

3D Dynamic Contrast-Enhanced Ultrasound for Personalized RRx-001 Dosing: A Preclinical and Clinical Pilot Study

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Background and Purpose: Tumor blood vessels are abnormal and contribute to therapy resistance. RRx-001, an NLRP3 inhibitor, has been shown to remodel tumor vascular properties, enhancing the effects of cytotoxic or immune-promoting agents. However, optimizing timing and dosing of combined treatment regimens is challenging on a patient-by-patient basis; tools for longitudinal imaging to guide such treatment strategy are necessary. Our aim was to evaluate the feasibility of 3D dynamic contrast-enhanced ultrasound (3D DCE-US) to detect acute and subtle tumor perfusion changes following RRx-001 therapy in both mice and humans.

Methods: RRx-001 was administered intraperitoneally at varying doses to determine its effect on perfusion in a mouse model of colon cancer. Blood flow and volume were assessed semi-quantitatively using 3D DCE-US within hours and at subsequent time-points. Additionally, we assessed 3D DCE-US in three patients enrolled in a Phase 2 RRx-001 clinical trial (NCT02096354) by imaging them longitudinally.

Results: In mice ($n = 38$), 6 mg/kg RRx-001 led to a 1.5% increase in AUC ($P = 0.02$) and MTT at 24 hours; 12 mg/kg showed no acute effect. In patients ($n = 3$), 66 mg weekly dosing produced variable perfusion changes within hours to days. Two patients with stable disease showed transient perfusion increases (survival 9-15 months); one with progressive disease showed early perfusion loss (survival 4 months). No grade 3/4 toxicities occurred.

Conclusion: These findings highlight the potential of 3D DCE-US as a noninvasive, bedside tool for real-time monitoring of RRx-001-induced perfusion changes, supporting the feasibility of personalized dosing strategies in both preclinical and clinical settings.

Key words: Ultrasound; Vascular normalization; Contrast-enhanced ultrasound; Oncology; Tumor vasculature

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Tumor blood vessels provide essential nutrients and oxygen for cancer cell survival and proliferation. However, because tumor angiogenesis, the process by which blood vessels are recruited and devel-

op in tumors, is highly dysregulated, vascular systems in the tumor microenvironment tend to be abnormal and poorly formed. These vascular inefficiencies result in suboptimal nutrient and oxygen delivery to tumor tis-

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sues, promote a highly heterogeneous tumor microenvironment, and limit the efficacy of many cancer therapies that depend on an adequate tumor blood supply [1].

Several antiangiogenic agents have been approved for cancer treatment, either as monotherapies or in combination with conventional treatments, including bevacizumab and ziv-aflibercept for metastatic colorectal adenocarcinoma [2,3]. A well-documented benefit of antiangiogenic therapy, which may account for the observed synergy with cytotoxic agents in cancers such as colorectal cancer and renal cell carcinoma, is its ability to induce vascular remodeling [4]. This hypothesis suggests that normalizing the chaotic, leaky, and immature tumor vasculature can improve drug and oxygen delivery [5]. However, because dosing and timing requirements vary between patients, therapies like bevacizumab must often be individualized and guided by longitudinal monitoring of tumor blood flow. Other emerging agents, such as RRx-001 [6], have also shown the ability to rapidly modulate tumor perfusion within minutes to hours. RRx-001 is a novel hypoxia-activated nitric oxide donor with radio- and chemo-sensitizing effects and evidence of vascular normalization properties [7–11]. It is currently under investigation in a phase 3 trial for small cell lung cancer (SCLC) [11] and a phase 2b/3 trial as an anti-mucositis agent in first-line head and neck cancer, where it has received FDA Fast Track designation [12,13]. Nevertheless, timing and dosing of such agents in combination therapies remain major challenges.

Vascular remodeling varies greatly between patients and depends on the baseline characteristics of the tumor microenvironment. Therefore, tools to monitor and detect acute and subtle perfusion changes indicative of vascular remodeling are needed to appropriately time secondary therapies. Non-invasive bedside monitoring techniques able to detect perfusion modifications during therapy have been extensively explored. These include several dynamic contrast-enhanced (DCE) imaging modalities such as magnetic resonance (MR), computed tomography (CT), and ultrasound (US). DCE-US, in particular, offers a safe, non-ionizing, cost-effective, and repeatable approach for monitoring tumor perfusion at the bedside [14]. Our group has recently expanded this approach to 3D dynamic contrast-enhanced ultrasound (3D DCE-US), using a volumetric probe to capture entire tumor volumes longitudinally, reducing operator-dependent variability and sampling errors [15].

In this pilot study, a preclinical and clinical component were conducted to assess the feasibility of using 3D DCE-US to monitor acute and subtle perfusion changes following RRx-001 administration. We demonstrate that 3D DCE-US can detect subtle dose-dependent changes,

including patient-specific responses, within hours to a day after treatment.

Methods

Ethics statement

All preclinical experimental procedures involving laboratory animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. All patients were recruited and gave informed consent as part of the Phase 2 randomized, open-label study of RRx-001 versus regorafenib in metastatic colorectal cancer (ROCKET) (NCT02096354) and also consented for 3D DCE-US imaging as part of an approved protocol by our institution's Institutional Review Board (IRB) and Scientific Review Committee (SRC).

RRx-001

RRx-001 was provided by EpicentRx, Inc (La Jolla, CA). The synthesis and characterization of RRx-001 is reported in detail elsewhere [8,16]. For preclinical studies, RRx-001 was dissolved in DMSO and then diluted with growth medium for a final concentration of DMSO at < 0.05%. RRx-001 solution preparation immediately preceded intraperitoneal (IP) administration. In clinical studies, RRx-001 was solubilized in a solvent co-mixture of 1-part dimethylacetamide (DMA) + 2 parts PEG-400 and diluted with water for injection (WFI) prior to infusion.

In Vivo Tumor Model and Experimental Design A total of 38 female nude mice (Charles River, Wilmington, MA; 6–8 weeks old; 20–25 g) were induced with murine (CT26; ATCC, Manassas, VA) colon cancer. CT26 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. At 70%–80% confluence and following trypsinization, 1×10^6 tumor cells were collected, suspended in 50 µL phosphate-buffered saline and 50 µL Matrigel (BD Biosciences, San Jose, CA), and injected subcutaneously into the right lower hind limb of the nude mice. Tumors were allowed to grow for 10 days to an average diameter of 10 mm (range: 6–14 mm) in the maximum direction (measured with electronic calipers). These tumors frequently exhibited central necrosis, providing an ideal model to assess whether RRx-001 can rapidly improve blood perfusion. Treatment responses were examined using tumor growth measurements. All animals were randomly stratified into one of three groups: Control ($n = 16$), Treatment Dose 1 (RRx-001 at 6 mg/kg; $n = 13$), Treatment Dose 2 (RRx-001 at 12 mg/kg; $n = 9$). Tumors were imaged with 3D

DCE-US at the following timepoints: baseline (before treatment), day 0 after treatment within 4 hours, and day 1 after treatment.

In-Human Treatment And Evaluation Three eligible patients with advanced colorectal cancer and liver metastases confirmed by pathological biopsy (Table 1), all of whom were enrolled in the ROCKET (NCT02096354) phase 2 clinical trial from 2012-2014 consented to undergo 3D DCE-US imaging. Patients were imaged at baseline (pre-therapy), within 2-4 hours after a first treatment dose, 7 days post-first treatment, and 2 weeks post-first treatment (when patients were available; only patients 2 and 3). All three patients had experienced progression following first-line and second- or third-line chemotherapy and subsequently received 66 mg of RRx-001 administered once weekly, followed by 180 mg/m² of irinotecan on a biweekly schedule. Patient-level quantitative DCE-US parameters are reported in table 2. This study was conducted with the approval of the local Institutional Review Board (IRB). Patient response was classified as Responder (R), Stable Disease (SD), or Progressive Disease (PD) based on clinical standard imaging after the full course of therapy.

3D DCE-US imaging

DCE-US uses FDA-approved intravascular ultrasound contrast agents, which consist of highly echogenic, micron-sized gas bubbles (microbubbles), stabilized by a shell made from biodegradable materials. This enables qualitative tissue perfusion assessment and quantitative parameterization of tissue blood flow and blood volume. Imaging was carried out in 3D with a Philips EPIQ7 coupled to a clinical X6-1 3D transducer using clinical grade contrast microbubble agents (Definity, Lantheus Medical Imaging, MA, USA) administered

as boluses. Definity microbubbles (FDA-approved perfluoro microbubbles) were diluted 1:4 in sterile 0.9% saline after activation as per the manufacturer. For all mice imaging procedures, mice were anesthetized with inhalation of 2% isoflurane in room air (administered at 2 L/min). Mice were placed prone on a heated imaging stage to maintain constant body temperature for the entire duration of the experiments. A 27G needle catheter (VisualSonics, Toronto, Canada) attached to an infusion pump (Kent Scientific, Torrington, CT) was placed into one of the two tail veins for contrast agent injection. The transducer was clamped in a fixed position to reduce motion artifacts. To further minimize artifacts in the near-field zone of the clinical transducer, a customized standoff ultrasound gel was placed on the skin of all mice. The distance between the transducer and the center of the tumor was set at 30 mm. The following imaging parameters were kept constant for 3D DCE-US imaging experiments in all tumors: center frequency, 3.2 MHz; mechanical index, 0.09; volume rate, 1 Hz; dynamic range, 52 dB; focus distance beneath the tumor, 40-60 mm from the transducer head. A suspension of 120 µL was administered over five seconds (at a constant injection rate of 24 µL/sec) with an infusion pump. Control and treated tumors were imaged at baseline and at four hours and one day following initiation of therapy. For each bolus, 3D DCE-US datasets were acquired in real time over four minutes per mouse using the ultrasound system's built-in Digital Navigation Link, which interfaced with custom in-house MevisLab modules written in C++ [17-19].

Imaging in patients followed a similar protocol. The same Philips EPIQ7 system and X6-1 transducer were used with the same imaging parameters as in mice, with the exception of custom adjustments to the focus and

Table 1 Patient Characteristics: stable disease (SD) and progressive disease (PD)

Patient number	Age (years)	Gender	Hb at baseline (g/dL)	ALT at baseline (U/L)	Albumin (g/dL) at baseline	Size of liver metastasis at baseline (mm)	Dose of RRx-001 (mg)	RECIST 1.1 relative change from day 0-56
1	75	M	9.2	60	3.3	67	66	0% SD
2	64	M	8.7	70	3.1	82	66	+26% PD
3	58	F	9.6	45	4.1	29	66	+15% SD

Table 2 Mean 3D-DCE-US patient measurements of regional blood volume (RBV) and regional blood flow (RBF)

Patient	Parameter ($\times 10^4$)	S00	S01	S02	S03
1	RBV	3.87 (3.75, 4.00)	18.9 (17.9, 19.9)	8.64 (8.31, 8.97)	NA
	RBF	0.267 (0.228, 0.308)	1.13 (0.868, 1.21)	0.700 (0.544, 0.867)	NA
2	RBV	6.99 (6.55, 7.43)	8.25 (7.78, 8.71)	7.86 (7.15, 8.56)	3.16 (2.98, 3.35)
	RBF	0.499 (0.331, 0.685)	1.21 (0.617, 1.87)	0.718 (0.445, 1.03)	0.787 (0.596, 0.997)
3	RBV	5.96 (5.60, 6.31)	6.32 (6.12, 6.52)	2.94 (2.72, 3.16)	0.923 (0.797, 1.05)
	RBF	2.50 (1.79, 3.28)	1.17 (0.956, 1.40)	0.111 (0.0831, 0.142)	0.158 (0.0635, 0.276)

Confidence interval (CI) presented in parentheses. NA indicates no scan was performed, and no measurements are available.

depth of imaging. We used the infusion-based disruption-replenishment approach described previously [17]. Patients were infused over a period of up to three minutes with a solution consisting of 0.9 mL of contrast agent diluted in 35.1 mL of saline, delivered at a constant rate of 0.08 mL/s using a syringe pump (Medfusion 3500; Smiths Medical, Dublin, OH) to achieve steady-state conditions. Microbubble solutions were freshly prepared, gently agitated before infusion, and infused using a syringe pump to maintain consistent concentration throughout the injection. The whole syringe pump was gently agitated by the operator throughout the entire infusion period; this was important to maintain a consistent concentration. A single disruption-replenishment sequence was then performed while the contrast agent infusion continued. The disruption (“flash”) event was based on a high-mechanical Index (MI of 0.77) through about three temporal 3D frames applied to the entire image volume, lasting 2–3 seconds in total. Cine imaging of 3D DCE-US contrast dynamics within the lesion was performed for about one minute immediately after the flash to capture the wash into steady-state, and as previously described [17], all data was recorded using the Digital Navigation Link. Patients were asked to breath-hold during the first 10–20 seconds of replenishment of contrast agent and were trained to recommence breathing slowly by an attending radiologist. All imaging was performed by sonographers (> 2 yrs experience). Imaging parameters were set per patient for depth, focus and width at baseline and maintained for subsequent scans [15,17].

3D DCE-US analysis

All 3D DCE-US imaging datasets were analyzed using custom Python-based software in a similar fashion in mice and in humans. To generate volumes of interest (VOI), we manually contoured the whole tumor visualized on sagittal, longitudinal, and coronal slices using ITKsnap to produce volumes of interest. Image analysis was performed by one reader with more than two years of DCE-US experience. The reader was blinded to the treatment information. All 3D DCE-US imaging datasets were analyzed with custom software as previously described [17–19]. Tumor perfusion was quantified using a first-pass kinetic analysis of the signal intensity-time curve within the VOI, based on the wash-in and wash-out dynamics of microbubbles following bolus injection. For conventional parameter extraction from mouse bolus data, a time-intensity curve (TIC) was generated by averaging the linearized intensities of all pixels within the VOI. A lognormal model was fitted to each perfusion curve to extract the following perfusion parameters in arbitrary units (a.u.): peak enhancement

(PE, measured in arbitrary units, i.e., au), area under the curve (AUC, au), mean transit time (MTT, seconds) and time to peak (TP, seconds). PE and AUC are generally related to blood volume while TP and MTT are estimates of perfusion rates. Perfusion parameters on days 0 and 1 were normalized to the baseline values to show percent changes [15,17]. In patients, we used the mono-exponential curve fit to the replenishment curve, as previously described [17,20]. Given the small number of patients, no parameters were extracted; instead, the full replenishment curve was visualized for this observational component.

Statistical analysis

All data are expressed as the mean + standard error of the mean (SEM). Data were analyzed using IBM SPSS statistics software version 22 (IBM Corp, Chicago, IL). A two-tailed Wilcoxon Rank Sum Test was performed to compare two datasets. A *p*-value of less than 0.05 was considered statistically significant. For mouse work, Δ AUC at 24 hours was designated as the primary endpoint. The effect size for Δ AUC between treated and control tumors was 0.85 (95% CI, 0.18–1.42). Because this was an exploratory pilot study, no formal adjustment for multiple comparisons was applied. In-human data was only qualitatively analyzed.

Results

Preclinical evaluation of tumor perfusion subtle change with 3D-DCE-US

A total of 38 animals were imaged. In figure 1A, we present semi-quantitative perfusion parameters derived from a bolus. These results suggest a trend toward increased perfusion parameters (blood flow and volume) within 4 hours of 6 mg/kg RRx-001 treatment, with effects persisting to 24 hours and supported by qualitative observations. Specifically, AUC and MTT, both reflective of overall blood flow and volume, showed an approximate 1.5% increase at 24 hours, which was statistically significant ($P = 0.02$). Non-significant increases in PE and TP were also observed at both 4 and 24 hours following the 6 mg/kg dose. In contrast, no acute increases in perfusion parameters were seen with the 12 mg/kg dose, which instead resembled the perfusion trends of control animals. A representative 3D CEUS image of a lesion is shown in figure 1B.

Patient evaluation of tumor perfusion subtle changes with 3D DCE-US

The responses of the three imaged patients with third-line advanced colorectal cancer, treated as part of the randomized Phase 2 ROCKET trial (NCT02096354)

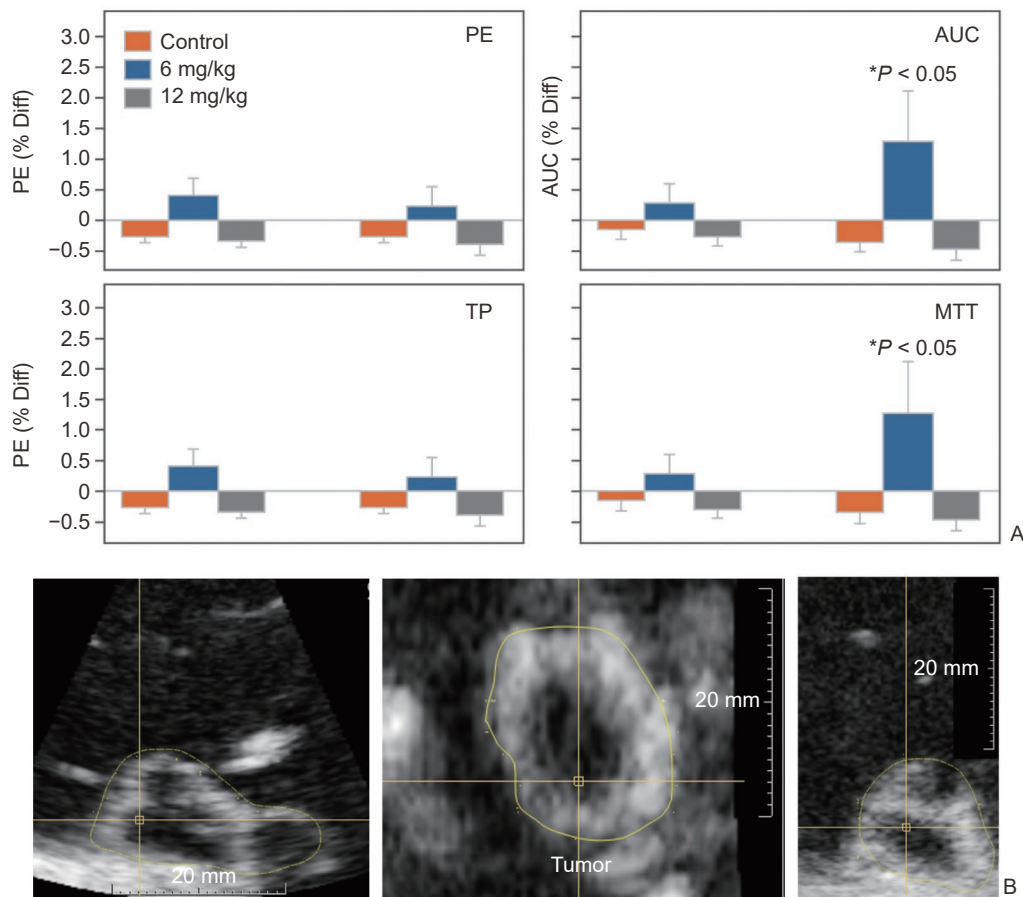


Figure 1 (A) 3D DCE-US detects dose-dependent changes in tumor perfusion parameters following RRx-001 treatment in mice. All animals available for control ($n = 16$) vs. 6 mg/kg ($n = 13$) vs. 12 mg/kg ($n = 9$) for each of 4 bolus-perfusion parameters shown as percent difference relative to baseline at 4 hrs and 24 hrs. Trend is noted in overall increases in perfusion parameters in mice receiving RRx-001. Peak enhancement (PE) reflects a measure of blood volume at the highest peak while area under the curve (AUC) represents total blood volume flowing in tumor from wash in to wash out. While a trend is noted for PE and AUC within 4 hours after treatment, AUC at 24 hours after treatment was the only one significant parameter ($P = 0.02$; compared to control at 24 hours after baseline). Time-to-peak (TP) is proportional to blood flow at wash-in and mean transit time (MTT) represents the total time it takes contrast to wash in and wash out (also a measure of blood flow). While increases in blood flow (i.e., faster wash-in and longer wash-out) are noted after RRx-001, these were not significant. Error bars are standard errors of the mean (SEM). Significance is represented as $*P = 0.02$ (Mann-Whitney test); (B) Contrast ultrasound image at peak enhancement of ~25 mm lesion on hind leg of animal viewed in sagittal, coronal, and axial planes. The yellow volume of interest designates the lesion in 3D.

evaluating RRx-001 ± irinotecan versus regorafenib ± irinotecan, are presented in [table 1](#): SD ($n = 2$) and PD ($n = 1$). No grade 3/4 toxicity was observed in these patients, and the most common grade 1/2 adverse events were localized infusion pain ($n = 3$) and fatigue ($n = 2$). Infusion pain was attributed to RRx-001 and not the ultrasound contrast agent (as it is well-documented in many clinical trials without ultrasound contrast agent involvement). The median overall survival of patients with SD approached 15 months and 9 months (patients 1 and 3, respectively) compared to 4 months for Patient 2 who had PD. We visually observed subtle perfusion changes in the imaging over the course of one week in all three patients, as illustrated in representative 2D snapshot images extracted from the 4D volume shown in [figure 2](#). [Figure 2C](#) presents the best fit to the TIC for all three patients: Patient 1 had an immediate increase in perfusion followed by a return to baseline perfusion by 7

days; Patient 2 had an increase in perfusion within 4 hours lasting 7 days, followed by complete cessation of blood flow within two weeks of treatment initiation; Patient 3 had no changes in perfusion within 4 hours, but cessation of flow was observed within 7 days following therapy.

Discussion

This study evaluated the ability of 3D DCE-US to quantitatively detect subtle and acute tumor perfusion changes in murine tumors before and after treatment with RRx-001, and in three colorectal cancer patients with liver metastases treated on the ROCKET clinical trial. In treated tumors, perfusion increased markedly within 24 hours compared with controls ($\Delta AUC +1.5\%$, $P = 0.02$), consistent with an acute vascular modulation effect of RRx-001. In contrast, early changes in some

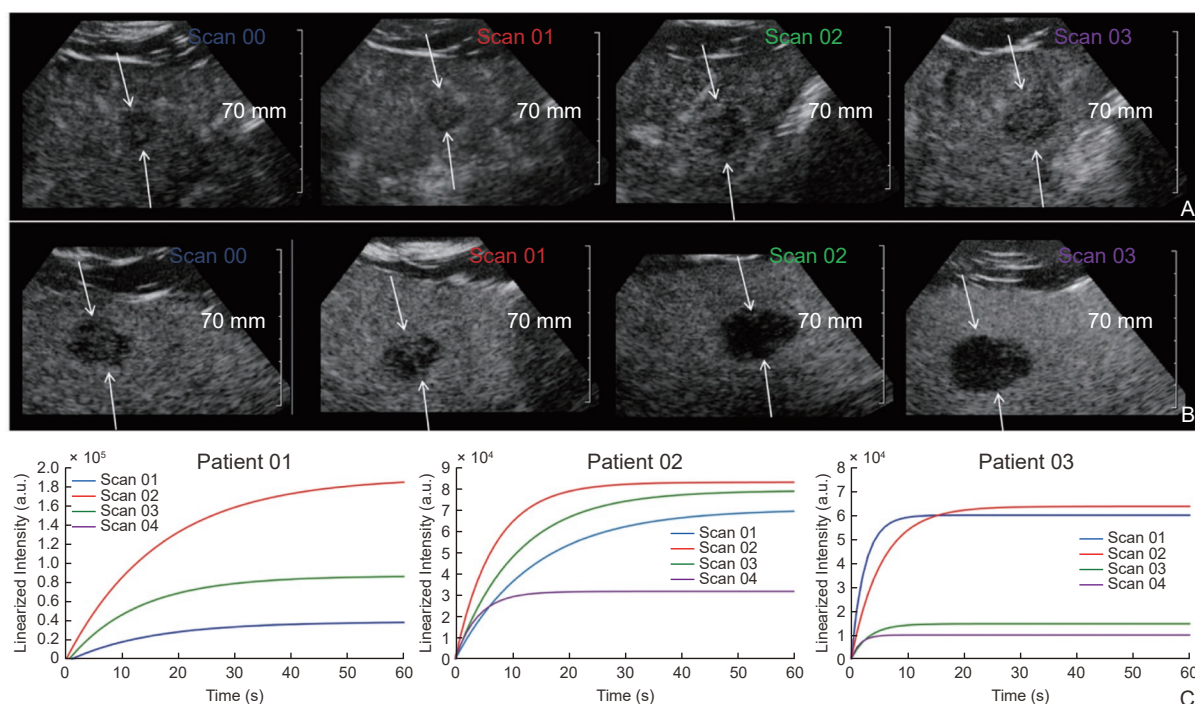


Figure 2 Longitudinal 3D DCE-US reveals heterogeneous patient-specific perfusion responses to RRx-001 over two weeks. Panel A shows representative snapshot images from scan 00 (baseline), scan 01 (2-4 hours), scan 02 (7 days) and scan 03 (2 weeks) from the 3D-DCEUS of patient 2, and Panel B shows the same of patient 3, and Panel C is the perfusion TIC for patients 1, 2 and 3. The blue curve represents baseline scans before any RRx-001 treatment administration, the red curve represents 2-4 hours post first treatment, the green curve is 7 days post treatment and the purple curve 2 weeks after initial treatment. Note from C the drastic differences in baseline perfusion, and the change in perfusion for each patient. The same Philips EPIQ7 system and X6-1 transducer were used with the same imaging parameters as in mice, with the exception of custom adjustments to the focus and depth of imaging.

tumors were more subtle (e.g., $\Delta\text{AUC} +5\text{-}10\%$, $P = 0.02$), reflecting heterogeneous baseline vascularity and response kinetics. To better understand the mechanism behind these perfusion changes, it is important to consider the biological activity of RRx-001. Intravenously administered RRx-001 binds to hemoglobin on red blood cells (RBCs) and induces nitro-oxidative modifications [21-23]. These modified RBCs express ligands that bind to endothelial adhesion molecules such as VCAM-1 and ICAM-1 [23], similar to sickled cells. Previous studies have shown that these RRx-001-loaded RBCs become entrapped within the dense microvascular network of tumors, leading to hemolysis, endothelial injury, vaso-occlusion, and regression of neovascularity [24]. It is therefore plausible that RRx-001 promotes vascular remodeling by pruning immature vessels, resulting in improved perfusion and more efficient delivery of chemotherapy [7] and oxygen—an effect observed in other preclinical and clinical studies. Notably, during this remodeling window, RRx-001 has demonstrated synergy with radiation, chemotherapy, and immunotherapy [24-26].

However, with repeated administration of RRx-001, especially at higher doses, there is a potential for excessive vessel regression, leading to reduced vascular supply and increased hypoxia, especially within the tumor

core. This occurs because immature tumor vessels are preferentially sensitive to RRx-001. Ideally, RRx-001 should be administered at a dose and frequency that promotes vascular normalization without compromising vascular access to the extent that it hinders drug delivery or triggers hypoxia-driven tumor adaptation and VEGF independence. Currently, RRx-001 is administered clinically for solid tumors at a dose of 4 mg once weekly, typically prior to chemotherapy or immunotherapy. At this dosing schedule, reductions in interstitial fluid pressure and increases in tumor perfusion have been observed, sufficient to enhance tumor sensitivity to radiation and chemotherapy. Importantly, these effects occur without the arterial thromboembolic events, hypertension, or proteinuria commonly associated with approved VEGF inhibitors [27].

In the ROCKET study, RRx-001 was administered as a single agent until disease progression, followed by intravenous irinotecan (180 mg/m^2) every two weeks. However, while the 66 mg dose of RRx-001 used in the ROCKET study may have initially normalized tumor neovessels, leading to the improved perfusion observed in Patients 1 and 2, continued administration at this dose may have led to excessive vascular pruning, potentially impairing drug delivery and reducing the efficacy of secondary treatment.

Similarly, in the mouse study, a 6 mg/kg dose of RRx-001 increased blood flow, whereas a 12 mg/kg dose reduced it. This suggests a dose-dependent effect: higher doses may cause excessive pruning and hypoxia, particularly in tumors with low microvascular density (MVD). Although MVD was not assessed in these studies, we and others have shown that 3D DCE-US can quantify parameters correlated with histological MVD [15,17]. Paradoxically, a higher baseline MVD may be associated with better outcomes following RRx-001 treatment. While increased MVD is generally associated with poor prognosis [28], as seen with bevacizumab, it may also serve as a “vascular reserve” that mitigates the effects of excessive vascular destruction by RRx-001.

Our results demonstrate that 3D DCE-US can detect subtle, acute perfusion changes in both mice and patients. Compared to traditional 2D imaging, 3D DCE-US offers potentially more accurate and quantifiable measurements by minimizing sampling error and operator variability during longitudinal imaging [15,17]. Notably, the 3D method is not more complex; the 2D and 3D workflows are similar [15,17,29]. In this pilot study, we explore basic volumetric quantification. Although ultrasound frequency limitations in small animals reduced sensitivity to subtle changes, clear perfusion differences were observed across treatment groups. The large SEM bars, however, suggest that vascular remodeling varies significantly on a tumor-by-tumor basis, reinforcing the need for individualized perfusion imaging to guide combination therapies involving agents such as RRx-001. In humans, we demonstrated in three patients that baseline perfusion and tumor-specific properties strongly influence both acute and longer-term (up to two weeks) perfusion changes on a patient-by-patient basis.

Nevertheless, this pilot feasibility study has several limitations, most notably the small number of animals and patients imaged, leaving several important questions unanswered. Importantly, immunohistochemistry was not performed, so it remains unknown whether the effects on blood flow observed with 3D DCE-US correlate with MVD or VEGF levels before and after RRx-001 treatment. Additionally, intratumoral uptake of chemotherapy following RRx-001 was not directly measured—an important limitation, as this is a key determinant of therapeutic efficacy.

Despite these limitations, these hypothesis-generating findings suggest that 3D DCE-US has potential as a tool to track patient-specific tumor perfusion responses to RRx-001, with the aim of identifying an optimal vascular remodeling window. The study also confirms that RRx-001 induces short-term functional changes in tumor perfusion and may enhance drug and oxygen

delivery, potentially improving the efficacy of established treatments such as chemotherapy and radiation therapy. Future planned work will incorporate immunohistochemistry and 3D DCE-US to examine the relationship between RRx-001 dose, perfusion, and vascular permeability—particularly in combination with various chemotherapies. Additional factors to investigate include the effects of tumor size and baseline perfusion on the timing and duration of the vascular normalization window, and whether the observed radio- and chemo-sensitization effects are linked to RRx-001-induced hyperperfusion. Comparative studies with bevacizumab are also planned to benchmark the magnitude of tumor blood flow changes induced by RRx-001. Additionally, voxel-wise heterogeneity analysis and histological correlation were not performed in this pilot study but are planned for future work to better characterize spatial perfusion patterns. Finally, longitudinal quality assurance and phantom calibration will be incorporated in all studies.

Altogether, these findings highlight the promise of 3D DCE-US as a translational imaging tool to guide timing and dosing of vascular-modulating agents like RRx-001, ultimately supporting more personalized and effective cancer therapy strategies.

Conclusions

This pilot study demonstrates the feasibility of 3D DCE-US to noninvasively capture acute, dose-dependent perfusion changes following RRx-001 therapy in both murine tumors and human liver metastases. The ability to detect subtle, individualized vascular responses within hours supports the utility of 3D DCE-US as a bedside imaging tool to guide dosing and optimize therapeutic timing. As interest grows in vascular-modulating agents across oncology, incorporating real-time perfusion imaging may be key to unlocking their full clinical potential.

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Not applicable.

Authors' Contributions

Oronsky designed the experiments and drafted the manuscript; Li conducted experimental work and performed data analysis; Wang supported experimental work, performed animal imaging, and contributed to analysis; Vo-Phamhi drafted and edited the manuscript; Caroen conceived the study, contributed to manuscript drafting, and provided financial support; Abrouk contributed to manuscript drafting and data analysis; Reid supervised the study, helped design the experiments, and reviewed the manuscript; Alizadeh reviewed and contributed to the drafting of the

manuscript; Hristov, Fisher, and El Kaffas supervised the study, contributed to data analysis and manuscript drafting, and participated in the design and conceptualization of experimental and clinical work.

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Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All pre-clinical experimental procedures involving laboratory animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. All patients were recruited and gave informed consent as part of the Phase 2 randomized, open-label study of RRx-001 versus regorafenib in metastatic colorectal cancer (ROCKET) (NCT02096354) and also consented for 3D DCE-US imaging as part of an approved protocol by our institution's Institutional Review Board (IRB) and Scientific Review Committee (SRC).

Conflict of interest

The authors report no conflicts of interest.

Consent for publication

Not applicable.

References

- [1] Chen Z, Han F, Du Y, Shi H, Zhou W. Hypoxic microenvironment in cancer: molecular mechanisms and therapeutic interventions. *Signal Transduct Target Ther* 2023;8:70.
- [2] Al-Husein B, Abdalla M, Trepte M, Deremer DL, Somanath PR. Antiangiogenic therapy for cancer: an update. *Pharmacotherapy* 2012;32:1095-1111.
- [3] Van Cutsem E, Tabernero J, Lakomy R, Prenen H, Prausová J, Macarulla T, et al. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. *J Clin Oncol* 2012;30:3499-3506.
- [4] Ganss R. Tumour vessel remodelling: new opportunities in cancer treatment. *Vasc Biol* 2020;2:R35-R43.
- [5] Kanter J, Oronsky B, Reid T, Caroen S, Stirn M, Brinkhaus F, et al. Explosive hazards identified during the manufacture and transportation of 1-Bromoacetyl-3-dinitroazetidine (RRx-001). *Org Process Res Dev* 2022;26:3010-3014.
- [6] Oronsky B, Scicinski J, Cabrales P, Minchinton A. RRx-001, an epigenetic-based radio- and chemosensitizer, has vascular normalizing effects on SCCVII and U87 tumors. *Clin Epigenetics* 2016;8:53.
- [7] Oronsky B, Reid T, Cabrales P. Vascular priming with RRx-001 to increase the uptake and accumulation of temozolomide and irinotecan in orthotopically implanted gliomas. *J Drug Target* 2021;29:998-1003.
- [8] Oronsky B, Guo X, Wang X, Cabrales P, Sher D, Cannizzo L, et al. Discovery of RRx-001, a Myc and CD47 downregulating small molecule with tumor targeted cytotoxicity and healthy tissue cytoprotective properties in clinical development. *J Med Chem* 2021;64:7261-7271.
- [9] Oronsky B. Profile EpicentRx, Inc. *Hum Vaccin Immunother* 2023;19:2184963.
- [10] Oronsky B, Carter C, Reid T, Brinkhaus F, Knox SJ. Just eat it: a review of CD47 and SIRP-alpha antagonism. *Semin Oncol* 2020;47:117-124.
- [11] Oronsky B, Reid TR, Larson C, Caroen S, Quinn M, Burbano E, et al. REPLATINUM phase III randomized study: RRx-001 + platinum doublet versus platinum doublet in third-line small cell lung cancer. *Future Oncol* 2019;15:3427-3433.
- [12] Oronsky B, Goyal S, Kim MM, Cabrales P, Lybeck M, Caroen S, et al. A review of clinical radioprotection and chemoprotection for oral mucositis. *Transl Oncol* 2018;11:771-778.
- [13] Bonomi M, Blakaj DM, Kabarriti R, Colvett K, Takiar V, Biagioli M, et al. PREVLAR: phase 2a randomized trial to assess the safety and efficacy of RRx-001 in the attenuation of oral mucositis in patients receiving head and neck chemoradiotherapy. *Int J Radiat Oncol Biol Phys* 2023;116:551-559.
- [14] Amioka A, Masumoto N, Gouda N, Kajitani K, Shigematsu H, Emi A, et al. Ability of contrast-enhanced ultrasonography to determine clinical responses of breast cancer to neoadjuvant chemotherapy. *Jpn J Clin Oncol* 2016;46:303-309.
- [15] El Kaffas A, Hoogi A, Zhou J, Durot I, Wang H, Rosenberg J, et al. Spatial characterization of tumor perfusion properties from 3D DCE-US perfusion maps are early predictors of cancer treatment response. *Sci Rep* 2020;10:6996.
- [16] Scicinski J, Oronsky B, Taylor M, Luo G, Musick T, Marini J, et al. Preclinical evaluation of the metabolism and disposition of RRx-001, a novel investigative anticancer agent. *Drug Metab Dispos* 2012;40:1810-1816.
- [17] El Kaffas A, Sigrist RMS, Fisher G, Bachawal S, Liau J, Wang H, et al. Quantitative three-dimensional dynamic contrast-enhanced ultrasound imaging: first-in-human pilot study in patients with liver metastases. *Theranostics* 2017;7:3745-3758.
- [18] Zhou J, Zhang H, Wang H, Lutz AM, El Kaffas A, Tian L, et al. Early prediction of tumor response to bevacizumab treatment in murine colon cancer models using three-dimensional dynamic contrast-enhanced ultrasound imaging. *Angiogenesis* 2017;20:547-555.
- [19] Wang H, Hristov D, Qin J, Tian L, Willmann JK. Three-dimensional dynamic contrast-enhanced US Imaging for early antiangiogenic treatment assessment in a mouse colon cancer model. *Radiology* 2015;277:424-434.
- [20] Wei, K. Assessment of myocardial viability using myocardial contrast echocardiography. *Echocardiography* 2005;22:85-94.
- [21] Oronsky B, Fanger GR, Oronsky N, Knox S, Scicinski J. The implications of hypoxia in cancer. *Transl Oncol* 2014;7:167-173.
- [22] Oronsky BT, Oronsky AL, Lybeck M, Oronsky NC, Scicinski JJ, Carter C, et al. Episensitization: defying time's arrow. *Front Oncol* 2015;5:134.
- [23] Jani VP, Asaro R, Oronsky B, Cabrales P. RRx-001 increases erythrocyte preferential adhesion to the tumor vasculature. *Int J Mol Sci* 2021;22:4713.
- [24] Kim MM, Parmar HA, Schipper M, Devasia T, Aryal MP, Kesari S, et al. BRAINSTORM: a multi-institutional phase 1/2 study of RRx-001 in combination with whole brain radiation therapy for patients with brain metastases. *Int J Radiat Oncol Biol Phys* 2020;107:478-486.

- [25] Tomita Y, Oronsky B, Abrouk N, Cabrales P, Reid TR, Lee MJ, et al. In small cell lung cancer patients treated with RRx-001, a down-regulator of CD47, decreased expression of PD-L1 on circulating tumor cells significantly correlates with clinical benefit. [Transl Lung Cancer Res](#) 2021;10:274-278.
- [26] Lee MJ, Tomita Y, Yuno A, Lee S, Abrouk NE, Oronsky B, et al. Results from a biomarker study to accompany a phase II trial of RRx-001 with reintroduced platinum-based chemotherapy in relapsed small cell carcinoma. [Expert Opin Investig Drugs](#) 2021;30:177-183.
- [27] Ratner M. Genentech discloses safety concerns over Avastin. [Nat Biotechnol](#) 2004;22:1198.
- [28] Hu X, Liu H, Ye M, Zhu X. Prognostic value of microvessel density in cervical cancer. [Cancer Cell Int](#) 2018;18:152.
- [29] Wang H, Lutz AM, Hristov D, Tian L, Willmann JK. Intra-animal comparison between three-dimensional molecularly targeted US and three-dimensional dynamic contrast-enhanced US for early antiangiogenic treatment assessment in colon cancer. [Radiology](#) 2017;282:443-452.