Novel Hyaluronan Formulation Enhances the Efficacy of Boron Neutron Capture Therapy for Murine Mesothelioma

MASAO SASAI[1], HIROYUKI NAKAMURA[2], NAGAKO SOUGAWA[4], YOSHINORI SAKURAI[3], MINORU SUZUKI[3] and CHUN MAN LEE[1,4]

[1]*Medical Center for Translational Research, Osaka University Hospital, Osaka, Japan;*
[2]*Department of Electronic Chemistry, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Tokyo, Japan;*
[3]*Particle Radiation Oncology Research Center Laboratory, Research Reactor Institute, Kyoto University, Osaka, Japan;*
[4]*Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan*

Abstract. *Background: Malignant pleural mesothelioma (MPM) is a refractory cancer of the pleura caused by asbestos exposure. MPM is difficult to treat because it easily disseminates. Boron neutron capture therapy (BNCT) is a radiotherapy in which cancer cells that selectively take up ^{10}Boron-containing compounds are destroyed, and normal cells are uninjured. Hyaluronan (HA) is a ligand of cluster of differentiation 44 (CD44), that is expressed on MPM cells. Materials and Methods: In order to enhance BNCT for MPM tumors, we developed a novel HA-containing ^{10}B (sodium borocaptate: BSH) formulation (HA-BND-S). We examined the efficacy of HA-BND-S using MPM cells and a mouse MPM model. Results: HA-BND-S preferentially bound MPM cells dose-dependently, and increased the cytotoxicity of BNCT compared to BSH in vitro. HA-BND-S administration significantly increased the survival of MPM tumor-bearing mice compared to BSH at the same ^{10}B dosage in BNCT. Conclusion: Modifying BSH with HA is a promising strategy for enhancing the efficacy of BNCT for therapy of MPM.*

Malignant pleural mesothelioma (MPM) is a fatal disease caused by asbestos exposure, and the disease appears after a long delay, about 40 years after exposure on average. Multimodal therapy is recommended for MPM because the disease easily disseminates. In its early stage, surgery is effective. However, carcinomatous pleuritis cannot be cured by surgery, and the aim of therapy for this stage of disease is to attain remission or dormancy by chemotherapy and radiation therapy; therefore, more effective therapies are needed.

Hyaluronan (HA) is a glycosaminoglycan with high viscosity and a high capacity for holding water. HA plays a crucial role in the progression and metastasis of tumor cells that express large amounts of receptors for HA. One such receptor, cluster of differentiation 44 (CD44), promotes tumor progression by signal transduction when it binds HA (1-3). MPM produces large amounts of HA, and a high concentration of HA in the pleural effusion is evidence for MPM diagnosis (4). HA binding to the CD44 induces MPM cell proliferation and hypotaxis (5). Therefore, various therapies have been designed to inhibit HA–CD44 interaction, including low molecular weight oligosaccharide and CD44 decoys, antibodies, and siRNA (6-8), but their efficacy *in vivo* was unsatisfactory. To combat CD44-expressing tumors, it is not only necessary to inhibit the HA–CD44 interaction, but also to kill the tumor cells. CD44 is expressed on MPM cells but not on normal mesothelial cells (9). Therefore, we hypothesized that HA could be used as a ligand in MPM-targeting therapies.

Boron neutron capture therapy (BNCT) is a cell-selective radiation therapy that uses alpha particles and lithium nuclei produced by the boron neutron capture reaction. These particles only destroy the cells that take up ^{10}B because they do not travel more than 10 μm, which corresponds to the size of a cell (10). BNCT is suitable for treating multiple and diffuse tumors, such as glioblastoma and head and neck tumors (11). Sodium borocaptate (BSH) is used to target MPM tumors in BNCT. To improve this therapy, various carriers for BSH, such as liposomes, have been investigated, but to date, an effective vector has not been developed. For this purpose, we focused on HA because it is stable in living organisms and liposomal formulations, and binds to the CD44 on MPM tumor cells. Herein we developed an HA-based formulation with BSH, and

Correspondence to: Chun Man Lee, Medical Center for Translational Research, Osaka University Hospital, 4-15 Yamada-oka, Suita, Osaka, 565-0871, Japan. Tel: +81 668796551, Fax: +81 668796549, e-mail: tg4c_1211@hp-mctr.med.osaka-u.ac.jp

Key Words: MPM, BNCT, BSH, HA, CD44, hyaluronan formulation.

evaluated its efficacy in BNCT using MPM cells and a murine model of pleural mesothelioma.

Materials and Methods

Mice. Female BALB/cAJcl mice at 6-8 weeks of age were obtained from CLEA Japan (Tokyo, Japan) and kept in standard housing. *In vivo* experiments were performed under a protocol approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine (#21-055-0).

Cell lines. The AB22 murine mesothelioma cell line was kindly provided by Dr. Cleo Robinson and Dr. Bruce WS Robinson, University of Western Australia, Australia (12). The cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum, 1% (v/v).

Animal model. One million AB22 cells were injected into the right intercostal parietal pleura of mice under general anesthesia with a mixture of agents (midazolam, butorphanol tartrate, and medeto-midine) (13). Seven days after injection, tumors were observed at the right intercostal parietal pleura by exploratory thoracotomy, and these tumors led to the death of mice within 20 days after tumor cell injection.

Reagents. HA formulation with BSH (HA-BND-S) was developed by Dr. Nakamura at the Tokyo Institute of Technology (14). Fluorescein-labeled HA was obtained from Iwai Chemicals Company (Tokyo, Japan). HA was obtained from R&D Systems (Minneapolis, MN, USA).

In vitro competitive binding assay for the HA receptor. AB22 cells were seeded into 96-well plates (1×10^4 cells /well). After 24-h incubation, the cells were washed and fixed with 1% paraformaldehyde for 1 h. After washing with washing buffer [phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA)], 3% BSA in PBS was added as a blocking agent, and the cells were treated with or without HA (2.0 mg/ml), a CD44-neutralizing antibody (10 μg/ml, IM7; Thermo Scientific, Rockford, IL, USA), BSH, or HA-BND-S (2 mg/ml or 0.2 mg/ml of HA) for 20 min at 37˚C. The cells were then incubated with 1 mg/ml fluorescein-HA for 20 min at room temperature. After washing, the fluorescence intensity was measured by a fluorescence plate reader, TriStar LB 941 (Berthold Technologies, Bad Wildbad, Germany).

In vitro cytotoxicity of BNCT with HA-BND-S. AB22 cells were seeded (2×10^6 cells/well) into 60-mm dishes, and incubated with HA-BND-S or BSH for 24 h or 1 h, respectively (each 600 ppm ^{10}B). After removing the boron compounds and washing, the cells were transferred into an Eppendorf tube, and neutron irradiation was performed at the Kyoto University Research Reactor for 35 min to a fluence of 1.8×10^{12} neutrons/cm^2. The cells were then washed with PBS and seeded into 96-well plates at 2,000 cells/200 μl/well. The cytotoxicity was assessed 3, 4, and 6 days after neutron irradiation by a tetrazolium assay with Cell Cycling kit-8 (Dojindo, Kumamoto, Japan).

Antitumor efficacy of BNCT with HA-BND-S for murine mesothelioma. Mice bearing mesothelioma were irradiated with a thermal neutron beam at the Japan Research Reactor 4 (JRR-4) 7 days after tumor cell inoculation. The neutron irradiation was performed in a single fraction using thermal beam mode I of the JRR-4 for 17 min to a total fluence of $2.1\text{-}2.4\times10^{12}$ neutrons/cm^2 (thermal neutron flux, $2.0\text{-}2.3\times10^9$ neutrons/cm^2/s; γ-ray absorbed dose, 3.6 Gy/h at a reactor power of 3.5 MW). To evaluate the effect of BNCT on the mesotheliomas, the mice were sacrificed 14 days after irradiation. In a separate experiment, the mice were subjected to various treatments, either irradiated or not, and their survival time after irradiation was recorded.

Statistical analyses. Differences between groups in the survival experiment were determined using the Kaplan–Meier log-rank test. A *p*-value less than 0.05 was considered statistically significant.

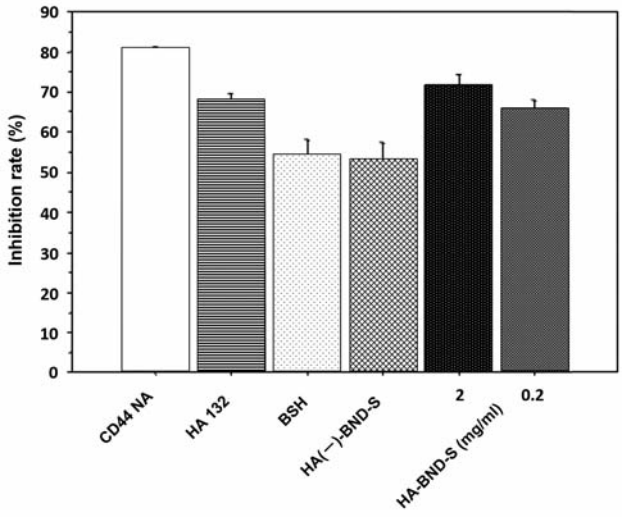

Figure 1. *Competitive inhibition of various compounds of the binding of fluorescein-labeled hyaluronan (HA) to malignant pleural mesothelioma cells. From left to right, the percentage inhibition of a cluster of differentiation 44-neutralizig antibody (CD44 NA), HA 132 kDa (2.0 mg/ml), sodium borocaptate (BSH), BND-S lacking HA [HA(−)-BND-S], HA-BND-S (2 mg/ml HA), and HA-BND-S (0.2 mg/ml HA). The binding was inhibited 20% more with HA-BND-S than with HA(−)-BND-S. The HA binding was inhibited by HA-BND-S in a concentration-dependent manner. Data are mean values±SEM.*

Results

HA-BND-S preferentially binds to MPM cells in vitro. AB22 cells have been previously shown to express large amounts of CD44 on their surface by flow cytometric analysis with IM7, whose epitope lies outside the HA-binding domain of CD44 (15). In an *in vitro* competitive binding assay, the binding of fluorescein-labeled HA to AB22 cells was markedly inhibited by a CD44 neutralizing antibody. The binding was more severely inhibited by a large excess of HA than by BSH or BND-S lacking HA. HA-BND-S inhibited the binding similar to HA, and in a dose-dependent manner (Figure 1). These results suggest that like HA, HA-BND-S binds MPM cells.

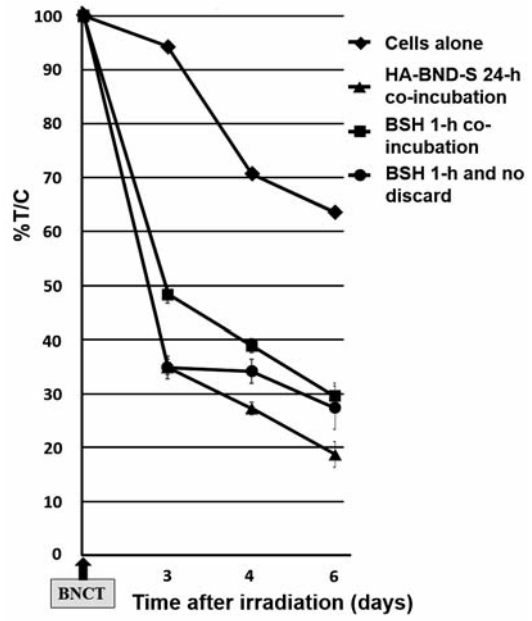

Figure 2. *Cytotoxicity of boron neutron capture therapy (BNCT) with each boron compound for malignant pleural mesothelioma (MPM) cells. The percentage of dead cells after BNCT(% T/C) is shown for MPM cells treated as follows: sodium borocaptate (BSH) 1-h incubation, not washed; BSH 1-h incubation, washed; hyaluronan containing BSH formulation (HA-BND-S) 24-h incubation, washed; and no-boron control. HA-BND-S led to the strongest cytotoxicity. Neutron irradiation was performed to a total fluence of 1.8×10^{12} neutrons/cm^2 at Kyoto University Research Reactor. Data are mean values±SEM.*

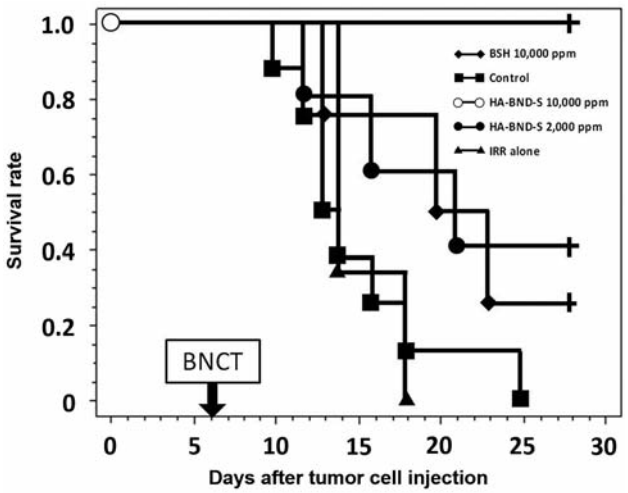

Figure 3. *Survival of malignant pleural mesothelioma (MPM)-bearing mice treated with boron neutron capture therapy (BNCT). All mice were observed for 28 days after tumor inoculation. Survival curves are shown for phosphate buffered saline-treated control, irradiation (IRR) alone, sodium borocaptate (BSH) (10,000 ppm), hyaluronan containing BSH formulation (HA-BND-S) (2,000 ppm) and HA-BND-S (10,000 ppm). The mean survival time was 15.1±1.7 days, 15.3±1.3 days, 19.8±2.5 days, 21.2±7.0 days, and 28 days, respectively. HA-BND-S (10,000 ppm) versus control group was significantly different at $p<0.05$. Data are mean values±SEM.*

BNCT with HA-BND-S is highly cytotoxic to MPM tumor cells in vitro. The cytotoxicity of BNCT with HA-BND-S *versus* BSH was examined *in vitro*. Cells treated with HA-BND-S that was washed-off before neutron irradiation exhibited more cytotoxicity than washed BSH-treated cells or unwashed BSH-treated cells at all time points examined after neutron irradiation (Figure 2). These results suggest that the boron concentration in tumor cells treated with HA-BND-S and then washed was higher than that of cells that remained in the presence of BSH.

BNCT with HA-BND-S results in complete remission of MPM in mice. HA-BND-S (50 or 10 mg ^{10}B/kg) was injected into the pleural cavity of MPM model mice 24 h before BNCT, or BSH (50 mg ^{10}B/kg), or PBS as control was injected into the pleural cavity 1 h before BNCT. The mean survival time of the control mice (PBS, irradiation alone, HA-BND-S alone) was 15.1 days after tumor inoculation, and there was no significant difference among them. Therefore, HA-BND-S (50 mg ^{10}B/kg) alone had no effect on these mice, even though they bore advanced mesothelioma. The mean survival times of mice treated with BSH and with a low dose of HA-BND-S were 19.8 and 18.2 days after tumor inoculation, respectively, and were not significantly different from that of the controls. Notably, however, BNCT with 50 mg ^{10}B/kg HA-BND-S significantly increased the mean survival time of the tumor-bearing mice compared to BNCT with 10 mg ^{10}B/kg HA-BND-S or 50 mg ^{10}B/kg BSH ($p<0.05$ and $p<0.001$, respectively; Figure 3).

Discussion

In this study, we demonstrated that HA-BND-S, like HA, preferentially binds CD44-expressing MPM tumor cells, and that BNCT with HA-BND-S is highly cytotoxic for MPM cells. We also showed that BNCT with HA-BND-S was far superior to BNCT with BSH in suppressing MPM lesions.

To enhance the efficacy of BNCT, it is important to increase the boron concentration in or on the target tumor cells (16). For this purpose, boron-containing liposomes were developed to enhance BNCT efficacy (17). HA, owing to its cationic charge, stabilizes liposomal formulations containing high concentration of boron, and binds CD44, that is abundantly expressed on MPM cells, but not on normal mesothelial cells (9). We, therefore, developed a formulation with HA and BSH to target MPM. HA-BND-S had the CD44-binding activity of HA, and bound MPM cells (Figure

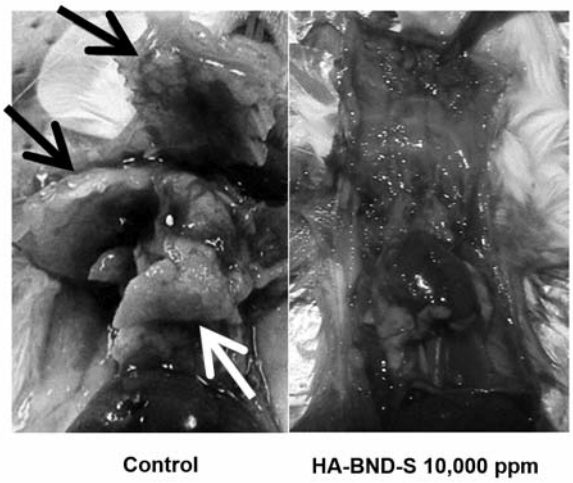

Figure 4. *Macroscopic views of the thorax of malignant pleural mesothelioma (MPM)-bearing mice with and without boron neutron capture therapy (BNCT) treatment with hyaluronan containing sodium borocaptate (BSH) formulation (HA-BND-S). In the untreated control group (left panel), pleural mesothelioma invaded the mediastinum, the heart, and the uninjected side of the pleural cavity. The black and white arrows indicate massive pleural mesotheliomas. In the high-dose HA-BND-S BNCT-treated group (right panel), no mesotheliomas were detected in the pleural cavity 28 days after tumor transplantation. In the BNCT-treated BSH group and low-dose HA-BND-S group, 28 days after tumor transplantation tumors were detected in the thorax of all mice (not shown).*

1). Moreover, BNCT treatment of MPM cells that had been incubated in HA-BND-S and then washed led to a higher cytotoxicity than that of BSH-treated cells that were kept in the BSH-containing medium (Figure 2). These results indicate that the boron concentration on the MPM cells of the HA-BND-S-treated group remained high, even after washing. In our *in vitro* experiments, BNCT killed most of the ^{10}B-treated cells. In addition, the PBS group showed some cell death, indicating that the γ-rays alone had a partially cytotoxic effect.

To examine the effect of BNCT with HA-BND-S *in vivo*, we used MPM-bearing mice. In this model, mice bearing MPM died within 20 days after tumor cell inoculation. Even BNCT with 50 mg ^{10}B/kg BSH, which is used clinically (18), did not demonstrate significant efficacy compared with the PBS control in these mice (p>0.05). Remarkably, BNCT with HA-BND-S resulted in complete remission in the mice bearing MPM for at least 28 days after tumor inoculation (Figure 4). These observations indicate that HA-BND-S administered into the pleural cavity accumulated at the MPM lesions and stayed at the tumors *in vivo*. This explains why none of the HA-BND-S-treated mice that underwent BNCT died during the observation period.

BNCT was performed 1 h after BSH administration, in order to follow the reported procedure for the clinical use of BNCT, and because the BSH concentration in the pleural cavity is thought to decrease over time due to its hydrophilic nature (19). On the other hand, HA-BND-S is hydrophobic, and therefore has bioaccumulation potential, so HA-BND-S was administered 24 h before BNCT treatment to give it time to accumulate at the target cells. The accumulation of fluorescently labeled analogs of the boron compounds was observed on the target cells (data not shown).

BNCT with conventional boron formulations is performed for patients suffering from relapsed MPM in Japan. The aim of this study was to improve the efficacy of the BNCT using a targeting device that is modification of the conventional boron formulation for MPM. Indeed, HA-BND-S successfully enhanced the efficacy of BNCT for MPM *in vivo*. HA compounds were developed as targeting devices for HA receptors (20), and as inhibitors of the interaction between intrinsic HA and CD44 that elicits MPM proliferation. HA is reported to play a crucial role in tumor progression and proliferation (5, 21-24). Furthermore, as the HA of HA-BND-S prevents leakage of negatively charged boron ions from liposomal formulations due to its cationic charge, the high boron content in HA-BND-S is maintained. Studies in the near future examining the selectivity of this compound, its pharmacokinetics, and the boron concentration around tumor cells, are required before its clinical use can be considered.

In summary, we developed a formulation with HA and BSH that markedly enhanced the efficacy of BNCT for MPM *in vivo*. BNCT with this novel boron CD44-tageting formulation has clinical potential as a drug delivery system for MPM therapy.

Acknowledgements

This work was supported, in part, by a grant for research and development of a Nanomedicine from the Health Labour Sciences Research Grant of the Ministry of Health Labour and Welfare, and a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

References

1 Li Y and Heldin P: Hyaluronan production increases the malignant properties of mesothelioma cells. Br J Cancer *85(4)*: 600-607, 2001.

2 Misra S and Heldin P: HA/CD44 interactions as potential targets for cancer therapy. FEBS J *278(9)*: 1429-1443, 2011.

3 Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H and Gunthert U: Expression and modulation of CD44 variant isoforms in humans. J Cell Biol *124(1-2)*: 71-82, 1994.

4 Fujimoto N, Gemba K, Asano M, Fuchimoto Y, Wada S, Ono K, Ozaki S and KishimotoT: Hyaluronic acid in the pleural fluid of patients with malignant pleural mesothelioma. Respiratory Investigat *51(2)*: 92-97, 2013.

5 Nasreen N, Mohammed KA, Hardwick J, Van Horn RD, Sanders K, Kathuria H, Loghmani F and Antony VB: Low molecular weight hyaluronan induces malignant mesothelioma cell proliferation and haptotaxis: role of CD44 receptor in MMC proliferation and haptotaxis. Oncol Res *13(2)*: 71-78, 2002.
6 Ghatak S, Misra S and Toole BP: Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. J Biol Chem *280*: 8875-8883, 2005.
7 Ghatak S, Misra S and Toole BP: Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/AKT cell survival pathway J Biol Chem *277*: 38013-38020, 2002.
8 Misra S, Hascall VC, De Giovanni C, Markwald RR and Ghatak S: Delivery of CD44 shRNA/nanoparticles within cancer cells: perturbation of hyaluronan/CD44v6 interactions and reduction in adenoma growth in $Apc^{Min/+}$ MICE J Biol Chem *284*: 12432-12446, 2009.
9 Asplund T and Heldin P: Hyaluronan receptor is expressed in human malignant mesothelioma cells but not on normal mesothelial cells. Cancer Res *54(16)*: 4516-4523, 1994.
10 Barth RF, Jeffrey A, Coderre M and Vicente GH: Boron neutron capture therapy of cancer: current status and future prospects. Clin Cancer Res *11*: 3987-4002, 2005.
11 Yamamoto T and Matsumura A: Boron neutron capture therapy for glioblastoma. Cancer Lett *262*: 143-152, 2008.
12 Davis MR, Manning LS, Whitaker D, Garlepp MJ and Robinson BW: Establishment of a murine model of malignant mesothelioma. Int J Cancer *52(6)*: 881-886, 1992.
13 Kawai S, Kaneko S and Kurosawa T: Effect of three types of mixed anesthetic agents alternate to ketamine in mice. Exp Anim *60(5)*: 481-487, 2011.
14 Tachikawa S, EL Zaria ME, Inamoto R, Sato S and Nakamura H: Protoporphyrin-lipids: Their synthesis, liposomal formulation and efficient delivery to tumor. Bioorg Med Chem *22(17)*: 4745-4751, 2014.
15 Mikecz K, Dennis K and Shi M: Modulation of hyaluronan receptor (CD44) function *in vivo* in a murine model of rheumatoid arthritis. Arthritis Rheum *42(4)*: 659-668, 1999.
16 Shelly K, Feakes DA, Hawthorne MF, Schmidt PG, Krisch TA and Bauer WF: Model studies directed toward the boron neutron-capture therapy of cancer: boron delivery to murine tumors with liposomes. Proc Natl Acad Sci USA *89*: 9039-9043, 1992.
17 Koganei H, Ueno H, Tachikawa S, Tasaki L, Ban HS, Suzuki M, Shiraishi K, Kawano K, Yokoyama M, Maitani Y, Ono K and Nakamura H: Development of High boron content liposomes and their promising antitumor effect for neutron capture therapy of cancers. Bioconjugate Chem *24*: 124-132, 2013.
18 Witting A, Malago M, Collette L, Huiskamp R, Buhrmann S, Nievaart V, Kaiser GM, Jockel KH, Schmid KW, Ortmann U and Sauerwein WA: Uptake of two ^{10}B-compounds in liver metastases of colorectal adenocarcinoma for extracorporeal irradiation with boron neutron capture therapy (EORTC Trial 11001). Int J Cancer *122(5)*: 1164-1171, 2008.
19 Mehta SC, Boudinot FD and Lu DR: Pharmacokinetics of sodium mercaptoundecahydrododecaborate after intravenous injection in rats. Drug Metab Dispos *23(12)*: 1368-1371, 1995.
20 Culty M, Nguyen HA and Underhill CB: The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan receptor. J Cell Biol *166*: 1055-1062, 1992.
21 Maharijan AS, Pilling D and Gomer RH: High and low molecular weight hyaluronic acid differentially regulates human fibrocyte differentiation PLOs One 6(10), 2011.
22 Wolf D, Schumann J, Koerber K, Kiemer AK, Vollmar AM, Sass G, Papadopoulos T, Bang R, Klein SD, Brune B and Tiegs G: Low-molecular-weight hyaluronic acid induces nuclear factor-kappaB-dependent resistance against tumor necrosis factor alpha-mediated liver injury in mice. Hepatology *34(3)*: 535-547, 2001.
23 Alaniz L, Rizzo M, Malvicini M, Jaunarena J, Avella D, Atorrasagasti C, Aquino JB, Garcia M, Matar P, Silva M and Mazzolini G: Low-molecular-weight hyaluronan inhibits colorectal carcinoma growth by decreasing tumor cell proliferation and stimulating immune response. Cancer Lett *278(1)*: 9-16, 2009.
24 Ropponen K, Tammi M, Pakkinen J, Eskelinen M, Tammi R, Lipponen P, Alhava UAE and Kosma VM: Tumor cell-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. Cancer Res *58(2)*: 342-347, 1998.

Received December 19, 2015
Revised February 5, 2016
Accepted February 12, 2016