Anti-angiogenic effect of heparin-taurocholate conjugate on mouse glioblastoma animal model

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ABSTRACT SUMMARY
Glioblastoma is the most common brain tumor in adult human. To treat the glioblastoma, we used low-molecular-weight-heparin derivative (LHT7) which was chemically modified with taurocholate, one of bile acids. We confirmed that LHT7 could reduce the vessel sprouting from rat aortic ring and tumor growth. So, LHT7 would be a promising anti-angiogenic drug for glioblastoma therapy.

INTRODUCTION
Glioblastoma is the most common malignancy of the central nerve system. Because of early infiltration of surrounding tissue and the high recurrence rate with fast progression, the overall survival time of patients is only 7-15 months. Over the past decades, surgical elimination and radiotherapy have been improved. However, it is difficult to completely eliminate the tumor, glioblastoma patients have been received chemotherapy. Temozolomide is one of the widely used chemotherapy agent. Temozolomide-mediated methylation in DNA could induce the death of glioblastoma cell. But tumor cells are able to repair this mutation by using MGMT (O-6-Methylguanine-DNA methyltransferase) repair system. Therefore, we need to treat the glioblastoma with different way. In general, most cancer cells can highly induce the angiogenesis for receiving the nutrients and oxygen. Glioblastoma has also higher vascularization. Inducing the death of thousands of cancer cells that is caused by anti-angiogenesis therapy is more efficient than that of single cancer cell that cytotoxic drugs cause. Therefore anti-angiogenesis therapy is promising therapy to cure glioblastoma.

In several studies, heparin has been reported as an anticancer drug by binding VEGF and VEGF receptor as well as an anti-coagulant agent. However, highly negative charged and hydrophilic heparin generally represents short half-life. Moreover, it also has side effect like a hemorrhage. To overcome these limitations, chemically modified heparin derivatives were developed. Here we prepared heparin-taurocholate conjugate (LHT7) to increase circulation time of heparin due to hydrophobic taurocholate and to reduce its adverse effect. In this study, we evaluated whether LHT7 could show anti-tumor and anti-angiogenic effect on mouse glioblastoma in brain of nude mice.

EXPERIMENTAL METHODS
Preparation of LHT7. Taurocholate sodium salt was mixed with N, N-dimethylformamide, triethylamine and 4-nitrophenylchloroformate to synthesize carbonate-taurocholate derivative. And then added 4-methylmorpholine and ethylenediamine to make taurocholate-ethylenamine. LMWH (MW 4.5 kDa) was mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride which was added in this solution for carboxylic group activating then was put N-hydroxysuccinimide. Then added taurocholate-ethylenamine slowly.

Animals and Cell line. 5-7 weeks old Sprague-Dawley (SD) male rats and BALB/C nu/nu nude male mice were purchased from Nara-Bio Company (Seoul, Korea). U87MG cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC, 2013-0228) of Hanyang University.

Ex vivo rat aortic ring assay. Aortas isolated from SD rats were cleaned with cold PBS and cut into 1 mm length separately. The aortic rings were randomly placed into 48 well plate which was precoated with 100 µl growth-factor-reduced (GFR) matrigel (BD Bioscience, USA) and then further added 100 µl of matrigel to overlay. The aortic rings were incubated in a final volume of 500 µl of serum free EBM-2 (Lonza) medium with or without 20 ng/ml VEGF165 (Perprotech) and different concentration of LHT7. The medium was exchanged once every other day. After 6 days, the sprouting microvessel was quantified by Image-Pro Plus 7.0.

Animal study. To establish glioblastoma mouse model, we used U87MG human glioblastoma cell line. Nude mice were anesthetized with an Isoflurane (Hana Pharm) and placed in a stereotactic apparatus modified for mice. The hole was drilled by an
electric drill. $5 \times 10^5$ U87MG cells in 8µl PBS were injected into the right striatum using a 10 µl Hamilton syringe. The needle was left in place for 3 min prior to removal to allow tumor cells to settle at the injection site. After surgery, injected mice were randomly divided into two groups. LHT7 (5 mg/kg b.w.) was intravenously administered via tail vein for 30 days. As control group, PBS was treated for same time. After 30 days, mice were sacrificed and brain sections were nissl stained.

RESULTS AND DISCUSSION
To confirm the effect of LHT7 on angiogenesis, we examined the sprouting of vessels from rat aortic ring assay. The treated VEGF induced sprouting of microvessel from aortic ring, leading to the robust tubular formation from aorta. However, when we co-treated LHT7, the length of sprouting microvessel was significantly decreased in a dose dependent manner. As shown Figure 1, only VEGF-treated group showed microvessel sprouting by 57.39% when compared to control group (no treatment of VEGF). However when VEGF and LHT7 with 1, 5, 10 and 50 µg/ml were co-treated, the percentage of microvessel sprouting was 71.11, 96.08, 115.19 and 144.02%, respectively. These results represent that sprouting vessel was reduced by LHT7 in a dose dependent manner.

![Figure 1: Ex vivo VEGF induced rat aortic ring assay with various concentrations of LHT7. The sprouting microvessel was quantified by Image-Pro Plus 7.0.](image)

To evaluate the effect of LHT7 in vivo model, we intravenously treated LHT7 to glioblastoma bearing mice for 30 days. After 30 days, resected brain sections were carried out with nissl stain. Results showed that the volume of brain tumor in LHT7 treated group was smaller than that of control group (Fig 2, arrow). These results indicated that LHT7 could inhibit tumor growth in brain. As on-going work, we are doing the mechanism study on brain tumor model after LHT7 treatment.

![Figure 2: Effect of LHT7 on glioblastoma model. Black arrow indicates glioblastoma.](image)

CONCLUSION
In this study, we firstly evaluated the effect of LHT7 on glioblastoma model. We confirmed that LHT7 effectively reduced the microvessel sprouting from rat aortic ring assay. Moreover, LHT7 also suppressed the tumor growth in glioblastoma model. These results demonstrated that LHT7 could cure the glioblastoma using anti-angiogenic effect.

REFERENCES

ACKNOWLEDGMENTS
This study was supported by Mid-Career Research program grant (NRF-2012R1A2A1A01012042) and partially by the Brain Korea 21 plus program grant (NRF-22A20130011095) through the National Research Foundation of Korea (NRF) funded by Korea Government.