Phytate-Complex as a Novel MRI Agent for Tumor Associated Macrophage (TAM)-specific Image Contrast and Drug Delivery

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INTRODUCTION

Tumor-associated macrophages (TAMs) accumulate in various cancers and promote tumor angiogenesis and progression [1], and thus might be ideal imaging targets for non-invasive detection of tumors with high specificity. Here, we developed an iron-calcium-phytate complex (ICPC) as an MRI agent that specifically targets TAMs, and validated its efficacy in an orthotopic murine model of metastatic melanoma. Beyond the capability of current TAM imaging methods, which is limited to visualization of TAMs [2], we also developed an ICPC variant by combining the ICPC with an anticancer drug. Using a Doxorubicin-ICPC complex (Dox@ICPC), we also present our preliminary results that strongly support the potential utility of this novel antitumor MR agent that achieves both TAM-specific MR imaging and tumor-specific drug delivery simultaneously.

MATERIALS AND METHODS

Determination of the Thermodynamics of Fe^{3+} Binding to Phytate and Preparation of ICPC: we performed isothermal titration calorimetry (ITC) experiments using a Microcal 200 isothermal titration microcalorimeter (Microcal, USA) [3]. Based on our ITC results, a 10 mM solution of Fe^{3+}-phytate was prepared by mixing equimolar concentrations of Fe^{3+} ions and phytate solution. To minimize the ability of Fe^{3+}-phytate to chelate Ca^{2+} from the blood, equimolar concentrations of Ca^{2+} ions were added to the Fe^{3+}-phytate solution (pH = 6.0).

Biodistribution of ICPC and Identification of the Cells that Absorbed ICPC: The clearance of ICPC from blood was investigated after i.v. injection of the agent (10 μmol·kg^{-1}) into Sprague–Dawley (SD) rats. The biodistribution and retention time of ICPC were examined in C57BL/6 mice. Immunohistochemical (IHC) staining using an antibody against F4/80 was performed to confirm the identity of the cells that absorbed ICPC.

Elucidation of ICPC Uptake by Macrophages: we used thioglycollate-elicited peritoneal macrophages (TEPM) from wild-type mice, which represent inflammation-activated cells [4].

Evaluation of ICPC as an Effective Macrophage-specific MRI Probe: ICPC alone and ICPC-labeled RAW264.7 macrophages were imaged for relaxation rate (R2*) mapping. RAW264.7 macrophages were cultured in 10-cm^2 plates at a density of 2×10^6 cells mL^{-1} in a medium (ICPC concentrations ranging 0 - 0.3 mM). After incubation for 12 h, the plates were washed with PBS, and ICPC-labeled cells were prepared in PCR tubes.

Inoculation of B16F1 Melanoma Mice: B16F1 melanoma cells were cultured in DMEM complete medium (Sigma-Aldrich, USA) with 10% heat-inactivated FBS (Gibco, USA) and 1% penicillin-streptomycin. B16F1 cells were transfected with an FG12 lentiviral vector that expressed green fluorescence protein (GFP) under the control of the ubiquitin C promoter. We inoculated 8-week-old C57BL/6 male mice with 4×10^5 B16F1 or B16F1-GFP melanoma cells per mouse *via* the left front footpad [5]. The tumor was allowed to grow to ~ 1.0 cm in diameter. MRI scans were then performed for metastatic lymph node (LN) imaging. Regional LNs (left axillary and brachial) and non-regional LNs were harvested from the mice.

MRI: MRI studies were performed on a 9.4 T MR scanner with a transmit-only volume coil for excitation and a phased array 4-channel surface coil for signal reception (Bruker, Germany). For the estimation of relaxation rate (R2*) and relaxivity (r2*) of ICPC in vitro, a gradient echo sequence (GE) was used with 8 different echo times (TEs) ranging 3.14 - 10.14 ms, with a step size of 1.0 ms. Other imaging parameters were: TR = 8000 ms; flip angle (FA) = 90º; FOV = 60×60 mm^2; matrix size = 256×256; 1 slice (0.5 mm); NEX = 4. R2* were calculated using Matlab (MathWorks Inc., USA). For in vivo LN imaging in animals, we performed MRI scans before and 4, 8, 16, and 24 h after intravenous (10 μmol·kg^{-1}) administration of ICPC (n=9). A GE and a spin echo sequence (SE) were used (respiratory-gated and fat-suppressed; TR/TE = 335/6.7 ms for GE and 4000/58 ms for SE), FA = 90º; FOV = 30×30 mm^2; matrix size = 384×384; 15 slices (0.5 mm); NEX = 8).

RESULTS

Determination of the Thermodynamics of ICPC: Fig. 1a illustrates the thermodynamics of ICPC obtained by ITC analyses. Titration of Fe^{3+} into phytate exhibited Fe^{3+} binding site saturation in accordance with a two-site set model, in which Fe^{3+} ions bind one set of binding sites with high affinity (4.62 μM) and bind another set with low affinity (31.25 μM). These data support the use of ICPC as an injectable MRI contrast agent, due to its stability under physiological conditions [3].

Biodistribution of ICPC and Identification of the ICPC-Absorbing Cells : The clearance of ICPC from blood after i.v. injection (10 μmol·kg^{-1}) into SD rats as well as its biodistribution and retention time in C57BL/6 mice are shown in Figs. 1c and 1d, respectively. ICPC clearance from the blood was rapid. Maximal ICPC uptake in the mice occurred at 4 h in the liver and at 8 h in the spleen. Other tissues, including the brain, heart, lung, kidney, lymph node, and thymus, showed very low levels of ICPC uptake, as verified by Prussian blue (PB) staining (data not shown). These results support the application of ICPC as an MRI agent for use *in vivo*. The IHC results clearly showed that ICPC was engulfed by tissue macrophages, in particular hepatic Kupffer cells (Fig. 1e) and sinusoidal lining cells of the spleen (Fig. 1f) [6].

Elucidation of ICPC Uptake by Macrophages: LPS-stimulated TEPM more efficiently engulfed ICPC in a dose-dependent manner, as compared to unstimulated TEPM (Fig. 2a). In contrast, residential peritoneal macrophages did not ingest ICPC. As an additional control, B16F1 melanoma cells did not take up ICPC (Fig. 2b), confirming that ICPC is macrophage-specific. These data strongly suggest that ICPC is selectively engulfed by activated macrophages.

Evaluation of ICPC as an Effective Macrophage-specific MRI Probe: Fig. 2c shows the MR images used for the estimation of R2* of ICPC in vitro. The resulting R2* maps are shown in Fig. 2d. The ICPC alone had an estimated r_2^* of 82.9 ± 3.1 mM^{-1}s^{-1}. Incubation of macrophages with various concentrations of ICPC resulted in an r_2^* of 1056.1 ± 105.9 mM^{-1}s^{-1}, i.e., 16-fold higher than that of ICPC alone (Fig. 2e).

Detection of Metastatic Lymph Nodes in B16F1-GFP Melanoma Mice In Vivo: Fig. 3a shows the pre- and post-contrast MR images of the B16F1-GFP melanoma mice where the hypo-intense LN metastasis is clearly detected. These findings were confirmed by GFP fluorescence and PB staining (Fig. 3b). IHC analysis demonstrated that ICPC was preferentially and predominantly engulfed by F4/80$^+$ macrophages within tumor regions, as the antibody staining completely overlapped the PB staining (Fig. 3b).

Doxorubicin-ICPC Complex (Dox@ICPC): Fig.3c illustrates the thermodynamics of Dox@ICPC obtained by ITC analysis. Titration of doxorubicin into phytate showed that phytate has high affinity for doxorubicin (22.7 nM). In Fig. 3d, fluorescence analysis clearly demonstrated that Dox@ICPC was selectively targeted in tumor regions of metastatic LNs, which were further confirmed by PB staining.

DISCUSSION

Phytate has long been used for the detection of sentinel LNs in cancer patients in the form of technetium-phytate (Tc-phytate), an established radiopharmaceutical agent for PET [7]. The chemical nature of phytate is ideally suited for simultaneously binding Fe^{3+} and Ca^{2+} ions and even an anticancer drug such as doxorubicin, subsequently forming insoluble particles under physiological conditions [3]. Activated macrophages are then able to engulf these insoluble ICPC particles [8]. The ability of ICPC to target activated macrophages within tumor regions is therefore distinct from that of iron-oxide nanoparticles, which can also localize to healthy LNs [9]. In summary, we have developed a tumor-specific MRI agent for use in the accurate and direct detection of tumor metastases. Our preliminary data also strongly support that the novel antitumor MR agent, Dox@ICPC, may potentially achieve both TAM-specific MR imaging and tumor-specific drug delivery simultaneously.

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Figure 1

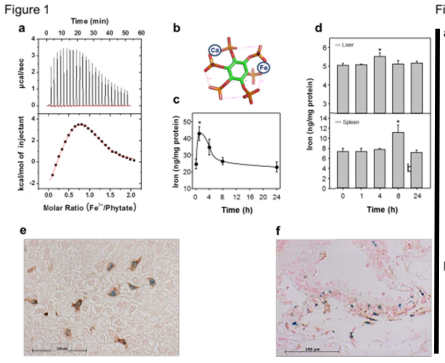

Figure 2

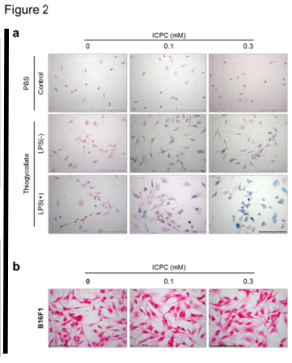

Figure 3