Peer Review File

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Reviewer A

The authors described that honokiol (HNK) inhibited glioblastoma (GBM) growth via the induction of macrophage polarization from M2 to M1. To achieve penetration through the blood brain barrier (BBB) to improve drug delivery, HNK was packed in lipophilic carriers such as liposomes.

I think this is a unique strategy for GBM therapy, and I would ask several questions to the authors.

1. Liposomes might be useful for manipulation to penetrate the BBB. The authors showed GBM growth in vivo by a subcutaneous model. However, this is not clinically relevant. I hope therapeutic efficacy would be represented in an intracranial model. Do the authors think that liposomal HNK would be expected to have therapeutic efficacy in an intracranial model? How much is the molecular weight of HNK?

Reply 1: Thank you very much for the reviewer's valuable comments. Lip-HNK has been proven to be able to pass through the blood-brain barrier in animal experiments in phase I clinical trials. In phase I and phase II clinical trials conducted in our center, it has also been observed that Lip-HNK can indeed reduce intracranial tumors. The valuable opinions of the reviewer will be addressed in our ongoing intracranial model experiments to further explore the mechanism and therapeutic efficacy, and we will provide this information after exploration.

2. M2 macrophages are known to grow vigorously under hypoxia, which might have a potent impact on macrophage polarization. Was the effectiveness of HNK affected by the tumor microenvironment (TME), especially hypoxia? What happens if macrophages are cultured under hypoxic condition? Did the authors compare the difference in M1/M2 polarization between normoxia and hypoxia?

Reply 2: We appreciate the reviewer's thought-provoking comments. In response to hypoxia, the tumor utilizes pathways involving angiogenesis, metastasis, glycolysis, and survival to overcome the unfavorable environment. Hypoxia-inducible factor-1a (HIF-1a) plays a pivotal role in the tumor's response to hypoxia. The TME is often characterized as hypoxic resulting from the extensive growth of tumor cells as well as disorganized tumor blood vessels. Cancer cells adapt to hypoxic conditions, allowing

tumors to survive and become more resistant to radiation and chemotherapy. Given the pivotal role of HIF-1a induced by either hypoxia or oncogenic pathways, inhibition of HIF-1a has been shown to sensitize tumor cells to chemotherapy and radiotherapy in numerous preclinical studies. Lan et al. have demonstrated the inhibitory effect of HNK on HIF-1a protein levels and its regulation of gene transcription (Honokiol inhibits hypoxia-inducible factor-1 pathway. Int J Radiat Biol. 2011 Jun;87(6):579-90.). Therefore, hypoxia will not affect the therapeutic effect of HNK according to this theory, but it may increase the sensitivity of the tumor to radiotherapy and chemotherapy, and can also be used as a potential chemotherapeutic sensitizer for the tumor. We will further explore this problem and confirm these findings in our next research.

We investigated the impact of hypoxic conditions on macrophages. RAW264.7 macrophage morphologies and phenotypes vary when cells are exposed to different hypoxic conditions. We cultured RAW264.7 cells for 7 days under normoxic or hypoxic conditions in the presence of LPS+IFN-γ or IL-4. We found that hypoxia did not influence the expression of M1 macrophage-related markers in the LPS+IFN-γ group, but it did increase the expression of M2 macrophage-related markers in the IL-4 group (Supplementary Figure 7). The mechanism was not studied in our experiments. In the future, we will continue our investigations into these areas.
Changes in the text: Supplementary Figure 7, line: 37-39

3. Is the regulation of macrophage polarization affected by IDH-1 status?

Reply 3: Although the brain is thought to be an immune-privileged site, gliomas have a substantial component of immune cells, where macrophages and microglia account for as many as 20%–30% of the total cells in some highly aggressive tumors (Badie and Schartner 2000; Kong et al. 2010). In addition to the differences in immune cell content, Zhang et al. previously described marked differences in effector immune cell functions across mutant IDH1 (muIDH1) and wtIDH1 gliomas (Zhang et al. 2016). Gene expression signatures of wtIDH1 gliomas are strongly associated with an inflammation and immunologic response (Ceccarelli et al. 2016) and high levels of several chemokines and interleukins that may stimulate infiltration of regulatory immune cells. There are few studies on the regulation of macrophage polarization affected by IDH status. Jha et al. found that M2 polarization activates glutamine catabolism and UDP-GlcNAc-associated modules. Correspondingly, glutamine deprivation or inhibition of N-glycosylation decreased M2 polarization and the production of chemokine CCL22. In M1 macrophages, they identified a metabolic

break at *IDH*, the enzyme that converts isocitrate to alpha-ketoglutarate, providing a mechanistic explanation for TCA cycle fragmentation.

#4. What is the mechanism of GBM growth when M2 macrophages became dominant in the current study? Did the authors examine whether angiogenesis and immunosuppressive TME were induced in M2 polarization as determined by in situ histological analyses including the proliferation index of tumor cells/endothelial cells, microvessel counting, and distribution of cytotoxic T cells?

Reply 4: Macrophage polarization is defined by the distinct functions activated by different sets of cytokines or extracellular signals and results in either M1 classically activated protective macrophages, or M2 alternatively activated pathogenic macrophages. In the TME, M2 TAMs are immunosuppressive and are induced by stimuli such as IL-4 and IL-13. Numerous studies revealed that M2 TAMs were activated and in turn promoted tumor progression under the guidance of tumor-released immunosuppressive cytokines and chemokines. Our results demonstrated that Lip-HNK in vivo significantly reduced the recruitment of tumor supportive GAMs (M2 type) and inhibited GBM tumor growth. Lip-HNK as an anti-tumor drug inhibits macrophage polarization to M2, so whether M2 polarization induces angiogenesis and immunosuppressive TME is not the focus of our study. However, as for the reviewer's constructive comments, we examined the impact of Lip-HNK on several aspects of tumor biology such as vessel density in mouse tissues, as shown in Supplementary Figure 4, and the expression of CD31 in the Lip-HNK group was significantly lower than that in the control group.
Changes in the text: Supplementary Figure 6, line: 32-36.

#5. How does macrophage polarization alter after temozolomide (TMZ) therapy? Do the authors expect a synergistic effect of combination therapy with HNK when GBM becomes TMZ resistant? Or should combination therapy be commenced before TMZ resistance?

Reply 5: Thank you very much for the constructive comments. At present, there are studies regarding alterations in macrophage polarization after TMZ therapy, so we did not repeat this experiment. Azambuja JH et al. found that mouse glioma cell line GL261 sensitive or GL261 TMZ resistant cells differentially modulated macrophage polarization. They also showed that macrophages exposed to GL261 TMZ resistance conditioned medium exhibited decreased production of nitric oxide (NO), as well as

increased arginase activity, CD206 expression, and IL-10 release when compared to the control macrophage conditioned medium of GL261 cells. In another parallel experiment, we are also exploring the beneficial effects of HNK on TMZ-induced death of drug sensitive and resistant malignant glioma cells. TMZ is a first-line chemotherapeutic drug for GBM patients. However, TMZ administration has multiple side effects such as nausea, vomiting, constipation, headaches, fatigue, loss of appetite, mouth sores, and hair loss. Human malignant gliomas are very aggressive, and the 5-year survival rate of malignant glioma patients is about 5%. Interestingly, our parallel experiment showed the enhanced effects of HNK on TMZ-induced death of human and murine glioma cells. As a result, HNK has specific benefits of reducing the complications of TMZ by decreasing its dosage and accordingly improving the quality of life of GBM patients. In addition, the current phase II clinical trials are aimed at TMZ-resistant patients, so Lip-HNK may also be a new choice for these patients.

#6. In vivo data showed that body weights did not change during treatment. What is the adverse effect of HNK?

Reply 6: Thank you very much for the reviewer's valuable comments. In preclinical safety research evaluation, the highest dose of Lip-HNK with no serious toxicity was 40 mg/kg, and there was no obvious toxicity to the heart and central nervous system in vivo. In addition, no adverse reactions were found in genotoxicity and reproductive toxicity. The main pre-clinical adverse reactions are irritation at the injection site and surrounding tissues. In a phase I clinical trial of glioma treatment, the main toxic reaction of Lip-HNK was the stimulation of blood vessels and surrounding tissues. Only 2 cases had mild hematological toxicity which could be improved after symptomatic treatment.

Reviewer B

This paper looks at liposomal honokiol and its effects on macrophage polarization and the growth of glioblastoma cells both in vitro and in vivo.

The authors stimulated macrophages towards M1 or M2 phenotypes and then looked at the effects of Lip-HNK treatment by qPCR. The authors state that Lip-HNK treatment can reverse M2 polarization but none of their experiments actually show this, but rather they show a reduction in the expression level of M2 markers (Arg1 and IL-4).

Comment 1: In the experiments in macrophages alone, decreased M2 phenotype by reduced Arg1 gene expression (statistically significant) is shown, along with a slight

non-significant increase in iNOS gene expression (compared to controls). The authors should be stimulating the macrophages towards M2 and then looking at increased release in the media of M1 markers after Lip-HNK treatment to truly show that Lip-HNK can repolarize them i.e. NO/TNF-a are released M1 factors. They should also be looking at arginase, IL-10, and TGF-β in the media of M2 polarized macrophages and reduced levels of these after Lip-HNK treatment.

Reply 1: We appreciate the reviewer's thought-provoking comments. As a matter of fact, we have already done this, and we have added the results according to the comments of the reviewers.
Changes in the text: Supplementary Figure 2, line: 8-14.

Comment 2: The authors also need to include additional controls in their macrophage only experiments i.e. Lip-HNK by itself and liposomes alone (as they have done as the control in the in vivo experiment). It is unknown what these do to macrophage polarization.

Reply 2: Thank you very much for the constructive comments. We have observed the effects of Lip-HNK and liposomes alone on macrophage polarization. Results showed that liposomes alone had no effect on macrophage polarization.

Comment 3: Figure 1 and 2 C and D show the same data as figure 1 and 2 E and F, no need for both.
The authors need to show references for what CD11c and MHC class II IA/IE are used for as they have not explained this. From most studies, CD80 would be a traditional M1 macrophage marker.

Reply 3: We appreciate the reviewer's thought-provoking comments. Considering the reviewer's suggestion, we have deleted Figure 1 and 2 E and F.
Changes in the text: see page 28, 29, line 486-494, 596-504

MHC (major histocompatibility complex) class II molecules are transmembrane glycoproteins expressed on the surface of professional antigen-presenting cells, such as macrophages, dendritic cells, and B cells. Before their exposition on the cell surface, MHC class II molecules react with endocytosed exogenous antigens, which are then presented to T cells. In our study, MHC class II IA/IE was used to identify macrophages, while CD11c was mainly used to identify M1 macrophages.

MHC class II (MHC II) molecules are mostly located on antigen presenting cells (APCs), such as macrophages. After macrophages ingest pathogens, they use MHC to prompt helper T cells to start the immune response. MHC class II IA/IE is used for macrophage identification (J K Pullen, L B Schook. Bone marrow-derived macrophage expression of endogenous and transfected class II MHC genes during differentiation in vitro. J Immunol. 1986 Aug 15;137(4):1359-65). (Jiurong Liang, Yoosun Jung, Robert M Tighe, Ting Xie, Ningshan Liu, Maura Leonard, Michael Dee Gunn, Dianhua Jiang, Paul W Noble. A macrophage subpopulation recruited by CC chemokine ligand-2 clears apoptotic cells in noninfectious lung injury. Am J Physiol Lung Cell Mol Physiol. 2012 May 1;302(9): L933-40). As early as 2004, it was reported that CD11c can be used to identify macrophages. The latest research on this topic is also available (Karim Vermaelen et al. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. Cytometry A. 2004 Oct;61(2):170-77. doi: 10.1002/cyto.a.20064), (Sistiana Aiello et al. Transplantation-Induced Ischemia-Reperfusion Injury Modulates Antigen Presentation by Donor Renal CD11c + F4/80 + Macrophages through IL-1R8 Regulation. J Am Soc Nephrol. 2020 Mar;31(3):517-531). Although CD80 is a traditional marker of M1 macrophages, CD11c has also been used to represent M1 macrophages in many studies.

Comment 4: Was there a wash out period after the macrophage stimulation? Given you are assessing IL-4 gene expression level, this is very important.

Reply 4: The population diversity and functional plasticity of macrophages are important characteristics of macrophages that play an important role in several diseases (Sica A, et al,J Clin Invest. 2012 Mar; 122(3):787-95). It is well-known that macrophages can switch phenotypes between M1 or M(LPS) and M2 or M(IL-4). Vichaya et al. found that IFN-γ prevented reverse polarization (Vichaya et al Front Immunol. 2019; 10: 2956). In our study, in order to avoid the subsequent detection of IL-4, cells were stimulated with LPS and IFN-γ or IL-4 to induce polarization for 48 hours followed by washout for 24 hours.

Comment 5: In the co-culture results, Lip-HNK is included this time and no differences are seen, which suggests that by itself Lip-HNK does nothing. While IFN-γ/LPS stimulation resulted in reduced growth as you would expect, IL-4 stimulation does not result in statistically significant increased growth as you would also expect, although higher levels are noted. What is seen, however, is that after stimulation towards M2, the addition of Lip-HNK does reduce growth, but there is no evidence that Lip-HNK does this by repolarizing the macrophages towards M1. Further experiments such as

assessing the conditioned media from these co-cultures for increased NO/TNF-α are needed to conclusively state this.

Reply 5: Macrophages are classified as classically activated, pro-inflammatory M1 and alternatively activated M2 cells, which possess different functions and transcriptional profiles with respect to inflammatory responses. As polarization is not fixed, macrophage functional plasticity is modulated by the microenvironment, allowing them to rapidly react to danger signals and maintain tissue homeostasis. In the co-culture results, although the addition of Lip-HNK did not demonstrate any differences, the current experimental evidence cannot show that Lip-HNK does not work. Because we eluted for 24 hours after IFN-γ/LPS and IL-4 stimulation, and then added Lip-HNK, pretreatment highly enhanced its ability to regulate macrophage characteristics and inhibit immunosuppression. Therefore, the potential benefit of preconditioned MSCs (Bone marrow mesenchymal stem cells) and IL-6 should be considered for future clinical treatment. Considering the reviewer's suggestion, we have added TNF-α mRNA expression in our study.
Changes in the text: Supplementary Figure 1, line 3-7

Comment 6: The STAT1/6 results are not very convincing, especially because you cannot see Fig 3A properly - and pY701 does not increase until 50 μM. The STAT6 results fit with the down regulation of the M2 phenotype but do not show a depolarization towards M1. However, even these results are not very convincing as 6.25 μM Lip-HNK treatment looks like (by the image) it increased pSTAT6. Other published papers have looked at HNK treatment in glioma (e.g. PMID: 30587839, 32817393) showing other mechanisms of action and these should also be looked at, as the results of the co-culture system only looked at absolute glioma cell growth and not anything else.

Reply 6: Thank you very much for the reviewer's valuable comments. We have adjusted Figure 3 as per the reviewer's comments. Although other published papers have looked at HNK treatment in glioma, and also show other mechanisms of action, our study looked at the results of the co-culture system on glioma cell growth. However, another parallel experiment is being carried out in vivo. We believe that in the near future, we will provide a complete study of the drug mechanism.
Changes in the text: line 518, page 35

Comment 7: As the authors point out themselves, an in vivo experiment being performed in the flank is a major limitation of the study. This, together with the low

numbers of mice per group, is not thorough. The authors even make note of the BBB and how important it is, and yet they did not perform their study in the brain. Additionally, in this experiment, what is the endpoint for this study? The methods suggest it is when tumors reach 2000 mm^3, but that is not what is shown Figure 5. A survival curve would be better if this is the endpoint, but if not, change the methods to match this. These results however do show a decrease in M2 macrophages by decreased CD206, but as I do not understand the significance of increased CD11c and MHC class II IA/IE cells, I do not understand the results.

Reply 7: We appreciate the reviewer's thought-provoking comments. The blood brain barrier (BBB) restricts the entry of toxic substances, drug molecules, and various proteins and peptides to maintain brain homeostasis. One of the most important factors in drug delivery is the selection of an appropriate drug delivery system, such as liposomes, which are a new drug delivery system and provide a potential way to solve this problem. Their unique phospholipid bilayer structure (similar to the physiological membrane) make them more compatible with the lipid bilayer of the BBB, which helps drugs enter the brain. Because of its poor solubility, HNK is not widely used in clinical cancer treatment. However, liposomes can overcome this limitation; thus, Lip-HNK has promising clinical applications. At present, phase I clinical trials of Lip-HNK in the treatment of advanced malignant solid tumors have been carried out in our hospital, demonstrating good safety, tolerance, and no obvious side effects. Before that, the State Key Laboratory of Biotherapy of Sichuan University carried out a series of pharmacodynamic studies in vitro and in vivo, and in vitro concentrations (10 μM) were effective in tumor tissues in vivo. Considering the reviewer's suggestion, we will increase the quantity of mice for verification. In the phase I clinical trial, the standard of administration was 28 consecutive days, and then the condition was evaluated. Therefore, we also used the same method in this study. Whether the tumor reached 2000 mm^3 was not the end point of the study, but was applied according to the time of the current clinical trial. At present, we are conducting an intracranial tumorigenesis experiment in another parallel experiment, and also expanding the number of mice for the subcutaneous tumorigenesis experiment. We will also evaluate the study endpoint according to the reviewers' suggestions. MHC class II (MHC II) molecules are mostly located on antigen presenting cells (APCs), such as macrophages. After macrophages ingest pathogens, they use MHC to prompt helper T cells to start the immune response. MHC class II IA/IE is used for macrophage identification. CD11c is used to identify M1 macrophages. Although CD80 is a traditional marker of M1 macrophages, CD11c has also been used to represent M1 macrophages in many studies.

Special thanks to you for your helpful comments.

Overall, while of interest, the specific point that Lip-HNK is able to repolarize towards M1 is not justified by their experiments. However, Lip-HNK does reduce M2 markers and this is what the authors should focus on.

Reviewer C

I would suggest this paper to be accepted with minor revision.

The authors mentioned that:

A considerable increase of STAT1 activation was observed, by contrast, STAT6 activation was suppressed, both by Lip-HNK. STAT1 and STAT6 are the key signaling molecules of M1 and M2 polarization, respectively.

My suggestion is: is it possible to make sure that STAT1 activation has a certain relationship with STAT6 inhibition? Using a STAT1 inhibitor to study the expression of STAT6?

Reply: STAT1 and STAT6 reportedly reciprocally regulate each other in T cells (Yu CR, et al J Immunol. 2004 Jul 15; 173(2):737-46.). However, Miyamoto et al. found that in macrophages, STAT6 regulates STAT1 activation, whereas STAT6 activation is STAT1-independent, suggesting that the regulation of STAT protein activity differs depending on cell type (Hiroya Miyamoto, et al, J Biol Chem. 2012 Sep 21; 287(39): 32479–32484.). However, there are no studies on the relationship between STAT1 activation and STAT6 inhibition. We appreciate the reviewer’s thought-provoking comments, and we will employ STAT1-deficient and STAT6-deficient mice for further study.