcade maintenance) with V-ND or V-CP. E-F) Re-growth of MMC clones from unstained MM.1S (E) and U266 (F) on DiI-stained (red) MM-BMSC, 10 days after V-NP +/- melphalan 20 μM. G-J) Isolation of osteotropic 5TGM1 cells from two KaLwRij mice with subcutaneous plasmacytomas; at sacrifice, whole-bone marrow was harvested and cultured in bulk in tissue-culture treated flasks or seeded at low concentration in 96w: G) diagram; H) Cell viability (by GFP fluorescence) of re-expanded cells from the bone marrow of two different mice (M#1 and M#2) +/- melphalan 10 μM; I) Percentage of GFP-positive colonies 3 weeks after treatment at seeding +/- continuous melphalan 10 μM; J) optical microscopy of colonies emerging from whole bone marrow culture. **p<0.01.

Fig 3. VLA4 nanoparticle (V-NP) biodistribution

Ex vivo NP distribution by optical fluorescence in non-tumor (NTu) and 5TMM mice A) Representative organ images 3h from the infusion of saline vs. V-DiI, B) DiI-NP by fluorescence (aRE) in NTu, non-targeted (DiI-NT) 3h after infusion vs. V-DiI at 3, 24, or 48h from infusion C-D) NTu vs. MM mice at 3h (C) and 48h (D) after infusion of V-DiI (aRE) (N=4/timepoint) E-G) Ex-vivo flow cytometry of cell suspensions 3h from injection of V-DiI vs. saline; E) liver, F) bone marrow and spleen G) MFI shows increased uptake of V-DiI in the BM of MM mice vs NTu or saline (NTu plus MM) (N=5-6 samples/group).

Fig 4. Increased VLA4 expression and V-NP uptake in R-MMC

A) DiI-NP uptake in WT RPMI8226 and DexR-8226 cells (mean fluorescence intensity), B-C) Flow cytometry of WT RPMI8226 (grey) vs DexR-8226 (green) for α_4 , β_1 , CD138, and CD38 (B) and co-expression of VLA4 subunits α_4 and β_1 (C), D) Sensitivity by MTT of WT (grey) vs. DexR-8226 (green) to dexamethasone or V-CP, E) VLA4 expression in RPMI8226 treated with melphalan (10 μ M 48h), F) 48h viability (live/dead) of RPMI8226 cells co-cultured with MM-BMSC from two newly diagnosed myeloma patients (#A and #B); co-cultures were either pre-treated with vehicle or dexamethasone 72h, then with V-ND or V-CP. G-K) In vivo uptake of V-DiI by melphalan-resistant MMC. G-K) 5TMM mice were treated from week 3 with melphalan 5mg/kg twice a week (N=3) or saline i.p. (N=2) and V-DiI three times a week i.v. and euthanized 48h later; G) experimental design; H) ex-vivo imaging for GFP and DiI representative images; I) fluorescent quantification as ratio of DiI to GFP aRE two-way ANOVA $p < 0.01$; J) flow cytometry of bone marrow from the pelvis of one Saline/V-DiI (top) and one HD-Mel/V-DiI (bottom) treated mice, showing gating of GFP+ MMC and (on the right) the histogram for DiI fluorescence of GFP+ gated cells; K) DiI MFI in GFP+ in all samples (1 femur and 1 hemipelvis per mouse, N=10 samples), L) ex-vivo treatment with V-NP of bone marrow whole-culture from a melphalan-

treated 5TMM mouse, GFP fluorimetry (growth of MMC only) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.01$ to control.

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Fig 5. V-CP prolong survival in mice

A-G) Female (N=3-4/group) and male (N=3-4/group) mice were inoculated with 5TGM1 i.v. and treated from day 5 with three V-ND (N=7), V-CP (N=8) or saline (N=6) injections per week until day 19 (7 injections) then followed for survival. A) Treatment scheme; B) Body weight over time; C) Hb in g/dL by CBC at the end of treatment; dotted lines: anemia (mild or severe, SA) D-E) serum ALT (D) and albumin (E) at the end of treatment; normal range: dotted lines. F) SPEP at week 4 G) Kaplan-Meier survival curve shows 2-3 day increase in median survival in V-CP ($p < 0.01$). H-L) Mature male mice (N=6-7/group) were inoculated i.v. with 5TGM1 cells and treated with vehicle or melphalan 5mg/kg i.p. twice a week from day 9 to day 24 and intravenous injections of V-ND or V-CP three times a week from day 11 to day 31. Groups were V-ND plus vehicle (grey), V-ND/HD-Mel (blue), or V-CP/HD-Mel (red). H) timeline and experimental scheme. I) M-protein by SPEP over four weeks of treatment. J) Disease specific survival (excluding 2 no-drug mice dead for pulmonary embolism after too rapid i.v. injection) as Kaplan-Meier curve, analyzed by Log-Rank test ($p < 0.001$ for V-CP). K) Serial CBC show Hb nadir after the end of melphalan treatment on week 4 ($p < 0.01$), but no significant difference after treatment discontinuation

on week 5. L) Weight monitoring as percentage of baseline over time. M) Liver weight at euthanasia shows no difference across treatments. * $p < 0.05$, ** $p < 0.01$.

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Fig 6. V-CP in combination with low-dose melphalan decrease tumor burden in MM mice
A-G) 5-7 week old female KaLwRij mice were inoculated with 5TGM1 i.v. and treated on days 8-19 with i.p. vehicle or melphalan 3mg/kg (Mel-LD) twice a week and V-NP (V-CP or V-ND) i.v. three times a week days 9-26, (3 groups, N=5-6/group) and sacrificed on day 27. A) Experimental scheme, timeline, and right knee in vivo optical imaging on day 26; B) M-protein by SPEP C) representative ex-vivo GFP fluorescence intensity of leg bones, pelvis, and spleen D-F) aRE of bones from legs (D), spine (E), and pelvis (F). G) Hb by CBC on day 27; mild or severe (SA) anemia as dotted lines. H-J) day 27 creatinine (H) BUN (I), ALT (J) and albumin (K), dotted lines: normal range. L) representative H&E (top) and TUNEL staining of liver sections at euthanasia (day 27) M) H&E histology of the spleen. N) Anti-mouse IgG IHC staining (DAB) of spleens, hematoxylin counterstaining. M-N) Representative bone sections stained with H&E (M) or IgG IHC (N; DAB and methyl green) * $p < 0.05$, ** $p < 0.01$, F=follicle, P=pulp, GP=growth plate, T=tumor, BM=bone marrow.