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Original Article

A radioactive and fluorescent dual modality cysteine cathepsin-B activity-based probe for the detection and treatment evaluation in rheumatoid arthritis

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Abstract: Activated macrophages are key effector cells and specific markers in patients with rheumatoid arthritis (RA). Cysteine cathepsin B (CTS-B) is highly expressed in macrophages and positively associated with RA activity and severity. This study aims to evaluate an activity-based multi-modality diagnostic agent, ^{68}Ga-BMX2, which targets CTS-B to visualize the arthritis activity and evaluate the treatment efficacy. A CTS-B activity-based probe, BMX2, was labeled efficiently with ^{68}Ga to produce ^{68}Ga-BMX2 for fluorescent and positron emission tomography (PET) multi-modality imaging. The affinity and specificity of BMX2 binding with the CTS-B enzyme in macrophages were determined by radioactive experiment using RAW 264.7 cell lines, with CA074 and BMX5 as the inhibitors to test the specificity of the binding. Then, PET and fluorescence imaging were acquired on collagen-induced arthritis (CIA) mice. Additionally, the treatment monitoring capability of ^{68}Ga-BMX2 PET/CT imaging was tested with methotrexate (MTX). RAW 264.7 macrophage cells showed significant uptake of ^{68}Ga-BMX2. The binding of BMX2 with CTS-B in RAW 264.7 macrophage cells is time-dependent and could be blocked by CA074 and BMX5. *In vivo* optical and PET imaging showed high signals in the right hind arthritis in CIA mice from ^{68}Ga-BMX2 and BMX2 accumulated for at least 120 h. Additionally, ^{68}Ga-BMX2 signals were significantly reduced in the MTX-treated CIA mice compared to the control group. The ^{68}Ga-BMX2, a radioactive and fluorescent dual-modality diagnostic agent targeting CTS-B, demonstrated a practical approach for CIA PET and fluorescence imaging. The ^{68}Ga-BMX2 multimodality imaging could significantly monitor the treatment response in the CIA mice.

Keywords: Cysteine cathepsin B, activity-based probe, multimodality imaging, rheumatoid arthritis, positron emission tomography

Introduction

Rheumatoid arthritis (RA) is a long-lasting and incapacitating condition characterized by uncontrolled inflammation in movable joints, impacting around 1% of the global population [1]. Untreated RA can result in the deterioration and impairment of the affected joints, as well as systemic problems and perhaps premature mortality [2]. The degradation of bone and cartilage in joints affected by RA is mainly caused by synovial fibroblasts and activated macrophages. These cells can affect the formation of osteoclasts by releasing enzymes that break down the extracellular matrix and substances that promote inflammation [3]. An earlier rheumatology visualization or the start of disease-modifying antirheumatic drug (DMARD) therapy, such as methotrexate, is linked to a milder progression of RA through radiographic assessment, as well as a greater likelihood of achieving early and long-lasting remission without the need for DMARDs [4]. Nevertheless, there are still patients who experience synovial inflammation while achieving clinical remission or low disease activity [5]. Persistent inflammation can result in the recurrence of RA and the development of bone erosion lesions [6]. Therefore, the assessment of the RA activity is crucial for the subsequent management of patients.

Many imaging modalities used in clinical practice, such as musculoskeletal ultrasonography (MSUS) or MRI, are sensitive but not specific in detecting musculoskeletal inflammation. This lack of specificity makes it difficult to distinguish between different types of diseases and hampers the development of targeted and personalized treatment strategies [7]. Functional imaging is a promising method that can be used to characterize inflammation in living organisms without the need for invasive procedures. It has the potential to greatly improve patient care by enabling early diagnosis, distinguishing between different types of diseases, and selecting personalized treatment options [8]. Optical molecular imaging is a highly promising tool for detecting early RA due to its ability to provide detailed images with great spatial resolution. Furthermore, Positron emission tomography/computed tomography (PET/CT) imaging is extensively utilized due to its ability to provide excellent image resolution and functional characterization [9, 10].

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Studies demonstrated that activated macrophages were the predominant cell type found in a synovial biopsy of RA and consistent with the degree of joint erosion [3] and the severity of RA [4]. Activated synovial macrophages are a crucial indicator for evaluating the level of disease activity in the initial phases and for tracking treatment efficacy in later stages of the disease [5, 6]. Therefore, molecular imaging specifically targeting activated macrophages is a promising method to detect RA activity. The advancement of (R)-[11C]PK11195 targeting macrophage probes enables RA imaging, but the significant absorption of (R)-[11C]PK11195 in the surrounding tissue near the joints hinders the ability to visually analyze minor variations in the uptake of the probe within the joints [11].

Hence, the utilization of agents capable of accurately identifying activated macrophages with a high level of sensitivity shows potential as contrast agents for the evaluation and monitoring of RA. Cysteine cathepsin (CTS) is a prominent family of proteases that are generated by activated macrophages. They have a crucial function in causing joint degradation and are therefore important targets for evaluating and monitoring disease treatment [16, 17]. CTS-B, a member of the CTS family, is significantly increased in activated macrophages and shows a favorable association between the proportion of activated macrophages and the disease activity of rheumatoid arthritis [12-14]. The particular inhibitor CA074 [15], which targets CTS-B, effectively reduced lipid peroxidation and mitochondrial dysfunction in activated macrophages. Additionally, it repressed macrophage pyroptosis, leading to the alleviation of inflammation [19, 20]. Isotope-labeled CTS-B probes show potential for the image-based evaluation of RA and may be useful for evaluating macrophage activation. Several CTS-specific probes have been developed for optical and PET imaging to track the advancement of tumors, cardiovascular disease, and lung inflammation [17-19]. Nevertheless, the efficacy of cathepsin protease probes containing optical and PET tracers has not been evaluated in RA models.

This study utilized CTS-B targeted agent, BMX2, which is a Cbz-Phe-Lys peptide coupled with a sulfo-cyanine 5.5 for fluorescence imaging. Additionally, it was labeled with Gallium-68 (^{68}Ga, half-life = 68 min, β^+ emitter) using a 1,4,7,10-Tetraazacyclododecane (DOTA) for radionuclide labeling. The efficacy of ^{68}Ga-BMX2 in detecting CTS-B was assessed both *in vitro* and *in vivo*. The ability of ^{68}Ga-BMX2 for CTS-B detection *in vitro* and *in vivo* was evaluated. Finally, the uptake difference between treated and control models of RA was tested to investigate the potential application of ^{68}Ga-BMX2 on treatment monitoring.

Methods

Materials

The sulfo-cyanine5.5-NHS ester was purchased from Lumiprobe Corporation (USA). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester (DOTA-NHS-ester) was bought from Macrocyclics, Inc. (USA). Ammonium acetate (NH_4OAc) and other chemicals were purchased from Sigma Aldrich. $^{68}GaCl_3$ was eluted from a ^{68}Ge-^{68}Ga generator (ITM, Germany) with 0.05 M super-pure Hydrogen chloride solution (HCl). The DMEM medium and penicillin-streptomycin were bought from Gibco (C11995500BT and 15070063, respectively). Hank's Buffer was purchased from Solarbio (H1025), and HEPES was purchased from Sigma-Aldrich (7365-45-9). RAW 264.7 macrophage cell lines were bought from SUNNLL (China). RIPA buffer was purchased from Beyotime (P0013B). Bovine type II collagen (2 mg/ml) and complete and incomplete Freund Adjuvant were purchased from Chondrex. Dimethyl sulfoxide (DMSO) was bought from Sigma-Aldrich (276855). CA074 was purchased from APExBIO (A1926). Methotrexate (MTX) was bought from APExBIO (A4347). The DBA/1 male mice were bought from Shouzheng Pharma (Wuhan) Biotechnology Co., Ltd.

Synthesis of ^{68}Ga-BMX2

The Cbz-Phe-Lys-AOMKs were produced using an improved synthesis route based on a previously documented approach [22]. Then, the amine terminal of the side chain was altered using lysine to attach sulfo-cyanine5.5-NHS ester and DOTA-NHS-ester conjugating to get the ultimate precursor BMX2. The synthesis scheme and more information can be found in the previous study [20].

To perform ^{68}Ga radiolabeling, the BMX2 solution (20 μg, 1 μg/μL in pure water) was mixed with 500 μL ammonium acetate buffer (NH_4OAc, 0.5 M, pH = 5.0) and was degassed for 3 min. Next, $^{68}GaCl_3$ (925-1295 MBq) was eluted with 4 mL HCl (0.05 M) and transferred into the reaction vial. It was then mixed with 280 μL of NH_4OAc buffer (1.0 M, pH 5.0) to be diluted. Subsequently, the BMX2 solution was transferred into the reaction vial, and the pH of the resulting mixture was measured as 4.5-5.0.

Following incubation at a temperature of 90°C for a duration of 10 minutes, the solution was initially diluted by 10-fold using sterilized water and injected through a Sep-Pak C18 Light Cartridge (Waters, USA), followed by a 10 mL water flush. The product that was retained on the C18 column was gradually washed out using a 1 mL solution of 70% ethanol and formulated with 10 mL saline containing 1% ascorbic acid. Prior to injection into the mice, the solution was passed through a 0.22 μm Millipore filter. The filtered solution was then transferred into a sterile vial and divided into many vials for either cell study or in vivo investigation.

Cellular uptake assay

The RAW 264.7 macrophage cell line was cultivated in DMEM media, which was enriched with 10% FBS and 1%

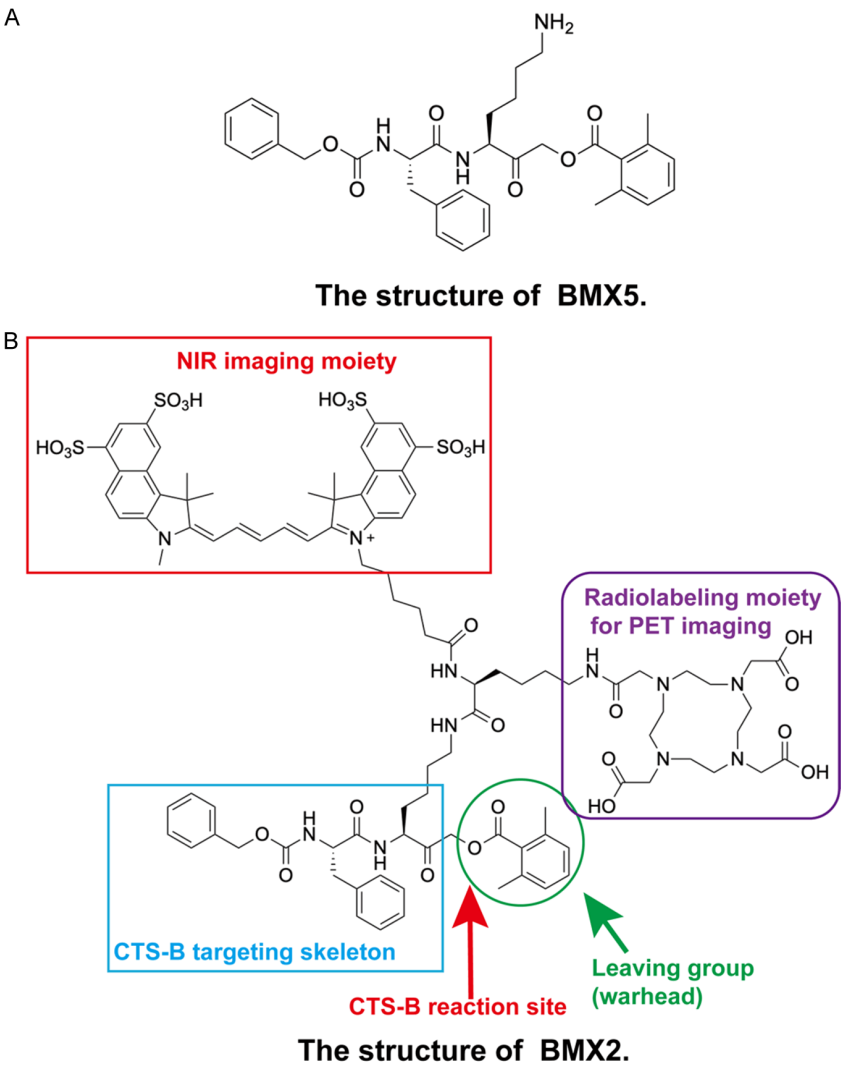

Figure 1. The structure of BMX5 and BMX2.

penicillin-streptomycin at 37°C in a humidified environment with 5% CO_2. The day prior to the cellular uptake test, 1×10^6 RAW 264.7 macrophage cells were individually placed in 6-well plates and left to incubate overnight. The initial batch of RAW 264.7 macrophage cells was subsequently incubated with ^{68}Ga-BMX2 (37 kBq/well) for 30 min, 60 min, 120 min, and 240 min in Hank's Buffer with HEPES. The second set of RAW 264.7 macrophage cells was then incubated with BMX5 (the precursor of BMX2, as depicted in **Figure 1**) and ^{68}Ga-BMX2 (BMX5 1 μM and ^{68}Ga-BMX2 37 kBq/well) for 30 min, 60 min, 120 min, and 240 min in Hank's Buffer with HEPES. The third set of RAW 264.7 macrophage cells was subsequently incubated with CA074 and ^{68}Ga-BMX2 (CA074 25 μM and ^{68}Ga-BMX2 37 kBq/well) 30 min, 60 min, 120 min, and 240 min in Hank's Buffer with HEPES. Subsequently, every cell sample was disrupted using 150 μL of RIPA buffer and gathered in an Eppendorf tube. The radioactivity and protein concentrations were assessed independently to ascertain cellular absorption. The results were expressed as the radioactivity added per gram of protein, represented as a percentage (%ID/g).

Type II collagen-induced arthritis (CIA) model

All animal experiments adhered to the Animal Care and Use Committee guidelines of the Department of Animal Resources, the Second Xiangya Hospital of Central South University (No. 20230596). Male DBA/1 mice were randomly allocated to either the CIA group or the healthy control (HC) group. In order to create the CIA model, bovine type II collagen was combined with an equal volume of complete Freund Adjuvant in a container filled with ice. To construct the CIA model, bovine type II collagen was combined with an equal volume of complete Freund Adjuvant in a container with an ice bath. Following complete emulsification, the mice were initially vaccinated by injecting 100 μL of the emulsion into the subcutaneous tissue of the right hind foot plantar. This was recorded as day (D) 0. On D14, the booster vaccination was conducted by mixing 2 mg/mL of bovine type II collagen with an equal volume of incomplete Freund Adjuvant and then phaco-emulsified in an ice bath. The mice were administered a 100 μL emulsion by injection at the plantar region of the right hind foot. Arthritis was observed in 90% of cases between 42 and 56 days. Healthy control mice were administered a simulated injection in the right hind foot plantar region under identical conditions. Following the booster vaccine, the erythema and edema of the ankle joint were documented every two days. The severity of arthritis inflammation was assessed using a grading scale ranging from 0 to 4. The grading criteria were as follows [21]: 0 indicated paws with no swelling or focal redness, 1 indicated paws with swollen joints, 2 indicated paws with mild swelling of ankle or wrist joints, 3 indicated paws with severe inflammation, and 4 indicated paws with deformity or ankylosis. Ultimately, the mice were euthanized after the study.

Optical/PET imaging and biodistribution

On day 21, the mice from the CIA were separated into four groups in a random manner for the PET imaging scan. These groups included two blocked groups, one with ^{68}Ga-BMX2 with CA074, and the other with ^{68}Ga-BMX2 with BMX5. There was also a non-blocked group with only ^{68}Ga-BMX2, as well as a healthy control group with only ^{68}Ga-BMX2. The mice were administered anesthesia and then received an intravenous injection of either CA074 or BMX5 (4 mg/kg body weight) or the equivalent volume of

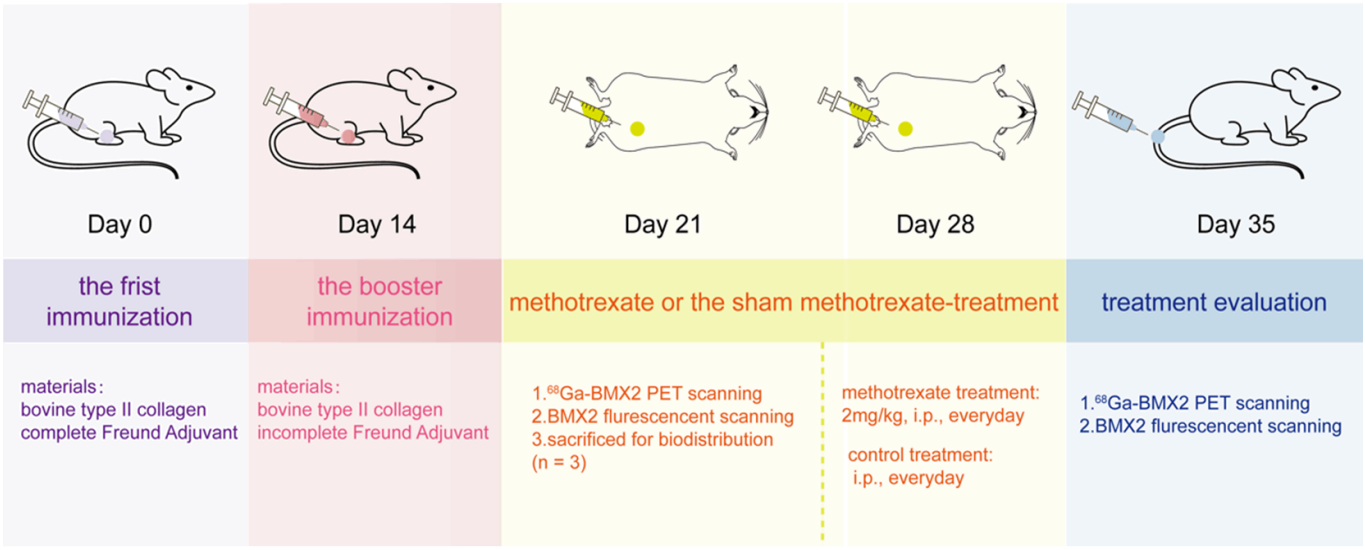

Figure 2. Experiment flowchart of this study. DBA/1 male mice were used to establish a CIA mouse model.

saline solution 60 min before ^{68}Ga-BMX2 injection. The mice were first anesthetized and then set on the micro-PET scanner (Inveon small-animal PET/CT, Siemens) for CT imaging, followed by intravenously injecting 200 μCi of ^{68}Ga-BMX2. Imaging acquisition commenced promptly for a 1-hour dynamic PET scan, followed by a static PET/CT scan at 2 hours and 4 hours after injection. Subsequently, quantitative imaging assays were performed. The level of radioactivity in the particular tissue was quantified as a percentage of the injected dose per gram of tissue, taking into account the decay over time (%ID/g).

The biodistribution of ^{68}Ga-BMX2 was assessed at 4 hours to evaluate the uptake and metabolic pathway of arthritis in the right hind arthritis after PET imaging. The mice (n = 3 for each group) were euthanized, sacrificed, and dissected at 4 hours after injection. All relevant organs were collected and weighed to determine the biodistribution of the ^{68}Ga-BMX2. The level of radioactivity in the tissues was quantified using a γ counter (CAPRAC-t well counter, USA). The results are expressed as the proportion of the administered dosage per gram of tissue (%ID/g).

Fluorescence imaging was conducted to assess the *in vivo* distribution and uptake of BMX2 in the four aforementioned groups. The mice were administered anesthesia and then received an intravenous injection of either CA074 or BMX5 (4 mg/kg body weight), or an equivalent volume of saline solution, 60 minutes prior to the injection of BMX2. Subsequently, BMX2 was administered intravenously via the tail vein, and fluorescent images were acquired by the ChemiDocTM MP imaging system (BIO-RAD) at several time points (pre-injection, 2, 24-, 48-, 96-, and 120-hour post-injection) to observe the prolonged accumulation of BMX2 in arthritis.

Hematoxylin and eosin (H&E) staining and Safranin O/Fast Green staining

After the mice were sacrificed, the ankle joints of the right hind limb were immersed in 10% formalin for 24 hours. After decalcification with 10% EDTA for 21 days, the joints were sectioned at 5 μm sections and stained with haematoxylin and eosin (H&E). Safranin O/Fast Green (O-S) staining sections of the ankle joint sections was used to evaluate the cartilage structure, chondrocytes, cartilage matrix, and the tidal line integrity.

Treatment evaluation of CIA mice

To evaluate the effectiveness of BMX2 in monitoring treatment, the CIA mice were divided into two groups: the group treated with MTX and the group treated with a fake MTX. MTX, at a concentration of 100 mg, was dissolved in 5 ml of DMSO, properly mixed, and then stored. When necessary, a suitable quantity of dilution was obtained and combined with ultrapure water, resulting in a final concentration of 0.2 mg/ml. CIA mice were administered intraperitoneal injections of MTX (2 mg/ml per day) or a placebo for a duration of 2 weeks. Subsequently, the degree of redness and swelling in the ankle joint was documented, followed by the execution of PET imaging. The experimental flowchart is shown in **Figure 2**.

Multiple immunofluorescence (MIF) staining

MIF staining was performed in the inflamed arthritis of CIA mice before and after the MTX treatment according to standard procedures. After decalcification with 10% ethylene diamine tetraacetic acid (EDTA) for 21 days, the joints were sectioned at 5 μm sections and stained with the above antibodies. Inflammatory arthritis synovial tissue was incubated with anti-CTS-B (1:900, ab214428;

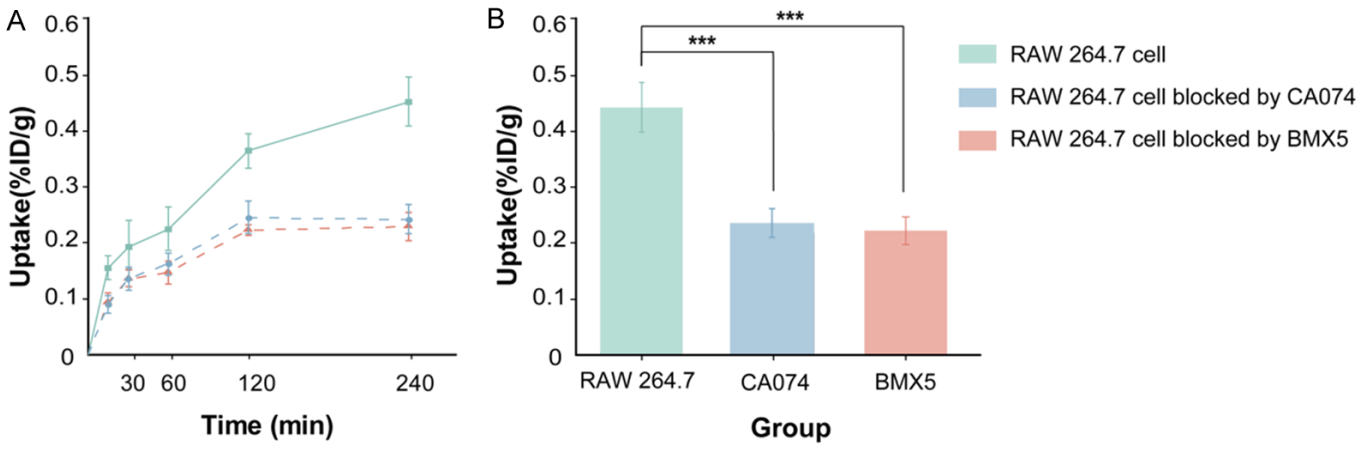

Figure 3. ^{68}Ga-BMX2 uptake in RAW 264.7 macrophage cells. A. Dynamic uptake of ^{68}Ga-BMX2 or ^{68}Ga-BMX2 with CA074 or BMX5 inhibition in RAW 264.7 macrophage cells at 30 min, 60 min, 120 min, and 240 min. B. Quantitative analysis of ^{68}Ga-BMX2 or ^{68}Ga-BMX2 with CA074 or unlabeled BMX2 inhibition in RAW 264.7 macrophage cells at 240 min incubation (*P < 0.05, **P < 0.01, ***P < 0.001).

Abcam) and anti-CD68 (1:500, ab201340; Abcam) antibodies. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and the images were viewed under a fluorescence microscope (Olympus, BX53). Finally, the images were processed using the ImageJ software (NIH, USA).

Statistical analysis

Data were expressed as mean ± SEM. Unpaired Student t-test was used to determine the statistical significance of differences between groups using Origin pro 2021 (OriginLab). Statistical significance was defined as a *p*-value less than 0.05.

Results

Cell uptake

Cell uptake experiments were conducted *in vitro* to evaluate the CTS-B-mediated uptake ^{68}Ga-BMX2 in RAW 264.7 macrophage cells. The findings demonstrated that the probes were absorbed by RAW 264.7 macrophage cells in a manner that depended on the duration of time. The highest absorption rate observed was 0.43 ± 0.07 %ID/g for ^{68}Ga-BMX2 after 4 hours. The uptake was markedly blocked by CA074 (0.24 ± 0.01 %ID/g) and BMX2 (0.22 ± 0.01 %ID/g) (**Figure 3**).

Optical/PET imaging and biodistribution

Collagen-induced arthritis (CIA) models, reflecting similar immunological and pathological features as human RA, are widely used for studying autoimmune mechanisms [22]. Thus, we employed DBA/1 male mice to construct the CIA model (**Figure 4**).

To assess the detective efficacy of ^{68}Ga-BMX2 for CIA, BMX2 was labeled with ^{68}Ga and utilized for PET imaging of CIA mice, CIA mice treated with CA074, BMX5 mice, and HC mice. The imaging of inflammatory arthritis in CIA mice was easily identified with strong contrast after the injection, in comparison to the background of the other side and the HC mice (**Figure 5A**, **5B**). The dynamic imaging revealed a persistent accumulation of ^{68}Ga-BMX2 in the inflammatory arthritis of CIA mice, which was inhibited by CA074 or BMX5 (**Figure 5C**, **5D**). The inflamed arthritis uptake and inflamed arthritis to muscle ratio of ^{68}Ga-BMX2 in the CIA group were 2.17 ± 0.29 %ID/g and 3.42 ± 0.62, respectively, at 4 h post-injection. Pre-injecting CA074 or BMX5 significantly inhibited the uptake of ^{68}Ga-BMX2 in inflamed arthritis. At 4 hours post-injection, the inflamed arthritis uptake and the ratio of inflamed arthritis to muscle for ^{68}Ga-BMX2 were 0.49 ± 0.07 %ID/g and 1.17 ± 0.11, respectively, when CA074 was used. When BMX5 was utilized at a dose of 4 mg/kg body weight, the inflamed arthritis uptake and the ratio of inflamed arthritis to muscle for ^{68}Ga-BMX2 were 0.78 ± 0.19 %ID/g and 0.84 ± 0.32, respectively. However, the arthritis uptake and arthritis-to-muscle ratio in HC mice were notably lower, measuring 0.36 ± 0.10 %ID/g and 0.49 ± 0.07, respectively (**Figure 6A**). The biodistribution patterns of the probe in normal organs were found to be similar in both mice groups, as demonstrated by dynamic and delayed static imaging.

Arthritis targeting and biodistribution properties of ^{68}Ga-BMX2 were assessed after the last PET scan. The biodistribution test revealed that the ^{68}Ga-BMX2 exhibited significant accumulation in the arthritis-affected area of the right hind limb of the CIA mice, with a value of 4.03 ± 0.24 %ID/g at 4 hours after injection. In comparison, the HC mice showed a much lower accumulation of 0.83 ± 0.11 %ID/g (**Figure 6B**).

Due to the short half-life of ^{68}Ga, PET imaging could only be acquired for ideal imaging within 4 h post-injection. Hence, fluorescence imaging was conducted to observe the long-term distribution of BMX2 *in vivo*. Results showed

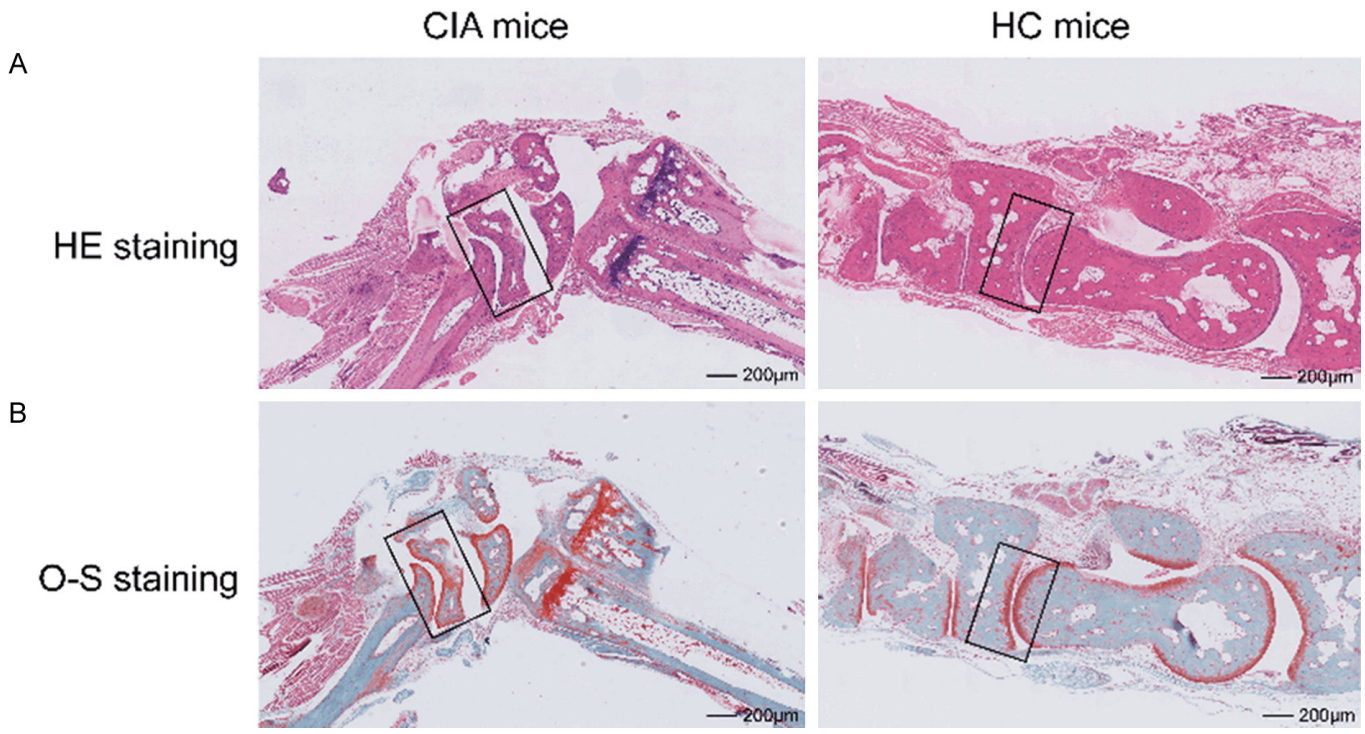

Figure 4. HE and O-S staining of CIA and HC mice. A. HE staining demonstrates that the synovial hyperplasia in the joint cavity of CIA mice was much severe than that of HC mice. Safranine O staining indicates that the cartilage destruction in the joint synovium of CIA mice were significantly ameliorated compared with those HC mice. B. By utilizing safranin O/Fast Green staining to illustrate the destruction of cartilage and bone damage in inflamed ankle joints, we found revealed significant destruction of the cartilage structure, notable reduction in chondrocyte count, and loss of the cartilage matrix in the CIA mice.

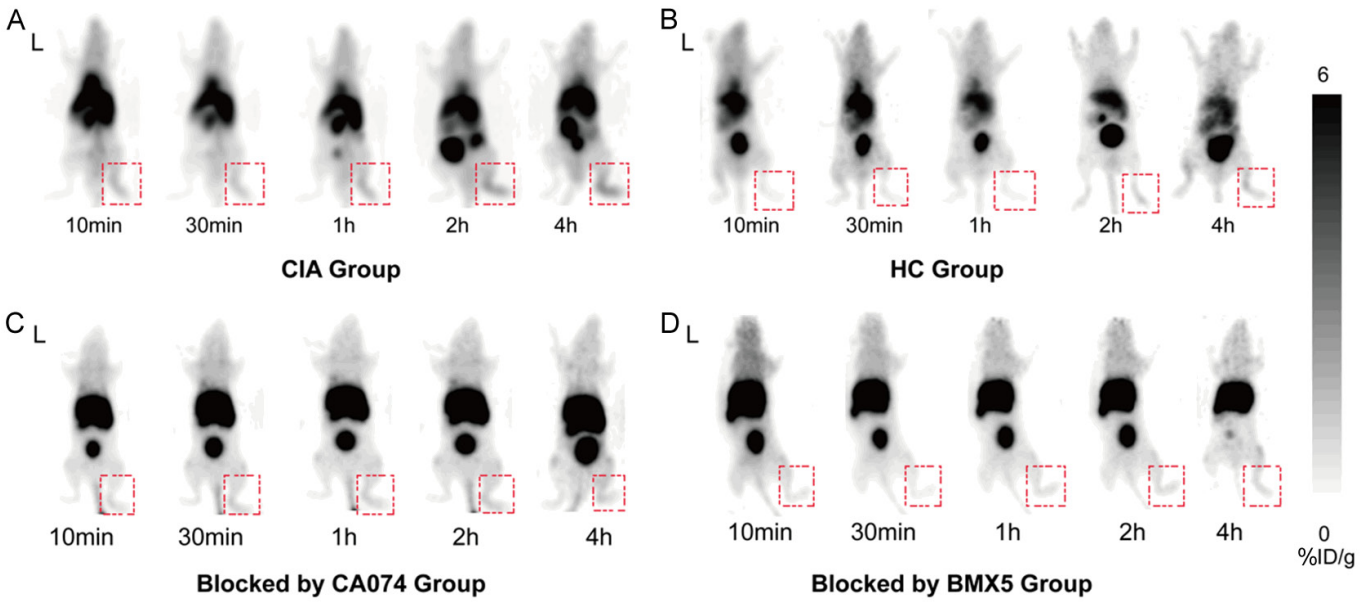

Figure 5. Representative PET images obtained at 10 min, 30 min, 1 h, 2 h, and 4 h after injection of ^{68}Ga-BMX2 in (A) CIA, (B) HC mice, (C) CIA + CA074 inhibited, and (D) CIA + BMX5 inhibited mice (the red block represents the arthritis of right rear foot).

that the BMX2 has the ability to accumulate in the inflamed arthritis for a minimum of 120 hours, showcasing its promising potential for radionuclide therapy in arthritis. The CA074 and BMX5 compounds effectively suppressed the fluorescence signal. In the HC mice group, the BMX2 accumulation is limited, as seen in **Figure 7**.

Treatment monitoring

In our study, MTX was employed as a modifying antirheumatic drug to validate the capability of ^{68}Ga-BMX2 in monitoring the treatment of CIA [23]. *In vivo* PET imaging was conducted in those with or without MTX-treated CIA mice.

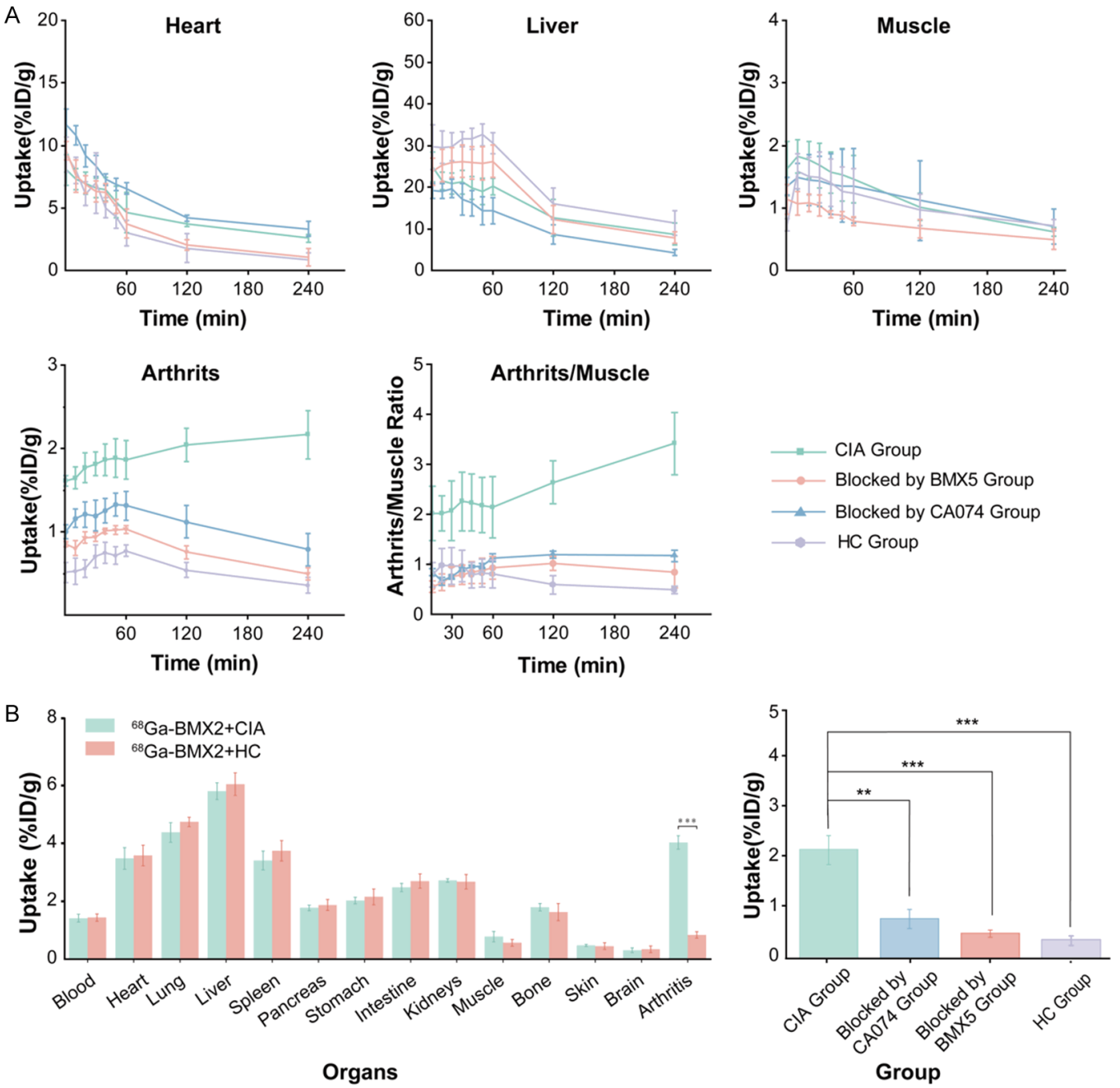

Figure 6. A. Quantitative analysis of the probe in the heart, liver, muscle, arthritis, and arthritis/muscle ratio from above groups in **Figure 2** (**P < 0.01, ***P < 0.001). B. Biodistribution of ^{68}Ga-BMX2 at 4 h after injection in CIA and HC mice and analysis of the arthritis/muscle ratio in 4 h after injection (*P < 0.05, **P < 0.01, ***P < 0.001).

The results demonstrated that MTX-treated CIA mice experienced a swelling reduction after the MTX treatment, as indicated by a decrease in the CIA score from 3 to 2 (**Figure 8A**). In contrast, the sham MTX-treated group showed no change in the CIA score, remaining at a score of 3 (**Figure 8B**). These findings were comparable with the results obtained from PET/CT scanning (**Figure 8C-F**), which showed a similar pattern of uptake. The PET imaging quantification revealed that the signal at 4 hours in cases of inflammatory arthritis, with or without MTX treatment, was measured at 3.14 ± 0.62 %ID/g and 2.18 ± 0.09 %ID/g, respectively (**Figure 8G**). Similarly, the ratios of inflammatory arthritis to muscle were 3.37 ± 0.35 and 1.32 ± 0.12, respectively, as shown in **Figure 8H**. Compared with the sham MTX-treated CIA mouse, MIF staining dedicated that the significantly decrease of CTS-B in activated macrophage in MTX-treated ones (**Figure 9**), which is consistent with our imaging results.

Discussion

Macrophages that have been activated, together with other immune cells, invade the synovium which has the potential to cause damage to both cartilage and bone,

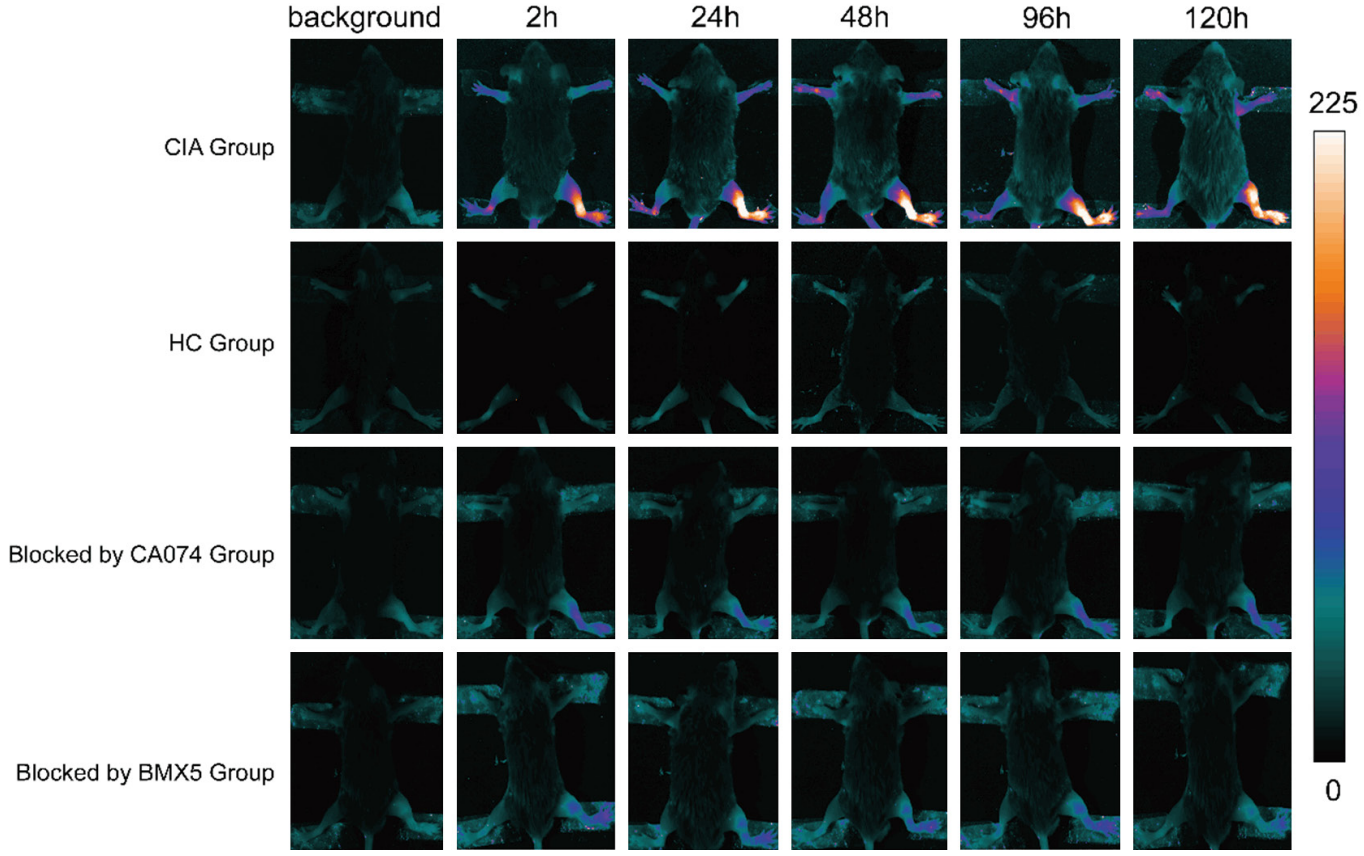

Figure 7. *In vivo* fluorescent imaging of CIA, HC, CIA blocked by BMX5, and CIA blocked by CA074 mice at pre-injection, 2 h, 24 h, 48 h, 96 h and 120 h post-injection of BMX2.

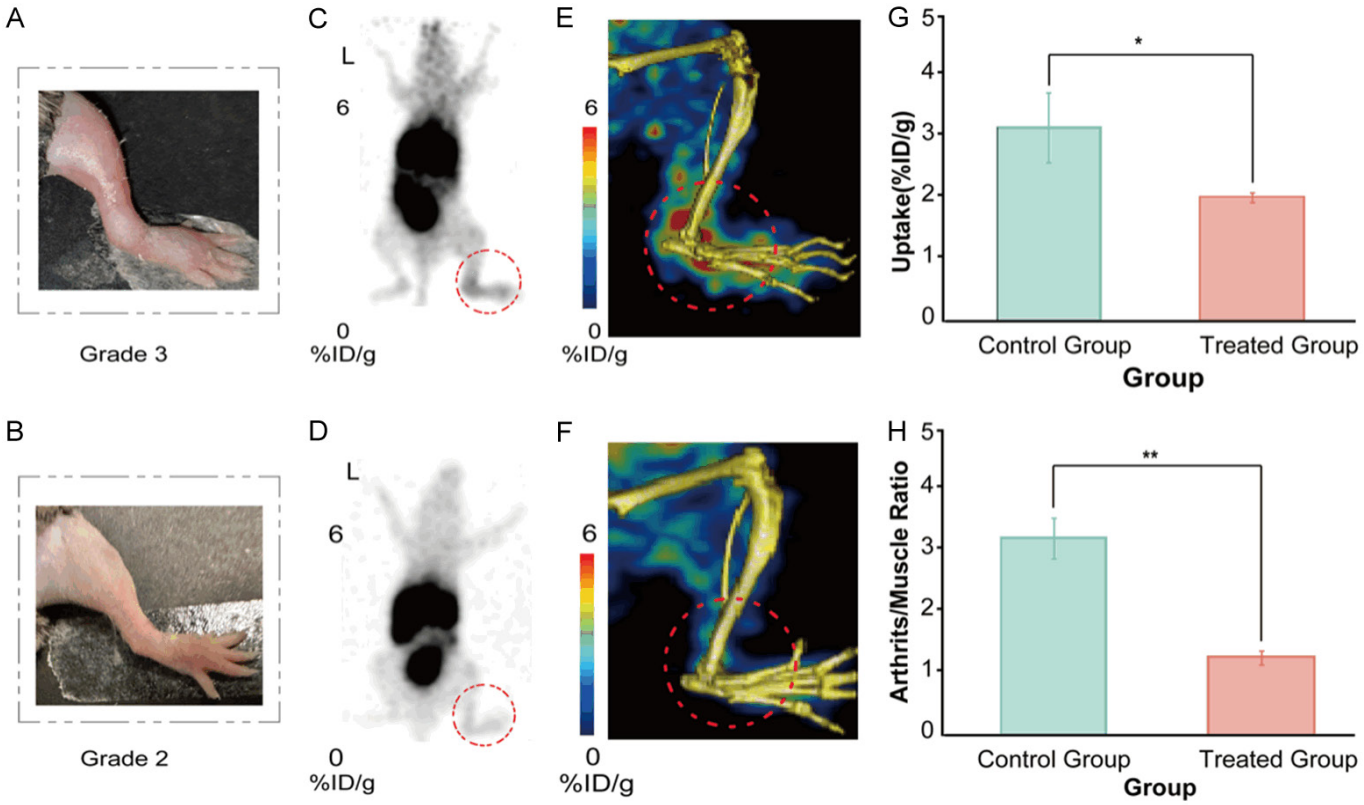

Figure 8. The inflamed arthritis score of (A) the sham MTX-treated CIA mice is 3 and (B) the MTX-treated CIA mice is 2. (C-F) Representative PET images were obtained at 4 h after injecting ^{68}Ga-BMX2 in these two groups (the red circle represents inflamed arthritis). (G, H) Quantitative analysis of the arthritis and arthritis-to-muscle ratio in 4 h after injection (*P < 0.05, **P < 0.05, ***P < 0.01).

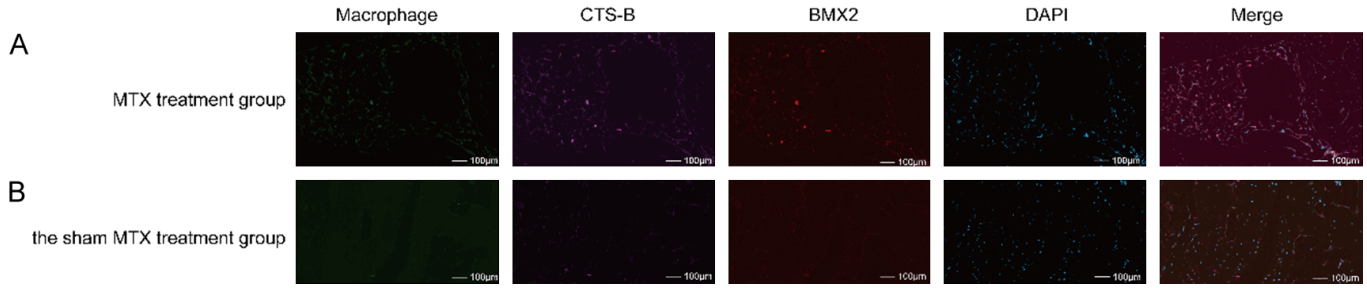

Figure 9. MIF staining was performed in the MTX and sham-MTX treated CIA mice. Tissues were labelled with the optical probe BMX2 (red) and co-stained with the macrophage activation marker CD68 (green) and CTS-B (purple). DAPI nuclear staining is shown in blue. The BMX2 signal colocalized well with the CTS-B distribution in macrophages detected by the specific antibodies in the sham-MTX treated mice, whereas the expression of CTS-B in activated macrophages decreased in MTX treated CIA mice.

leading to incapacity and the inability to work [20, 21]. Activated synovial macrophages are a crucial biomarker for evaluating the level of disease activity starting from the initial phases of the disease and for tracking the effectiveness of treatment in the later stages of the disease [29, 30]. CTS-B in the activated macrophage plays a significant role in the cartilage invasion and is directly related to the activation of macrophages and the severity of disease activity in RA [14].

Therefore, the non-invasive imaging of CTS-B *in vivo* has the potential to become a new and innovative imaging technique. Currently, a limited number of ^{64}Cu labeled enzyme activity-based probes (ABPs) have undergone initial testing for fluorescent and PET multimodality imaging of CTS-B activity in models of living cancer xenografts, atherosclerotic disease, and idiopathic pulmonary fibrosis. These tests have shown the potential of CTS-B targeting probes for disease imaging [24, 31]. Nevertheless, there is still a substantial amount of work required to enhance the pharmaceutical kinetics and imaging quality.

Further, there has been a lack of focus on the possibilities of using radiolabeled CTS-B tracers for RA imaging. Additionally, it is important to note that only the active or functional version of the proteases is capable of effectively operating under pathological or physiological settings [27]. It has been verified that the strength of the BMX2 signal is strongly influenced by the concentration of the active form of the CTS-B enzyme, which is of greater importance in biological research and molecular diagnosis [25].

Therefore, a CTS-B activity-based multimodality probe, BMX2, which includes a CTS-B activity-based peptide, a sulfo-cy5.5 for fluorescence imaging, and a DOTA for chelating radionuclides, was labeled with ^{68}Ga for CIA detection and MTX treatment monitoring. The BMX2 possesses a notable CTS-B specificity and strong binding affinity, allowing it to accumulate in inflammatory joints for a duration exceeding 120 hours. Consequently, it effectively measures the CTS-B activity *in vivo*.

Our previous study has shown that BMX2 has a significantly high rate of ^{68}Ga labeling yield (95.2 ± 2.6%) and product purity (higher than 99.5%). Furthermore, BMX2 was successfully tagged with ^{68}Ga for *in vivo* PET imaging of CTS-B activity [20]. In this study, we selected RAW 264.7 macrophage cell line to determine the reaction activity with BMX2 [26]. Results showed that the signal intensity ^{68}Ga-BMX2 gradually increases with time in RAW 264.7 macrophage cell lines and is blocked by CA074 and BMX5. Those results indicated that BMX2 has a high affinity with CTS-B in activated macrophage, which is time-dependent and concentration-dependent.

For *in vivo* imaging studies and therapy evaluations, we employed the CIA animal model, which is commonly used to study autoimmune mechanisms, reflecting similar immunological and pathological features as human RA [22]. The CIA mice underwent imaging to assess the sensitivity of BMX2 in detecting CTS-B activity *in vivo* and monitoring the treatment effectiveness. The results showed BMX2 obtained excellent detection and treatment evaluation capability.

MTX is the predominant disease-modifying antirheumatic medication utilized for the treatment of RA [23]. However, the extended use of MTX is impeded by its frequent adverse reactions and potential toxic effects (such as hepatotoxicity, nephrotoxicity, or even cancer) [28]. RA, similar to other chronic inflammatory conditions, exhibits a fluctuating trajectory and presents varying prognoses across different people [29]. With the advent of targeted immunotherapies, it is imperative that the clinical practice for RA transitions to customized medicine [8]. A system that can simultaneously exert effective therapeutic activity, in addition to precise RA activity, would greatly enhance the treatment processes for RA. Molecular imaging plays a crucial role in precision medicine due to its noninvasive nature and its ability to be easily used at every level of patient care. Targeted molecular therapy for CTS-B is a promising treatment. Several studies have documented the use of CTS-B-specific inhibitor, CA074, reduced lipid peroxidation and mitochondrial dysfunction in activated macrophages and suppressed macrophage pyroptosis to alleviate inflammation [15, 16]. Neverthe-

less, the utilization of this technique in clinical settings has not been implemented thus far [30]. This may be attributed to the complex physiological conditions of humans and the potential adverse effects of CTS-B-specific inhibitors [31].

Developing a particular radionuclide therapeutic probe is crucial for rheumatoid arthritis (RA) due to the vital role of CTS-B and the necessity of personality treatment in RA. In our preliminary study, BMX2 exhibits a significant and particular affinity for CTS-B, resulting in its accumulation in the inflammatory arthritis of CIA mice for a duration exceeding 120 hours. These findings suggest that BMX2 has the potential to be used as a CTS-B inhibitor for RA therapy. However, further study is needed to determine its treatment efficacy.

One limitation of this study is the use of a murine model of CIA, which does not fully encompass the complexity of human RA. Therefore, we will perform further *in vivo* human studies to strengthen the validity of our animal model results. Additionally, as activated macrophages are involved in many inflammatory diseases, further studies will verify the broader implications of BMX2 in other inflammatory diseases such as myocardial infarction and osteoarthritis, with the aim of completing the clinical transformation and better assisting in the assessment of inflammatory activity, efficacy testing, etc.

Conclusions

The CTS-B activity-based radioactive and fluorescent dual-modality diagnostic agent, ^{68}Ga-BMX2, was utilized for PET and fluorescence imaging of CIA, which demonstrated an effective detective approach and treatment monitoring for RA.

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Disclosure of conflict of interest

The authors declared that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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