In Vivo Quantitative Assessment of Therapeutic Response to Bortezomib Therapy in Disseminated Animal Models of Multiple Myeloma With [18F]FDG and [64Cu]LLP2A PET

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Research Article

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Abstract

Background

Multiple myeloma (MM) is a disease of cancerous plasma cells. Current treatments have improved the survival rate; however, most MM patients relapse. Imaging based timely determination of therapeutic response is critical for improving outcomes in MM patients. Very late antigen-4 (VLA4) is over expressed in MM cells. Here, we evaluated [^{18}F]FDG and VLA4 targeted [^{64}Cu]LLP2A for quantitative PET imaging in MM models of variable VLA4 expression, and following bortezomib therapy.

Methods

In vitro and *ex vivo* VLA4 expression was evaluated by flow cytometry. Human MM cells, MM.1S-CG and U266-CG (CG: luciferase and green fluorescent protein), were injected intravenously in NOD-SCID gamma mice. Tumor progression was monitored by bioluminescence imaging (BLI). Treatment group received bortezomib (1mg/kg, twice/week) intraperitoneally. All cohorts (treated, untreated and no-tumor) were longitudinally imaged with [^{64}Cu]LLP2A (2-3 MBq; Molar Activity: 44.14 ± 1.40 MBq/nmol) and [^{18}F]FDG (7.4-8.0 MBq) PET respectively.

Results

Flow cytometry confirmed high expression of CD49d in U266 cells (>99%) and moderate expression in MM.1S cells (~52%). BLI showed decrease in total body flux in treated mice. In MM.1S-CG untreated versus treated mice, [^{64}Cu]LLP2A localized with a significantly higher SUV_{mean} in spine (0.58 versus 0.31) and femur (0.72 versus 0.39) at week 4 post tumor inoculation. In U266-CG treated versus untreated mice, there was a 4-time percent [^{64}Cu]LLP2A increase in spine at week 3. Compared to [^{64}Cu]LLP2A, [^{18}F]FDG PET detected treatment related changes at later time points.

Conclusion

[^{64}Cu]LLP2A is a promising tracer for *in vivo* assessment of therapeutic response in disseminated models of MM.

Background

Multiple myeloma (MM) is a cancer of abnormal plasma cells in the bone marrow. It is a complex disease characterized by intratumoral heterogeneity, inflammatory tumor microenvironment, and genetic instability [1, 2]. Patients with MM are showing significantly improved survival rates due to successful transplantation and evolving novel therapies; however, most relapse with refractory disease [3]. There remains an urgent need to develop techniques for identifying patients whose disease is progressing aggressively and patients who could benefit from a change in their therapy [4, 5]. Prognostic classification of patients solely based on genetics can be challenging at the time of diagnosis. Therefore,

monitoring of therapeutic response is considered an important approach to identify patients who are progressing quicker and should be monitored closely [6]. Furthermore, the International Myeloma Working Group (IMWG) has proposed the evaluation of minimal residual disease (MRD) as one of the key criteria for defining response [7]. Techniques such as whole-body imaging that can improve the accuracy of MRD detection are therefore critical for comprehensive assessment of complete response [8].

Positron emission tomography (PET) is a positron-emitting radionuclide based functional imaging technique that is highly sensitive for detecting disease burden of varying degree [9]. Molecularly targeted PET radiopharmaceuticals have been successfully utilized for assessing biological processes at cellular and molecular levels, enabling early and accurate identification of cancerous cells [10]. PET fused with advanced anatomic imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI), provides a whole-body, high-resolution and sensitive platform for probing the biology of disease, achieving early diagnosis, disease staging, treatment planning and evaluation of therapeutic response [11, 12]. Multiple myeloma (MM) is a radiologically defined disease [13]. PET/CT or PET/MR imaging facilitates longitudinal and sensitive detection of myeloma induced bone lesions, bone marrow infiltration, compositional changes in the microenvironment, and extra medullary disease [14, 15].

[¹⁸F]FDG is a radiolabeled glucose analogue and a biomarker of glucose metabolism. [¹⁸F]FDG is the most utilized metabolic PET imaging tracer and has been used to assess therapy response in patients with hematological malignancies like lymphoma [16–18] and MM [19, 20]. It is internalized into the cells *via* glucose transporter protein 1 (GLUT1) and metabolized by hexokinase 2 enzyme; both molecules generally overexpressed in tumor cells. The field of hematological imaging has traditionally been dominated by [¹⁸F]FDG and to a very good effect. However, despite being the PET gold standard for MM imaging, [¹⁸F]FDG imaging has certain limitations in MM. [¹⁸F]FDG is unable to distinguish between malignant cells and inflammation following therapy (flare reaction to steroids), leading to false positive results [21, 22]. Contrarily, in metabolically low MM lesions, [¹⁸F]FDG/PET invariably results in underestimation of tumor burden [22]. [¹⁸F]FDG/PET also has less than optimal efficacy for detecting diffuse bone marrow infiltration in patients with MM [20]. Furthermore, Rashe et al showed that low expression of hexokinase-2 is associated with false-negative FDG–positron emission tomography in multiple myeloma [23]. To overcome these limitations, a more specific and sensitive PET imaging agent is desirable in MM patients.

Recently there have been considerable advancements in the development of molecularly targeted PET imaging agents that are closer to clinical use than ever before [24, 25]. Molecular imaging of specific receptors/proteins overexpressed on tumor cells will be key to realizing the concept of precision health [26]. As promising new tracers are getting traction in clinic, their evaluation relative to the existing gold standard contrast agents can provide pertinent information regarding the unique and complementary information that is rendered by these agents. Very late antigen-4 (VLA4) is a non-covalent, heterodimeric integrin receptor that is upregulated in MM [27]. Previous studies suggested that VLA4 integrin (also known as $\alpha_4\beta_1$) is responsible for tumor proliferation and metastasis [28, 29]. It also plays a crucial role in drug resistance in hematological malignancies such as MM [30] and acute myelogenous leukemia [29].

We and others have shown the efficacy of a VLA4 targeted radiotracer, [^{64}Cu]LLP2A, in several pathologies including MM, melanoma and inflammation [27, 31–34]. There is currently an ongoing clinical trial to assess the safety and dosimetry of [^{64}Cu]LLP2A in healthy and MM patient volunteers [NCT03804424] [35]. In order to ascertain the qualitative and quantitative differences and similarities with [^{18}F]FDG, it is rational to perform fundamental investigations that will highlight the unique features of these tracers in a medullary myeloma setting.

In this study, we compared the efficacy of the gold standard metabolic tracer, [^{18}F]FDG, with VLA4 targeted [^{64}Cu]LLP2A PET for qualitative and quantitative longitudinal imaging of disease progression and bortezomib therapy response in disseminated preclinical models of human myeloma. Bortezomib is an FDA approved reversible proteasome inhibitor for the treatment of MM [36]. Previous studies have showed that bortezomib can downregulate VLA4 expression in myeloma cells and help reduce cell adhesion mediated-drug resistance (CAM-DR) [37]. Furthermore, recent work suggested that bortezomib-refractory myeloma cells have higher VLA4 expression as compared to the parental cells [38].

Two human myeloma models expressing different levels of VLA4 protein were evaluated to assess tracer performance *in vivo*. Standard uptake values (SUV) from PET data provide a reliable semi-quantitative measure of the tumor uptake and kinetics in various tissues, and help assess therapy response [39, 40]. Here, the radiotracer uptake was quantified in a longitudinal setting (weekly sequential imaging with both tracers) using the SUV in different organs and complemented with BLI and *ex vivo* flow cytometry; with a focus on the intramedullary disease.

In summary, we evaluated the quantitative and qualitative features of these two promising PET tracers in MM biology. The underlying hypothesis is that molecular imaging of plasma cell receptor VLA4 combined with the metabolic status can provide promising synergistic information on disease progression and therapeutic response.

Material And Methods

Ethics statement

All the experiments involving the use of radioactive materials were done at Washington University and conducted under the authorization of the Radiation Safety Commission in accordance with the University's Nuclear Regulatory Commission license. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals under the auspices of the Animal Studies Committee of Washington University.

Reagents

The chemicals and reagents used in the present study were of highest commercially available purity and all the solutions were prepared using ultrapure water (18 MV-cm resistivity; Millipore system). The proteasome inhibitor bortezomib, was purchased from Sigma-Aldrich. LLP2A-CB-TE1A1P (LLP2A)

peptide was purchased from Auspep (Tullamarine Victoria, Australia) and all other chemicals used in radiolabeling were purchased from Sigma Aldrich unless otherwise noted. Copper 64 ($t_{1/2} = 12.7$ h) was produced on a CS-15 biomedical cyclotron at Washington University School of Medicine. Radiochemical purity of the labeled peptide was evaluated by analytical reversed-phase high performance liquid chromatography (HPLC), that was performed on 1200 Infinity series chromatography system, purchased from Agilent Technologies (Santa Clara, CA). The XB-C18 Kinetex column which was procured from Phenomenex was used with mobile phases of 0.1% TFA in water (aqueous phase) and 0.1% TFA in acetonitrile (organic phase). [^{18}F]FDG was produced in compliance with good manufacturing practices (GMP) by Washington University Cyclotron facility.

Cell culture

The human myeloma cell lines, MM.1S and U266 were obtained from Professor Katherine N. Weilbacher (Department of Medicine, Washington University School of Medicine) and American Tissue Culture Collection (ATCC) respectively. MM.1S and U266 cell lines were modified to carry click beetle red luciferase (CBR; C) and green fluorescent protein (GFP; G) by Professor John DiPersio's group (Department of Internal Medicine, Bone Marrow Transplant Division, Washington University School of Medicine). The cells were maintained in suspension at 10^6 cells/mL in complete Roswell Park Memorial Institute medium (RPMI) 1640 medium (Thermo Fischer Scientific). The RPMI media was supplemented with 10 % fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Thermo Fischer Scientific). The cells were cultured in a jacketed humidified CO_2 (5%) incubator at 37°C .

MM.1S-CG/NSG and U266-CG/NSG MM mouse models

NOD-SCID gamma (NSG) mice purchased from Jackson laboratories (USA) were used to develop the MM mouse models utilized in this study. Mice were housed in ventilated cages and allowed food and water. MM.1S-CG (5×10^6 cells in 100 μL) and U266-CG (2×10^6 cells in 100 μL) cells were injected into the NSG mice *via* tail vein to establish disseminated MM disease. Tumors were allowed to grow for 1–2 weeks before initiation of bortezomib therapy. Tumor progression was monitored by weekly bioluminescent imaging (BLI). All tumor inoculation and imaging procedures were conducted under isoflurane anesthesia (1–2% vaporized in O_2).

Bioluminescent imaging (BLI)

The tumor progression *in vivo* was monitored by BLI using Caliper IVIS Imager (PerkinElmer, Waltham, MA, USA; Living Image 3.2, 1-300 sec exposures, binning 2–8, FOV 12.5 cm). Mice were injected with 150 mg/kg D-luciferin in PBS intraperitoneally and imaged 10 min post injection under isoflurane anesthesia. The total photon flux (photons/sec) was measured from regions of interest (ROIs) over the entire dorsal/ventral side of the mouse, using LivingImage 2.6 (Xenogen, CA, U.S.A). The optical signal was normalized to average radiance expressed in photons per second per centimeter square per steradian ($\text{p/s/cm}^2/\text{sr}$). Once the total body flux from each mouse reached 1×10^6 $\text{p/s/cm}^2/\text{sr}$, longitudinal PET imaging was initiated followed by bortezomib therapy (Fig. 1). The first PET imaging time point was

considered baseline for all the cohorts, including the therapy cohort (pre-therapy imaging time point (Week 0)).

Bortezomib therapy

NSG mice bearing MM.1S-CG and U266-CG tumors and non-tumor bearing control mice were divided into three groups: no tumor (control mice with no tumor burden; $n = 4$), untreated (tumor bearing, no treatment; $n = 6$) and treated (tumor bearing, bortezomib treatment; $n = 6$). Mice from the treated cohort received bortezomib (1mg/kg) intraperitoneal dose, twice a week, starting at week 1 (relative to the week 0 imaging time point) (Fig. 1). The therapy response in the treated mice was evaluated by quantification of luciferase signal (BLI imaging). The disease burden in tumor bearing untreated mice was also monitored by BLI.

[^{64}Cu] labeling of LLP2A-CB-TE1A1P

LLP2A-CB-TE1A1P peptide was radiolabeled with [^{64}Cu] as previously described [27]. Briefly, [^{64}Cu]chloride was diluted with 0.1M ammonium acetate solution (pH 5.5). LLP2A-CB-TE1A1P peptide (2.5 μg ; 1.61 nmol) was incubated with 74 MBq of [^{64}Cu] at 70°C for 30–40 min with shaking. After the incubation, the radiochemical purity was evaluated by analytical radio-High Performance Liquid Chromatography (HPLC).

Small animal PET imaging and image analysis

Non-tumor bearing control, MM.1S-CG and U266-CG myeloma tumor bearing (treated and untreated) mice were imaged with [^{18}F]FDG and [^{64}Cu]LLP2A PET/CT at baseline (Week 0) and then sequentially every week for up to 6 weeks. While we were able to image a sub-set of treated mice up to 6 weeks, the mice in other two cohorts either died earlier due to disease burden and stress due to multiple imaging sessions. Survival plot is included in the supplementary information (Fig. S2). In preparation for the [^{18}F]FDG PET imaging session, mice were fasted for 4 h with access only to water. Prior to the [^{18}F]FDG PET imaging session, mice were first injected with 7.4-8 MBq *via* tail vein, and imaged for 10 min at 1 h post injection of the radiotracer on the small animal INVEON PET/CT scanner (Siemens Medical Solutions, Knoxville, TN). Next day after the radioactivity from FDG had decayed, the same group of mice were injected with the VLA4 targeted radiotracer, [^{64}Cu]LLP2A (2–3 MBq) *via* tail vein. Whole body PET/CT static imaging (20 min static) was performed at 4 h post injection. Images were reconstructed using a 2DOSEM algorithm. CT and PET images acquired with [^{18}F]FDG and [^{64}Cu]LLP2A were co-registered on Inveon Research Workplace (IRW) software (Siemens Medical Solutions, Knoxville, TN). The reconstructed PET/CT images were viewed on IRW software, which allowed trans-axial, coronal, and sagittal displays of the slices and maximum intensity projection (MIP) PET/CT images. The volumetric regions of interest (ROI) were manually drawn using a 2-dimensional tool on the sagittal attenuation-corrected (using CT anatomical guidelines) PET slices. A semi-quantitative analysis of [^{18}F]FDG and [^{64}Cu]LLP2A activity was performed by calculating the mean standard uptake values (SUV_{mean}) within a ROI using the formula:

$SUV = (A [nCi/mL] \times [weight (g)]/[dose (nCi)])$ where A is the average activity in nCi/mL, in the specified volume of interest and decay corrected to the scan start time. Dose is the activity injected in nCi at the injection time, decay corrected to the imaging time, and weight is the whole animal in grams.

In vitro and ex vivo flow cytometry

The human myeloma cell lines MM.1S and U266 without any reporters were analyzed for CD49d ($\alpha 4$ subunit of VLA4, $\alpha 4\beta 1$) expression by flow cytometry. In preparation for cell surface staining, cells were suspended in 100 μ L buffered (pH $\sim$ 7.4) phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Cells were incubated with the phycoerythrin-cyanine 5 conjugated anti CD49d antibody or isotype control antibody for 30 min at 4°C in dark. After the incubation, cells were washed twice with 1 X PBS buffer and analyzed on a FACS Calibur 3 system (BD Biosciences). Data were analyzed using FlowJo software (BD Biosciences, San Jose, CA, USA).

To confirm and quantify the VLA4 expression ex vivo, cells were extracted by flushing the bone marrow from tibia, femur and pelvis of MM.1S-CG and U266-CG IV tumor bearing mice. Utmost care was taken to preserve the viability of the cells. Briefly, cells were washed with fluorescence-activated cell sorting (FACS) buffer (1 X PBS, 0.5 M EDTA, and 0.5% BSA), stained, and immediately analyzed using LSR Fortessa (BD Biosciences). The cells were stained for human CD29 (APC anti human CD29 antibody; BioLegend), mouse CD45 (BV510 Rat anti mouse CD45; BD Biosciences), and human CD49d (PE mouse anti-human CD49d antibody; BD Biosciences) and incubated for 30 min at 4°C in the dark. 7-Aminoactinomycin D (Thermo Fischer Scientific) (7AAD)⁻/GFP⁺ population was considered viable tumor cells. Blue laser (Ex. 488 nm) was used to detect 7AAD (Em. 695/40 nm) and GFP (Em. 530/30 nm), red laser (Ex. 640 nm) was used to detect APC anti-CD29 (Em. 670/30) while violet laser (Ex. 405 nm) was used to detect BV510 anti CD45 (Em. 525/50). PE anti CD49d (Em. 585/15) was detected by yellow/green laser (Ex. 552 nm). Flow cytometry data was analyzed with FlowJo software (BD, San Jose, CA, USA).

Statistical analysis

All the data is presented as mean $\pm$ standard deviation. The statistical analysis was performed by GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance between cohorts was calculated using one way ANOVA followed by Tukey's multiple comparison tests. P values less than 0.05 were considered statistically significant.

Results

[⁶⁴Cu] labeling of LLP2A-CB-TE1A1P

The VLA4 targeted peptide, LLP2A-CB-TE1A1P (LLP2A), was successfully radiolabeled as previously described with [⁶⁴Cu], resulting in a molar activity of 44.14 ± 1.40 MBq/nmol [27]. The radiochemical purity of $> 99 \pm 0.03\%$ was confirmed by analytical radio-HPLC (Fig S1).

Bioluminescent imaging (BLI)

Small animal BLI was crucial for validating the establishment of the disseminated disease and for the longitudinal monitoring of the systemic disease progression in MM.1S-CG and U266-CG disseminated (intravenous) tumor models. BLI images confirmed the presence of tumor in the bone marrow rich sites such as the spine, long bones (femur and tibia) and pelvis. The representative BLI images from the MM.1S-CG (Fig. 2a) and U266-CG (Fig. 2b) tumor bearing mice, no treatment cohort showed increased whole body luciferase activity, especially in the spine and femurs as compared to the mice treated with bortezomib. In the MM.1S-CG myeloma mouse model (Fig. 2a), the qualitative BLI signal in both the treated and untreated mice showed similar tumor burden at the baseline imaging time point (Week 0, defined as the week prior to the start of bortezomib therapy). Bortezomib treatment was started post baseline imaging (week 0). Quantitative and qualitative BLI signal showed significant percent increase ($p < 0.05$) in tumor burden in mice with no treatment as compared to the ones treated with bortezomib over the period of time (week 0 to week 4) (Fig. 2c). Post initiation of bortezomib therapy at week 4, the percent increase in tumor signal increased more than 10-fold in untreated mice as compared to the treated mice.

Similar to MM.1S-CG, the BLI data for U266-CG tumor bearing mice (Fig. 2b) demonstrated the expected pattern of tumor engraftment confirming comparable tumor burden in both treated and untreated mice at week 0 (i.e., prior to start of treatment). The luciferase activity/BLI signal in bortezomib treated mice decreased with time, whereas in the no treatment mice cohort, the BLI activity increased significantly post week 3 of tumor inoculation.

Small animal PET imaging

Longitudinal small animal PET imaging study was performed in both the MM.1S-CG and U266-CG mouse models (treated and untreated cohorts) as well as in the non-tumor bearing healthy control cohort. The goal was to compare the performance of the clinical gold standard metabolic radiotracer, [^{18}F]FDG, with the VLA4 targeted imaging agent, [^{64}Cu]LLP2A, in evaluating bortezomib therapy response in mice with MM.1S-CG and U266-CG disseminated myeloma disease. In the MM.1S-CG tumor bearing, treated and healthy control mice, [^{18}F]FDG/PET showed normal physiological uptake in the metabolically active organs like brain, gastrointestinal tract and heart. [^{18}F]FDG/PET also showed background uptake in the leg muscles and brown fat (Fig. 3a). The MIP images of [^{64}Cu]LLP2A/PET showed robust uptake of this radiotracer in the tumor rich skeletal sites such as spine, pelvis and femurs (confirmed by BLI) whereas mice with no tumor burden did not show significant [^{64}Cu]LLP2A uptake in these sites (Fig. 3a). The SUV_{mean} [^{18}F]FDG/PET and [^{64}Cu]LLP2A/PET data at the pre-therapy imaging time point showed no significant differences in spine, right, and left femur uptake among the tumor bearing (mice divided into two groups - treated that were later treated with bortezomib and untreated with no treatment) and no tumor mice (Fig. 3b). Figure 3c shows the comparison of percent change in SUV_{mean} among treated, untreated and no tumor cohorts of mice with both [^{18}F]FDG/PET and [^{64}Cu]LLP2A/PET over a period of time (week 1–6). Bortezomib therapy was initiated post baseline imaging, and then longitudinal imaging was done with both the tracers up to 6 weeks for treated mice and up to week 3 and 4 for no tumor and

untreated mice respectively (Fig. 1). The SUV_{mean} data from [^{18}F]FDG/PET imaging showed 3-fold increase in the spine uptake of untreated mice as compared to treated mice at week 4 (Fig. 3c). The SUV_{mean} uptake of [^{18}F]FDG in both the femurs was comparable in all three groups throughout the study. The data from [^{64}Cu]LLP2A imaging showed significant increase in spine uptake for the untreated group of mice as compared to the treated cohort (0.58 ± 0.13 vs 0.31 ± 0.05) at week 4. No significant changes were observed in the spine uptake among treated and no tumor mice. The right and left femurs started showing changes in the SUV_{mean} by week 4 between the treated and untreated cohorts with 2-fold increase in [^{64}Cu]LLP2A uptake respectively (Fig. 3c).

Figure 4a shows representative MIP small animal PET images of [^{18}F]FDG at 1h and [^{64}Cu]LLP2A at 4h post injection of the respective tracers in the U266-CG tumor bearing mice. [^{64}Cu]LLP2A/PET identified the tumor burden in spine and leg bones of tumor bearing mice while [^{18}F]FDG showed background uptake in heart, brain, leg muscles and brown fat. [^{18}F]FDG and [^{64}Cu]LLP2A SUV data at week 0 confirmed comparable tumor burden in spine, right femur, and left femur of U266-CG tumor bearing mice (grouped as treated and untreated) and no tumor mice (Fig. 4b). The SUV_{mean} quantification and comparison data with [^{18}F]FDG PET showed increased [^{18}F]FDG uptake in spine of untreated mouse (SUV_{mean} of 0.93) which was almost twice as high as that of mice treated with bortezomib (SUV_{mean} 0.54 ± 0.16) at week 3 (Fig. 4c). Similarly, [^{64}Cu]LLP2A/PET data showed significantly higher SUV_{mean} uptake of 1.56 in spine of untreated mouse, as compared to 0.59 ± 0.27 in case of treated mice. However, both the femurs of untreated mice showed low uptake (non-significant) of [^{18}F]FDG as compared to the treated and no tumor mice. The comparison of SUVs from all the three groups (treated, untreated and no tumor age-matched) for right and left femurs using [^{64}Cu]LLP2A showed no significant changes in the uptake of the radiotracers in these tissues.

In vitro and ex vivo flow cytometry

In vitro-cultured MM.1S and U266 cells were analyzed for surface expression of CD49d (α_4 sub unit of VLA4/ $\alpha_4\beta_1$) and CD29 (β_1 sub unit of VLA4/ $\alpha_4\beta_1$) using flow cytometry, which showed expression in both cell lines, albeit at different levels (Fig. 5a-b). The mean fluorescent intensity (MFI) of CD49d in U266 cells was ~ 5 times higher than the MM.1S cells. Similarly, the MFI values for CD29 was significantly higher in U266 cells as compared to MM.1S cells (Fig. 5c-d).

Analysis of bone marrow from the tibial, femoral, and pelvic bones showed the persistence of GFP/human CD-45 positive MMC in bortezomib treated mice. Gating of these tumor cells showed expression of human CD49d (α_4) and CD29 (β_1), while bone marrow mouse CD45+ cells remain unstained (Fig. 5e-f). This indicates that, in vivo, U266-CG and MM.1S-CG cells surviving therapy with bortezomib may still be detected by VLA4 imaging.

Discussion

Hematological malignancies can greatly benefit from molecularly targeted whole-body advanced imaging methods. Imaging plays an indispensable role in the drug development process as well as in translation of preclinical findings into patient care [41]. Recently there have been exciting developments in the sphere of MM imaging. Non-invasive imaging modalities such as PET/CT or PET/MRI have been extensively used to investigate bone lesions and metastasis in MM [22, 42, 43]. Our group has previously showed that antibody-based PET nuclear agents, [^{89}Zr]daratumumab and [^{89}Zr]elotuzumab targeted to overexpressed proteins CD38 and CS1 respectively on myeloma cells can be used as companion diagnostics for preclinical imaging of MM [22, 42]. Ulaner et al demonstrated the first-in-human use of [^{89}Zr]daratumumab in measuring MM burden, evaluating minimal residue disease (MRD), and assessing daratumumab therapy response [44]. Previous studies used [^{68}Ga]Pentixafor for assessing chemokine receptor 4 (CXCR4) that is overexpressed on myeloma cells and plays an imperative role in tumor proliferation, tumor invasiveness and metastasis [25]. PET imaging with CXCR4 targeted [^{68}Ga]Pentixafor enabled detection of myeloma lesions that could not be detected with [^{18}F]FDG and paved way for selecting patients for CXCR4 directed therapies [43].

One of the most commonly used imaging agent for MM is [^{18}F]FDG, that assesses the metabolic activity of myeloma cells [45]. [^{18}F]FDG is the gold standard MM imaging agent for MM patient management largely due to its ability to differentiate between metabolically active and inactive sites of MM [46, 47]. Despite the frequent use of [^{18}F]FDG in MM imaging, this metabolic tracer has limited use in therapy response monitoring. PET imaging using [^{18}F]FDG can lead to false positive signal due to increased uptake in a treatment setting due to flare, and false negatives due to decreased uptake in hypometabolic MM lesions [22, 48]. To overcome these limitations, we propose the use of a non-[^{18}F]FDG PET tracer to evaluate therapy response in MM.

The activated form of VLA4 (also known as the $\alpha_4\beta_1$ integrin) is overexpressed in MM, and plays an important role in ensuring myeloma cell adhesion and survival within the bone marrow (BM) [49]. Previous studies showed that ^{64}Cu labeled high affinity peptidomimetic ligand, LLP2A ([^{64}Cu]LLP2A) can be used to target VLA4 in murine melanoma mouse model and can efficiently detect small metastatic lesions [32]. Another recently published study demonstrated the use of [^{64}Cu]LLP2A as a PET imaging agent to detect VLA4-mediated hyper adhesion in transgenic sickle cell disease mice [50]. Our group has previously evaluated the potential of [^{64}Cu]LLP2A to image VLA4 expression in MM mouse models [27, 31]. In this study we sought out to evaluate the efficacy of these two radiotracers, [^{18}F]FDG and [^{64}Cu]LLP2A, with different targeting modes of action in MM cells for monitoring bortezomib (proteasome inhibitor) therapy response using longitudinal small animal PET imaging.

Flow cytometry analysis of human myeloma cell lines, MM.1S and U266 demonstrated that > 99% of U266 cells expressed CD49d (α_4) and CD29 (β_1) when normalized to the isotype control. Whereas, only 52% of MM.1S cells stained positive for CD49d and ~ 30% stained for CD29 (Fig. 5). Therefore, these two

cell lines with variable CD49d/ $\alpha 4$ surface expression were used to evaluate myeloma mouse models for longitudinal imaging studies with [^{18}F]FDG and VLA4 targeted [^{64}Cu]LLP2A (Fig. 1).

Disseminated myeloma was induced in NOD SCID Gamma (NSG) mice by injecting MM.1S-CG and U266-CG cells *via* tail vein, and the tumor progression was monitored weekly by BLI. Bioluminescence imaging (BLI) confirmed the presence of tumor in the bone marrow rich skeletal sites such as spine, pelvis and leg bones. Bioluminescence imaging (BLI) data also showed that the spine had higher tumor burden relative to the femurs and pelvis in both the tumor models. We postulated that the observed differential tumor burden *in vivo* could be because of the heterogeneous nature of the disseminated myeloma disease. The comparison of BLI data between treated and untreated cohorts of mice with MM.1S-CG and U266-CG tumors showed decreasing luciferin signal in mice treated with bortezomib as compared to tumor bearing mice with no treatment.

Longitudinal small animal PET imaging with [^{18}F]FDG and [^{64}Cu]LLP2A respectively was performed to evaluate bortezomib therapy response in these two human MM models. [^{18}F]FDG small animal PET/CT imaging at 1 h post-injection of the radiotracer showed normal physiological uptake in the brain, GI tract, brown fat and leg muscle [51]. [^{18}F]FDG is a biomarker for glucose metabolism. It requires GLUT1 protein on the cell surface for the internalization and hexokinase-2 enzyme for retention in tumor cells; but the expression of GLUT 1 transporters and hexokinase 2 is inconsistent in MM cells [23, 52, 53]. In both the MM.1S and U266 tumor models, no significant changes in [^{18}F]FDG uptake were observed in right and left femurs among the bortezomib treated, untreated, and no tumor cohorts. However, [^{18}F]FDG started showing significant difference in spine uptake among the treated and untreated cohorts at week 4 and week 3 for MM.1S-CG and U266-CG myeloma models respectively (Figs. 3 and 4).

The SUV_{mean} data from [^{64}Cu]LLP2A PET imaging showed that VLA4 targeted [^{64}Cu]LLP2A agent can detect changes in the tumor burden in spine as early as week 2 among the treated, untreated and no tumor cohorts. The femurs of MM.1S-CG tumor bearing mice with no treatment showed comparatively increased uptake at week 4 as compared to the MM.1S-CG tumor bearing mice treated with bortezomib. Previous studies showed that use of bortezomib downregulates the VLA4 expression on myeloma cells [37]. This is consistent with reduced translation of membrane proteins during proteotoxic stress. A later study, however, suggested that myeloma cells resistant to bortezomib may increase expression of VLA4 and its function *in vivo* [38]. In the femurs of U266-CG tumor bearing mice however there were no significant differences. This aligned with the BLI data that showed that majority of tumor burden was concentrated in the spine. The SUV PET data of spine for U266-CG mice (treated and untreated) demonstrated similar trend as that of BLI signal of spine in these mice. [^{64}Cu]LLP2A uptake was comparable between the treated and no-tumor cohorts in both the mouse models. Finally, [^{64}Cu]LLP2A binds to the activated form of VLA4, and normal cells such as leukocytes, B-cells, T-cells, and hematopoietic progenitors express also express VLA4 on their surface [54]. Therefore, healthy mice also showed some [^{64}Cu]LLP2A uptake expectedly, but it was significantly less compared to the mice with myeloma tumors.

Conclusion

This study demonstrated significant qualitative and quantitative differences in the uptake of standard-of-care [^{18}F]FDG and VLA4 targeted [^{64}Cu]LLP2A tracers in human models of disseminated MM. Based on the *in vitro*, *ex vivo* and *in vivo* data, we posit that [^{64}Cu]LLP2A is a promising PET imaging agent for treatment monitoring in MM patients.

Abbreviations

MM

Multiple myeloma

VLA4

Very Late Antigen 4

BM

Bone Marrow

[^{18}F]FDG

Fluorine-18-fluorodeoxy glucose

[^{64}Cu]LLP2A

Copper 64 labelled precursor LLP2A-CB-TE1A1P

NSG

NOD SCID Gamma

PET

Positron Emission Tomography

IMWG

International Myeloma Working Group

MRD

Minimal Residual Disease

GLUT1

Glucose Transporter Protein 1

FDA

US Food and Drug Administration

CAM-DR

Cell Adhesion Mediated Drug-Resistance

SUV_{mean}

Mean Standard Uptake Value

BLI

Bioluminescent Imaging

ROI

Regions of Interest

MFI

Declarations

Availability of data and material - all data generated and analyzed during the current study are included in this main article, and its supplementary information files.

Ethical approval: All applicable institutional guidelines for the care and use of animals were followed. All the animal studies were performed at Washington University School of Medicine under the guide for the Care and Use of Laboratory animals.

This article does not contain any studies with human participants performed by any of the authors.

Consent for publication- Not applicable

Conflict of Interest: Anchal Ghai has no conflict of interest.

Nikki Fettig has no conflicts of interest.

Francesca Fontana has no conflicts of interest.

John DiPersio has no conflicts of interest.

Michael Rettig has no conflicts of interest.

Julie O'Neal has no conflicts of interest.

Samuel Achilefu is co-founder of Sarya LLC

Kooresh Shoghi has no conflicts of interest.

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No other potential conflict of interest relevant to this article was reported.

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No other potential conflict of interest relevant to this article was reported.

Authors' contributions

AG performed the radiolabeling, PET and BLI data analysis, interpretation of data, and writing of the manuscript.

NF has acquired the PET and BLI data.

FF has analyzed the flow data and contributed in figure production for flow data.

JFD provided general guidance.

MR & JN helped acquire the flow data.

SA provided general guidance.

KS helped in study design, data analysis and reviewed the manuscript.

MS led the study design, interpretation of data, and manuscript write up and review.

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References

1. Chapman MA, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011;471(7339):467–72.
2. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer*. 2012;12(5):335–48.
3. Barlogie B, et al. Treatment of multiple myeloma. *Blood*. 2004;103(1):20–32.
4. Barlogie B, et al. Curing myeloma at last: defining criteria and providing the evidence. *Blood*. 2014;124(20):3043–51.
5. Martinez-Lopez J, et al. Long-term prognostic significance of response in multiple myeloma after stem cell transplantation. *Blood*. 2011;118(3):529–34.
6. Fulciniti M, Munshi NC, Martinez-Lopez J, *Deep Response in Multiple Myeloma: A Critical Review*. *Biomed Res Int*, 2015. **2015**: p. 832049.
7. Kumar S, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol*. 2016;17(8):e328–46.
8. Rasche L, et al. Combination of flow cytometry and functional imaging for monitoring of residual disease in myeloma. *Leukemia*. 2019;33(7):1713–22.

9. Fiordelisi MF, et al., *Preclinical Molecular Imaging for Precision Medicine in Breast Cancer Mouse Models*. Contrast Media Mol Imaging, 2019. **2019**: p. 8946729.
10. Mankoff DA. *A definition of molecular imaging*. J Nucl Med, 2007. 48(6): p. 18N, 21N.
11. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. Genes Dev. 2003;17(5):545–80.
12. Incoronato M, et al., *Radiogenomic Analysis of Oncological Data: A Technical Survey*. Int J Mol Sci, 2017. 18(4).
13. Barwick T, et al. Imaging in myeloma with focus on advanced imaging techniques. Br J Radiol. 2019;92(1095):20180768.
14. Cai W, et al. How molecular imaging is speeding up antiangiogenic drug development. Mol Cancer Ther. 2006;5(11):2624–33.
15. Pan D, et al. Nanomedicine: perspective and promises with ligand-directed molecular imaging. Eur J Radiol. 2009;70(2):274–85.
16. Cheson BD, et al. Revised response criteria for malignant lymphoma. J Clin Oncol. 2007;25(5):579–86.
17. Juweid ME, et al. Use of positron emission tomography for response assessment of lymphoma: consensus of the Imaging Subcommittee of International Harmonization Project in Lymphoma. J Clin Oncol. 2007;25(5):571–8.
18. Kazama T, et al. FDG PET in the evaluation of treatment for lymphoma: clinical usefulness and pitfalls. Radiographics. 2005;25(1):191–207.
19. Sundaram S, et al. FDG PET imaging in multiple myeloma: implications for response assessments in clinical trials. Am J Nucl Med Mol Imaging. 2018;8(6):421–7.
20. Sachpekidis C, Goldschmidt H, Dimitrakopoulou-Strauss A. *Positron Emission Tomography (PET) Radiopharmaceuticals in Multiple Myeloma*. Molecules, 2019. 25(1).
21. Ehlerding EB, et al. Molecular Imaging of Immunotherapy Targets in Cancer. J Nucl Med. 2016;57(10):1487–92.
22. Ghai A, et al. Development of [(89)Zr]DFO-elotuzumab for immunoPET imaging of CS1 in multiple myeloma. Eur J Nucl Med Mol Imaging. 2021;48(5):1302–11.
23. Rasche L, et al. Low expression of hexokinase-2 is associated with false-negative FDG-positron emission tomography in multiple myeloma. Blood. 2017;130(1):30–4.
24. Ulaner GA, et al. (18)F-FDG PET/CT for Systemic Staging of Newly Diagnosed Breast Cancer in Men. J Nucl Med. 2019;60(4):472–7.
25. Lapa C, et al. [(68)Ga]Pentixafor-PET/CT for imaging of chemokine receptor CXCR4 expression in multiple myeloma - Comparison to [(18)F]FDG and laboratory values. Theranostics. 2017;7(1):205–12.
26. Sikkandhar MG, et al., *Theranostic Probes for Targeting Tumor Microenvironment: An Overview*. Int J Mol Sci, 2017. 18(5).

27. Soodgupta D, et al., *Very late antigen-4 (alpha(4)beta(1) Integrin) targeted PET imaging of multiple myeloma*. PLoS One, 2013. **8**(2): p. e55841.
28. Holzmann B, Gossler U, Bittner M, *alpha 4 integrins and tumor metastasis*. Curr Top Microbiol Immunol, 1998. **231**: p. 125 – 41.
29. Matsunaga T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med. 2003;9(9):1158–65.
30. Damiano JS, Dalton WS. Integrin-mediated drug resistance in multiple myeloma. Leuk Lymphoma. 2000;38(1–2):71–81.
31. Soodgupta D, et al. Ex Vivo and In Vivo Evaluation of Overexpressed VLA-4 in Multiple Myeloma Using LLP2A Imaging Agents. J Nucl Med. 2016;57(4):640–5.
32. Beaino W, Anderson CJ. PET imaging of very late antigen-4 in melanoma: comparison of ⁶⁸Ga- and ⁶⁴Cu-labeled NODAGA and CB-TE1A1P-LLP2A conjugates. J Nucl Med. 2014;55(11):1856–63.
33. Mattila JT, et al. Positron Emission Tomography Imaging of Macaques with Tuberculosis Identifies Temporal Changes in Granuloma Glucose Metabolism and Integrin alpha4beta1-Expressing Immune Cells. J Immunol. 2017;199(2):806–15.
34. Peng L, et al. Combinatorial chemistry identifies high-affinity peptidomimetics against alpha4beta1 integrin for in vivo tumor imaging. Nat Chem Biol. 2006;2(7):381–9.
35. <https://ClinicalTrials.gov/show/NCT03804424>.
36. Kouroukis TC, et al. Bortezomib in multiple myeloma: systematic review and clinical considerations. Curr Oncol. 2014;21(4):e573–603.
37. Noborio-Hatano K, et al. Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. Oncogene. 2009;28(2):231–42.
38. Sevilla-Movilla S, et al. Upregulated expression and function of the alpha4beta1 integrin in multiple myeloma cells resistant to bortezomib. J Pathol. 2020;252(1):29–40.
39. Wahl RL, et al. From RECIST to PERCIST: Evolving Considerations for PET response criteria in solid tumors. J Nucl Med. 2009;50(Suppl 1):122S-50S.
40. Mankoff DA, Katz SI. PET imaging for assessing tumor response to therapy. J Surg Oncol. 2018;118(2):362–73.
41. Vanderheyden JL. The use of imaging in preclinical drug development. Q J Nucl Med Mol Imaging. 2009;53(4):374–81.
42. Ghai A, et al. Preclinical Development of CD38-Targeted [(89)Zr]Zr-DFO-Daratumumab for Imaging Multiple Myeloma. J Nucl Med. 2018;59(2):216–22.
43. Pan Q, et al. Chemokine receptor-4 targeted PET/CT with (68)Ga-Pentixafor in assessment of newly diagnosed multiple myeloma: comparison to (18)F-FDG PET/CT. Eur J Nucl Med Mol Imaging. 2020;47(3):537–46.
44. Ulaner GA, et al. CD38-targeted Immuno-PET of Multiple Myeloma: From Xenograft Models to First-in-Human Imaging. Radiology. 2020;295(3):606–15.

45. Hillengass J, et al. International myeloma working group consensus recommendations on imaging in monoclonal plasma cell disorders. *Lancet Oncol.* 2019;20(6):e302–12.
46. Anderson KC, et al. The Role of Minimal Residual Disease Testing in Myeloma Treatment Selection and Drug Development: Current Value and Future Applications. *Clin Cancer Res.* 2017;23(15):3980–93.
47. Durie BG, et al. Whole-body (18)F-FDG PET identifies high-risk myeloma. *J Nucl Med.* 2002;43(11):1457–63.
48. Zamagni E, et al. Prognostic relevance of 18-F FDG PET/CT in newly diagnosed multiple myeloma patients treated with up-front autologous transplantation. *Blood.* 2011;118(23):5989–95.
49. Harzschel A, et al., *VLA-4 Expression and Activation in B Cell Malignancies: Functional and Clinical Aspects.* *Int J Mol Sci,* 2020. 21(6).
50. Perkins LA, et al. Integrin VLA-4 as a PET imaging biomarker of hyper-adhesion in transgenic sickle mice. *Blood Adv.* 2020;4(17):4102–12.
51. Cook GJ, Fogelman I, Maisey MN. Normal physiological and benign pathological variants of 18-fluoro-2-deoxyglucose positron-emission tomography scanning: potential for error in interpretation. *Semin Nucl Med.* 1996;26(4):308–14.
52. Baschnagel AM, et al. The association of (18)F-FDG PET and glucose metabolism biomarkers GLUT1 and HK2 in p16 positive and negative head and neck squamous cell carcinomas. *Radiother Oncol.* 2015;117(1):118–24.
53. Ghai A, et al. Development of [(89)Zr]DFO-elotuzumab for immunoPET imaging of CS1 in multiple myeloma. *Eur J Nucl Med Mol Imaging.* 2021;48(5):1302–11.
54. Hosen N. Integrins in multiple myeloma. *Inflamm Regen.* 2020;40:4.

Figures

Figure 1

Imaging and therapy study design. Nod-SCID Gamma (NSG) mice were injected with human myeloma cell lines, MM.1S-CG and U266-CG via tail vein. Tumor progression was monitored weekly by

bioluminescence imaging (BLI). Mice were divided into three cohorts – tumor bearing, untreated (n=6), tumor bearing, treated (n=6) and no tumor mice (n=4). The treatment group was injected with bortezomib (1mg/kg) twice a week intraperitoneally starting at week 1 post inoculation of MM.1S-CG and U266-CG cells respectively. Mice were imaged weekly with small animal [18F]FDG PET and [64Cu]LLP2A PET respectively. Untreated tumor bearing mice died due to disease related complications or were euthanized at the onset of paralysis.

Figure 2

(a-b) Representative BLI images of mice injected with MM.1S-CG cells and U266-CG cells respectively (treated and untreated cohorts) showing systemic tumor progression and response to bortezomib therapy over time. Bortezomib therapy was started for mice in the treated cohort between weeks 0-1, after tumor inoculation. (c-d) Percent increase in bioluminescence signal intensity showing tumor progression in treated and untreated cohorts plotted as a function of time (number of weeks post implantation).

Figure 3

(a) Small animal maximum intensity projection (MIP) images with ^{18}F -FDG and ^{64}Cu -LLP2A tracers in mice injected with MM.1S-CG cells (treated and untreated cohort) via tail vein over time. Healthy mice with no tumors were also imaged with ^{18}F -FDG and ^{64}Cu -LLP2A respectively. Bortezomib therapy was started in tumor bearing mice in the treated cohort between weeks 0-1. (b) SUVmean quantification using ^{18}F -FDG and ^{64}Cu -LLP2A in different organs of MM.1S-CG systemic tumor bearing and healthy mice at week 0; before therapy was started (baseline). Percent change in SUV of different organs in MM.1S-CG tumor (treated and untreated) and no tumor mice with (c) ^{18}F -FDG, and (d) ^{64}Cu -LLP2A, plotted as a function of time. * P value ≤ 0.05 , ** P value ≤ 0.01

Figure 4

(a) Small animal maximum intensity projection (MIP) images with [18F]FDG and [64Cu]LLP2A tracers in mice injected with U266-CG cells (treated and untreated cohort) via tail vein over time. Healthy mice with no tumors were also imaged with [18F]FDG and [64Cu]LLP2A respectively. Bortezomib therapy was started in mice in the treated cohort between weeks 0-1. (b) SUVmean quantification using [18F]FDG and [64Cu]LLP2A in spine, right and left femur of U266-CG disseminated tumor bearing and healthy mice at week 0 respectively at baseline (before initiation of therapy). Percent change in SUV of different organs in U266-CG tumor (treated and untreated) and no tumor mice with (c) [18F]FDG and (d) [64Cu]LLP2A plotted as a function of time. * P value ≤ 0.05

Figure 5

Representative CD49d/ $\alpha 4$ (upper panel) and CD29/ $\beta 1$ (lower panel) surface expression levels in (a) MM.1S (b) U266 cell lines analyzed by flow cytometry. (c) Comparison of CD49d mean fluorescence intensity (MFI) values among MM.1S and U266 cell lines normalized to the isotype control. (d) Comparison of CD29 MFI values among MM.1S and U266 cell lines normalized to the isotype control. Representative CD49d/ $\alpha 4$ and CD29/ $\beta 1$ surface expression levels in BM cells of (e) MM.1S-CG cells injected mice (f) U266-CG cells injected mice analyzed by flow cytometry.

Supplementary Files

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