Supplementary Material

Material and methods

Chemicals and reagents

Doxorubicin (MedKoo Biosciences Inc., USA), bortezomib (LC Laboratories, Canada), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were both purchased from Lipoid GmbH, Germany, 18:1 Liss Rhod PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Avanti Polar Lipids Inc, USA), Cholesterol (Sigma Aldrich, Germany), SATA-modified Very Late Antigen-4 targeting peptide (SATA-VLA-4 targeting peptide) was synthesized by DGpeptidesCo., China. Internal standard (IS) bortezomib D8 major was purchased from Toronto Research Chemicals.

Doxorubicin loaded liposomes

Liposomes were prepared as described in the main material and methods. Ammonium sulfate 240 mM and EDTA 1 mM solution was used to hydrate the lipid film. After preparation of liposomes, the extra-liposomal buffer was replaced with HBS pH 7.5 by dialysis using a 10 000 MWCO dialysis membrane. For remote loading, doxorubicin solution in HBS pH 7.5 was added to the liposomes to get a final doxorubicin concentration 0.2 mg/mL followed by incubation at 60 °C for 1 hour. Free (unloaded) doxorubicin was removed by dialysis against HBS pH 7.4 for 24 hours at 4°C.

Encapsulated doxorubicin was determined by Ultra High Pressure Liquid Chromatography (UPLC) (Waters Corporation, USA) using a C18 column (ACQUITYUPLC®BEHC18 1.7 µm, 2.1 x 50 mm). Liposomes were diluted and dissolved in 25% acetonitrile in HBS. Water/acetonitrile/perchoric acid 75/25/1 was used as mobile phase. Flow rate was set at 0.500 mL/min. Injection volume was 7.5 µL. Excitation and emission wavelengths were 480 nm and 565 nm respectively. Run time was 3 minutes.

Bortezomib loaded liposomes

Bortezomib liposomes were prepared as described previously[1, 2] with some modifications. The lipid film was hydrated with a solution of mannitol, meglumine, acetic acid, and HEPES at 200 mM, 50 mM, 30 mM, and 20 mM respectively at pH 8.5. After extrusion, extra liposomal buffer was replaced with 140 mM NaCl and 20 mM HEPES buffer pH 6.5 by dialysis using a 10 000 MWCO dialysis membrane. Appropriate amount of bortezomib solution in DMSO was added to liposomes to get a final bortezomib concentration 22 µg/mL and incubated overnight at room temperature to remote loading. Un-encapsulated bortezomib was removed by dialysis against HBS pH 7.4 for 24 hours at 4°C.

To determine encapsulated bortezomib concentration, liposomes were dissolved in 35% acetonitrile in HBS. Bortezomib concentrations was measured by Ultra High Pressure Liquid Chromatography (UPLC) (Waters Corporation, USA) using a C18 column (ACQUITYUPLC®BEHC18 1.7 µm, 2.1 x 50 mm). Mobile phase water/acetonitrile/perchloric 65/35/0.1 was used. Absorbance was detected at 270 nm at flow rate of 0.600 mL/min and injection volume of 7.5 µL. Run time was set to 1 minute.

VLA-4 targeted liposomes

For preparation of VLA-4 targeting liposomes (Supplementary Figure 1), the in the main material and methods described lipid composition was used, to which equal amounts of $mPEG_{2000}$-DSPE and mal-PEG_{2000}-DSPE were added (each at 0.075 mM of total lipids). Freshly prepared liposomes were mixed with 1 mg/mL SATA-VLA-4 targeting peptide (activated by 0.05 M HEPES/0.05 M hydroxylamine-HCl/ 0.03 mM EDTA for approximately 60 minutes at room temperature) and incubated at 4 °C overnight. Unconjugated peptide was removed by gel permeation chromatography using PD-10 desalting columns (GE Healthcare). Doxorubicin or bortezomib was remotely loaded into untargeted and VLA-4 targeted liposomes as described above.

The amount of conjugated VLA-4 peptide was estimated indirectly by UPLC by measuring unconjugated peptide trapped in the PD-10 column during gel permeation. To recover unconjugated peptide from the column, 25 mL of HBS was passed through the column and collected in 5 mL fractions. VLA-4 peptide was determined in all 5 fractions. The peptide was detected at 220 nm with a BEH300 C18 1.7 µm, 2.1 x 50 mm column and a mobile phase of water/acetonitrile/trifluoroacetic acid 5:95:0.1 (eluent A), and acetonitrile/trifluoroacetic acid 100/0.1 (eluent B). The eluent gradient was set from 100% eluent A to 85% eluent A / 15% eluent B after 0.1 minute and subsequently to 60% A/ 40% B in the 3rd minute and finally to 100% A after 3.1 minutes.

Cell lines and primary cells

All myeloma cell lines were cultured in MM medium (advanced RPMI 1640 medium, 10% (v/v) fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all Gibco, ThermoFisher, USA)). MSCs were isolated from the bone marrow by adherence to the tissue culture plastic, and cultured in MSC medium (α-minimal essential media (αMEM, Gibco, USA),10% (v/v) FBS, 0.2 mM L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 µg/mL streptomycin). All MSCs were used at passage 3-5.

EPCs were isolated from the cord blood by density-gradient centrifugation of mononuclear cells using Ficoll-paque, seeded on collagen I (BD Biosciences)-coated wells and expanded in EPC medium (EGM-2 medium (Lonza, Switzerland), SingleQuots™ Kit (Lonza, Switzerland), 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin). All EPCs were used at passage 4-7.

Cytotoxicity of free drugs on myeloma cells in 2D and 3D cultures

L363-GFP and MM1S-mCherry were single cultured in 2D or co-cultured with MSCs-DiD and EPCs-DiD in 50% (v/v) Matrigel. The initial number of myeloma cells per well was equal in all conditions. The cells were cultured for either 1, 5 or 7 days before the drugs were added. The final concentration of bortezomib in each well was 2.5, 5.0 and 7.5 nM, and 0.5, 1 and 2 nM for L363 and MM1S cells respectively. Doxorubicin was added at final concentration 0.3, 1 and 3 µM, and 0.5, 1, 2 µM in each well of L363 and MM1S cells respectively. Equal amount of medium was added in the control wells. Each concentration was performed in duplicate. All conditions were live imaged 48 hours after adding the treatments. Ethidium homodimer-1 was added to all L363-GFP cultures to identify dead cells and calcein was added to all MM1S-mCherry cultures to identify living cells, both following manufacturer's

instructions (LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells, Thermo Fisher, USA). Images were taken using a Leica SPX8 Laser Scanning Confocal Microscope.

Porosity of the 3D model by Scanning electron microscopy (SEM)

50% (v/v) Matrigel plugs (hydrogel only or containing MSCs, EPCs and L363 cells) were fixed with 4% formaldehyde (VWR chemicals, United Kingdom) overnight. Tissue Tek OCT, (Sakura Finetek Europe) diluted with distilled water (1:1), was added overnight to the wells containing the gels to minimize shape change. Samples were snap-frozen with liquid nitrogen. The frozen gels were cross-sectioned (5 and 10 µm) using a cryostat (Thermo Scientific, CryoStar NX70) and air-dried overnight. Next, samples were dehydrated in an ethanol series (15 min. per step; 10%, 20%, 40%, 60%, 80% in distilled water, and finally 100% ethanol). Subsequently, samples were incubated in 50%-50% ethanol-hexamethyldisilazane and 100% hexamethyldisilazane after which the samples were air-dried, attached to a 0.5" aluminium specimen stub (Agar Scientific, United Kingdom) and sputtered with 2 nm gold. Specimens were finally analyzed on a Phenom Pro desktop SEM (Phenom World, The Netherlands).

Effect of liposome size on diffusion & intracellular uptake in 3D

3D co-cultures of L363-GFP, MSCs and EPCs (30 µL plugs) were pre-cultured for 7 days. At day 7, 5 µL of DiD labelled liposomes (small, intermediate and large) were added either to the medium, or introduced into the middle of the 3D co-culture with a thin pipet tip. All cultures were live imaged directly after liposome addition (0 hours), and after 24 and 48 hours. The total fluorescence intensity of the DiD labelled liposomes was quantified at the injection site (I), the 3D co-culture directly surrounding the injection site (3D-C), the border of the 3D co-culture next to the medium (3D-B), and in the medium (M).

After 48 hours, all 3D cultures were washed 3x with PBS to remove all free liposomes. The 3D cultures were fixated overnight using 4% formaldehyde (VWR chemicals, United Kingdom). The fixated co-cultures were stained for phalloidin and DAPI (both FAK100 kit, Merck Millipore, USA) according to the manufacturer's protocol. Images were taken using a Leica SPX8 Laser Scanning Confocal Microscope. The total fluorescence intensity of intracellular DiD labelled liposomes was quantified.

Intracellular uptake of untargeted & VLA-4 targeted liposomes in 3D

3D co-cultures of L363-GFP, MSCs-DiD and EPCs-DiD (30 and 50 µL plug volumes) were pre-cultured for 7 days. At day 7, 5 µL of rhodamine labelled untargeted or VLA-4 targeted liposomes (intermediate size) were injected into the center of the 3D co-culture. The liposome distribution was analyzed after 0, 24 and 48 hours, quantifying the total fluorescence intensity of the rhodamine labelled liposomes throughout the 3D co-culture.

After 48 hours, all 3D cultures were washed 3x with PBS to remove all free liposomes. The 3D cultures were fixed overnight using 4% formaldehyde (VWR chemicals, United Kingdom). The total fluorescence intensity of intracellular rhodamine labelled liposomes, and their co-localization with either myeloma or supporting cells, was quantified. Images were taken using a Leica SPX8 Laser Scanning Confocal Microscope.

Confocal imaging

All fluorescence images were taken with a Leica SP8X Laser Scanning Confocal Microscope using a white light laser (470-670 nm) and Leica LASX acquisition software. Cytotoxic activity of the drugs within the 3D model was live imaged: hybrid detectors collected fluorescence signal from either calcein (494/500-525) and mCherry (587/592-640), or from GFP (488/493-525) and ethidium homodimer-1 (528/600-640), both combinations were given the pseudocolors green and red.

The supporting MSCs and EPCs cultured in the 3D model were also live imaged: hybrid detectors collected fluorescence signal from either DiI (549/565-605) or DiD (644/665-705) which were given the pseudocolors cyan or red.

Fixated cells and co-cultures: hybrid detectors collected fluorescence signal from DAPI (405/430-480) and phalloidin-TRITC (532/540-575) which were given the pseudocolors blue and red. Liposomes were imaged collecting fluorescence signal from rhodamine B (560/570-590) or DiD (644/665-705) which were both given the pseudo color cyan.

All z-stack images were processed using ImageJ 1.51h software to create single maximum projections. Images of culture overviews were made using the mosaic function of the Leica LASX software, stitching the images together using smooth and linear blending.

References

1. Zuccari G, Milelli A, Pastorino F, Loi M, Petretto A, Parise A, et al. Tumor vascular targeted liposomal-bortezomib minimizes side effects and increases therapeutic activity in human neuroblastoma. J Control Release 2015; 211:44-52.
2. Yang X, Pang J, Shen N, Yan F, Wu LC, Al-Kali A, et al. Liposomal bortezomib is active against chronic myeloid leukemia by disrupting the Sp1-BCR/ABL axis. Oncotarget 2016; 7:36382-94.

Supplementary Figure 1 Schematic representation of the preparation of Very Late Antigen-4 (VLA-4) targeted stealth liposomes