

Basic Study

Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver cancers

Ye-Wei Liu, Frederik De Keyzer, Yuan-Bo Feng, Feng Chen, Shao-Li Song, Johan Swinnen, Guy Bormans, Raymond Oyen, Gang Huang, Yi-Cheng Ni

Ye-Wei Liu, Gang Huang, Shanghai Key Laboratory for Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai 201318, China

Ye-Wei Liu, Frederik De Keyzer, Yuan-Bo Feng, Feng Chen, Johan Swinnen, Guy Bormans, Raymond Oyen, Yi-Cheng Ni, Biomedical Group, Campus Gasthuisberg, KU Leuven, Leuven 3000, Belgium

Ye-Wei Liu, Shao-Li Song, Gang Huang, Institute of Clinical Nuclear Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

Ye-Wei Liu, Gang Huang, Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine (SJTUSM) & Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), Shanghai 200025, China

ORCID number: Ye-Wei Liu (0000-0002-4360-0132); Frederik De Keyzer (0000-0002-1855-3274); Yuan-Bo Feng (0000-0002-4235-3491); Feng Chen (0000-0001-8490-0054); Shao-Li Song (0000-0003-2544-7522); Johan Swinnen (0000-0002-7720-5077); Guy Bormans (0000-0002-0335-7190); Raymond Oyen (0000-0003-2256-5910); Gang Huang (0000-0003-1939-3978); Yi-Cheng Ni (0000-0002-8553-7565).

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Correspondence to: Yi-Cheng Ni, MD, PhD, Full Professor, Theragnostic Laboratory, Department of Imaging and Pathology, Biomedical Group, KU Leuven, Herestraat 49, Leuven 3000, Belgium. yicheng.ni@kuleuven.be
Telephone: +32-16-330165
Fax: +32-16-343765

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Abstract**AIM**

To compare therapeutic responses of a vascular-

disrupting-agent, combretastatin-A4-phosphate (CA4P), among hepatocellular carcinomas (HCCs) and implanted rhabdomyosarcoma (R1) in the same rats by magnetic-resonance-imaging (MRI), microangiography and histopathology.

METHODS

Thirty-six HCCs were created by diethylnitrosamine gavage in 14 rats that were also intrahepatically implanted with one R1 per rat as monitored by T2-/T1-weighted images (T2WI/T1WI) on a 3.0T clinical MRI-scanner. Vascular response and tumoral necrosis were detected by dynamic contrast-enhanced (DCE-) and CE-MRI before, 1 h after and 12 h after CA4P iv at 10 mg/kg (treatment group $n = 7$) or phosphate-buffered saline at 1.0 mL/kg (control group $n = 7$). Tumor blood supply was calculated by a semiquantitative DCE parameter of area under the time signal intensity curve (AUC30). *In vivo* MRI findings were verified by postmortem techniques.

RESULTS

On CE-T1WIs, unlike the negative response in all tumors of control animals, in treatment group CA4P caused rapid extensive vascular shutdown in all R1-tumors, but mildly or spottily in HCCs at 1 h. Consequently, tumor necrosis occurred massively in R1-tumors but patchily in HCCs at 12 h. AUC30 revealed vascular closure (66%) in R1-tumors at 1 h ($P < 0.05$), followed by further perfusion decrease at 12 h ($P < 0.01$), while less significant vascular clogging occurred in HCCs. Histomorphologically, CA4P induced more extensive necrosis in R1-tumors (92.6%) than in HCCs (50.2%) ($P < 0.01$); tumor vascularity heterogeneously scored +~+++ in HCCs but homogeneously scored ++ in R1-tumors.

CONCLUSION

This study suggests superior performance of CA4P in metastatic over primary liver cancers, which could guide future clinical applications of vascular-disrupting-agents.

Key words: Hepatocellular carcinoma; Combretastatin A4 phosphate; Rhabdomyosarcoma; Vascular-disrupting agent; Magnetic resonance imaging; Rats

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Core tip: Complex animal models combining primary and secondary liver malignancies proved feasible in rats. The therapeutic efficacy of the leading vascular disrupting agent combretastatin-A4-phosphate (CA4P) could be intra-individually compared between primary and secondary liver malignancies in the same cirrhotic rats. Clinical 3.0T magnetic resonance imaging allowed real-time monitoring of *in vivo* therapeutic responses within 12 h, and *ex vivo* microangiography and histopathology could validate the CA4P-induced tumoricidal effects. The therapeutic responses appeared

superior with secondary liver tumors over that with primary hepatocellular carcinomas, which are of translational significance for planning future clinical trials of CA4P in cancer patients.

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INTRODUCTION

As a first vascular disrupting agent (VDA), combretastatin-A4-phosphate (CA4P) targets the cytoskeletal tubulin of abnormal tumor endothelial cells, leading to a rapid but often reversible vascular occlusion^[1-3]. Theoretically, this may cause ischemic tumor necrosis by depriving malignant cells from the blood supply^[1-3]. Clinically, CA4P has been undergoing phase II/III trials in the setting of ovarian, thyroid and lung cancers alone or in combination with other chemotherapeutic agents^[4-6], and a good safety profile has also been shown in the first phase I clinical trial among a Chinese patient population^[7]. In the majority of transplanted tumor models, CA4P consistently induced massive central tumor necrosis, leaving only a few layers of peripheral viable tumor cells culpable for the incomplete treatment and cancer relapse^[8,9], which is also attributed to the unsatisfactory clinical outcomes^[3]. To tackle this bottleneck problem with all VDAs, a plausible solution has been proposed^[10].

On the other hand, diverse and paradoxical tumor responses to CA4P have been recently noticed in a few preclinical studies based on a carcinogen-induced primary liver cancer model^[11,12]. By gavage administration of diethylnitrosamine (DEN) in rodents, multifocal hepatomas of a full spectrum of tumor vascularity and cellular differentiation superimposed on various degrees of liver cirrhosis could be generated^[11-14]. Compared with the ectopically and orthotopically transplanted tumors, this primary HCC model is considered to be more clinically relevant for evaluating therapeutic drugs because of the heterogeneity in tumoral microenvironment similar to that of humans^[13,14], if an imaging platform can be available to accurately trace individual tumors^[14,15]. In this model, CA4P simultaneously caused not only tumor necrosis but also regional parenchymal necrosis in the cirrhotic liver^[11,12].

Tumor susceptibility to VDA therapy could be largely influenced by vascular features such as vessel density, diameter, regional instabilities in blood flow, vascular permeability and interstitial fluid pressure^[16,17]. Lines of evidence have shown that, rather than larger tumor vessels, smaller or thinner ones are more susceptible

to completely shut down in response to VDAs^[11,12,17]. Apart from the intrinsic properties of tumor vasculature, different tumor implantation sites and their dissimilar host-organ blood supplies may attribute to such variable efficacies of CA4P therapy as well^[18,19]. Take the ectopically implanted rhabdomyosarcoma (R1) as an example; intra-individual comparisons demonstrated that hepatic R1-tumors in the intact liver responded to CA4P much better than their subcutaneous and pancreatic counterparts did^[18,19]. However, issues still remain unknown as to whether R1-tumors would grow in the cirrhotic liver and whether R1-tumors growing in the cirrhotic liver are also good responders to CA4P, as they presented in the normal liver^[9,10,18-21].

So far, experimental analyses of CA4P have yielded all superior results in implanted liver tumors from animals with healthy liver^[9,10,18-21] and all inferior results on primary HCCs from rats with liver cirrhosis^[11]. Therefore, in order to assess this potential micro-environmental impact, it would be interesting to experimentally compare the therapeutic outcomes of CA4P between primary HCCs and secondary liver tumors in the same subjects with cirrhotic livers, though such a scenario is rarely seen in clinic^[22]. Accordingly, in this study we employed a DENA-induced HCC model in Wistar albino Glaxo/Rijswijk (WAG/Rij) rats that received intrahepatic transplantation of a R1-tumor to intra-individually compare the responses of different tumors to CA4P administration under the same micro-environment of liver cirrhosis. Clinical 3.0T magnetic resonance imaging (MRI) was applied for *in vivo* real-time therapeutic monitoring within 12 h, while *ex vivo* microangiography and histopathology were performed to validate the CA4P-induced outcomes.

MATERIALS AND METHODS

Animals and reagents

Male WAG/Rij rats, which are syngeneic for the cell-line of rhabdomyosarcoma (R1), weighing 300-350 g were purchased from Charles River Breeding Laboratories, Inc. (St. Aubain les Elbeuf, France). DENA (N0258) was purchased from Sigma-Aldrich (St. Louis, MO, United States). CA4P (C643025) was procured from Toronto Research Chemical Inc. (Toronto, Canada). MRI contrast agent Dotarem® (Gd-DOTA, Gadoterate meglumine; Guerbet, Villepinte, France), barium sulfate suspension (Micropaque®; Guerbet) and gas anesthetic isoflurane (Forane®; Baxter Healthcare, Deerfield, IL, United States) were also commercially obtained.

Experimental design

All animal experiments were approved by the ethics committee of KU Leuven University and performed in compliance with European and national regulations. *In vivo* procedures including gavage feeding, drug injection and MRI were carried out under gas anesthesia with 2% isoflurane (Harvard Apparatus, Holliston, MA, United States), while the laparotomy of intrahepatic R1-tumor

implantation was carried out under general anesthesia with intraperitoneal injection of pentobarbital (Nembutal; Sanofi Sante Animale, Brussels, Belgium) at 50 mg/kg.

As illustrated in Figure 1, multifocal primary hepatomas superimposed on liver cirrhosis were induced in rats by 14-wk oral gavage of DENA at 5 mg/kg/d using a 16 cm-long flexible plastic esophageal gastric tube (Fuchigami Kikai, Kyoto, Japan)^[13]. Tumor growth was monitored weekly by T2WI and T1WI from the 9th week until the largest liver tumor diameter reached more than 5 mm. A R1-tumor tissue block of 1 mm³ was implanted into the lower part of median liver lobe by laparotomy. Tumor growth was monitored weekly by MRI until R1 reached more than 5 mm in diameter. Next, all recruited tumor-carrying rats were randomly divided into sham group and CA4P-treated group. Seven rats in the CA4P group were intravenously injected with CA4P at 10 mg/kg, while the other 7 rats in the sham group intravenously received phosphate buffered saline (PBS) at 1 mL/kg. Multiparametric MRI was performed 4 h before and 1 h and 12 h after the CA4P/PBS treatment. Rats were sacrificed immediately after the last time point of MRI scanning for postmortem microangiography and histopathology.

In vivo MRI

A clinical 3.0T scanner (MAGNETOM Prisma; Siemens, Erlangen, Germany) and a human wrist coil (Hand/Wrist 16, A 3T Tim coil; Siemens) were used for imaging acquisition. To monitor tumor growth, T2-weighted (repetition time, 4000 ms; echo time, 70 ms; flip angle, 150°; field of view, 75 × 56 mm²; matrix, 256 × 192; acquisition time, 3.4 min) and T1-weighted (repetition time, 626 ms; echo time, 15 ms; flip angle, 160°; field of view, 75 × 56 mm²; matrix, 256 × 192; acquisition time, 3.8 min) turbo spin echo images (T2WI, T1WI) were performed weekly. Sixteen axial images with a slice thickness of 2.2 mm and a gap of 0.4 mm were acquired.

To evaluate tumor responses to CA4P treatment, T2WI, T1WI, dynamic contrast-enhanced (DCE) and consecutive CE-T1WIs were performed. DCE was conducted by a T1-weighted gradient echo (GE) sequence (repetition time, 7 ms; echo time, 2.45 ms; flip angle, 15°; field of view, 61 × 89 mm²; and matrix, 132 × 192) with 60 measurements in total acquisition time of 7.3 min. During DCE, an intravenous bolus of 0.02 mmol/kg Gd-DOTA was injected after the first 17 precontrast baseline measurements that were continued with 43 postcontrast measurements. Then, an intravenous bolus of 0.2 mmol/kg Gd-DOTA was injected, followed by consecutive CE-T1WI measurements.

MRI analyses

Images were analyzed with an off-line Siemens workstation and MeVisLab (version 2.6.2; MeVis Medical Solutions AG, Bremen, Germany). All the following measurements were conducted by three authors with

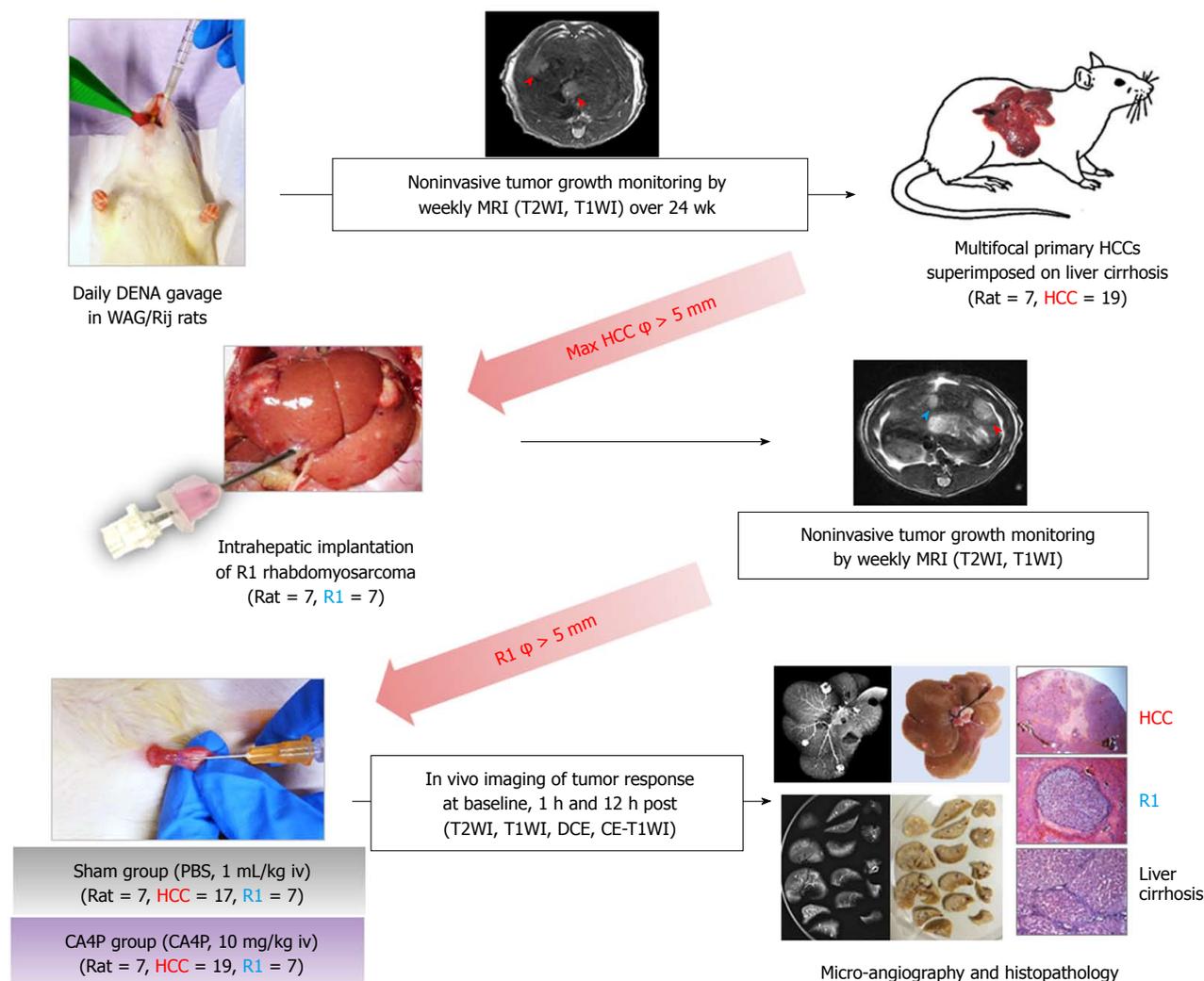


Figure 1 Flow chart of experimental protocol. ϕ : Diameter; CA4P: Combretastatin-A4-phosphate; CE: Contrast-enhanced; DCE: Dynamic contrast-enhanced; DENA: Diethylnitrosamine; HCC: Hepatocellular carcinoma; iv: Intravenous(ly); MRI: Magnetic resonance imaging; PBS: Phosphate-buffered saline; R1: R1 rhabdomyosarcoma; T1WI: T1-weighted imaging; T2WI: T2-weighted imaging; WAG/Rij rat: Wistar Albino Glaxo/Rijswijk rat.

consensus.

Tumor diameter: On T2WI, the tumor was manually contoured on the lesion-containing slices and tumor volume was automatically generated by the software, on which the tumor diameter was obtained.

Semiquantitative analysis of T1-weighted DCE: For DCE analysis, namely AUC30 calculation, the operator-defined region of interest (ROI) of tumor was freehand delineated on all tumor-containing slices. ROI of abdominal aorta was delineated from four consecutive slices for defining arterial input function. ROI of the liver was delineated on four representative slices each from median, left, right and caudate lobes. All ROIs were automatically copied to all measurements. Because of a low gadolinium dose, a linear relation between the amount of contrast agent in the tissue and the resultant difference in relaxation time could be assumed^[23]. As a robust semiquantitative DCE parameter against movements, area under the time signal intensity curve

(AUC30) was calculated to reflect tumor blood flow^[24].

Digital microangiography

After the last MRI scan, rats were anesthetized by an intraperitoneal injection of pentobarbital at 50 mg/kg. A laparotomy was performed with abdominal aorta cannulated, through which barium suspension was injected before the entire tumor-bearing liver was excised. Postmortem hepatic arteriography was conducted by a digital mammography unit (Em-brace; Agfa-Gevaert, Mortsel, Belgium) at 26 kV and 32 mAs. Then, the livers were fixed and sliced into 3-mm sections in the axial plane corresponding to the MR images, before being radiographed at 26 kV and 18 mAs for qualitative validation of tumor vascularity.

Histopathology

After microangiography, the tissue sections were paraffin-embedded, sliced and stained with hematoxylin and eosin (HE) for microscopic analyses using an Axiovert 200M microscope equipped with an AxioCam MR monochrome

digital camera (Carl Zeiss Inc, Gottingen, Germany) and AxioVision 4.8 software.

Calculation of CA4P-induced intratumoral necrosis: Microscopic images of H&E-stained tumor slices at a magnification of 12.5 were used to estimate the percentage of intratumoral necrosis by using ImageJ software^[25]. To obtain the 'necrotic ratio on each section', ROIs around the entire tumor and the necrotic tumor were manually delineated, respectively. The sectional tumor area of each 3-mm tumor section was measured and represented as the axial side of this tumor block with the largest diameter. Tumor necrosis was estimated independently by two pathologists, and calculated with the equation: Intratumoral necrosis ratio (%) = \sum [Necrotic ratio on each section (%) \times section area (mm²)] \times section thickness (mm) / $[4/3\pi r^3]$ (mm³).

Grading of HCC differentiation: In view of the high analogy to histopathological features in human liver cancer, rat HCCs were diagnosed according to the classical histomorphologic features of malignant hepatocytic tumors, often well vascularized, with wide trabeculae (> 3 cell layers), noticeable acinar pattern, small cell changes, cytologic atypia, prominent nucleoli, mitotic activity, vascular invasion, absence of Kupffer cells, lack of portal triad, and loss of the reticulin network^[26]. The differentiation of rat HCCs was further graded using a modified 4-scale Edmondson and Steiner system^[26] as the standard criteria, as follows: grade I, highly differentiated, consisting of tumor cells of moderate size arranged in thin trabeculae; grade II, larger cells with active nuclear mitosis and possible pseudoglandular structures often with steatosis; grade III, larger nuclei and more hyperchromatic or increased mitotic figures, granular and acidophilic cytoplasm, often with giant tumor cells; and grade IV, much less differentiated tumor cells with hyperchromatic nuclei and loss of trabecular pattern often with angioinvasion^[26].

Grading of tumor vascularity: To characterize variable degrees of tumoral vascularity, a semiquantitative vascular scoring system was adopted to classify HCCs as follows: +, similar vascular density to the liver parenchyma; ++, dense vasculature without vascular lakes; +++, denser vasculature with variously sized vascular lakes; and +++++, full of enlarged vascular lakes^[11,12].

Statistical analysis

Numerical data were expressed as the mean \pm standard error of the mean (SEM) and a significant difference was concluded for $P < 0.05$. *In vivo* imaging biomarker AUC30 at different time points and postmortem tumoral necrosis were compared between HCC and liver R1 by unpaired two-tailed *t*-test using GraphPad Prism (version 7.02; GraphPad Software Inc., La Jolla, CA, United States).

RESULTS

General aspects

In general, all rats tolerated the experimental procedures well, including gas anesthesia, DENA gavage, MRI scanning, laparotomy of intrahepatic tumor implantation, contrast administration and intravenous CA4P/PBS treatment. In total, 19 primary HCCs and 7 hepatic R1 allografts were successfully established in the 7 rats of the CA4P group (Table 1), while 17 primary HCCs and 7 R1-tumors were generated in the 7 rats of the sham group. The rats were sacrificed 12 h after CA4P/PBS treatment when CA4P-induced tumor necrosis was most evident.

Uniform versus variable vascularity between hepatic R1 allografts and primary HCCs

Similar to the previous findings in Sprague Dawley rats^[27], various tumoral vascularity and cellular differentiation of primary HCCs were discovered in the WAG/Rij rats (Table 1). Yet, vascularity of HCCs mainly appeared as grade +~+++, probably due to a lower-dosed DENA gavage (5 mg/kg/d vs 10 mg/kg/d) but a prolonged administration period (150 d vs 90 d) in addition to the different species. In contrast, vascularity of intrahepatic R1 allografts was uniformly identified as grade ++ (Table 1), similar to that of other tumor studies on different animal strains^[9,10,18-21].

Tumoricidal effects in metastatic R1-tumors versus heterogeneous responses in primary HCCs

In vivo real-time responses of primary HCCs and R1 allografts were visualized by multiparametric MRI prior to, and 1 and 12 h posttreatment. At baseline for the CA4P group and all time points for the sham group, hepatic R1 nodules appeared highly hyperintense on T2WIs (Figures 2A1, 3A1, 2D1), iso- to slightly hyperintense on precontrast T1WIs (Figures 2A2, 3A2, 2D2) and homogeneously hyper-enhanced on CE-T1WIs (Figures 2A3, 3A3, 2D3) compared with the liver parenchyma. Additionally, spontaneous necrosis existing in hepatic R1 of Rat 3 was indicated by the unenhanced area on CE-T1WI at baseline (Figure 3A3). Intra-individually, their paired primary HCCs on the same imaging slice appeared moderately hyperintense on T2WIs (Figures 2A1, 3A1, 2D1') as well as on precontrast T1WIs (Figures 2A2, 3A2, 2D2'), and hyper-enhanced on CE-T1WIs (Figures 2A3, 3A3, 2D3').

At 1 h after CA4P treatment, despite nearly unchanged intensities of hepatic R1 allografts on T2WIs (Figures 2A1', 3A1') and T1WIs (Figures 2A2', 3A2'), signals on CE-T1WIs were distinctly altered by an unenhanced central region surrounded by a positively enhanced periphery (Figures 2A3', 3A3'), indicative of ongoing extensive vascular shutdown. Nevertheless, the contrast of the primary HCC counterparts was slightly enhanced in a heterogeneous pattern (Figures 2A3', 3A3').

At 12 h, massive central necrosis occurred in

Table 1 Intra-individual comparison of induced tumor necrosis (%) between primary hepatocellular carcinomas and intrahepatically implanted R1 rhabdomyosarcomas in combretastatin-A4-phosphate-treated group

Rat	Primary HCC					Implanted hepatic R1				
	Tumor code	CA4P-induced necrosis, %	Tumor diameter in mm	Tumor vascularity ¹	Tumor differentiation ²	Tumor code	CA4P-induced necrosis, %	Tumor diameter in mm	Tumor vascularity ¹	
1	HCC_1	21.8	9.7	++	II	R1_1	72.3	12.1	++	
	HCC_2	16.4	6.5	++	III-IV					
	HCC_3	0	10.9	++	III					
2	HCC_4	43.1	6.4	+	III	R1_2	84.5	12.6	++	
	HCC_5	23.3	8.5	++	III					
3	HCC_6	92.3	8.1	+	I-II	R1_3	99.2	10	++	
	HCC_7	96.5	6.2	+	II					
	HCC_8	19.8	10	+	I					
	HCC_9	98.9	10	+	II					
4	HCC_10	99.2	14.3	+	I-II	R1_4	96.8	9.8	++	
5	HCC_11	27.6	18.3	+	III	R1_5	99.4	8.3	++	
	HCC_12	4.9	7.8	++	II-III					
	HCC_13	62.7	13	+	I-II					
6	HCC_14	47.6	14.2	+, +++ ³	I, III ⁴	R1_6	97.7	9	++	
	HCC_15	46.4	14.2	+, +++ ³	I, III ⁴					
7	HCC_16	76.1	12.5	+	II-III	R1_7	98.3	6.2	++	
	HCC_17	552.6	11.9	+	III					
	HCC_18	33.4	10.4	+	III					
	HCC_19	91.2	9	+	I-II					
Mean ± SD		50.2 ± 1.8	10.6 ± 0.2	/	/		92.6 ± 1.5	9.7 ± 0.3	/	

¹A vascular scoring system for rat liver tumor: vascular density similar to that of liver parenchyma (+), denser vasculature without vascular lakes (++), denser vasculature with small-sized vascular lakes (+++), and full of large vascular lakes (++++); ²A 4-scale grading system for HCC differentiation in rats: Well (I), moderately (II), poorly (III) and un-(IV) differentiated HCC lesions; ³Tumor vascularity was graded as + in the necrotic tumor, and +++ in the residual viable part; ⁴HCC differentiation was scored by I in the necrotic tumor, and III in the residual viable part. HCC: Hepatocellular carcinoma; SD: Standard deviation.

all the hepatic R1 tumors, as reflected by extreme hyperintensity on T2WIs (Figure 2A1''), isointensity on T1WIs (Figures 2A2'', 3A2'') and an unenhanced core surrounded by a hyperenhanced rim on CE-T1WIs (Figures 2A3'', 3A3''). Meanwhile, by comparison, patchy necrosis was heterogeneously induced in primary HCCs, shown as generally increased hyperintensity on T2WIs (Figures 2A1'', 3A1''), mingled hyper- and isointensities on T1WIs (Figures 2A2'', 3A2'') and regional unenhancement scattering in extremely hyperenhanced lesions on CE-T1WIs (Figures 2A3'', 3A3'').

These *in vivo* imaging findings were eventually confirmed by postmortem microangiography and histopathology. At 12 h, complete absence of tumor vessels was particularly identified in the center of hepatic R1 (Figures 2B and 3B), whereas in primary HCCs, generally denser vasculature was mixed with patchy avascular areas (Figures 2B and 3B). From HE-stained slices, massive hemorrhagic necrosis and focal necrosis were indicated in hepatic R1 and in primary HCCs, respectively (Figures 2C and 3C).

Meanwhile, in the sham group (Figure 2D), *in vivo* MRI did not show any obvious difference 4 h before, and 1 and 12 h after PBS injection. From postmortems, no vascular changes were microangiographically identified, and no acute tumoral necrosis was histopathologically discovered.

Quantitative changes of tumor blood supply in correlation to CA4P-induced necrosis

Real-time changes of tumor blood supply after CA4P

administration were monitored *in vivo*.

DCE-MRI: As reflected by AUC30 (Figure 4A), blood flow in hepatic R1-tumors dropped by 66% at 1 h due to vascular shut-down, followed by a further reduction of 7.3% at 12 h as a result of massive tumoral necrosis (Figure 4B). Nevertheless, in primary HCCs, only 11% tumor blood flow was reduced at 1 h because of vascular clogging, followed by a slight resumption of tumor perfusion at 12 h (Figure 4B), which was a heterogeneous combination of partial tumoral necrosis and reopening of large intra-tumoral vessels in residual tumor. As validated by histopathological analysis, tumoral necrosis in liver R1 allografts (92.6%) was more extensive than that in primary HCCs (50.2%) at 12 h after CA4P treatment (Figure 4C, Table 1).

Taken together, these intra-individual comparisons demonstrated that in general CA4P caused more extensive tumor vascular destruction and consequent tumoral necrosis in intrahepatically implanted R1-tumors than in the primary HCC lesions, both under the same cirrhotic liver background.

DISCUSSION

To the best of our knowledge, this is the first study where (1) a rat tumor model combining primary HCCs and an implanted R1-tumor in the same cirrhotic liver has thus been established and (2) the therapeutic efficacies of a VDA CA4P on distinct tumor types have been intra-individually compared. This, together with

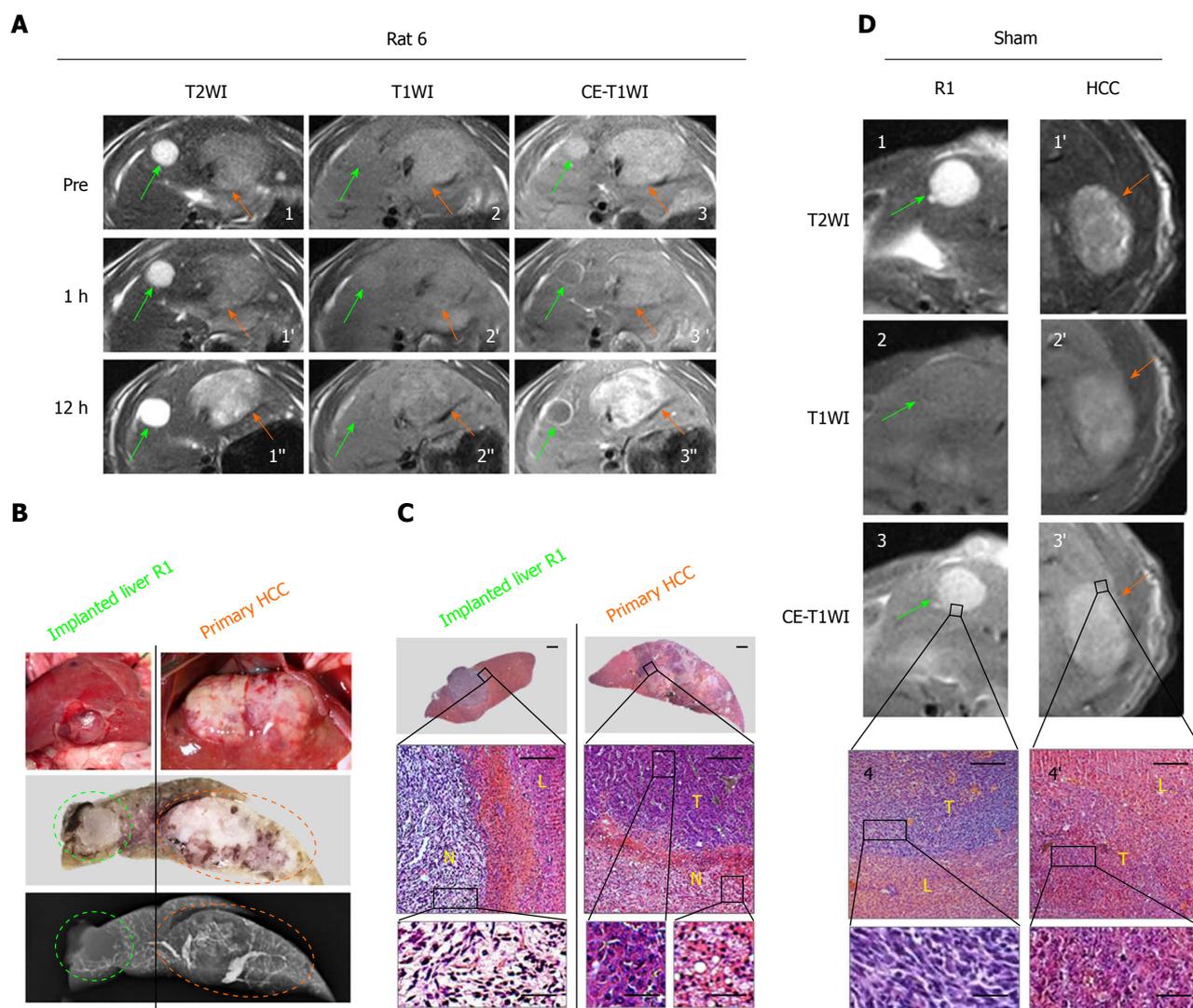


Figure 2 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft located in different liver lobes. A: T2WIs (1-1''), T1WIs (2-2') and CE-T1WIs (3-3'') of an implanted R1-tumor (green arrows) and a primary HCC (orange arrows) located in the median and left liver lobes, respectively, at baseline and 1 h and 12 h after CA4P therapy; B: Corresponding photomacrographs of median and left liver lobes (top panels), photomacrograph of liver blocks (middle panel) in 2-mm thickness corresponding to the transversal MRI, and microangiogram (bottom panel) of tumor-bearing liver blocks, revealing one R1-tumor (green circle) and one primary HCC (orange circle); C: Corresponding photomicrographs of R1-tumor (left column) and primary HCC (right column) in the median and left lobes, respectively. (HE staining; upper panels, $\times 12.5$ original magnification, scale bar = 800 μm ; lower panels, $\times 100$ original magnification, scale bar = 100 μm , $\times 400$ original magnification, scale bar = 25 μm); D: Sham control: T2WIs (1, 1'), T1WIs (2, 2') and CE-T1WIs (3, 3') of R1-tumor (green arrows) and primary HCC (orange arrows) located in the median and left liver lobes, respectively, at 12 h post PBS treatment, and corresponding photomicrographs (4, 4'); HE staining $\times 100$ original magnification, scale bar = 100 μm , $\times 400$ original magnification, scale bar = 25 μm). HCC: Hepatocellular carcinoma; L: Liver; N: Tumoral necrosis; PBS: Phosphate-buffered saline; T: Viable tumor.

the applied MRI-microangiography-histology platform, could be regarded as methodological advances for conducting more efficient theragnostic investigations on spontaneous vs metastatic liver malignancies.

This unique rat model of primary and secondary liver tumors induced by a carcinogen and surgery was employed not only to closely mimic the synchronous primary and metastatic liver malignancies seen in clinical patients, though of rarity^[22], but also to better compare such complex liver cancers, especially in terms of different tumor differentiation, angiogenesis and vasculature, towards the same therapeutics of CA4P.

Based on the fact that the target of CA4P is tumoral vasculature rather than cancer cells, transplanted

R1 rhabdomyosarcoma is a suitable model of secondary hepatic tumor because of the similar tumor neovascularization process and the existing vasculature pattern to those intrahepatic metastases^[15]. Transplanted R1-tumor is a type of homogeneous, hypervascularized, solid tumor, with abundant microvessels^[14]. Although in patients intrahepatic metastases occurring *via* the hematogenous route, they always end up with the same consequence of tumor neovascularization. Therefore, the derived results are representative of that in other metastatic liver tumors from different original sites.

Unlike ectopically and orthotopically transplanted tumor models that yield reproducible outcomes, experimental models of primary liver malignancies tend

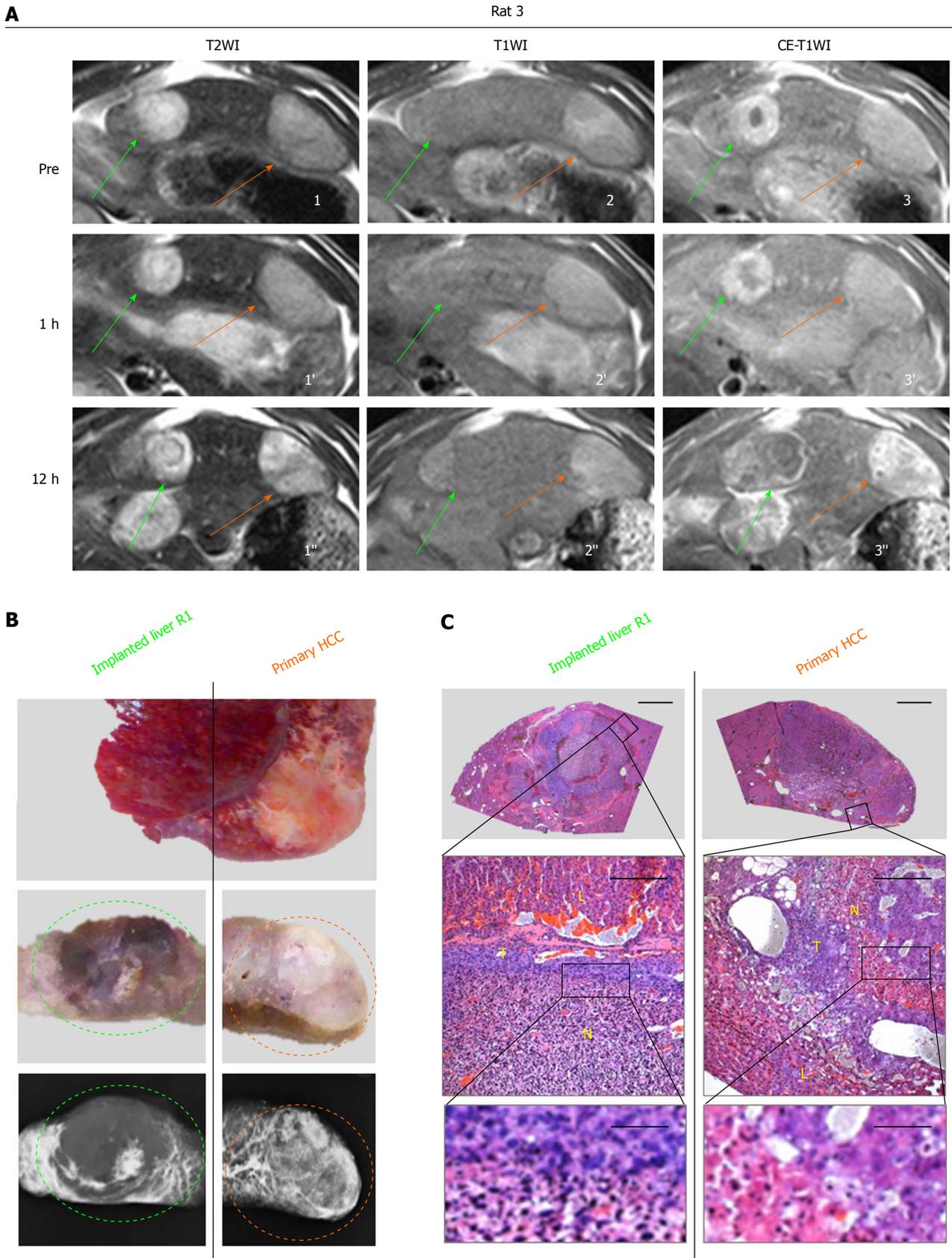


Figure 3 Intra-individual comparison of therapeutic responses to combretastatin-A4-phosphate between a primary hepatocellular carcinoma and a hepatic R1 allograft distributed in the same liver lobe. **A:** T2WIs (1-1''), T1WIs (2-2'') and CE-T1WIs (3-3'') of an implanted R1-tumor (green arrows) and a primary HCC (orange arrows) both located in the same left liver lobe at baseline and 1 h and 12 h after CA4P therapy; **B:** Corresponding macroscopic photographs of the left liver lobe (top panel) and liver blocks (middle panels) in 2-mm thickness corresponding to the transversal MRI, and microangiograms (bottom panels) of tumor-bearing liver block, revealing a R1-tumor (green circle) and a primary HCC (orange circle); **C:** Corresponding photomicrographs of R1-tumor (left column) and primary HCC (right column). (HE staining; upper panels, $\times 12.5$ original magnification, scale bar = 800 μm ; lower panels, $\times 100$ original magnification, scale bar = 100 μm , $\times 400$ original magnification, scale bar = 25 μm). HCC: Hepatocellular carcinoma; L: Liver; N: Tumoral necrosis; T: Viable tumor.

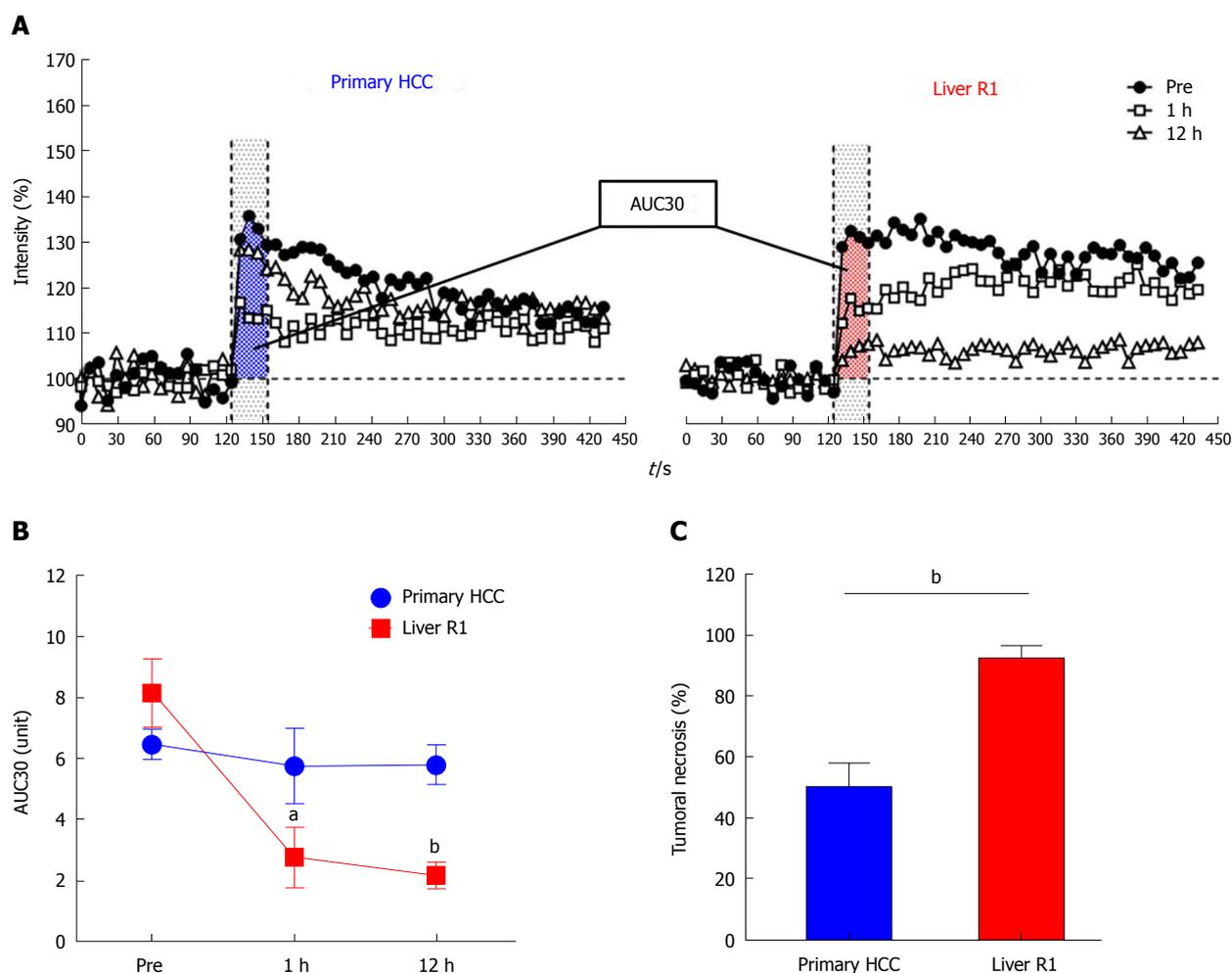


Figure 4 Changes of semiquantitative dynamic contrast-enhanced parameter of primary hepatocellular carcinomas and implanted liver R1-tumors and quantification of combretastatin-A4-phosphate-induced tumoral necrosis. A: Representative contrast enhancement-time curves of a primary HCC and a secondary liver R1-tumor before, and 1 h and 12 h after CA4P treatment, for calculating tumor AUC30 at different time points; B: Quantitative changes of tumor blood supply between HCCs and R1-tumors at baseline and 1 h and 12 h after CA4P treatment indicated by AUC30; C: Bar chart comparing the percentile tumoral necrosis between primary HCCs and implanted liver R1 at 12 h after CA4P therapy, which was estimated by postmortem HE staining. ^a*P* < 0.05, ^b*P* < 0.01. AUC30: Area under the time signal intensity curve; HCC: Hepatocellular carcinoma.

to be more therapeutically and histologically unpredictable owing to intra- and inter-tumoral heterogeneity^[11,12]. Particularly, despite undergoing similar carcinogenesis, DENA-induced primary HCCs exhibit huge diversities in carcinoma development, neovascularization or tumor vascularity, microenvironment and cellular differentiation in addition to varied degrees of liver cirrhosis^[11,12,14]. Therefore, while constructing both primary and implanted tumors could be more time-consuming and technically challenging^[13], this complex liver tumor model appears more clinically relevant for mimicking miscellaneous human cancers^[14,22].

In this study, distinct responses to CA4P, namely more complete tumoricidal effect on implanted R1-tumors *versus* variable outcomes in primary HCCs, simultaneously occurred in the same rats with cirrhotic livers. These findings are in alignment with the previous studies conducted in either DENA-induced primary HCC model on cirrhotic liver^[11,12] or implanted R1-tumor model in normal liver^[9,10,20,21]. Thus, the role of cirrhotic or normal liver background in the therapeutic impact

of CA4P could be basically excluded. It was more likely that the intrinsic vasculature of the individual tumors eventually determined various outcomes of CA4P therapy. Indeed, as a widely accepted notion, implanted liver tumors resemble more closely the secondary or metastatic liver cancer^[15]. Therefore, our results strongly indicate that, in general, CA4P exerts more potent therapeutic effects on the metastatic liver tumors, rather than the primary liver tumors.

In principle, tumor angiogenesis switches on when a tumor reaches 1 mm³ in volume, since this is the limited size of diffusion within which solid tumor cells can grow^[28]. Apart from the basic type of angiogenesis, namely endothelial sprouting, there are several nonangiogenic tumor vascularization mechanisms, including vasculogenic mimicry, intussusception and vascular co-option^[29,30]. Vasculogenic mimicry refers to tumor cells mimicking endothelial cells and directly participating in blood vessel formation, while intussusception and vascular co-option are both vascularization modes that essentially take advantage of the existing vasculature in the surrounding

benign tissue^[29,30]. For instance, in experimental liver metastatic model produced by splenic injection of CD38 colon carcinoma cells in mice, enlarged sinusoidal lakes were discovered to be developed by fusion of the normal structure of sinusoids^[31]. Since primary HCCs are generally hypervascularized tumors^[32], vascularization based on remodeling of the existing blood vessels is more complicated, especially in terms of enlarged vascular lakes. These lines of evidence may explain to some extent the heterogeneous vasculature observed in our primary HCC model that developed gradually in the context of cirrhotic liver^[11]. In support of this, by treating rats with DENA in a lower dose and a longer exposure period, less severe liver cirrhosis along with lower grades of tumor vascularity and HCC differentiation were identified in this study, as compared to a previous study^[11].

Liver cirrhosis is considered as a precancerous condition since over 80% HCCs arise on a background of cirrhosis^[26,33]. In fact, the progression of cirrhosis is accompanied by a deformation of the hepatic vasculature in regenerated lobules^[34]. Consequent hepatic vascular alterations include shunting of the portal and arterial blood directly into the central vein, compromising exchange between hepatic sinusoids and the adjacent liver parenchyma, and disturbed hepatobiliary excretion^[32,34]. In the context of cirrhosis, distorted neovasculature not only functioned as a unique mode of blood supply but also appeared to be responsive to CA4P treatment, leading to patchy necrosis in cirrhotic liver parenchyma^[12]. Hence, vigilance should be exercised when using VDAs in patients with extensive liver cirrhosis, since acute necrosis in liver parenchyma could further impair hepatic function.

Currently, although a series of phase II/III clinical trials have aimed at evaluating the treatment of CA4P in combination with chemotherapy in ovarian cancer^[4], anaplastic thyroid cancer^[5] and nonsquamous non-small cell lung cancer patients^[6], CA4P still literally remains an investigational medicine. The fetter that prevents CA4P from being ultimately adopted as a clinical anticancer therapy lies in tumor regrowth after monotherapy^[35], despite its prompt, effective and generic responses in almost all solid tumors. Hence, combining CA4P with sequential treatments like chemotherapy, conventional radiotherapy, internal targeted radiotherapy and antiangiogenic therapy could reinvigorate these VDAs and provide better long-term outcomes. In fact, a dual-targeting pan-anticancer theragnostic approach called OncoCiDia using CA4P sequentially with a radioiodinated necrosis avid compound, ¹³¹I-hypericin, has been proposed to achieve CA4P-induced necrosis-oriented internal targeted radiotherapy^[10,36]. In this context, prior to setting serial VDA-centric anticancer protocols, the present synchronous multiple liver cancer model in rodents could be a stepping-stone to help predict the diverse responses that may occur in patients, and to further address more complicated clinically relevant questions^[22]. For instance, to those patients with the

HCCs less responsive to CA4P, alternatives such as radiofrequency ablation, microwave ablation and high intensity focused ultrasound can be applied to massively necrotize the tumor before systemic administration of a necrosis-avid radiopharmaceutical in the OncoCiDia strategy^[10,36].

In conclusion, this study suggests distinct responses to CA4P, namely more complete tumoricidal effect on implanted R1-tumors vs variable outcomes in primary HCCs, simultaneously occurring in the same rats with cirrhotic livers, which could help to guide future clinical applications of VDAs.

ARTICLE HIGHLIGHTS

Research background

Previously, all favorable responses to the vascular disrupting agent (VDA) combretastatin-A4-phosphate (CA4P) on implanted liver tumors were derived from animals with healthy liver. Yet, the diverse and paradoxical responses to CA4P on primary hepatomas have been from rats with cirrhotic liver.

Research motivation

Therapeutic responses of CA4P between primary and secondary hepatic tumors had never been compared intra-individually in the same rats with underlying liver cirrhosis. And, the potential microenvironmental impact from the surrounding liver parenchyma needed to be assessed further.

Research objectives

We aimed to compare therapeutic responses of CA4P among carcinogen-induced primary hepatocellular carcinomas (HCCs) and surgically implanted rhabdomyosarcoma (R1) in the same rats by magnetic resonance imaging (MRI), microangiography and histopathology.

Research methods

We performed diethylnitrosamine gavage to induce primary HCCs and simultaneous intrahepatic implantation of R1 to create secondary liver tumor in the same rats. Tumor growth was monitored by T2-/T1-weighted images on a 3.0T MRI scanner. Rats were then intravenously treated with CA4P. Vascular response and tumoral necrosis before and after treatment were compared by dynamic contrast-enhanced (DCE-) and CE-MRI. Tumor blood supply was further calculated by a semiquantitative DCE parameter of area under the time signal intensity curve (AUC30). Eventually, *in vivo* MRI findings were validated by postmortem techniques.

Research results

In total, 19 primary HCCs and 7 hepatic R1 allografts were successfully established in the 7 rats of the CA4P group, while 17 primary HCCs and 7 R1-tumors were generated in the 7 rats of the sham group. Uniform and variable vascularity were identified, respectively, in hepatic R1 allografts and primary HCCs. As documented by *in vivo* MRI and postmortem histopathology, vascular shutdown generally occurred at 1 h after CA4P treatment; at 12 h after treatment, tumoricidal effects were observed in secondary R1 tumors, while heterogeneous responses were seen in the primary HCCs. Quantitatively, tumor blood supply reflected by AUC30 showed vascular closure (66%) in R1-tumors at 1 h ($P < 0.05$), followed by further perfusion decrease at 12 h ($P < 0.01$); less significant vascular clogging occurred in HCCs. Histomorphologically, CA4P induced more extensive necrosis in R1-tumors (92.6%) than in HCCs (50.2%) ($P < 0.01$); tumor vascularity heterogeneously scored ++-+++ in HCCs but homogeneously scored ++ in R1-tumors.

Research conclusions

To verify our original hypothesis that primary and secondary liver cancers may respond differently to VDA therapy due to the dissimilar tumor vascularity, a complex rat tumor model combining carcinogen-induced primary HCCs

and a surgically implanted R1-tumor in the same cirrhotic rats has thus been established to compare CA4P therapeutic responses intra-individually under the same microenvironment. Indeed, our hypothesis was verified by the superior performance of CA4P in metastatic over primary liver cancers. This could help to design future clinical trials and guide applications of VDAs.

Research perspectives

The merit of this study is that the present synchronous multiple liver cancer model in rodents could be a stepping-stone to help predict the diverse responses that may occur in patients, and to further address more complicated clinically relevant questions. The lesson that could be learnt from this study lies in the fact that although HCCs are generally hypervascularized, we should not take it for granted that the rich abnormal blood vessels naturally serve as plentiful drug targets for the VDA to inevitably induce massive tumor necrosis. This preclinical study's findings help in preparing a novel dual targeting pan-anticancer therapeutic strategy OncoCiDia in human liver cancers where CA4P could be applied as the first step.

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