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Preclinical Development of Near-Infrared-Labeled CD38-Targeted Daratumumab for Optical Imaging of CD38 in Multiple Myeloma

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Abstract

Purpose: Cluster of differentiation 38 (CD38) is a promising therapeutic target in multiple myeloma (MM) patients and has resulted in the development of several CD38 immunotherapies. Current methods to evaluate CD38 expression in the preclinical setting include *ex vivo* flow cytometry and immunohistochemistry, which can be cumbersome and do not give whole-body information. *In vivo* imaging technologies such as positron emission tomography rely on decay of radioisotopes, limiting the number of molecular interactions observed at any given time point. Here, we demonstrate the use of near-infrared (NIR) fluorescence imaging for spatiotemporal monitoring of CD38 expression in preclinical MM using the anti-CD38 daratumumab (DARA) conjugated to the NIR fluorophore IRDye800CW (DARA-IRDye800).

Procedures: Stability studies with human serum and binding assays with human myeloma cells were performed with DARA-IRDye800. Immunocompromised mice with intra- and extramedullary tumors ($n = 5/\text{group}$) were administered with DARA-IRDye800 for *in vivo* imaging up to 7 days after injection. *Ex vivo* biodistribution and flow cytometry studies were performed to validate *in vivo* imaging results. A separate therapy study was performed in mice with intramedullary tumors that were treated and not treated with DARA at a therapeutic dose ($n = 7/\text{group}$). DARA-IRDye800 was administered for subsequent *in vivo* and *ex vivo* imaging in both cohorts of mice.

Results: DARA-IRDye800 maintained stability and had high affinity for CD38 ($K_D = 3.5 \pm 0.05$ nM). DARA-IRDye800 demonstrated a 5- and 18-fold increase in contrast in tumor-bearing regions of mice with extra- and intramedullary MM. Finally, mice treated with therapeutic

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Author Contributions. Nicholas Cho contributed to the design and execution of all studies and data analysis and prepared the draft of the manuscript. Sooah Ko independently executed serum stability assay and provided additional support in conducting other studies. Dr. Monica Shokeen led the study, helped design experiments, and reviewed and revised the subsequent versions of the manuscript.

Compliance with ethical standards

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doses of DARA and imaged with DARA-IRDye800 showed an 11-fold decrease in fluorescence intensities *in vivo* compared with untreated controls.

Conclusions: Our studies establish DARA-IRDye800 as a promising contrast agent for preclinical evaluation of CD38 expression and for further investigating myeloma engraftment and kinetics in relation to anti-CD38 therapies.

Keywords

Near-infrared (NIR) fluorescence; Multiple myeloma (MM); Daratumumab (DARA); Cluster of differentiation 38 (CD38); IRDye800CW; Bone marrow

Introduction

Cluster of differentiation 38 (CD38) is a surface receptor expressed on myeloid, lymphoid, and non-hematopoietic cells [1] and is overexpressed on malignant cells in patients with multiple myeloma (MM), leukemia, and lymphoma [2, 3]. CD38-targeted monoclonal antibodies (mAbs) have either been approved for clinical use (*i.e.*, daratumumab (DARA)) or are undergoing evaluation (*i.e.*, isatuximab and MOR-202) in relapsed and refractory MM (RRMM) patients [4]. Each of these immunotherapies exhibits their own unique mechanisms of apoptosis and binds to different epitopes on the CD38 receptor. While mAbs such as DARA rely on recruitment of effector cells, removal of CD38⁺ immune-suppressor cells and apoptotic activity via secondary cross-linking of mAbs [5], isatuximab uniquely inhibits the enzymatic activity of CD38 and directly induces apoptosis without cross-linking [6].

As new CD38 mAbs with novel mechanisms of action (MoA) are being developed for MM and other diseases, understanding the predictive value of CD38 expression and its relation to therapeutic efficacy will be key for identifying the ideal candidate CD38 antibodies for new therapy regimens. While DARA has been extensively studied [7, 8], the MoA of CD38 in relation to other anti-CD38 therapies are still limited. Therefore, a strategy to evaluate CD38 expression spatiotemporally in preclinical models could inform physicians on implementing similar strategies for other mAbs in the pipeline. Traditional means of detecting receptor expression, such as *ex vivo* multicolor flow cytometry and histopathology, can provide valuable information on CD38 with respect to individual immune cells and other cellular markers, but can be time-consuming and labor-intensive. Such methods can also make it difficult to temporally assess CD38 expression during therapy in relation to processes such as angiogenesis and cell migration in the presence of an intact bone marrow microenvironment. There is an unmet need for a reliable whole-body imaging technique to assess whole CD38 expression quickly and accurately in these diffuse tumors for preclinical drug evaluation.

Recently, antibody-based imaging has emerged as a powerful tool for non-invasive, longitudinal imaging of functional markers due to the low toxicity and high specificity of such probes [9]. Our group has previously demonstrated the use of DARA as a companion diagnostic for positron emission tomography (PET) imaging in preclinical mouse models of MM [10]. While PET is a highly sensitive imaging modality in MM diagnosis, it allows

for monitoring of one molecular species and does not allow for the simultaneous tracking of interactions between molecular targets [11]. Near-infrared (NIR) fluorescence overcomes this limitation and is routinely used for imaging in preclinical animal models due to its reduced autofluorescence in the visible wavelength range (400–600 nm) and its ability for multiplexing of fluorescence signals at different emission wavelengths between contrast agents and fluorescently transfected cells [12]. This provides valuable functional information on biological processes involved in MM and associated therapies in preclinical animal models.

In this study, we evaluated DARA conjugated to the NIR dye IRDye800CW (Ex./Em. 774 nm/810 nm) (Li Cor) (DARA-IRDye800) for fluorescent imaging in MM animal models. DARA was chosen as the candidate anti-CD38 for these studies due to its approval in the clinic in addition to its high specificity to CD38 and low toxicity [13]. We hypothesized that the enhanced specific expression of CD38 glycoprotein on malignant plasma cells will favor increased DARA-IRDye800 uptake. The proof-of-principle *In vitro* and *in vivo* data in CD38⁺ human myeloma cells and MM mouse models demonstrated the potential of DARA-IRDye800 for both *in vivo* and *ex vivo* evaluation of CD38 expression in response to DARA therapy. These studies will contribute toward providing tools for monitoring CD38 expression in relation to novel anti-CD38 therapies evaluated in preclinical models for MM and other diseases in general.

Materials and Methods

Fluorescent Labelling of Monoclonal Antibody

DARA (Darzalex, Janssen, Beerse, Belgium) was generously donated by the Center of Advanced Medicine pharmacy at Washington University School of Medicine. Control IgG (Sigma Aldrich, St. Louis, MO, USA) and DARA were conjugated to the NIR dye, IRDye800CW NHS ester (Li Cor, Lincoln, NE, USA) according to the manufacturer's instructions. Briefly, antibodies were reacted at an antibody concentration of 5 mg/ml in 0.1 M potassium phosphate buffer (pH 8.5) for 2 h. Dye-to-antibody molar ratio of 3 to 1 was used. Unconjugated dye was removed by desalting Zeba spin columns (Sigma Aldrich). The degree of labelling (DOL) was determined using the DU-640B spectrophotometer (Beckman Coulter, Brea, CA, USA) to measure fluorophore absorbance at 774 nm and antibody absorbance at 280 nm, corrected for the fluorophore (Supplemental Fig. S1). The DOL is defined as the average dye-to-protein concentration ratio. After purification, conjugates were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) in the presence of human serum (Sigma Aldrich) at 37 °C at incubation intervals of 1, 3, 7, and 8 days. Gels were scanned using the Odyssey CLx (Li Cor) measured at the 800 nm channel, and images were analyzed in Image Studio version 5.2 software (Li Cor).

Cell Culture

The human myeloma MM.1S cells were obtained from ATCC and modified to express click beetle red luciferase and green fluorescent protein (MM.1S-GFP-luc) by the DiPersio laboratory (Professor John F. DiPersio, Washington University School of Medicine, St

Louis, MO, USA). Cells were cultured in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Chicago, IL, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% gentamicin (Sigma Aldrich).

In vitro Saturation Binding Assay

DARA-IRDye800 at various concentrations (0.675–33.76 nM) was incubated with 2.5×10^5 MM.1S-GFP-luc cells in triplicate for 3 h at 37 °C. Our group has previously confirmed high expression of the CD38 antigen on MM.1S-GFP-luc cells [10]. Cells were then washed twice in 1× phosphate-buffered saline (PBS). Flow cytometry was performed on the LSR Fortessa (BD) to calculate mean fluorescence intensities (MFI) of bound DARA-IRDye800. 7-Aminoactinomycin D (Thermo Fisher Scientific) (7AAD)[−]/GFP⁺ population was considered viable tumor cells and used for statistical analysis. Blue laser (Ex. 488 nm) was used to detect 7AAD (Em. 695/40 nm) and GFP (Em. 530/30 nm) while red laser (Ex. 640 nm) was used to detect IRDye800 (Em. 780/60 nm). Non-specific binding was determined by incubating MM.1S-GFP-luc cells with excess unlabeled (100-fold) DARA. Flow cytometry data was analyzed with FlowJo version 10.6.2 software (BD, San Jose, CA, USA). Competitive binding curves were fitted to obtain the K_D of DARA-IRDye800 using GraphPad Prism version 7 based on the non-linear fitting curve that compensates for the non-specific binding (one-site total and non-specific binding) (GraphPad Software Inc., La Jolla, CA, USA).

Animal Models

All animal studies were performed in accordance with the Institutional Animal and Use Committee of Washington University School of Medicine. Mice were anesthetized for all treatments and imaging with 2% (v/v) isoflurane/100% O₂. Female 1–3-month-old fox chase severely combined immunodeficient (SCID) mice (Charles Rivers Laboratories, Wilmington, MA, USA) were injected with 3×10^6 MM.1SGFP-luc cells in 100 µl 1× PBS subcutaneously (MM.1S SQ) or intravenously (MM.1S IV) *via* lateral tail vein. Tumor burden was monitored every 3–4 days *via* bioluminescence imaging (BLI) (Supplemental Fig. S2a), and mice were weighed (Supplemental Fig. S2b) prior to administration of DARA-IRDye800 in both mouse models. For imaging studies in MM.1S SQ and IV models, mice were randomized into respective cohorts when a mean photon flux of 1×10^9 photons/s was achieved. Healthy, female SCID mice of the same age were used for biodistribution studies as non-tumor mouse controls.

In Vivo and Ex Vivo Fluorescent Imaging

100 µg of DARA-IRDye800 and IgG-IRDye800, respectively, was administered intravenously (IV) in 1× PBS in MM.1S SQ and MM.1S IV mice ($n = 5$ /group). Daily optical imaging in the GFP (Ex./Em. 480 nm/535 nm) and IRDye800 channels (Ex./Em. 785 nm/820 nm) was performed up to 7 days following injection using the Optix MX3 time-domain diffuse optical imaging system (Advanced Research Technologies, Montreal, Canada). Prior to imaging, hair was removed by gentle clipping and depilatory cream to improve light transmission. Following imaging, mice were sacrificed at appropriate time points and tissue was excised. GFP (Ex./Em. 480 nm/535 nm) and IRDye800 (Ex./Em. 780 nm/820 nm) fluorescent images of excised tissue were acquired on the MX FX Pro In-Vivo

System (Bruker, Billerica, MA, USA) and Pearl Small Animal Imager (Li Cor), respectively. Tumor-to-background ratios (TBRs) were calculated from IRDye800 fluorescent images of MM.1S SQ mice by dividing the MFIs from regions of interest (ROIs) drawn in ImageJ (NIH, Bethesda, MD, USA) software around GFP⁺ tumor regions by the MFIs from non-tumor (background) regions on the contralateral side of the mouse. For MM.1S IV images, MFIs were also measured from ROIs drawn around GFP⁺ tumor regions using ImageJ software and compared with MFIs prior to injection of DARA-IRDye800. *Ex vivo* GFP and IRDye800 fluorescent images were analyzed in ImageJ and Pearl Cam version 2.0 (Li Cor) software, respectively, by measuring MFIs from ROIs drawn around bone and muscle tissue. Tissue-to-muscle ratio was calculated by dividing MFIs of bone tissue by MFIs of muscle tissue of each respective mouse.

Plasma Clearance

Plasma clearance was measured in MM.1S IV mice ($n = 5/\text{group}$). Prior to administration of DARA-IRDye800, whole blood was obtained through cheek bleeding of mice. At 2 and 7 days post-administration of DARA-IRDye800, whole blood was collected from mice by terminal cardiac puncture procedure. Plasma was obtained by mixing 15 μl of the whole blood with 15 μl of PBS-ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (10 mM) (Corning Life Sciences), at 3000 $\times g$ centrifugation for 1 min and freezing the resulting plasma at -80°C . The concentration of fluorescent antibody was determined by scanning 15 μl of plasma on the Odyssey CLx at the 800 nm channel. Normalized clearance was determined by dividing the signal intensity of plasma at the different time points by the intensity from plasma obtained pre-administration of DARA-IRDye800.

Biodistribution

MM.1S SQ, MM.1S IV and non-tumor SCID mice were anesthetized, bled, and dissected 2 and 7 days after injection of 100 μg of DARA-IRDye800 IV. Whole tumor and non-tumor tissues were dissected for biodistribution studies to quantify IRDye800 fluorescent tracer in units of percentage of injected dose per gram of tissue (%ID/g) as previously described [14]. Briefly, organs were suspended in 1 ml 1 $\times$ PBS and then homogenized by mechanical disruption using the Mini Beadbeater 24 (Biospec Products, Bartlesville, OK, USA) for 1 min. After homogenization, 150 μl of organs were pipetted into clear bottom 96-well black plates (Corning Life Sciences, Corning, NY). The NIR fluorescence intensity of the samples was measured at the 800 nm channel with the Odyssey CLx. The signal intensity was compared with a calibration curve and normalized to organ weight and homogenate volume to compute %ID/g. Calibration standards were made with increasing concentrations of DARA-IRDye800 in 1 $\times$ PBS.

Ex Vivo Flow Cytometry

Viable cells were obtained from tibial and femoral bone marrow flush from MM.1S IV mice, washed in fluorescence-activated cell sorting buffer (made with 1 $\times$ PBS, 0.5 M EDTA, and 0.5% bovine serum albumin (Sigma Aldrich)), stained, and immediately analyzed with LSR Fortessa. For GFP⁺ tumor graft viability, 7AAD⁻/GFP⁺ population was considered viable tumor cells. Laser channels and FlowJo version 10.6.2 software were used as previously described.

DARA Therapy Study

Weekly BLI was performed on MM.1S IV mice until they reached a mean photon flux of 3×10^6 photons/s. Mice were then randomized into untreated and treated cohorts ($n = 7/\text{group}$). Treated mice were provided DARA intraperitoneally (IP) at a therapeutic dose of 16 mg/kg of body weight in $1 \times \text{PBS}$. Treatment was administered weekly for 6 weeks in accordance with DARA treatment guidelines [15]. Additional BLI was performed twice per week during therapy. Relative bioluminescence was calculated for each time point by dividing photon flux by baseline photon flux prior to therapy. After 6 weeks, mice from treated and untreated cohorts were injected IV with 100 μg of DARA-IRDye800. Mice were imaged on Optix MX3 time-domain diffuse optical imaging system and immediately sacrificed at 7 days post-administration for *ex vivo* biodistribution and flow cytometry studies as previously described. Analysis of *in vivo* fluorescent images was performed in ImageJ by measuring MFIs from whole-body ROIs drawn around mice. Normalized fluorescence intensities were calculated by dividing MFIs post-contrast by MFIs prior to DARA-IRDye800 administration. *Ex vivo* images and flow cytometry data were analyzed as previously described with an additional stain performed with PE mouse anti-human CD38 (BD) for flow cytometry.

Statistical Analyses

All data is presented as mean $\pm$ standard deviation and statistical analysis was performed using GraphPad Prism version 7 software. Statistical significance between cohorts was calculated using Student's *t* test and two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test, unless specified otherwise. *p* values of less than 0.05 were considered statistically significant.

Results

In vitro Serum Stability and Saturation Binding Assay

DARA-IRDye800 maintained 99.5% of its initial fluorescent signal by day 8, indicating minimal dye deconjugation and formation of free dye during incubation (Fig. 1a). DARA-IRDye800 also demonstrated saturable binding to CD38⁺ MM.1S human myeloma cells. The dissociation constant (K_D) of DARA-IRDye800 was calculated to be 3.5 ± 0.05 nM and the receptor density (B_{max}) of DARA-IRDye800 was calculated to be 5.6 ± 0.13 nM (Fig. 1b), validating the high binding affinity of DARA-IRDye800 to MM.1S myeloma cells.

High Specificity of DARA-IRDye800 Conjugate to CD38⁺ Extramedullary Myeloma Tumors

To quantify contrast and the optimal imaging time point of DARA-IRDye800, MM.1S SQ mice were injected IV with the fluorescent conjugate. Significant uptake of DARA-IRDye800 was observed in GFP⁺ tumor-bearing regions by day 7 of fluorescent imaging (Fig. 2a) with a peak TBR of 5.84 ± 0.85 (Fig. 2b). This time point was later used for imaging intramedullary tumors in MM.1S IV mice. Tissue bio distribution studies were in agreement with the *in vivo* fluorescent imaging data (Supplemental Fig. S3). Tumor uptake was calculated to be 9.3 ± 2.95 %ID/g at day 7. Clearance of the conjugate was observed primarily through the liver with peak uptake of 6.04 ± 1.58 %ID/g at day 2 (Fig. 2c) and

2.56 ± 1.15 %ID/g at day 7. Liver uptake in non-tumor mice was more than 3-fold greater at both time points (day 2: 20.62 ± 1.99 %ID/g; day 7: 8.24 ± 6.5 %ID/g). No statistically significant differences were observed in other non-tumor tissue between MM.1S SQ and non-tumor mice.

High Specificity of DARA-IRDye800 Conjugate to CD38⁺ Intramedullary Myeloma Tumors

In vivo fluorescent imaging of DARA-IRDye800 was also performed in MM.1S IV mice. DARA-IRDye800 showed high specificity to GFP⁺ tumors in marrow-rich regions of the tibia (Fig. 3a), skull (Fig. 3b), and spine (Fig. 3c) with an 18-fold increase in MFIs by day 7 (Fig. 4a). *Ex vivo* fluorescent imaging of bones and non-tumor tissue from MM.1SIV mice administered with non-specific IgG conjugated to IRDye800CW (IgG-IRDye800) showed a 5-fold difference in MFIs in tumor-bearing regions compared with mice administered with DARA-IRDye800 (Supplemental Fig. S4; Fig. 4b). Tissue biodistribution studies demonstrated clearance of DARA-IRDye800 primarily in liver and spleen with peak uptake of 17.64 ± 3.69 and 16.75 ± 7.53 %ID/g, respectively (Fig. 4c, d). Statistically significant differences in uptake between non-tumor and MM.1S IV mice were observed in spleen at day 2 (non-tumor: 4.86 ± 1.73 %ID/g; MM.1S IV: 16.75 ± 7.53 %ID/g), liver at day 7 (non-tumor: 8.24 ± 6.5 %ID/g; MM.1S IV: 2.73 ± 1.23 %ID/g) and kidney at day 2 (non-tumor: 6.17 ± 2.82 %ID/g; MM.1S IV: 1.49 ± 0.9 %ID/g) and day 7 (non-tumor: 5.0 ± 2.57 %ID/g; MM.1S IV: 0.49 ± 0.18 %ID/g). A 4-fold decrease in fluorescence intensity of plasma was observed by day 7 (Fig. 4e). Viable, GFP⁺ tumor cells flushed from tibial and femoral bone marrow of mice administered with DARA-IRDye800 and IgG-IRDye800 were analyzed by flow cytometry and showed significantly increased expression of DARA-IRDye800 (MFI: $12,651 \pm 2134$) relative to IgG-IRDye800 (MFI: 1842 ± 35.13) (Fig. 4f).

DARA-IRDye800 Detects Changes in CD38 Expression During DARA Therapy Treatment

A proof-of-principle therapy study was conducted to validate the use of DARA-IRDye800 in non-invasively monitoring CD38 expression following CD38-targeted therapies. Mice treated with DARA showed an 11-fold decrease in normalized IRDye800 fluorescence intensities *in vivo* compared with untreated mice (Fig. 5a, b). Efficacy of DARA therapy in MM.1S IV mice was validated with BLI (Fig. 5c). *Ex vivo* imaging of bones from both mouse cohorts confirmed our *in vivo* imaging results. Tissue-to-muscle ratios of bones from IRDye800 and GFP channels were significantly higher in untreated mice (IRDye800: 8.51 ± 5.1 ; GFP: 1.36 ± 0.5) compared with treated mice (IRDye800: 2.07 ± 1.16 ; GFP: 0.58 ± 0.18) (Fig. 6a, b). Biodistribution of DARA-IRDye800 was statistically not significant across other non-tumor tissue (Fig. 6c). Flow cytometry on excised bone marrow validated *in vivo* and *ex vivo* images and showed a statistically significant decrease in MFIs of DARA-IRDye800 between treated (MFI: $12,841 \pm 1627$) and untreated (MFI: 2790 ± 2419) mice (Fig. 6d). No significant difference in MFIs of the commercial PE-conjugated CD38 antibody was observed between treated (MFI: 101.84 ± 75.81) and untreated (MFI: 133.66 ± 58.5) mice.

Discussion

To date, there are few NIR fluorescent contrast agents for use in hematologic malignancies such as MM *in vivo*. Most *in vivo* imaging strategies are primarily focused on transfection of fluorescent encoding vectors such as enhanced green fluorescent protein (eGFP) for tracking of whole tumor cell populations [16]. While retroviral transfection of fluorescent proteins is routinely used to monitor cell and protein expression, these reporter constructs require time-consuming methods for modulation of the native protein and typically use fluorescent proteins in the visible range (400–645 nm) that may not have sufficient brightness and contrast for *in vivo* imaging [8]. Molecular imaging in the NIR I range (650–950 nm) is an alternative strategy for preclinical evaluation of expression levels in the presence of therapy [17].

Antibody-based probes directed toward tumor-associated antigens have become promising contrast agents. Optically-labelled F(ab')₂ fragments from panitumumab (PaniF(ab')₂) and bevacizumab (BevF(ab')₂) have been developed for non-invasively measuring epidermal growth factor receptor and vascular endothelial growth factor, respectively, in the presence of different anti-angiogenic therapies in animal models [18, 19]. We reasoned that DARA-IRDye800 could be similarly used as a non-invasive, relatively inexpensive means of rapidly validating CD38 expression in animal models of MM and other hematologic diseases. The ease of functional fluorescence imaging, compared with PET, can potentially increase experimental throughput compared with other methods such as *ex vivo* flow cytometry on excised bone marrow tissue and can allow for spatiotemporal monitoring of CD38 expression in the presence of the bone marrow microenvironment.

We were able to demonstrate that the conjugation of the dye to the native antibody did not affect the specificity of DARA in both MM.1S SQ and IV mouse models. The K_D of DARA-IRDye800 to MM.1S was calculated to be 3.5 ± 0.05 nM, which is consistent with the affinity of native DARA (4.36 nM) [5, 15]. Furthermore, our plasma stability studies demonstrate that there was little fallout of free dye following conjugation, suggesting that fluorescence intensities are due to binding of DARA-IRDye800 and not due to non-specific uptake of unbound free dye. Our *in vivo* imaging results in both MM.1S SQ and IV mice showed that DARA-IRDye800 had high specificity to GFP⁺ tumor lesions. MM is a plasma cell disorder that causes significant skeletal morbidity and disruption of osteoclast and osteoblast activity within the bone marrow niche [20]. As anticipated, DARA-IRDye800 uptake was observed in bone marrow regions in MM.1S IV mice including skull, pelvis, spine, and long bones. Our *in vivo* results demonstrate the potential for non-invasive, whole-body imaging of CD38 in diffuse tumors within the bone marrow of MM mice.

Ex vivo biodistribution studies showed significant clearance through liver, spleen, and kidney by day 7 in MM.1S SQ and non-tumor mice. Due to their large size (~ 150 kDa), IgG mAbs such as DARA are primarily catabolized *via* the liver [21] with some catabolism in the spleen and kidney [22]. In addition, DARA has a half-life of 18 days [15], which matches the long half-life of DARA-IRDye800. Both factors may have contributed to high uptake of the fluorescent probe in spleen and kidney in MM.1S SQ and non-tumor mice.

While SQ injection of myeloma cells into SCID mice typically results in tumor growth at the sight of injection with rarely any systemic disease, IV injection of human myeloma cells results in diffuse tumor growth in variable regions of bone marrow in mice [23]. Normalized GFP fluorescence intensities from *ex vivo* organ images demonstrated greater tumor burden in the MM.1S SQ mice than in the bone tissue of MM.1S IV mice (Supplemental Fig. S5). Additionally, circulating tumor cells, abnormal paraprotein secretion in the serum and subsequent renal impairment, and tumor engraftment in the liver have been demonstrated following IV injection of human myeloma cells in SCID mice [23]. These factors are reflective of clinical MM and are likely contributing to greater uptake of DARA-IRDye800 in the liver and spleen with poor kidney clearance in the MM.1S IV mice.

We have demonstrated that DARA-IRDye800 can be used for monitoring CD38 expression in the presence of anti-CD38 therapy. A significant decrease in IRDye800 fluorescence intensities was observed between treated and untreated cohorts *in vivo* following DARA treatment and imaging with DARA-IRDye800. BLI and *ex vivo* imaging of tumor and non-tumor tissue in the IRDye800 and GFP fluorescent channels confirmed our *in vivo* imaging results. Biodistribution of DARA-IRDye800 showed no significant difference in uptake in other non-medullar tissue, suggesting that clearance was not altered due to DARA therapy between untreated and treated mice. *Ex vivo* flow cytometry on excised bone marrow showed significant decrease in MFIs of DARA-IRDye800 and no significant difference in MFIs with the commercial CD38 antibody. CD38 receptor was available on myeloma tumors and binding of DARA-IRDye800 to CD38 was not altered after multiple doses of DARA therapy, demonstrating that DARA-IRDye800 is a promising tool for non-invasive, preclinical evaluation of CD38 expression in medullar myeloma. While we anticipated that the therapeutic DARA and DARA-IRDye800 would bind to the same epitope of CD38 and observe a subsequent decrease in expression levels following DARA therapy [7], this is not the case for other CD38 mAbs. Isatuximab, another CD38-targeting mAb, binds to different amino acid groups on the CD38 receptor, leading to differences in physical binding to CD38 and a distinct MoA [4]. Continuous exposure to effective concentrations of isatuximab, unlike DARA, does not induce release of CD38 following binding on MM cells [6]. Future studies will focus on evaluating DARA-IRDye800 in immunocompetent MM mice in the presence of DARA and other anti-CD38 therapies such as isatuximab. While immunocompromised mice injected with human MM cells are routinely used to test novel imaging agents and therapeutics [23], we recognize that such models do not fully recapitulate the microenvironmental interactions with MM tumors. Further studies investigating DARA-IRDye800 uptake in relation to structural and functional markers of the bone marrow microenvironment *in vivo* are warranted.

Conclusions

Our studies establish DARA-IRDye800 as a promising contrast agent for evaluating CD38 expression and activity in medullar MM, and can be used to further investigate myeloma engraftment, progression and kinetics. This agent may lead to better understanding of the MoA of novel CD38-targeted immunotherapies under investigation in animal models of MM and other hematologic malignancies. These future studies can help in developing novel

therapy regimens to enhance response and overcome resistance in the treatment of these cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest

Mr. Cho, Ms. Ko, and Dr. Shokeen all report grants from National Cancer Institute, grants from National Cancer Institute Center of Multiple Myeloma Nanotherapy, grants from Washington University Imaging Sciences Pathway Fellowship, grants from Washington University Molecular Imaging Core, grants from Siteman Cancer Center Small Animal Cancer Imaging shared resource, and grants from Washington University Nutrition Obesity Research Center during the conduct of the study.

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Fig. 1.

In vitro evaluation of stability and binding of DARA-IRDye800. **a** SDS-PAGE of DARA-IRDye800 incubated in human serum between 1 and 8 days. Free dye represents unmodified IRDye800CW NHS ester. **b** Flow cytometric saturation binding assay showing specific and non-specific binding of DARA-IRDye800 to CD38⁺ MM.1S cells. $n = 3/\text{group}$.

Fig. 2. High contrast observed with DARA-IRDye800 at longer time points in MM.1S SQ mice. a Representative GFP and IRDye800 *in vivo* images of MM.1S SQ mice 2 and 7 days post-administration of DARA-IRDye800. b Calculated TBRs in MM.1S SQ mice across individual time points of DARA-IRDye800. c Normalized biodistribution (%ID/g) at 2 and 7 days post-administration of DARA-IRDye800 in non-tumor and MM.1S SQ mice. $n = 5$ /group. Two-way ANOVA followed by Sidak's multiple comparisons test was performed on biodistribution data. ** $p < 0.01$; **** $p < 0.0001$. a.u., arbitrary units.

Fig. 3. DARA-IRDye800 allows for non-invasive imaging of cancerous bone marrow regions in MM.1S IV mice. Representative images of GFP and IRDye800 fluorescence *in vivo* of **a** femur, **b** skull, and **c** spine in separate MM.1S IV mice 7 days post-administration of DARA-IRDye800. a.u., arbitrary units.

Fig. 4. Biodistribution and flow cytometric analysis of DARA-IRDye800 in MM.1S IV mice. **a** MFIs of DARA-IRDye800 in GFP⁺ tumor regions from *in vivo* animal images 7 days post-administration. **b** Tissue-to-muscle ratios of DARA-IRDye800 and IgG-IRDye800 from *ex vivo* images of GFP⁺ bones. **c** Representative GFP and IRDye800 fluorescent images of (1) blood, (2) heart, (3) lung, (4) liver, (5) spleen, (6) kidney, (7) bones (skull, spine, pelvis, and leg bones), (8) muscle, and (9) brain. **d** Normalized biodistribution (%ID/g) at 2 and 7 days post-administration of DARA-IRDye800 in non-tumor and MM.1S IV mice. **e** Normalized plasma clearance of DARA-IRDye800 2 and 7 days post-administration. **f** Flow cytometric analysis of IRDye800 MFIs from excised bone marrow of DARA-IRDye800 and IgG-IRDye800 control. $n = 5/\text{group}$. Two-way ANOVA followed by Sidak's multiple comparisons test was performed on biodistribution data. Student's t test was performed on all other datasets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. a.u., arbitrary units.

Fig. 5. Changes in DARA-IRDye800 fluorescence intensities observed *in vivo* with respect to therapy. **a** Representative *in vivo* images of IRDye800 fluorescence in mice treated and untreated with DARA therapy. **b** MFIs of bone regions normalized to precontrast MFIs of DARA-IRDye800 from *in vivo* animal images of untreated and treated mice. **c** Longitudinal BLI of DARA therapy prior to mouse killing. $n = 7/\text{group}$. Student's t test was performed on normalized fluorescence intensities. *** $p < 0.001$. a.u., arbitrary units.

Fig. 6. Biodistribution and flow cytometric analysis of changes in DARA-IRDye800 with respect to therapy. **a** Representative IRDye800 and GFP fluorescent images of excised (1) heart, (2) lung, (3) liver, (4) spleen, (5) kidney, (6) leg bones, and (7) muscle from MM.1S IV mice untreated and treated with DARA at a therapeutic dose. **b** MFIs of bone regions normalized to MFIs of muscle tissue for IRDye800 and GFP between untreated and treated cohorts. **c** Normalized biodistribution (%ID/g) of DARA-IRDye800 in untreated and treated mouse cohorts following sacrifice. **d** Flow cytometric analysis of DARA-IRDye800 and commercial PE-conjugated human anti-CD38 (PE-CD38 mAb) MFIs in GFP⁺ viable tumor cells from excised tibial and femoral murine bone marrow. $n = 7/\text{group}$. Two-way ANOVA followed by Sidak's multiple comparisons test was performed on biodistribution data. Student's t test was performed on all other datasets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. a.u., arbitrary units.