

Monitoring Combretastatin A4–Induced Tumor Hypoxia and Hemodynamic Changes Using Endogenous MR Contrast and DCE-MRI

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Purpose: To benchmark MOBILE (Mapping of Oxygen By Imaging Lipid relaxation Enhancement), a recent noninvasive MR method of mapping changes in tumor hypoxia, electron paramagnetic resonance (EPR) oximetry, and dynamic contrast-enhanced MRI (DCE-MRI) as biomarkers of changes in tumor hemodynamics induced by the antivascular agent combretastatin A4 (CA4).

Methods: NT2 and MDA-MB-231 mammary tumors were implanted subcutaneously in FVB/N and nude NMRI mice. Mice received 100 mg/kg of CA4 intraperitoneally 3 hr before imaging. The MOBILE sequence (assessing R1 of lipids) and the DCE sequence (assessing K^{trans} hemodynamic parameter), were assessed on different cohorts. pO₂ changes were confirmed on matching tumors using EPR oximetry consecutive to the MOBILE sequence. Changes in tumor vasculature were assessed using immunohistology consecutive to DCE-MRI studies.

Results: Administration of CA4 induced a significant decrease in lipids R₁ ($P=0.0273$) on pooled tumor models and a reduction in tumor pO₂ measured by EPR oximetry. DCE-MRI also exhibited a significant drop of K^{trans} ($P<0.01$) that was confirmed by immunohistology.

Conclusion: MOBILE was identified as a marker to follow a decrease in oxygenation induced by CA4. However, DCE-MRI showed a higher dynamic range to follow changes in tumor hemodynamics induced by CA4. **Magn Reson Med** 000:000–000, 2015. © 2015 Wiley Periodicals, Inc.

Key words: tumor; hypoxia; hemodynamics; CA4; MOBILE; DCE-MRI

INTRODUCTION

The chaotic vascular network in tumors is a well-known target of antitumor treatment. Antiangiogenic agents and vascular disrupting agents (VDAs) are part of the vascular targeting agents family (1). Whereas antiangiogenic agents are designed primarily to slow down angiogenesis, VDAs are used to target the already established vasculature (2).

Combretastatin A4 (CA4) is a VDA derived from the bark of *Combretum caffrum* (3) that has been known to induce a decrease in tumor perfusion within the first hours after its administration (4,5). CA4 phosphate (CA4P), a prodrug that directly metabolizes into CA4 (6), is one of numerous VDAs that are currently undergoing clinical trials (1,7–11). Considering the tumor as a whole, a vascular shutdown occurs rapidly after CA4 administration, inducing a decrease in tumor perfusion and tumor oxygenation at early first times (4,5,12), resulting in an extensive tumor necrosis in 24 hr. Those parameters recover after 1 day, despite an increase in tumor necrosis. However, a rim of viable cells at the periphery of the necrotic tissue remains untreated by CA4 and turns out to be sufficient for tumor regrowth (13). Consequently, combinations with other therapies—including radiotherapy (14,15), hyperthermia (16), and chemotherapy (17)—are currently being investigated, as these conventional methods will target the residual well-perfused and oxygenated peripheral cells. Preclinical studies have shown that the efficiency of radiotherapy combined with CA4P therapy depends on timing and scheduling (14,18). Indeed, the treatment is more efficient when CA4P is administered at the same time or after radiotherapy, as it reinforces the damages to vasculature caused by irradiation (15) whereas there is no synergistic effect when the CA4P is administered before irradiation, probably because VDA administration causes an increase in tumor hypoxia, leading to radioresistance.

Accordingly, it is relevant to identify markers able to monitor changes in tumor hemodynamics induced by CA4 treatment. Dynamic contrast-enhanced MRI (DCE-MRI) is a noninvasive method of monitoring the early effects of VDAs on tumor vasculature (19). Decreases in the volume transfer constant between blood plasma and extravascular extracellular space (K^{trans}) and the blood plasma volume per unit volume of tissue (v_p) have been shown to be relevant markers of CA4 efficacy (20–22). The effects of CA4P on oxygenation have also been investigated recently with invasive techniques such as diffuse reflectance spectroscopy (23), near infrared

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spectroscopy and pimonidazole staining (24), and ^{19}F MRI oximetry (25). Recently, a new noninvasive MRI technique based on the high solubility of oxygen in lipids was developed to map changes in tumor hypoxia. The technique, known as mapping oxygenation by imaging lipids relaxation enhancement (MOBILE) (26), allows the monitoring of R_1 relaxation rate changes of the lipid peak induced by a change in tissue oxygenation. MOBILE has been applied on animal models to follow a change in oxygenation induced by an hyperoxic challenge in brain, muscles, and mammary tumors and has also been implemented in clinical settings (27,28). The present study benchmarks MOBILE, electron paramagnetic resonance (EPR) oximetry, and DCE-MRI as biomarkers of changes in tumor hemodynamics induced by the antivascular agent CA4. Immunohistological experiments confirmed the effects of CA4 *ex vivo*.

METHODS

Tumor Models

NT2 cells (provided by E. Jaffee) or MDA-MB-231 cells (LGC Promochem) were injected subcutaneously in 6-week-old FVB/N or nude NMRI female mice (Janvier), respectively. Animal studies were undertaken in accordance with national and local ethical committee regulations (agreement number UCL/2010/MD/001). Experiments were performed when the tumors reached a diameter

of 5.5 ± 0.5 cm. Mice were anesthetized via inhalation of isoflurane (Forene, Abbot, UK) mixed with air, as this form of anesthesia has been shown to not interfere with tissue hemodynamics (29). Body temperature was maintained at $37^\circ\text{C} \pm 1^\circ\text{C}$ with a circulating water blanket and monitored together with respiration during the experiments.

Imaging Protocol

Because of the injection of the contrast agent for DCE-MRI, we could not obtain MOBILE images for the same mice. Consequently, we randomly separated mice into two distinct cohorts of five tumors per model to undergo either DCE-MRI or both MOBILE and EPR experiments. Each mouse received an intraperitoneal injection of a solution of CA4 dissolved in dimethyl sulfoxide (DMSO; 100 mg/kg of CA4). Three EPR measurements were acquired at baseline before MOBILE imaging and were repeated 3 hr after CA4 administration immediately following the MOBILE acquisitions. DCE-MRI was also performed before and 3 hr after CA4 injection. The complete imaging protocol is described in Figure 1.

MRI Experiments

All MR experiments were performed on an 11.7T MRI system (Bruker, Ettlingen, Germany).

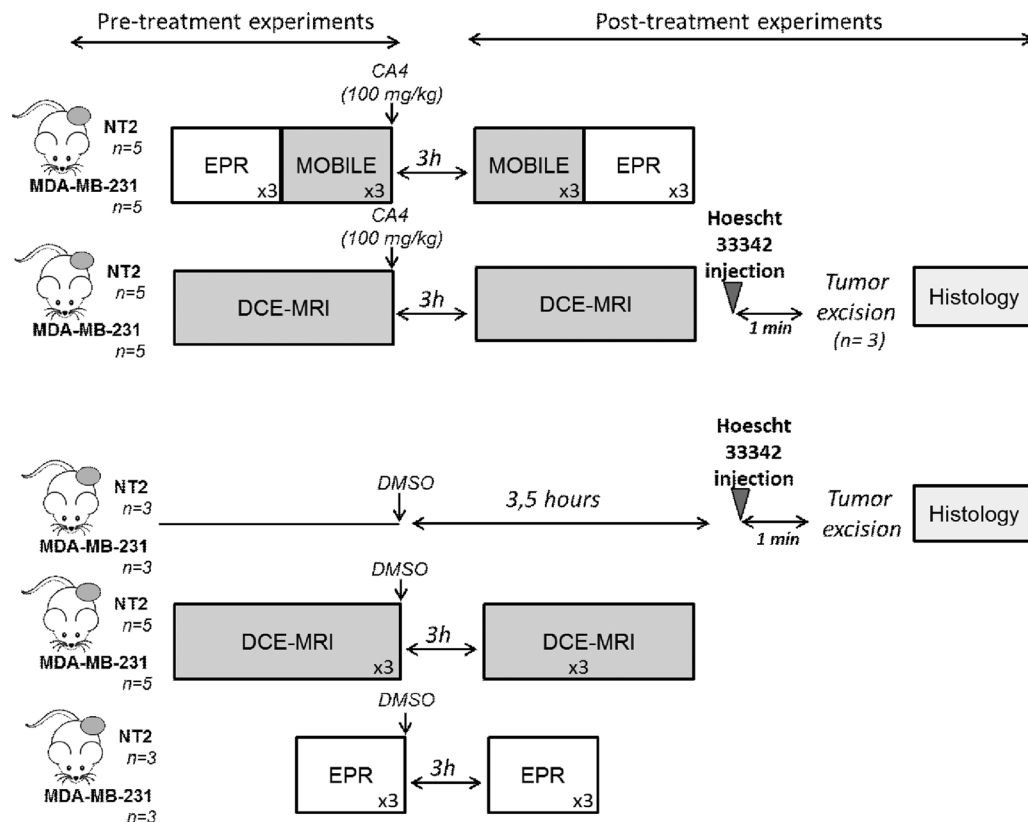


FIG. 1. Experimental protocol. Three EPR measurements were acquired at baseline before MOBILE imaging and were repeated 3 hr after CA4 administration immediately following MOBILE acquisitions. DCE-MRI was also performed before and 3 hr after CA4 injection. For 3 of 5 animals per model in the DCE-MRI cohort, the perfusion marker Hoechst 33342 was administered intravenously 1 min before sacrifice. The staining was compared qualitatively with control mice ($n = 3$ mice/model), which received only DMSO vehicle.

DCE-MRI

A quadrature whole body coil was used for radiofrequency transmission and reception. First, anatomical images were acquired using a T_2 -weighted axial turbo spin echo RARE sequence (repetition time [TR]=2500.0 ms; echo time [TE]=33.0 ms; RARE factor=8). Two transverse, 1-mm-thick slices were selected: one centered on the tumor and one positioned on the aorta/vena cava. For DCE-MRI, T_1 -weighted gradient echo images were obtained with a fast low angle shot sequence using the following parameters: TR/TE=15/2.074 ms; flip angle=40°; field of view=37.5 mm; matrix=128 × 64; and zero-fill acceleration factor=1.4. A first set of 400 scans with a temporal resolution of 1.19 s was acquired (number of averages=1), with gadoterate meglumine administered intravenously after the twentieth scan over 2 s (0.17 and 0.26 mmol Gd/kg for MDA-MB-231 and NT2 tumors, respectively). Afterward, a slower DCE data set was acquired with a temporal resolution of 10.1 s to monitor the washout of the contrast agent (150 images; number of averages: 10). A proton density-weighted image was acquired before T_1 -weighted sequences using the following parameters: TR/TE=6000/2.074 ms; flip angle=40°; field of view=37.5 mm; and matrix=128×64. The data analysis is provided in the Supporting Information.

MOBILE Experiments

The MOBILE experiments were performed with a 1H transmit/receive MRI CryoProbe. Parametric images of T_1 relaxation time were acquired using a segmented inversion recovery fast imaging with steady state free precession sequence in free induction decay mode. The extensive list of parameters used for this sequence has been described previously (26). Images were acquired at baseline and 3 hr after intraperitoneal administration of CA4. Briefly, the acquisition parameters were TR/TE=4/1.2 ms; flip angle=5°; bandwidth=100 kHz; matrix=64 × 64; four segments; and a total acquisition time of 1 min, 20 s. A single pulse sequence was performed to assess the offset between water and lipid peaks that was then used as an imaging frequency offset. The lipid peak of interest was ~4.0 ppm. A saturation pulse was added to spoil the water signal. A series of 40 images (spaced by scan TR=100 ms) with a slice thickness of 2 mm were acquired with a spatial resolution of 0.344 × 0.344 mm.

The data analysis is provided in the Supporting Information.

EPR Oximetry

Because EPR oximetry requires the injection of an oxygen-sensitive probe (30), 40 μ L of charcoal suspension (100 mg/mL) were injected within the tumor 24 hr before the experiment. EPR spectra were recorded using a 1.1-GHz EPR spectrometer (Magnetech, Berlin, Germany) as described previously (26). According to a previously acquired calibration curve, the line width of each spectrum has been translated into an absolute pO_2 value. This quantitative technique provides a mean oxygenation level from the whole tumor.

Statistical Analysis

All values are presented as the mean $\pm$ standard error. All statistical analyses were performed using GraphPad Prism 5. Because of the small sample size, we submitted our data to nonparametric tests. Wilcoxon matched paired tests were performed to 1) evaluate the difference in lipid relaxation rates induced by CA4, 2) compare the R_1 values obtained at baseline and 3 hr after VDA administration; and 3) compare the relative changes of K^{trans} between baseline and 3 hr after CA4 administration. We applied the Mann-Whitney test to investigate whether the lipid R_1 values acquired at baseline were significantly reduced 3 hr after the administration of CA4. Results were considered significant when two-tailed P values were less than 0.05.

RESULTS

Oxygenation Follow-up

Mean lipid R_1 values have been calculated within each ROI at baseline and 3 hr after CA4 administration. Figure 2A presents typical parametric maps obtained with an NT2 tumor. All NT2 tumors and four MDA-MB-231 tumors out of 5 exhibited a reduction in lipid R_1 values caused by CA4 injection. A decrease of 1% up to 7% was observed in MDA-MB-231 models, whereas a lower effect was observed in lipid R_1 values recorded in NT2 tumors in which the decrease in lipid R_1 values did not exceed 3% (Fig. 2B). On pooled tumor models, lipid R_1 values recorded at baseline were significantly lowered by CA4 administration ($P=0.0273$) (Fig. 2C). Histogram analysis showed that the distribution of lipid R_1 values shifted significantly to lower values in both models ($P=0.0024$ for the NT2 tumor model; $P<0.0001$ for the MDA-MB-231 tumor model) (Fig. 2D and 2E).

EPR oximetry provides comprehensive information about tumor oxygenation levels. We excluded one tumor from each tumor model because of their low signal-to-noise ratio (cutoff=4). NT2 tumors with a mean pO_2 of 3.9 ± 0.8 mm Hg appeared to be more hypoxic than MDA-MB-231 tumors whose basal pO_2 was 10.9 ± 2.7 mm Hg. Both models displayed a systematic fall in their oxygenation level 3 hr after CA4 administration, with a mean pO_2 of 2.7 ± 0.8 mm Hg and 7.3 ± 1.8 mm Hg for NT2 and MDA-MB-231, respectively.

Hemodynamics Follow-up

Figure 3 presents DCE-MRI data. Representative signal enhancement time curves before and after treatment are shown for MDA (Fig. 3A) and NT2 (Fig. 3B) tumors. In both models, a rapid and large enhancement after contrast agent injection was observed before treatment. After treatment, enhancement curves displayed smaller amplitude and slower washout.

For both models, the mean K^{trans} values decreased after treatment compared with pretreatment (from 0.63 ± 0.1 to 0.09 ± 0.02 min⁻¹ for NT2 and from 0.61 ± 0.1 to 0.08 ± 0.02 min⁻¹ for MDA-MB-231), showing a trend toward significance ($P=0.0625$ for each model). When considering pooled models, the relative change of K^{trans} observed between pretreatment and

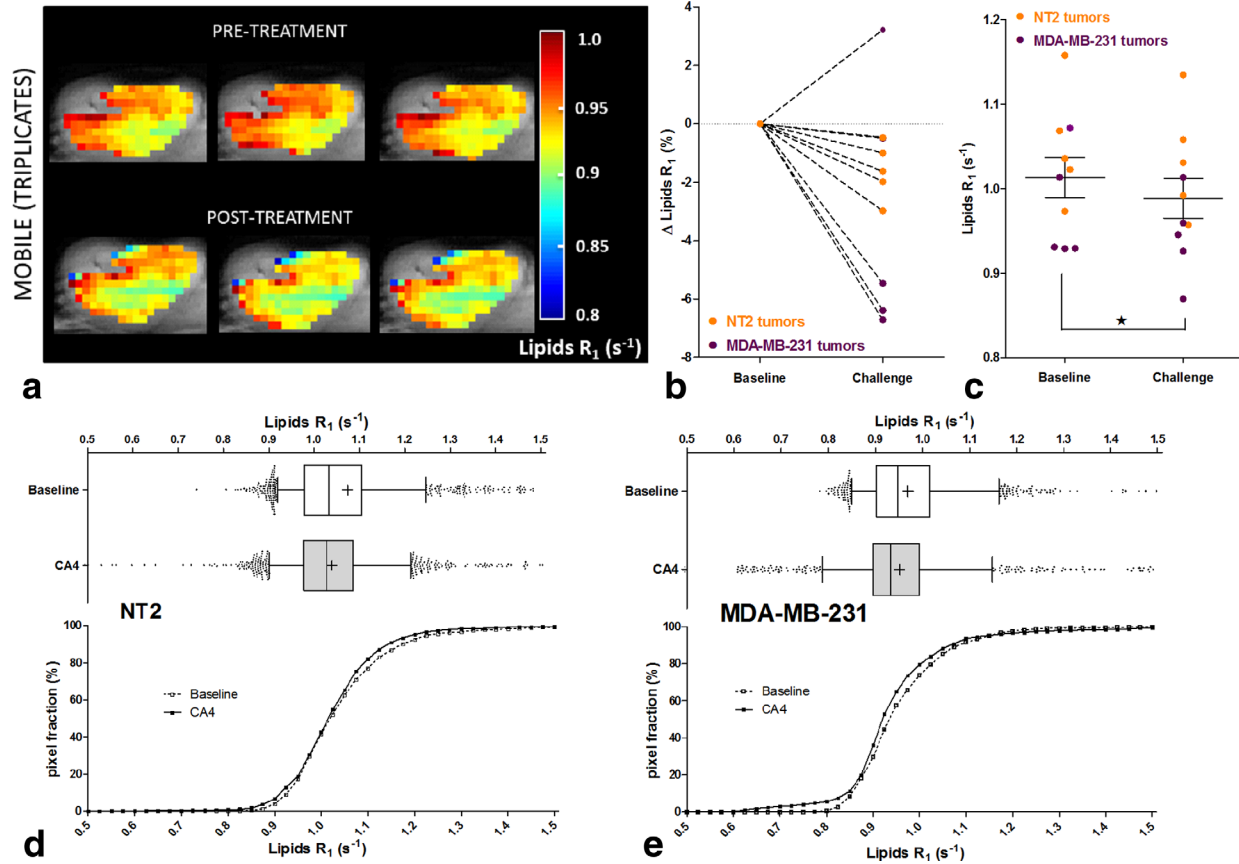


FIG. 2. MOBILE experiments. **A:** Three parametric maps of lipid R_1 values were recorded at baseline and three other maps were recorded 3 hr after CA4 administration. Tissue containing charcoal was excluded from the analysis. Considering the tumor as a whole, a decrease in mean lipid R_1 values was recorded: from 1.02 s^{-1} at baseline to 0.96 s^{-1} 3 hr after CA4 injection. It is also important to note that on this tumor, the center of the tumor is more sensitive to CA4 than the peripheral part. **B:** Relative changes in lipid R_1 values induced by CA4 3 hr after its administration in NT2 and MDA-MB-231 tumors ($n=5/\text{tumor model}$). **C:** Mean lipid R_1 values recorded for each tumor at baseline and after the hypoxic challenge. There was a significant decrease in lipid R_1 values in pooled data from both tumor models. **D:** Distribution of lipid R_1 values recorded at baseline and 3 hr after CA4 administration in the NT2 tumor model. Box and whisker plots show a decrease in the median value, as well as a reduction in the values of the first and third quartiles. We also observed a decrease in the mean value (represented by the small cross) after CA4 administration. The whiskers represent the dispersion of 90% of the lipid R_1 values measured. A graph of cumulative frequencies of lipid R_1 is shown at the bottom for similar data. **E:** Distribution of lipid R_1 values recorded at baseline and 3 hr after CA4 administration in the MDA-MB-231 tumor model. Box and whisker plots show a decrease in the median value, as well as a reduction in the values of the first and third quartiles. We also observed a decrease in the mean value after CA4 administration. The whiskers represent the dispersion of 90% of the lipid R_1 values measured within the tumors. A graph of cumulative frequencies of lipid R_1 is shown at the bottom for similar data.

posttreatment conditions was statistically significant ($P < 0.01$) (Fig. 3C). Typical K^{trans} maps obtained for an MDA-MB-231 tumor are presented in Figure 3D.

DISCUSSION

CA4 is the leading compound of VDAs, inhibiting tubulin assembly into microtubules in endothelial cells. As a result, a vascular collapse occurs, reducing blood flow and the supply of oxygen and nutrients (2). In the current study, we monitored the changes in hemodynamics and oxygenation in NT2 tumors and MDA-MB-231 tumors because their lipid content is sufficient to measure lipid T_1 . We focused on a lipid peak of 4.0 ppm, corresponding to the protons that make up the glycerol backbone of triglycerides. Our results show that MOBILE can reflect the increase of hypoxia that occurs 3 hr after CA4 administra-

tion. Indeed, a significant decrease in recorded lipid R_1 values reflects the sensitivity of the technique to address an increase in tumor hypoxia from 1% to 7%. According to a previous publication (31), the changes in lipid R_1 values were lower in the most hypoxic tumor model. Although T_1 measurements reflect oxygenation in tissues, oxygenation in the vascular compartment can also be assessed using blood oxygen level-dependent MRI or functional MRI (32). However, McPhail et al. (33) demonstrated recently that an increase in the effective transverse relaxation rate observed 24 hr after CA4 injection was caused by a change in hemodynamics rather than a change in oxygenation. The hypoxic effect of CA4 on the tumor microenvironment was confirmed via EPR oximetry, which we used as an invasive but quantitative technique of reference. Despite a difference in the basal oxygen level within the two tumor models, both were

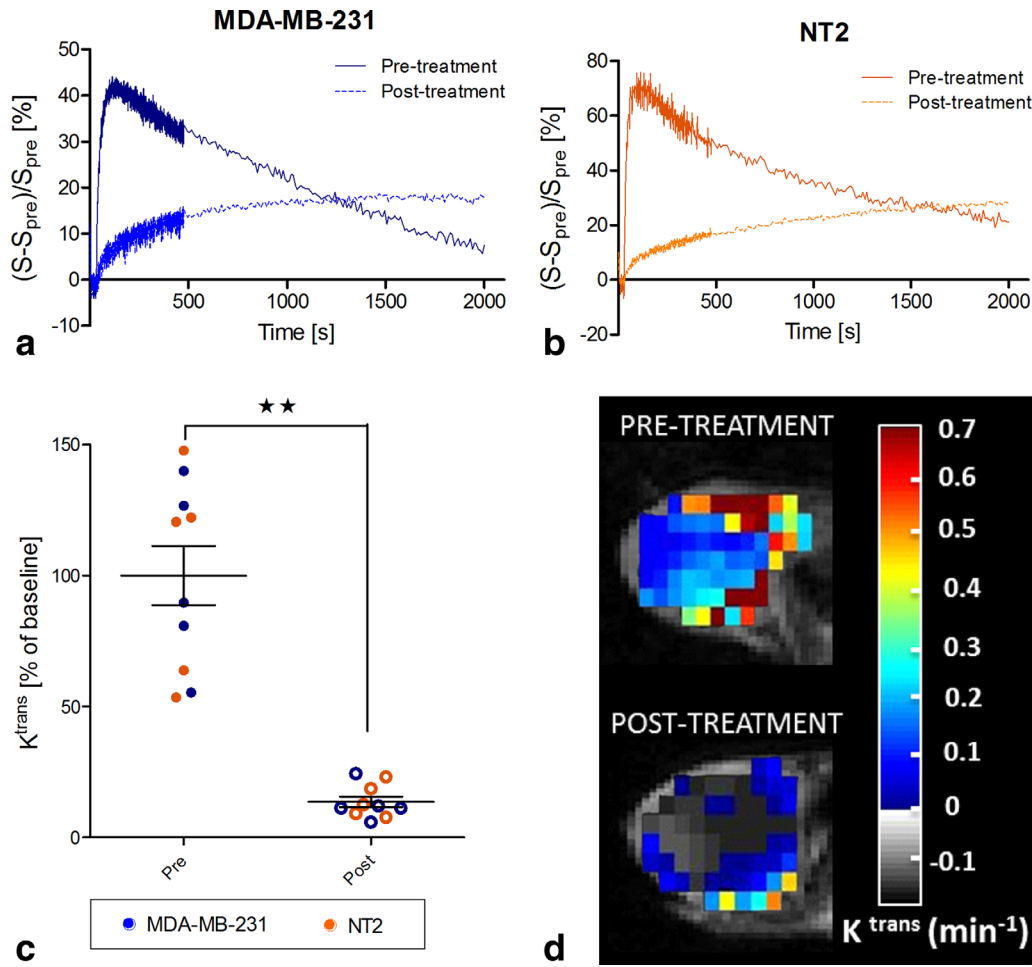


FIG. 3. DCE-MRI experiments. **A, B:** Typical signal enhancement in % observed before and 3 hr after CA4 treatment in MDA-MB-231 (A) and NT2 (B) tumors. A smaller enhancement and a slower washout were observed after CA4 treatment. **C:** Relative changes in K^{trans} induced by CA4 treatment in MDA-MB-231 and NT2 tumors ($n=5/\text{tumor model}$). There was a significant decrease of K^{trans} in pooled data from both tumor models. $**P < 0.01$. **D:** Typical K^{trans} obtained at baseline and 3 hr after treatment. After treatment, K^{trans} values decreased and a high proportion of pixels were nonperfused ($K^{trans} < 0$, gray region).

influenced by CA4 administration. This finding is consistent with previous histological observations: in an immunohistological pimonidazole study from El-Emir et al. (34), an increase in hypoxic areas of 23.7% in both peripheral and central parts of the tumor was revealed 1 hr after CA4 administration. In the present study, monitoring pO_2 levels with EPR 3 hr after DMSO administration did not reveal any effect on pO_2 levels 3 hr after the injection. These findings suggest that the observed changes in the treated group were due to CA4 administration (see Supporting Information for details).

With regard to tumor hemodynamics, DCE-MRI also allowed the detection of early effects of acute CA4 treatment. After contrast agent injection, tumors exhibited a smaller enhancement and a slower washout after CA4 treatment compared with pretreatment signal enhancement. Pharmacokinetic modeling confirmed the hemodynamic changes induced by CA4, as reflected by the significant decrease of K^{trans} observed between pre- and posttreatment conditions. Indeed, when pooling results obtained from both tumor models, K^{trans} showed a reduction of 86.3% 3 hr after CA4 treatment. K^{trans} represents the rate of transfer of the contrast agent from the blood

to the interstitial space, and its physiological interpretation depends on the balance between capillary permeability and blood flow in the tumor (35): higher permeability makes K^{trans} more reflective of blood flow (flow-limited situation), whereas lower permeability makes K^{trans} more reflective of vascular permeability (permeability-limited situation) (19,35,36). Although caution should be exercised in the interpretation of K^{trans} , the K^{trans} changes observed in our study are likely related to tumor blood flow changes. Indeed, in a previous study investigating the effects of CA4 administration in a hepatocellular carcinoma model using DCE-MRI, we showed that the magnitude of changes in K^{trans} determined with gadoterate was similar to blood flow changes determined in a parallel group of mice with the perfusion marker Hoescht 33342 (36). Additionally, Maxwell et al. (22), in a study of rat P22 sarcomas, found a strong correlation between mean K^{trans} values (determined with Gd-DTPA, a similar contrast agent) and tumor blood flow determined by radiolabeled iodoantipyrine uptake (determined in parallel groups of animals). Some pixels are nonenhancing in posttreatment condition, as they are no longer perfused (Fig. 3D). These pixels were set to 0

for the calculation of mean values to avoid outliers affecting the mean (36). Other types of analysis (eg, fraction of enhancing voxels) could also be useful to detect the early effects of CA4 treatment.

Histological analysis supported our MRI findings (see Supporting Information). Although no quantitative assessment was achieved, a reduction of perfused vessels was clearly observed in treated tumors in both models compared with a separate group of untreated tumors. Zhao et al. (37) made the same observation in MDA-MB-231 tumors 2 hr after CA4P treatment.

VDAs are cytostatic drugs that induce no reduction size immediately after administration. Cytotoxic drugs are currently used in combination with VDAs to target the residual cells of the viable rim. Moreover, because tumor size is not a good indicator of early treatment response, functional imaging is required to assess early changes in tumor microenvironment (38) that could influence the timing of cotreatment administration. For example, if CA4 administration is to be combined with x-ray therapy, it is important to administer it during or after the irradiation in order to strengthen the damages induced by radiotherapy and to avoid radioresistance that could be induced by a CA4 administration before radiotherapy (15). On the contrary, a recent study involving a radiopharmaceutical compound highlighted the importance of administering CA4 24 hr before the injection of ¹³¹I-hypericin, a necrosis avid contrast agent that accumulates in necrosis areas and irradiates cells of the viable rim (39). However, a tumor's response to VDA will still depend on its vasculature, microenvironmental characteristics, host-tumor interactions, and level of expression genes (39). Consequently, it is necessary to monitor those changes to individualize treatment and improve its efficiency. Despite its widespread use in pre-clinical experiments and its invasive properties, it is important to note that immunohistology is not a useful tool for predicting tumor response to VDA therapy (40).

In addition to the limitations of DCE-MRI described by Fruytier et al. (36), it is also important to note that MR experiments could not have been performed on the same mice because the administration of the contrast agent would have affected the lipid R1 measurements. Further work on other tumor models is needed to investigate whether MOBILE can monitor decreases in tumor oxygenation when the lipid content is lower.

In conclusion, our DCE-MRI results and MOBILE observations combined to highlight a substantial change in the tumor microenvironment, validating the use of MOBILE to monitor decreases in tumor oxygenation. However, a higher dynamic range was observed with DCE-MRI that recorded a drastic fall in K^{trans} parameters, reflecting the vascular shutdown. Consequently, DCE-MRI has a higher dynamic range to observe changes in the tumor microenvironment at early time points of CA4 treatment of NT2 and MDA-MB-231 tumors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supporting Figure S1. Histological results. Representative pictures of tumor sections from MDA-MB-231 (A-B) and NT2 tumors (C-D) stained with CD31 antibody for endothelial cells (red). The perfusion marker Hoechst 33342 (blue) was injected 1 min before sacrifice. The CA4 effects were clearly visualized by a less presence of perfused vessels in treated tumors (B-D) compared to untreated tumors (A-C).