Investigating \textit{in vitro} and \textit{in vivo} αvβ6 integrin receptor-targeting liposomal alendronate for combinatorial γδ T cell immunotherapy

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\section*{ABSTRACT}
The αvβ6 integrin receptor has been shown to be overexpressed on many types of cancer cells, resulting in a more pro-invasive and aggressive phenotype, this makes it an attractive target for selective drug delivery. In tumours that over-express the αvβ6 receptor, cellular uptake of liposomes can be enhanced using ligand-targeted liposomes. It has previously been shown in both \textit{in vitro} and \textit{in vivo} studies that liposomal alendronate (L-ALD) can sensitise cancer cells to destruction by Vγ9Vδ2 T cells. It is hypothesised that by using the αvβ6-specific peptide A20FMDV2 as a targeting moiety for L-ALD, the therapeutic efficacy of this therapy can be increased in αvβ6 positive tumours. Targeted liposomes (t-L) were formulated and the targeting efficacy of targeted liposomes (t-L) was assessed by cell uptake and cytotoxicity studies in the αvβ6 positive cell lines A375Pβ6. Bio-distribution of both L and t-L were carried out in αvβ6 positive (A375P and PANC0403) and αvβ6 negative (A375Ppuro and PANC-1) subcutaneous tumour mouse models. Immuno-compromised mice bearing A375P experimental metastatic lung tumours were treated with L-ALD or t-L-ALD as monotherapies or in combination with \textit{ex vivo}-expanded Vγ9Vδ2 T cells. \textit{In vitro}, αvβ6-dependent uptake of t-L was observed, with t-L-ALD being more effective than L-ALD at sensitising A375Pβ6 to γδ T cells. Interestingly, t-L-ALD led to slightly higher but not significant reduction in tumour growth compared to L-ALD, when used as monotherapy \textit{in vivo}. Moreover, both L-ALD and t-L-ALD led to significant reductions in tumour growth when used in combination with γδ T cells \textit{in vivo} but t-L-ALD offered no added advantage compared to L-ALD.

1. Introduction

Integrins are heterodimeric glycoproteins composed of non-covalently linked α and β subunits and are involved in a variety of cell processes including proliferation \cite{1}, survival \cite{2}, migration \cite{3} and invasion \cite{4}, giving them a key role in cancer. The αvβ6 integrin receptor is expressed on epithelia, usually only in the process of tissue remodelling \cite{5} and wound healing \cite{6}. However, overexpression of this receptor has been detected in many types of tumours including colon cancer \cite{7}, gastric carcinomas \cite{8}, oral squamous cell carcinomas \cite{9} and breast cancer and is typically associated with a more pro-invasive and aggressive phenotype \cite{10,11}. Therefore, this integrin provides an attractive molecular target for targeted drug delivery to cancer cells. The 20 amino acid peptide, A20FMDV2, (NAVPNLRGDQVLQAVKT), derived from the VPI coat-protein of the foot-and-mouth disease virus, has shown very good specificity for αvβ6 \cite{12}. This peptide has been shown to effectively and specifically target αvβ6-expressing cancers \cite{13}. A20FMDV2 has not previously been conjugated to any type of nanocarrier and its ability to target nanoformulations has not been tested. Only one other study has targeted the αvβ6 integrin receptor using a nanoparticle formulation. In that study, liposomes were targeted using an alternative peptide, H2009.1 \cite{14}.

Alendronate (ALD), is a nitrogen containing bisphosphonate (N-BP) that can sensitise tumour cells to killing by Vγ9Vδ2 T cells in both \textit{in vitro} \cite{15-19} and \textit{in vivo} studies \cite{20-27}. The encapsulation of ALD in liposomes (L-ALD), has been shown to increase its therapeutic efficacy \cite{24}. Long-circulating liposomes passively target the tumour due to the enhanced permeation and retention (EPR) effect \cite{28}, leading to a greater amount of the encapsulated drug reaching the tumour cells.

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The aim of this study is to formulate αvβ6 integrin targeted ALD liposomes (t-L-ALD), using the peptide A20FMDV2. It is hypothesised that A20FMDV2 conjugation to liposomal alendronate will promote αvβ6-receptor mediated endocytosis and improved therapeutic efficacy in combination with γδ T cell immunotherapy in vitro and possibly in vivo.

2. Materials and methods

Additional methods are described in Supplementary information.

2.1. Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dipalmityl 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were obtained from Lipoid (Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (CF-DPPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-diethylentriaminepentaacetic acid (ammonium salt) (DSPE-DTPA) and DSPE-PEG(2000) maleimide were purchased from Avanti Polar Lipids, Inc. (USA). The cysteine-modified A20FMDV2 peptide (Sequence: NAVPNLRGLQVLQAVKVRT-Cysteine, Purity: > 95%) was purchased from GenerScript Limited (Hong Kong). Dextrose, cholesterol, sodium chloride, phosphate buffered saline (PBS) tablets, N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), methanol (Analytical reagent grade), chloroform (Analytical reagent grade), and Sepharose 2B were purchased from Sigma (UK). LavaPep™ Protein and Peptide quantification kit was obtained from Web Scientific (UK). PD-10 desalting column was obtained from GE Healthcare Life Sciences (UK). Snake Skin® dialysis tubing (MWCO 10000 Da) was purchased from Thermo-Fisher (USA). Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Glutamax™ and antibiotic-antimycotic solution were purchased from Invitrogen (UK). Foetal Bovine Serum was purchased from First Link (UK). Human AB serum (male) was obtained from Sigma (UK). Thiazolyl blue tetrazolium bromide (MTT), tropolone and alendronate sodium trihydrate were obtained from Alfa Aesar (UK). DMSO was obtained from Fisher (UK). Human IFN-γ ELISA Ready-set-go kit was purchased from eBiosciences (UK). The 10D5 antibody was obtained from Abcam (UK). FITC labelled secondary antibody was obtained from eBiosciences (UK). The per cent peptide conjugated to the liposomes was calculated by quantifying the amount of peptide in the liposome sample before and after purification.

2.2. Preparation of liposomes

Stock solutions of 40 mg/ml were prepared in chloroform/methanol (4:1 v/v). To avoid degradation, lipid solutions were stored at −20 °C under nitrogen. Untargeted Liposomes (L) were prepared by thin film hydration (TFH). DSPC, cholesterol and DSPE-PEG2000 (55:40:5 mole ratio) were added to a 25 ml round-bottom flask and 2 ml chloroform/methanol (4:1 v/v) was added. A thin lipid film was formed upon removal of the solvent under reduced pressure using a rotary evaporator (Rotavapor® R-210, Buchi UK). The lipid film was flushed with nitrogen to remove any remaining traces of organic solvent. The film was then hydrated by adding 1 ml of PBS, adjusted to pH 7.4. The liposome suspension was left for 1 h at 60 °C and was vortexed (Vortex genie 2, Scientific Industries Inc., USA) every 15 min [29]. Liposomes were prepared at a final concentration of 25 mM total lipid. The size and polydispersity (PDI) of the liposomes was reduced with serial extrusion using the mini-extruader (Avanti Polar Lipids, USA) through polycarbonate membranes (Avanti Polar Lipids, USA) with pore sizes of 0.8 μm (5 ×), 0.2 μm (5 ×), 0.1 μm (10 ×) and 0.08 μm (15 ×) at 60 °C. The resulting suspension was stored at 4 °C.

For targeted liposomes (t-L), the liposomes were formed as above with the addition of 0.5–2% mol of DSPE-PEG2000-maleimide. The overall formulation of the liposomes was DSPC:cholesterol:DSPE-PEG2000:DSPE-PEG2000-maleimide (55:40:3:4.5:0.5–2 molar ratio). The DSPE-PEG2000 was reduced in order to keep the overall percentage of PEG2000 at 5%. After the liposomes were formed, they were flushed with nitrogen and incubated with A20FMDV2 peptide at RT overnight (25–100 μg peptide/μmol lipid) under nitrogen. Excess peptide and ALD was removed by eluting the liposomes through a Sephadex 2B column with PBS; or via overnight dialysis against PBS using a dialysis bag with a MWCO of 10,000 kDa at room temperature.

For cellular uptake studies, fluorescent liposomes were formed as above but with the inclusion of 1% mol CF-DPPE to give a final liposome composition of DSPC:CF-DPPE:cholesterol:DSPE-PEG2000:DSPE-PEG2000-maleimide (54:1:40:4:1 molar ratio).

Liposomes containing alendronate (L-ALD and t-L-ALD) were prepared as above, but the lipid film was hydrated with 1 ml of 100 mM solution of ALD in HEPES Buffered Saline (HBS, 20 mM HEPES, 150 mM NaCl). Un-encapsulated ALD was removed by overnight dialysis against HBS using a dialysis bag with a MWCO of 10,000 kDa.

2.3. Peptide quantification

The amount of peptide conjugated to the liposomes was determined by LavaPep™ Protein and Peptide quantification kit. A calibration curve was obtained in the range 0.122–500 μg/ml using free A20FMDV2. Liposomes were diluted 100 times in deionised water and the amount of peptide quantified according to the manufacturer’s instructions. Briefly, 50 μl of the diluted sample was incubated with 50 μl of LavaPep working solution for 60 min in the dark at RT. The fluorescence intensity was then measured using 540 ± 10 nm and 630 ± 10 nm excitation and emission filters, respectively (FLUOSStar Omega, BMG Lab Tech). The per cent peptide conjugated to the liposomes was calculated by quantifying the amount of peptide in the liposome sample before and after purification.

2.4. Cell culture conditions

The cell lines Panc-1 (CRL-1469™, pancreatic), PANC0403 (CRL-2555™, pancreatic) and 4T1 (CRL-2539™, breast) were obtained from ATCC®. A375Ppuro and A375Pβ6puro cell lines were created using the human melanoma cell line A375P (CRL-3224™, melanoma), which was infected with plb8e retroviruses encoding puromycin resistance alone or in combination with cDNA for human β6, as previously reported [12]. The A375Ppuro and A375Pβ6 cell lines were a kind gift from Prof. John Marshall (QMUL). The A375Pβ6 cell line was subsequently transfected with firefly luciferase (luc) using an SFG retroviral vector whereby luc was co-expressed with dsTomato red fluorescent protein. Transduced cells were then flow sorted for red fluorescence to obtain a pure A375Pβ6-luc cell line [24]. All cell lines were maintained at 37 °C, 5% CO2 and 5% relative humidity. Advanced RPMI (PANC-1, PANC0403, 4T1) or DMEM media (A375Ppuro, A375Pβ6puro) were used, both of these were supplemented with 10% FBS, 1% GlutaMax™ and 1% Penicillin/Streptomycin.

2.5. Characterisation of cell lines for αvβ6 integrin expression

αvβ6 integrin receptor expression was confirmed by 10D5 antibody staining and flow cytometry. Cells (1 × 10^6/100 μl) were incubated with 5 μl of 10D5 or the isotype control (IgG FITC) for 30 min at 4 °C, washed twice with 1 ml PBS before 30 min incubation with 2.5 μl of the FITC labelled IgG secondary antibody at 4 °C then washed with PBS. Using the FL1 detector, 10,000 cells were gated and the fluorescence was analysed under live gating. The cells were read on a BD FACS
Calibur™ flow cytometer obtained from BD Bioscience (US) and analysed using FlowJo software.

2.6. Cellular uptake of liposomes using flow cytometry

Cells were plated in a 24 well plate at a density of 50,000 cells/well and left overnight to allow the cells to attach. Cells were treated with 32.5–130 μM CF-DOPC containing liposomes (L or t-L) in complete media (10% FBS) for 1 or 4 h. In order to determine if the increased uptake of t-L was αvβ6 receptor specific, peptide inhibition studies were carried out. Cells were incubated at 4 °C for 10 min and then treated with 0.2 ml of 50 μg/ml free peptide in complete media for a further 10 min on ice. The fluorescently labelled L and t-L (32.5–130 μM) were then additionally incubated with the cells for 1 or 4 h. Additional peptide (50 μg in 25 μl PBS) was added after 1 h to ensure that the αvβ6 receptors remained blocked. Upon completion of the incubation period, the cells were washed with PBS, trypsinised and transferred into BD flow cytometer tubes. The cells were washed in PBS and re-suspended in 500 μl of PBS. The fluorescence was analysed in triplicates for each condition using the FL1 detector with 10,000 cells gated. All flow cytometry data was acquired using a BD FACs Calibur™ flow cytometer obtained from BD Bioscience (US) and analysed using FlowJo software.

2.7. Treatment of cancer cell lines with L-ALD/t-L-ALD and γδ T cells in combination therapy studies

The cell lines A375Ppuro, A375Pβ6, PANC-1 and PANC0403 were seeded at 50,000 cells/well in 96 well plates. Cells were treated for 24 h with 30 or 60 μM of ALD, L-ALD or t-L-ALD. Empty-liposomes (EL) and targeted EL (t-EL) were used as controls at lipid concentrations equivalent to that of the L-ALD and t-L-ALD, depending on the drug loading. The treatments were removed and replaced with 2.5 × 10⁴ ex vivo expanded Vγ9Vδ2 T cells, or γδ T cell culture media as a control, for a further 24 h. The γδ T cells were then removed and the cell monolayers were washed with PBS before cell viability was assessed with MTT as described below.

2.8. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was prepared in PBS at a concentration of 5 mg/ml and was diluted in media (1:6) prior to use. The supernatant of each well was removed and MTT solution (120 μM) was added to each well. The plates were then incubated at 37 °C and 5% relative humidity for 3 h. The MTT solution from each well was removed and DMSO (200 μl) was added and incubated for 30 min at RT. The reaction was quenched by the addition of 0.1 M EDTA solution to the mixture (5% v/v of the reaction mixture) to chelate free ¹¹¹In. Unbound ¹¹¹In:EDTA was removed using NAP-5 desalting columns equilibrated with PBS and the liposomes were collected in fraction 1–3 (~150 μl per injection dose).

2.11. Efficiency and stability of the radiolabelling in serum

Samples of the radiolabelled liposomes or ¹¹¹In:EDTA were spotted in glass microfibrew Chromatography paper impregnated with silica gel. These strips were then developed using a mobile phase of 50 mM EDTA in 0.1 M ammonium acetate. Strips were placed on a multi-purpose storage phosphor screen (Cyclone®, Packard, Japan) and kept in an autoradiography cassette (Kodak Biomax Cassette®) for 10 min. Quantitative autoradiography counting was then carried out using a cyclone phosphor detector (Packard®, Australia). The labelling stability was tested by incubation of the radio-conjugates in the presence or absence of FBS. Samples were diluted in 50% FBS or PBS (1:2 (v/v)), and incubated for 24 h at 37 °C. The percentage of ¹¹¹In:EDTA (imobile spot) remained conjugated to the liposomes was evaluated by TLC, using the same protocol as described above.

2.12. Animal models

All animal experiments were performed in compliance with the UK Home Office (1989) Code of Practice for the housing and care of Animals used in Scientific Procedures. Female SCID/Beige (SPECT/CT studies) and male NOD SCID gamma (NSG) mice (bio-distribution and therapy studies), 4–6 weeks old, were obtained from Charles River (UK). Female BALB/c mice, 4–6 weeks old, were obtained from Harlan Laboratories (UK). For the human xenograft tumour models, subcutaneous (s.c.) tumours were established by injecting 5 × 10⁶ cells in 100 μl PBS into each of the rear-flanks of SCID/Beige. For the murine tumour model, 1 × 10⁶ cells in 100 μl PBS were injected into each of the rear flanks of BALB/c mice. The size of the tumour was measured using callipers and tumour volume were determined using the equation:

\[ \text{Tumour Volume (mm³)} = \frac{A^2 \times B}{6} \]

where A and B represent the width and the length of the tumours, respectively [31]. Experiments commenced when tumours reached ~300 mm². For the lung model, 5 × 10⁵ cells were injected into the tail vein of NSG mice. This tumour model was monitored via s.c. injection of the mice with 100 μl 30 mg/ml luciferin/20 g mouse and subsequent scanning after 20 min using an IVIS Lumina series III In Vivo Imaging system (Perkin-Elmer). Images were quantitatively analysed by drawing regions of interest (ROI) around the tissues using Living Image 4.3.1 Service Pack 2 software (Perkin-Elmer, USA).

2.13. Whole body SPECT/CT imaging of radiolabelled liposomes in tumour-bearing mice

Mice were injected with liposomes containing 2 μmol lipid and radiolabelled with 1 MBq or 10–15 MBq, for bio-distribution and SPECT/CT studies, respectively, via tail vein injection. Mice were imaged with nanoSPECT/CT scanner (BioScan®, USA) 0–30 min, 4 h and 24 h post i.v. injection. For each mouse, a tomography was initially performed (45 Kvp; 1000 ms) to obtain parameters required for the SPECT and CT scanner, including the starting line, finish line and axis of rotation of the acquisition for each mouse. SPECT scans were obtained using a 4-head scanner with 1.4 mm pinhole collimators and the...
following settings: number of projections: 24; time per projection: 60 s and duration of the scan 60 min. CT scans were obtained at the end of each SPECT acquisition using 45 Kvp. All data were reconstructed with MEDISO (medical Imaging System) and the combining of the SPECT and CT acquisitions were performed using PMOD® software.

2.14. Gamma counting of radiolabelled liposomes in tumour-bearing mice

After 24 h, the mice were perfused with ~25 ml of 1000 U/l heparin in 0.9% sodium chloride in order to remove any liposomes remaining in the blood. The major organs (brain, lung, liver, spleen, kidney, heart, stomach and intestine), muscle, skin, bone (femur), carcass and tumours were collected, weighed and placed in scintillation vials. Additionally, 5 μl blood samples taken at various time points (5, 10, 30, 60, 240 and 1440 min), and urine and faeces collected with the aid of metabolic cages were also placed in scintillation vials for analysis. Each sample was analysed for [111In] specific activity using an automated gamma counter (LKB Wallac 1282 Compugamma, PerkinElmer, UK) together with dilutions of injected dose with dead time limit below 60%. The gamma rays emitted by the radioisotope were detected, quantified and corrected for physical radioisotope decay by the gamma counter. Radioactivity readings (counts per minute - CPM) were plotted as percentage of injected dose per organ or percentage of injected dose per gram of tissue.

2.15. Therapy study

Male NSG mice (4-6 weeks) were inoculated with 5 × 10^6 A375P86.1uc cells by i.v. injection to form experimental metastatic lung tumours. Bioluminescence imaging of mice was carried out on day 6 as described above and mice were divided into 4 treatment groups: naïve, L-ALD, γδ T cells and L-ALD and γδ T cells combination treatment. Doses in therapy experiments were 0.5 μmol of ALD/mouse (L-ALD) and 1 × 10^7 cells/mouse (γδ T cells), all injected via the tail vein. Three doses of each treatment were given at one-week time intervals on days 7, 14 and 21. In the case of the combination treatment, mice were pre-injected with L-ALD (days 6, 13, and 20) then injected with γδ T cells (days 7, 14, and 21). Tumour growth was monitored by bioluminescence imaging twice weekly, as described above.

2.16. Determination of IFN-γ concentration with ELISA

Animals from the therapy study were sacrificed and sera was analysed for human IFN-γ. Sera were diluted 1:2 and analysed using a human IFN-γ ELISA Ready-set-go-kit as per the manufacturer’s protocol.

2.17. Pre-incubation of liposomes with mouse sera

Mouse serum was obtained by allowing blood obtained from a terminal bleed of SCID/Beige mice to clot. The blood was centrifuged to pellet the clotted cells, and the serum was removed for further use. t-L were incubated with the mouse serum for various periods of time (10 min, 1 h, 4 h) at 37°C. Any proteins that had not interacted with the surface of the liposomes were removed using a 10 cm Sepharose 2B column and eluted using PBS. t-L were incubated with cells without any further processing and their uptake analysed by flow cytometry as described above.

2.18. Statistics

For all experiments, data were presented as mean ± SD, except for therapy experiments where data were presented as mean ± SEM; n denotes the number of repeats. Independent variable Student t-tests were performed using IBM SPSS version 20 for in vitro cytotoxicity studies. For in vivo studies, significant differences were examined using one-way ANOVA. The t-value, degrees of freedom and two-tailed significance (p-value) were determined. *p < 0.05, **p < 0.01 and ***p < 0.001.

3. Results

3.1. Preparation and characterisation of αvβ6 integrin-targeted liposomes (t-L)

DSPC:Chol:DSPE-PEG2000 liposomes were prepared using lipid film hydration method and extrusion. t-L were prepared by incorporating DSPE-PEG2000-maleimide (0.5–2 mol%) in the liposomes, then linked directly to αvβ6-targeting peptide. Free peptide was removed before further characterisation. The effects of %mol DSPE-PEG2000-maleimide and the peptide A20FMDV2 (25 μg/μmol lipid) on the physicochemical characteristics of the liposomes were examined. There was no significant difference in the hydrodynamic size [157.8–159.1 nm], PDI (0.080–0.098) or zeta potential (−14.6 to −14.6 mV) of the liposomes when DSPE-PEG2000-maleimide (1 mol%) or A20FMDV2 were included in the formulation (Table 1). The size of the liposomes is in the range reported to be extravasated in regions of leaky vasculature as part of the EPR effect [32]. The low PDI values indicated that the liposomes were homogenous in size and the absence of liposome aggregation. The identical physicochemical characteristics of the L and t-L ensure that any differences in behaviour of these liposomes are solely due to the ability of the A20FMDV2 peptide to bind to the αvβ6 integrin receptor. Liposomes containing 0.5 or 2% DSPE-PEG2000-maleimide with or without A20FMDV2 were also characterised and no significant change to the physicochemical characteristics of the liposomes were observed (Table S1).

3.2. Optimisation of peptide loading on liposomes surface

The effect of different initial addition of peptide on the final peptide loading on the t-L was examined. The LavaPep peptide assay was used to quantify the amount of peptide conjugated to the t-L and confirmed the purification of the t-L from the unconjugated A20FMDV2 using a Sepharose 2B column (Fig. S1). In the case of 1%mol DSPE-PEG2000-maleimide, the amount of A20FMDV2 added to the liposomes was adjusted from 25 to 100 μg/μmol lipid (10–40 nmol per mol lipid) (Table 2). Final peptide loading achieved ranged from 17.7 to 21.7 μmol of ALD/μmol lipid (initial peptide − final peptide/μmol lipid) on the physicochemical characteristics of the liposomes were observed (Table 1).

<table>
<thead>
<tr>
<th>Initial peptide added (μg/μmol lipid)</th>
<th>Size (nm)a,b</th>
<th>PDIc,d</th>
<th>Zeta Potential (mV)c,d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>159.1 ± 1.7</td>
<td>0.098 ± 0.025</td>
<td>−14.6 ± 1.53</td>
</tr>
<tr>
<td>25</td>
<td>157.8 ± 2.5</td>
<td>0.08 ± 0.013</td>
<td>−12.5 ± 1.08</td>
</tr>
</tbody>
</table>

a Liposomes containing 1% mol DSPE-PEG2000-maleimide.
b Hydrodynamic diameter measured by dynamic light scattering.
c Analysed by electrophoretic light scattering using 10 mM NaCl.
d Data are represented as mean ± SD.
change in the presence of free A20FMDV2. For A375Ppuro, a small increase in the amount of t-L when compared to L was observed in some conditions and was affected by the presence of A20FMDV2, despite their lack of the integrin receptor. This t-L/L ratio was lower than that of αv6 positive cell lines, however, and only occurred sporadically.

3.5. t-L-ALD are more effective than L-ALD at sensitising an integrin positive cell line to destruction by γδ T cells

In order to test the therapeutic benefit of using liposomes targeted with the A20FMDV2 peptide, ALD was encapsulated into L and t-L formulations. The ability of the resulting L-ALD and t-L-ALD to sensitize cancer cell lines to destruction by Vγ9Vδ2 T cells was then tested by assessing cell viability and γδ T cell-derived IFN-γ production. The αv6 positive cell line A375Pβ6 was used in this assay. As shown in Fig. 3, none of the treatments in isolation caused toxicity. However, when the cells were pre-treated with free or liposomal ALD, and were subsequently treated with γδ T cells, a significant decrease in cell viability was observed. t-L-ALD in combination with γδ T cells led to significantly lower cell viability than L-ALD at both 30 μM (p < 0.001) and 60 μM (p < 0.01) concentrations. To further confirm the increased sensitivity of αv6 positive cancer cells to γδ T cells when treated with t-L-ALD as compared to L-ALD, the IFN-γ release from the γδ T cells was quantified. Significantly higher amounts of IFN-γ were released when the γδ T cells were co-cultured with cells pre-treated with t-L-ALD as compared to L-ALD (p < 0.001 and p < 0.001 for 30 μM and 60 μM, respectively). This finding is in agreement with the results obtained by the MTT assay. In the case of the αv6 negative cell line, A375Ppuro, no difference in the ability of L-ALD and t-L-ALD to sensitise the cells to γδ T cells was seen in terms of either cytotoxicity or IFN-γ release (Fig. S6).

3.6. Whole body SPECT/CT imaging of mice injected with 111In labelled L and t-L

L and t-L were formulated with 1% mol DSPE-PEG2000-maleimide and radiolabelled with 111In. Labelling efficiencies of 86.3% and 61.9% were obtained for L and t-L, respectively (Fig. S7). Stability studies were performed after 24 h at 37 °C in both PBS and 50% FBS in order to replicate in vivo conditions. For L, 87.8% of the 111In remained bound to the liposomes after 24 h incubation with PBS. On incubation of L with serum, 91% stability was seen. For t-L, 80.7% and 78.2% of 111In remained bound after 24 h incubation with PBS and FBS, respectively.

Tumour-bearing SCID/Beige mice were imaged by SPECT with computed tomography (CT) scanning to study the organ bio-distribution of radiolabelled L and t-L. The mice were inoculated with either the two melanoma or the two pancreatic human xenograft models on their back left flank (αv6 negative tumour: A375Ppuro or PANC-1) (right of the image) or right flank (αv6 positive tumour: A375Pβ6 or PANC0403) (left of the image). They were imaged immediately after injection, and 4 and 24 h post-injection by SPECT/CT. As shown in Fig. 4, no differences in the whole body bio-distribution for [111In]L and [111In]t-L were observed. At early time-points, good blood circulation was observed for both types of liposomes as indicated by signals seen in the heart and head of the mice. Over time, the liposomes accumulated in the liver and spleen. Slight accumulation of the liposomes in the tumours could also be seen at later time-points, but this could not be clearly observed in these images, possibly due to masking by prolonged blood circulation of the liposomes. L and t-L gave comparable signals in both the positive and negative tumours. Gamma counting was used to quantify the tumour uptake of L and t-L.

3.7. Comparable tumour distribution for both L and t-L was obtained in integrin negative and positive models

The organ bio-distribution and tumour uptake of [111In]L and
Fig. 1. The effect of the extent of expression of αvβ6 integrin receptor on the uptake of t-L. (A) Representative images of cell lines incubated with the antibody 10D5 (specific to αvβ6) or an isotype control. Cells were analysed for αvβ6 integrin receptor expression using flow cytometry and FL-1 detector. In a comparison of the αvβ6 expression of different cell lines using the % positive cells gated in M1 or the MFI, the 4T1 cancer cell line showed the lowest receptor expression, while A375Pβ6 has the highest expression both in terms of % of positive cells and the number of receptors per cell. (B) Comparison of the MFI fold increase of fluorescently labelled t-L to L. Cells were treated with 32.5 µM L or t-L for 1 or 4 h. With increasing expression of αvβ6, the uptake of t-L in relation to L further increases. This is a further indication of the specificity of the t-L to the αvβ6 integrin receptor. Values are expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01 (ANOVA).

[111In]-L in tumour-bearing SCID/Beige mice, was then quantitatively determined by gamma counting. No significant differences were observed in the blood circulation profiles of [111In]L and [111In]-t-L (Fig. 5A). At 1 h post administration, ~66%ID of both formulations remained in the blood, decreasing to 31–39%ID and ~8%ID after 4 and 24 h, respectively. L and t-L also had similar excretion profiles (Fig. 5B), with only small fraction of the liposomes excreted within 24 h in the urine (1.0–1.5%ID) and faeces (0.30–0.37%ID). In agreement with the SPECT imaging studies, liposomes accumulated mainly in the liver (L: 43 ± 3 %ID/g t-L; 29 ± 9 %ID/g) and the spleen (L: 255.5 ± 89.7 %ID/g t-L: 219.4 ± 78.8 %ID/g) (Figs. 5C and S8). No significant differences in the organ bio-distribution of [111In]L and [111In]-t-L were observed. Additionally, no difference in uptake of t-L and L was observed in the αvβ6 positive tumours. A comparison of the uptake of both [111In]L and [111In]-t-L among the different tumour models showed that when the tumours were normalised by weight, there was no significant difference in the uptake of the liposomes among different tumour models (1.7–3.2 %ID/g tumour). Once again no difference in bio-distribution was observed between [111In]L and [111In]-t-L.

3.8. Both L-ALD and t-L-ALD sensitise tumours to γδ T cell therapy in vivo

L-ALD and t-L-ALD were used as a monotherapy and in combination with ex vivo-expanded Vγ9Vδ2 T cells in an experimental metastatic lung model with the αvβ6 positive A375Pβ6 melanoma cell line in NOD-SCID gamma (NSG) mice. On day 6, all six groups had the same average tumour size (~1.3 × 106 photons, as determined by bioluminescence imaging). L-ALD, t-L-ALD or γδ T cells as monotherapies did not result in a significant reduction in tumour growth. Monotherapy with t-L-ALD showed a trend towards greater effectiveness than L-ALD (4.70 × 108 ± 1.78 × 108 vs. 1.24 × 109 ± 4.65 × 108 photons, day 27, p = 0.15). However, this difference was not significant due to variability in tumour size. Mice pre-treated with L-ALD or t-L-ALD 24 h prior to injection of γδ T cells showed a significant reduction in tumour growth, with tumour sizes of 7.53 × 107 ± 2.02 × 107 and 9.06 × 107 ± 3.33 × 107 photons, respectively, compared to 1.42 × 109 ± 6.38 × 108 photons for naïve tumours on day 27 (Fig. 6). No improvement on the ability of t-L-ALD to sensitise the tumours to γδ T cells compared to L-ALD was observed. IFN-γ serum levels were measured on day 27. Mice pre-treated with L-ALD or t-L-ALD prior to γδ T cells had levels of 32.6 ± 19.3 and 12.3 ± 4.4 pg/ml (p < 0.05), respectively, compared to only 6.5 ± 0.9 pg/ml in γδ T cells-only treated group, mirroring the significant reduction in tumour growth observed in these groups.
3.9. Use of mouse sera in cell uptake studies reduces receptor-mediated cell uptake of t-L in vitro

In order to explore one possible explanation for the lack of improvement in therapeutic efficacy of t-L-ALD compared to L-ALD when used in combination with γδ T cells in therapy studies, cell uptake studies were performed in vitro, whereby t-L were pre-incubated with mouse serum and excess serum and unbound proteins were removed by size exclusion chromatography, prior to their 1 h incubation with cell lines (Fig. 7). Interestingly, this study showed the reduction in receptor-mediated uptake was indeed dependent on the incubation times with mouse serum. This result suggests that mouse serum played a direct role in reducing active targeting efficiency in vivo. Unfortunately, whether this was a direct effect of the enzymes present in the serum on A20FMDV2 peptide’s stability or a protein corona effect, could not be precisely established.

4. Discussion

In our study, αvβ6 positive cells were successfully targeted in vitro. Higher uptake of t-L versus L was observed in the A375Pβ6 and PANC0403 αvβ6 positive cell lines. This increased uptake of t-L led to an increased therapeutic efficacy of t-L-ALD at sensitising A375Pβ6 to γδ T cells when compared to L-ALD. The amount of receptor present on the surface of cells is a key consideration when evaluating a targeted drug delivery system. The expression of αvβ6 receptor on A375Pβ6 cells was confirmed in vivo in a flank mouse tumour model in a previous study carried out by co-authors of this paper. SPECT/CT imaging showed that the radiolabelled peptide A20FMDV2 had seven times higher retention in the αvβ6 positive A375Pβ6 solid tumours than the αvβ6 negative A375Puro tumour implanted in the same mouse (Saha et al., 2010). This demonstrates the expression of the αvβ6 receptor in vivo. In our study, it has been shown that the targeting efficiency of t-L was linked to αvβ6 integrin receptor expression on the cell surface. As
expected, the targeting efficiency was directly proportional the level of receptor expression, when comparing the three αβ6 positive cell lines with A375P6 > PANC0403 > 4T1 in terms of both αβ6 integrin receptor expression and targeting efficiency. This correlation has been previously reported by Elias et al. [33], with the density of the cell-surface HER2/neu receptor shown to be an important factor in the targeting capability of HER2-targeted nanoparticles.

While the peptide A20FMDV2 has previously been shown to selectively target the integrin receptor αβ6 in vitro [12,13,34], it has not been conjugated to a nanoparticle before. In this work we provide evidence of successful conjugation of the peptide A20FMDV2 to the surface of liposomes for the first time, and have shown using in vitro peptide inhibition studies that the t-L are taken up by cells in a receptor-specific manner. Only one study by Grey et al. has specifically targeted the αβ6 integrin receptor using a liposomal formulation [14]. In this study, liposomes were conjugated to the αβ6-specific peptide, H2009.1. Interestingly, authors have shown that in vitro targeting using the αβ6-positive cell line, H2009, could be achieved only when the peptide was incorporated in the tetrameric form and not the monomeric form. Our results have shown that in vitro targeting could be achieved despite using the monomeric form of the peptide. Future work investigating the use of multivalent forms of the peptide A20FMDV2 to further increase targeting efficiency is therefore worth investigating.

In our study, while active targeting could be achieved in vitro using t-L, no increase in %ID/g tumour, could be seen in αβ6 positive tumours during in vivo bio-distribution analysis. There have been previous reports in the literature demonstrating that peptide-targeted liposomes showed no improvement in tumour accumulation compared to untargeted liposomes in vivo [35]. It was therefore not surprising to see comparable tumour uptake data in vivo for L and t-L in all tumours tested. However, we hypothesised that targeting L-ALD to the αβ6 integrin receptor, known to be overexpressed on cancer cells and absent on healthy cells, will result in increased uptake of t-L-ALD due to receptor-mediated endocytosis in vivo and hence better therapeutic outcomes, when combined with γδ T cell-based immunotherapy. Kırpotin et al. demonstrated that despite no increase in tumour uptake of HER2 targeted liposomes in subcutaneous HER-2 positive breast cancer tumours in vivo, when excised tumours were studied using flow cytometry and microscopy, a 6-fold increase in cell internalisation of these targeted liposomes was observed [36]. Similarly, in another study, transferrin-targeted siRNA nanoparticles did not show an increase in tumour accumulation but resulted in a ~50% decrease in tumour growth compared to non-targeted nanoparticles due to increased cellular uptake [37]. Our therapy in experimental metastatic melanoma A375P6-lung model results showed no added advantage using t-L-ALD over L-ALD when used in combination with γδ T cells, following our treatment protocols. There could be several explanations to the results obtained; the most obvious one is that L-ALD, in combination with immunotherapy, resulted in efficient tumour growth delay, decreasing the scope for any further improvements, using the targeted delivery approach to be detected. Using lower doses of L-ALD and t-L-ALD may reveal a difference in therapeutic efficacy of the two formulations. Additionally, when L-ALD and t-L-ALD were used as monotherapies, mice treated with t-L-ALD had an average tumour size that was 2.6 times lower than that of mice treated with L-ALD. We hypothesise that this may be as results of higher affinity of t-L-ALD than L-ALD to A375P6 cells as demonstrated during in vitro studies. Another possibility could be that ALD experiences better endosomal escape from t-L-ALD than L-ALD, facilitated by the receptor-mediated uptake of the former. Unfortunately however, large variations in tumour size meant that this reduction in tumour size was not significant. Using higher doses of L-ALD and t-L-ALD as monotherapies or increasing the number of mice per treatment group may allow the difference in therapeutic efficacy to be observed.

The lack of correlation between the in vitro and in vivo targeting efficacy that we observed agrees with a previous study by Gray et al. [14]. As discussed above, liposomes targeted using the αβ6-specific peptide H2009 in its tetrameric form demonstrated encouraging findings in vitro, with a 5–10 fold increase in uptake for targeted liposome. However, targeted liposomes did not lead to an improvement in targeting or efficacy in vivo using an αβ6 positive H2009 subcutaneous tumour model [35]. Gray and his colleagues, concluded that it is the properties of the liposome (size and PEGylation) and not the targeting peptide that determines in vivo bio-distribution. Poor tumour penetration due to the dense extracellular matrix and high interstitial pressure in solid tumours may have prevented liposomes from exerting a targeting effect within the tumour itself [38,39]. Likewise, a folate-targeted formulation of L-ALD demonstrated in vitro targeting but no therapeutic advantage was observed during in vivo studies with a folate receptor-α positive ovarian tumour model [24]. The presence of folate receptors on endogenous non-tumour cells leading to accelerated systemic clearance was hypothesised to be responsible for the lower therapeutic efficacy of the targeted formulation.

Recent studies have revealed that the formation of a protein corona on the surface of liposomes can lead to an inhibition of targeting
efficacy, as the targeting-peptides can be hidden from their receptors [40–43]. We have therefore conducted a study to see if incubation of t-L-ALD with mouse serum will compromise the targeting efficiency, in an attempt to correlate the in vitro targeting efficiency with in vivo active targeting in a living mouse. Our results indeed showed that incubation with mouse serum reduced the targeting efficiency of t-L, in a time-dependent manner. This suggests that the direct interaction of mouse serum and t-L adversely affects active targeting of t-L-ALD. Further studies to explore types of protein corona involved are warranted and as it has been reported that protein corona may differ based on what species the serum is from, extrapolation of data in mice to humans is not possible [40]. Another explanation of the mouse serum data could be the enzymatic degradation of A20FMDV2. A study performed by Saha et al. has shown that only 50% of the peptide A20FMDV2 [13] remained intact after 4 h incubation with mouse serum. Amino acid modifications may help improve resistance to enzymatic degradation [44–46]. To understand the exact effect of serum on active targeting in our study needs further investigation.

5. Conclusion

In this work, A20FMDV2 peptide was successfully conjugated to PEGylated liposomes and active targeting was confirmed in vitro in melanoma and pancreatic cells lines. In vitro targeting efficiency was directly linked to levels of αvβ6 receptor expressions and was blocked by addition of excess peptide, suggesting receptor-mediated endocytosis as a mechanism of internalisation. αvβ6-targeted liposomal alendronate led to significant improvement in sensitisation of αvβ6 positive cancer cell line to γδ T cells, and improved cell kill in vitro. Despite the slight promise in using t-L-ALD as a monotherapy, no added advantage was observed when combined with γδ T cells immunotherapy, in an experimental metastatic lung mice model. Future therapy studies with modified treatment protocols need to be performed to determine if a significant improvement in therapeutic efficacy using t-L-ALD can be

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Fig. 4. *In vivo* whole body 3D SPECT/CT imaging of 111In labelled liposomes in tumour-bearing immunocompromised mice. Mice were i.v. injected with 111In labelled liposomes at a dose of 2 μmol lipid/mouse. Mice were inoculated bi-focally with the αvβ6 positive tumour on the left of the image (A375Pβ6) (right flank) and the negative tumour (A375Ppuro) on the right of the image (left flank). Whole body 3D SPECT/CT imaging were performed at 0–30 min, 4 and 24 h post-injection with scanning time of 40–60 min each. SPECT data is displayed in colour scale and CT data is in grey scale, respectively (high to low signals: top to bottom).
achieved. In vivo peptide stability and the protein corona effect may be responsible for the decrease in in vivo targeting efficacy compared to the in vitro results obtained, which also needs to be investigated further.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.04.025.

References

