UZ LEUVEN | RADIOLOGIE

March 15th, 2018

Dear Dr. Lian-Sheng Ma, the Editor-in-Chief of *World Journal of Gastroenterology*,

Thank you for your instructions regarding our submitted manuscript (Manuscript No: 38594) entitled "Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver tumors".

The comments from the three reviewers have been carefully addressed point-by-point both in the manuscript (yellow highlighted) and in this revision letter (see the appended pages).

We hope that this revised version should meet the high requirements for publishing our paper in *World Journal of Gastroenterology* and eventually contribute to the ever-enhancing impact of your journal.

Sincerely yours

With my best regards,

Yicheng Ni

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Responses to the Editors' Instructions

We would like to express our sincere gratitude to you and the reviewers for your valuable and constructive evaluation and guidance in the revision of this article. Below, we have seriously responded to the reviewer's comments and detailed the corresponding changes by yellow-highlighting in the revised manuscript.

Responses to the Reviewer #1's Comments

The authors investigated vascular damage effects with combretastatin A4 phosphate (CA4P) against rat models of implanted rhabdomyosarcoma (R1) or chemically induced hepatocellular carcinoma (HCC). They found that vascular damage effects were more severe in R1 as compared to HCC.

1) CA4P was applied to R1 and HCC in the same rats. Rationality of this model should be explained. What human situation did this model correspond to?

Reply: Thank you for indicating our insufficient explanations to this unique tumor model in rats. The complex model of primary and secondary liver tumors induced by a carcinogen and surgery, respectively, in the same cirrhotic livers of the same rats was employed not only to simulate the rare synchronous primary and metastatic liver malignancies in clinical patients (supported by a new reference), but also to better compare such diverse liver cancers, especially in terms of different tumor angiogenesis and vasculature, towards a same therapeutics.

Given the fact that CA4P targets tumor vessels instead of tumor cells, the microenvironment of surrounding liver tissue may influence the pattern of tumor angiogenesis and, consequently, the responses of liver tumors to CA4P. So, we generated this rat liver tumor model, where both primary and transplanted secondary liver tumors co-exist in the same cirrhotic liver background. This enabled the more efficient intra-individual comparisons of CA4P efficacy by excluding other influential factors, from which the findings could help to foretell the prognostics among the subjects with primary and secondary liver tumors.

The corresponding explanations and a pertinent reference have been added and highlighted in Discussion.

2) In Introduction, CA4P targets cytoskeletal tubulin of abnormal tumor endothelial cells. How did the authors define abnormal tumor endothelial cells? Were there any differences

between abnormal endothelial cells and endothelial cells in healthy tissues? These parts are basis of this study.

Reply: Indeed, difference in the architecture, cellular and biochemical composition between normal and tumoral blood vessels provide the basis of the selectivity of CA4P. Unlike the endothelial cells in normal tissues where both intracellular tubulin and actin coexist to form healthy cytoskeleton, the endothelial cells of immature tumor neovasculature lacks of actin with tubulin alone to exert cytoskeletal function. Tubulin is known to be the target of CA4P that can depolymerize the former, leading to balloonization of the otherwise flat-shaped endothelium resulting in vascular obstruction and ischemic tumor necrosis. Besides, immature smooth muscle and pericyte contact are also sensitive to tubulin-binding VDAs like CA4P, causing disrupting of junctions between endothelial cells, rounding up and surface blebbing of these cells, increasing vessel leakage, increasing vascular resistance, stagnation of flow, stacking of red blood cells and vessels occlusion in tumors (Dana M, et al. *Gynecologic Oncology*. 145 (2017) 393–406.).

3) Human HCC occurs mainly in liver infected with HBV or HCV. The authors analyzed chemically induced HCC. There might be differences in characters between human HCC and this study. How were the pathological characters in HCC in this study? Were vascular structures the same as human HCC? This part affects the applicability of this study to human.

Reply: We agree that DENA-induced primary HCC is etiologically classified as a toxicity caused chronic liver disease, while the leading cause of human HCC is hepatitis B/C infection and alcohol intoxication. However, independently of the original underlying causes, similar pathophysiological process including liver injury, regeneration, cirrhosis and eventually malignant mutation occurs.

As reported previously by our group (Ni Y, et al. J Magn Reson Imaging. 1994;4(3):355–363) using the same DENA-induced primary liver cancer model, histopathological similarity between rat and human HCCs could be identified, eventually this rat model has successfully predicted the drug behaviors of new contrast agents as proven by later clinical applications of such new imaging diagnostics. For instance, a full spectrum of cellular differentiation and tumoral vascularity are the common features. Similarly to human HCCs, rat primary HCCs presented a strong heterogeneity of vasculature and diverse components ranging from hypo-perfused areas (cysts, thrombus and spontaneous necrosis) to variable sized blood vessels and even vascular lakes. Therefore, we believe the results derived from this animal model is of high clinical relevance and translational value for VDA study.

This corresponding part has been added and highlighted in Discussion.

4) How to calculate AUC30 should be described. It was hard to imagine AUC30. Figure 2 C and D, Figure 3 C.

Reply: Thanks for pointing out this insufficient explanation. Representative contrast enhancement-time curves (CTCs) of primary and secondary liver tumors from DCE MRI used for calculating AUC30 has been added and highlighted in Figure 4A.

5) It was hard to evaluate cell morphology. In addition to the photos, the other ones would be necessary in more magnification.

Reply: Thanks for your suggestion. Microscopic images at a higher magnification of 400 have been added in Figure 2 and 3.

Responses to the Reviewer #2's Comments

This is an interesting paper looking at the effect of a vascular disrupting agent on primary and secondary hepatic tumors in the same animal. Could the authors please respond to the following questions/comments:

Reply: Thanks for your appreciation on the concept of our study.

1) How were the "36 HCCs created in 14 rats"? Was the distribution deliberate or a matter of chance?

Reply: Unlike the implanted R1 tumor that was well under control, spontaneous HCCs developed randomly in different liver lobes in rats, where the number of tumors per animal is unpredictable, which however could be monitored by in vivo noninvasive MRI as described.

2) Why did the authors choose only up to 12 hrs as a time point, ie how do we know that the action of the vascular disrupting agent does not continue (and even maybe increase) more so over time?

Reply: The endpoint of experiment was set up based on the pharmacokinetics of CA4P. With a short half-life of 3.6 minutes, CA4P exerts a rapid but reversible effect of causing vascular collapse in solid tumors. The consequence of reducing tumor blood flow was evident as early as 10 minutes post administration (Siemann DW, et al. *Expert Opin Investig Drugs*. 2009;18(2):189-97.), and maximal during the following 1-6 hours (West CM, et al. *Anticancer Drugs.* 2004;15(3):179-87.). According to the findings in our previous studies, CA4P-induced tumor necrosis was most evident at 12 hours, and tumor relapses from the viable residuals shortly after few days due to the stimulation of hypoxia.

The translational relevance of this observation window for CA4P-induced acute necrosis lies in that this preclinical study is for preparing CA4P as the first step of a novel dual targeting pan-anticancer theragnostic strategy called OncoCiDia in human liver cancers. The protocol of the second step of treatment (targeted intratumoral radiotherapy) is to intravenously administer a radiolabeled necrosis-avid compound ^{131}I-Hypericin at 12 hours post CA4P treatment, when CA4P-caused necrosis is most evident. Therefore, we set up 12 hours as the endpoint to observe acute CA4P effects in the current study, as reasoned in the text.

3) It appears that the authors consider the rhabdomyosarcoma as a metastatic lesion. If so, what is the primary? The authors may want to consider that there are primary hepatic rhabdomyosarcomas (although extremely rare).

Reply: As we know, the target of CA4P is tumor vasculature rather than tumor cells. The reason why we use transplanted R1 rhabodomyosarcoma as a tumor model of secondary hepatic tumors is because of the similar tumor neovascularization process and the existing vasculature pattern. Specifically, transplanted R1 tumor is a type of homogeneous, hypervascularized, soild tumor, with abundant micro-vessels. Therefore, the derived results should be representative of that in other metastatic liver tumors from different original sites.

In addition, there are also several other advantages of R1 model in the application of comparing therapeutic efficacy of anticancer treatments: 1) R1 tumor is free of large spontaneous necrosis before exceeding 3 g, which helps to distinguish CA4P-induced acute tumoral necrosis; 2) as the host of allograph R1 tumor, there is no evidence of an immune response from WAG/Rij rats to R1, which makes the results also relevant to human patients. This has been discussed and added in Discussion.

4) Why were there only male rats used?

Reply: We agree that, rigorously, VDA efficacy should be evaluated in both animal genders. However, as liver cancer occurs independently of gender, we choose male rats in the current study for the following reasons: 1) male rats have more venous routes including penis and tail veins, which facilitate multiple and frequent intravenous administrations like injection of contrast agent and CA4P; 2) male rats are generally larger in body size, which brings better MR imaging qualities; 3) male rats are tougher under the long-term experimental processes including carcinogenesis, MR imaging diagnosis and CA4P therapy.

5) Regarding the rhabdomyosarcoma, one could argue that there is a different vascularity pattern when we are referring to an “implant” versus a metastatic lesion that metastasized through a hematogenous route for example. How would this change the action of the vascular disrupting agent?

Reply: We agree that tumor distant spread may occur via different routes including hematogenous and lymphatic metastasis, but tumorigenesis always ends up by the same consequence of tumor neovascularization. There is a general consensus that transplanted tumor model could mimic metastatic lesion. Therefore, VDA efficacy in secondary tumors is considered to be independent of their spread routes. This notion has been supported by the extensive clinical trials of CA4P. This has been discussed and added in Discussion.

6) How do the authors account for the difference in the necrosis pattern between the primary and the metastatic tumor?

Reply: Tumor susceptibility to VDA therapy could be largely influenced by intrinsic vascular features. Evidences have shown that, rather than larger tumor vessels, smaller or thinner ones are more susceptible to completely shut down in response to VDAs.

As systematically discussed in another recently accepted paper by our group, the strong heterogeneity of vasculature presents inside primary liver tumors, and diverse components ranging from hypo-perfused areas (cysts, thrombus and spontaneous necrosis) to variable sized blood vessels and even vascular lakes, contribute to the diverse and relatively inferior therapeutic responses to CA4P in primary HCCs.

By comparison, metastatic tumors is generally more homogeneous and hypervascularized with abundant micro-vessels, which tends to shut-down completely upon CA4P treatment leading to massive necrosis in tumor parenchyma.

7) The authors show a possible different pattern here using an implant of a rare tumor. One could argue that it is not necessarily easy to generalize with other types of metastatic lesions to the liver given the fact that they may follow other routes and other biological behavior.

Reply: Like we mentioned in the answer to Question 5 and 3, no matter which way a secondary tumor spreads through, it always ends up by the similar pattern of tumor neovascularization that is the real target of VDAs including CA4P. The homogeneous, hypervascularized micro-vessel pattern in the transplanted R1 tumor could be considered as a representative vascular feature in secondary or metastatic tumors, which has been proven by the outcomes from extensive clinical trials on VDAs for non-hepatic indications. As this study was aimed at evaluating tumor responses to a vascular disrupting agent CA4P, tumor vasculature is a more critical factor rather than other biological features. This has been discussed and added in Discussion.

Responses to the Reviewer #3's Comments

The manuscript entitled “Intra-individual comparison of therapeutic responses to vascular disrupting agent CA4P between rodent primary and secondary liver cancers” has been evaluated. It is a well-written and presented manuscript. The aouthors did extensive work to show the effect of combretastatin-A4-phosphate (CA4P) among hepatocellular carcinomas (HCCs). In this particular study, the authors compared therapeutic responses of a vascular-disrupting-agent (VDA) Combretastatin-A4-phosphate (CA4P) among hepatocellular carcinomas (HCCs) and implanted rhabdomyosarcoma (R1) in the same rats by magnetic-resonance-imaging (MRI), microangiography and histopathology. They were able to show that thirty-six HCCs were created by diethylnitrosamine gavaged in 14 rats that were also intrahepatically implanted with one R1 as monitored by T2-/T1-weighted images (T2WI/T1WI) on a 3.0T MRI-scanner. Vascular response and tumoral necrosis were detected by dynamic-contrast-enhanced (DCE-) and CE-MRI before, 1h and 12h after CA4P iv at 10 mg/kg (treatment group n=7) or PBS at 1.0 ml/kg (control group n=7). Tumor blood-supply was calculated by a semi-quantitative DCE parameter of area-under-the-time-signal-intensity-curve (AUC30). In vivo MRI findings were verified by postmortem techniques. The authors found that 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 1h. In that tumor necrosis occurred massively in R1-tumors but patchily in HCCs at 12h. AUC30 revealed vascular closure (66%) in R1-tumors at 1h ($P<0.05$), followed by further perfusion decrease at 12h ($P<0.01$); while less significant vascular clogging occurred in HCCs. In conclusion, in this study, they revealed effective performance of CA4P in metastatic over primary liver cancers, leading to future clinical applications of VDAs.

Reply: Many thanks for the Reviewer’s appreciation of our work.