Pharmaceutical Research, Vol. 26, No. 12, December 2009 (© 2009)
DOI: 10.1007/s11095-009-9971-6

Research Paper

Leucine-Aspartic Acid-Valine Sequence as Targeting Ligand and Drug Carrier for Doxorubicin Delivery to Melanoma Cells: *In Vitro* Cellular Uptake and Cytotoxicity Studies

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Received April 29, 2009; accepted September 14, 2009; published online October 6, 2009

Purpose. To study the feasibility of *Leucine-Aspartic Acid-Valine* (LDV) as targeting ligand and drug carrier for targeted delivery to integrin $\alpha_4\beta_1$ over-expressing cancer cells.
Methods. Poly(L,D,V) was randomly copolymerized using N-carboxyanhydrides of leucine, β-benzyl-aspartic acid, and valine. Oligo(LDV), consisting of 2-6 LDV units, were synthesized by solid phase protein synthesis (SPPS) method. Binding of Leu-Asp-Val, Val-Asp-Leu, and Leu-Asn-Val, and internalization of FITC labeled LDV by wild-type and integrin α_4 knock-down A375 cells were studied. Cytotoxicity of poly(L,D,V)-Dox, oligo(LDV)-Dox, and doxorubicin (Dox) was also determined on wild-type, integrin α_4 knock-down A375 cells, and normal human epithelial keratinocytes (NHEK).
Results. LDV was essential for the specific binding and internalization by cells expressing integrin $\alpha_4\beta_1$. Cytotoxicity of poly(L,D,V)-Dox and oligo(LDV)-Dox was integrin α_4-dependent, while free Dox did not show this differential effect. No observable cytotoxicity trend was found when increasing LDV repeating unit. Poly(L,D,V) was relatively more effective than oligo(LDV) for the delivery of Dox to A375.
Conclusion. LDV containing moieties bind specifically to integrin $\alpha_4\beta_1$ expressing cancer cells. The binding, internalization, and cytotoxicity depend on the level of integrin $\alpha_4\beta_1$ expression. Poly(L,D,V) and oligo(LDV) were both effective in the *in vitro* targeted delivery of Dox to integrin $\alpha_4\beta_1$ over-expressing A375 cells.

KEY WORDS: binding specificity; cellular uptake; LDV; oligo(LDV); targeted delivery.

INTRODUCTION

The goal of cancer chemotherapy is to suppress or eradicate cancer using effective, safe, and well-tolerated medications. A major problem in cancer chemotherapy is the collateral damage to the normal cells due to the anti-cancer agents. Based on the biochemical, and/or physiological difference between cancer and normal cells or tissues, targeted cancer drug delivery is designed to preferentially deliver the anti-cancer agents to the cancer cells, so as to increase the therapeutic efficacy and decrease the side-effects. The targeted delivery can be achieved by active drug targeting and passive drug targeting. The difference of receptors expression on cell membrane between cancer cells and normal cells provides a basis for active targeting by not only specific interaction between drug delivery system and cells, but also facilitated cellular uptake via receptor-mediated endocytosis (1). Antibodies (2), folic acid (3), low-density lipoprotein mimics (4), lectins (5), transferrin (6), and integrins-targeted peptides have been utilized for targeted delivery of anti-cancer agents based on their specific bindings between ligands and receptors.

The integrins are a family of transmembrane glycoproteins that mediate cell–cell and cell–matrix adhesion. There are 18 α- and 8 β- subunits that associate in various combinations to form 24 known mammalian integrin heterodimers (7). Integrin expression pattern changes during tumor growth, metastasis, and angiogenesis (8). Some peptide fragments were found to bind to integrins specifically, for example, LDV binding to integrin $\alpha_4\beta_1$ (9) and RGD binding to integrin $\alpha_v\beta_3$ (10), $\alpha_v\beta_5$ (11), and $\alpha_5\beta_1$ (12). Based on the over-expression pattern of integrins on the cancer cells and the specific binding between peptides and integrins, targeted drug delivery systems have been designed. Current integrin-targeted cancer chemotherapy can be classified into three categories: 1) integrin antagonist, for example, Vitaxin®, an antibody to integrin $\alpha_v\beta_3$, was once in phase II clinical trial as an anti-cancer agent by inhibiting the tumor metastasis when integrin $\alpha_v\beta_3$ is over-expressed (13); 2) integrin-targeted anti-cancer drug delivery, such as the cyclic RGD-Dox-nanoparticle formulation, which was reported to actively target Dox to tumor site *in vivo* (14), and RGD

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attached to poly(ethylene glycol) (15) or poly(l-lactide) (16) for integrin-targeted drug delivery, which have produced promising *in vitro* and *in vivo* results; 3) integrin-targeted gene delivery, for example, RGD was inserted into the HI loop of adenovirus to enhance the gene transfection efficiency (17).

Small peptides that have been reported to have specific binding to integrins include RGD, RYD, LDV, RHDS, etc. However, most of the current integrin-targeted delivery studies have been based on RGD or its receptors. RGD can target to $\alpha_v\beta_3$ or $\alpha_v\beta_5$, which not only over-express in cancer but also in endothelial cells (18). Compared to RGD, LDV has not been widely studied in the targeted drug delivery field. Based on the reported binding specificity, LDV has a more specific binding pattern (mostly binding to integrin $\alpha_4\beta_1$) (19, 20). In addition, integrin $\alpha_4\beta_1$ has a more specific expression pattern (21, 22) in cancer types when compared to $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The specificity may endow LDV as a better targeting ligand to cancer cells that over-express integrin $\alpha_4\beta_1$, such as melanoma and leukemia (23). Our lab has presented the preliminary results to demonstrate the potential of using LDV as a targeting moiety to integrin $\alpha_4\beta_1$ (24). However, the understanding of the basic parameters for using LDV to target integrin $\alpha_4\beta_1$, such as binding specificity, cellular uptake, and response to different levels of integrin $\alpha_4\beta_1$ expression, is still needed for rational design of a targeted drug delivery system.

In this study, LDV will be used as an active targeting ligand to study the integrin $\alpha_4\beta_1$-targeted anti-cancer drug delivery system. Three amino acids—L,D,V— will be randomly polymerized to form poly(L,D,V), or constructed orderly as oligo(LDV) to serve as drug carrier for integrin-targeted drug delivery with Dox as anti-cancer agent and melanoma cells as model cancer cells.

MATERIALS AND METHODS

Materials

A375 and Jurkat were purchased from American Type Culture Collection (ATCC) (Manassas, VA). NHEK was a Clonetics® product (Lonza Inc, Walkersville, MD). A375 cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen Co., Carlsbad, CA) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. NHEK cells were cultured in keratinocyte basal medium supplemented with SingleQuot Kit containing supplements and growth factors. Jurkat cells were grown in RMPI (Roswell Park Memorial Institute) medium containing 25 mM HEPES, 2 mM L-glutamin, 50 IU/mL of Penicillin, and 50 μg/mL of Streptomycin.

Fmoc-leucine, Fmoc-aspartic acid-butyl ester, Fmoc-valine, and Fmoc-asparagine were purchased from VWR (Batavia, IL). Leucine, valine, β-benzyl-aspartic acid, and RIPA buffer were obtained from Sigma-Aldrich (St. Louis, MO). FITC and Dox were purchased from MP Biomedicals (Solon, OH). Epicentre MasterPure RNA purification kit was obtained from Epicentre biotechnologies (Madison, WI). All other chemicals and solvents (HPLC grade) were purchased from Sigma-Aldrich or VWR, and used without further purification.

Synthesis and Characterization of LDV/VDL/LNV on Magnetic Beads

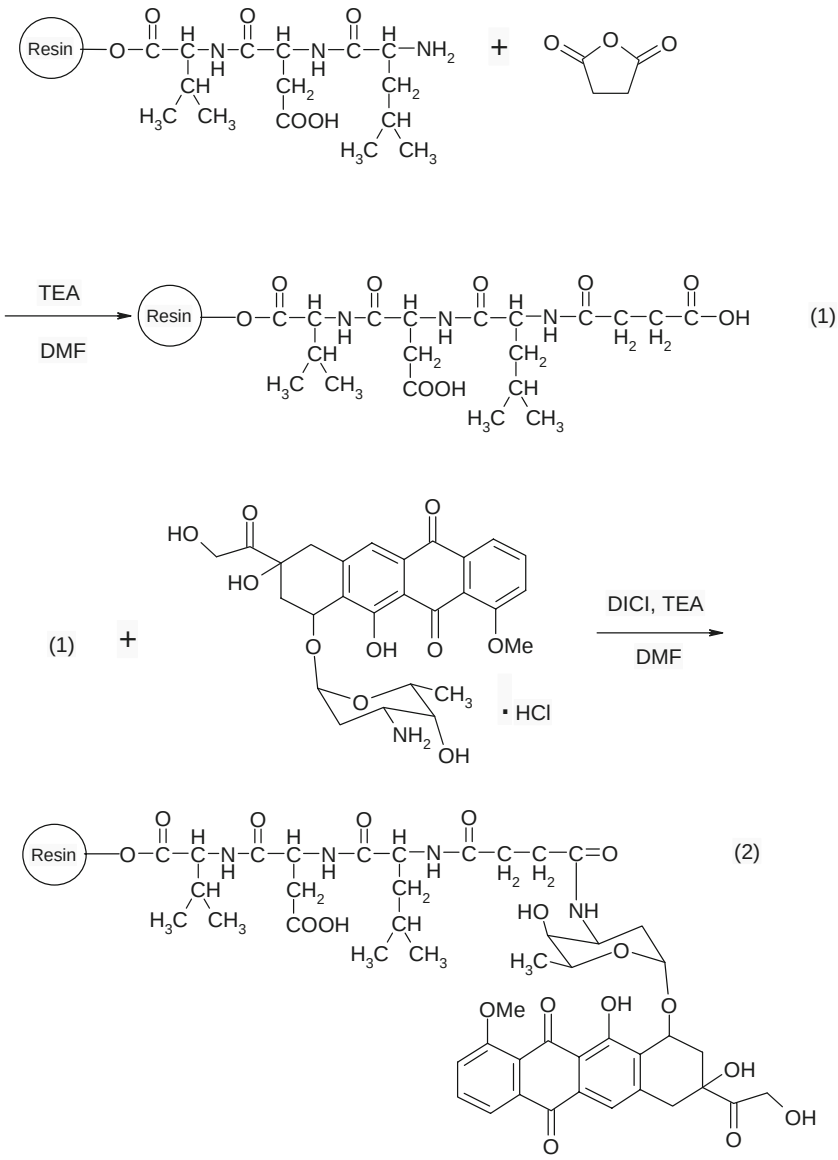
Magnetic beads embedded with Fe_3O_4 and containing hydroxyl groups on the surface were prepared by the following procedure: Polyvinylalcohol (PVA) was dissolved in DI water with high speed stirrer. Fe_3O_4 powder, styrene, divinylbenzol, and benzoyl peroxide were mixed and added to PVA solution. The temperature was then raised to 70–75°C and maintained for 3 h. Acrylic acid was added, and the reaction continued for ~18 h untill a gray or black mixture was obtained. The beads with ~30 μm in diameter were collected by sieving through micro-mesh screens (BMC Inc, MI). The surface hydroxyl group of the beads was determined by acetylation method and measured to be~1 mmol/g. Leu-Asp-Val (LDV), Leu-Asn-Val (LNV), and Val-Asp-Leu (VDL) were conjugated to magnetic beads using standard Fmoc solid phase protein synthesis (SPPS) method. Briefly, magnetic beads (120 mg) were soaked in dimethylformamide (DMF) for 30 min; Fmoc-Val (204 mg), 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (228 mg), and collidine (90 μL), were added and incubated overnight, washed with DMF and CH_2Cl_2, capped with 0.5 M acetic anhydride and 0.5 M triethylamine in DMF for 1 h, washed with DMF and CH_2Cl_2, de-Fmoc with 20% piperidine in DMF for 30 min, and washed with DMF and CH_2Cl_2. Fmoc-Asp(OtBu) (124 mg), hydroxybenzatriazole (41 mg), and diisopropylcarbodiimide (DICI) (48 μL) were added and incubated for 1 h, washed, de-Fmoc, washed, and then the same steps as Fmoc-Asp(OtBu) were repeated with Fmoc-Leu (106 mg). VDL and LNV were synthesized in similar procedures except different order or protected amino acid derivative was used. Peptides were cleaved from the beads using 20% trifluoroacetic acid (TFA) in CH_2Cl_2. After most of solvents were rotavapped, isopropylether was added to precipitate the product, which was collected by centrifuge, and then lyophilized for characterization. ESI-MS spectra of the peptides were recorded on a Varian 1200 ESI-triple-quadruple mass spectrometer.

Synthesis and Characterization of FITC Labeled LDV

LDV was synthesized using Fmoc SPPS method with Wang resin, followed by FITC conjugation in pyridine/DMF/CH_2Cl_2 (12:7:5) overnight (25). FITC-labeled LDV was cleaved and collected using the same method mentioned above.

Synthesis and Characterization of Dox Conjugated Oligo (LDV)

Oligo(LDV) were synthesized using Fmoc SPPS method on Wang resin, followed by *in situ* Dox conjugation (Scheme 1). Briefly, after building oligo(LD(OtBu)V) on the resin, succinic anhydride was added to activate the N-terminal of oligo(LD(OtBu)V) and served as a linker for Dox attachment. Oligo(LD(OtBu)V)-Dox was then de-protected and at the same time cleaved from the resin by using 20% of TFA in CH_2Cl_2. The products were collected, lyophilized, and characterized by MALDI-TOF mass spectrometer (Axima Kratos, Shimadzu). The repeating LDV units in oligo(LDV)-Dox ranging from 1 to 6 were prepared.



Scheme 1. Synthetic scheme for doxorubicin conjugation to LDV.

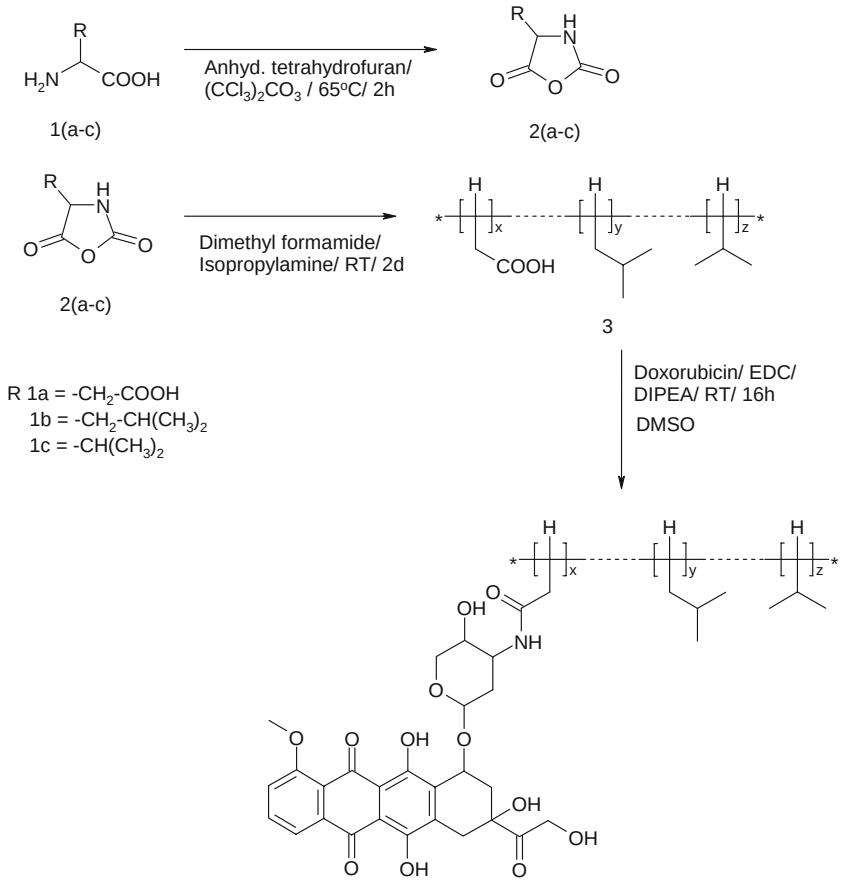
Synthesis and Characterization of Poly(L,D,V) and Dox Conjugated Poly(L,D,V)

Poly(L,D,V) was prepared by isopropyl amine-initiated random polymerization of N-carboxyanhydrides of leucine, β-benzyl-aspartic acid, and valine in DMF at room temperature for two days, followed by debenzylation with 6% trifluoromethane sulfonic acid in TFA at 0ºC until the clear polymer solution became turbid (Scheme 2). The reaction mixture was evaporated in a rotavap to a pasty mass. The residue was treated with water, and the pH of the polymer suspension was adjusted to 3.0 with sodium hydroxide. The product was washed free from TFA salts and lyophilized. The molecular weight of poly(L,D,V) was determined by size exclusion chromatography on Waters HPLC system (Millipore, Billerica, MA) using an Ultrastyragel column with tetrahydrofuran as a mobile phase.

Dox was conjugated to a fraction of aspartic acid residues in poly(L,D,V) by using *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) (Scheme 2) in dimethyl sulfoxide (DMSO). The unreacted drug was separated by dialysis of a colloidal suspension of poly(L,D,V)-Dox against water. The particle size of the conjugate in water was measured on a 3000 HS_A Zetasizer (Malvern Instruments, UK). Fluorometric assay of Dox with 470 nm excitation and 585 nm emission was conducted on a QM-2000-6 fluorescence spectrophotometer (Photon Technology International) to analyze the drug loading of the conjugate dissolved in DMF.

Integrin α4 Expression in Wild-Type/Knock-Down A375 Cells, and NHEK Cells

A375 cells (80% confluent) were transfected with 20 pmol of integrin α4-specific siRNA or scrambled siRNA (Santa Cruz Biotech) using Lipofectamine 2000 according to manufacturer's protocol. Cells were harvested after 24-h culture in DMEM without antibiotics. Integrin a4 knock-down was characterized by Western blot and PCR. Wild-type/



Scheme 2. Synthetic scheme for poly(L,D,V) and poly (L,D,V)-Dox.

knock-down A375 or NHEK Cell lysates for Western blot analysis were prepared by subjecting the cell pellets to 3 freeze-thaw cycles in HEPES buffer and extracted by RIPA buffer. SDS-PAGE (10%) gel was used, and integrin α4 was detected by using rabbit polyclonal anti-α4 antibody (Santa Cruz Biotech). Total RNA was extracted using Epicentre MasterPure RNA purification kit. First strand cDNA was synthesized using random primers. Reverse transcription mixture was used as a template for real-time PCR (My iQ™ single color real-time PCR detection system, iCycler Thermal Cycler) with the gene-specific primers. Gene expression level was normalized with GAPDH.

Binding Study

LDV /VDL /LNV conjugated magnetic beads (2.5 mg blank beads-equivalent) were added to standard cell counter vials. Cells (8×10^6) in 10 mL of cell culture medium were added to the vials containing ligand-bearing beads and incubated at 37°C for 30 min, with gentle agitation on a shaker. Magnetic field was applied to separate the bound and unbound cells, followed by counting the unbound cells in supernatant using a Coulter Z1 Particle Counter. Wild-type and integrin α4 knock-down A375 cells were used in the binding studies. Free LDV (~2 μg/mL) was also added to compete with LDV-anchored magnetic beads in A375 cells binding study.

Internalization Study

Wild-type or integrin α4 knock-down A375 cells were cultured to 80% confluence on 12-well cell culture plates. LDV-FITC in HBSS (equivalent to 1 μg/mL of FITC) was added to the cells and incubated for 3 h. EDTA (10 mM) was added and incubated for 15 min to wash off the surface-bound LDV-FITC by inhibiting the calcium-dependent integrin-LDV interaction (26). Cells were collected and lysed using RIPA buffer. The internalized LDV-FITC or FITC was determined by a fluorescence spectrophotometer (Photon Technology International) with 488 nm excitation and 518 nm emission wavelengths. Results were normalized based on protein concentrations in each well, which were measured by bicinchoninic acid (BCA) protein assay.

Cytotoxicity Study

Sulforhodamine B (SRB) colorimetric assay was used for the *in vitro* cytotoxicity study of free Dox, poly(L,D,V)-Dox, and oligo(LDV)-Dox on wild-type, integrin α4 knock-down A375 cells, and NHEK cells. Briefly, cells were seeded in 96-well plates. Upon 60% confluence, cells were treated with free Dox, poly(L,D,V)-Dox, or oligo(LDV)-Dox for 48 hours, then fixed with trichloroacetic acid. Free Dox, poly (L,D,V)-Dox, or oligo(LDV)-Dox with ~1 μM to 1 mM of Dox equivalence were used. The cellular proteins in each well were stained with 50 μl of 0.4% SRB in 1% acetic acid.

Unbound SRB was washed away by DI water; then, 200 μl of 10 mM Tris buffer solution were added to each well to dissolve the SRB bound to cellular protein. SRB absorbance was measured at 550 nm wavelength using a TriStar LB 941 Plate Reader (Berthold Technology, Oak Ridge, TN).

RESULTS

Synthesis and Characterization of LDV/LNV/VDL, LDV-FITC, Oligo(LDV)-Dox

MS-MS spectra signals were recorded as the follows: LDV (346.1, M–H^+; 223.0, y_2; 228.9, b_2; 201.0, a_2; 118.0, y_1; 86.0, L); VDL (346.4, M–H^+; 328.6, MH–H_2O; 247.1, y_2; 229.3, y_2-H_2O; 214.8, b_2; 200.6, b_2-H_2O; 187.0, a_2; 132.7, y_1; 85.4, L; 71.3, V); LNV (345.0, M–H^+; 328.0, M–NH_3; 232.0, y_2; 227.9, b_2; 211.0, b_2-NH_3; 199.9, a_2; 182.9, a_2-NH_3; 117.8, y_1; 86.1, L; 71.7, V); LDV-FITC (705.6, M–H^+); LDV-Dox (978.2, M–Na^+); $(LDV)_2$-Dox (1313.7, M–H^+); $(LDV)_3$-Dox (1708.4, M–K^+); $(LDV)_4$-Dox (2028.7, M–H^+); $(LDV)_5$-Dox (2385.6, M–H^+); $(LDV)_6$-Dox (2743.0, M–H^+). TLC showed the Rf values of free FITC and LDV-FITC were 1, 0.84; free Dox and oligo(LDV)n-Dox (n=1 ~ 6) were 1, 0.82, 0.73, 0.51, 0.49, 0.44, 0.42, respectively.

Synthesis and Characterization of Poly (L,D,V)-Dox

The molecular weight of poly (L,D,V) was found to be ~3 kD. Poly(L,D,V)-Dox formed a colloidal suspension in water with a z-average particle size of 230 nm. For the cytotoxicity assays, a colloidal suspension of poly(L,D,V)-Dox containing 387 μM of Dox was used as a stock, from which various dilutions of polymer-drug conjugate were prepared.

Integrin α4 Expression in Wild-Type/Knock-Down A375 Cells, and NHEK Cells

After A375 cells were transfected by integrin α4-specific siRNA, the mRNA level of integrin α4 in A375 cells was reduced by 48±3.2 % (Fig. 1), and integrin α4 protein level was also decreased by 52±10% (Fig. 2). No significant difference of integrin α4 mRNA and protein expression was observed after treating the cells with scramble (non-specific) siRNA (P>0.05). Integrin α4 expression in NHEK was much less when compared to wild-type A375, with only ~1% of mRNA and ~18% of protein expression.

Binding Study

The binding of LDV to A375 cells showed a significant decrease (P<0.05) when the integrin α4 expression was reduced (Fig. 3). Compared to wild-type A375 cells without any treatment, the binding of LDV to integrin α_4 knock-down A375 was decreased by 65%. The binding of LDV to A375 cells was significantly higher than the binding of VDL or LNV to A375 cells (p<0.05). However, the binding of VDL or LNV to A375 cells had no significant difference (p>0.05) compared to the blank beads, and knock-down of integrin α4 in A375 cells did not significantly change their binding (p>0.05). Competitive binding of free LDV (~2 μg/mL) reduced the binding of LDV-anchored magnetic beads to A375 cells by ~32% (p<0.05).

Internalization Study

The internalization of LDV-FITC reached plateau after 3 h of incubation. As shown in Fig. 4, about 30% lower internalization of LDV-FITC was observed on the integrin α4 on knock-down A375 when compared to the wild-type A375 at 3 h of incubation. Free FITC had higher internalization (~2 folds) when compared to LDV-FITC on both cell lines; however, no significant difference (P>0.05) of FITC internalization was observed before and after the knock-down of integrin α4.

Cytotoxicity Study

The cytotoxicity profiles of free Dox, poly(L,D,V)-Dox, and oligo(LDV)-Dox on wild-type A375, integrin α4 knock-down A375, and NHEK are shown in Figs. 5, 6 and 7, respectively. Free poly(L,D,V) or oligo(LDV) did not show significant cytotoxic effects on the cell lines up to ~1 mM (data not shown).

Free Dox had similar cytotoxicity effects on wild-type (IC_{50} 0.11 μM) and integrin α4 knock-down (IC_{50} 0.09 μM) A375 cells, and higher cytotoxicity on NHEK (IC_{50} 0.02 μM). Free Dox also had similar cytotoxic effects on wild-type A375

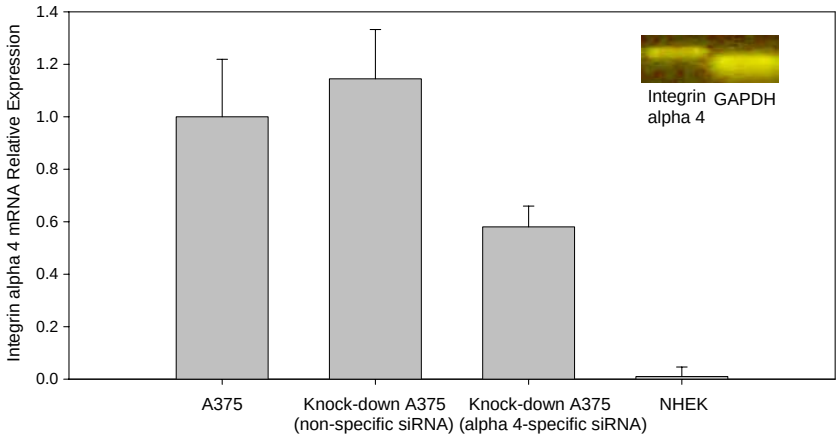


Fig. 1. Integrin α4 mRNA expression by real-time PCR. (n=3).

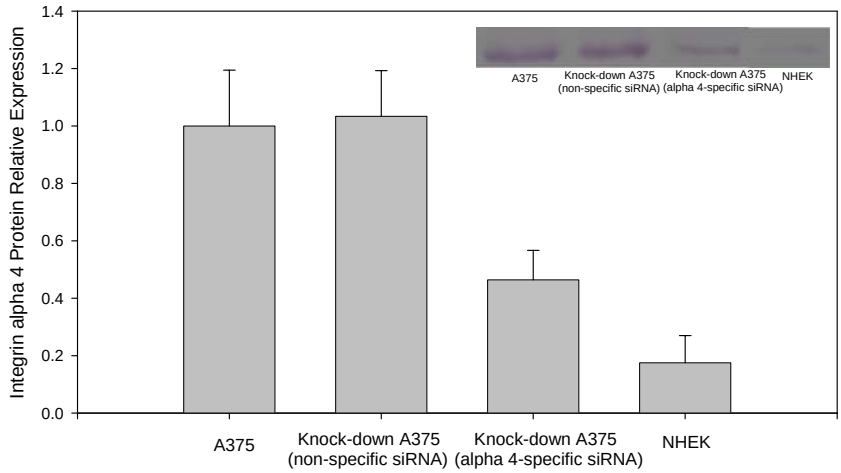


Fig. 2. Integrin α4 protein expression by western blot. (n=3).

cells as poly(L,D,V)-Dox (IC_{50} 0.09 μM), followed by oligo (LDV)-Dox (IC_{50} 0.32~1.3 μM). While on NHEK, Dox was much more toxic than poly(L,D,V)-Dox (IC_{50} 5.31 μM) and oligo(LDV)-Dox (IC_{50} 3.24~6.08 μM). Free Dox was found more toxic than the conjugates on integrin α4 knock-down A375 as well. A significant decrease (3~8 folds) in the cytotoxicity of poly(L,D,V)-Dox and oligo(LDV)-Dox on A375 cells was observed when the integrin α4 expression was knocked down. Cytotoxicity of poly(L,D,V)-Dox and oligo (LDV)-Dox on NHEK was the lowest among the tested cell lines, which has the lowest integrin α4 expression among three cell lines. For example, compared to free Dox, LDV-Dox was about 3 times less toxic on A375 cells (IC_{50} 0.355 μM), but was about 23 times less toxic on integrin α_4 knock-down A375 cells (IC_{50} 2.13 μM), and about 162 times less toxic on NHEK (IC_{50} 3.24 μM). Altering the number of repeating LDV units in oligo(LDV)-Dox did not show any trend on their cytotoxic effects on all the cell lines.

Compared to free Dox, poly(L,D,V)-Dox had similar toxicity on wild-type A375 cells (IC_{50} 0.093 μM), but was about 28.9 times less toxic on integrin α4 knock-down A375 cells (IC_{50} 2.66 μM), and 266 times less toxic on NHEK cells (IC_{50} 5.31 μM). Compared to oligo(LDV)-Dox, poly(L,D,V)-Dox had higher toxicity on wild-type A375 cells, similar toxicity on integrin α_4 knock-down A375 cells, and generally less toxicity on NHEK cells, as depicted by the IC_{50} values (Table 1).

DISCUSSION

LDV was found to be recognized by integrin $\alpha_4\beta_1$ (also called very late antigen-4 (VLA-4) or CD49d/CD29 (19, 20)), which is involved in the adhesion of lymphocytes, dendritic cells, and stem cells to extracellular matrix and endothelial cells (21), as well as in the migration of white blood cells to sites of inflammation. (22) Integrin $\alpha_4\beta_1$ receptors have been observed to over-express in melanoma and leukemia cells (23), also found in the pathology of a variety of diseases including asthma, multiple sclerosis, rheumatoid arthritis, and virus infection (27–29). Natalizumab (Tysabri), a humanized recombinant monoclonal antibody (MAb) that binds to integrin α_4, was the first adhesion molecule antagonist

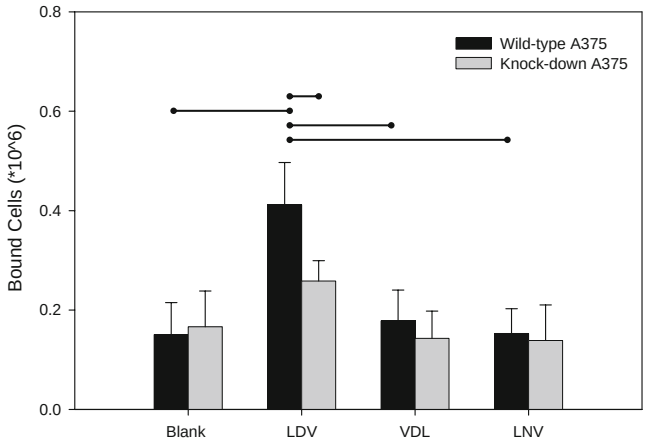


Fig. 3. Cell-ligand binding study on wild-type and integrin α_4 knock-down A375 Cells. In x-axis, blank = beads without any ligand; LDV/VDL/LNV = beads immobilized with LDV/VDL/LNV. Y-axis represents the cells bound to the ligand-immobilized magnetic beads. The *bars* represent means (n=3) and *error bars* represent standard deviation. ANOVA and *t*-test: ●—● significantly different (P<0.05).

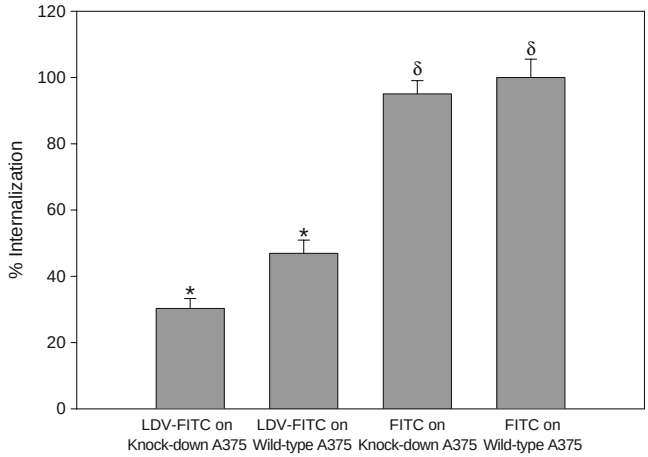


Fig. 4. Percent internalization of FITC labeled LDV on wild-type and integrin α_4 knock-down A375 cells. The *bars* represent means (n=3) and *error bars* represent standard deviation. *T*-test: * = significantly different (P<0.05), δ=no significant difference (P>0.05).

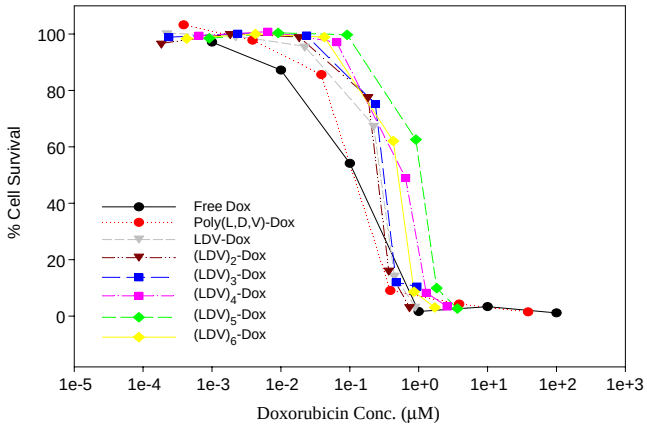


Fig. 5. Cytotoxicity profiles of Dox, poly(L,D,V)-Dox, and oligo (LDV)-Dox on A375 cells.

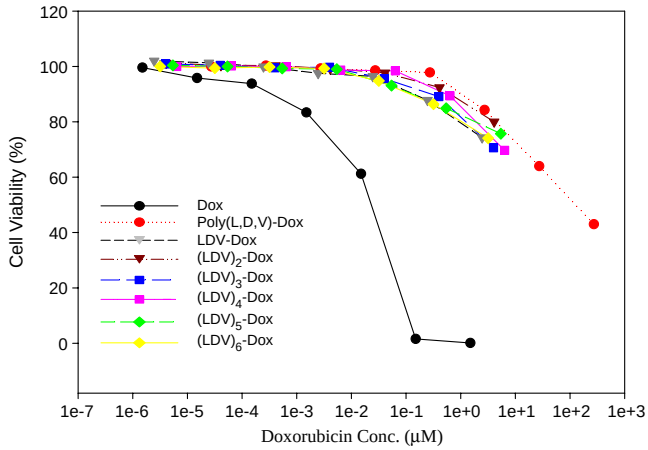


Fig. 7. Cytotoxicity profiles of Dox, poly(L,D,V)-Dox, and oligo (LDV)-Dox on NHEK cells.

proceeded to clinical trial for patients with multiple sclerosis and other inflammatory disorders (30).

Small molecule integrin $\alpha_4\beta_1$ inhibitors derived from LDV have been investigated as anti-inflammatory agents (31). For example, cyclo(ILDV-NH(CH2)5CO) showed 5-fold more potent than CS-1 peptide inhibiting the adhesion of human T-lymphoblastic leukemia cells on fibronectin *in vitro* (32). Dutta *et al.* synthesized a series of small cyclic LDV mimetics using SPPS method, including monomeric, dimeric, hexa-, hepta- and octa-peptides such as cyclo(MeIle/MePhe-LDV-X) and evaluated by *in vitro* cell adhesion assays and *in vivo* inflammation models (33, 34). LDV-derived small peptides were also used to inhibit tumor metastasis *in vivo*. Kaneda *et al.* studied the anti-metastatic potency of Glu-Ile-Leu-Asp-Val (EILDV), which showed an inhibitory effect on the metastasis induced by B16-BL6 melanoma cells (35). LDV-integrin $\alpha_4\beta_1$ interaction was also used to develop a blood filtration membrane for the retention of leukocyte during blood filtration (36). However, very few studies have been reported on the use of LDV as a targeting ligand to integrin $\alpha_4\beta_1$ in the targeted drug delivery field.

The hypothesis of this study is that LDV polymers and oligomers can be used as targeting ligand as well as drug carrier to deliver low molecular weight anti-cancer agents (e.g. doxorubicin) to tumor sites that over-express integrin $\alpha_4\beta_1$. Conjugation of a low molecular weight anti-cancer drug to a polymeric carrier could bring a drastic change in the *in vivo* behavior of the drug by one or more of the following mechanisms (37): 1) increase drug solubility; 2) prolong blood circulation time; 3) passively target through accumulation in tumor by enhanced permeation and retention (EPR) effect; 4) actively target through directing drug to specific site by targeting ligand; 5) change cellular uptake pathway from passive diffusion to endocytosis; 6) control drug release by introducing pH- or enzyme-sensitive spacers. In order to form the basis for using LDV as targeted delivery carrier via one or more of the above mentioned mechanisms, the verification of the binding specificity, binding response to the level of integrin $\alpha_4\beta_1$ expression, and the differential cytotoxicity between cancer cells and normal cells is essential.

In this study, the expression of integrin $\alpha_4\beta_1$ in different cell lines was determined by western blot and PCR. Binding specificity studies were carried out to verify that the interaction between LDV and these cell lines was integrin α_4-dependent. Various types of methods have been employed in the literature to study the ligand-receptor binding behavior, including radio-labeled ligand method (38), cell adhesion on the immobilized ligands on plate surface (39), as well as other ligand-receptor research (40–42). Coating of ligands depends on factors such as ligand concentration, coating buffer, temperature and duration of coating. Dissolution of water-soluble ligands in direct coating method often results in stability or reproducibility issue. Covalently linking the

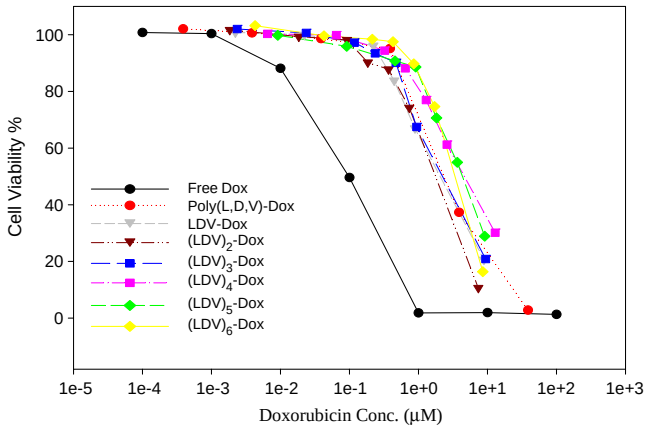


Fig. 6. Cytotoxicity profiles of Dox, poly(L,D,V)-Dox, and oligo (LDV)-Dox on integrin α_4 knock-down A375 cells.

Table 1. IC_{50} Values of Dox, Poly(L,D,V)-Dox, and Oligo(LDV)-Dox on Wild-Type and Integrin α_4 Knock-Down Cells

IC_{50} estimated (mM)	A375	Knock-down A375	NHEK
Free Dox	0.11±0.03	0.09±0.01	0.02±0.01
Poly(L,D,V)-Dox	0.09±0.01	2.66±0.35	5.31±0.32
LDV-Dox	0.36±0.01	2.13±0.20	3.24±0.69
$(LDV)_2$-Dox	0.32±0.01	1.55±0.25	3.8±0.50
$(LDV)_3$-Dox	0.38±0.01	2.38±0.24	3.91±0.32
$(LDV)_4$-Dox	0.71±0.06	4.72±0.65	5.43±1.10
$(LDV)_5$-Dox	1.3±0.21	4.16±0.52	6.08±0.79
$(LDV)_6$-Dox	0.63±0.02	3.06±0.54	5.72±0.31

ligands to a solid surface is one of alternative approaches to overcome the dissolving problem. In this study, this is achieved by covalently linking ligand on magnetic beads as presented in the cell-ligand binding study. The process to synthesize peptides on magnetic beads is similar to that on the Wang resin except the solid phase resin is embedded with Fe_3O_4. Instead of cleaving the ligands off the beads after completing the peptide synthesis, the ligand-conjugated beads were incubated with the cells for a given time period, and then magnetic field was applied to separate the bound and unbound cells. Cells used in the study were in suspended state, which provided a 3-dimensional exposure to the ligands other than plate-attached cells with some cell surface area unavailable for binding. To avoid the endocytosis of the ligands, the beads were prepared with a diameter of ~30 μm, which is significantly larger than the size of cells (11 to 22 μm) used in the experiments. Therefore, the uptake of ligand is prevented, and the result of binding study reflects only the binding of the ligands.

The binding study using different derivatives of LDV was designed to confirm the structure specificity of LDV as targeting ligand to integrin $\alpha_4\beta_1$. VDL, one of the derivatives with an opposite sequence to LDV, starts with valine at the N-terminal, and ends with leucine at the C-terminal. The other derivative is LNV, in which the D is substituted with N (asparagine); the only structural difference between D and N is that the carboxylic acid group in D is replaced by an amide group. LDV showed higher binding to A375 cells when compared to LNV and VDL. Knock-down of integrin α4 in A375 cells reduced the binding of LDV to the cells, but did not alter the binding behavior of LNV or VDL, confirming the requirement of aspartic acid residue and the sequence of the three amino acids in the specific cellular binding process. This experiment demonstrated that LDV is the minimum required structure for the specific binding to integrin $\alpha_4\beta_1$, and the binding depends on the amount of integrin. Because integrin α4 has been reported to be essential for LDV binding (43), the response of LDV derivatives binding to a modulated integrin α4 expression level provides fundamental information on their binding specificity. The internalization difference of FITC-labeled LDV on wild-type and knock-down A375 cells further proved the involvement of LDV and integrin $\alpha_4\beta_1$ interaction in the cellular uptake process of LDV by A375 cells. Therefore, our hypothesis is supported by the findings, and LDV can be utilized for the design of targeted drug delivery.

To access the contribution of number of LDV repeating units in a drug carrier, LDV was randomly polymerized as well as orderly constructed to make two different types of carriers for drug delivery. Cytotoxicity profiles of free Dox, poly(L,D,V)-Dox, and oligo(LDV)-Dox were obtained on wild-type A375, integrin α4 knock-down A375, and NHEK cell lines using SRB assay. In contrast to free Dox, the conjugates were able to differentiate between cancerous and normal cells, showing different levels of cytotoxic effects. It was also found that oligo(LDV) (orderly assembled LDV derivatives) did not show better binding properties when compared to poly(L,D,V) (randomly polymerized LDV derivatives). Instead, poly(L,D,V) has a better ability than oligo(LDV) and monomer LDV for their selectivity of cancer cells over normal cells, as shown by IC_{50} values on the basis of Dox equivalence. For oligo(LDV), ligand-receptor interaction might be hindered by their ordered structures, thus the molecular flexibility and mobility could be hampered and consequently lead to a decrease in the affinity or binding between the peptide and the specific receptors. The random poly(L,D,V) showed better targeting effect, which could be attributed to its polymer nature that facilitates the endocytosis process of the polymer-drug conjugate in the cellular uptake. Different hydrophobicity of the conjugates and different linkages between drug and carrier may also contribute to the observed differences. While a succinic acid linker was used in the preparation of oligo(LDV)-Dox, doxorubicin was conjugated directly to the beta-carboxylic group of aspartic acid residues in poly(L,D,V)-Dox. Both linkages are considered relatively stable linkage. Most macromolecule-Dox conjugates are generally less cytotoxic than free Dox. However, since poly(L,D,V)-Dox contains targeting ligands in the macromolecule backbone, it would enhance the cellular uptake of this conjugate. This enhancement may result in a similar cytotoxicity profile as free Dox. The higher cytotoxicity may be also caused by the poly(L,D,V) sensitization of the cells. The synthesis of an ordered poly(LDV) was not pursued, due to limited water solubility with the increase of LDV unit and the cytotoxicity results from oligo(LDV). When comparing poly(RGD), oligo(RGD), and RGD monomers in terms of their inhibitory effects through the specific interaction with integrins, poly(RGD) was able to competitively inhibit the binding of radio-labeled fibronectin to the melanoma cell surface (44), or inhibit experimental and spontaneous lung metastasis of BL6 cells (45) more potently than oligo(RGD) or RGD alone on a molar basis. It was reported that the inhibitory effect of poly(RGD) decreased with a decrease in repeating RGD unit (46). However, in this study, oligo(LDV) targeting property or differential capability did not increase with the increase of LDV unit (up to 6 units) based on cytotoxicity study. This can be explained by comparing the conformational difference between LDV and RGD derivatives. It has been reported that secondary structure of poly(RGD) was a beta-turn based on the CD spectra data and its amino acid sequence, which suggested that poly(RGD)-mediated inhibition of cell adhesion was due to its potent binding capacity to fibronectin receptors on cell surface probably through its conformational properties (44). Studies on other RG-containing polymers, such as $(GRGDS)_4$ and $GRGDS(GRGES)_2GRGDS$, also showed the importance of secondary structure of the polymers in their targeting capability, which means the targeting capability of RGD derivatives was not determined solely by the number of RGD units but also their secondary structure (47). Unlike RGD, LDV derivatives were unable to form a beta turn because of the different structures and properties of the three composite amino acids. Therefore, increasing the LDV unit did not bring in conformational change to improve the ligand-receptor interaction; thus, no significant change was observed in terms of their differential capability towards cell lines with different levels of integrin $\alpha_4\beta_1$ expression.

CONCLUSION

In this study, LDV was identified as an essential peptide sequence for targeting integrin $\alpha_4\beta_1$ expression cells; the

binding of LDV with different cell lines was integrin α_4-dependent; and the internalization of LDV decreased when integrin α_4 expression was reduced. Based on the findings, LDV derivatives such as poly(L,D,V) and oligo(LDV) were designed and evaluated as anti-cancer drug carriers for cancer cells over-expressing integrin $\alpha_4\beta_1$. *In vitro* cytotoxicity results revealed that unlike free Dox, poly(L,D,V)-Dox and oligo (LDV)-Dox were able to differentiate cancerous and normal cells. Among the tested targeted delivery systems, poly(L,D,V)-Dox showed better targeting capability than oligo(LDV)-Dox to A375. The results further support the targeted drug delivery systems built upon the concept of peptide-integrin interaction and encourage the future exploration of other integrin-specific ligands as targeting ligand or drug carrier.

ACKNOWLEDGEMENTS

Authors would like to thank Dr. Jianhua Ren and Xiaoning Zhao for providing us with magnetic beads, and Dr. Der Thor for his help on the siRNA knock-down technique. This study is supported by NIH 1R15CA100182-01A2.

REFERENCES

1. Rihova B. Receptor-mediated targeted drug or toxin delivery. Adv Drug Deliv Rev. 1998;29:273–89.
2. Hudson PJ, Souriau C. Engineered antibodies. Nat Med. 2003;9:129–34.
3. Leamon CP, Reddy JA. Folate-targeted chemotherapy. Adv Drug Deliv Rev. 2004;56:1127–41.
4. Chung NS, Wasan KM. Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. Adv Drug Deliv Rev. 2004;56:1315–34.
5. Fischer D, Kissel T. Histochemical characterization of primary capillary endothelial cells from porcine brains using monoclonal antibodies and fluorescein isothiocyanate-labelled lectins: implications for drug delivery. Eur J Pharm Biopharm. 2001;52:1–11.
6. Qian ZM, Li H, Sun H, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev. 2002;54:561–87.
7. Arnaout MA. Integrin structure: new twists and turns in dynamic cell adhesion. Immunol Rev. 2002;186:125–40.
8. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med. 1999;222:124–38.
9. Makarem R, Humphries MJ. LDV: a novel cell adhesion motif recognized by the integrin alpha 4 beta 1. Biochem Soc Trans. 1991;19:380S.
10. Smith JW, Cheresh DA. Integrin (alpha v beta 3)-ligand interaction. Identification of a heterodimeric RGD binding site on the vitronectin receptor. J Biol Chem. 1990;265:2168–72.
11. Pedchenko V, Zent R, Hudson BG. Alpha(v)beta3 and alpha(v) beta5 integrins bind both the proximal RGD site and non-RGD motifs within noncollagenous (NC1) domain of the alpha3 chain of type IV collagen: implication for the mechanism of endothelia cell adhesion. J Biol Chem. 2004;279:2772–80.
12. Suehiro K, Mizuguchi J, Nishiyama K, Iwanaga S, Farrell DH, Ohtaki S. Fibrinogen binds to integrin alpha(5)beta(1) via the carboxyl-terminal RGD site of the Aalpha-chain. J Biochem. 2000;128:705–10.
13. MedImmune initiates phase I/II clinical study with Vitaxin. Expert Rev Anticancer Ther. 2001;1:166.
14. Bibby DC, Talmadge JE, Dalal MK, Kurz SG, Chytil KM, Barry SE, *et al.* Pharmacokinetics and biodistribution of RGD-targeted doxorubicin-loaded nanoparticles in tumor-bearing mice. Int J Pharm. 2005;293:281–90.
15. Maeda M, Izuno Y, Kawasaki K, Kaneda Y, Mu Y, Tsutsumi Y, *et al.* Amino acids and peptides. XXX. Preparation of Arg-Gly-Asp (RGD) hybrids with poly(ethylene glycol) analogs and their antimetastatic effect. Chem Pharm Bull (Tokyo). 1997;45:1788–92.
16. Chen R, Curran S, Curran JM, Hunt JA. The use of poly(l-lactide) and RGD modified microspheres as cell carriers in a flow intermittency bioreactor for tissue engineering cartilage. Biomaterials. 2006;27:4453–60.
17. Koizumi N, Mizuguchi H, Hosono T, Ishii-Watabe A, Uchida E, Utoguchi N, *et al.* Efficient gene transfer by fiber-mutant adenoviral vectors containing RGD peptide. Biochim Biophys Acta. 2001;1568:13–20.
18. Niu JX, Zhang WJ, Ye LY, Wu LQ, Zhu GJ, Yang ZH, *et al.* The role of adhesion molecules, alpha v beta 3, alpha v beta 5 and their ligands in the tumor cell and endothelial cell adhesion. Eur J Cancer Prev. 2007;16:517–27.
19. Wayner EA, Kovach NL. Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin. J Cell Biol. 1992;116:489–97.
20. Guan JL, Hynes RO. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1. Cell. 1990;60:53–61.
21. Chigaev A, Zwartz GJ, Buranda T, Edwards BS, Prossnitz ER, Sklar LA. Conformational regulation of alpha 4 beta 1-integrin affinity by reducing agents. :"Inside-out" signaling is independent of and additive to reduction-regulated integrin activation. J Biol Chem. 2004;279:32435–43.
22. Singh J, Adams S, Carter MB, Cuervo H, Lee WC, Lobb RR, et al. Rational design of potent and selective VLA-4 inhibitors and their utility in the treatment of asthma. Curr Top Med Chem. 2004;4:1497–507.
23. Garcia-Gila M, Cabanas C, Garcia-Pardo A. Analysis of the activation state of alpha4beta1 integrin in human B cell lines derived from myeloma, leukemia or lymphoma. FEBS Lett. 1997;418:337–40.
24. Zhong S, Li Y, Chan W, Jasti B, Li X. Integrin a4ß1-dependent Binding of Leucine-Aspartate-Valine (LDV) to A375 Cells: a Rationale for Targeting Drug Delivery. Vienna: Cotrolled Release Society; 2006.
25. Liu M, Xie C, Pan H, Pan J, Lu W. Separation of polyethylene glycols and their fluorescein-labeled compounds depending on the hydrophobic interaction by high-performance liquid chromatography. J Chromatogr A. 2006;1129:61–6.
26. Hyduk SJ, Chan JR, Duffy ST, Chen M, Peterson MD, Waddell TK, *et al.* Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. Blood. 2007;109:176–84.
27. Luissint AC, Lutz PG, Calderwood DA, Couraud PO, Bourdoulous S. JAM-L-mediated leukocyte adhesion to endothelial cells is regulated in cis by alpha4beta1 integrin activation. J Cell Biol. 2008;183:1159–73.
28. Andersson EC, Christensen JP, Marker O, Thomsen AR. Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. J Immunol. 1994;152:1237–45.
29. Singh J, Van Vlijmen H, Liao Y, Lee WC, Cornebise M, Harris M, *et al.* Identification of potent and novel alpha4beta1 antagonists using in silico screening. J Med Chem. 2002;45:2988–93.
30. Stuve O, Bennett JL. Pharmacological properties, toxicology and scientific rationale for the use of natalizumab (Tysabri) in inflammatory diseases. CNS Drug Rev. 2007;13:79–95.
31. Jackson DY, Quan C, Artis DR, Rawson T, Blackburn B, Struble M, *et al.* Potent alpha 4 beta 1 peptide antagonists as potential anti-inflammatory agents. J Med Chem. 1997;40:3359–68.
32. Haworth D, Rees A, Alcock PJ, Wood LJ, Dutta AS, Gormley JJ, *et al.* Anti-inflammatory activity of c(ILDV-NH(CH2)5CO), a novel, selective, cyclic peptide inhibitor of VLA-4-mediated cell adhesion. Br J Pharmacol. 1999;126:1751–60.
33. Dutta AS, Gormley JJ, Coath M, Hassall L, Hayward CF, Gellert PR, *et al.* Potent cyclic peptide inhibitors of VLA-4 (alpha4beta1 integrin)-mediated cell adhesion. Discovery of compounds like cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (ZD7349) compatible with depot formulation. J Pept Sci. 2000;6:398–412.
34. Dutta AS, Crowther M, Gormley JJ, Hassall L, Hayward CF, Gellert PR, *et al.* Potent cyclic monomeric and dimeric peptide inhibitors of VLA-4 (alpha4beta1 integrin)-mediated cell adhesion based on the Ile-Leu-Asp-Val tetrapeptide. J Pept Sci. 2000;6:321–41.

35. Kaneda Y, Yamamoto Y, Okada N, Tsutsuml Y, Nakagawa S, Kakiuch M, *et al.* Antimetastatic effect of synthetic Glu-Ile-Leu-Asp-Val peptide derivatives containing D-amino acids. Anticancer Drugs. 1997;8:702–7.
36. Momtaz M, Rerat V, Gharbi S, Gerard E, Pourcelle V, Marchand-Brynaert J. A graftable LDV peptidomimetic: design, synthesis and application to a blood filtration membrane. Bioorg Med Chem Lett. 2008;18:1084–90.
37. Nori A, Kopecek J. Intracellular targeting of polymer-bound drugs for cancer chemotherapy. Adv Drug Deliv Rev. 2005;57:609–36.
38. Nowak G. Methods and data analyses in radioligand-receptor binding: unnecessary transformations may cause biased results. Pol J Pharmacol. 1998;50:101–5.
39. Steinitz M, Baraz L. A rapid method for estimating the binding of ligands to ELISA microwells. J Immunol Methods. 2000;238:143–50.
40. Dardik BN, Schwartzkopf CD, Stevens DE, Chatelain RE. A quantitative assay for the non-covalent association between apolipoprotein[a] and apolipoprotein B: an alternative measure of Lp[a] assembly. J Lipid Res. 2000;41:1013–9.
41. Sriramarao P, Mendler M, Bourdon MA. Endothelial cell attachment and spreading on human tenascin is mediated by alpha 2 beta 1 and alpha v beta 3 integrins. J Cell Sci. 1993;105 (Pt 4):1001–12.
42. Schraa AJ, Kok RJ, Berendsen AD, Moorlag HE, Bos EJ, Meijer DK, *et al.* Endothelial cells internalize and degrade RGD-modified proteins developed for tumor vasculature targeting. J Control Release. 2002;83:241–51.
43. Mould AP, Komoriya A, Yamada KM, Humphries MJ. The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin alpha 4 beta 1. Inhibition of alpha 4 beta 1 function by RGD peptide homologues. J Biol Chem. 1991;266:3579–85.
44. Murata J, Saiki I, Ogawa R, Nishi N, Tokura S, Azuma I. Molecular properties of poly(RGD) and its binding capacities to metastatic melanoma cells. Int J Pept Protein Res. 1991;38:212–7.
45. Saiki I, Murata J, Matsuno K, Ogawa R, Nishi N, Tokura S, *et al.* Anti-metastatic and anti-invasive effects of polymeric Arg-Gly-Asp (RGD) peptide, poly(RGD), and its analogues. Jpn J Cancer Res. 1990;81:660–7.
46. Saiki I, Iida J, Murata J, Ogawa R, Nishi N, Sugimura K, *et al.* Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. Cancer Res. 1989;49:3815–22.
47. Tressler RJ, Belloni PN, Nicolson GL. Correlation of inhibition of adhesion of large cell lymphoma and hepatic sinusoidal endothelial cells by RGD-containing peptide polymers with metastatic potential: role of integrin-dependent and -independent adhesion mechanisms. Cancer Commun. 1989;1:55–63.