Dynamic contrast-enhanced magnetic resonance imaging studies in preclinical settings and beyond

Septian Hartono

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Dynamic Contrast-Enhanced Magnetic Resonance Imaging Studies in Preclinical Settings and Beyond

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Summary

Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) with tracer kinetic modeling has been proposed as a biomarker for assessment of tumor angiogenesis in clinical trials.

In this work, we developed and implemented a high spatial and temporal resolution (2 s) DCE-MRI protocol for small animal preclinical studies. Microcirculatory parameters derived were validated with corresponding immunohistochemistry staining of murine human xenografts. Results show that the technique is potentially applicable as a biomarker for pre-clinical drug development, as it might allow monitoring of anti-angiogenic therapy in murine models.

We also explored the feasibility of applying the technique for perfusion imaging of mice liver. We observed a low value of interstitial volume fraction and permeability-surface area product in normal mouse liver cases, which corresponded with murine liver physiology and the results previously obtained in the human studies. These results indicated potential for the use of DCE MRI as a biomarker for sinusoidal capillarization and fibrosis in preclinical liver studies.

We further implemented the developed DCE MRI technique and analysis method for pre-clinical drug studies with mice implanted with hepatocellular carcinoma xenograft and treated with bevacizumab, a monoclonal antibody which was used in the clinic for anti-angiogenic therapy. Dose-related effects of bevacizumab are observed, suggesting potential of using the technique as a reliable non-destructive testing method for time course observation of microcirculatory changes in animals. This would also facilitate translation and clinical application to early time points after anti-angiogenic therapy, as our current study showed early decrease in micro-vascular density and blood flow after an anti-angiogenic treatment.

Finally, we also propose an alternative approach for analyzing DCE-MRI data of necrotic regions based on the diffusional transport of solutes in the tumor interstitium. Gadolinium diffusivity might serve as an alternative interstitial transport parameter in necrotic tumor regions and complement the other microcirculatory parameters derived from DCE MRI for a more complete analysis of the entire tumor.

1. Introduction

1.1 Background

1.1.1 Angiogenesis and Anti-angiogenic Therapy

Formation of new blood vessels, more commonly called angiogenesis, is important for various physiological processes, e.g., in reproductive cycle, organ development, and tissue injury repair (1). Angiogenesis results from a complex process involving activation of endothelial cell receptors, which will phosphorylate tyrosine kinase to induce new endothelial cells and in the end would result in new blood vessels.

It had been long suggested that tumor growth is angiogenesis dependent (2). Formation of new blood vessels is essential for tumor growth. It is also well acknowledged that tumor is not able to grow beyond 1-2 mm³ in complete absence of angiogenesis, since nutrients could not reach targeted tissues beyond the distance of 100-200 μ m (3). Moreover, angiogenesis is also essential for tumor metastatic mechanism, in which the host tumor spreads to other organs by entering the blood circulation through its nearest capillary. Thus, Folkman suggested targeting the new blood vessels for cancer therapy (4). This is the aim of anti-angiogenic therapy, which intends to inhibit new blood vessel formation. It was postulated that the tumor would starve and eventually die if its food supply was cut off.

Angiogenic processes are also crucial for cancer therapy for at least another reason. Tumor vasculature is irregular and leaky, in contrast with the more regular structure found in normal tissues. Moreover, the complex network of irregular neo-vessels in the tumor is highly congested, which results in a high interstitial pressure and inhibits effectiveness of chemotherapeutic agents. Hence, it is also hypothesized that a normalized vasculature would improve drug delivery to the tumor.

This goal can be accomplished by applying anti-angiogenic agents, which would not only inhibit new blood vessel formation but also normalize the pericyte coverage of neo-vessels. This would result in a decrease in interstitial fluid pressure and consequently improve drug delivery to the tumor (5). It is also postulated that the normalization period (window) is the best time to administer concomitant chemotherapy or radiation therapy.

Various anti-angiogenic agents have been developed, and two anti-angiogenic agents were used in this thesis.

ABT-869 is an oral multi-targeted receptor tyrosine kinase (RTK) inhibitor active against both the VEGF and the PDGF families of receptors (Abbott Laboratories, Illinois). The PDGF family of RTK improves pericyte coverage and the inhibition of PDGF together with VEGF stimulates vessel regression, manifesting as an anti-vascular effect with the reduction in tumor vascularity and capillary permeability.

Bevacizumab (trade name: Avastin, F. Hoffman-La Roche, Basel, Switzerland) is a monoclonal antibody which binds with the vascular endothelial growth factor (VEGF) so that it would not be phosphorylated and trigger the subsequent angiogenesis processes (6).

The choice of drug in the studies (ABT-869 for the Phase I clinical study and Bevacizumab for the preclinical studies) was done together with the pharmaceutical companies who provide the drug for respective studies.

The emergence of anti-angiogenic therapy demands a new paradigm in assessing the effectiveness of anti-angiogenic drugs. For instance, morphological imaging might not be able to adequately characterize the drug effect since anti-angiogenic drug does not necessarily reduce the size of the tumor. Hence, traditional structural imaging criteria like Response Evaluation Criteria in Solid Tumors (RECIST), which relies on tumor size measurement (7), might not be appropriate for anti-angiogenic therapy assessment. Hence, various functional imaging techniques have been developed to address this issue, one of which is dynamic contrast-enhanced (DCE) imaging.

1.1.2 Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) & Tracer Kinetic Modeling

Dynamic contrast-enhanced (DCE) imaging involves the intravenous injection of a contrast tracer and sequential imaging to monitor the flow of tracer in the tissue of interest and a feeding artery. With advances in fast imaging techniques and developments in tracer kinetic modeling, *in vivo* assessment of organ microcirculation using DCE imaging by computed tomography (DCE-CT) or magnetic resonance imaging (DCE-MRI) has become more realistic in the clinical settings (8-10).

Recently, DCE-MRI with tracer kinetic modeling has been proposed as a biomarker for assessment of tumor angiogenesis in clinical trials (9, 11-13). DCE-MRI datasets are analyzed using a mathematical model, which describes the kinetics of the tracer inside the tissue of interest. These tracer kinetic models yield various blood flow parameters that characterize tissue microcirculation, e.g., plasma flow (F) and permeability (PS). These microcirculatory parameters are then employed to assess the effects of anti-angiogenic agents.

A commonly used tracer for DCE imaging is Gd-DOTA, a complex of gadolinium with tetraazacyclododecanetetraacetic acid (14). The agent is able to diffuse through the capillary pore membranes into the interstitium. However, it remains outside the cells such that there are two spaces traversed by the agent: the intravascular space and the extravascular extracellular (or, interstitial) space.

Thus, consider a bi-compartmental capillary-tissue system where the first compartment represents the vascular space and the second compartment represents the interstitial space. The tracer concentration in each compartment following an impulse input of tracer can be given by (15)

- in compartment 1:

$$v_1 \frac{\partial}{\partial t} C_1(t, z) dz = -\rho FL \frac{\partial}{\partial z} C_1(t, z) dz - K_{21} C_1(t, z) dz + K_{12} C_2(t, z) dz + L \delta_t(t) \delta_z(z) dz \quad (1.1a)$$

- in compartment 2:

$$v_2 \frac{\partial}{\partial t} C_2(t, z) dz = K_{21} C_1(t, z) dz - K_{12} C_2(t, z) dz \quad (1.1b)$$

where C_1 and C_2 denote tracer concentration in the vascular and interstitial space, respectively. z denotes the axial coordinate along the length (L) of the cylindrical capillary, and radial concentration gradients are assumed negligible due to the small radial dimension of the capillary. K_{ij} denotes the transfer constant from compartment j to compartment i , v_1 is the fractional vascular volume, and v_2 is the fractional interstitial volume. $\delta(t)\delta(z)$ denotes a unit impulse input of tracer into the vascular space at $t=0$ and $z=0$.

The operational equation for analysis of the DCE imaging data can be expressed as

$$C_{tiss}(t) = F C_{in}(t) \otimes R(t) \quad (1.2)$$

where C_{tiss} denotes the tracer concentration in the tissue voxel, $C_{in}(t)$ denotes the arterial input concentration and $\otimes$ denotes the convolution operator. $R(t)$ is the impulse residue response function, which describes the fractional amount of tracer remaining in the tissue due to an impulse input. The impulse residual function, $R(t)$, is given by

$$R(t) = \frac{1}{L} \left(v_1 \int_0^L C_1(z, t) dz + v_2 \int_0^L C_2(z, t) dz \right). \quad (1.3)$$

The generic form of $R(t)$ is obtained by solving Equations 1.1 (a) and (b). The various microcirculatory parameters are acquired by deconvoluting Equation 1.2 (16).

Standard Two-Compartmental Model

Assuming well-mixed (homogeneous) compartments, spatial concentration gradients along z can be neglected and tracer concentrations in each compartment at time t from Equations 1.1 (a) and (b) can be simplified and are given as follows (17-19):

$$v_1 \frac{dC_1(t)}{dt} = -\rho F C_1(t) - K_{21} C_1(t) + K_{12} C_2(t) + \delta_t(t) \quad (1.4a)$$

$$v_2 \frac{dC_2(t)}{dt} = K_{21} C_1(t) - K_{12} C_2(t) . \quad (1.4b)$$

$R(t)$ is then given by

$$R_{CC}(t) = \frac{s_1 + k_{21} + k_{12}}{s_1 - s_2} \exp(s_1 t) - \frac{s_2 + k_{21} + k_{12}}{s_1 - s_2} \exp(s_2 t) \quad (1.5)$$

where s_1 and s_2 are the solutions of the following quadratic equation:

$$s^2 + \left(k_{21} + k_{12} + \frac{1}{t_1}\right)s + \frac{k_{12}}{t_1} = 0 \quad (1.6)$$

$k_{ij} = K_{ij}/v_j$ denotes the rate constant from compartment j to compartment i and t_1 denotes the vascular phase transit time ($=v_1/F$). The permeability-surface area product PS can be estimated by the following relationship:

$$PS = k_{21} v_1 = k_{12} v_2 . \quad (1.7)$$

Distributed Parameter Model

Distributed parameter model does not assume well-mixed compartments (15, 20-22), and solving Equation 1.3 with 1.1 yields the impulse residual function,

$$R_{DP}(t) = \begin{cases} 1, & 0 < t \leq t_1 \\ 1 - \exp(-k_{21} t_1) \cdot [1 + X_2(t - t_1)], & t > t_1; \end{cases} \quad (1.8)$$

where

$$X_2(t) = \int_0^t \exp(-k_{12} \tau) \sqrt{\frac{t_1 k_{12} k_{21}}{\tau}} \cdot I_1(2\sqrt{t_1 k_{12} k_{21} \tau}) d\tau \quad (1.9)$$

and I_1 is the modified Bessel function, given by

$$I_1(x) = \left(\frac{x}{2}\right) \sum_{m=0}^{\infty} \frac{1}{m!(m+1)!} \left(\frac{x}{2}\right)^{2m} \quad (1.10)$$

The extraction ratio of the tracer from the vascular space to the interstitial space, E , is given by

$$E = 1 - \exp(PS/F) \quad (1.11)$$

Adiabatic Tissue Homogeneity Model

The adiabatic tissue homogeneity (ATH) model assumes an adiabatic approximation for the distributed parameter model and the impulse residual function $R(t)$ is given by (23)

$$R_{ATH}(t) = \begin{cases} 1, & 0 < t \leq t_1 \\ E \exp(-k_{adb}(t - t_1)), & t > t_1; \end{cases} \quad (1.12)$$

where k_{adb} denotes the clearance rate constant.

Generalized Kinetic model

The generalized kinetic (GK) model assumes a negligible vascular space (24), and the operational equation is given by

$$C_{tiss}(t) = K^{trans} C_{in}(t) \otimes R_{GK}(t) \quad (1.13)$$

The impulse response function is given by

$$R_{GK}(t) = \exp\left(-\frac{K^{trans}}{v_e} t\right) \quad (1.14)$$

where K^{trans} is the volume transfer constant between blood plasma and the interstitial space and v_e is the fractional interstitial volume. K^{trans} approximates F and PS under flow-limited (high permeability) and permeability-limited (high flow) conditions, respectively (25).

Uptake Integral Approach

The uptake integral approach (26) integrates the tissue tracer concentration time curve and postulates that the total tracer concentration in the capillary reflects the leakiness of the capillary (27). It also assumes that the degree of leakiness reflects the state of the blood vessel and hence can be used to assess the effectiveness of an anti-angiogenic therapy. The main parameter, initial area under the signal-time curve (IAUC), is given by

$$IAUC_t = \int_0^t C_{tiss}(\tau) d\tau \quad (1.15)$$

1.2 Objectives

This thesis builds on previous works done in human studies. DCE-CT studies have been previously performed in various types of tumors (28-30) and the blood perfusion derived by the distributed parameter model was validated by a study with $\text{H}_2^{(15)}\text{O}$ positron emission tomography (PET) measurements (31). It was followed by several DCE-MRI studies using a distributed parameter model in hepatic metastases (8, 32). More recently, the technique has been applied to assess the effectiveness of an anti-angiogenic therapy and it was found that permeability correlated with drug exposure and might predict patient outcome (9). It indicated the potentials of this technique to serve as a biomarker for anti-angiogenic therapy.

It also demonstrated the need to perform a similar study to explore anti-angiogenic drug mechanism in animal setting. We would like to explore the feasibility of translating the works that have been done in humans back to animals, i.e., from bedside to the bench.

The main goal of this thesis is elucidate the feasibility of translating and applying high temporal and spatial resolution DCE-MRI technique in animal settings.

This goal is broken into several steps:

1. To illustrate the application of high temporal and spatial resolution DCE-MRI as a biomarker of an anti-angiogenic therapy in human setting and highlight the potentials in translating back the technique for anti-angiogenic drug mechanism exploration in animal setting
2. To design a similarly fast DCE-MRI sequence for animal setting and validate the microcirculatory parameters obtained from the standard two-compartment model employed in the technique
3. To apply the technique to characterize the microcirculatory of normal mice liver
4. To apply the technique to evaluate the effectiveness of an anti-angiogenic therapy in animal setting
5. To devise an alternative technique to analyze DCE-MRI data in necrotic regions of the tumor which were often encountered in xenograft studies and fitted poorly by compartmental tracer kinetic models

Objectives 1: High temporal and spatial resolution DCE-MRI in a Phase I study of an anti-angiogenic drug

Three tracer kinetics methods for analysis of dynamic contrast-enhanced (DCE) MRI data, namely generalized kinetics (GK) model, the distributed-parameter (DP) model, and initial area under tumor tracer curve (IAUC) method, were compared in a Phase I study of an anti-angiogenic drug ABT 869 to explore their utility as biomarkers. DCE MRI performed at baseline and 2 weeks post-treatment was analyzed using all three methods, yielding percentage changes for various tracer kinetics parameters. Correlation analyses between these parameters and with drug exposure were performed. The association of these parameters with time-to-progression were respectively examined using receiver operating characteristic and Kaplan-Meier curves. All three tracer kinetics methods were found to have biomarker potential for assessing the effects of anti-angiogenic therapy. It also highlighted the potentials in translating back the technique for anti-angiogenic drug mechanism exploration in animal setting.

Objectives 2: Validation of microcirculatory parameters derived from standard two-compartment model in animal setting

The feasibility of translating the DCE-MRI technique and the tracer kinetic modeling previously used for human studies in animal MR machine were demonstrated. A similarly fast DCE-MRI sequence was designed and the appropriate tracer kinetic model was used to characterize the microcirculatory of the tissue of interest. Phantom studies were performed to validate the T_1 estimates derived by the proposed protocol before applying it in mice studies. The technique was then employed in various types of xenografts in mice. Microcirculatory parameters such as blood flow (F), permeability-surface area product (PS), fractional intravascular space (v_1), and fractional extracellular extravascular space (v_2), obtained using a standard two-compartmental model were validated against other established analysis methods and histological indices. Decent correlations were observed and it indicated the possibility to use the technique as biomarkers for drug discovery and drug development. Validation of these parameters might allow their use to monitor anti-angiogenic therapy in murine models. Accurate estimation of these microvascular parameters of plasma flow, permeability, fractional intravascular space, and fractional interstitial space might allow better understanding of mechanism of anti-angiogenic agents.

Objectives 3: Characterization of the microcirculatory of normal mice liver

The potentials of using the technique were exhibited by a DCE-MRI study in mice liver. The DCE-MRI datasets of mice liver were amendable to tracer kinetic analysis using a dual-input standard two-

compartment model. Estimated micro-circulatory parameters were consistent with liver physiology, indicating viability of applying the technique for liver cirrhosis study in animal setting.

Objectives 4: Application of the technique to evaluate the effectiveness of bevacizumab in mice implanted with human hepatocellular carcinoma xenograft

The technique was applied to assess the effectiveness of bevacizumab, an anti-angiogenic agent approved for clinical use, in mice implanted with human hepatocellular carcinoma xenograft. Many studies have demonstrated with histopathology a decrease in microvascular density after anti-angiogenic therapy, but only a few have incorporated DCE-MRI (33, 34). The DCE-MRI signature of the microcirculatory changes that result from bevacizumab therapy with a human HCC xenograft model was described. Particularly, we are interested to see whether the knowledge of these parameters might allow us to design *in vivo* imaging of normalization window, as it would make possible better design of drug scheduling that allow administration of concomitant cytotoxic drug during this normalization window to exploit benefits from improved drug delivery to the tissue.

Objectives 5: An alternative technique to analyze DCE-MRI data in necrotic regions of the tumor

Compartmental tracer kinetic models currently employed for analysis of dynamic contrast-enhanced (DCE) MRI data yield poor fittings or parameter values that are unphysiological in necrotic regions of the tumor, as these models only describe microcirculation in perfused tissue. The use of Fick's law of diffusion was explored as an alternative method for analysis of DCE-MRI data in the necrotic regions. Poorly perfused and necrotic tumor regions exhibiting delayed and slow enhancement were identified using a k-means clustering algorithm. Tracer behavior in necrotic regions was shown to be consistent with Fick's diffusion equation. The use of gadolinium diffusivity was proposed as an alternative parameter for quantifying tracer transport within necrotic tumor regions.

2. A Comparative Study of Dynamic Contrast-Enhanced MRI Parameters as Biomarkers for Anti-angiogenic Drug Therapy¹

2.1 Introduction

Dynamic contrast-enhanced (DCE) MRI is a functional imaging technique that has been used to assess the effects of anti-angiogenic and anti-vascular drugs in clinical trials (11, 35-42). The technique of DCE MRI involves the intravenous injection of a tracer (contrast agent) and subsequent imaging at various time points (dynamic imaging) to monitor the tracer time course within the tissue of interest. The initial area under the tumor concentration-time curve (IAUC) is a semi-quantitative parameter commonly used as an indicator for neo-angiogenesis (35). The Generalized Kinetics (GK) model provides quantitative estimates for the tissue transfer constant (K^{trans}) and fractional interstitial (extravascular extracellular) volume (v_e) (24). Previous studies of anti-angiogenic drug trials have shown correlation of DCE MRI parameters with drug exposure, tumor size and treatment response (11, 35-42). Despite being a quantitative parameter, the transfer constant K^{trans} incorporates both the effects of blood flow and capillary permeability. According to the GK model, K^{trans} reflects permeability when blood flow is much higher than permeability, and reflects blood flow when permeability dominates (24). It is generally accepted that for tumors prior to treatment, K^{trans} reflects blood flow because the capillaries within these tumors are highly permeable (24). However, novel vascular targeting drugs could reduce both permeability and blood flow through vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) inhibition; and the mechanism of action of these novel agents remains to be investigated (5, 43-44). Therefore, following therapy, the question arises as to whether K^{trans} still measures flow or permeability, or a combination of both.

The distributed-parameter (DP) model offers the possibility of estimating flow (F) and permeability (PS) separately (8, 15, 21, 31), and the fractional vascular volume v_1 , and fractional interstitial volume v_2 . Since anti-angiogenic drugs might exert different pharmacodynamic effects on tumor flow and permeability, a method that can separately estimate flow and permeability may potentially be useful. In this study, we compared the use of IAUC, GK and DP models in a Phase I anti-angiogenic drug trial, and examined their correlation with drug level and disease progression, to explore their utility as potential biomarkers. We also studied the possible correlation between parameters of different DCE MRI analysis methods, to understand the relationships between these parameters.

¹ Parts of this study were presented as a poster in the 2010 International Society of Magnetic Resonance in Medicine Annual Meeting, Stockholm, Sweden and published in *NMR in Biomedicine* (2011) 24(9):1169-80.

2.2 Materials and methods

2.2.1 Phase I Trial and Patients

ABT-869 is an oral multi-targeted receptor tyrosine kinase (RTK) inhibitor active against both the VEGF and the PDGF families of receptors (45). The PDGF family of RTK improves pericyte coverage and the inhibition of PDGF together with VEGF stimulates vessel regression, manifesting as an anti-vascular effect with the reduction in tumor vascularity and capillary permeability (45). Institutional review board approval was obtained for a phase I study of ABT-869 (Abbott Laboratories, Illinois) (9) and written informed consent was obtained from each patient recruited. The Phase I trial was conducted in three segments (A, B and C). Segment A was a sequential dose-escalation study with the primary intent of defining the maximum tolerable dose and to recommend a tolerable Phase 2 dose (RPTD). Segment B involved a cohort expansion at the RPTD and segment C studied the tolerability and pharmacodynamics of a lower dose cohort for comparison with the RPTD. In all three segments, patients received ABT-869 until evidence of tumor progression or dose-limiting toxicity.

ABT-869 was administered as lipid solution formulation diluted with 60 ml of Ensure Plus® (Abbott Laboratories, Illinois). It was given as a continuous daily oral dosage at night in treatment cycles of 21 days, except on Days 1 and 15 when drug was administered in the morning for pharmacokinetics studies. Thirty-three patients formed the Phase I study population. For the present correlative study, 5 patients without DCE MRI were excluded, leaving 28 patients suitable for analysis. Patient demographics and tumor types are shown in Table 2.1. DCE MRI was performed at baseline (within 1 week before commencement of drug), and subsequently on Day 3 and Day 15 for Segments A and B. Patients in Segment C only had baseline and Day 15 scans. A flowchart summarizing patient inclusion and exclusion numbers for the various statistical correlative analyses is provided in Figure 2.1.

Tumor Type	Patient ID	Sex	Age	Size on CT (Diameter mm)	Outcome at Cycle 4
Non small cell lung	1006	M	73	39	AE/SD
	1103	F	47	113	PD
	1111	F	61		AE/NA
	1112	M	73	45	AE/NA
	1202	M	62	159	SD
	1203	M	76	90	AE/SD
	1511	M	64	272	AE/SD
Colorectal	1001	F	50	346	SD
	1101	F	49	132	PD
	1102	F	65	153	SD
	1109	M	56	83	SD
	1201	F	62	70	AE/SD
	1503	F	45	17	SD
Hepatocellular	1004	M	56	188	PD
	1005	M	57	120	SD
	1501	M	69	124	PD
	1506	M	53	67	SD
Ovary	1108	F	74	65	SD
	1505	F	48	96	PD
	1509	F	56	58	SD
Endometrial sarcoma	1002	F	30	195	SD
	1105	F	47	92	PD
Breast	1507	F	57	430	PD
Neuroendocrine	1104	M	33	46	AE/SD
Kidney	1110	M	68	130	SD
Head and Neck	1113	M	65	55	PD
Bladder	1510	M	41	68	SD
Malignant nerve sheath tumor	1508	F	71	51	AE/NA

Table 2.1. Demographics of the 28 patients with baseline and Day 15 DCE MRI scans.

SD: Stable disease (late progressor)

PD: Progressive disease (early progressor)

AE: Adverse event

AE/NA: Adverse event before cycle 4, not applicable for classification as early or late progressor

AE/SD: Adverse event after cycle 4, stable disease, late progressor

Figure 2.1. A flowchart depicting the inclusion and exclusion of patients for various analyses in the Phase I trial. Five patients were excluded from DCE MRI analysis as MRI was not performed. Six patients were excluded from correlation with drug exposure as they had their pharmacokinetics evaluated while they were on a tablet formulation whereas the majority of patients had their pharmacokinetics evaluated while on lipid solution formulation. These patients were not excluded from correlation with progression as they were maintained on the lipid solution formulation for the rest of the study after completion of pharmacokinetic analysis.

2.2.2 DCE-MRI Protocol

DCE MRI was performed using a 1.5-Tesla MR scanner (Avanto; Siemens, Erlangen, Germany) with phased array surface coils (TIM; Siemens, Erlangen, Germany). An oblique coronal or oblique sagittal scan plane was selected along a line joining a single dominant lesion to the aorta or its major branch. The length of the aorta or supplying artery was included in the scan plane whenever possible, to minimize inflow artifacts. The dominant lesion is kept at the center of the slab.

A three-dimensional fast low-angle shot (3D-FLASH) sequence was used (TR 3.15 msec, TE 1 msec, flip angle 6° and 10°, 8mm slice thickness, 256×256 matrix, 10 slices per slab, acquisition time 4 sec,

FOV 40×40 cm). Ten pre-contrast acquisitions of each flip angle (6° and 10°) were obtained during quiet respiration. Dynamic post-contrast scans were acquired with the same sequence and with a flip angle of 10°. 0.2 mmol/kg of gadolinium contrast agent (Gadodiamide, Omniscan, Nycomed, Oslo, Norway; or Gadopentetic acid, Magnevist, Schering, Berlin, Germany) was injected after the 10th set of dynamic images at 3 mL/sec followed by a 20 mL saline flush. A total of 90 consecutive scans were obtained for the dynamic series with temporal resolution of 4 sec over 6 min with the patient maintaining quiet respiration.

2.2.3 Imaging Data Analysis

Post-processing was performed off-line on a Pentium IV personal computer with Matlab™ (MathWorks, Natick MA). For reduced inflow effects and wrap, only the 6 central slices from the imaging volume (of 10 slices) were selected for processing. For each patient, one region-of-interest (ROI) consisting of the largest visible tumor in the central 6 slices was manually outlined by a radiologist.

Gadolinium contrast concentration was estimated using the dual-flip angle method (46-48). The arterial input function (AIF) was obtained by either sampling a 3×3 (voxel-averaged) window placed within the aorta or a smaller ROI consisting of voxels that clearly reside within a major feeding artery visible on the MR images. For a liver lesion, an additional ROI is placed over the portal vein or its major branches and a dual-input approach (8) was used to analyze the DCE MR images.

The tissue concentration-time curve $C_{\text{tiss}}(t)$ for each voxel within the lesion ROI was separately fitted using the GK and DP models, which can be respectively given by

$$C_{\text{tiss}}(t) = \text{AIF} \otimes K^{\text{trans}} \exp\left(-\frac{K^{\text{trans}}}{v_e} t\right) \quad (2.1a)$$

and

$$C_{\text{tiss}}(t) = \text{AIF} \otimes F \left\{ \begin{aligned} &u(t) - u(t - t_1) + \\ &u(t - t_1) \left[1 - \exp\left(-\frac{PS}{F}\right) \left[1 + \int_0^{t-t_1} \exp\left(-\frac{PS}{v_2} \tau\right) \sqrt{\frac{PS}{v_2} \frac{PS}{F} \frac{1}{\tau}} I_1\left(2 \sqrt{\frac{PS}{v_2} \frac{PS}{F} \tau}\right) d\tau \right] \right] \end{aligned} \right\} \quad (2.1b)$$

where $\otimes$ denotes the convolution operator, In Eq. (1b), t_1 denotes the vascular mean transit time which is related to blood flow F and the fractional vascular volume v_1 by $v_1 = Ft_1$. $u(t)$ denotes the Heaviside unit-step function and I_1 is the Bessel function. Model-fitting was performed using a

constrained nonlinear optimization algorithm ('Fmincon', MatlabTM, The Mathworks, Natick, MA) which yields the sum-of-squared residues (SSR) as a measure of the goodness-of-fit, and output flags reflecting the status of the optimization search. Poor fittings with exceedingly large SSR (greater than a stipulated threshold) and negative output flags (indicating no feasible solution) were excluded. The median parameter value in the tumor ROI was taken as the representative parameter value for the tumor (36).

IAUC for each tumor voxel can be calculated by summing the area under the concentration-time curve for the first 60 sec following contrast arrival. The contrast arrival time for IAUC was visually estimated based on the AIF. As IAUC is dependent on the cardiac output which varies between patients, it can be normalized to account for this dependence by dividing it with the area under the AIF for the same period (IAUC_{norm}). Again, the median tumor IAUC values were taken as the representative values.

2.2.4 Statistical Analyses

A. Correlation of DCE MRI Parameters with Drug Exposure

It is of interest to investigate whether any of the DCE MRI parameters would reflect differences in drug levels and correlate with drug exposure. A measure for patient drug exposure can be derived from the pharmacokinetics studies (performed at Days 3 and 15 of treatment), which involved the sampling of ABT-869 drug concentrations in plasma at the following time points: baseline, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 24 hours post-dosing. The sampled ABT-869 plasma concentration-time curve was extrapolated to infinity using at least the last 3 concentration points (WINNONLIN, Pharsight Corp., Cary, NC). The area under the drug plasma concentration-time curve extrapolated to infinity, AUC, was used as a measure of drug exposure. The Spearman rank correlation coefficient (r) was used to evaluate the strength of relationship between the percentage change from baseline of DCE MRI parameters at Day 15 with drug plasma AUC. During this Phase I trial, in order to explore the pharmacokinetic profile of a tablet formulation of ABT-869, half the cohort of segment C (i.e., 6 patients) received a single dose of ABT-869 in tablet formulation 2 days before the start of treatment, and were subsequently continued on the lipid solution formulation similar to the other patients. These 6 patients were excluded from the correlation analysis of DCE MRI parameters with drug exposure (i.e., drug plasma AUC). Therefore, the patient sample size for correlation analysis of change in DCE MRI parameters with drug plasma AUC was $n=28-6=22$ (Fig. 2.1).

B. Early and Late Progression

In order to explore whether changes in DCE MRI parameters could be predictive of patient outcome (hence drug efficacy), we classified patients as early or late progression based on whether they progress before or after cycle 4, similar to other exploratory studies (11, 41). The RECIST (Response Evaluation Criteria in Solid Tumors) criteria were adopted for imaging response, i.e., a 20% increase in the sum of the longest diameter of target lesions was considered as progressive disease (tumor progression). For tumor size assessments, baseline CT was performed within 4 weeks before ABT-869 treatment, and repeated every 2 cycles (i.e., 6 weeks) and at the final visit. Patients demonstrating progressive disease in the first 2 evaluation scans (cycle 2 or 4) were considered early progressors. All other patients were considered late progressors. Time-to-progression was measured as the number of days between the start of cycle 1 and tumor progression.

Receiver operating characteristic (ROC) analysis (49) was performed to examine the ability of each DCE MRI parameter to discriminate between the early and late progressors. The ROC curve can be used to assist in identifying a cut-off that yields optimal sensitivity and specificity. The area under the ROC curve, A_z , gives a measure of the predictive accuracy of the parameter. A_z and its 95% confidence interval (CI) were estimated for each DCE MRI parameter. For ROC analysis, 3 patients who did not complete 4 cycles of treatment due to adverse events, were considered indeterminate in treatment outcome and were excluded ($n=28-3=25$).

C. Kaplan-Meier Curves and Time-to-Progression Analysis

For each of the DCE MRI parameters that show correlation with drug exposure and a significant A_z , we further studied whether the parameter could inform on the risk of disease progression and patient survival. Using the optimal parameter cut-off identified from ROC analysis, which stratified the patients into two groups, Kaplan-Meier curves were plotted to depict patient survival probability with time-to-progression for each group. To compare the two groups, the hazard ratio (HR) was used as an estimate of the relative likelihood (risk) of disease progression (hazards of progression) between the two groups. For the Kaplan-Meier time-to-progression analysis, an additional 5 patients were excluded as they encountered an adverse event before disease progression (i.e., their time-to-progression were indeterminate) and the sample size was $n=28-3-5=20$ (Fig. 2.1).

D. Correlation between DCE MRI parameters

To explore the possible relationship between DCE MRI parameters of different methods, correlation analysis using Spearman rank correlation coefficient was performed to compare the parameters K^{trans} ,

v_e and $IAUC_{norm}$, with the parameters F , PS , v_1 and v_2 . To understand how the relationships of these parameters would change with drug treatment, comparisons were performed at baseline (i.e., pre-treatment) and at Day 15 post-treatment. All statistical analyses were performed using STATA v. 10 (StataCorp LP, TX), assuming a two-sided test at the conventional 0.05 level of significance. For the correlation analysis between DCE MRI parameters of different methods, data from all 28 patients with baseline and Day 15 scans were included.

2.3 Results

Examples of pre- and post-treatment DCE MRI results for a late progressor and a early progressor are shown in Figures 2.2 and 2.3, respectively. Examples of voxel fittings using the GK and DP models are provided in Figure 2.4.

A. Correlation of DCE MRI Parameters with Drug Exposure

The Spearman's rank correlation coefficients (r) relating percentage change in DCE MRI parameters (at Day 15 relative to baseline) with drug plasma AUC are shown in Table 2.2. Significant inverse correlation was found for the parameters PS , v_1 , v_e and $IAUC_{norm}$. Correlation plots of these parameters with drug plasma AUC are shown in Figure 2.5. The correlation between percentage change in K^{trans} , F and $IAUC$ with drug plasma AUC was not statistically significant.

B. Early and Late Progression

Median values of the various parameters at baseline and Day 15 for the patients involved in ROC analysis are shown in Fig 2.6. Results of ROC analysis examining the change in DCE MRI parameters as predictors of patient outcome in terms of progression status are shown in Table 2.3. Of the 4 parameters that showed significant correlation with drug plasma AUC (i.e PS , v_1 , v_e and $IAUC_{norm}$), only PS ($Az = 0.779$; 95% CI 0.549 to 0.906) and v_e ($Az = 0.853$; 95% CI 0.639 to 0.955) can be considered effective predictors. The difference between the Az values of PS and v_e was not statistically significant ($p=0.523$). The parameters K^{trans} , v_1 , and F did not yield significant Az values for their ROC curves. An optimal cut off point of 1.7% decrease in v_e can be identified on the ROC curve of v_e that yielded 88.2% and 87.5% in sensitivity and specificity, respectively, for predicting late progressors. Similarly, an optimal cut off point of 25.1% drop in PS gives a sensitivity of 64.7% and a specificity of 87.5%. These optimal cut off points for v_e and PS were applied in the time-to-progression analysis.

Figure 2.2. Example of a late progressor (lung cancer) with the baseline and Day 15 DCE MRI results shown in (a) and (b) respectively. In both (a) and (b), examples of pre-contrast and post-contrast images are shown together with the various parameter maps. Baseline ROI median (and mean $\pm$ standard deviation) values are: $F=20.5$ (29.2 ± 38.9) ml/100ml/min, $PS= 5.4$ (11.1 ± 29.2) ml/100ml/min, $v_1=8.2$ (9.7 ± 7.1) ml/100ml, $v_2=16.2$ (25.3 ± 25.1) ml/100ml, $K^{trans}=0.20$ (0.3 ± 0.5) /min, $v_e= 20.4$ (23.8 ± 17.4) ml/100ml, $IAUC=8.05$ (15.3 ± 18.6) mMsec, and $IAUC_{norm}=5.81$ (8.8 ± 10.8)%. Day15 ROI median (mean $\pm$ std) values are: $F=11.9$ (25.1 ± 35.9) ml/100ml/min, $PS= 3.6$ (8.2 ± 30.3) ml/100ml/min, $v_1=3.2$ (3.8 ± 2.6) ml/100ml, $v_2=14.2$ (27.6 ± 30.5) ml/100ml, $K^{trans}=0.07$ (0.3 ± 0.4) /min, $v_e= 14.9$ (17.2 ± 14.7) ml/100ml, $IAUC=2.44$ (5.2 ± 8.9) mMsec, and $IAUC_{norm}=1.6$ (2.7 ± 4.6)%.

C. Kaplan-Meier Curves and Time-to-Progression Analysis

The Kaplan-Meier curves relating survival probability with time-to-progression for the patients grouped according to the optimal cut off points for v_e (1.7%) and PS (25.1%), are shown in Figs. 2.7(a) and (b), respectively. Figure 2.7(a) shows that a decrease in v_e of more than 1.7% at Day 15 reduces the hazards of progression (HR=0.24; 95% CI 0.08 to 0.68; $p=0.008$). Similarly, a decrease in PS of more than 25.1% reduces the hazards of progression but the reduction is not statistically significant (HR=0.50; 95% CI 0.19 to 1.27; $p=0.145$) (Figure 2.7b).

Figure 2.3. Example of an early progressor (colorectal cancer) with the baseline and Day 15 DCE MRI results shown in (a) and (b) respectively. In both (a) and (b), examples of pre-contrast and post-contrast images are shown together with the various parameter maps. Baseline ROI median (and mean $\pm$ standard deviation) are: $F=28.9$ (34.6 ± 26.8) ml/100ml/min, $PS=9.5$ (12.5 ± 10.4) ml/100ml/min, $v_1=9.7$ (10.8 ± 7.0) ml/100ml, $v_2=16.7$ (20.3 ± 14.2) ml/100ml, $K^{trans}=0.23$ (0.4 ± 1.7) /min, $v_e=24.0$ (27.0 ± 15.0) ml/100ml, $IAUC=8.38$ (12.0 ± 8.3) mMsec, and $IAUC_{norm}=7.7$ (8.8 ± 6.1)%. Day15 ROI median (mean $\pm$ std) values are: $F=15.0$ (20.7 ± 18.0) ml/100ml/min, $PS=7.1$ (12.3 ± 16.5) ml/100ml/min, $v_1=5.6$ (8.6 ± 8.4) ml/100ml, $v_2=28.2$ (39.7 ± 28.0) ml/100ml, $K^{trans}=0.12$ (0.2 ± 0.2) /min, $v_e=24.8$ (33.6 ± 22.7) ml/100ml, $IAUC=2.21$ (4.1 ± 3.8) mMsec, and $IAUC_{norm}=3.7$ (5.5 ± 5.2)%.

D. Correlation between DCE MRI parameters

K^{trans} was more strongly correlated with F ($r=0.830$, $p<0.001$) and v_1 ($r=0.849$, $p<0.001$) at baseline (pre-treatment) than at Day 15 post-treatment (Table 2.4). This is consistent with the fact that in untreated tumor, blood vessel permeability is high and K^{trans} reflects F in the regime of high permeability. However, with anti-angiogenic treatment and the decrease in vessel permeability, K^{trans} no longer correlated with F ($r=0.464$, $p=0.013$ at Day 15). On the other hand, v_e not only correlated with v_2 at baseline and Day 15, it also correlated strongly with PS and v_1 . The parameter v_e is more strongly associated with PS ($r=0.929$; $p<0.001$) and v_1 ($r=0.738$; $p<0.001$) on Day 15 as compared to baseline. Hence, v_e is potentially a useful parameter to complement K^{trans} . $IAUC_{norm}$ on Day 15 showed

strong correlation with F ($r=0.755$, $p<0.001$), PS ($r=0.8$, $p<0.001$) and v_1 ($r=0.87$, $p<0.001$). A similar trend was observed at baseline.

Figure 2.4. Examples of voxel fittings at baseline (a) and Day 15 (b) for the study case in Figure 2.3. (a) $F=37.2$ ml/100ml/min, $PS= 4.5$ ml/100ml/min, $v_1=14.1$ ml/100ml, $v_2=2.2$ ml/100ml, $K^{trans}=0.32$ /min, $v_e= 35.1$ ml/100ml. (b) $F=6.3$ ml/100ml/min, $PS= 11.6$ ml/100ml/min, $v_1=2.2$ ml/100ml, $v_2=2.8$ ml/100ml, $K^{trans}=0.04$ /min, $v_e= 15.0$ ml/100ml.

DCE-MRI Parameter	Spearman's r (p -value)
<i>Distributed parameter model</i>	
F	-0.387 ($p = 0.076$)
v_1	-0.501 ($p = 0.018$)*
v_2	-0.387 ($p = 0.076$)
PS	-0.542 ($p = 0.009$)*
<i>Generalized kinetics model</i>	
K^{trans}	-0.106 ($p = 0.640$)
v_e	-0.614 ($p = 0.002$)*
<i>Integral uptake approach</i>	
IAUC	-0.152 ($p = 0.499$)
IAUC _{norm}	-0.519 ($p = 0.013$)*

Table 2.2. Spearman's rank correlation coefficient r -relating percentage change in DCE-MRI parameters (between baseline and Day 15) with drug plasma AUC. Cells with significant Spearman's correlation $|r| > 0.5$ and $p < 0.05$ are indicated by*. DCE, dynamic contrast-enhanced; AUC, area under drug plasma concentration curve.

Figure 2.5. Correlation plots of change in median parameter values (between baseline and Day15) with drug plasma AUC (indicative of drug exposure). Only parameters with significant Spearman's correlation coefficients are shown.

2.4 Discussion

The GK model is a simplified tracer kinetics model which involves two independent parameters, K^{trans} and v_e . The present results show that in a high permeability state such as the untreated tumors at baseline, K^{trans} correlated strongly with F . However, after therapy K^{trans} did not correlate with F or PS , because K^{trans} does not specifically reflect F or PS in mixed states. These observations are in support of the GK model theory (24). A recent simulation study of the GK model revealed that v_e has a strong positive dependence on both blood volume and interstitial volume, and a marginal positive dependence on flow and permeability (25). Therefore, it is plausible that a reduction in blood volume and permeability by anti-angiogenic treatment could result in a decrease in v_e . The present correlation results in Table 2.4 show a positive association between v_e and PS at baseline and Day 15. This is likely due to the fact that a decrease in permeability results in the flattening of the tissue concentration-time curve (25), which can translate into a decrease in K^{trans} and v_e . Consequently, a marginal positive dependence of v_e on permeability is shown in simulations (25). Conversely, an

Figure 2.6. Median values of the various parameters at baseline and Day 15 for the cohort of patients involved in receiver operating characteristic (ROC) analysis ($n=25$). Late and early progressors are represented by open (blue) and filled (red) circles, respectively.

increase in blood volume and permeability due to blood vessel proliferation could result in the increase of v_e observed in 5 of the 8 early progressors (Fig. 2.6).

The DP model is mathematically more complex and separately estimates parameters with specific pathophysiological meaning. As v_1 is indicative of tumor vascularity, the decrease in v_1 and PS were found to correlate with drug exposure, which is consistent with the postulated effects of the drug (45). The normalized IAUC ($IAUC_{norm}$) is superior to IAUC in reflecting drug exposure as $IAUC_{norm}$ takes into account differences in the cardiac output of the patients as manifested in the AIF. $IAUC_{norm}$ is a measure of contrast uptake and it correlated with the parameters F , PS and v_1 , which are related to the effects of neo-angiogenesis, although it does not specifically reflect F or PS (35). Thus, $IAUC_{norm}$ is indicative of the overall effects neo-angiogenesis, with the added strengths of simplicity and robustness in implementation.

DCE MRI parameter	Az (95% CI)
<i>Distributed parameter model</i>	
F	0.596 (0.387 to 0.789)
v_1	0.654 (0.425 to 0.820)
v_2	0.794 (0.593 to 0.932)*
PS	0.779 (0.549 to 0.906)*
<i>Generalized kinetics model</i>	
K^{trans}	0.397 (0.211 to 0.613)
v_e	0.853 (0.639 to 0.955)*
<i>Integral uptake approach</i>	
IAUC	0.559 (0.349 to 0.756)
IAUC _{norm}	0.721 (0.506 to 0.879)*

Table 2.3. Results of receiver-operating characteristic (ROC) analysis examining the change in DCE MRI parameters. Area under the ROC curve, Az, and its 95% confidence interval (CI) are listed for each parameter. For an effective predictor (*), the 95% CI for Az should be greater than the value of 0.5 which indicates random guessing.

All 3 methods for processing DCE MRI datasets can potentially serve as biomarkers, since each method yielded at least one parameter that showed correlation with drug exposure and/or patient outcome. Positive results were shown for the use of cut-off limits for v_e and PS to predict late progression in this Phase I trial. Other investigators working with PTK787 have shown significant difference in K^{trans} between progressors and non-progressors but have not provided sensitivity and specificity values or specific cut-off points (41). The present results show that a 1.7% drop in v_e (from baseline at Day 15) could predict late progressors with a sensitivity of 88.2% and specificity of 87.5%; and a 25.1% drop in PS could yield a corresponding sensitivity of 64.7% and specificity of 87.5%. However, the current low cut-off for v_e might not be within the reproducibility limits of DCE MRI (50). Future studies in Phase II/III trials with homogenous tumor types would be useful to determine if current correlations to progression free survival are reproducible and whether DCE MRI performed at week 2 can predict good outcome and act as a surrogate marker for response.

It is known that anti-angiogenic agents decrease capillary permeability by increasing pericyte coverage and decreasing basement membrane pores. We found that a decrease in PS correlated with both drug exposure and patient outcome. Although F has been previously validated (31), it is difficult to validate PS derived from DCE imaging studies. The present results may indirectly indicate that PS

Figure 2.7. Kaplan-Meier progression-free survival probability. (a) Comparing $> 1.7\%$ vs. $\leq 1.7\%$ decrease in v_e . (b) Comparing $> 25.1\%$ vs. $\leq 25.1\%$ decrease in PS .

		F	PS	v_1	v_2
K^{trans}	Baseline	0.830 ($p < 0.001$)*	0.343 ($p = 0.074$)	0.849 ($p < 0.001$)*	-0.172 ($p = 0.380$)
	Day 15	0.464 ($p = 0.013$)	0.026 ($p = 0.898$)	0.395 ($p = 0.038$)	-0.140 ($p = 0.479$)
v_e	Baseline	0.485 ($p = 0.009$)	0.681 ($p < 0.001$)*	0.706 ($p < 0.001$)*	0.555 ($p = 0.002$)*
	Day 15	0.484 ($p = 0.009$)	0.929 ($p < 0.001$)*	0.738 ($p < 0.001$)*	0.696 ($p < 0.001$)*
$\text{IAUC}_{\text{norm}}$	Baseline	0.709 ($p < 0.001$)*	0.529 ($p = 0.004$)	0.889 ($p < 0.001$)*	0.0389 ($p = 0.844$)
	Day 15	0.755 ($p < 0.001$)*	0.800 ($p < 0.001$)	0.870 ($p < 0.001$)*	0.386 ($p = 0.043$)

Table 2.4. Correlation between K^{trans} , v_e , $\text{IAUC}_{\text{norm}}$, and F , PS , v_1 , and v_2 , at baseline and Day 15. Cells with significant Spearman's correlation $r > 0.5$ and $p < 0.05$ are indicated by*.

can be reasonably estimated as there is correlation with drug exposure, consistent with pre-clinical observations of the drug effects (45). The ability of DCE MRI to separately assess blood flow and capillary permeability would be of significance in a clinical drug trial. It would not only increase our understanding of the mechanism of action of the novel vascular targeting drugs, it may also allow for the identification of the so-called "normalization window" following anti-angiogenesis treatment, where a decrease in permeability and increase in blood flow is postulated (5), such that concomitant cytotoxic and radiation therapy can be administered.

There are limitations of our study. The DCE MRI datasets were acquired at high temporal resolution (4 sec) for more rapid sampling of the arterial and tumor concentration-time curves, and organ coverage was reduced. The reduction in organ coverage may imply that not all metastatic lesions were assessed and that effects of tumor heterogeneity might not be fully accounted for. A repeatability study at baseline to assess the reproducibility of the various DCE MRI parameters should provide further insights on their utility as effective biomarkers (50). However, this was not performed in the current trial to reduce the burden of an additional imaging visit for the patients. The analysis and measures of patient outcome in this Phase I trial are exploratory in nature and are not conclusive as many tumor types are included. Further studies in a Phase II or Phase III setting with a more homogenous patient population are required for validation.

In conclusion, all three tracer kinetics methods were found to yield feasible biomarkers for assessing the effects of anti-angiogenic therapy. $\text{IAUC}_{\text{norm}}$, v_e , v_1 and PS correlated with drug exposure, while v_e and PS were predictive of late progression. Analysis by the DP model offers the possibility to separately evaluate blood flow and capillary permeability in tumors. The parameters v_e and PS can complement the predictive value of K^{trans} and $\text{IAUC}_{\text{norm}}$ which have been shown to be useful in other drug trials. Further studies are required to determine if DCE MRI parameters can serve as a surrogate marker for clinical benefits.

3. Validation of Microcirculatory Parameters Derived from the Standard Two-Compartment Model with Murine Xenografts Model²

3.1 Introduction

It is important to validate the parameters derived from DCE MRI against established techniques and to ascertain that the DCE MRI parameters reflect the actual microcirculatory state and pathophysiology of the tumors imaged. Previous DCE MRI validation studies (51-54) have attempted to correlate GK model parameters, namely the transfer constant K^{trans} and the fractional interstitial volume v_e , with histological indices of the tumor microvasculature. However, according to GK model theory, K^{trans} incorporates both the effects of blood flow and vessel permeability (24) and a recent simulation study (25) has shown that K^{trans} has a significant positive correlation with blood flow, permeability and blood volume, as well as a significant negative correlation with interstitial volume. With such ambiguities in interpretation, it might be difficult to identify appropriate markers for validation of K^{trans} .

The standard two-compartment model (17-18, 55-56) describes tissue microcirculation with distinct parameters, namely blood flow (F), vessel permeability-surface area product (PS), fractional vascular volume (v_1) and fractional interstitial volume (v_2). These physiological parameters can be readily validated by comparison with appropriate histological markers or other established tracer techniques. More complex tracer kinetic models such as the distributed parameter models (56) are also capable of separately estimating blood flow and permeability. However, the distributed parameter models require rapid sampling of tissue tracer concentration curves and might not be suitable for use in the preclinical setting where the temporal resolution of the DCE-MRI scans is low compared to the rapid murine circulation.

In this study, we attempt to validate parameters of the standard two-compartment model derived from DCE MRI of human cancer xenografts in mice by comparing v_p and v_e with histological markers; and by comparing F and PS with the corresponding estimates obtained using a macromolecular tracer (Galbunin) with established tracer analysis techniques, namely, the initial slope method and Patlak plot.

² Parts of this study were presented as a poster for the 2011 International Society of Magnetic Resonance in Medicine Annual Meeting, Montreal, Canada and submitted for publication in *Applied Magnetic Resonance*.

3.2 Materials and methods

3.2.1 Mice and tumor cell line

This study was approved by the Institutional Animal Care and Use Committee and all mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. Male non-obese diabetic (NOD) mice ($n = 28$, 9-10 weeks old) with severe combined immunodeficiency (SCID, Animal Resources Centre, Canning Vale, Western Australia) were used as host animals for various types of human cancer xenografts. Details of the xenograft model and the formation of cancer cell lines can be found in Ref. (57). Eight different types of human xenografts (Table 3.1) were implanted subcutaneously in the SCID mice. These tumors are known to differ in degree of angiogenesis.

Type of xenograft	Number
Hepatocellular carcinoma (HCC)	7
Gastric/stomach cancer (GC)	5
Lung cancer (LC)	4
Renal cell carcinoma (RCC)	3
Gastrointestinal stromal tumor (GIST)	3
Colorectal cancer (COL)	3
Pancreatic cancer (PAN)	2
Squamous cell carcinoma (SCC)	1

Table 3.1. Study population

3.2.2 DCE-MRI Protocol

DCE MRI experiments were performed on a 7-T MR scanner (Bruker ClinScan, Bruker BioSpin MRI GmbH, Ettlingen, Germany) using a three-dimensional (3D) spoiled gradient recalled sequence (FLASH 3D) with the following parameters: repetition time $TR = 3.04$ ms, echo time $TE = 1.23$ ms, field of view $FOV = 36 \times 36$ mm², 128×128 matrix half-Fourier reconstructed to 256×256 , 8×1 mm² slices, final image resolution of $0.14 \times 0.14 \times 1$ mm², and acquisition time of 2 s.

Tissue precontrast and postcontrast T1 values were estimated using the variable flip angle technique (46-47). To estimate the tissue native T1 values, precontrast images were acquired with different flip angles: 2°, 4°, 6°, 8°, 10°, 12°, 14°, 16°, and 18°. For each flip angle, 10 repetitions were performed and averaged. The dynamic imaging protocol consists of 10 baseline acquisitions with flip angles 6° and

14° (five times each), followed by the manual injection of 50 mL of gadoterate meglumine, INN (Dotarem, Guerbet S.A., Villepinte, France) through the tail vein and 50 mL saline flush. One-hundred and thirty postcontrast acquisitions were performed with flip angle 14° over a period of 260 s, with the contrast injected at the start of the first postcontrast acquisition.

	Multiple FAs estimation of pre-contrast (native) tissue T_1									Baseline		Post-contrast
Flip angle	2°	4°	6°	8°	10°	12°	14°	16°	18°	6°	14°	14°
Number of acquisition	10	10	10	10	10	10	10	10	10	5	5	130

Table 3.2. DCE-MRI protocol in Bruker ClinScan 7T machine

Contrast injection at the first acquisition

3.2.3 Phantom Validation

At low concentrations, contrast agent concentration C can be estimated by the difference in longitudinal relaxation rates,

$$r_1 C = \frac{1}{T_1^C} - \frac{1}{T_1^0} \quad (3.1)$$

where T_1^C and T_1^0 are the post-contrast and pre-contrast (native) T_1 values, respectively, and r_1 denotes the longitudinal relaxivity. A phantom study was performed to validate the T_1 values estimated by the above variable flip angle protocol by comparing with those derived from inversion recovery measurements (inversion time $TI = 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 4000$ ms; repetition time $TR = 5000$ ms). The phantom consists of tubes of saline diluted with 0.05 g/L of copper sulphate to mimic T_1 value in human body tissue at 1.5 sec and filled with various concentration of Dotarem at 0.01, 0.05, and 0.1 mM.

3.2.4 Galbumin-enhanced imaging protocol

Galbumin (BioPal, USA) is gadolinium-labeled bovine albumin with a molecular weight of ~74 kDa. Galbumin-enhanced scans commenced 15 min after the Gd-DOTA-enhanced scans, when the tissue enhancement due to Gd-DOTA has visibly faded. The Galbumin dynamic imaging protocol consists of two phases: an initial rapid imaging phase consisting of 15 acquisitions at 2 s temporal resolution using the 3D VIBE sequence mentioned above and a delayed phase of 15 acquisitions, with 58 s delay between each acquisition. A dose of 0.1mL of Galbumin at 25 mg/mL was manually injected through the tail vein after the first set of dynamic images and followed by a 50 μ L saline flush.

3.2.5 Data Processing

Post-processing was performed off-line on an Intel® Core™2 Duo personal computer with Matlab™ (MathWorks, Natick, MA). For reduced inflow effects and wrap, only the 4 central slices from the imaging volume (of 8 slices) were selected for processing. Regions-of-interest (ROIs) corresponding to the lesions were manually outlined on the 4 central slices.

Galbunin concentration was estimated from the Galbunin-enhanced dynamic scans by the change in longitudinal relaxation rates after injection of Galbunin.

3.2.6 Tracer kinetic modeling

For the Gd-DOTA-enhanced scans, tissue tracer concentration-time curve $C_{\text{tiss}}(t)$ corresponding to each voxel was fitted using the standard two-compartment model, as explained in Chapter 1.1.2, Eq. 1.5-1.9.

3.2.7 Galbunin kinetic analysis

The Galbunin-enhanced scans were analyzed using model-independent methods to derive alternative estimates for blood flow and vessel permeability. Galbunin concentration-time data $C_{\text{tiss}}^{\text{Gal}}(t)$ corresponding to the first rapid imaging phase was analyzed using the maximal slope method (58) to yield estimates for blood flow,

$$F_{\text{max}} = \frac{\left. \frac{d}{dt} C_{\text{tiss}}^{\text{Gal}}(t) \right|_{\text{max}}}{\left. C_{\text{A}}^{\text{Gal}}(t) \right|_{\text{max}}}, \quad (4.1)$$

where $C_{\text{A}}^{\text{Gal}}(t)$ is the arterial input function sampled from the Galbunin-enhanced scans.

Galbunin concentration-time data for the delayed imaging phase was analyzed using the Patlak plot method to derive the Galbunin influx rate constant K_i , which is indicative of vessel permeability (59):

$$\frac{C_{\text{tiss}}^{\text{Gal}}(t)}{C_{\text{A}}^{\text{Gal}}(t)} = K_i \frac{\int_0^t C_{\text{A}}^{\text{Gal}}(\tau) d\tau}{C_{\text{A}}^{\text{Gal}}(t)} + v_0, \quad (4.2)$$

where v_0 is the ordinate intercept of the Patlak plot.

3.2.8 Histopathology

a. Tissue sectioning

The tumor was manually dissected from the subcutaneous tissue. It was orientated in a plane similar to the imaging plane. The superior, inferior, left and right borders were stained with different colors to allow orientation. The tumor was manually sectioned and subsequently fixed and embedded. A microtome section was obtained from the superior aspect of each section.

b. CD-31 stain

Tumor cryosections (5 μm thick) were pretreated using optimized antigen retrieval methods and then immunohistochemical staining performed. Vasculature was stained using a platelet/endothelial cell adhesion molecule/CD31 antibody and CD34 antibody (1:100 dilution; BD PharMingen, San Diego, CA). Visualization was done with automated stainer and biotin–avidin complex.

c. Masson's trichrome

Interstitial collagen tissue was used as a marker of the interstitial space and was stained with the Masson's trichrome stain. The digital image was threshold for interstitial collagen. The percentage of area stained for collagen is used as a measure of the interstitial space.

3.2.9 Microscope/image acquisition

Digital images of the tumor slices were created at a magnification of x400 with a Nikon microscope (Nikon Instruments, Melville, NY). Image analysis for vessel counting was done using Nikon imaging software (NIS-Elements Basic Research 3.0, Nikon Instruments, Melville, NY). The software allowed the application of standardized computational algorithms as well as review and refinement of the results of those algorithms by human operators (60). A camera resolution of 1300 x 1028 pixels would result in a pixel size of 0.240 μm /pixel. The pixel size of the DCE-MRI image was calculated by dividing the field-of-view with the resolution of the matrix. A grid sized to the DCE-MRI pixel is overlaid onto the digital histopathology image. Subsequent analysis (such as vessel count or percentage area of staining) is calculated with respect to each grid box (which correspond in size to the DCE-MRI pixel).

3.3 Results

Parameters of DCE-MRI sequences used in human studies and its translated version in animal studies were given in Table 3.3. The sequences were based on a similar basic FLASH sequence. The values of TR and TE were set to be as similar as possible within the allowances given by the machine.

Parameters	Human	Animal
Machine	Avanto (1.5T, Siemens)	ClinScan (7T, Bruker)
Platform	syngoMR	syngoMR
Sequence	FLASH 3D	FLASH 3D VIBE
FoV	40 x 40 cm	36 x 36 mm
TR	3.03 ms	3.04 ms
TE	1.17 ms	1.23 ms
Temp. resolution	1.38 s	2 s
Slice	6	8
Slice thickness	8 mm	1 mm
Base resolution	256 x 256	128 x 128
Flip angle	6° & 18°	6° & 14°

Table 3.3. Comparison of DCE-MRI parameters in clinical and preclinical settings

Comparison between T_1 values derived by the inversion recovery sequence with those estimated by the dual flip angle method was given in Figure 3.1. A linear correlation with slope of 1.22 and negligible ordinate intercept was obtained. Hence a reasonable precision of T_1 values estimated by the latter method was acquired and indicated the reliability of the estimated concentration values in the mouse tissue.

Figure 3.1. (a) Phantom setup, positioned as followed from the bottom left corner, moving counter-clockwise: saline, saline with 0.05 g/L of copper sulphate (solution ‘A’), ‘A’ added with 0.01, 0.05, and 0.01 mmol/mL of Dotarem, respectively, and (b) comparison between T_1 derived by the inversion recovery sequence with T_1 estimated by multiple flip angle method

The various microcirculatory parameters estimated by the standard two-compartment model for each type of xenograft are presented in Table 3.4. Blood flow F is highest in the renal xenografts, reaching 164.15 ± 1.05 ml/100ml/min, and lowest in the HCC, GIST, and colorectal xenografts at around 25-30 ml/100ml/min.

Correlations between the microcirculatory parameters derived by the standard two-compartmental model with their respective immunohistochemistry staining were presented in Figure 3.2. Statistically significant correlations ($p < 0.001$) were found for each parameter.

An example of a hepatocellular carcinoma xenograft is shown in Figure 3.3. Histological sections of the tumor with CD31 and Masson’s Trichrome staining are shown in Figures 3.3 (b) and 3.3 (e). The hotspots of each staining are shown in Figures 3.3 (c) and 3.3 (f). The blood vessels and the interstitial collagen tissue are outlined in light green in the hotspot. The percentage of area of blood vessels is 3.3% and the interstitial volume is 18.55%. The values are similar to those obtained with DCE-MRI, as shown in Figures 3.3 (a) and 3.3 (d), with percentage of blood volume is measured at 3.499% and interstitial volume at 18.435%.

Type of Xenograft	F_p (ml/min/100ml)	PS (ml/min/100ml)	v_p (%)	v_e (%)
HCC	28.73 ± 6.74	3.98 ± 2.00	0.89 ± 0.44	7.18 ± 2.75
Gastric	60.59 ± 30.32	4.22 ± 1.50	1.70 ± 0.86	7.90 ± 1.53
Lung	51.25 ± 18.99	6.00 ± 2.43	4.06 ± 1.03	17.29 ± 4.06
RCC	164.15 ± 1.05	6.27 ± 4.17	4.56 ± 0.08	10.87 ± 7.95
GIST	26.83 ± 4.01	3.07 ± 1.13	0.82 ± 2.28	22.92 ± 2.28
Colorectal	27.86 ± 9.29	3.68 ± 1.61	2.79 ± 1.60	14.78 ± 17.81
Pancreas	34.64 ± 4.00	7.39 ± 0.21	2.02 ± 0.05	12.80 ± 5.84
SCC	98.99	11.8	5.18	22.75

Table 3.4. Microcirculatory parameters (mean $\pm$ standard deviation) estimated by the standard two-compartment model for each type of xenograft

The maps of maximum slope $F_{\max}$ and influx rate constant K_i derived from Galbumin-enhanced scans are shown in Figures 3.4 (b) and 3.4 (d). The maps of blood flow and permeability derived from DCE-MRI are shown in Figure 3.4 (a) and 3.4 (c).

3.4 Discussion

Validation studies of plasma flow in DCE-CT have been performed using microspheres. Stewart et al (61) derived the hepatic arterial blood flow (H_ABF) from a dual-input version of the adiabatic tissue homogeneity model and compared it with the result obtained from radiolabeled microsphere studies. A strong correlation was observed between H_ABF values from both techniques, with $r^2 = 0.81 \pm 0.05$ ($p < 0.05$).

Water PET has been used to validate plasma flow in DCE-CT. Bisdas et al (31) used a distributed parameter (DP) model for tracer kinetic analysis in human brain studies to validate the derived perfusion values with $H_2^{15}O$ PET scans and significant correlation was found with $r = 0.50$ to 0.79 (r^2 -values ranged from 0.45 to 0.79) and $0.01 \leq p \leq 0.05$. $H_2^{15}O$ -PET has also been used to validate tumor blood flow derived from DCE-MRI. Nevertheless, K_{trans} was often used as a surrogate of blood flow and not F directly (62). Similarly, in another study using human melanoma xenografts, images of $E \cdot F$ (where E is the extraction fraction) were obtained by subjecting DCE-MR images to Kety analysis and compared to $Na^{99m}TcO_4$ as a perfusion tracer (63).

Figure 3.2. Correlation between (a) blood flow derived by the standard two-compartmental model with the maximum slope of Galbumin uptake curve, (b) vascular permeability with k_{slow} derived from Galbumin uptake curve, (c) intravascular space volume with % of CD34 stain, and (d) interstitial space volume with % of Masson-Trichrome stain

Unlike in DCE-CT, there has not been a study that validates F estimated by DCE MRI directly. In this study, we attempted to use F directly (and not its surrogate, K_{trans}) with a macromolecule Galbumin used as a substitute for the microspheres.

K_{trans} has also been previously used to validate permeability. Ferrier et al (51) tried to validate blood flow derived from DCE-MRI by using K_{trans} as a estimates of vascular permeability and compared them to estimates K obtained using [^{14}C]aminoisobutyric acid quantitative autoradiography ([^{14}C]AIB QAR), which was an established method of evaluating blood-tumor barrier permeability. Significant correlation was found between K_{trans} and K ($r = 0.905$, $p = .035$).

Figure 3.3. Fractional intravascular space volume of a mouse with HCC xenograft as derived by (a) the standard two-compartmental model and (b) by the CD34 staining as shown in the inset of the whole tumor. The blood vessels are outlined in light green in (c) the hotspot. Fractional extracellular space volume of a mouse with HCC xenograft as derived by (d) the standard two-compartmental model and (e) by the Masson-Trichrome staining as shown in the inset of the whole tumor. The blood vessels are outlined in light green in (f) the hotspot.

Figure 3.4. Blood flow of a mouse with HCC xenograft as derived by (a) the standard two-compartmental model and (b) the slope of the Patlak plot. Vascular permeability of a mouse with HCC xenograft as derived by (c) the standard two-compartment model and (d) the k_{slow} of the Patlak plot.

Nevertheless, it is more difficult to validate vascular permeability in general. The blood-to-brain influx rate constant (K_i) as derived from the Patlak plot was the established tracer analysis technique as the estimate for vascular permeability and it was used as well in this study (59).

So, instead of using K_{trans} which can mean either flow or permeability, in this study we have shown the ability to measure flow and permeability separately and validate them independently.

Egeland et al (64) compared the fractional volume of the extravascular extracellular space (EESF) derived by DCE-MRI with histology. They found that the numerical values of the DCE-MRI-derived parameters were not significantly different from the absolute values of tumor blood perfusion or fractional volume of the extravascular extracellular space in any of the tumor lines. Similarly,

Benjaminsen et al (65) investigated whether Gd-DTPA-based DCE-MRI can be used to assess the EESF of tumors using amelanotic human melanoma xenografts. Images of λ (the partition coefficient of Gd-DTPA) were obtained by Kety analysis of DCE-MRI data. Positive correlations were found between λ and EESF obtained by invasive imaging.

Gaustad et al (66) investigated whether Gd-DTPA-based DCE-MRI can be a useful method for characterizing vascularity of tumors by comparing the images of $E\cdot F$ as obtained by Kety analysis with Blood Supply Time (BST) images (i.e., images of the time from when arterial blood enters a tumor through the supplying artery until it reaches a vessel segment within the tumor) and morphologic images of the microvascular network which were produced by intravital microscopy. They found that the $E\cdot F$ images mirrored the morphology (microvascular density) and the function (BST) of the microvascular networks well.

In this study, we compared both v_p and v_e directly with immunohistological chemistry stainings and not to invasive or morphological imaging.

This study suffered from the following limitations. As the parameters derived from DCE-MRI were compared with histology, there might be some distortions to the tissue during stain preparation. The orientation of scans might not exactly correspond with the histology slice as well. Difficulty in finding exact stains for flow and permeability also forced us to use a rather less than ideal substitute in the form of Galbumin (and its respective K_i from Patlak plot).

3.5 Conclusion

We have shown that the parameters derived from the standard two-compartmental model correlated with the respective values derived from histopathology.

It indicates the reliability of the proposed technique and potentials of applying this technique as a biomarker for pre-clinical drug developments, as it might allow monitoring of anti-angiogenic therapy in murine models.

4. Dynamic Contrast-Enhanced MRI of Mouse Liver³

4.1 Introduction

Liver cirrhosis constitute a final common pathway for a myriad of liver diseases including Hepatitis B, C and fatty liver, resulting in significant morbidity and mortality. Although advances in the last 20 years have led to availability of many efficacious drugs that can control Hepatitis B and C, treatment regimens are still suboptimal with high cost, significant side effects and incomplete response. Most treatment guidelines have thus advocated treatment only for those at highest risk of developing cirrhosis while ‘masterly inactivity’ is recommended for those with early liver disease.

The problem in real life scenario however, is the lack of good non-invasive tool that can accurately predict early onset fibrosis and progression to cirrhosis. While non-invasive tests including fibroscan, magnetic resonance elastography and serum based tests have been reported to show fairly good correlation with fibrosis, the sensitivity and specificity of most of these tests are still suboptimal at F2 to F3 fibrosis (metavir score), which unfortunately, is the range upon which clinical decisions are made for treatment (67-68). In addition, most of these tests are targeted at the degree of fibrosis, which is a marker of end point damage but does not reflect disease onset or direct pathogenesis.

Accumulating evidence indicates that one of the early pathogenetic processes in liver cirrhosis is capillarisation of liver sinusoidal endothelium or loss of fenestrae. This change is believed to alter the liver sinusoidal dynamics and result in ischemia of hepatocytes and drive the chronic process of injury. By capitalizing on the physical concepts of mono-compartment and dual-compartment kinetics, DCE imaging is able to detect the Space of Disse to indicate whether fibrosis has occurred. Indeed, human studies have shown that patients with normal livers returned a near-zero fractional interstitial space (v_2) due to large fenestrae allowing free exchange of low-molecular weight compounds such as gadolinium-chelate contrast between the vascular space (sinusoids) and interstitial space (Space of Disse) while cirrhotic patients demonstrate a measurable value for the interstitial Space of Disse (8, 10, 69).

In this work, we explore the feasibility of applying a high spatial and temporal resolution (2 s) DCE-MRI protocol for arterial concentration estimation and perfusion imaging in mice liver. We carried

³ Parts of this study were presented as a poster in the 2011 Radiological Society of North America Annual Meeting, Chicago, IL, the 2011 Engineering in Medicine and Biology Conference, Boston, MA, the 2013 International Society for Magnetic Resonance in Medicine Annual Meeting, Salt Lake City, UT, published in the proceedings of 2011 Engineering in Medicine and Biology Conference, page 2788-2791, and submitted for publication in *Journal of Biomedical Science and Engineering*.

out a pilot study using DCE-MRI to assess the kinetics of normal mouse liver to compute a reliable model that can be subsequently applied in the cirrhotic mice.

4.2 Materials and Methods

4.2.1 Mice

Five male C57BL/6 mice (6 weeks old, 30 ± 2 g) were used in this study. Mouse core body temperature was monitored with a rectal probe and maintained at 37 ± 0.3 °C by a feedback-controlled warm-air heating system. The mice were kept under 1-2 % isoflurane anesthesia during MR scanning.

This study was approved by the local Institutional Animal Care and Use Committee. All mice were maintained according to the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. They were provided with sterilized food and water ad libitum and housed in ventilated cages with air and oxygen.

4.2.2 DCE-MRI Protocol

The DCE-MRI protocol employed in this study has been explained earlier in Chapter 3.2.2.

4.2.3 Tracer Kinetic Modeling

For the Gd-DOTA-enhanced scans, tissue tracer concentration-time curve $C_{\text{tiss}}(t)$ corresponding to each voxel was fitted using the standard two-compartment model and the generalized kinetic model as explained in Chapter 1.1.2, Eq. 1.5-1.9 and Eq. 1.15-1.16.

4.2.4 Dual Input Model

Consider two sources of blood flow to the liver: flow from the hepatic artery F_A and flow from the portal vein F_{PV} . Assuming that the tracer concentration-time curves for the hepatic artery $C_A(t)$ and the portal vein $C_{PV}(t)$ can be sampled from dynamic MR images, the total input concentration-time curve $C_{\text{in}}(t)$ can be expressed as follows (8):

$$C_{\text{in}}(t) = \alpha C_A(t) + (1 - \alpha) C_{PV}(t) \quad (3.2)$$

where $\alpha (= F_A/F)$ denotes the arterial fraction. The total hepatic blood flow, F , then is given as the sum of F_A and F_{PV} .

4.2.5 Data Processing

Post-processing was performed off-line on an Intel® Core™2 Duo personal computer with Matlab™ (MathWorks, Natick, MA). For each case, region of interest corresponding to the liver within the central six (of eight) imaging slices were manually outlined by an experienced radiologist.

The hepatic arterial input $C_A(t)$ was approximated by sampling the concentration-time curve at the aorta and the portal venous input $C_{PV}(t)$ was sampled from the portal vein. $C_A(t)$ and $C_{PV}(t)$ were divided by a factor of $(1 - Hct)$, where Hct is the fractional hematocrit for mice and was set at 45% (70-71).

4.3 Results

A typical set of signal-time concentration profiles in the artery, the portal vein, and the liver tissue was given in Figure 4.1. The profiles were similar with the findings previously reported in human studies (8). (Koh *et al.*, 2008) The order of arrival of the contrast was as follow: in the artery, in the portal vein, and in the liver tissue. It showed that the two vascular inputs mix in the sinusoids at different time intervals. However, current temporal resolution of image acquisition of two seconds might not be adequate in delineating the difference clearly.

Microcirculatory parameters derived from the conventional compartmental model and the generalized kinetic model were presented in Tables 4.1 and 4.2.

Extravasation parameters derived from the conventional compartmental model such as v_2 and PS were zero or close to zero for all cases. They agreed with the murine physiology (72-75) and corresponded with the results previously observed in human studies (8). The liver is a highly vascular organ that consists of a series of fenestrated sinusoids which function as the vascular space. The sinusoids are predominantly supplied by the portal vein (80%) and supplemented by the hepatic artery (20%) (76, 77). They are separated from the liver cords by the Space of Disse, which are comprised of two rows of closely apposed hepatocytes. The Space of Disse may then be considered as an interstitial space

Figure 4.1. Signal concentration time-curves in the artery, portal vein, and liver tissue of Mouse 01

within the liver. However, due to the large size of the fenestrae of the sinusoids, low-molecular weight compounds such as gadolinium contrast can trace freely between the vascular space (sinusoids) and the interstitial space (Space of Disse). Both of them then effectively function as a single compartment system (10, 78). Hence low values of fractional interstitial volume and permeability-surface area product in normal liver were expected, as were observed in this study.

Parametric maps of Mouse 03 were given in Figure 4.2 and a relatively monotonic distribution of v_2 and PS could be observed in Figures 4.2 (b) and 4.2 (d), thus confirming the single compartment system of normal liver.

Mean values for vascular perfusion (F) as calculated by the standard two-compartment model ranged from 381.34 mL/100mL/min to 835.54 mL/100mL/min. There is over-estimation of the hepatic blood flow as the mean of blood flow for mice liver was previously estimated in other studies at 180 mL/100mL/min (22). (Davies and Morris, 1993) The mean values for fractional blood volume (v_1) as calculated by the standard two-compartment model ranged from 5.91% to 16.38%. There is under-estimation of the values of the percentage of mice hepatic blood volume, as this was previously estimated at 23%-36% (79).

CC model		Mouse 01	Mouse 02	Mouse 03	Mouse 04	Mouse 05
F	(ml/100ml/min)	596.09 ± 350.58 (512.65)	835.54 ± 530.91 (682.41)	334.45 ± 215.31 (285.52)	381.34 ± 234.06 (326.31)	850.38 ± 522.97 (674.17)
t_1	(s)	2.00 ± 2.11 (1.73)	1.82 ± 2.64 (1.33)	1.59 ± 1.23 (1.37)	1.98 ± 2.26 (1.47)	1.64 ± 1.56 (1.47)
v_1	(%)	13.91 ± 6.50 (13.91)	15.99 ± 9.87 (14.62)	5.91 ± 3.15 (6.25)	8.03 ± 4.42 (8.04)	16.38 ± 9.00 (15.53)
v_2	(%)	6.10 ± 16.64 (0.01)	8.38 ± 19.68 (0.01)	8.67 ± 25.75 (0.00)	2.71 ± 9.75 (0.35)	5.16 ± 15.37 (0.00)
PS	(ml/100ml/min)	17.22 ± 49.53 (0.29)	11.71 ± 38.56 (0.26)	4.07 ± 12.31 (0.00)	13.26 ± 22.79 (3.31)	19.15 ± 62.62 (0.09)
α	(%)	66.80 ± 37.07 (82.13)	43.76 ± 38.11 (38.89)	59.68 ± 28.79 (56.94)	54.29 ± 37.75 (60.56)	55.98 ± 33.50 (55.32)

Table 4.1. Microcirculatory parameters estimated by the standard two-compartmental model (mean values ± standard deviations, with median values in parentheses)

GK model		Mouse 01	Mouse 02	Mouse 03	Mouse 04	Mouse 05
K_{trans}	(min ⁻¹)	9.10 ± 9.62 (5.71)	12.99 ± 12.66 (9.07)	4.40 ± 4.56 (3.02)	4.72 ± 4.89 (3.31)	15.10 ± 13.87 (10.32)
v_e	(%)	14.66 ± 9.55 (13.96)	15.89 ± 11.88 (13.92)	6.99 ± 4.35 (7.11)	8.97 ± 5.19 (9.34)	15.06 ± 10.17 (14.57)
α	(%)	56.74 ± 40.50 (64.31)	43.88 ± 38.35 (37.36)	45.17 ± 26.86 (43.82)	46.70 ± 36.67 (48.62)	44.27 ± 30.51 (41.22)

Table 4.2. Microcirculatory parameters values estimated by the generalized kinetic model (mean values ± standard deviations, with median values in parentheses)

Figure 4.2. Parametric maps of (a) blood flow, (b) permeability-surface area product, (c) fractional intravascular space, and (d) fractional extravascular space for Mouse 03 (slice 2 to 7)

Mean values for α derived by the conventional compartmental model ranged from 43.76% to 66.80%. There is over-estimation of α as Davies and Morris (77) (Davies and Morris, 1993) reported arterial fraction of 19.44%, while Brown reported arterial fraction of 28.77% (79).

The mono-compartmental system can be inferred as well from the signal concentration-time curve profile in the liver tissue in Figure 4.3, as a contrast uptake enhancement pattern of a rapid increase which was followed by rapid washout was observed (32). Figure 4.3 also showed an example of fitting the dual-input conventional compartmental model to liver tissue concentration-time curve and the impulse residual function $R(t)$ corresponding to the fitting of the enhancement pattern.

Figure 4.3. (a) Example of fitting the dual-input standard two-compartmental model to liver tissue concentration-time curve and (b) impulse residual function $R(t)$ corresponding to the fitting of the enhancement pattern. $F = 220.6$ mL/100mL/min, $t_1 = 2.58$ sec, $\alpha = 25.7\%$, $v_1 = 9.5\%$, $v_2 = 0\%$, $PS = 0$.

4.4 Discussion

In this study we observed the low values of interstitial space and permeability in all mice, which are concordant with the normal liver physiology.

The shape of the signal concentration-time curve in the liver tissue is also consistent with a single-compartment system, as is shown in Figure 4.3 (a). A contrast uptake enhancement pattern of a rapid increase was followed by rapid washout (32). (Koh *et al.*, 2011) Figure 4.3 (b) also showed an example of fitting the dual-input two-compartment model to liver tissue concentration-time curve and the impulse residual function $R(t)$ that is the result of the fitting of the enhancement pattern. It showed an impulse residual function which immediately dropped to zero after the vascular phase, consistent with the behavior of a single-compartment system.

Compared to previously published values of mice hepatic microcirculation, the current model over-estimates hepatic blood flow and under-estimates percentage of mice hepatic blood volume. An over-estimation of arterial fraction, α , was also observed.

Mouse 04		Liver	Muscle
F	(ml/100ml/min)	381.34 ± 234.06 (326.31)	112.07 ± 71.01 (93.83)
t_1	(s)	1.98 ± 2.26 (1.47)	5.26 ± 7.09 (2.86)
v_1	(%)	8.03 ± 4.42 (8.04)	5.68 ± 4.14 (4.71)
v_2	(%)	2.71 ± 9.75 (0.35)	9.29 ± 4.74 (8.85)
PS	(ml/100ml/min)	13.26 ± 22.79 (3.31)	14.11 ± 10.18 (13.00)

Table 4.3. Microcirculatory parameters in liver and muscle for Mouse 04 (mean values $\pm$ standard deviations, with median values in parentheses)

The over-estimation of α might be due to difficulty in finding a voxel which corresponds to the portal vein for all dynamic images and hence a worse signal-to-noise ratio from the portal vein, which possibly increased the contribution from the arterial input.

To illustrate this problem, the conventional compartmental model was applied as well to analyze the parameters in muscle of Mouse 04 to show the difference of the tracer kinetic behaviors and microcirculatory parameters values in liver and muscle. The values of the microcirculatory parameters were compared in Table 3.3. The value of blood flow in muscle was significantly less than in liver. The values of v_2 and PS were not zero or close to zero. Davies and Morris (77) (Davies and Morris, 1993) reported that the blood flow in muscle was estimated at 91 mL/100 mL/min. It was similar to what was observed in Mouse 04 at 112.07 ± 71.01 mL/100 mL/min. Brown (79) reported that the percentage of vascular space in muscle was estimated at 3%-5%. It also agrees with what is observed in Mouse 04 at $5.68 \pm 4.14\%$. The relatively smaller error in the estimation of F and v_1 in muscle perhaps indicated a higher technical difficulty in applying tracer kinetic modeling in liver since it incorporated not one input signal as is in the muscle but two input signals, aorta and portal vein, for data fitting, which might also be exacerbated by the similar shape of arterial and venous input curves, as was shown in Figure 4.1.

Although there are technical challenges to this technique, the low values of the mice interstitial space are concordant with observations in humans and lend potential for the use of this quantitative technique as a potential biomarker for sinusoidal capillarization and fibrosis in the Space of Disse. Currently, ultrasound and MR elastography have been used as non-invasive techniques for the assessment of fibrosis and cirrhosis. Although advanced fibrosis and cirrhosis can be accurately determined with both modalities, challenges remain for the assessment of early fibrosis.

Our current findings set the stage for future studies with thioacetamide induced liver fibrosis and cirrhosis to determine if DCE-MRI can serve as a imaging biomarker for this important process.

4.5 Conclusion

We have shown that the parameters derived from the standard two-compartmental model correlated with the respective values derived from histopathology.

It indicates the reliability of the proposed technique and potentials of applying this technique as a biomarker for pre-clinical drug developments, as it might allow monitoring of anti-angiogenic therapy in murine models.

5. Assessment of Drug Activity in the Preclinical Settings: A Pilot Study with Bevacizumab⁴

5.1 Introduction

Avastin (bevacizumab), an anti-VEGF humanized monoclonal antibody, has demonstrated positive clinical treatment effect and is currently approved for treatment of colorectal carcinoma and NSCLC in combination with standard chemotherapy. Nevertheless, recently the FDA has revoked the approval of bevacizumab as a treatment for breast cancer, as the clinical trials had shown that the drug was not helping breast cancer patients to live longer or to meaningfully control their tumors, but exposed them to potentially serious side effects like severe high blood pressure and hemorrhaging. Moreover, even though blocking the VEGF pathway by bevacizumab has been shown to prevent tumor progression in the short-term, eventual progression of disease in the presence of VEGF therapy has been observed in clinical studies. Resistance develops quickly when patients are treated with bevacizumab as monotherapy. Therefore, improvement of our understanding of the mechanism of bevacizumab is needed.

Although many studies have demonstrated with histopathology a decrease in microvascular density after anti-angiogenic therapy, only two studies have incorporated DCE-MRI. Kiessling studied VEGFR2 blocker and validated the Brix model findings with histopath (80). Troiani studied a VEGF-EGFR dual antibody and used K^{trans} but did not have histopathology to validate the DCE MRI findings (81). We are not aware of preclinical DCE MRI studies with bevacizumab.

We aim to determine if DCE-MRI as evaluated by the standard two-compartment model is a sensitive enough technique to detect changes in microcirculatory parameters after administration of a single dose of bevacizumab. We hope to determine what changes are seen when the therapy is effective, as this knowledge would enable translation to human and allow monitoring of therapeutic effect and may potentially act as a biomarker.

We would also like to compare the parameters derived by the two-compartment model, which is more complex and requires high temporal resolution so that it can be properly analyzed, with the parameters derived by the generalized kinetic model to see whether the more complex two-compartment model could give additional information which could not be obtained from the simpler generalized kinetic model.

⁴ Parts of this study were as posters in the 2012 Annual Meeting of International Society of Magnetic Resonance in Medicine, Stockholm, Sweden and submitted for publication in *Journal of Cancer Research*.

5.2 Materials and Methods

5.2.1 Mice

Twenty-seven male NOD SCID mice (6 weeks old, 30 ± 2 g) formed the study population & were under 1-2 % isoflurane during the scanning. Mouse core body temperature was monitored with a rectal probe and maintained at 37 ± 0.3 °C by a feedback-controlled warm-air heating system. This study was approved by the local Institutional Animal Care and Use Committee. All mice were maintained according to the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. They were provided with sterilized food and water ad libitum and housed in ventilated cages with air and oxygen. They were implanted with human-derived hepatocellular carcinoma xenograft line, sensitive to bevacizumab therapy.

Six mice were used for characterization study. They were imaged longitudinally at the following number of days after implantation: 13, 16, 20, 23, 27, and 34. They were administered with Xolair (omalizumab, a human anti-IgE antibody) at 10 mg/kg at day 15 after implantation.

The rest of the mice were used for dose-dependent effect study. Two dose levels of Avastin were investigated: low dose (n = 4) at 1 mg/kg and high dose (n=8) at 10 mg/kg. The drug was administered intraperitoneally. Mice were scanned at baseline, day 1, day 5, and day 8 after therapy. Nine control mice were similarly imaged after receiving omalizumab at 10 mg/kg. The experimental design was outlined in Table 5.1.

Group	Number of days after implantation							IHC
	13 (BL)	15 (D0)	16 (D1)	20 (D5)	23 (D8)	27	34	
Characterization study								
Characterization (n = 6)	MRI	Xolair 10mg/kg	MRI	MRI	MRI	MRI	MRI	No
Dose-dependent effect study								
Control (n = 9)	MRI	Xolair 10mg/kg	MRI	MRI	MRI+IHC			Yes
Treated, low dose (n = 4)	MRI	Avastin 1mg/kg	MRI	MRI	MRI+IHC			Yes
Treated, high dose (n = 8)	MRI	Avastin 10mg/kg	MRI	MRI	MRI+IHC			Yes

Table 5.1. The overall experimental design for the bevacizumab study

5.2.2 DCE-MRI Protocol

The DCE-MRI protocol used in this study has been explained earlier in Chapter 3.2.2.

5.2.3 Histopathology

Tumors were sectioned and stained immunohistochemically to identify CD34 for quantification of microvessel density.

5.2.4 Data Processing

Post-processing was performed off-line on an Intel® Core™ i5 personal computer with Matlab™ (MathWorks, Natick, MA, USA). The six central slices from the imaging volume of eight slices were selected for processing. Region of interests corresponding to the xenograft and major artery were manually outlined.

Gadolinium contrast concentration was estimated using the dual-flip angle method (46-47), as explained in Chapter 3.2.2. The arterial input function (AIF) was obtained by sampling a small window placed within the aorta.

For the Gd-DOTA-enhanced scans, tissue tracer concentration-time curve $C_{tiss}(t)$ corresponding to each voxel was fitted using the standard two-compartment model and the standard two-compartment model, as explained in Chapter 1.1.2, Eq. 1.5-1.9 and Eq. 1.15-1.16.

Model-fitting was performed using a constrained nonlinear optimization algorithm ('Fmincon', Matlab™; Mathworks) which yields the sum-of-squared residues (SSR) as a measure of the goodness-of-fit and output flags reflecting the status of the optimization search. Poor fittings with exceedingly large SSR (greater than a stipulated threshold) and negative output flags (indicating no feasible solution) were excluded. The median parameter value in the tumor ROI was taken as the representative parameter value for the tumor.

5.2.5 Statistical Analyses

Spearman's rank correlation coefficient, r , was used to evaluate the strength of the relationship between the microvasculature parameters.

5.3 Results

Characterization study

Values of microcirculatory parameters derived by the standard two-compartment model and the generalized kinetic model for the characterization study are presented in Table 5.2. Baseline (Day 13 after implantation) blood volume of the characterization group was 5.15 ± 1.49 %, which increased to 6.91 ± 2.82 % at Day 20 before it dropped to 1.18 ± 0.41 % at Day 23. The low level of blood volume was sustained until the terminal time point (Day 34) at the level of 1.23 ± 0.39 %. An example of such drop between Day 20 and Day 23 was shown in Figure 5.1 and the time course graphs of the microcirculatory parameters were displayed in Figure 5.2.

Similarly, blood flow increased from 54.91 ± 12.13 ml/100ml/min at baseline to 64.16 ± 9.44 ml/100ml/min at Day 20 and dropped to 33.23 ± 9.25 ml/100ml/min at Day 23. This low level of blood flow was sustained until the terminal time point at the level of 32.44 ± 4.99 ml/100ml/min (Figure 5.2 (a)). The same trend was also observed in the generalized kinetic model. The baseline value of K_{trans} was 0.17 ± 0.03 min⁻¹, which increased to 0.33 ± 0.12 min⁻¹ at Day 20 before dropping to 0.10 ± 0.03 min⁻¹ at Day 23 (Figure 5.2 (e)).

Characterization	Day 13	Day 16	Day 20	Day 23	Day 27	Day 34
<i>Standard two-compartment model</i>						
F (ml/100ml/min)	54.91 ± 12.13	62.20 ± 16.49	64.16 ± 9.44	33.23 ± 9.25	34.55 ± 7.18	32.44 ± 4.99
v_1 (%)	5.15 ± 1.49	5.50 ± 2.05	6.91 ± 2.82	1.18 ± 0.41	1.98 ± 0.52	1.23 ± 0.39
v_2 (%)	13.76 ± 3.96	13.25 ± 5.67	9.79 ± 1.57	8.70 ± 2.69	8.64 ± 2.15	10.75 ± 2.12
PS (ml/100ml/min)	7.96 ± 0.96	6.88 ± 1.64	10.48 ± 1.79	5.99 ± 3.07	7.10 ± 2.89	9.39 ± 3.19
<i>Generalized kinetic model</i>						
Ktrans (/min)	0.17 ± 0.03	0.22 ± 0.16	0.33 ± 0.12	0.10 ± 0.03	0.13 ± 0.03	0.11 ± 0.04
v_e (%)	16.85 ± 3.13	16.14 ± 3.74	15.57 ± 2.47	9.85 ± 2.37	10.82 ± 2.23	12.43 ± 2.55
<i>Tumor size</i>						
Tumor size (cm ³)	0.10 ± 0.03	0.16 ± 0.06	0.38 ± 0.13	0.40 ± 0.30	1.03 ± 0.27	2.06 ± 0.53

Table 5.2. The microcirculatory parameters as derived by the standard two-compartment model and the generalized kinetic model for the characterization study (mean $\pm$ standard deviation) at various days after implantation

Figure 5.1. Map of v_1 (above) and mean of contrast enhancement (below) of Mouse 02 in the characterization group before (left) and after (right) the switching point. It showed a drop in blood volume and less contrast enhancement after the switching point.

Dose-dependent effect study

Values of microcirculatory parameters derived by the standard two-compartment model and the generalized kinetic model for the dose-dependent effect study are presented in Table 5.3. The time course graphs of these parameters were shown in Figure 5.3.

Baseline blood flow and volume for the control group was 95.20 ± 24.02 ml/100ml/min and 8.77 ± 3.72 %, respectively. The values were 133.73 ± 27.9 ml/100ml/min and 9.67 ± 1.72 % for the group treated with bevacizumab 1mg/kg and 95.26 ± 26.69 ml/100ml/min and 10.02 ± 3.85 % for the group dosed with bevacizumab 10mg/kg.

A sharp reduction of 46.18 ± 15.39 % and 37.88 ± 37.31 % in blood flow at Day 1 was observed in the group treated with bevacizumab 1mg/kg and 10mg/kg, respectively (Figure 5.3 (a)). An increase of 28.18 ± 58.16 % in blood flow at Day 1 was observed in the control group. A further reduction of 31.55 ± 38.03 % at Day 5 was found in bevacizumab 10mg/kg, but not in 1mg/kg, which saw an increase of 11.90 ± 33.06 %.

Figure 5.2. Time course graphs of microcirculatory parameters for the characterization study

A reduction of 52.69 ± 24.77 % in blood volume at Day 1 was observed in the group treated with bevacizumab 10mg/kg, while a reduction of 6.10 ± 31.86 % was observed in those treated with bevacizumab 1mg/kg. An increase of 29.31 ± 66.84 % at Day 1 was observed in the control group (Figure 5.3 (b)). A strong correlation was found between percentage of vessel area derived by CD34 staining with percentage of blood volume derived by the two-compartment model ($r = 0.577$, $p = 0.003$, Figure 5.4 (a)).

The control group also saw a drop in blood flow and blood volume in Day 8 after dosing, which corresponded to 23 days after implantation, the same switching point found in the characterization study (Figure 5.2 (a), (b), and (e)). They dropped to 63.34 ± 17.69 ml/100ml/min and 4.53 ± 2.77 %, respectively, which is similar to the values found in the group treated with bevacizumab 1mg/kg at 64.08 ± 22.58 ml/100ml/min and 4.19 ± 2.47 % (Figure 5.3 (a)). The individual plots of blood volume trends for the control group and the 10 mg/kg bevacizumab group were displayed in Figure 5.5. Blood volume of some mice in the control group dropped to the similar level found in the treated group. It illustrated the switch which occurred at Day 8 after dosing, or at 23 days after implantation.

A similar reduction of 22.17 ± 45.16 %, 35.28 ± 57.28 %, and 50.81 ± 20.60 % in interstitial volume at Day 1 was observed in the control group, the group treated with bevacizumab 1mg/kg and 10mg/kg, respectively (Figure 5.3 (c), (f)). There is no significant difference for the trend of permeability between the control group and the treated groups (Figure 5.3 (d)).

Xolair 10mg/kg	BL	D1	D5	D8
<i>Standard two-compartment model</i>				
F (ml/100ml/min)	95.20 ± 24.02	124.36 ± 59.16	105.59 ± 33.39	63.34 ± 17.69
v_1 (%)	8.77 ± 3.72	10.27 ± 2.28	8.68 ± 3.59	4.53 ± 2.77
v_2 (%)	27.56 ± 9.80	17.82 ± 4.55	10.67 ± 3.29	11.03 ± 7.78
PS (ml/100ml/min)	15.92 ± 6.30	12.46 ± 3.96	9.10 ± 3.69	12.26 ± 6.83
<i>Generalized kinetic model</i>				
K_{trans} (/min)	0.26 ± 0.07	0.40 ± 0.16	0.39 ± 0.10	0.23 ± 0.09
v_e (%)	33.38 ± 9.57	23.73 ± 4.65	16.27 ± 3.04	15.30 ± 9.08
Tumor size (cm ³)	0.063 ± 0.023	0.127 ± 0.06	0.265 ± 0.155	0.435 ± 0.283

Avastin 1 mg/kg	BL	D1	D5	D8
<i>Standard two-compartment model</i>				
F (ml/100ml/min)	133.73 ± 27.91	70.22 ± 19.35	77.85 ± 23.77	64.08 ± 22.58
v_1 (%)	9.67 ± 1.72	9.29 ± 4.15	4.51 ± 1.62	4.19 ± 2.47
v_2 (%)	33.86 ± 13.57	16.11 ± 5.85	12.18 ± 4.27	11.21 ± 2.49
PS (ml/100ml/min)	11.67 ± 2.78	14.25 ± 10.97	10.56 ± 5.28	9.79 ± 0.83
<i>Generalized kinetic model</i>				
K_{trans} (/min)	0.29 ± 0.05	0.30 ± 0.15	0.22 ± 0.13	0.17 ± 0.05
v_e (%)	31.78 ± 10.70	22.46 ± 7.39	15.42 ± 4.72	14.35 ± 2.51
Tumor size (cm ³)	0.054 ± 0.01	0.092 ± 0.01	0.180 ± 0.04	0.322 ± 0.07

Avastin 10 mg/kg	BL	D1	D5	D8
<i>Standard two-compartment model</i>				
F (ml/100ml/min)	95.26 ± 26.69	51.82 ± 16.15	39.45 ± 14.14	33.90 ± 8.74
v_1 (%)	10.02 ± 3.85	4.35 ± 1.77	2.15 ± 0.64	1.82 ± 0.66
v_2 (%)	28.43 ± 7.43	13.13 ± 4.15	12.55 ± 4.85	10.98 ± 2.73
PS (ml/100ml/min)	14.25 ± 9.47	11.39 ± 3.33	8.44 ± 1.64	8.40 ± 2.50
<i>Generalized kinetic model</i>				
K_{trans} (/min)	0.27 ± 0.08	0.16 ± 0.06	0.10 ± 0.02	0.09 ± 0.03
v_e (%)	27.71 ± 9.33	15.88 ± 4.65	13.40 ± 4.26	12.56 ± 3.28
Tumor size (cm ³)	0.069 ± 0.031	0.141 ± 0.068	0.182 ± 0.081	0.225 ± 0.109

Table 5.3. Microcirculatory parameters estimated by the standard two-compartment model and the generalized kinetic model for the dose-dependent effect study at baseline and various days (1, 5, and 8) after dosing

Figure 5.3. Time course graphs of microcirculatory parameters for the dose-dependent effect study in control (blue) and treated groups (1mg/kg: green, 10mg/kg: red)

An increase of 65.98 ± 85.25 % in K_{trans} at Day 1 after dosing was observed in the control group, while a reduction of 0.34 ± 39.59 % and 35.05 ± 29.83 % was observed in those treated with bevacizumab 1mg/kg and 10mg/kg, respectively (Figure 5.3 (e)).

The tumour growth rate in the group treated with bevacizumab 10mg/kg seemed to be slower than those in control group or those treated with bevacizumab 1mg/kg, as was shown in Figure 5.3 (g), but the difference was not significant and inconclusive, as what was expected from a single-dose study.

Comparison of tracer kinetic models

Correlation coefficients between microcirculatory parameters derived from the standard two-compartment model and the generalized kinetic model at each time point and combining all groups (control, treated with bevacizumab 1mg/kg, treated with bevacizumab 10mg/kg) were presented in Table 5.4. Correlation coefficients for the control group and the group treated with bevacizumab 10mg/kg were presented in Table 5.5 and 5.6, respectively. The correlation coefficients for the group treated with bevacizumab 1mg/kg was not shown as the number of samples ($n = 4$) were too small to render the data meaningful.

It was generally accepted that for tumors before treatment, K_{trans} reflected blood flow as the capillaries within these tumors were highly permeable. Combining all groups, K_{trans} showed a moderate correlation with F ($r = 0.389$, $p = 0.081$) and very strong correlation with v_1 ($r = 0.766$, $p < 0.001$) at baseline. However, there is a large variability of correlation coefficient values between K_{trans} and F in the control group ($r = 0.059$, $p = 0.887$) and the treated group ($r = 0.611$, $p = 0.118$). Caution is

Figure 5.4. Correlation between percentage of vessel area derived by CD34 staining with (a) blood volume as derived by the standard two-compartment model at Day 8, (b) blood volume as derived by the standard two-compartment model at Day 5, and (c) K_{trans} as derived by the generalized kinetic model.

Figure 5.5. Individual plots of blood volume in control group (blue) & 10 mg/kg bevacizumab group (red) at various days after dosing

advised in interpreting these correlation values in the control and treated groups as it may be affected by the relatively small number of samples in both groups.

Novel vascular targeting drugs could affect both permeability and blood flow through inhibition of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Therefore, after therapy, the question arose as to whether K_{trans} still measured flow or a combination of both flow and permeability. In the control group, there is no correlation between K_{trans} and PS at all time points. In the treated group, K_{trans} showed no correlation with PS ($r = 0.180$, $p = 0.672$) and moderate correlation with F ($r = 0.611$, $p = 0.118$) at baseline. This is again consistent with the fact that in untreated tumor, blood vessel permeability is high and K_{trans} reflects F in the regime of high permeability.

K_{trans} showed a stronger correlation with PS after treatment of bevacizumab 10mg/kg ($r = 0.542$, $p = 0.170$ at Day 1, $r = 0.929$, $p = 0.007$ at Day 5, and $r = 0.883$, $p = 0.033$ at Day 8). K_{trans} also showed a moderate correlation with F at Day 8 after treatment of bevacizumab 10mg/kg ($r = 0.677$, $p = 0.156$). It illustrated the difficulty of interpreting K_{trans} after treatment of anti-angiogenic agents as it might represent both flow and permeability.

Parameter	Time point	F	PS	v_1	v_2
K^{trans}	Baseline	0.389 (p = 0.081)	0.062 (p = 0.790)	0.766 (p < 0.001)*	0.286 (p = 0.209)
	Day 1	0.884 (p < 0.001)*	0.181 (p = 0.433)	0.892 (p < 0.001)*	0.335 (p = 0.138)
	Day 5	0.842 (p < 0.001)*	0.094 (p = 0.702)	0.919 (p < 0.001)*	-0.328 (p = 0.170)
	Day 8	0.788 (p < 0.001)*	0.485 (p = 0.067)	0.888 (p < 0.001)*	0.029 (p = 0.919)
v_e	Baseline	0.300 (p = 0.186)	0.566 (p = 0.009)*	-0.025 (p = 0.917)	0.631 (p = 0.003)*
	Day 1	0.638 (p = 0.002)*	0.535 (p = 0.014)*	0.791 (p < 0.001)*	0.758 (p < 0.001)*
	Day 5	0.356 (p = 0.135)	0.164 (p = 0.502)	0.481 (p = 0.039)*	0.548 (p = 0.015)*
	Day 8	0.404 (p = 0.137)	0.518 (p = 0.051)	0.593 (p = 0.022)*	0.843 (p < 0.001)*

Table 5.4. Correlation between K_{trans} , v_e , and F , PS , v_1 , and v_2 , at each time point, combining all groups (control, treated with bevacizumab 1mg/kg, treated with bevacizumab 10mg/kg). Cells with significant Spearman's correlation $r > 0.5$ and $p < 0.05$ are indicated by *.

Xolair (n = 9)	Time point	F	PS	v_1	v_2
K^{trans}	Baseline	0.059 (p = 0.887)	0.209 (p = 0.589)	0.770 (p = 0.019)*	0.184 (p = 0.634)
	Day 1	0.932 (p < 0.001)*	0.034 (p = 0.946)	0.441 (p = 0.239)	0.051 (p = 0.914)
	Day 5	0.333 (p = 0.385)	-0.167 (p = 0.678)	0.683 (p = 0.05)*	-0.083 (p = 0.843)
	Day 8	0.393 (p = 0.396)	0.214 (p = 0.662)	0.964 (p = 0.003)*	0.214 (p = 0.662)
v_e	Baseline	0.367 (p = 0.336)	0.200 (p = 0.613)	-0.033 (p = 0.948)	0.633 (p = 0.076)
	Day 1	0.133 (p = 0.744)	0.467 (p = 0.213)	0.500 (p = 0.178)	0.367 (p = 0.336)
	Day 5	0.400 (p = 0.291)	-0.333 (p = 0.385)	0.733 (p = 0.031)*	0.400 (p = 0.291)
	Day 8	0.500 (p = 0.267)	0.786 (p = 0.048)*	0.393 (p = 0.396)	0.929 (p = 0.007)*

Table 5.5. Correlation between K_{trans} , v_e , and F , PS , v_1 , and v_2 , at each time point for control mice. Cells with significant Spearman's correlation $r > 0.5$ and $p < 0.05$ are indicated by *, n = 7 at Day 8.

Bv 10 (n = 8)	Time point	F	PS	v_1	v_2
K^{trans}	Baseline	0.611 (p = 0.118)	0.180 (p = 0.672)	0.802 (p = 0.022)*	-0.120 (p = 0.779)
	Day 1	0.434 (p = 0.284)	0.542 (p = 0.170)	0.723 (p = 0.050)*	0.193 (p = 0.654)
	Day 5	0.536 (p = 0.236)	0.929 (p = 0.007)*	0.429 (p = 0.354)	-0.214 (p = 0.662)
	Day 8	0.677 (p = 0.156)	0.883 (p = 0.033)*	0.736 (p = 0.122)	0.795 (p = 0.067)
v_e	Baseline	0.000 (p = 1.000)	0.643 (p = 0.096)	0.000 (p = 1.000)	0.691 (p = 0.069)
	Day 1	0.405 (p = 0.327)	0.643 (p = 0.096)	0.810 (p = 0.022)*	0.833 (p = 0.015)*
	Day 5	0.107 (p = 0.840)	0.071 (p = 0.906)	0.250 (p = 0.595)	1.000 (p < 0.001)*
	Day 8	0.600 (p = 0.242)	0.543 (p = 0.297)	0.771 (p = 0.103)	1.000 (p = 0.003)*

Table 5.6. Correlation between K_{trans} , v_e , and F , PS , v_1 , and v_2 , at each time point for mice treated with bevacizumab 10mg/kg. n = 7 at Day 5 & n = 6 at Day 8.

5.4 Discussion

Switching point

A switching point could be appreciated in the blood flow time course graph of the characterization group in Figure 5.2 (a), as there was a sharp drop in blood flow between day 20 and 23 after implantation. The switch could also be observed in the blood volume and K_{trans} time course graphs at the same time point (Figure 5.2 (b), (e)). Further studies were needed to elucidate the biological processes responsible for this drop. As decrease in DCE-MRI measured blood volume and flow could be observed in mice without any treatment, DCE-MRI studies should be carefully designed in order to identify drug effects that also reduce blood flow and volume.

This switching point could also be seen as a drop in blood flow and blood volume found in the control group in the dose-dependent effect study at Day 8 after dosing, which was equivalent to 23 days after implantation and hence was consistent with the data found in the characterization group (Figures 5.3 (a), (b)). The correlation coefficient value between percentage of vessel area and blood volume at Day 8 after dosing ($r = 0.577$, $p = 0.003$, Figure 5.4 (a)), when the mice were sacrificed, was actually worse than the value at Day 5 ($r = 0.764$, $p < 0.001$, Figure 5.4 (b)), the immediate preceding time point. This is due to the low level of estimated blood volume by DCE-MRI in the control group at Day 8 after dosing, which approached similar values with the mice treated with bevacizumab (Figures 5.3 (b), 5.5).

Although the measured blood volume was similar for all groups at Day 8 after dosing, CD34 staining for vessels, as shown in Figure 5.6, actually showed that the control group has more stained vessels than the group treated with bevacizumab 10 mg/kg. While the low DCE-MRI reading for blood volume was related to vessels shut down in the treated group, we postulated that the low blood volume measured by DCE-MRI in the control group was related to increased proportions of non-perfused vessels possibly due to increase in interstitial fluid pressure. We postulated that this was the cause of the switch seen in the characterization study and the control group in the dose-dependent effect study. Caution then is advised for interpretation of DCE-MRI data post-switching point as it might be obscured by other biological processes driving the tracer kinetics inside the tumor, especially if no characterization study has been done before hand or if no sham controls were included.

It is also still to be seen whether this switching point hypothesis applies for all types of xenografts, as this study only dealt with one type of xenograft. In xenografts with switching point as seen in this study, DCE-MRI studies are best performed before the switching point to avoid this confounding effect. This also highlights the need of an alternative tracer kinetics method to analyze the DCE-MRI data post-switching point that considers the biological processes governing the new state of the tumor microcirculatory.

Figure 5.6. CD34 staining for control mice (above) and mice treated with bevacizumab 10 mg/kg (bottom) and their respective measured blood volume by DCE-MRI at Day 8 after dosing, which showed the inaccuracies of measurement of blood volume as derived by the DCE-MRI after the switching point.

Differential effect of bevacizumab

The trends of blood flow, blood volume, and K_{trans} illustrated the dose-dependent effects of bevacizumab (Figures 5.3 (a), (b), and (e)). A more gradual drop of blood volume was observed in group treated with bevacizumab 1mg/kg. On the other hand, a steep drop of blood volume was observed in group treated with bevacizumab 10mg/kg. It indicated faster effect and larger degree of vascular reduction in group treated with higher dose of bevacizumab. Significant reductions of blood volume and K_{trans} in group treated with lower dose of bevacizumab not at Day 1 after dosing, but only at Day 5 after dosing (Figures 5.3 (b) and (e)). As we have noted before, blood volume derived by the two-compartment model and K_{trans} correlated strongly with microvascular density (MVD) derived by CD34 staining (Figure 5.4). These results suggested potentials of this technique as a sensitive biomarker of MVD and as a reliable non-destructive testing method for anti-angiogenic agents like bevacizumab.

Concordance with known biology

VEGF expression results in increased numbers of leaky neovessels. It promotes the survival and mitosis of endothelial cells and increases mobilization of progenitor endothelial stem cells (82-83).

Antagonism of VEGF by bevacizumab reverses this effect. Lee et al found that bevacizumab decreases tumor vascular density and interstitial fluid pressure in human GBM and colonic carcinoma xenografts (84). Yuan et al visually observed the effects of bevacizumab on similar tumors grown in direct observation models and found that there is decrease in permeability to rhodamin labeled bovine albumin (85).

Willet et al found that a single infusion of the VEGF- specific antibody bevacizumab decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of viable, circulating endothelial and progenitor cells in rectal carcinoma patients (86). Our results are concordant with human data. We found that bevacizumab therapy results in decrease in microvascular density (MVD) histopathologically (Figure 5.7). There is a good correlation between the degree of MVD with the fractional intravascular volume as evaluated by the standard two-compartment model (Figure 5.4 (a)). There is also a good correlation between the degree of MVD with K_{trans} as evaluated by the generalized kinetic model as well (Figure 5.4 (c)).

Figure 5.7. Microvascular density (MVD) as measured by CD34 stainings for each treatment group. It showed that bevacizumab therapy resulted in decrease in MVD.

Tumor growth and biomarkers

Tumor growth might affect the interpretation of extravasation parameters such as v_2 and v_e . There was no difference found in trend of the interstitial space volume in control and treated groups. All of them showed the same trend of reduction in interstitial volume over time (Figure 5.3 (c) and (f)). It perhaps indicated the proliferation of tumor cells, which filled the interstitial space when the tumors grew.

Thus, even if the drug caused some change in the interstitial volume, it would be difficult to measure as it was confounded by this tumor growth factor.

Signature of normalization

It has been postulated that anti-angiogenic drugs may result in the normalization of blood vessels. However, these preclinical studies were performed with VEGF receptor antibodies, though these observations may theoretically be extrapolated to bevacizumab (87-88). Direct observation of decrease interstitial fluid pressure has been demonstrated with bevacizumab in humans (89). Willet et al found an increase in the fraction of vessels with pericyte coverage in human rectal cancer. In support of normalization, improvement of tumor drug concentration has been reported by Dickson et al and Yanagisawa et al in animal studies (90, 91).

There is no significant change in PS in the treated and control animals (Figure 5.3 (d)). This observation is similar to human studies (86, 89). Willet et al postulated that PS would increase, due to decrease in interstitial fluid pressure, but explained that they did not see a change because there was an overall decrease in absolute number of vessels, and hence the overall vessel surface area (86, 89). Hence, the permeability-surface area product would not change as an increase in permeability is balanced by a decrease in total surface area. Hence, though PS is an interesting parameter that can be derived from the standard two-compartment model, caution is advised in its interpretation as it may be affected by both morphological change in the capillary as well as the interstitial fluid pressure.

It also indicated that although this technique might be able to evaluate the anti-vascular effect of an anti-angiogenic agent (as reflected in the results of blood flow, blood volume, and K_{trans}), it might not be adequate to evaluate the normalization process (if any) inside the tumor.

6. Gadolinium diffusivity in mice xenografts⁵

6.1 Introduction

Histologically, microcirculation in a heterogeneous tumor may be broadly divided into two or more overlapping zones, which could include a necrotic zone, semi-necrotic zone, stabilized tumor circulation, an advancing tumor front or normal tissue (92). On dynamic contrast-enhanced (DCE) MRI scans, a heterogeneous tumor may consist of voxels, which exhibit a spectrum of tracer uptake behaviors, such as a rapid increase followed by washout, an initial increase followed by a plateau or progressively increasing trends. Tracer kinetic analyses by compartmental modeling have provided insights into the differences in tumor microvasculature that could result in these patterns (25, 32).

However, fitting of tracer uptake curves by compartmental models often fails in necrotic regions of the tumor, which exhibit delayed enhancement curves that increase very slowly and almost linearly with time (93-94). It has been shown that fitting of such enhancement curves associated with the necrotic voxels yield un-physiological values for the kinetic parameters, which should be excluded from subsequent statistical analysis of the kinetic parameters (93-94). As the necrotic regions of the tumor are devoid of functional capillaries and effective blood flow, the late arrival and slow accumulation of tracer in necrotic tissues could be indicative of a mechanism of transport that differs from the viable tissues. In this study, we explore an alternative approach for analyzing DCE MRI data of such necrotic regions based on the diffusional transport of solutes in the tumor interstitium (95).

6.2 Materials and Methods

6.2.1 Theory

The movement of solute molecules in the interstitium can be driven by concentration gradients (diffusion) and pressure gradients (convective motion of interstitial fluid) (95). In normal tissues, it is believed that convection is the dominant mechanism for interstitial transport of large molecules, whereby fluid movement from the capillaries to lymphatic channels within the interstitium is driven by pressure differences between these structures (95-96). However, most tumors lack anatomically well-defined lymphatic vessels and poor lymphatic drainage leads to an accumulation of fluid and

⁵ Parts of this study were published in Koh T. S., Hartono S., Thng C. H., and Ng Q. S. In vivo Measurement of Gadolinium Diffusivity by Dynamic Contrast-Enhanced MRI: A Preclinical Study using Human Xenografts, *Magnetic Resonance in Medicine* (2013) 69(1):269-76.

increase in interstitial pressure, which reduces the difference in pressure between the capillaries and interstitial space.

Consequently, convective transport can be hindered in the tumor interstitium, especially in central regions of the tumor that exhibited elevated interstitial fluid pressure. In such situations, diffusion may become the dominant mechanism of transport in the interstitial space (95-96). Consider a tumor with a central necrotic core surrounded by gradually more perfused tissues that are in turn enclosed by an outer rim of viable blood vessels. On intravenous injection of a gadolinium contrast agent, the gadolinium tracer delivered to the tumor outer rim by blood flow can diffuse into the interstitial space and establishes a tracer concentration gradient between the outer and central regions of the tumor interstitium. The diffusion of gadolinium tracer across this spatial concentration gradient within the tumor interstitium can be described by Fick's diffusion equation (95-97)

$$\frac{\partial}{\partial t} C = D_{Gd} \nabla^2 C \quad (6.1)$$

where C denotes tracer concentration, D_{Gd} refers to the tracer diffusivity or diffusion constant, and ∇^2 denotes the spatial Laplacian operator. Equation 1a can be derived from the continuity equation (97), which states that the rate of change in concentration is given by the influx and outflux of tracer:

$$\frac{\partial}{\partial t} C + \nabla \cdot J = 0 \quad (6.2)$$

where J is the diffusive flux (mass of tracer per area per time) and ∇ denotes the del operator. Next, we consider Fick's law (97):

$$J = -D_{Gd} \nabla C \quad (6.3)$$

which assumes that the tracer flux J is proportional to the concentration gradient and moves from regions of high concentration toward regions of low concentration. The proportionality constant is given by the tracer diffusivity D_{Gd} , which is assumed to be the same in all directions. Substituting Eq. 6.3 into Eq. 6.2, and by further assuming that D_{Gd} is independent of position and concentration, we arrive at Eq. 6.1.

If the mechanism of delayed and slow tracer uptake within the necrotic core is predominantly due to diffusion (driven by surrounding spatial concentration gradients), Eq. 6.1 states that the slope of the tissue concentration curve with respect to time would be directly proportional to its spatial Laplacian. Therefore, corresponding to the portion of the slow uptake curve that increases linearly with time, Fick's equation predicts that the Laplacian would remain at a relatively constant value, which can be readily verified on DCE MRI data of advanced tumors with poorly perfused or necrotic cores.

6.2.2 Mice

Male NOD mice ($n = 24$, 6 weeks old, 30 ± 2 g) with severe combined immunodeficiency were used as host animals for various types of human cancer xenografts. Details of the xenograft model and the formation of cancer cell lines can be found in Ref. 57. For each mouse, two tumors of the same cancer cell line were implanted at the flanks (one tumor at each flank). This study was approved by the Institutional Animal Care and Use Committee. Sterilized food and water were provided ad libitum and the mice were housed in ventilated cages with oxygen and at constant temperature and humidity.

During MR scanning, the mice were kept under 1–2% isoflurane anesthesia. Mouse core body temperature was monitored with a rectal probe and maintained at $37 \pm 0.3^\circ\text{C}$ by a feedback-controlled warm-air heating system.

About 6 weeks after implantation, the mice were subjected to DCE-MRI before being sacrificed.

6.2.3 DCE-MRI

The DCE-MRI protocol used in this study has been explained earlier in Chapter 3.2.2.

6.2.4 Histopathology

The tumors were harvested and processed using routine formalin fixed paraffin embedding protocols. Sections were cut at 5-mm-thickness and stained with hematoxylin and eosin (HE). CD31 (Dilution 1:50, Dako, Mo823, JC70A) immunohistochemical staining was performed using a Leica BondMax automated machine (Leica Microsystems) with Epitope Retrieval solution 2. All samples were read by a pathologist who recorded the tumor type, areas of necrosis, cystic change and distribution of CD31 staining. Digital images of the sections were obtained using a high-resolution scanner (ScanScope XT; Aperio) at 40x magnification.

6.2.5 Data Processing

Post-processing was performed off-line on an Intel® Core™2 Duo personal computer with Matlab™ (MathWorks, Natick, MA). For reduced inflow effects and warp, only one of the four central slices from the imaging volume (of eight slices) was selected for processing. For each mouse, a region-of-interest (ROI) consisting of the larger tumor was outlined on one of the central slices, which clearly revealed a poorly perfused core on the DCE scans.

The FLASH signal S can be written in a linear form (98)

$$\frac{S}{\sin \alpha} = \frac{S}{\tan \alpha} E_1 + M_0(1 - E_1) \quad (6.4)$$

where α denotes flip angle, $E_1 = \exp(-TR/T_1)$ and M_0 is a factor that accounts for machine gain and proton density. By plotting $S/\sin \alpha$ against $S/\tan \alpha$ for the various flip angles (2° to 18°) acquired in the precontrast scans, native T_1 (T_1^0) and M_0 values for each voxel can be estimated from the slope and ordinate intercept of the linear plot. Assuming that M_0 for each voxel remains unchanged with the arrival of the contrast agent, postcontrast T_1 (T_1^C) being the only unknown in S can be estimated at each dynamic postcontrast scan. An additional 10 dynamic baseline scans (with 6° and 14°) were executed before the injection of contrast agent to check that T_1^0 remains consistent at baseline before the arrival of contrast agent.

Gadolinium concentration C in each voxel was estimated by the change in longitudinal relaxation rates

$$r_1 C = \frac{1}{T_1^C} - \frac{1}{T_1^0} = \Delta R_1 \quad (6.5)$$

where r_1 denotes the longitudinal relaxivity. The linear relationship between C and the change in longitudinal relaxation rate ΔR_1 was validated in phantom experiments for low gadolinium concentrations (< 0.5 mM) corresponding to $\Delta R_1 < \sim 17 \times 10^{-4} \text{ ms}^{-1}$. Although Eq. 6.5 may be used to estimate concentrations in the poorly and moderately perfused tissue regions, the linear relationship may not hold for higher concentrations reached in the highly perfused regions and blood pools due to strong susceptibility effects at 7 T. Corresponding to each imaging slice, a 3D dataset of $C(x,y,t)$ consisting of two spatial coordinates (x,y) and a time coordinate (t) can be obtained.

From an image processing perspective, the Laplacian (in Eq. 6.1) is a derivative operator that highlights discontinuities (edges) in the image and de-emphasizes regions with slowly varying values (99). The two-dimensional Laplacian can be approximated by the discrete second-order derivative in the x - and y -directions, as

$$\nabla^2 C(x,y) = \frac{[C(x+1,y)+C(x-1,y)+C(x,y+1)+C(x,y-1)]-4C(x,y)}{\Delta^2} \quad (6.6)$$

where Δ denotes the in-plane voxel dimension, which is equal in the x - and y -directions in the present case. A discrete approximation of the Laplacian can be implemented using the convolution mask

$$M_{xy} = \frac{1}{\Delta^2} \begin{pmatrix} 0 & 1 & 0 \\ 1 & -4 & 1 \\ 0 & 1 & 0 \end{pmatrix} \quad (6.7)$$

Contributions from the diagonal directions can be further incorporated into the discrete approximation of the Laplacian by assuming isotropic weights with the convolution mask (99)

$$M_{Diag} = \frac{1}{\Delta^2} \begin{pmatrix} 1 & 1 & 1 \\ 1 & -8 & 1 \\ 1 & 1 & 1 \end{pmatrix} \quad (6.8)$$

Although Eq. 6.8 is commonly used for simplicity (99), more accurate estimates of the Laplacian can be obtained by accounting for the differences in weights due to the diagonal distance. For each time point t_i , M_{Diag} was applied on $C(x,y,t_i)$ to estimate $\nabla^2 C(x,y,t_i)$, which again yielded a 3D dataset of $\nabla^2 C(x,y,t_i)$ for each imaging slice.

Instead of manually stepping through each voxel within the tumor ROI to identify necrotic voxels with delayed and slow contrast uptake curves, we used a semiautomated k-means clustering algorithm commonly used in the analysis of signal-time behavior in DCE MRI datasets (100-101). Tumor voxels on the postcontrast MR images were classified into subsets (clusters) by the clustering algorithm such that each subset consists of voxels that exhibit a similar contrast enhancement behavior. As an input to the k-means clustering algorithm, the number of distinct clusters within each tumor was initially stipulated as 10, which could result in some of the output clusters having similar contrast enhancement patterns. As our aim was to isolate the cluster of necrotic voxels with delayed and slow enhancement, stipulating a larger number of initial clusters (10 in this case) would allow the clustering algorithm more flexibility in separating the necrotic voxels from the other enhancement patterns. If a small number of initial clusters were used, the algorithm may end up mixing the necrotic voxels with other voxels exhibiting progressively increasing trends. Once the necrotic voxels were identified, the other clusters with similar enhancement patterns can be manually combined into fewer larger clusters.

As the necrotic voxels were associated with low signal-to-noise ratios, the median contrast concentration at each time point was used to derive a representative concentration–time curve $C_{\text{slow}}(t)$ for the necrotic cluster, as the median is less sensitive to outliers than the mean. Similarly, the median value of $\nabla^2 C_{\text{slow}}$ in the necrotic cluster as a function of time was computed. To explore whether contrast uptake in the necrotic cluster follows Fick’s equation, we may check whether the linear portion of $C_{\text{slow}}(t)$ corresponds to a constant value of its Laplacian $\nabla^2 C_{\text{slow}}$. Further, if Fick’s equation could account for the behavior of contrast uptake within the necrotic core, then a simple and robust estimate of the gadolinium diffusivity constant D_{Gd} in vivo can be given by the ratio of the slope of $C_{\text{slow}}(t)$ and the constant value of $\nabla^2 C_{\text{slow}}$ (Eq. 6.1).

6.3 Results

Tumors extracted from all 14 mice were found to develop necrosis on histological examination. An example of a gastric cancer xenograft is shown in Figures 6.1 and 6.2. As T_1 and gadolinium concentration were estimated at every post-contrast imaging time point, only the maps of their median

values over all time points are shown in Figure 6.1(c) and (d), respectively. Comparison of the pre- and post-contrast T_1 maps show that post-contrast T_1 values in the surrounding tissues decrease with the arrival of gadolinium while T_1 values for the central regions remain high due to the lack of gadolinium delivery. Correspondingly, estimated median gadolinium concentrations in the central regions remain low (Fig.6.1d) due to late and very slow contrast uptake.

Histological sections of the larger (left) tumor in Figure 6.1 are shown in Figure 6.2 with HE (a) and CD31 (b) staining. Necrotic regions of the tumor are identified and outlined (green) on the HE section (Fig. 6.2a) by a pathologist. Comparison of Figures 6.1 and Fig. 6.2b (CD31 staining) show that areas of higher signal intensity on the MR scans corresponded with increased CD31 staining in the tumor periphery, while the central loss of signal intensity correlated with lack of CD31 staining in the central zone of the tumor.

Within the tumor, voxel locations that are associated with different contrast uptake patterns are depicted in Figure 6.2(c) and color-mapped to their respective median concentration-time patterns in Figure 6.2(d). The central region of the tumor (black) exhibited a delayed and slow increase in contrast concentration $C_{\text{slow}}(t)$ that is almost linear in time towards the later part of the curve from about 130s to 260s after contrast injection. As illustrated in Figure 6.2(e), the linear portion of $C_{\text{slow}}(t)$ corresponds to a period of time when the Laplacian $\nabla^2 C_{\text{slow}}$ flattens and remained relatively constant. This behavior is consistent with Fick's equation and supports the hypothesis that diffusion might be the dominant mechanism of transport in the central necrotic regions. In vivo gadolinium diffusivity D_{Gd} was estimated to be $3.44 (=0.0157/45.59) \cdot 10^{-4} \text{mm}^2/\text{s}$ in this case. The coefficient of determination R^2 for the linear regression fit of $C_{\text{slow}}(t)$ was 0.92 and the F -test indicated acceptance of linearity with a p -value < 0.001. Another example of estimating D_{Gd} for a xenograft of hepatocellular carcinoma is shown in Figure 6.3. Consistently in the 14 study cases, $C_{\text{slow}}(t)$ in the necrotic regions exhibited a linear behavior with time from 130s to 260s post-contrast, with R^2 for the linear fits ranging from 0.84 to 0.96 and p -values < 0.001 for the F -test.

Values of D_{Gd} estimated for the 14 study cases of various cancer xenografts are plotted against their respective in-plane tumor sizes in Figure 6.4. These values of D_{Gd} range from 0.99 to $3.44 \cdot 10^{-4} \text{mm}^2/\text{s}$ with a mean ($\pm$ standard deviation) of $2.08 (\pm 0.88) \cdot 10^{-4} \text{mm}^2/\text{s}$. Spearman correlation analysis (two-tailed) yielded correlation coefficient $r = 0.50$ and p -value = 0.07, indicating a marginal positive correlation between D_{Gd} and tumor size, which is statistically not significant.

Figure 6.1. Example of a mouse with gastric cancer xenografts on both flanks. Outline of the larger tumor on the left flank is shown in (a). Comparison of the pre-contrast T_1 (b) and median post-contrast T_1 (c) maps show that the central regions are poorly supplied with the gadolinium tracer, such that post-contrast T_1 values in the central voxels remain high after contrast arrival in the surrounding tissue. Correspondingly, the estimated median tracer concentrations in the central voxels are low (d).

Figure 6.2. Histological sections of the left tumor (in Fig. 1) with HE and CD31 staining are shown in (a) and (b), respectively. (a) The low power picture shows a rim inked in green which corresponds to necrosis on histology. A magnified view (20X) in the central region clearly reveals areas of complete necrosis which appear as pinkish amorphous zones. (b) There is a peripheral rim of increased CD31 density which indicates the presence of blood vessels in the periphery. However, the central area shows patchy loss of staining which corresponds to the lack of vascular network. (c) Tumor voxels were clustered into subsets according to their contrast enhancement patterns and their median concentration-time curves are shown in (d). Each colored location in (c) corresponds to the concentration-time curve of the same color in (d). The central necrotic region (black) exhibited a delayed and slow uptake in concentration $C_{\text{slow}}(t)$ that subsequently increases linearly with time. $C_{\text{slow}}(t)$ is magnified and shown together with the median Laplacian $\nabla^2 C_{\text{slow}}$ in (e). The linear portion of $C_{\text{slow}}(t)$ corresponds to a nearly constant value of $\nabla^2 C_{\text{slow}}$, in accordance with Fick's law. Dividing the slope of $C_{\text{slow}}(t)$ and the average value of $\nabla^2 C_{\text{slow}}$ yields a gadolinium diffusivity D_{Gd} of $3.44 (=0.0157/45.59) \cdot 10^{-4} \text{mm}^2/\text{s}$. Coefficient of determination R^2 for the linear regression fit of $C_{\text{slow}}(t)$ was 0.92 and the F -test for linearity yielded a p -value < 0.001 .

Figure 6.3. Example of a study case with hepatocellular carcinoma xenograft. (a) The central and lateral areas marked in green show coagulative necrosis with cystic degeneration. In this case, although there is cell death (cytoplasm and nuclei breakdown), the overall structure of the tissue remains like a scaffolding. Areas of cystic degeneration are shown in the magnified view (20X) where the necrotic material has undergone further ingestion by histiocytes and has dissolved to leave a void. (b) CD31 reveals a rim of strong staining in the periphery with the central areas showing a lower staining intensity (marked in green). This corresponds to decreased signal intensity on the MR scans. Each colored location in (c) corresponds to the concentration-time curve of the same color shown in (d). In (e), the estimated D_{Gd} was $2.27 \times 10^{-4} \text{ mm}^2/\text{s}$, R^2 for the linear regression fit of $C_{\text{slow}}(t)$ was 0.96 and the F -test yielded a p -value < 0.001 .

6.4 Discussion

Analysis of DCE MRI data is usually performed using compartmental tracer kinetic models which describe microcirculation of perfused tissues and yield quantitative parameters pertaining to blood flow, capillary permeability, fractional intravascular volume and interstitial volume (25, 32). However, these models might not adequately account for tracer transport in the necrotic tumor cores that are poorly perfused (93-94). In tumors with high interstitial fluid pressure, it has been postulated that convective transport in the tumor interstitium is negligible and the movement of solutes is predominantly driven by diffusion along concentration gradients (95-96). In this study, we found that DCE MRI data of necrotic tumor regions can be modeled using Fick's equation for diffusion, yielding in vivo estimates of gadolinium diffusivity D_{Gd} as a parameter for tracer transport.

Figure 6.4. Gadolinium diffusivity D_{Gd} estimated for various cancer xenografts are plotted against their respective in-plane tumor sizes estimated on MR scans. Spearman correlation analysis (two-tailed) yielded $r=0.5$ and $p\text{-value}=0.07$.

In contrast to compartmental tracer kinetic models, the application of Fick's equation does not require the sampling of an arterial input function as the forcing function because the movement of tracer into the central necrotic regions of the tumor is driven by the surrounding spatial concentration gradients instead of blood flow. Tracer delivered to the peripheral regions of the tumor by blood flow diffuses into the interstitial space and establishes a concentration gradient between the peripheral and central interstitial regions of the tumor. Because tracer concentration is initially higher in the peripheral interstitial regions than the central region, spatial differences in concentration between these regions constitute the 'source' that drives tracer flux towards the central region. Examples of tracer concentration-time profiles in the surrounding regions of the necrotic core are depicted in Figure 6.2(d) and Figure 6.3(d). In regions at the immediate vicinity of the necrotic core (i.e., regions colored green and magenta in Figure 6.2(c) and Figure 6.3(c)), the concentration-time curves somewhat plateau after about 130s (see the corresponding green and magenta concentration-time curves in Figure 6.2(d) and Figure 6.3(d)), and only increase slightly till the end of the imaging experiment at 260s after contrast injection. These concentration-time curves estimated by DCE MRI depict the boundary conditions for diffusion of tracer into the necrotic core.

Rofstad and co-workers (93-94) showed by comparison with histological sections that Gd-DTPA concentration increased slowly and nearly linearly with time in voxels consisting primarily of necrotic tissue, which is consistent with the behavior of $C_{\text{slow}}(t)$ reported here. They further showed that fitting Tofts model to such concentration-time curves yielded either very high values for interstitial volume v_e (>1), or very low v_e values (<0.01) (94). However, voxels in viable tissue showed physiological v_e values, usually $0.05 < v_e < 0.8$ (94). They suggested that such threshold values for v_e can be determined by establishing v_e frequency distributions for tumor regions consisting exclusively of viable tissue. Thus, necrotic voxels yielding unphysiological values for v_e outside these threshold values can be identified and excluded from subsequent statistical analysis of tracer kinetic parameters. Such voxels which exhibit $C_{\text{slow}}(t)$ and fail model-fitting can be further analyzed using Fick's equation to yield DGd as a tracer transport parameter.

It is important to make a distinction between gadolinium diffusivity and apparent diffusion coefficient (ADC) of water as quantified by diffusion weighted imaging (DWI). DWI and ADC are useful biomarkers of tumor response based on the hypothesis that tumor cells would initially swell (restricting water diffusion) and subsequently decrease in volume with a resultant increase in interstitial space (improving water diffusion) (102). Gadolinium diffusivity refers to the diffusion properties of the gadolinium tracer and not the water molecules, and interrogates a process of gadolinium delivery in the tumor interstitium distinct from water diffusion revealed by DWI. The diffusivity of a solute in the tissue interstitium can be affected by factors such as its molecular weight, configuration, charge, binding, as well as the physiochemical properties of the interstitial matrix (95).

Phantom studies of Gd-DTPA diffusivity in a tissue mimic material (polyvinyl alcohol-cryogel) have been previously performed using MRI (103). Using a fast inversion-recovery spin-echo sequence, Gordon et al (103) estimated Gd-DTPA concentration by identifying regions of MR signal null corresponding to a particular choice of inversion time (which is related to T1 and hence concentration). Because simple boundary and initial conditions can be specified for the phantom geometry in a single spatial dimension, Fick's equation can be solved analytically in one dimension and fitted to the concentration data to yield estimates for diffusivity. The diffusivity of Gd-DTPA through tissue mimic material was estimated to be $2.6 \pm 0.04 \times 10^{-4} \text{ mm}^2/\text{s}$ (103). Considering that Gd-DTPA and INN are of similar molecular weights (590 and 560 Da, respectively), the present estimates of $2.08 (\pm 0.88) 10^{-4} \text{ mm}^2/\text{s}$ for INN diffusivity in various tumors are in good agreement with those estimated by Gordon et al (103).

Measurements of macromolecule diffusivity through the extracellular matrix of tumor tissues have been performed using an optical microscopy approach called fluorescence recovery after photobleaching (FRAP) (101). The technique of FRAP is based on the movement of a fluorescent marker into a tissue region containing the same marker that has been previously rendered non-

fluorescent by photobleaching. The acquired FRAP images can be analyzed using Fourier transform to yield the characteristic diffusion coefficient of the fluorescent molecule, typically fluorescently labeled bovine serum albumin and nonspecific mouse immunoglobulin. In vivo measurements of diffusion coefficients using FRAP have been performed in tumors implanted in the dorsal skinfold chamber of SCID mice (104). However, this technique might not be directly translatable to humans and the in vivo measurement of diffusion coefficient in patients by FRAP is currently not feasible (104).

The present approach of applying Fick's equation with DCE MRI allows an alternative MRI technique for measurement of tracer diffusivity in tumors, which is translatable to the clinic. As gadolinium diffusivity is an indicator of the transport of gadolinium tracer to necrotic regions of high interstitial pressure within the tumor, it can serve as a surrogate indicator of drug delivery into these regions for anti-cancer drugs of a similar molecular size, charge and other relevant properties. Drug delivery is an important factor in determining the efficacy of anti-cancer therapy. Drug failure could be attributed to inefficient delivery of drug to the tumor or poor drug biological effects. Non-invasive monitoring of tumor drug transport with gadolinium diffusivity could be a useful tool for studying drug failure, by providing further information on whether drug failure could be due to poor delivery.

The present methodology can potentially be applied to other imaging modalities such as DCE computed tomography (CT) or positron emission tomography (PET), to study tracer delivery. For PET imaging, labeling the drug with an appropriate imaging tracer could allow drug delivery to be directly studied. In DCE CT, tracer concentration is directly proportional to CT attenuation (i.e CT number in Hounsfield units) and the present methodology of estimating tracer diffusivity would be directly applicable. For DCE CT datasets with high signal-to-noise ratios, it might be possible to calculate the concentration gradients and Laplacian reliably at the voxel level and map tracer diffusivity within the tumor.

The current DCE MRI experiments interrogated tracer distribution in the poorly perfused tissues over a period of less than 5 minutes. It is conceivable that if the experiments had interrogated the system over a longer period, more voxels could have exhibited a plateau in their concentration-time curves and hence allowed for fitting with other kinetic models. Further studies with a longer examination period are required to find the maximum time window for plateau and fitting of other kinetic models.

A limitation of the present methodology is that it is only applicable to poorly perfused tumor regions where interstitial convective transport is negligible. Also, it may not be useful in areas of moderate interstitial fluid pressure where both diffusive and convective transport processes are possible. However, gadolinium diffusivity may serve as an alternative interstitial transport parameter in

necrotic tumor regions and complement the other microcirculatory parameters derived from DCE MRI for a more complete analysis of the entire tumor.

7. Conclusions

Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) with tracer kinetic modeling has been proposed as a biomarker for assessment of tumor angiogenesis in clinical trials. In this study, we have successfully elucidated the feasibility of applying high temporal and spatial resolution DCE-MRI technique in preclinical settings and beyond.

In the phase I trial of ABT-869, we have found that all three tracer kinetics methods (generalized kinetic model, distributed parameter model, and initial area under tumor tracer curve method) yielded feasible biomarkers for assessing the effects of anti-angiogenic therapy. $IAUC_{norm}$, v_e , v_l and PS correlated with drug exposure, while v_e and PS were predictive of late progression. Analysis by the distributed parameter model offers the possibility to separately evaluate blood flow and capillary permeability in tumors. The parameters v_e and PS can complement the predictive value of K^{trans} and $IAUC_{norm}$ which have been shown to be useful in other drug trials.

Translating these findings into animal studies, we have shown that the microcirculatory parameters derived from the standard two-compartmental model correlated with the values derived from immunohistochemistry staining. It indicated the reliability of the proposed technique and potentials of applying this technique as a biomarker for pre-clinical drug developments, as it might allow monitoring of anti-angiogenic therapy in murine models.

We have also illustrated the feasibility of utilizing DCE-MRI as a biomarker for normal mouse liver. We observed a low value of interstitial space volume fraction and permeability-surface area product in normal mouse liver, which corresponded with murine liver physiology and the results previously obtained in the normal human liver studies. It indicates potential for applying this technique for further studies in cirrhotic mice liver, to investigate whether DCE-MRI could be used as a biomarker for cirrhosis characterization.

We have shown that a hepatocellular carcinoma (HCC) xenograft would reach a switching point where there would be a significant and sharp drop in blood volume presumably related to increase in interstitial fluid pressure and decrease in perfused vessels. We concluded that a DCE-MRI characterization study should be performed for each type of xenograft in order to understand the baseline perfusion characteristics of the xenograft before any drug treatment. In studies with HCC xenograft, DCE-MRI are best performed before the switching point to avoid this confounding effect. Caution is advised for interpretation of DCE-MRI results in these studies, especially if no characterization study has been done before hand or if no sham controls were included.

In a study of bevacizumab, an anti-angiogenic agent, in murine models, we have also showed that the effects of bevacizumab are dose-related. The results suggested potential of DCE MRI as a sensitive biomarker of microvascular density. This facilitates translation and clinical application to early time points after anti-angiogenic therapy. This study also validated DCE-MRI as a reliable non-destructive testing method for time course observation of MVD changes in animals and facilitates future experiments to study drug resistance. We have demonstrated viability of using this model for pre-clinical drug developments, i.e., to assess drug efficacy and plan for treatment, as the results obtained with DCE-MRI strongly correlated with the blood vessel staining.

Finally, we have developed a new technique to analyze DCE-MRI data particularly in necrotic regions. We proposed that gadolinium diffusivity might serve as an alternative interstitial transport parameter in necrotic tumor regions and complement the other microcirculatory parameters derived from DCE MRI for a more complete analysis of the entire tumor.

List of Publications

1. Koh T. S., Thng C. H., Lee P. S., **Hartono S.**, Rumpel H., Goh B. C. and Bisdas S. Hepatic Metastasis: In vivo assessment of perfusion parameters in hepatic metastases with dynamic contrast-enhanced MRI with dual-input two-compartment tracer kinetics model, *Radiology* (2008) 249:307-20.
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8. **Hartono S.**, Thng C. H., Tan C. H., Thian Y. L., Yong C. X., Yang C. T., Huynh T. H., Chuang K. H., and Koh T. S., Dynamic Contrast-Enhanced MRI of Mouse Liver: A Feasibility Study using a Dual-Input Two-Compartment Tracer Kinetic Model. Submitted for publication in *Journal of Biomedical Science and Engineering*.
9. **Hartono S.**, Thng C. H., Ong R. W. J., Ng Q. S., Lim T. K. H., Yu S. W. K., Chuang K. H., Huynh T. H., Martarello L., and Koh T. S., Validation of Microcirculatory Parameters Derived from the Standard Two-Compartment Compartment Model with Murine Xenografts Model. Submitted for publication in *Applied Magnetic Resonance*.
10. **Hartono S.**, Koh T. S., Ng Q. S., Ong R. W. J., Huynh T. H., Martarello L., and Thng C. H., Value of conventional compartment DCE MRI modeling in demonstrating microvascular changes of human xenografts treated by Bevacizumab. Submitted for publication in *Journal of Cancer Research*.

Conference Proceedings/Posters

1. **Hartono S.**, Koh T. S., Zhou L., Ng Q. S., Lee P. S., Chuang K. H., Dan Y. Y., Martarello L., and Thng C. H. Dynamic Contrast-Enhanced MRI of Mouse Cirrhotic Liver: A Pilot Study, *Proceedings of International Society of Magnetic Resonance in Medicine* 2013;21:2220. Salt Lake City, UT.
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Other Publications

1. Koh T. S., Thng C. H., **Hartono S.**, Choo S. P., Ng Q. S., Khoo J. B. K., Bisdas S., and Koh D-M., Deconvolution Assessment of Splenic and Splanchnic Contributions to Portal Venous Blood Flow in Liver Cirrhosis, *Medical Physics* (2011) 38:2768-2782.
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1. Thng C. H., Koh T. S., **Hartono S.**, Lee P. S., Miyazaki K., Collins D., Leach M. O., Lewington V., and Koh D-M., Contrast uptake enhancement patterns in neuroendocrine liver metastases, *ISMRM Annual Meeting 2010*, Stockholm, Sweden.
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