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HAL Id: hal-02269970
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Submitted on 23 Aug 2019

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Molecular Magnetic Resonance and Ultrasound Imaging of Tumor Angiogenesis

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Abstract
Seeking out and identifying imaging biomarkers for early cancer diagnosis and the evaluation of patient response to therapy requires an improvement in the specificity of imaging techniques. This study explores in vivo neo-angiogenesis assessment using molecular mechanisms through target molecular Magnetic Resonance (MR) and Ultrasound (US). In this context, our study examines and compares the use of both imaging technics, targeting the same integrin in a mouse xenograft tumor model. Following xeno transplantation of human renal cell carcinoma (Human A498), thirteen nude mice were injected with αvβ3-targeted and non-targeted Contrast Agents (CA) for MR and US use, respectively. CA binding to the targeted receptor was measured through Dynamic Susceptibility Contrast MR imaging and Differential Targeted Enhancement (DTE) US imaging. The specificities and co location of both targeted CAs were studied throughout the tumor, in both hypo- and hyper-vascularized areas. One hour postinjection, a significant difference was observed between the signals of targeted and non-targeted MR CA (p = 0.03). The DTE-US targeted CA was significantly enhanced compared to non-targeted CA (p = 0.002). In the hypo- and hyper-vascularized regions of interest, a strong correlation was observable between both modalities, with values of r 10min = 0.86 (p = 0.0003), r 30min = 0.85 (p = 0.0004) and r 60min = 0.87 (p = 0.0001). This study highlights the specificity of each targeted CA. A high correlation was noted between MR and US molecular imaging for angiogenesis assessment. These two molecular imaging modalities may be used interchangeably to monitor patient response to anti-angiogenic treatment.

Keywords
Bimodal molecular imaging DCE-US/MRI, Avβ3 - Targeted contrast agent, USPIO nanoemulsion, US microbubbles

Abbreviations
CA: Contrast Agents; US: Ultrasound; DTE: Differential Targeted Enhancement; MR: Magnetic Resonance; SE: Standard Error; Fps: Frames per second; DSC: Dynamic Susceptibility Contrast; ROI: Region of Interest

Introduction
Molecular imaging using targeted contrast agents has been used to highlight fundamental biological mechanism for some time now, and facilitates the development of new drugs [1]. It has also proved to be a valuable tool for the early detection of molecular and cellular events, and for the characterization of molecule expression levels involved in inflammation and angiogenesis process [2-5]. The development of new contrast agents in the oncology field focuses on their specific targeting of angiogenesis and their binding to receptors/molecules such as the αvβ3 integrin, which is highly expressed on activated endothelial cells in tumor vessels [6].

Molecular Ultrasound (US) is an recent imaging approach that has rapidly found its niche among other molecular imaging modalities thanks to its high tempo-

Received: June 26, 2017; Accepted: October 09, 2017; Published: October 11, 2017
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ral resolution and ease of use [2]. Dynamic Susceptibility Contrast (DSC)-Magnetic Resonance (MR) imaging uses Ultra-Small Super Paramagnetic Iron Oxide (USPIO) Contrast Agent (CA) to provide information on tumor vascularization and hemodynamic status.

With the development of targeted agents binding to specific receptors, DSC-MR imaging can also provide molecular information in intra- and extra-vascular conditions; this has the advantage of requiring low CA concentrations making it possible to probe specific molecular events [7].

The increasing development of novel targeted therapies to treat tumors necessitates a parallel development of technologies that can determine their potential efficacy, and thus facilitate therapeutic protocol planning. Efforts are also being made to assess and quantify biological effects induced by USPIO.

The aims of this work were to evaluate ανβ3-targeted contrast agents and characterize their affinity to the ανβ3 sites.

Materials and Methods

All experiments followed the guidelines of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes, and were approved by the Animal Research Committees of the relevant laboratories, C2EA-26 and C2EA-44 (both registered at the French research ministry).

Mice and tumor model

Ten female immune deficient nude mice (6-8 weeks old) were bred and housed in the Animal Care Facility at the Institute Gustave Roussy (Villejuif, France), in accordance with institutional guidelines for animal welfare. Human A498 renal cell carcinoma (ATCC-HTB-44, American Type Culture Collection, Manassas, USA) were cultivated in EMEM (Eagle’s Minimum Essential Medium, Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS (Fetal Bovine Serum). The experiments started 30 days after 3 × 10⁶ cells in 0.2 mL matrigel (Corning Corporated, Tewksbury, USA) were subcutaneously inoculated into the right flank of the mice.

Imaging protocol

Imaging sessions were performed using MR and US, each combined with targeted and non-targeted CA that had been injected intravenously in the systemic circulation (Figure 1). Targeted and non-targeted protocols were performed on the same day (D1) for US experiments, and systematically at a 2-day interval for MR experiments (i.e. non-targeted contrast agent injection was made on D3 or D5 and finally the targeted contrast agent injection was carried out on D3 or D5) to avoid any interaction between the MR- and US-targeted CAs.

Mice were anesthetized before each imaging session by the inhalation of isoflurane (2%) in room air (1.5 L/min). Their body temperature was monitored and maintained constant using a heated platform during US acquisitions or a constant warm air flow during MR acquisitions. Table 1 shows the number of mice used to evaluate ανβ3-targeted contrast agents and characterize their affinity to the ανβ3 sites.

Molecular MR imaging

MR imaging was performed using a clinical 1.5 T scanner (Achieva, Philips Healthcare, Best, The Netherlands) equipped with a 60 cm bore and a conventional 23 mm diameter microscopy surface coil for reception. Relaxation rate $R_2^*$ measurements were made using a 3D $T_2^*$-weighted gradient-echo multi-echo sequence, selected for its sensitivity to apparent transverse relaxation effects induced by USPIO. Dynamic Susceptibility

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<th>Mouse</th>
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*Same mice for US specificity, MR specificity, and targeting protocols; x Same mice for MR and US CA co-localization; + Dead mouse.
MHz center frequency (MS-250, Visual Sonics). Tumor volumes were measured using 2D B-mode imaging, as described in the literature [9]. 2D Differential Targeted Enhancement Ultrasound (DTE-US) imaging sessions were performed on the maximal transverse section of each tumor using contrast pulse sequencing technology, to reach the nonlinear signal produced by microbubbles. Images were collected at a high spatial resolution (lateral and axial resolution of 165 and 75 \( \mu \)m, respectively), with 4% transmission power, 5 frames per second (fps), and a dynamic range of 40-dB [9].

Micro Marker \( \alpha\beta3 \)-targeted and control (IgG) microbubbles (Visual Sonics, Toronto) with a diameter of approximately 2-3 \( \mu \)m were manually injected via the retro-orbital vein (50 \( \mu \)L per injection, injection time: 2 seconds). A 30-minute interval between the two injections allowed the first CA to be cleared from the vasculature [9]. After each CA injection, the signal intensity was recorded as a function of time during the wash-in contrast enhancement. CA targeting was then observed 10 minutes after the bolus injection in order to allow the targeted microbubbles to bind to their endothelial molecular receptors [9], and a series of images was recorded over 30 seconds. Using the destruction/replenishment approach [10], a continuous high-powered destructive pulse (100% transmit power for 1 s) was then applied to destroy all microbubbles within the US beam elevation. Images were recorded while freely circulating CA replenished the tumor vessels. Data were then ana-

Contrast (DSC)-MR was performed repeatedly for one hour after the CA injection. \( \text{R}_2^* \) mapping (3D 0.5 mm isotropic sequence, TR/TE/echo spacing = 90/5.9/9.7 ms, 6 echoes, 0.3 \( \times \) 0.3 \( \times \) 0.5 mm\(^3\) reconstruction voxel size, 220 Hz/pix, Tacq = 4.2 min) was carried out before and after the injection in the caudal vein of 100 \( \mu \)mol Fe/kg USPIO-based nanoemulsion functionalized with RGD-binding \( \alpha\beta3 \) (P4000, referred to as E1 in patent application US 2015/0320889 A1, entitled 'Vectorised magnetic emulsion', and filed on Nov. 12, 2015). Identical \( \text{R}_2^* \) mapping protocol was also carried out before and after the injection of a non-functionalized control nanoemulsion (P3999, referred to as E1T in patent application US 2015/0320889). Nanoparticle diameters were approximately 190 nm. These contrast agents are also commercialized by Chematech (France). As generally assumed in a first approximation, transverse relaxation rate \( \text{R}_2^* = 1/T_2^* = \text{R}_{2,0}^* + \text{r}_{2,\text{Fe}}^* \) is linear with concentration, and relaxivity is \( \text{r}_{2,\text{Fe}}^* \sim 210 \text{ mM}^2\text{s}^{-1} \) (patent application US 2015/0320889 A1). All images were processed with Matlab software (2011B, Math works, USA). Mean pre- and post-injection variations, or \( \Delta \text{R}_2^* (\text{R}_2^* \text{pre} - \text{R}_2^* \text{post}) \), were calculated and monitored for 1 hour to quantify CA concentration, which directly reflects the binding to the targeted receptor.

**Contrast-enhanced molecular US**

All US acquisitions were performed on a dedicated system for small animal imaging (VEVO’2100, Visual Sonics, Canada), using a linear array probe with a 21
alcohol (Cryomatrix OCT compound, Thermo Fisher Scientific, USA), then frozen in liquid nitrogen. The tumors were cut into 7-8 μm-thick sections located as close as possible to the maximal transverse section imaged by DTE-US and DSC-MR. Sections were stained with Hema-toxylin, Eosin and Saffron (HES) and observed to verify tissue integrity.

To verify the presence of β3 on the cells of the vascular endothelium of renal carcinoma, cover slips were incubated overnight at 4 °C with anti-human rabbit β3 (1:1000; 04-1060, Millipore) in TNB buffer as a primary antibody, then incubated for 2 h with HRP-conjugated goat anti-mouse IgG (1:1000; 111-036-047, Jackson ImmunoResearch) in TNB buffer as a secondary antibody. Given that the tumor tissue could also express the β3 integrin, β3/CD31 double labeling was carried out to demonstrate the labeling of the integrin in the vascular endothelium. CD31 was revealed using anti-mouse rat CD31 (1:50; 553370, BD Biosciences) then goat anti-rat IgG coupled to Alexa Fluor 555 (1:200; A-21434, Life Technologies). The β3 was amplified using the TSA kit (NEL749A001KT, Perkin Elmer) and revealed by FITC-Streptavidin (1:200, STAR2B, AbD Serotec). The nuclei were labeled with DAPI (Atlantic Bone Screen, France). Nuclear staining with DAPI allowed the identification of tissue structure and quality. Tissue fluorescence was observed under a multiphoton microscope.

Evaluation of targeted MR and US contrast agent colocalization

MR data were colocalized on the US 2D acquisitions to choose the closest slice in the 3D MR-acquisition volume (R2*-weighted images, first echo); to compare DTE-US imaging to DSC-MR imaging, mean values of DTE and relaxation rates ΔR2* were determined in whole tumor ROI (ROI-1) and multiple intratumoral ROI (ROI 2 to 7) to reflect the heterogeneity of intratumoral molecular expression. ROI 2 to 5 was manually drawn to facilitate the location (Figure 2b). The two remaining ROIs (ROI 6 and 7) were determined as the two extrema of targeted contrast agent imaging, i.e. they were hypo- (ROI-6) and hyper- (ROI-7) contrast enhanced. ROIs all measured 3.14 mm2 (circle with a radius of 1 mm).

Immunofluorescence

Immunofluorescence

Immediately after the last MR imaging session, all five tumors were extracted, embedded in a OCT gel-like medium consisting of polyethylene glycol and polyvinyl alcohol (Cryomatrix OCT compound, Thermo Fisher Scientific, USA), then frozen in liquid nitrogen. The tumors were cut into 7-8 μm-thick sections located as close as possible to the maximal transverse section imaged by DTE-US and DSC-MR. Sections were stained with Hema-toxylin, Eosin and Saffron (HES) and observed to verify tissue integrity.

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Statistical analysis

All continuous measurements were expressed as mean ± standard deviation. Correlations were statistically analyzed using the Pearson’s correlation coefficient (r) for normally distributed data, and the Spearman rank coefficient correlation was used in the absence of normal distribution. The significance level of the correlation was determined with corresponding Pearson and Spearman tests. The ΔR₂* and DTE values were not nor-
mally distributed and were therefore compared using a non-parametric Wilcox on signed-rank test. A p-value of less than 0.05 was considered statistically significant.

**Results**

Figure 3 shows an example of the same tumor imaged by MR ($R_2^*$-weighted scan, first echo with a Signal to Noise Ratio of 70) and DTE-US before and after the targeted CA injection. Although the signal was homogeneous before injection in both MR and US images, strong heterogeneity was observed after the injection of the CA, with signal enhancement in US sessions and an $R_2^*$ increase in MR imaging indicating molecular $\alpha\nu\beta_3$ expression.

**MR and US contrast agent specificity**

DTE-US measurements at the tenth minute postinjection were compared to $\Delta R_2^*$ measurements post-injection from 10 to 60 minutes at intervals of 7 minutes, in order to determine the optimal time at which MR imaging parameters should be compared in terms of CA binding specificity. Figure 4 shows the $\Delta R_2^*$ mean values ($\pm$ Standard Error SE) for targeted (square) and non-targeted (diamond) CA as a function of time. $\Delta R_2^*$ values became significantly different between targeted and non-targeted CA for all acquisitions performed 37 minutes postinjection and later ($p = 0.03$), thus providing the imaging window to capture specific binding. At 1 hour postinjection, the $\Delta R_2^*$ value for targeted CA was $16.5 \pm 3.9$ s$^{-1}$; significantly different to that of non-targeted CA, i.e. $5.8 \pm 1.8$ s$^{-1}$ ($p = 0.03$).

Figure 5 shows the mean ($\pm$ SE) US contrast signal intensities evaluated 10 minutes after the CA injection, before and after targeted and non-targeted CA destruction. Non-significant difference was observed in the signal for non-targeted CA, whereas a significant decrease was observed for the targeted CA ($p = 0.03$, Wilcoxon on test), indicating the binding of the specific CA. DTE values (mean $\pm$ SE), calculated as the difference between US signal intensities before and after CA destruction, were $260.5 \pm 66.0$ a.u. for the non-targeted CA and $2601.5 \pm 488.3$ a.u. for the targeted CA. Significantly different changes in contrast were observed between US targeted and non-targeted CA ($p = 0.002$, Wilcoxon on test for paired samples).

Figure 6 shows the mean ($\pm$ SE) US contrast signal intensities evaluated 10 minutes after the CA injection, before and after targeted and non-targeted CA destruction. Non-significant difference was observed in the signal for non-targeted CA, whereas a significant decrease was observed for the targeted CA ($p = 0.03$, Wilcoxon on test), indicating the binding of the specific CA. DTE values (mean $\pm$ SE), calculated as the difference between US signal intensities before and after CA destruction, were $260.5 \pm 66.0$ a.u. for the non-targeted CA and $2601.5 \pm 488.3$ a.u. for the targeted CA. Significantly different changes in contrast were observed between US targeted and non-targeted CA ($p = 0.002$, Wilcoxon on test for paired samples).
The mean values of $\Delta R^*_{2}$ and DTE measured for ROI 1 to 5 were not significantly correlated, even for different post-injection times ($r < 0.71$). The strong correlation was observed on the specific ROIs, i.e., the hypo- and hyper-vascularized areas (ROI 6 and ROI 7, Figure 7 with respectively $r_{10\text{min}} = 0.86$ ($p = 0.0003$); $r_{30\text{min}} = 0.85$ ($p = 0.0004$) and $r_{60\text{min}} = 0.87$ ($p = 0.0001$).

Immunofluorescence

The observations acquired by optical microscopy in tissue images (Figure 8) indicated the colocalization of $\beta3$ with CD31, confirming the presence of integrin on the endothelial cells of this A498 RCC tumor model. Several integrins are involved in cancer progression but most of them, such as $\alpha v\beta3$, are over expressed, making them difficult to quantify [6,11,12]. We successfully evaluated colocalization using Imaris (Bit plane Scientific Software) to quantify the percentage of colocalization between the 2 CD31 and $\beta3$ markers, and found that colocalized $\beta3$/CD31 areas made up 30.6% of the vasculature area.

Discussion

The contrast agents, i.e. iron oxide nanoemulsion (DSC-MR) and microbubbles (DTE-US), were both functionalized to target the same integrin ($\alpha v\beta3$) for pre-clinical oncology applications, and both were shown to provide specific targeting [13]. The novelty of this work was to make a fair comparison of molecular imaging approaches seeking to identify the colocalization of angiogenesis target, using different contrast agents with different fixation mechanisms. These results highlight the reliability of molecular imaging for angiogenesis.

An entire analysis protocol was specifically developed here to colocalize the US 2D acquisition to the closest slice in the 3D MR-acquisition volume. This protocol was combined with a systematic segmentation of the different tumor areas where $\Delta R^*_{2}$ and DTE were calculated. In this way, we ensured the closest and thus fairest possible comparison between MR and US results, enabling region-based comparison among imaging modalities.

First, the comparison of these specific contrast agents...
with the non-targeted contrast agents within each imaging session yielded significant differences (MR - Figure 4, US - Figure 5). The dynamic contrast provided by MR imaging allowed the evaluation to take place over a sufficiently long time scale to observe the CA kinetic, which was not the case for US acquisitions. US experiments gave access to measurement of the quantity of targeted microbubbles at only one time point, i.e. 10 minutes postinjection, as recommended by Van Rookie, et al. [14]. The optimal window to perform MR imaging was assessed (Figure 4), and the targeted MR signal became significantly different from the non-targeted one after 37 minutes. The MR CA injected for this study had larger diameters and stronger relaxivity than those commonly used, and appears to be a promising tool for molecular imaging. These molecular MR CAs, based on nano-objects and containing a high payload of contrast-generating metals (USPIO in this study), are now sensitive enough for use in target-specific imaging. In light of the sensitivity ($r_1$~210 mM$^{-1}$s$^{-1}$) and the observed average specific $\Delta R_2^*$ (close to 10 s$^{-1}$) recorded, we can consider that approximately 50 µM concentration of iron (or a 50 pM concentration of droplets) was measurable, which is within the range of receptor concentrations.

Secondly, a good correlation was observed between specific targeted MR and US signal, with a high correlation coefficient of 0.87 at 60 minutes after injection Figure 7.

The results highlighted the high colocalization of MR and US targeted contrast agents in vivo, despite differences in their respective sizes (190 nm vs. 2-3 µm diameters) and fixation mechanisms. MR nanoeulsion was targeted with RGD, while US CA was targeted with antibodies. Thus, the high colocalization observed in this study demonstrated the reliability of molecular imaging, whatever imaging modalities, MR or US. However, simultaneous imaging by both modalities does not provide any evidence that could explain the fundamental biological fixation mechanism. In particular, the different CA sizes led us to expect the observation of some integrons expressed exclusively on activated endothelial cells from vessels with the DTE-US, and integrons expressed on tumor cells after extravasation of the MR AC. These differences in CA behavior might explain observations made in other ROIs.

The localization of $\beta_3$ was assessed and confirmed by immunofluorescence within the vessels and within the extracellular matrix. The difficulty of implementing integrin labelling and observation makes it impossible to study slices thicker than 20 to 30 µm, which would allow a more robust observation and possible quantification within the vessels. Nevertheless, the multi-staining DAPI/CD31/$\beta_3$ was optimized to allow high quality visualization.

Finally, our study highlights the equivalence of molecular DTE-US and DSC-MR imaging in terms of specificity and CA localization. This approach has already allowed the prediction of response to targeted therapies by the use of MR and US imaging to evaluate the early response of different therapeutic agents in renal cell carcinoma [15]. Some studies suggested that RGD peptide could not necessarily reflect the change of integrin $\alpha$v$\beta_3$ expression on neoangiogenesis detection [16,17]. Consequently, the real potential of RGD peptide in therapy response monitoring needs to be confirmed with well-designed clinical investigations. Beyond this limit of targeted-CAs specificity, the main benefits of the molecular US approach are its accessibility and low cost. Limitations linked to operator dependency can often be overcome with practice. The evaluation of early response to treatment could be achieved by improving methods for the detection and evaluation of tumors and identifying the expression levels of cell surface receptor such as integrins, thus permitting doctors to select the most efficient type of treatment for each case. Combining this early response to treatment with an accessible imaging technique such as molecular US would be a major gain for personalized care.

Acknowledgments

This work was performed on platforms affiliated to the France Life Imaging network (grant ANR-11-INBS-0006), and was partially funded by the network.

References


