Published in final edited form as:

J Control Release. 2017 October 10; 263: 57–67. doi:10.1016/j.jconrel.2017.03.030.

A quantitative method for screening and identifying molecular targets for nanomedicine

Peng Guo^{1,2,3}, Jiang Yang^{2,3}, Diane R. Bielenberg^{2,3}, Deborah Dillon⁴, David Zurakowski⁵, Marsha A. Moses^{2,3,†}, and Debra T. Auguste^{1,†,‡,*}

¹Department of Biomedical Engineering, The City College of New York, 160 Convent Avenue, New York, NY 10031, United States

²Vascular Biology Program, Boston Children's Hospital, 1 Blackfan Circle, Boston, MA, 02115, United States

³Department of Surgery, Harvard Medical School and Boston Children's Hospital, 300, Longwood Avenue, Boston, MA 02115, United States

⁴Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75, Francis Street, Boston, MA 02115, United States

⁵Department of Anesthesia, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA, 02115, United States

Abstract

Identifying a molecular target is essential for tumor-targeted nanomedicine. Current cancer nanomedicines commonly suffer from poor tumor specificity, “off-target” toxicity, and limited clinical efficacy. Here, we report a method to screen and identify new molecular targets for tumor-targeted nanomedicine based on a quantitative analysis. In our proof-of-principle study, we used comparative flow cytometric screening to identify ICAM-1 as a potential target for metastatic melanoma (MM). We further evaluated ICAM-1 as a MM targeting moiety by characterizing its (1) tumor specificity, (2) expression level, (3) cellular internalization, (4) therapeutic function, and (5) potential clinical impact. Quantitation of ICAM-1 protein expression on cells and validation by immunohistochemistry on human tissue specimens justified the synthesis of antibody-functionalized drug delivery vehicles, which were benchmarked against appropriate controls. We engineered ICAM-1 antibody conjugated, doxorubicin encapsulating immunoliposomes (ICAM-Dox-LPs) to selectively recognize and deliver doxorubicin to MM cells and simultaneously neutralize ICAM-1 signaling via an antibody blockade, demonstrating significant and simultaneous inhibitory effects on MM cell proliferation and migration. This paper describes a

*Corresponding author. dauguste@ccny.cuny.edu, Tel: +1-212-650-6634, Fax: +1-212-650-6727.

†These authors contributed equally to this work.

‡Current address: Department of Chemical Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, United States

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

novel, quantitative metric system that identifies and evaluates new cancer targets for tumor-targeting nanomedicine.

Graphical Abstract

Keywords

cancer target; nanomedicine; metastatic melanoma; immunoliposome; ICAM-1; drug delivery

Introduction

Tumor-targeted nanomedicines have the potential to mitigate the spatial and temporal challenges of therapeutic delivery to primary tumors and metastatic lesions via tumor recognition [1–4]. For example, MM-302 (human epidermal growth factor receptor 2 (HER2) antibody-conjugated liposomal doxorubicin), a receptor targeting immunoliposome, has recently demonstrated clinical benefits for HER2-positive metastatic breast cancer patients by significantly improving median progression free survival by 7.6 months and demonstrating an overall patient response rate of 11% [5–9]. However, other aggressive cancers, such as metastatic melanoma (MM) and pancreatic cancer, have no or limited clinically effective targets [10–14]. On these cancer cell membranes, hundreds of different proteins are upregulated or downregulated to promote cancer development and progression. Choosing an appropriate target is a critical parameter, therefore, in the development of tumor-targeted nanotherapeutics, which impacts therapeutic biodistribution, efficacy, and safety. To date, there remains a lack of quantitative studies to systematically identify, evaluate, and validate cancer targets for nanotherapeutics.

The goal of our study is to develop an unbiased method to screen and identify molecular targets that may be useful for tumor-targeting. A flowchart of the procedures for this method is shown in Figure 1. First, we applied comparative flow cytometric screening to profile the expression of cancer-related cell surface antigens on cancer cells and their non-cancerous (control) counterparts. Candidates were ranked and selected based on the level of overexpression. Second, the screened candidate was evaluated as a molecular target for tumor-targeted nanomedicine by characterizing its (1) tumor specificity, (2) expression level, (3) cellular internalization, (4) therapeutic function and (5) potential clinical impact. Third, we validated the effectiveness of the identified target by constructing tumor-targeted immunoliposomes conjugated with antibodies recognizing the target and measuring their tumor-specific affinity and half maximal inhibitory concentration (IC50).

In this study, we focused our attention on MM, the most fatal skin cancer [15–18]. The methodology presented here identifies parameters necessary for evaluating a potential

therapeutic target to disrupt cancer pathogenesis, and offers the opportunity to systematically and quantitatively, in an unbiased way, discover molecular targets for nanomedicine applications.

Experimental Section

Materials

Dulbecco's phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), 0.25% trypsin/2.6 mM ethylenediaminetetraacetic acid (EDTA) solution, Gibco® Dulbecco's Modified Eagle Medium (DMEM), Gibco®DMEM/F12(1:1) were purchased from Invitrogen (Carlsbad, CA, USA). Quantum Simply Cellular microbeads were purchased from Bangs Laboratory (Fishers, IN, USA). Mouse anti-human ICAM-1 monoclonal antibody, immunoglobulin G (IgG) isotype controls were purchased from R&D Systems (Minneapolis, MN, USA). For antibodies used in flow cytometric analysis, Phycoerythrin (PE)-conjugated mouse anti-human VEGFR1, VEGFR2, and FLOR1 antibodies were purchased from R&D Systems, and all other PE-conjugated antibodies and PE-conjugated mouse or rat IgG isotype controls were purchased from BioLegend (San Diego, CA, USA). Human melanoma tissue (ME2080b, T382a and T386) and normal tissue (BN00011 and BN1002a) arrays were purchased from US Biomax (Rockville, MD, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Slide-A-Lyzer™ Dialysis Cassette (MWCO 20 kDa), Corning Costar Transwell Permeable Supports and Lab-Tek II Chamber Slide System were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Fluorogel with tris buffer was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Quantum Simply Cellular microbeads were purchased from Bangs Laboratories (Fishers, IN, USA). RNeasy mini kit was purchased from Qiagen (Valencia, CA, USA).

Cell culture

A375SM human metastatic melanoma was obtained from Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX, USA) [19,20]. C32 human metastatic melanoma was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) [21–23]. Adult human epidermal melanocytes from lightly-pigmented skin (HEMa-LP, normal human melanocytes) were purchased from Cascade Biologics (Invitrogen). A375SM and C32 cells were cultured in DMEM, normal human melanocytes in MGM™-4 melanocyte growth medium (Lonza), with all recommended supplements, respectively. All cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Flow cytometry measurement

Cell membrane expression of molecular target candidate was evaluated by a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) as described previously [24]. Quantification of the molecular target density on the cell surface was determined with reference to Quantum Simply Cellular microbeads, using the protocol as provided by the manufacturer. Briefly, 10⁶ cells were collected and rinsed twice through suspension-spin cycles. Cells were blocked by 1% bovine serum albumin (BSA) in PBS for

30 min in an ice bath. After BSA blocking, cells were incubated with PE-conjugated antibodies for 1 h at RT. Cells were rinsed with 1% BSA in PBS three times, resuspended in PBS, and evaluated by flow cytometry.

Quantitative analysis of MM cell binding and uptake of synthesized liposomes was studied by flow cytometry analysis. 10^6 cells were placed in each well of a 6-well cell culture plate and incubated for 2–4 h at 37°C with (1) rhodamine-dextran (RD)-encapsulating, nonspecific IgG conjugated liposome (IgG-RD-LP), (2) RD-encapsulating ICAM-1 antibody conjugated liposome (ICAM-RD-LP) at a final concentration of 1 μ M lipids per 10^6 cells. All liposome-treated cells were washed with PBS, harvested using a 0.25% trypsin/2.6 mM EDTA solution, and washed with PBS three times. Binding data were acquired using a BD FACSCalibur flow cytometer and analyzed using FlowJo software. The specific cell uptake of ICAM-RD-LP with reference to non-specific IgG-RD-LPs was calculated by dividing the mean fluorescence intensity of ICAM-RD-LP stained cells by that of the IgG-RD-LP stained cells.

Immunohistochemistry

162 human melanoma tissue samples and 178 normal human tissue samples from 20 different organs were stained and evaluated for ICAM-1 expression level and tumor specificity. Immunohistochemical staining was performed on paraffin-embedded human melanoma tissue microarrays (ME2080b, T382a and T386) and normal tissue microarrays (BN00011 and BN1002a). The individual tissue cores in the microarrays were scored by an independent pathologist, with no knowledge of sample identity, for no staining (0), weak staining (1+), moderate staining (2+), or strong staining (3+). Photomicrographs were taken on an Olympus BX41 microscope by using an Olympus Q-Color5 digital camera (Olympus America Inc.).

Immunofluorescent staining

A375SM, C32 or human melanocytes (2×10^5 cells) were seeded in a Lab-Tek II Chamber Slide System with 2 mL media overnight at 37°C. After media was removed, cells were rinsed with PBS three times and fixed with 4% formaldehyde in PBS at RT for 10 min, followed by three rinses with PBS. Samples were blocked with 1% BSA in PBS for 30 min in an ice bath. After BSA blocking, samples were stained with PE-conjugated ICAM-1 antibodies or PE-conjugated control IgG for 1 h and rinsed with PBS. DAPI was used to stain the cell nuclei. Immunofluorescent stained samples were dried overnight in the dark and examined under a Leica TCS SP5 confocal fluorescent microscope (Leica Microsystems, Buffalo Grove, IL, USA). Digital images were captured with AxioVision digital image processing software.

qRT-PCR

The mRNA expression levels of ICAM-1 in MM cells were analyzed using qRT-PCR. A375SM, C32 and human melanocyte cells were cultured at 10^6 cells/well in 6-well cell culture plates overnight. Cells were then removed from each well by incubating with a trypsin/EDTA solution for 3 min. The cells were washed with PBS for three times. RNA was extracted, purified using the Qiagen RNeasy mini kit, and quantified using a

SpectraMaxPlus 384 UV-Visible Spectrophotometer (Molecular Devices Corp, Sunnyvale, CA, USA). Reverse transcription was conducted using the Applied Biosystems Taqman RT protocol. Detection and quantification of mRNA were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). All PCR samples were referenced to the gene expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Cell proliferation

Human MM cells (A375SM and C32) were pre-seeded in 96-well plates (5×10^3 cells/well). After 24-h incubation, cells were treated with 10 μ g/mL of IgG or ICAM-1 antibodies in DMEM with 10% fetal bovine serum (FBS) for 24 h. Then IgG or ICAM-1 antibody-treated cells were rinsed twice with PBS, and grown in DMEM with 10% FBS. At day 1, 3, and 5 after treatment, cell viability was determined by a Dojindo cell counting kit using the protocol from the manufacturer (Rockville, MD). To evaluate the cytotoxicity of immunoliposomes, cells were treated with (1) PBS, (2) Free Dox, (3) non-specific IgG-conjugated, Dox-encapsulating liposomes (IgG-Dox-LPs), (4) ICAM-1 antibody-conjugated, Dox-encapsulating liposomes (ICAM1-Dox-LP) at the equivalent Dox dosage of 0, 0.08, 0.4, 2, 10, 50 μ g/mL for 6 h. Cells were rinsed twice with PBS and grown for 48 h before the Dojindo assay.

Cell migration

Human MM cell migration was measured as described previously [25]. Briefly, A375SM and C32 cells were pre-treated with the following samples: IgG, ICAM-1 antibody, IgG-LP, ICAM-LP for 24 h, and then seeded onto COSTAR migration inserts with permeable support polycarbonate membrane (8 μ m pore size) in a 24-well plate at a cell density of 10^5 cells per well in DMEM without FBS. DMEM with 10% FBS was added to the lower wells. The cells were allowed to migrate for 20 h. The inserts were stained with Diff-Quik Stain Set and the cells that did not migrate through the insert membranes were removed using cotton swabs. Four fields/insert were counted for each sample.

ICAM-Dox-LP synthesis and characterization

The ICAM-1 targeted, Dox-encapsulating immunoliposome was prepared by the transmembrane gradient assay as described previously [26,27]. Briefly, a lipid formulation consisting of DOPC:DSPE-PEG-COOH (95:5, mol:mol) was used to prepare liposomes. 50 mmol lipid mixture was solubilized in chloroform and dried under a dry nitrogen stream. The resulting lipid film was dissolved in 1 mL DMSO:EtOH (7:3, v:v). The lipid solution was injected in 9 mL of 240 mM PBS while being rigorously agitated to yield a 5 mM lipid solution. After 10 freeze-thaw cycles, lipid solution was extruded via a NorthernLipids Extruder with a 100 nm polycarbonate nanoporous membrane. After extrusion, the liposome solution was dialyzed in PBS using a Slide-A-Lyzer dialysis cassette (MWCO 20 kDa) overnight at room temperature (RT). Then Dox was added to liposome solution to reach a final concentration of 200 μ g/mL, and incubated for 6 h to facilitate active loading. The resulting Dox-encapsulating liposome solution was dialyzed in PBS using a Slide-A-Lyzer dialysis cassette (MWCO 20 kDa) overnight at RT.

The surface of Dox-encapsulating liposomes was modified with the ICAM-1 antibodies via the DSPE-PEG-COOH anchor. EDC (2 mg) and NHS (3 mg) were mixed with 1 mmol of lipid (liposomes) in PBS and incubated for 6 h at RT. A Slide-A-Lyzer dialysis cassette (MWCO 10 kDa) was used to remove unreacted EDC and NHS. Next, ICAM-1 antibody or the IgG isotype was added to EDC-modified liposomes at a molar ratio of 1:1000 (antibody:phospholipid) and incubated overnight at RT. Unreacted antibodies were removed by using a FLOAT-A-LYZER G2 dialysis tubing (MWCO 300 kDa). In liposome binding experiments, non-cytotoxic rhodamine-dextran encapsulating liposomes (ICAM-RD-LPs) were prepared and tested to replace the cytotoxic ICAM-Dox-LPs. The preparation process was similar as ICAM-RD-LP with the exception being that the 1 mL lipid solution was added to a 9 mL rhodamine-dextran solution (1 mg/mL).

The density of ICAM-1 antibodies conjugated on liposomes was quantified via microbead assay as described previously [25–27]. Liposomes cannot be detected by flow cytometry because of their size, therefore, 2 μ m borosilicate beads were encapsulated within DOPC: DSPE-PEG-COOH (95:5, mol:mol) liposomes by sonicating small unilamellar liposomes with microbeads in PBS for 6 h. Microbeads were rinsed three times in PBS via suspension-spin cycles to separate free liposomes. Conjugation of PE-ICAM-1 antibody or PE-IgG (nonspecific binding) to microbead encapsulating liposomes was performed using EDC/NHS chemistry. The surface densities and ratios of ICAM-1 and EGFR antibody conjugated to each microbead were determined with reference to Quantum Simply Cellular microbeads, which have defined numbers of antibody binding sites per bead. Liposome size and zeta potential were measured by dynamic light scattering on a Zeta-PALS analyzer (Brookhaven Instruments, Holtsville, NY) in PBS.

Statistical analysis

All experimental data were obtained in triplicate unless otherwise mentioned and are presented as mean $\pm$ standard deviation. Statistical comparison by analysis of variance was performed at a significance level of $P < 0.05$ using Student's t-test.

Results and Discussion

Screening of targets of MM cell lines

In order to identify targets for MM-specific nanomedicine, we constructed a cancer target panel that consists of 44 cancer-related cell membrane proteins (Table S1). These cancer target candidates were selected by three criteria: (1) cell membrane proteins, which are more desirable targets in comparison to intracellular or secreted proteins because they can be directly accessed by the nanomedicine for tumor recognition and may aid in internalization of the nanomedicine via receptor-mediated endocytosis (2) low expression in healthy vital organs (e.g. lung, liver, and heart), and (3) involvement in cancer development and progression. This panel includes several clinically-used cancer targets (e.g. HER2, epidermal growth factor receptor (EGFR) [28,29], and vascular endothelial growth factor receptors (VEGFRs)) and recently identified MM targets (e.g. CD44 [30,31] and Integrin α v β 3 (ITGA5B3) [32,33]). We used comparative flow cytometric analysis to profile the surface expression levels of these cell membrane proteins in two human MM cell lines (A375SM

and C32) [19–23] and normal human melanocytes (control). We chose to use comparative flow cytometry to screen potential targets for MM-specific nanomedicine over other conventional screening assays (e.g., PCR array and mass spectrometry) because this method is unique in utilizing antibody-antigen interactions to precisely quantify the surface expression of cell membrane proteins on live cancer cells. This approach closely recapitulates the interaction between tumor and antibody-directed nanomedicine *in vivo*. Such information cannot be obtained by other screening methods. Obtained MM target candidates were ranked based on their average overexpression levels on MM cells relative to normal human melanocytes (Figure 2a). The top 10 most overexpressed candidates on A375SM and C32 cells are listed in Figures 2b and c, respectively. ICAM-1, TFRC, ITGAVB3, and ITGA6 emerged as four highly overexpressed cell surface targets on both A375SM and C32 cells. We also compared the expression levels of these two sets of top 10 candidates on normal human melanocytes (Figure 2d). ICAM-1, TFRC, and ITGA6 demonstrated lower cell surface densities in normal melanocytes, in comparison with ITGAVB3 and other candidates, suggesting that ICAM-1, TFRC and ITGA6 are three potential MM targets. We focused on ICAM-1 because it has the lowest expression in normal human tissues (13 of 45 types of organs) compared with TFRC (39 of 45) and ITGA6 (33 of 45) according to Human Protein Atlas database (www.proteinatlas.org). ICAM-1 is a cell membrane glycoprotein involved in immunological cascades with important roles in regulating cell adhesion [24,34,35], and previous work in human tissues also suggested that ICAM-1 may play an important role in melanoma metastasis development [36–40].

Evaluation of ICAM-1 for MM-specific nanomedicine

(1) Tumor specificity—The primary role of a targeting moiety is to discriminate between the tumor and the healthy tissues. We evaluated the tumor specificity of ICAM-1 by quantitatively analyzing its expression level in 162 cases of human melanoma (including 67 cases of MM (Stage IV) and 95 cases of non-metastatic melanoma (non-MM, Stages I–III)) and 178 cases of normal human tissues (covering 20 types of organs) via immunohistochemical (IHC) staining. As shown in Figure 3a, ICAM-1 was significantly upregulated in melanoma tissues as compared with most normal tissues. ICAM-1 was also present in 6 of 20 normal human organs: bone, lung, lymph node, spleen, testis, and thymus. Though this is not optimal, our findings correlate with the Human Protein Atlas database, which reported that ICAM-1 expression in normal organ tissues is substantially less than those of CD44 and ITGAVB3, two widely accepted melanoma targets [41,42]. Overexpression of ICAM-1 in 162 human melanoma tissues provides clinically-relevant evidence supporting ICAM-1 as a promising molecular target for melanoma.

We also compared the tumor specificity of ICAM-1 protein expression (IHC scores) with ICAM-1 gene expression (Figure 3b) in an independent validation cohort of MM patient tumors (n=44) and various normal organs (n=486) via R2: Genomics Analysis and Visualization Platform database (r2.amc.nl/). High ICAM-1 expression was found in normal lung tissues at both protein and gene levels, which may be a potential off-target site for ICAM-1 antibody-guided nanomedicine. Our previous animal studies demonstrated that ICAM-1 antibody-guided iron oxide nanoparticles did not target normal lung during

circulation[24]. In addition, we also evaluated the potential interaction between ICAM-1 antibody-guided nanomedicine and the immune system by using the same database. In Figure 3c, we analyzed the ICAM-1 gene expression levels on 13 different types of immune cells including both innate and adaptive leukocytes. Among these cell types, only monocytes demonstrated a comparative ICAM-1 gene expression to MM tumors (approximately 71%). Due to the low number of monocytes in the blood circulation (usually comprising 2–8% of leukocytes), it may not have a strong interference on the MM tumor targeting activity of ICAM-1 antibody-guided nanomedicine. We also found that hematopoietic stem cells from the bone marrow, which give rise to all leukocytes, have a significantly lower gene expression of ICAM-1 compared with MM tumors.

(2) Expression level—Overexpression of the target molecule is indicative of preferential nanomedicine binding to the desired cell type and therefore increased effectiveness of tumor-specific drug delivery [25,26,43–47]. The overexpression of ICAM-1 was quantified in 162 cases of human melanoma tumor tissues and two human MM cell lines (A375SM and C32). ICAM-1 in human melanoma tissues was stained and scored by an independent pathologist. As shown in Figure 4a, strong ICAM-1 staining was observed in both non-metastatic and metastatic melanoma tissues, but was absent in normal skin tissues. Enlarged images in Figure 4a further revealed that ICAM-1 predominantly localized on melanoma cell membranes, which is accessible for nanomedicine binding. Quantified IHC scores in Figure 4b show that ICAM-1 was significantly overexpressed in 95 cases of human non-metastatic and 67 cases of metastatic melanoma relative to normal skin tissues. There is no significant difference in ICAM-1 levels between non-metastatic and metastatic melanoma tissues, suggesting that ICAM-1 may play important oncogenic roles at both early and late stages of this disease.

We measured ICAM-1 expression levels in two human MM cell lines (A375SM and C32) as well as normal human melanocytes via qRT-PCR (Figure 4c). mRNA levels of ICAM-1 in A375SM and C32 cells were 6 and 11-fold higher than that of normal human melanocytes, respectively. ICAM-1 surface density on A375SM and C32 was measured by flow cytometric analysis as $697,000 \pm 8,250$ and $1,059,000 \pm 4,600$ molecules/cell, respectively (Figure 4d). In contrast, normal melanocytes only expressed $58,000 \pm 1,500$ molecules/cells, more than 12-fold less than either of the MM cell lines. We validated the overexpression and surface localization of ICAM-1 on these two MM cells by immunofluorescent staining where A375SM, C32 and normal human melanocytes were incubated with fluorophore-conjugated ICAM-1 antibodies (Figure 4e). Strong ICAM-1 staining was observed in A375SM and C32 cells but not in normal human melanocytes, indicating the selective binding of the ICAM-1 antibody to MM cells. The combined data from human melanoma tissues and cell lines consistently support that our identified target, ICAM-1, is abundantly expressed in MM at both protein and mRNA levels and may serve as a highly specific target for MM.

(3) Internalization—Many therapeutic agents (e.g. proteins or nucleic acid drugs) are unable to spontaneously enter cancer cells [48,49]. The capability of a nanomedicine to deliver its cargo across a cell membrane and access intracellular targets has a direct impact on its efficacy. Receptor-mediated internalization of ICAM-1 antibodies was detected in

both A375SM and C32 cells via two independent measurements: immunofluorescent staining and flow cytometry. Confocal fluorescent images (Figures 5a and b) show that at the 10 min incubation time, fluorophore-conjugated ICAM-1 antibodies were mainly localized at the MM cell surface. At 120 min, ICAM-1 antibodies were endocytosed and distributed within endosomes in the perinuclear region. Flow cytometry data (Figures 5c and d) also confirmed the increased internalization of ICAM-1 antibodies over time in both A375SM and C32 cells. Internalization of the ICAM-1 antibody in MM cells suggests it may mediate intracellular drug delivery. Because cell endocytosis is an ATP-driven process that is dramatically affected by temperature [50,51], we incubated MM cells at 4°C for 120 min after 10 min ICAM-1 antibody staining and found that the internalization process was significantly inhibited at 4°C (Figures 5c and d). This confirmed cell endocytosis of ICAM-1 antibodies.

(4) Therapeutic function—ICAM-1 has previously been found to have an important role in promoting melanoma progression and metastasis [36–40], we reasoned that ICAM-1 may not only serve as a target for MM recognition, but may also hinder disease progression. Using neutralizing antibodies to block ICAM-1 may therefore abrogate adhesion and subsequently a pro-metastatic phenotype. Similar effects were observed in our previous studies [24,25]. For example, we have demonstrated that immunoliposomes modified with the CXCR4 neutralizing antibodies not only guided immunoliposomes to metastatic breast cancer cells but also impeded cancer cell migration via blockade of CXCR4-mediated signaling pathways [25]. Here, we examined the therapeutic function of ICAM-1 in two predominant malignant behaviors of MM: proliferation and migration. First, in MM cell proliferation studies (Figures 6a and b), treatment with the ICAM-1 antibody (10 µg/mL) did not alter A375SM and C32 cell proliferation. However, as shown in Figures 6c and d, the ICAM-1 antibody (10 µg/mL) did exhibit potent activity in inhibiting both A375SM and C32 cell migration via blocking ICAM-1. The number of migrated A375SM and C32 cells was significantly reduced by 74% and 85%, respectively, in comparison to cells treated with non-specific IgG. These findings demonstrate that an ICAM-1 antagonist such as a neutralizing antibody may be useful in blocking metastasis of MM.

(5) Potential clinical impact—Due to the complexity and heterogeneity of tumors, it is essential to evaluate a broad array of patient specimens for our identified target. Tissue specimens were obtained from 162 melanoma patients and 25 normal controls for IHC staining. Demographic details of the patients included in the study are shown in Table 1. The melanoma patient group consisted of 88 men and 74 women with a median age of 54.5 years (ranging from 7 to 88 years). Melanoma patients were divided into two groups: non-MM patient group and MM patient group, with a similar gender profile and median age. IHC was scored as follows: 0 for no staining; 1+ for partial membrane immunoreactivity in >10% of cells; 2+ for weak to moderate complete membrane immunoreactivity in >10% of cells; and 3+ for strong complete membrane immunoreactivity in >10% of cells. Positive ICAM-1 staining was defined as an equivalent IHC score of 2+; which was observed in 27 of 95 (28%) non-MM patients and 24 of 67 (36%) MM patients. Conversely, 0 of 25 (0%) normal skin tissue controls evaluated showed positive ICAM-1 staining. Overall, positive ICAM-1 staining was detected in 28% non-MM patient samples and 36% MM patient samples,

whereas EGFR, another established target in the field, was only detected in approximately 11.4% of patient's tumors [28]. The melanoma patient population which presented positive ICAM-1 staining (32% in total) may potentially benefit from ICAM-1 targeted therapy. Importantly, soluble ICAM-1 protein levels were significantly elevated in the serum of melanoma patients compared to those of healthy controls and correlated with the stage of disease [52,53]. We therefore hypothesize that ICAM-1 may be used as a diagnostic biomarker to identify patients that may benefit from ICAM-1 targeted therapy.

We next assessed the impact of ICAM-1 expression on MM patient survival in R2: Genomics Analysis and Visualization Platform metastatic melanoma datasheet (n=44). Tumor ICAM-1 expression levels showed an inverse correlation with MM patient overall survival (OS, Figure 7). High tumor ICAM-1 remarkably correlated with poor OS, suggesting that ICAM-1 is closely associated with the clinical outcome of MM.

Validation of ICAM-1 as both a delivery and therapeutic target for MM using engineered immunoliposomes

We engineered an ICAM-1-targeted, doxorubicin (Dox) encapsulating immunoliposome (ICAM-Dox-LP) by covalently conjugating ICAM-1 antibodies to the surface of liposomal doxorubicin (scheme shown in Figure 8a). Non-targeting liposomal doxorubicin (Doxil®) has previously failed in Phase II clinical trials for MM therapy due to its insufficient efficacy [54]. To be clinically successful, our ICAM-Dox-LPs were designed to improve efficacy by enabling them to selectively recognize and target MM cells (and spare normal cells) via the ICAM-1 antigen-antibody interaction between ICAM-Dox-LP and MM cells. Dox was loaded within liposomes using the transmembrane gradient method [26], leading to an encapsulation efficiency of greater than 90%. Non-specific IgG conjugated, Dox encapsulating liposomes (IgG-Dox-LP) were also prepared as non-targeting controls. As-synthesized ICAM-Dox-LP and IgG-Dox-LP exhibited similar hydrodynamic diameters of approximately 100 nm with a polydispersity index of approximately 0.1, indicating uniformity (Table 2). Liposomes with diameters less than 200 nm can avoid capture by the reticuloendothelial system (RES), extending their *in vivo* circulation time [55]. Additionally, their size is small enough so that they can accumulate in tumors by leaking through pores in the vasculature, coined the enhanced permeability and retention effect [56]. The zeta potential of ICAM-Dox-LPs and IgG-Dox-LPs exhibited anionic surface charges, which was less than -10 mV. The surface densities of ICAM-1 antibody and IgG on liposomes were quantified as $3,040 \pm 20$ molecules/ μm^2 for ICAM-Dox-LP and $3,100 \pm 28$ molecules/ μm^2 for IgG-Dox-LP, equivalent to approximately 67 molecules per liposome.

We next quantified the MM-specific binding and uptake of ICAM-Dox-LP relative to IgG-Dox-LP. We encapsulated a non-cytotoxic fluorescent molecule, rhodamine-dextran (RD), to replace the highly cytotoxic Dox in the immunoliposome preparation. Binding of RD-encapsulating, ICAM-1 or non-specific IgG conjugated liposomes (ICAM-RD-LPs or IgG-RD-LPs) to A375SM, C32, and normal human melanocytes were quantified by measuring the cellular fluorescence via flow cytometry. As shown in Figure 8b, ICAM-RD-LPs demonstrated significantly higher MM binding activity relative to its non-specific counterpart, IgG-RD-LPs, in both A375SM and C32 cells. No significant differences in

binding of ICAM-RD-LPs and IgG-RD-LPs were observed with normal human melanocytes. These results demonstrate that ICAM-RD-LPs can selectively bind MM cells relative to normal human melanocytes via targeting overexpressed ICAM-1 on MM cells.

MM-specific cytotoxicity of ICAM-Dox-LP was evaluated by measuring its half maximal inhibitory concentration (IC₅₀) in human MM cells. In dose-dependent cytotoxicity studies with A375SM and C32 cells (Figures 8c and d), ICAM-Dox-LPs showed significantly increased cytotoxicity than non-specific IgG-Dox-LP in both human MM cell lines. Empty immunoliposome vehicles (ICAM-LP) did not demonstrate any cytotoxicity, suggesting the ICAM-1 antibody conjugated immunoliposome itself was not cytotoxic. IC₅₀s for ICAM-Dox-LP, IgG-Dox-LP, and free Dox, calculated by SigmaPlot 12.0 software, were 0.53, 1.74, and 0.71 µg/mL for A375SM, and 0.02, 0.33, and 0.01 µg/mL for C32, respectively. These data indicate that ICAM-Dox-LPs effectively killed MM cells via enhanced delivery of Dox to MM cells as compared to non-targeted liposomes, which was achieved by improving liposome binding and uptake via the specific ICAM-1 antibody-antigen interaction.

We also evaluated the therapeutic benefits of ICAM-1 blockade from ICAM-Dox-LP. Empty ICAM- or IgG-LPs (without Dox, at a liposome-conjugated antibody concentration of 10 µg/mL) were used in this study to assess the effects of ICAM-1 blockade on MM cell migration without interference from Dox cytotoxicity. Consistent with the results from free ICAM-1 antibody treatment (as shown in Figure 6d), migration of ICAM-LP treated cells was significantly reduced compared to that of IgG-LP treated cells for both A375SM and C32 (Figures 8e and f). ICAM-LPs efficiently inhibited A375SM and C32 cell migration by over 60% and 72%, respectively. These results demonstrate that our engineered ICAM-Dox-LPs may simultaneously block tumor metastasis by inhibiting MM cell migration through the neutralizing ICAM-1 antibody and kill cancer cells effectively with MM-selective delivery of Dox, which may lead to synergistic therapeutic effects in MM treatment.

Conclusion

In summary, we developed a general method to screen and identify molecular targets for tumor-targeted nanomedicine. In this proof-of-principle study, we applied flow cytometric screening in MM cells to compare 44 cancer-related cell membrane proteins and identified ICAM-1 as a promising target for MM-specific nanomedicine. We further determined ICAM-1's targeting and therapeutic potential by measuring its tumor specificity, expression level, receptor-mediated cellular internalization, therapeutic functions and clinical heterogeneity with both human specimens and cancer cell lines. Importantly, our engineered ICAM-1-targeted liposomal doxorubicin, simultaneously inhibited MM cell proliferation and migration, validating the potential effectiveness of ICAM-1-targeted immunoliposome in MM treatment. Considering the fact that liposomal doxorubicin (Doxil® and Myocet®) are clinically safe and effective nanomedicines for metastatic ovarian and breast tumors, our target discovery platform holds potential in clinical translatability by identifying targets to increase the selectivity, while reducing the cytotoxicity and therefore improving the therapeutic efficacy of liposomal doxorubicin for MM and other cancers. Moreover, this quantitative screening and targeting strategy may be extended to other nanomedicine systems including solid lipid nanoparticles and polymeric nanocapsules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

D. Auguste acknowledges the support of NIH 1DP2CA174495. M. Moses acknowledges the support of the Breast Cancer Research Foundation and the Advanced Medical Foundation.

References

1. Tong R, Kohane DS. New Strategies in Cancer Nanomedicine. *Annu Rev Pharmacol Toxicol*. 2016; 56:41–57. [PubMed: 26514197]
2. Tong R, Langer R. Nanomedicines Targeting the Tumor Microenvironment. *Cancer J*. 2015; 21:314–321. [PubMed: 26222084]
3. Schroeder A, Heller DA, Winslow MM, Dahlman JE, Pratt GW, Langer R, Jacks T, Anderson DG. Treating metastatic cancer with nanotechnology. *Nat Rev Cancer*. 2011; 12:39–50. [PubMed: 22193407]
4. Wang AZ, Langer R, Farokhzad OC. Nanoparticle Delivery of Cancer Drugs. *Annu Rev Med*. 2012; 63:185–198. [PubMed: 21888516]
5. Espelin CW, Leonard SC, Geretti E, Wickham TJ, Hendriks BS. Dual HER2 targeting with trastuzumab and liposome-encapsulated doxorubicin (MM-302) demonstrates synergistic antitumor activity in breast and gastric cancer. *Cancer Res*. 2016; 76(6):1517–27. [PubMed: 26759238]
6. Geretti E, Leonard SC, Dumont N, Lee H, Zheng J, De Souza R, Gaddy DF, Espelin CW, Jaffray DA, Moyo V, Nielsen UB, Wickham TJ, Hendriks BS. Cyclophosphamide-Mediated Tumor Priming for Enhanced Delivery and Antitumor Activity of HER2-Targeted Liposomal Doxorubicin (MM-302). *Mol Cancer Ther*. 2015; 14:2060–2071. [PubMed: 26162690]
7. LoRusso P, Krop I, Miller K, Ma C, Siegel BA, Shields AF, Molnar I, Wickham T, Reynolds J, Campbell K, Hendriks B, McClure T, Moyo V, Munster P. Abstract CT234: A phase I study of MM-302, a HER2-targeted PEGylated liposomal doxorubicin, in patients with HER2+ metastatic breast cancer. *Cancer Res*. 2015; 75:CT234-CT234.
8. Munster P, Krop I, Miller K, Dhindsa N, Niyijiza C, Nielsen U, Oduyungbo A, Rajarethinam A, Marande M, Campbell K, Geretti E, Reynolds J, Hendriks B, Wickham T, Moyo V, LoRusso P. Abstract P4-12-29: Assessment of safety and activity in an expanded phase 1 study of MM-302, a HER2-targeted liposomal doxorubicin, in patients with advanced HER2-positive (HER2+) breast cancer. *Cancer Res*. 2013; 73 P4-12-29-P4-12-29.
9. Miller K, LoRusso PM, Munster P, Krop I, Ma C, Lee H, Reynolds J, Campbell K, Moyo V, Hendriks B, Wickham T, Siegel BA, Shields AF. Abstract P4-15-04: Effect of pre-treatment with cyclophosphamide on MM-302 (HER2-targeted liposomal doxorubicin) deposition in HER2-positive metastatic breast cancer patients assessed by ⁶⁴Cu-MM-302 PET/CT. *Cancer Res*. 2015; 75 P4-15-04-P4-15-04.
10. Johnson DB, Sosman JA. Update on the targeted therapy of melanoma. *Curr Treat Options Oncol*. 2013; 14:280–292. [PubMed: 23420410]
11. Kwong LN, Davies MA. Targeted therapy for melanoma: rational combinatorial approaches. *Oncogene*. 2014; 33:1–9. [PubMed: 23416974]
12. Paulson AS, Tran Cao HS, Tempero MA, Lowy AM. Therapeutic Advances in Pancreatic Cancer. *Gastroenterology*. 2013; 144:1316–1326. [PubMed: 23622141]
13. Michl P, Gress TM. Current concepts and novel targets in advanced pancreatic cancer. *Gut*. 2013; 62:317–326. [PubMed: 23112132]
14. Costello E, Greenhalf W, Neoptolemos JP. New biomarkers and targets in pancreatic cancer and their application to treatment. *Nat Rev Gastroenterol Hepatol*. 2012; 9:435–444. [PubMed: 22733351]
15. Finn L, Markovic SN, Joseph RW. Therapy for metastatic melanoma: the past, present, and future. *BMC Med*. 2012; 10:23. [PubMed: 22385436]

16. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M. Nivolumab plus Ipilimumab in Advanced Melanoma. *N Engl J Med*. 2013; 369:122–133. [PubMed: 23724867]
17. Flaherty KT. Targeting Metastatic Melanoma. *Annu Rev Med*. 2012; 63:171–183. [PubMed: 22034865]
18. Lee C, Collichio F, Ollila D, Moschos S. Historical review of melanoma treatment and outcomes. *Clin Dermatol*. 2013; 31:141–147. [PubMed: 23438377]
19. Bielenberg DR, Hida Y, Shimizu A, Kaipainen A, Kreuter M, Kim CC, Klagsbrun M. Semaphorin 3F, a chemorepellent for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. *J Clin Invest*. 2004; 114:1260–1271. [PubMed: 15520858]
20. Kozlowski JM, Hart IR, Fidler IJ, Hanna N. A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. *J Natl Cancer Inst*. 1984; 72:913–917. [PubMed: 6584666]
21. Chen TR, Shaw MW. Stable chromosome changes in human malignant melanoma. *Cancer Res*. 1973; 33:2042–2047. [PubMed: 4725368]
22. Amin DN. Tumor Endothelial Cells Express Epidermal Growth Factor Receptor (EGFR) but not ErbB3 and Are Responsive to EGF and to EGFR Kinase Inhibitors. *Cancer Res*. 2006; 66:2173–2180. [PubMed: 16489018]
23. Scott GA, McClelland LA, Fricke AF, Fender A. Plexin C1, A Receptor for Semaphorin 7A, Inactivates Cofilin and Is a Potential Tumor Suppressor for Melanoma Progression. *J Invest Dermatol*. 2009; 129:954–963. [PubMed: 18987670]
24. Guo† P, Huang† J, Wang L, Jia D, Yang J, Dillon DA, Zurakowski D, Mao H, Moses MA, Auguste DT. ICAM-1 as a molecular target for triple negative breast cancer. *Proc Natl Acad Sci U S A*. 2014; 111:14710–14715. † Contributed equally. [PubMed: 25267626]
25. Guo P, You JO, Yang J, Jia D, Moses MA, Auguste DT. Inhibiting Metastatic Breast Cancer Cell Migration via the Synergy of Targeted, pH-triggered siRNA Delivery and Chemokine Axis Blockade. *Mol Pharm*. 2014; 11:755–765. [PubMed: 24467226]
26. Guo P, You JO, Yang J, Moses MA, Auguste DT. Using breast cancer cell CXCR4 surface expression to predict liposome binding and cytotoxicity. *Biomaterials*. 2012; 33:8104–8110. DOI: 10.1016/j.biomaterials.2012.07.043 [PubMed: 22884683]
27. Guo† P, Yang† J, Jia D, Moses‡ MA, Auguste‡ DT. ICAM-1-Targeted, Lcn2 siRNA-Encapsulating Liposomes are Potent Anti-angiogenic Agents for Triple Negative Breast Cancer. *Theranostics*. 2016; 6:1–13. † Contributed equally. ‡ Contributed equally. [PubMed: 26722369]
28. Boone B, Jacobs K, Ferdinande L, Taideman J, Lambert J, Peeters M, Bracke M, Pauwels P, Brochez L. EGFR in melanoma: clinical significance and potential therapeutic target. *J Cutan Pathol*. 2011; 38:492–502. [PubMed: 21352258]
29. Girotti MR, Pedersen M, Sanchez-Laorden B, Viros A, Turajlic S, Niculescu-Duvaz D, Zamboni A, Sinclair J, Hayes A, Gore M, Lorigan P, Springer C, Larkin J, Jorgensen C, Marais R. Inhibiting EGF Receptor or SRC Family Kinase Signaling Overcomes BRAF Inhibitor Resistance in Melanoma. *Cancer Discov*. 2013; 3:158–167. [PubMed: 23242808]
30. Bombelli FB, Webster CA, Moncrieff M, Sherwood V. The scope of nanoparticle therapies for future metastatic melanoma treatment. *Lancet Oncol*. 2014; 15:e22–e32. [PubMed: 24384491]
31. Guo Y, Ma J, Wang J, Che X, Narula J, Bigby M, Wu M, Sy MS. Inhibition of human melanoma growth and metastasis in vivo by anti-CD44 monoclonal antibody. *Cancer Res*. 1994; 54:1561–1565. [PubMed: 7511044]
32. Trabocchi A, Menchi G, Cini N, Bianchini F, Raspanti S, Bottoncetti A, Pupi A, Calorini L, Guarna A. Click-Chemistry-Derived Triazole Ligands of Arginine–Glycine–Aspartate (RGD) Integrins with a Broad Capacity To Inhibit Adhesion of Melanoma Cells and Both in Vitro and in Vivo Angiogenesis. *J Med Chem*. 2010; 53:7119–7128. [PubMed: 20809642]
33. Benezra M, Penate-Medina O, Zanzonico PB, Schaer D, Ow H, Burns A, DeStanchina E, Longo V, Herz E, Iyer S, Wolchok J, Larson SM, Wiesner U, Bradbury MS. Multimodal silica nanoparticles

- are effective cancer-targeted probes in a model of human melanoma. *J Clin Invest.* 2011; 121:2768–2780. [PubMed: 21670497]
34. Roland CL, Harken AH, Sarr MG, Barnett CC. ICAM-1 expression determines malignant potential of cancer. *Surgery.* 2007; 141:705–707. [PubMed: 17560245]
 35. Maurer CA, Friess H, Kretschmann B, Wildi S, Müller C, Graber H, Schilling M, Büchler MW. Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer. *Int J Cancer J Int Cancer.* 1998; 79:76–81.
 36. Johnson JP, Stade BG, Holzmann B, Schwäble W, Riethmüller G. De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. *Proc Natl Acad Sci.* 1989; 86:641–644. [PubMed: 2643120]
 37. Alexander CL, Edward M, MacKie RM. The role of human melanoma cell ICAM-1 expression on lymphokine activated killer cell-mediated lysis, and the effect of retinoic acid. *Br J Cancer.* 1999; 80:1494–1500. [PubMed: 10408388]
 38. Ghislin S, Obino D, Middendorp S, Boggetto N, Alcaide-Loridan C, Deshayes F. LFA-1 and ICAM-1 expression induced during melanoma-endothelial cell co-culture favors the transendothelial migration of melanoma cell lines in vitro. *BMC Cancer.* 2012; 12:455. [PubMed: 23039186]
 39. Zhang P, Goodrich C, Fu C, Dong C. Melanoma upregulates ICAM-1 expression on endothelial cells through engagement of tumor CD44 with endothelial E-selectin and activation of a PKC - p38-SP-1 pathway. *FASEB J.* 2014; 28:4591–4609. [PubMed: 25138157]
 40. Hamai A, Meslin F, Benlalam H, Jalil A, Mehrpour M, Faure F, Lecluse Y, Vielh P, Avril MF, Robert C, Chouaib S. ICAM-1 has a critical role in the regulation of metastatic melanoma tumor susceptibility to CTL lysis by interfering with PI3K/AKT pathway. *Cancer Res.* 2008; 68:9854–9864. [PubMed: 19047166]
 41. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigartyo CAK, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, Ponten F. Tissue-based map of the human proteome. *Science.* 2015; 347:1260419–1260419. [PubMed: 25613900]
 42. Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S, Wernerus H, Björling L, Ponten F. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol.* 2010; 28:1248–1250. [PubMed: 21139605]
 43. Wang B, Guo P, Auguste DT. Mapping the CXCR4 receptor on breast cancer cells. *Biomaterials.* 2015; 57:161–168. [PubMed: 25916504]
 44. Liu D, Auguste DT. Cancer targeted therapeutics: From molecules to drug delivery vehicles. *J Controlled Release.* 2015; 219:632–643.
 45. Gunawan RC, Almeda D, Auguste DT. Complementary targeting of liposomes to IL-1 α and TNF- α activated endothelial cells via the transient expression of VCAM1 and E-selectin. *Biomaterials.* 2011; 32:9848–9853. [PubMed: 21944721]
 46. Gunawan RC, Auguste DT. Immunoliposomes That Target Endothelium In Vitro Are Dependent on Lipid Raft Formation. *Mol Pharm.* 2010; 7:1569–1575. [PubMed: 20666515]
 47. Gunawan RC, Auguste DT. The role of antibody synergy and membrane fluidity in the vascular targeting of immunoliposomes. *Biomaterials.* 2010; 31:900–907. [PubMed: 19879646]
 48. Bareford L, Swaan P. Endocytic mechanisms for targeted drug delivery★. *Adv Drug Deliv Rev.* 2007; 59:748–758. [PubMed: 17659804]
 49. Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: relevance to drug delivery. *Cell Mol Life Sci CMLS.* 2009; 66:2873–2896. [PubMed: 19499185]
 50. Batzri S, Korn ED. Interaction of phospholipid vesicles with cells. Endocytosis and fusion as alternate mechanisms for the uptake of lipid-soluble and water-soluble molecules. *J Cell Biol.* 1975; 66:621–634. [PubMed: 1174130]
 51. Mukherjee S, Ghosh RN, Maxfield FR. Endocytosis. *Physiol Rev.* 1997; 77:759–803. [PubMed: 9234965]

52. Harning R, Mainolfi E, Bystryn JC, Henn M, Merluzzi VJ, Rothlein R. Serum levels of circulating intercellular adhesion molecule 1 in human malignant melanoma. *Cancer Res.* 1991; 51:5003–5005. [PubMed: 1680025]
53. Banks RE, Gearing AJ, Hemingway IK, Norfolk DR, Perren TJ, Selby PJ. Circulating intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in human malignancies. *Br J Cancer.* 1993; 68:122–124. [PubMed: 7686390]
54. Smylie MG, Wong R, Mihalcioiu C, Lee C, Pouliot JF. A phase II, open label, monotherapy study of liposomal doxorubicin in patients with metastatic malignant melanoma. *Invest New Drugs.* 2007; 25:155–159. [PubMed: 16957835]
55. Liu D, Mori A, Huang L. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. *Biochim Biophys Acta BBA - Biomembr.* 1992; 1104:95–101.
56. Maruyama K. Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. *Adv Drug Deliv Rev.* 2011; 63:161–169. [PubMed: 20869415]

Figure 1.
Workflow of procedures to screen and identify cancer targets for nanomedicine.

Figure 2. Expression profiles of 44 cancer-related cell membrane proteins as potential metastatic melanoma targets. (a) Expression levels of these membrane proteins on the surface of two metastatic melanoma cell lines (A375SM and C32) were determined by flow cytometric analysis and compared with those of normal human melanocytes. Top 10 most overexpressed target candidates on A375SM (b) and C32 cells (c) were ranked by their overexpression with two-tailed *P* values (* *P*<0.0001). (d) Expression levels of membrane proteins from (b) and (c) on normal human melanocytes.

Figure 3.

Tumor specificity of ICAM-1 protein and gene expressions. (a) Expression levels of ICAM-1 in human melanoma tissues (162 cases) and normal tissue samples from 20 different normal organs (178 cases). The expression level of ICAM-1 was determined by the IHC staining score and presented as box-and-whisker plot. Gene expression of ICAM-1 in the validation cohort (b, 44 cases of metastatic melanoma tissues and 486 cases of various normal organ tissues) and 13 types of immune cells (c, 176 cases). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant, Mann-Whitney test)

Figure 4.

Quantification of ICAM-1 expression levels in human melanoma tissues and cell lines. (a) Representative microscopic images of human melanoma tissues and normal human skin tissues stained with an anti-human ICAM-1 antibody. (The dashed box illustrates the area subjected to increased magnification in the lower panel; scale bars represent 50 μm .) (b) Quantification of ICAM-1 staining scores at different stages of melanoma and in non-neoplastic skin tissues. Data are presented as box-and-whisker plot. (***) $P < 0.001$, Mann-Whitney test) (c) ICAM-1 gene expression in melanoma and normal melanocytes as quantified by qRT-PCR. ICAM-1 fold change is relative to GAPDH. (d) ICAM-1 surface levels in human melanoma cell lines (A375SM and C32), and normal human melanocyte cells as measured by flow cytometry. (e) Representative fluorescence microscopic images of ICAM-1 immunofluorescent staining in A375SM, C32, and normal melanocytes. DAPI was used to stain the cell nuclei. Phycoerythrin (PE)-conjugated ICAM-1 antibody was used to stain ICAM-1. Scale bars represent 20 μm . (***) $P < 0.001$ versus normal human melanocytes.

Figure 5. Confocal microscopy demonstrating the internalization of PE-conjugated ICAM-1 antibodies into metastatic melanoma cells, A375SM (a) and C32 (b). The amount of internalized PE-conjugated ICAM-1 antibodies was quantified by flow cytometry for A375SM (c) and C32 (d).

Figure 6.

Evaluation of therapeutic functions by blocking ICAM-1 on metastatic melanoma cells (A375SM and C32) with its neutralizing antibodies. After treating all cells with non-specific IgG, or ICAM-1 neutralizing antibodies, A375SM and C32 cell proliferation (a and b) was determined at day 1, 3, 5 by Dojindo cell viability assay. Representative microscopic images (c) and quantitative analysis (d) of metastatic melanoma cell migration in a transwell migration assay after incubating with non-specific IgG or ICAM-1 neutralizing antibodies. All scale bars are 50 μm. (* $p < 0.001$).

Figure 7. Correlation between ICAM-1 expression and overall survival of metastatic melanoma patients (n=44) displayed by Kaplan-Meier curves (percentile cutoff=0.23, chi=2.70, log-rank test).

Figure 8.

ICAM-1 functions as an efficient cancer target for immunoliposome to facilitate metastatic melanoma-targeted drug delivery. (a) Schematic illustration of ICAM-Dox-LP. (b) Cell binding and uptake of ICAM-RD-LP and non-specific IgG-RD-LP in metastatic melanoma cells and normal melanocytes were quantified via flow cytometry. Cytotoxicity profiles of ICAM-Dox-LP in metastatic melanoma cells, A375SM (c) and C32 (d), were evaluated by Dojindo cell viability assay. All cells were treated with ICAM-LP (without Dox), free Dox, and non-specific IgG-Dox-LP as controls. Representative microscopic images (e) and quantitative analysis (f) of metastatic melanoma cell migration in a transwell migration assay after incubating with non-specific IgG-LP or ICAM-LP (without Dox). All scale bars are 50 μm. (* $p < 0.001$ versus non-specific IgG labeled groups).

Table 1

Clinical heterogeneity of ICAM-1 in melanoma samples

Variable	Normal (N=25)	Non-metastatic Melanoma (N=95)	Metastatic Melanoma (N=67)	Total (N=162)
Median age (range) — yr	37 (18–50)	54 (7–88)	55 (31–80)	54.5 (7–88)
Sex — no. (%)				
Male	13 (52)	55 (58)	33 (49)	88 (54)
Female	12 (48)	40 (42)	34 (51)	74 (46)
ICAM-1 Expression Score — no. (%)				
0	24(96)	29 (30)	22(33)	51(31)
1+	1(4)	39 (41)	21(31)	60(37)
2+	0(0)	15 (16)	19(28)	34(21)
3+	0(0)	12 (13)	5(8)	17(11)
Positive ICAM-1 Expression — no. (%)				
Male	0(0)	15 (27)	13 (39)	28 (32)
Female	0(0)	12 (30)	11 (32)	23 (31)
Total	0(0)	27 (28) ***	24 (36) ***	51 (32) ***

Fisher's exact test: *** $P < 0.001$ versus normal

Table 2

Characterization of ICAM-1-targeted immunoliposomes

Sample	Size (nm)	Polydispersity Index	Zeta Potential (mV)	Encapsulation Efficiency (%)	Antibody Density (molecules/ μm^2)
ICAM-Dox-LP	105 $\pm$ 31	0.113	-8.8 $\pm$ 6.7	92.0 $\pm$ 1.6	3,040 $\pm$ 20
IgG-Dox-LP	101 $\pm$ 24	0.071	-4.8 $\pm$ 4.1	91.5 $\pm$ 0.5	3,100 $\pm$ 28
ICAM-RD-LP	124 $\pm$ 46	0.096	-6.6 $\pm$ 3.2	37.0 $\pm$ 0.8	3,040 $\pm$ 20
IgG-RD-LP	118 $\pm$ 19	0.085	-6.5 $\pm$ 3.8	38.6 $\pm$ 1.0	3,100 $\pm$ 28