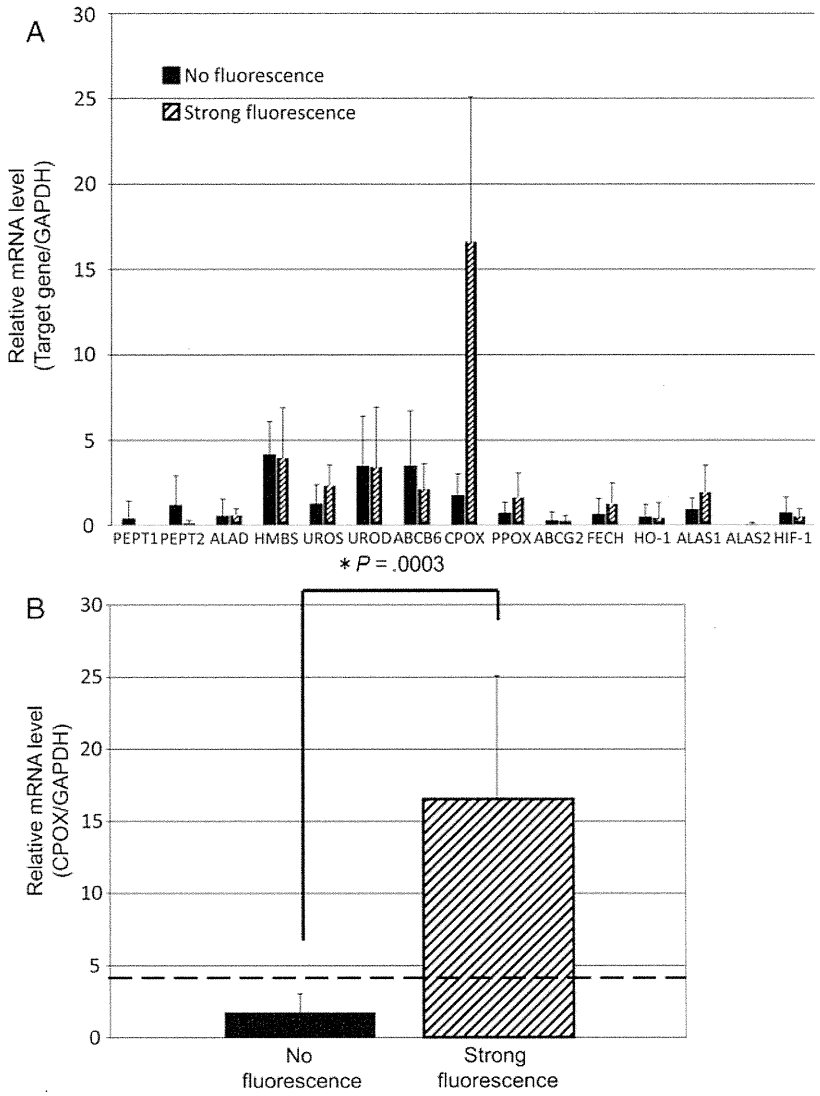

Fig. 4. (A) Comparison of the mRNA levels of the PEPT1, PEPT2, ALAS1, ALAS2, ALAD, HMBS, UROS, UROD, ABCB6, CPOX, PPO, FECH, ABCG2, HIF-1, and HO-1 genes between the no-fluorescence and strong-fluorescence groups. The mRNA levels of these genes are normalized to the mRNA level of GAPDH. Data are expressed as mean ± SD (N = 10). (B) Statistical analysis of CPOX mRNA levels between the no-fluorescence and the strong-fluorescence groups. Data are expressed as mean ± SD (N = 10). A horizontal broken line indicates the best cutoff value for CPOX expression levels that discriminates between strong-fluorescence and no-fluorescence groups.

of malignant gliomas infiltrated by live clonogenic tumor cells and is helpful for the precise resection of those regions. ALA is converted to PpIX in the body (Fig. 2) and emits red fluorescence with the excitation of blue-violet light (Fig. 1B). As PpIX accumulates preferentially in the tumor tissue in comparison with normal tissue, this red fluorescence becomes a good hallmark for discrimination between normal and tumor tissues, especially in malignant gliomas, which have infiltrative characteristics.

Approximately 80% to 90% of malignant gliomas show this red fluorescence in surgery, while only a limited number of brain tumor cases do not. In the surgery for metastatic brain tumors and lesionectomy for radiation necrosis and neurodegenerative disease, white matter around the lesion shows weak and vague fluorescence that also provides us with a hallmark for the surgery.[17] Additionally, in meningioma, some tumors showed the PpIX fluorescence, which is especially helpful for the removal of the infiltrative portion in bone and normal parenchyma.[7] Clinical data indicate that ALA-PDD-assisted resection of malignant gliomas may result in statistically significant prolongation of progression-free survival.[9]

It has previously been suggested that brain tumor fluorescence induced by 5-ALA is correlated to (*a*) cellular density, (*b*) an MIB-1 labeling index as an indicator of tumor cell proliferative activity, (*c*) the area of CD-31 staining as a measure for neovascularity of the tumor,[6] and (*d*) blood-brain barrier permeability.[15] In many cases, however, we have experienced that despite all of the 4 factors being fulfilled, malignant brain tumors did not always exhibit 5-ALA–induced PpIX fluorescence. In fact, we found

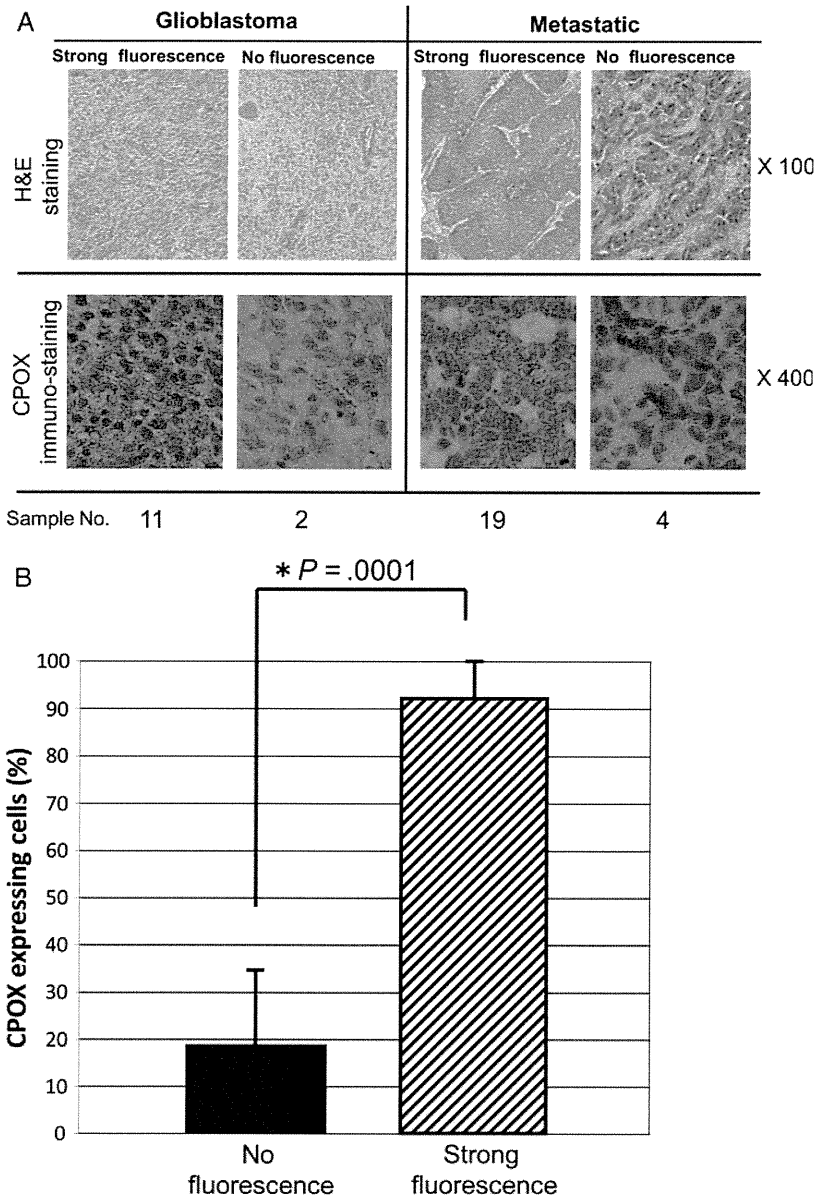

Fig. 5. (A) H&E staining and CPOX-immunohistochemical staining for specimens of metastatic tumors and glioblastomas with strong fluorescence or no fluorescence. CPOX-positive cells were immunologically stained a brown color. Nuclei were stained a blue color by H&E. CPOX is highly expressed in both metastatic tumors and glioblastomas with strong fluorescence. (B) Statistical analysis of CPOX-positive cells (stained brown) between the no-fluorescence and the strong-fluorescence groups. Data are expressed as mean ± SD (N = 10). The percentage of CPOX-positive cells was significantly higher in the strong-fluorescence group than in the no-fluorescence group ($P = .0001$).

that 67% of the metastatic brain tumors and some glioblastomas did not exhibit 5-ALA–induced PpIX fluorescence. In this context, we analyzed the expression levels of major enzymes and transporters involved in the biosynthesis and metabolism of porphyrin to find a new biomarker for the 5-ALA–induced PpIX fluorescence in brain tumors.

The present study provides evidence that the level of mRNA encoding CPOX was remarkably high in malignant brain tumors that exhibited strong red fluorescence of PpIX after administration of 5-ALA (Fig. 4B). Immunohistochemical studies with the CPOX-specific antibody support our findings (Fig. 5B). To our knowledge, the present study is the first report showing that the induction of *CPOX* gene expression is one of the key molecular mechanisms underlying 5-ALA–induced fluorescence of malignant brain tumors. The transcriptional upregulation of the CPOX gene is considered a pivotal step in enhancing the biosynthesis of protoporphyrinogen, which is readily converted to PpIX by the action of protoporphyrinogen oxidase (Fig. 2).

Induction of CPOX *Gene Expression as a Key Molecular Mechanism Underlying 5-ALA-induced Fluorescence*

Our data from qRT-PCR analysis have clearly demonstrated that the high levels of CPOX mRNA are well correlated with the high intensities of 5-ALA-induced fluorescence. CPOX is a mitochondrial enzyme involved in the porphyrin metabolism pathway and plays a role in oxidizing coproporphyrinogen III to produce protoporphyrinogen. It has been demonstrated that transfection of prostate cancer LNCaP cells with a CPOX-expressing vector increases the intracellular accumulation of PpIX in vitro. Moreover, methotrexate (MTX) at nontoxic low concentrations increases the expression of CPOX at both mRNA and protein levels, and MTX pretreatment followed by ALA exposure results in a 3-fold increase in the intracellular PpIX level.[18] As a consequence, the effect of photodynamic therapy was enhanced in MTX-preconditioned cells. Similar results were also observed in skin cancer cell lines in vivo.[19] Although these in vitro experiments do not directly reflect the in vivo situations in brain tumor patients, such observations indirectly support the notion that the induction of CPOX gene expression is one of the key molecular mechanisms underlying 5-ALA-induced fluorescence of malignant brain tumors. Little is known, however, about the tumor-associated transcriptional activation of the *CPOX* gene. The mechanisms involved in the induction of the *CPOX* gene in malignant brain tumors should be elucidated in future studies.

Porphyrins play critical roles in diverse biological processes, such as respiration and oxidative metabolism. Both porphyrin biosynthesis and its intracellular concentration are tightly regulated (Fig. 2). In recent years, evidence has been accumulating to show that ABCB6, one of the human ABC transporters, transports coproporphyrinogen III from the cytoplasm to the mitochondria;[20,21] whereas another ABC transporter, ABCG2, is responsible for the cellular homeostasis of porphyrins and their related compounds.[22,23] Recent studies suggest that ABCG2, a porphyrin efflux pump, is downregulated in tumors, and thereby PpIX is facilitated to accumulate in colorectal and cervical cancers.[24] In the present study, the mRNA level of ABCG2 was somewhat lower in the brain tumors with high levels of ALA-induced fluorescence compared with those without ALA-induced fluorescence (Fig. 2A). However, there was no statistical significance. On the other hand, imatinib mesylate and other protein kinase inhibitors reportedly enhance the efficacy of photodynamic therapy by inhibiting ABCG2.[23,25,26] Therefore, both the induction of the *CPOX* gene and the inhibition of ABCG2 would increase 5-ALA-induced PpIX accumulation and thereby enhance the efficacy of 5-ALA/pophyrin-based PDD and therapy of malignant brain tumors.

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References

1. Lipson RL, Baldes EJ. The photodynamic properties of a particular hematoporphyrin derivative. *Arch Dermatol*. 1960;82:508–516.
2. Lipson RL, Baldes EJ, Olsen AM. Hematoporphyrin derivative: a new aid for endoscopic detection of malignant disease. *J Thorac Cardiovasc Surg*. 1961;42:623–629.
3. Lipson RL, Baldes EJ, Olsen AM. Further evaluation of the use of hematoporphyrin derivative as a new aid for the endoscopic detection of malignant disease. *Dis Chest*. 1964;46:676–679.
4. Lipson RL, Baldes EJ, Gray MJ. Hematoporphyrin derivative for detection and management of cancer. *Cancer*. 1967;20(12):2255–2257.
5. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. *J Neurosurg*. 2000;93(6):1003–1013.
6. Stummer W, Reulen HJ, Novotny A, Stepp H, Tonn JC. Fluorescence-guided resections of malignant gliomas—an overview. *Acta Neurochir Suppl*. 2003;88:9–12.
7. Kajimoto Y, Kuroiwa T, Miyatake S, et al. Use of 5-aminolevulinic acid in fluorescence-guided resection of meningioma with high risk of recurrence. Case report. *J Neurosurg*. 2007;106(6):1070–1074.
8. Lacroix M, Abi-Said D, Fourney DR, et al. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *J Neurosurg*. 2001;95(2):190–198.
9. Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol*. 2006;7(5):392–401.
10. Bottomley SS, Muller-Eberhard U. Pathophysiology of heme synthesis. *Semin Hematol*. 1988;25(4):282–302.
11. Kennedy JC, Pottier RH, Pross DC. Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B*. 1990;6(1–2):143–148.
12. Kaneko S. Intraoperative photodynamic diagnosis of human glioma using ALA induced protoporphyrin IX. *No Shinkei Geka*. 2001;29(11):1019–1031.
13. Stummer W, Stocker S, Wagner S, et al. Intraoperative detection of malignant gliomas by 5-aminolevulinic acid-induced porphyrin fluorescence. *Neurosurgery*. 1998;42(3):518–525; discussion 525–516.

14. Utsuki S, Oka H, Sato S, et al. Possibility of using laser spectroscopy for the intraoperative detection of nonfluorescing brain tumors and the boundaries of brain tumor infiltrates. Technical note. *J Neurosurg*. 2006;104(4):618–620.
15. Ennis SR, Novotny A, Xiang J, et al. Transport of 5-aminolevulinic acid between blood and brain. *Brain Res 10*. 2003;959(2):226–234.
16. Krishnamurthy P, Ross DD, Nakanishi T, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem*. 2004;279(23):24218–24225.
17. Miyatake S, Kuroiwa T, Kajimoto Y, Miyashita M, Tanaka H, Tsuji M. Fluorescence of non-neoplastic, magnetic resonance imaging-enhancing tissue by 5-aminolevulinic acid: case report. *Neurosurgery*. 2007;61(5): E1101–1103; discussion E1103–1104.
18. Sinha AK, Anand S, Ortel BJ, et al. Methotrexate used in combination with aminolaevulinic acid for photodynamic killing of prostate cancer cells. *Br J Cancer*. 2006;95(4):485–495.
19. Anand S, Honari G, Hasan T, Elson P, Maytin EV. Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells in vitro and in vivo. *Clin Cancer Res*. 2009;15(10): 3333–3343.
20. Krishnamurthy P, Xie T, Schuetz JD. The role of transporters in cellular heme and porphyrin homeostasis. *Pharmacol Ther*. 2007;114(3): 345–358.
21. Krishnamurthy PC, Du G, Fukuda Y, et al. Identification of a mammalian mitochondrial porphyrin transporter. *Nature*. 2006;443(7111):586–589.
22. Wakabayashi K, Tamura A, Saito H, Onishi Y, Ishikawa T. Human ABC transporter ABCG2 in xenobiotic protection and redox biology. *Drug Metab Rev*. 2006;38(3):371–391.
23. An R, Hagiya Y, Tamura A, et al. Cellular phototoxicity evoked through the inhibition of human ABC transporter ABCG2 by cyclin-dependent kinase inhibitors in vitro. *Pharm Res*. 2009;26(2):449–458.
24. Gupta N, Martin PM, Miyauchi S, et al. Down-regulation of BCRP/ABCG2 in colorectal and cervical cancer. *Biochem Biophys Res Commun*. 2006;343(2):571–577.
25. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. *Cancer Biol Ther*. 2005;4(2):187–194.
26. Liu W, Baer MR, Bowman MJ, et al. The tyrosine kinase inhibitor imatinib mesylate enhances the efficacy of photodynamic therapy by inhibiting ABCG2. *Clin Cancer Res*. 2007;13(8):2463–2470.

硼素中性子捕捉療法について

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1 はじめに

近年、定位放射線治療に代表される高精度放射線照射技術の開発は目覚ましく、強度変調放射線治療（Intensity modulated radiotherapy(IMRT)）やサイバーナイフ等、最先端の医療機器を使用した放射線治療が可能な施設が国内で増加している。また、動体追跡放射線治療（Image-guided radiotherapy（IGRT））は、わが国が世界に先駆けて開発した誇るべき放射線治療技術でもあり、動きのある体幹部腫瘍などに対しても空間的線量集中が可能となっている。しかしながら原発性悪性脳腫瘍である悪性神経膠腫など、明確な輪郭を持たず正常組織に浸潤性に発育する腫瘍に対しては、いかに高精度に線量集中を行っても制御は難しい。

そこで我々は、悪性神経膠腫に対する治療に、腫瘍細胞選択的粒子線治療である硼素中性子捕捉療法（Boron neutron capture therapy(BNCT)）を積極的に取り入れ臨床研究を行ってきた[1]。BNCTで利用する抗腫瘍効果は、硼素－10の中性子捕獲反応から得られる粒子線であり、粒子線治療の一種とされるが、陽子線や炭素線治療とは全く異なる原理を有した治療法である。本稿ではBNCTの最近の話題と今後の展望について紹介する。

2 BNCTの概念と適応疾患

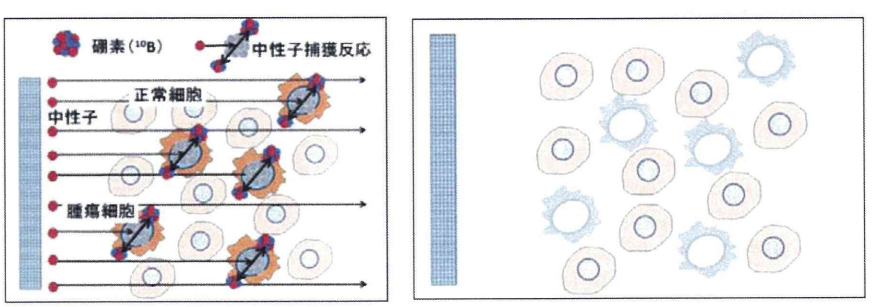
BNCTでは、硼素化合物に与えられた腫瘍探索性と、腫瘍細胞選択照射という他の放射線治療が持ち得ない特長を生かすことにより、浸潤性に発育する腫瘍や腫瘍体積が大きくかつ形状が不整な腫瘍、既放射線治療例など、通常の放射線治療や最先端の放射線治療をもってしても適応が困難である疾患を、治療の対象とすることが可能である。

硼素（Boron、B）の安定同位体である^{10}Bは、エネルギーの低い中性子である熱中性子を高率に捕獲し、ヘリウム原子核（α粒子）とリチウム反跳核に分裂する。この反応を中性子捕獲反応という（図1）。この反応によって生じるヘリウム原子核、リチウム反跳核は、分裂後それぞれ9μm、4μmと腫瘍細胞1個に相当する飛程で動き停止し、その間に全エネルギーを放出する高LET（linear energy transfer）の粒子線であり、殺細胞効果は非常に大きい。この反応を治療に応用したのがBNCTである。あらかじめ腫瘍に集積性を有する硼素化合物を投与し、その後に中性子を患部に照射すれば、腫瘍内で生じた中性子捕獲反応により放出される高LET粒子線の飛程が腫瘍細胞の大きさを超えないため、腫瘍細胞周囲の正常組織は温存され、腫瘍細胞のみが死滅する[2]。

悪性神経膠腫、特に膠芽腫は、治療抵抗性を示すきわめて予後不良の原発性脳腫瘍である。その平均生存期間は診断から約1年とされ、過去20年間生存率に大きな改善はみられていない。その原因として血液脳関門や薬剤耐性機構の存在などが指摘されているが、最も大きな原因は腫瘍の浸潤性性格にあると言える。悪性神経膠腫の辺縁は明瞭ではなく、腫瘍細胞は画像上の造影域を越え、少なくとも周囲脳2cmまでは存在するとされる。そのため、腫瘍の造影域を外科的に全摘出行い得ても、浸潤部からの再発は必至であり、放射線・化学療法を組み合わせる必要がある。BNCTは悪性神経膠腫を最適な適応疾患として発展してきた。

3 海外におけるこれまでのBNCT

現在、本邦を中心に発展途上を遂げているBNCTであるが、臨床試験はかつて欧米でも盛んに行われた。BNCTの原理が提唱されて以後、臨床応用へ向けた開発研究が急速に進められ、1951年には医療用原子炉（ブルックヘブン国立研究所（BNL）研究炉、米国）が作られた。1953年から脳腫瘍患



A 硼素中性子捕捉療法（BNCT）では、あらかじめ腫瘍選択性を有するホウ素（^{10}B）化合物の投与を行う。硼素（^{10}B）化合物投与後に、低エネルギーの中性子を照射することで、^{10}Bが中性子と核反応を生じ、そこから生じたヘリウム原子核（アルファ粒子）とリチウム反跳核で、腫瘍細胞を選択的に破壊する。

B この中性子捕獲反応を腫瘍細胞で選択的に生じさせることで、浸潤部においても正常細胞を温存しつつ腫瘍細胞のみが破壊される。

図1　硼素中性子捕捉療法（BNCT）における細胞選択的照射の理論図

者に対するBNCTが開始され、BNLおよびその後のマサチューセッツ工科大学炉（MITR）での臨床研究は1961年に終了し、当時のホウ素化合物が腫瘍選択性に乏しかったこと、熱中性子線の深達性が悪いことなどから、血中ホウ素濃度が高く、正常組織の障害が生じた。その後中性子線・硼素化合物に改良が行われ、米国では単剤のホウ素化合物（BPA）を用い、組織深達性で勝る熱外中性子を用いた非開頭照射が1999年まで行われた。しかし本試験での生存期間は13～15ヵ月と治療効果がわずかであり、中性子照射線量の増加を試みたところ、生存期間が延長したが深刻な中枢神経合併症が生じたため、現在米国でのBNCTは困難となっている。この米国での臨床試験には硼素化合物の投与プロトコール以外に、照射後の評価に大きな問題があったと考えられている。すなわち、当時の試験では、放射線障害・壊死の画像評価が不十分で、画像上の造影域が増大した症例がすべて再発症例と評価された結果、これをBNCTでの線量不足とみなし、線量増加が行われた可能性が高い。

欧州においは、これまでにオランダ、チェコでの BSH を用いた臨床試験、スウェーデン、フィンランドでの BPA を用いた臨床試験等がある。注目すべきは最近スウェーデンのグループが行った BPA の投与量増量試験（900mg/kg）であり、これによってより均一に高濃度のホウ素を腫瘍に集積させる試みである[3]。2001 年から 2003 年に本手法で新規診断膠芽腫を治療し、生存期間中央値（MST）が 17.7 ヵ月（N=29）と、BNL の成績（BPA（250～330mg/kg）、MST 12.8 ヵ月、 N=53）[4]に比較して有意に良好であった。またこの試験では、現在標準治療となった化学療法剤・テモゾロミドを BNCT に併用することで、治療成績が向上することも示している。

4 これまでの当施設での臨床経験

これまでに臨床試験で使用されてきた硼素化合物は、BSH（sodium borocaptate）と必須アミノ酸フェニルアラニンの誘導体であるBPA（boronophenylalanine）の2種類のみである。BSHは通常、血液脳関門を通過できないため、正常の脳組織には浸透しないが、悪性脳腫瘍では血液脳関門が破壊されているためにBSHが浸透し、周囲脳組織の間に硼素濃度の集積勾配が形成される。BPAはアミノ酸トランスポーターを介して、増殖の盛んな腫瘍細胞により多く取り込まれる。BPAは正常脳へも集積することや増殖の停止した休止期腫瘍細胞には取り込まれにくい弱点を有していた。我々はこれら2種類の硼素化合物を併用するプロトコールを考案し、腫瘍内の硼素分布の不均一を低減させることを試みてきた[5]。また医療照射設備の進歩から、中性子線の組織深達性は格段に改善し、現在は原子炉内での開頭手術は不要となっている。BNCTが成功するか否かは、硼素化合物の腫瘍細胞選択的集積に負うところが非常に大きい。開頭術中中性子照射を行う場合には、硼素化合物投予後に腫瘍組織を採取し、硼素の集積を実測できたが、非開頭BNCTが可能となってからは実測値が得られなくなった。照射線量は正常脳組織により規定されるが、BPAの集積はわずかながら個体差もあり、特に再発腫瘍、既放射線治療例では注意が必要となる。これを解決したのがPET検査である。BNCT用治療薬BPAを^{18}Fでラベルした^{18}F-BPAをトレーサーとした ^{18}F-BPA-PET検査（図２）を施行することで、BNCT施行前に腫瘍と正常組織の硼素の取り込み比（Tumor/Normal brain ratio: T/N ratio）を求めることが可能である[6]。これまでの我々の解析によると、BPA-PETにおける膠芽腫のT/N比は、新規診断例で4.3、腫瘍再発例で3.9と活動性の病態においては有意差がみられず、全体でのL/N比は4.1であった。また、既放

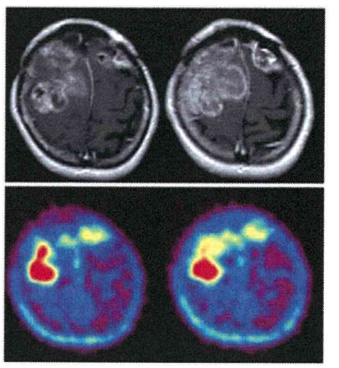


図２　硼素中性子捕捉療法施行前の ^{18}F-BPA PET
再発悪性髄膜腫症例のガドリニウム造影 MRI（上段）と ^{18}F-BPA PET（下段）の対比を示す。本例は、複数回の定位的放射線治療を受けた後に再発を生じている。造影領域が PET トレーサーの集積に一致しないことがポイントであり、BNCT では高集積を認める病変のみに治療効果が及び、単に過去の治療の影響から造影を受けた部位は照射されない。

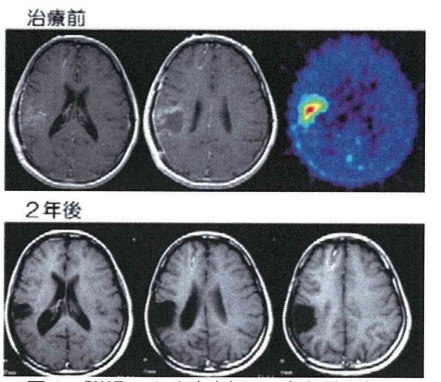


図３　BNCT による治療経過（新規診断例）
新規診断膠芽腫症例に対して長期間の局所制御が得られている。照射前の ^{18}F-BPA PET は高集積を示し、２年後の MRI でも局所再発なく経過している。

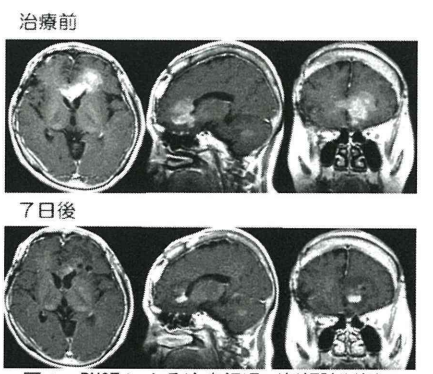


図４　BNCT による治療経過（新規診断例）
新規診断膠芽腫症例に対して、BNCT 後早期から著名な腫瘍縮小効果が得られている。

射線治療例では腫瘍再発と放射線壊死の鑑別も可能であり、本PET検査はBNCTの適応判断・治療計画のみならず治療後の病態診断に対しても有用であることがわかってきた[7]。

2007年までの新規診断膠芽腫の生存期間は、従来の開頭摘出手術+X線分割外照射の成績を有意に上回り、BNCT治療例の生存期間中央値（MST）は15.6ヵ月（ハザード比：0.4）であった。また、既存の硼素化合物では前述の我々のプロトコールにおいても深部線量の不足、細胞レベルでの不均一性が解消されたとは言い難く、X線分割外照射の併用およびBPAの増量・長時間投与を導入し、MSTは23.5ヵ月とさらなる生存期間の延長傾向を示すことができた[8]（図3、4）。また最近では、YamamotoらがBNCTの自験例から、新規診断膠芽腫における開頭・術中照射群と非開頭・外照射群の治療成績を比較し報告している。これによれば、開頭・術中照射群ではBSH単剤を用い、非開頭・外照射群では我々と同様、BSH・BPA（250mg/kg）の併用にX線分割外照射を組み合わせ、MSTがそれぞれ23.3（N=7）、27.1（N=8）ヵ月と非常に良好である[9]。新規診断例におけるX線分割外照射の併用は、既放射線治療例における再照射（図5）と同様、腫瘍細胞選択性を有するBNCTならではの強みであり、標準治療に上乗せすることも可能な放射線治療である。BNCTの治療回数は年々増加傾向にあったが、国内2箇所の医療用原子炉（日本原子力研究開発機構4号炉（JRR4、茨城県・東海村）、京都大学原子炉実験所（KUR、大阪府・熊取町））は、共に長期間の補修・メンテナンスに入り、本邦でのBNCTは休止状態にあった。昨年になってようやくJRR4（2010年3月）、KUR（2010年6月）が再稼働し、医療照射は本格的に再開した。

5 悪性髄膜腫に対する挑戦

悪性髄膜腫は膠芽腫と同様に、周囲脳への浸潤性発育を伴う制御困難な脳腫瘍である。我々はこれまでに、悪性髄膜腫に対してもBNCTによる挑戦を行ってきた[10]。再発悪性髄膜腫12例に対して20回のBNCTを施行し、全例複数回の手術や放射線治療後の再発患者である。適応の判断にはBPA-PETを使用し、L/N比は平均3.8であった（図2）。初回BNCT時の最小腫瘍線量は18.8〜50.7Gy-Eqと計算され、全例で腫瘍体積の縮小を認めた。BNCT後の全体の生存期間は平均15ヵ月、初発時からの生存期間は94ヵ月であった。局所制御は良好であったが、全身への転移・髄腔内播種が主な死因となった。髄膜腫は発生母地が脳表に近く、BNCT治療においては比較的有利な例が多いが、多発性に再発・増大を繰り返す例が多く、単独・単回照射での制御は困難である。我々のこれまでの治療経験から、悪性髄膜腫がBNCTに良好に反応する腫瘍群であることを示したが、再発例では既に複数回の放射線治療を受けていることが多く、今後は初回治療時にBNCTを用いることで予後の改善に期待できると考えている。

6 多施設共同第2相臨床試験について

将来的な展望として、BNCTがさらなる発展を遂げるためには、より腫瘍選択性を有する強力な硼素化合物の開発が重要な課題と指摘される。しかしながら、そもそも原子炉を用いて治療を行う限り、BNCTは臨床研究の域を脱することはできず、試薬開発にとどまる現状では、薬剤開発という創薬シーズを刺激することは困難である。そこでBNCTが

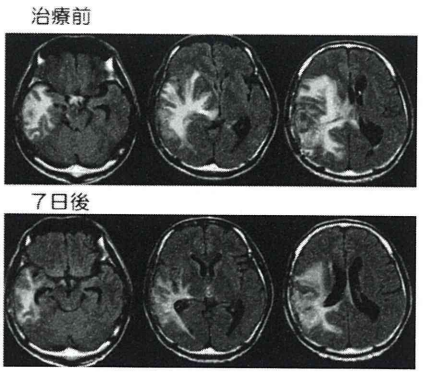


図5 BNCTによる治療経過（再発既放射線治療例）
既放射線治療、再発膠芽腫症例に対しても、BNCT後早期から脳浮腫が軽減しているのがわかる。同時に正中線変位は解消され、神経学的症状も改善が得られた。

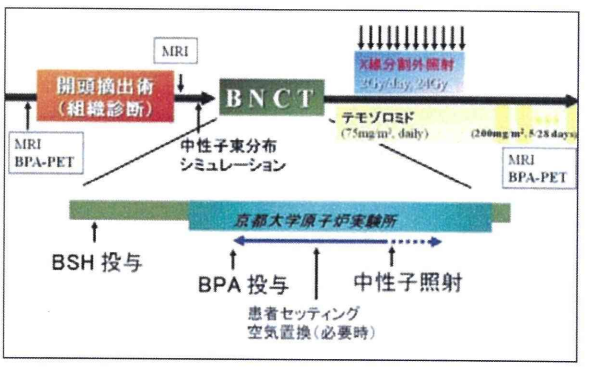


図6 原子炉BNCTによる多施設共同第2相臨床試験（進行中）のプロトコール

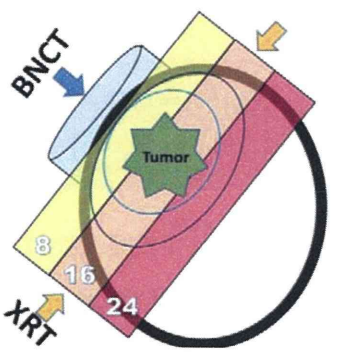


図7 BNCTとX線分割外照射併用治療の概略図

医療として認知されるには、まず原子炉から脱却しなければならない。最近、脳腫瘍での成績の向上や他臓器への応用など多方面からの注目もあり、加速器中性子源の開発研究に拍車がかかっている。医療用中性子源としての加速器が実現すれば、医療機器としての申請が可能となり、BNCTが医療承認を目指す“治験”という枠組みに参入できるようになる。現在、世界中で医療用加速器が開発研究されているが、国内では京都大学原子炉実験所内に設置されており、2010年秋の治験開始を目指し準備が進められている。

我々はその準備段階として、これまでの臨床経験をふまえた新規治療プロトコールを立案し、原子炉BNCTによる多施設共同第2相臨床試験（UMIN000002385, NCT00974987）を立ち上げ、症例登録を開始した。

本試験は、新規診断の膠芽腫を対象とし、プロトコールにはこれまでの我々の経験が集約されている（図6）。初発膠芽腫を対象として、主要評価項目を全生存期間、副次評価項目を腫瘍縮小効果と有害事象の発現とし、BNCT及びX線分割外照射（24Gy）（図7）にテモゾロミドを併用した放射線化学療法の治療効果を検討することを目的とした。登録症例の主な適格規準は次のように定めた。

1）手術により病理組織学的に膠芽腫の診断が得られている患者

2）MRI画像において、次のことが確認されている患者。A. 腫瘍がテント上、一側半球に限局し、最深部が頭皮より6 cm以内の症例（最深部が6 cm以上であっても、腫瘍摘出腔への空気置換により照射可能と判断した症例は適応とする）、B. 単発であり、播種を認めない。

3）同意取得時年齢が15歳以上75歳以下の患者

4）Karnofsky Performance Scale（KPS）が60%以上の患者

また治療のプロトコールは、1）BNCT、2）X線分割外照射：2Gy/日x12日、3）テモゾロミド併用投与（X線分割外照射終了まで75mg/m^{2}連日）、4）テモゾロミド維持療法（X線分割外照射終了後150-200mg/m^2、5日間投与23日休薬を繰り返す）で行い、2年間の患者登録の後、2年間の追跡調査を行うこととした。以上の実施計画を作成し、臨床研究情報センターに登録および参加施設間での調整を行い、本年3月から施設・症例登録を開始している。目標症例数は、第2相臨床試験であるがテモゾロミドによる標準治療との比較を視野に、これまでの我々のBNCT治療成績をもとに統計学的解析を行い、45例とした。

7 BNCTの今後

加速器BNCTが臨床応用されれば、照射の自由度は増し、現時点では困難な、分割照射や複数回照射、多門照射など様々な応用が可能となる。また今後は、動物実験でその有効性が示されている、分子標的薬やナノテクノロジー、ドラッグデリバリーシステムなどの手法を用いたホウ素化合物の臨床応用も期待される[2]。現在BNCTは、悪性脳腫瘍に加え、再発頭頸部腫瘍、多発肝腫瘍、胸膜中皮腫に対しても、その適応を拡げている状況にある。脳腫瘍はもちろん、これらの新規適応疾患に対しても、BNCTが有用な新規治療法になり得るという科学的根拠を、臨床試験を通じて明らかにする必要がある。

参考文献

1)川端信司ほか: PET journal 6: 26-9, 2009.
2)Barth RF, et al: Clin Cancer Res 11: 3987-4002, 2005.
3)Skold K, et al: Acta Neurol Scand, 2009.
4)Diaz AZ: J Neurooncol 62: 101-9, 2003.
5)Kawabata S, et al: J Neurooncol 65: 159-65, 2003.
6)Imahori Y, et al: Clin Cancer Res 4: 1825-41, 1998.
7)Miyashita M, et al: J Neurooncol 89: 239-46, 2008.
8)Kawabata S, et al: J Radiat Res (Tokyo) 50: 51-60, 2009.
9)Yamamoto T, et al: Radiother Oncol 91: 80-4, 2009.
10)Miyatake S, et al: Neurosurgery 61: 82-90, 2007.

Current Organ Topics:

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Ⅱ．悪性脳腫瘍に対するホウ素中性子捕捉療法

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〔*Jpn J Cancer Chemother* **38**(6): 927-932, *June*, 2011〕

はじめに

ホウ素中性子捕捉療法（boron neutron capture therapy，BNCT）は原理上腫瘍に対する細胞選択的照射が可能な唯一の放射線治療法である。本稿ではBNCTの原理，悪性脳腫瘍（悪性神経膠腫と悪性髄膜腫）の治療例，治療成績，放射線壊死の治療，今後の展望等を述べていきたい。

1．BNCTの原理

BNCTは抗がん剤による化学療法と粒子線による放射線療法の双方の特徴をもつ治療法である。その原理を図1に示す[1]。まず腫瘍細胞に選択的に硼素化合物を集積させ，そこに中性子を照射する。硼素化合物には毒性はなく，治療に用いる熱もしくは熱外中性子にもほとんど細胞を壊す作用はないが，硼素原子（^{10}B）に中性子が衝突したときに発生するアルファ線とリチウム線（粒子線）ががんを破壊する。この粒子の飛距離ががん細胞1個に相当するので，硼素化合物をがん細胞に集積できれば，がん細胞のみを破壊し，硼素の入っていない正常の細胞は破壊を免れて残ることが可能となる。BNCTでがんを破壊するのは中性子ではなく，この粒子線であり，このような細胞選択的な放射線による癌の破壊はBNCT以外には存在しない。

BNCTの成否は硼素化合物の腫瘍への選択的集積と中性子の腫瘍への到達が決定する。硼素化合物の腫瘍への選択的集積は脳腫瘍の場合，以下の2点を利用して可能となる。まず使用する硼素化合物のうちBSH（sodium borocaptate）は静脈内投与により，破綻した血液脳関門（BBB）から腫瘍に受動的に集積され，正常脳ではBBBが保たれるため，BSHの集積は起こらない。今一つの化合物がBPAであり，これは必須アミノ酸であるphenylalanineを硼素で修飾した化合物である。よって蛋白代謝の亢進した腫瘍組織では能動的に集積する。この治療用化合物をフッ素ラベルしたものをトレーサーとして利用するのがF-BPA-PETである。このPETでBPAの集積が確認できれば，その腫瘍のX線に対する感受性を問わず，BNCTは必ず効果を発揮し，その適応決定および線量評価に本PETは有用である。悪性神経膠腫でのF-BPA-PETの代表例を図2Aに示す。図2Bに示すGd造影T1強調画像にほぼ一致して，この症例では対側正常脳の7倍のBPAの集積を認めた。つまり，同部位に腫瘍と正常細胞が混在しておれば，BPA単独でも，正常細胞に比べて腫瘍には7倍の粒子線が付与できることを示している。

2．悪性神経膠腫に対する治療効果

この症例はbiopsyでgrade 3以上の悪性神経膠腫と診断され，BNCTを目的に紹介された。図2にPETと造影MRIを示した。本例の治療経過を図3に示す。BNCT後1週間で大部分の造影域が消失し，この結果からも先のPETによる治療効果の予測が有用であることが確認できる。別の再発膠芽腫の症例に対するBNCT

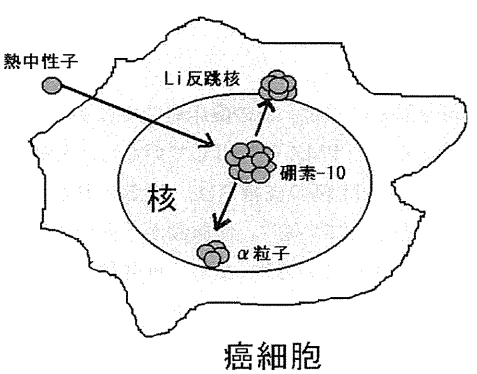

図1　BNCTの原理

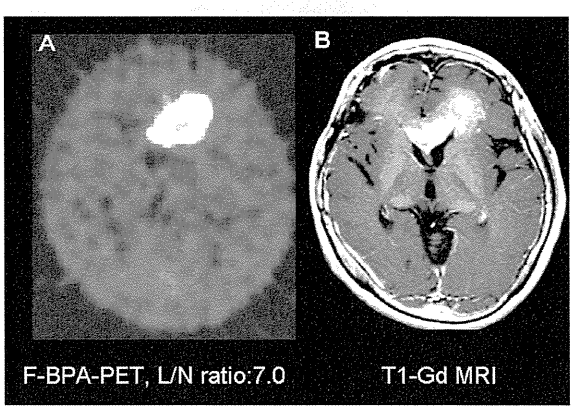

図2

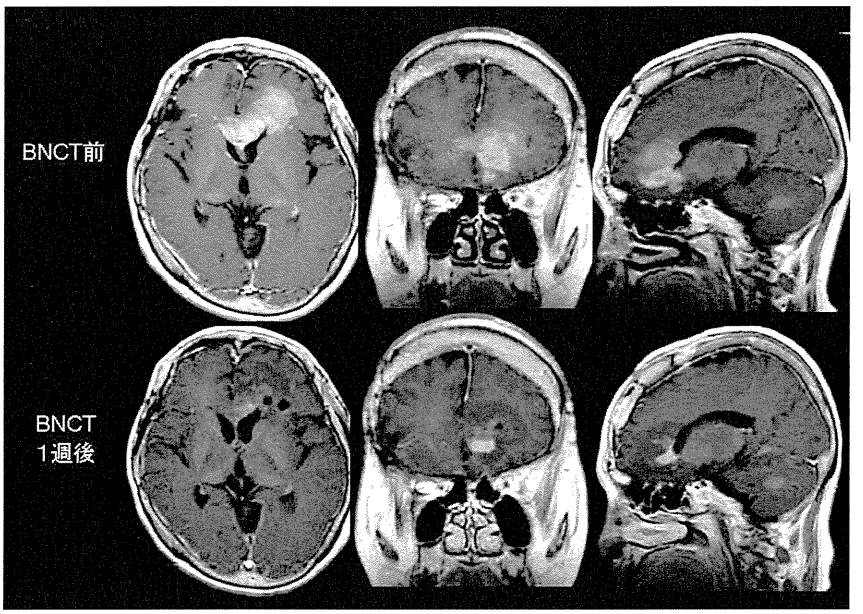

図 3

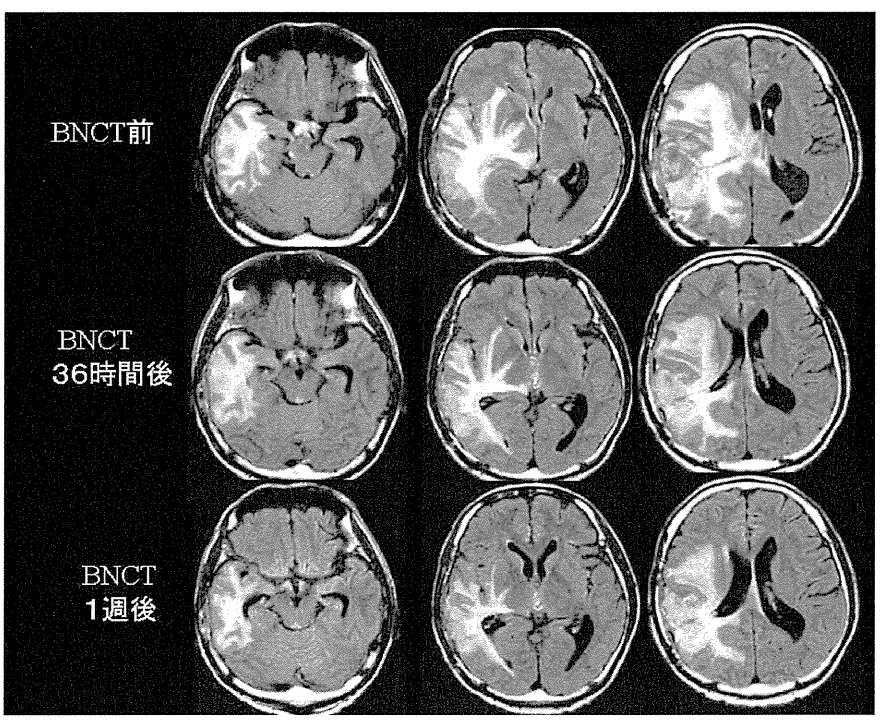

図 4 再発膠芽腫

の早期治療効果を図4に紹介する。この症例も1週間という短期間で麻痺，失語症の改善を認めた[2]。

新規診断膠芽腫におけるBNCTの効果を図5に示す。この症例はgross total resection後，BNCTと30 GyのX線外照射を加え，様子を観察している。脳放射線壊死を発症し，右上肢の麻痺が悪化したものの，テモゾロミド（TMZ）の服用なしで，5年間再発を認めていない。

2007年までにBNCTにて治療した新規診断膠芽腫の生存曲線を図6に示す。この臨床研究では明らかな再発を確認するまで，TMZを使用していない。X線外照射を加えた後半の11例の成績では，23.5か月という生存期間中央値を示している[3]。他施設からもほぼ同様の治療成績が公表されている[4]。また，再発神経膠腫に対しても優れた腫瘍制御を経験している[5]。

3．悪性髄膜腫に対する治療効果

悪性神経膠腫とともに悪性髄膜腫も難治性悪性脳腫瘍

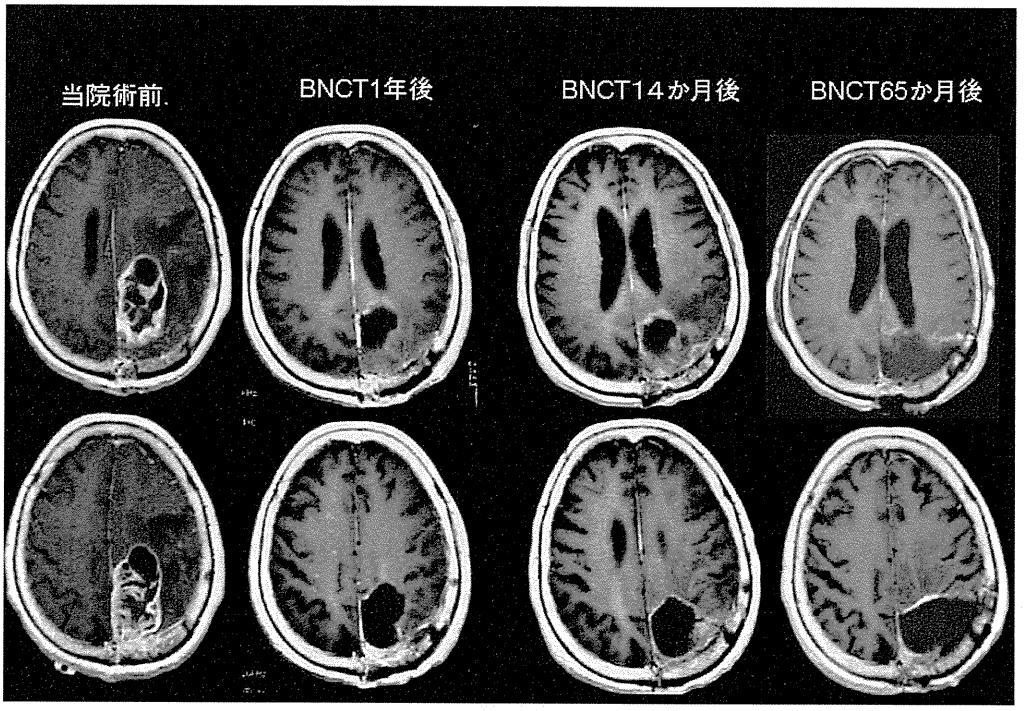

図 5　新規診断膠芽腫

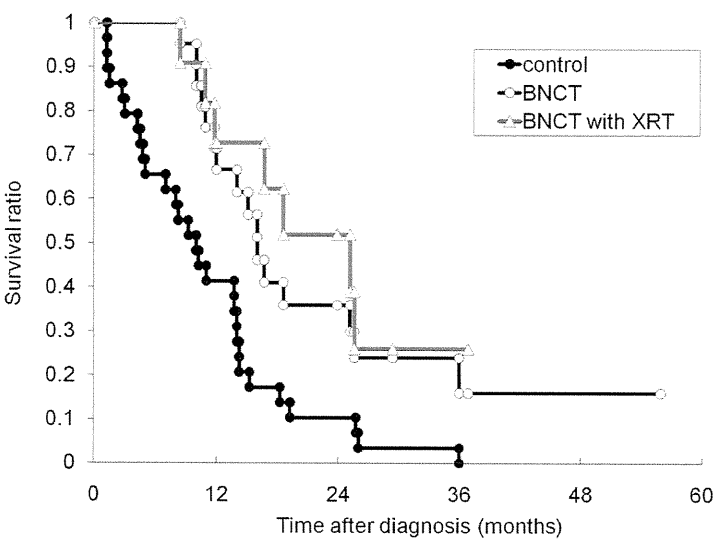

図 6　新規診断膠芽腫の生存曲線

であり，治療に難渋することも多い。図7に anaplastic meningioma に対する BNCT の治療例を示す。この症例は数回の手術，SRS 後に多発性に再発を認めた症例であるが，BNCT により見事に腫瘍制御が可能となり，また自立歩行も可能となった[6]。われわれは 2011 年 2 月現在で 18 例の悪性髄膜腫に本法を実施している。すべての症例で腫瘍の縮小効果を認めているが[7]，全身転移や照射外再発が問題である。

4．症候性脳放射線壊死の治療

高線量放射線治療の宿命として，脳放射線壊死が問題となる。もちろんこの放射線壊死を避けるような線量計画が重要であるが，腫瘍選択的な放射線治療である BNCT といえど，すでに放射線治療歴のある再発症例に本治療を行えば，症候性脳放射線壊死を惹起することはやむを得ない。最近，脳放射線壊死に対して，抗血管内皮増殖因子抗体であるベバシズマブ(商品名アバスチン)が著効を示すという報告がなされ[8]，われわれも積極的に使用したところ，多くの症例で著効を認めた。図8に BNCT 後 30 Gy の X 線照射を加えた膠芽腫の症例に発生した症候性脳放射線壊死に対するベバシズマブの効果

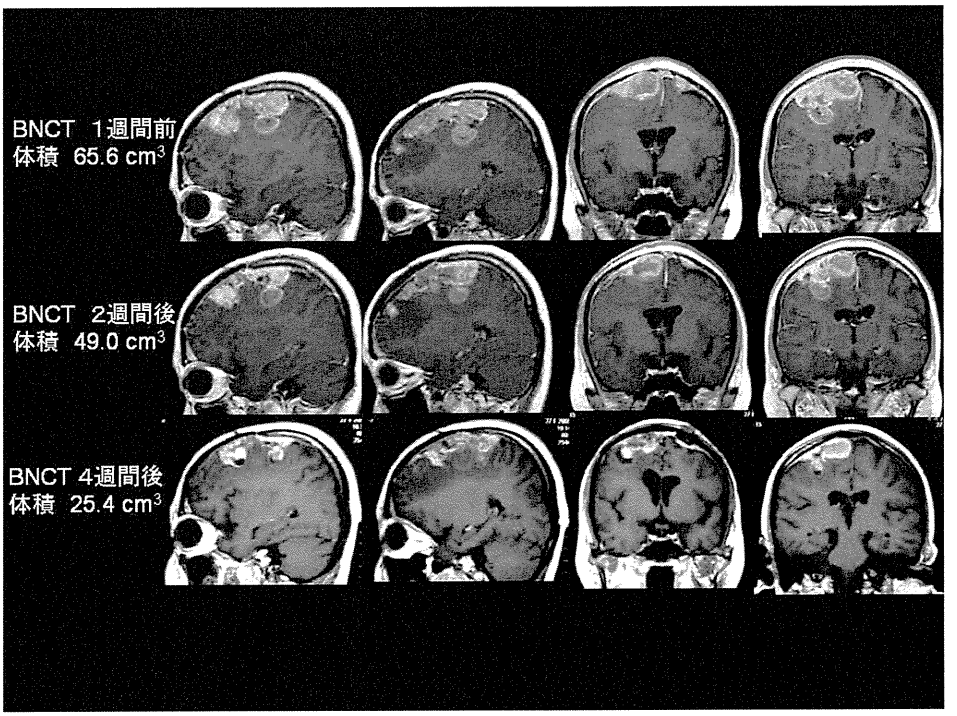

図 7 悪性髄膜腫
BNCT 待機中 1 か月間で腫瘍体積は 2 倍に急増大し，歩行不能となる。
BNCT 後 1 週間で歩行可能へ改善。

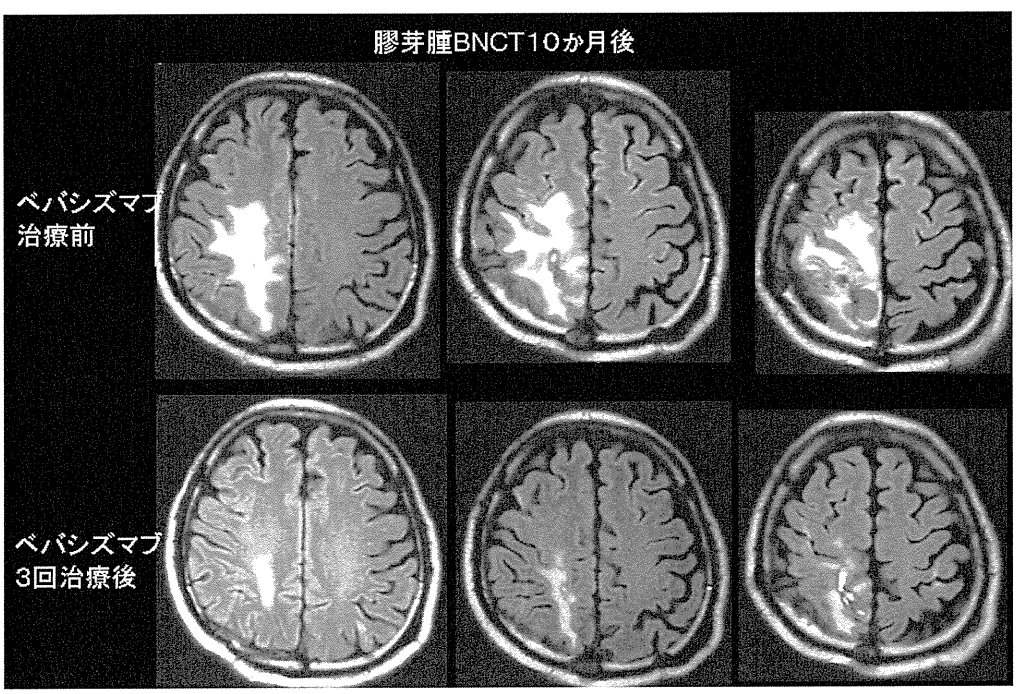

図 8 放射壊死に対するベバシズマブの効果

を示す[9]。まだ脳放射線壊死に対する保険適応のない本剤ではあるが，最近高度医療評価制度での承認が得られたので，本病態に難渋されておられれば，ぜひご相談いただきたい。

今後の展望

本稿で紹介した脳腫瘍以外にも，頭頸部がん，悪性黒色腫，難治性中皮腫等のがん腫に対して著効を示すBNCT ではあるが，一般化には大きな問題が存在する。現状で使用しうる中性子源は原子炉しか利用できない。

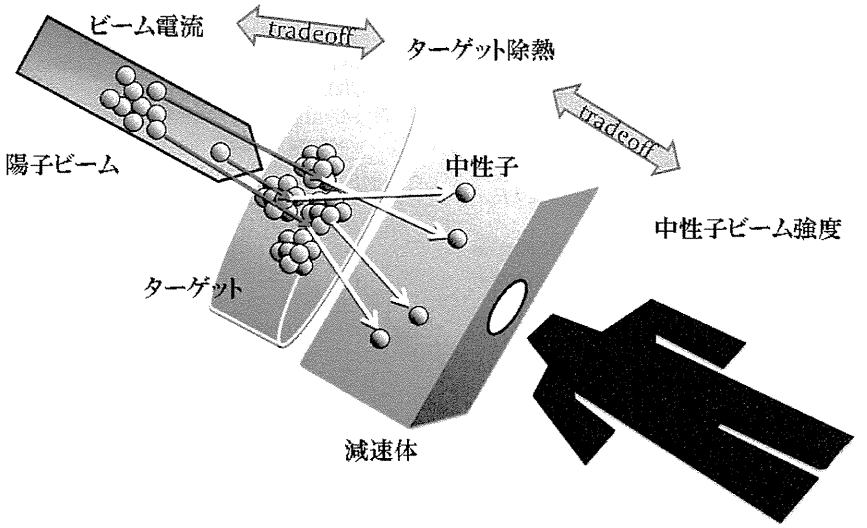

図 9　加速器中性子源

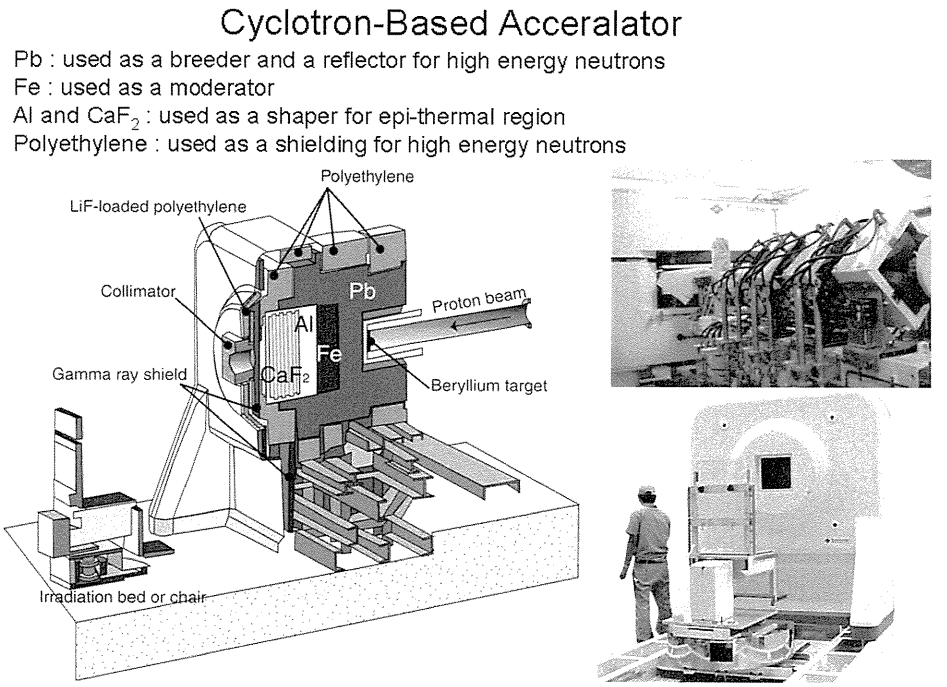

図 10　サイクロトロン型小型加速器

原子炉は広大な施設が必要であり，かつその燃料にウランを使用することにより，燃料廃棄も大きな問題となる。そこで新規中性子源として加速器が注目されている。その原理を図9に示す。陽子線を金属ターゲットに照射することによりさまざまなエネルギーの中性子が発生し，本治療に最適化したエネルギーの中性子を選別して照射に使用することができる。

ベリリウムターゲットを用い，冷却装置を工夫することにより，ターゲットの冷却という問題がクリアーでき，住友重機械がサイクロトロン型小型加速器による中性子発生装置の開発に成功した（図10）。2009年の脳腫瘍学会で実器をご案内したように，すでに必要十分な中性子を発生し，スーパー特区のサポートにより，われわれはまさに医薬品医療機器総合機構（PMDA）に治験申請を行おうとしている。

この装置を用いることにより，院内BNCTが可能となり，本法がより一般的な治療として普及するものと期待する。

文　献

1) Coderre JA and Morris GM: Review: The radiation biology of boron neutron capture therapy. *Radiation Res* **151**: 1-18, 1999.

2) Miyatake S, Kawabata S, Kajimoto Y, *et al*: Modified boron neutron capture therapy (BNCT) for malignant gliomas using epithermal neutron and two boron compounds with different accumulation mechanisms—Effectiveness of BNCT on radiographic images—. *J Neurosurgery* **103**: 1000-1009, 2005.
3) Kawabata S, Miyatake S, Kuroiwa T, *et al*: Boron neutron capture therapy for newly diagnosed glioblastoma. *J Rad Res* **50**: 51-60, 2009.
4) Yamamoto T, Nakai K, Kageji T, *et al*: Boron neutron capture therapy for newly diagnosed glioblastoma. *Radiother Oncol* **91**: 80-84, 2009.
5) Miyatake S, Kawabata S, Yokoyama K, *et al*: Survival benefit of boron neutron capture therapy for recurrent malignant gliomas. *J Neuro-Oncol* **91**: 199-206, 2009.
6) Tamura Y, Miyatake S, Nonoguchi N, *et al*: Boron neutron capture therapy for recurrent malignant meningioma. Report of first trial. *J Neurosurgery* **105**: 898-903, 2006.
7) Miyatake S, Tamura Y, Kawabata S, *et al*: Boron neutron capture therapy for malignant tumors related to meningiomas. *Neurosurgery* **61**: 82-91, 2007.
8) Gonzalez J, Kumar AJ, Conrad CA, *et al*: Effect of bevacizumab on radiation necrosis of the brain. *Int J Radiat Oncol Biol Phys* **67**: 323-326, 2007.
9) Furuse M, Kawabata S, Kuroiwa T, *et al*: Repeated treatments with bevacizumab for recurrent radiation necrosis in patients with malignant brain tumors: a report of 2 cases. *J Neuro-Oncol* (in press)

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Application of a Novel Boronated Porphyrin (H_2OCP) as a Dual Sensitizer for Both PDT and BNCT

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Background and Objective: Boronated porphyrins have emerged as promising dual sensitizers for use in both photodynamic therapy (PDT) and boron neutron capture therapy (BNCT), by virtue of their known tumor affinity, low cytotoxicity in dark conditions, and easy synthesis with high boron content. Octa-anionic 5,10,15,20-tetra[3,5-(nido-carboranylmethyl)phenyl] porphyrin (H_2OCP) is a boronated porphyrin having eight boron clusters linked to the porphyrin ring. To evaluate H_2OCP's applicability to both PDT and BNCT, we performed an in vitro and ex vivo study using F98 rat glioma cells.

Materials and Methods: We examined the time-dependent cellular uptake of H_2OCP by measuring the boron concentration over time, and compared the cellular uptake/clearance of boron after exposure to H_2OCP in conjunction with boronophenylalanine (BPA) and sodium borocaptate (BSH), both of which are currently used in clinical BNCT studies. We evaluated the cytotoxicity of H_2OCP-mediated PDT using a colony-forming assay and assessed the tumorigenicity of the implantation of pre-treated cells using Kaplan–Meier survival curves. Fluorescence microscopy was also performed to evaluate the cellular uptake of H_2OCP.

Results: H_2OCP accumulated within cells to a greater extent than BPA/BSH, and H_2OCP was retained inside the cells to approximately the same extent as BSH. The cell-surviving fraction following laser irradiation (8 J/cm^2, 18 hours after exposure to 10 μg B/ml H_2OCP) was <0.05. The median survival times of the pre-treated cell-implanted rats were longer than those of the untreated group ($P < 0.05$). The fluorescence of H_2OCP was clearly demonstrated within the tumor cells by fluorescence microscopy.

Conclusions: H_2OCP has been proven to be a promising photosensitizer for PDT. H_2OCP has also been proposed as a potentially effective replacement of BPA or BSH, or as a replacement of both BPA/BSH. Our study provides more evidence that H_2OCP could be an effective novel dual sensitizing agent for use in both PDT and BNCT. Lasers Surg. Med. 43:52–58, 2011. © 2011 Wiley-Liss, Inc.

Key words: boron neutron capture therapy; boronated porphyrin; F98 rat glioma cells; H_2OCP; photodynamic diagnosis; photodynamic therapy

INTRODUCTION

The prognosis of patients with malignant glioma, especially glioblastoma (GB), is poor. The median survival of GB patients is <2 years after the initial diagnosis [1], with most recurrence occurring at the site of the original tumor. Therefore, more aggressive local therapies are necessary to eradicate unresectable tumor cells that invade adjacent normal brain tissue. Two adjuvant therapies with the potential to destroy these cells are photodynamic therapy (PDT) [2–4] and boron neutron capture therapy (BNCT) [5–8]. Both are bimodal therapies, the individual components of which are non-toxic in isolation but tumoricidal in combination. Boronated porphyrins have emerged as promising dual sensitizers for both PDT and BNCT by virtue of the following characteristics: tumor affinity by the porphyrin ring; ease of synthesis with a high boron content; low cytotoxicity in dark conditions; and desirable photophysical properties, including strong light absorption in the visible and near infrared regions, the ability to generate singlet oxygen upon light activation, and fluorescence properties [9,10]. Several boronated porphyrins have been synthesized and evaluated in cellular and animal studies [9,10]. Among these, boronated porphyrins BOPP [11,12] and CuTCPH [13], each containing four boron clusters, have been extensively investigated. This type of boronated porphyrin was found to selectively deliver therapeutic concentrations of boron into tumor cells with low cytotoxicity in dark conditions and with long retention times within tumors. Boronated porphyrins having high boron content (up to 16 boron clusters) have been reported, [9,14] and it has been postulated that this type of compound could potentially deliver higher amounts of boron to tumors at the same dose.

There is no conflict of interest with any of the authors.

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In particular, the synthesis and cellular evaluation of the octa-anionic 5,10,15,20–tetra[3,5–(nido–carboranylmethyl)phenyl] porphyrin (H_2OCP), containing eight boron clusters (38% boron by weight), have been reported previously by the authors [15]. In that study, H_2OCP was shown to deliver high amounts of boron to human glioma T98G cells with low cytotoxicity in dark conditions. In this study, we evaluated the potential of H_2OCP as a dual sensitizer for both PDT and BNCT using F98 rat glioma cells. Although several boronated porphyrins have been proposed as boron delivery agents for BNCT, only a few have been investigated as dual sensitizers for both PDT and BNCT of tumors [9,16].

MATERIALS AND METHODS

Boron Delivery Agents

The H_2OCP was prepared as previously described [15]. Boronophenylalanine (BPA) (L-isomer) was kindly supplied by the Stella Chemifa Corporation (Osaka, Japan) and was prepared as a fructose complex [17]. Sodium borocaptate (BSH) was purchased from Katchem Ltd. (Katchem, Prague, Czech Republic) and dissolved in sterile saline.

Cell Culture

F98 rat glioma cells produce infiltrating tumors in the brains of Fischer rats [18]. The tumors have been shown to be refractory to a number of treatment modalities, including radiation therapy [19]. Based on their in vivo histology, the F98 rat glioma cells have been characterized as anaplastic or undifferentiated glioma [20]. In the present study, F98 rat glioma cells were kindly obtained from Dr. Barth (Department of Pathology, the Ohio State University, Columbus, OH). They were routinely cultivated in our laboratory in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin at 37°C in an atmosphere of 5% CO_2. All the materials for the culture medium were purchased from Gibco Invitrogen (Grand Island, NY).

Cellular Uptake/Clearance of Boron

The F98 rat glioma cells were seeded in 100 mm dishes (BD Falcon™, Franklin Lakes, NJ), and the culture medium without H_2OCP was exchanged for the H_2OCP-containing culture medium just before confluence. In all cellular studies, three 100 mm dishes for each cellular study were used. H_2OCP was dissolved in DMSO prior to dilution into the culture medium; the final DMSO concentrations never exceeded 1%. After the completion of exposure, the H_2OCP-containing culture medium was removed and the cells were washed twice with 4°C phosphate-buffered saline (PBS). Finally, the cells were retrieved using trypsin and fed 60% nitric acid in the cellular solution to extract intracellular boron. In order to evaluate the time-dependent boron uptake, the cells were exposed to 20 μg B/ml of H_2OCP for 6, 12, or 18 hours. Cellular uptake/clearance experiments were conducted using culture media containing 20 μg B/ml boron from either the H_2OCP, BPA, or BSH stock solutions and were exposed to the cells for a 12-hour period, followed by clearance times of 0, 2, and 6 hours. The boron concentrations were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using an iCAP6000 emission spectrometer (Hitachi High-Technologies, Tokyo, Japan). PBS and trypsin were purchased from Gibco Invitrogen, and the 60% nitric acid was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Colony-Forming Assay

F98 rat glioma cells were incubated in culture media with two different doses of H_2OCP (5 and 10 μg B/ml) and without H_2OCP (control) for 18 hours in 150 cm^2 flasks (TPP®; Zollstrasse, Trasadingen, Switzerland). Following incubation, the cells were retrieved from the flasks, seeded onto 60 mm dishes (BD Falcon™) with 10^4 cells each and irradiated with visible light of 405 nm from a diode laser (Ball Semiconductor, Frisco, TX). The cells were evenly irradiated at powers of 0 (control), 2, 4, and 8 J/cm^2. Following laser irradiation, the cells were seeded onto dishes; each with the same predetermined number of cells, iteratively. After 7 days, all of the colonies (>50 cells) were counted and assessed by calculating the cell-surviving fraction.

Tumorigenesis of In Vitro Pre-Treated Tumor Cells

In the treated group, F98 cells were exposed to 20 μg B/ml H_2OCP for 12 hours at 37°C prior to laser irradiation (4 J/cm^2), after which the tumor cells were implanted into 10 male Fischer rats. As a control study, untreated F98 cells were prepared and implanted in five male rats. Dead cells were stained with trypan blue just before implantation, counted under microscope, and expressed as a percentage of total cells per field-of-view segment. Viable cells were counted and were implanted into the rat brains. All male Fischer rats (200–250 mg) were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg) and placed in a stereotactic frame (Model 900, David Kopf Instruments, Tujunga, CA). A midline scalp incision was made and the bregma was identified. A 1 mm burr hole was made in the right frontal region of the skull and a 22-gauge needle attached to a 25 μl syringe was inserted into the caudate nucleus using the same stereotactic coordinates, with the needle tip inserted 5 mm into the dura. An injection of 10^5 cells in 10 μl of serum free medium was administered at a rate of 1 μl/minute. After the infusion, the needle was left in place for 3 minutes and the burr hole was then covered with bone wax. After implantation surgery, the body weight and neurological function of the rats were monitored daily. One day before death became imminent (defined by significant weight loss and a lack of activity or severe neurological deficits), the rats were euthanized and Kaplan–Meier survival curves were plotted and analyzed.

Cytotoxicity of H_2OCP in Dark Conditions

We examined the cytotoxicity of H_2OCP in the dark with a viable cell-counting method and a colony-forming assay. F98 rat glioma cells were seeded in 100 mm dishes and were incubated in culture media containing two different concentrations of H_2OCP (0, 20 μg B/ml) for cell counting. After exposure to H_2OCP for 24 hours, the cells were counted

using the trypan blue dye exclusion method. This assay was performed in triplicate. The cytotoxicity in the cell count was assessed by the percentage of viable of cells. For the colony-forming assay, F98 rat glioma cells were seeded in 100 mm dishes and were incubated in culture media containing five different concentrations of H_2OCP (0, 5, 10, 20, 40 μg B/ml). After exposure to H_2OCP for 24 hours, the cells were retrieved from the dishes and were seeded onto 100 mm dishes, each with the same predetermined number of cells. This assay was also performed in triplicate. After 7 days, all of the colonies (>50 cells) were counted and assessed by calculation for the cell-surviving fraction.

Fluorescence Microscopy

F98 rat glioma cells were seeded in a two-well chamber mounted on glass slides with a cover (Nalge Nunc International, Rochester, NY) and the culture medium without H_2OCP was exchanged for the H_2OCP-containing culture medium just before confluence. The cells were exposed to 20 μg B/ml H_2OCP for 24 hours. After exposure, the glass slides were washed with 4°C PBS and the two-well chamber was removed. The nucleus-specific hoechst dye (Hoechst 33342, Lonza, Maryland, MD) was added (10 μg/ml) and the glass slides mounted onto cover glasses using DPX Mountant for histology (44581, Fluka Biochemika, Darmstadt, Germany). The two-well chamber slides were observed using an inverted fluorescence microscope system (BZ-8000, Keyence, Tokyo, Japan).

RESULTS

Cellular Uptake/Clearance of Boron

The measured cellular boron concentrations obtained by in vitro cellular delivery using H_2OCP were 158.2 ± 3.8, 272.2 ± 15.3, and 405.1 ± 22.6 ng B/ml 10^6 cells after 6, 12, and 18 hours of exposure, respectively. Nearly three times more boron was found within cells after 18 hours of exposure than after 6 hours of exposure (Fig. 1). The determined cellular boron concentrations for in vitro cellular uptake/clearance of boron in response to exposure to H_2OCP, BPA, and BSH for 12 hours were 272.2 ± 15.3, 239.7 ± 12.3, and 85.2 ± 2.0 ng B/ml 10^6 cells, respectively. In contrast, at 6 hours after exposure the cellular boron concentrations were 246.7 ± 14.6, 84.9 ± 0.7, and 67.0 ± 4.6 ng B/ml 10^6 cells, respectively. At the same boron dose, H_2OCP delivered significantly higher amounts of boron to cells than did BPA or BSH (log-rank test, $P < 0.05$). Furthermore, while BPA cleared rapidly from cells, both H_2OCP and BSH showed high cellular retention of boron for up to 6 hours (Fig. 2).

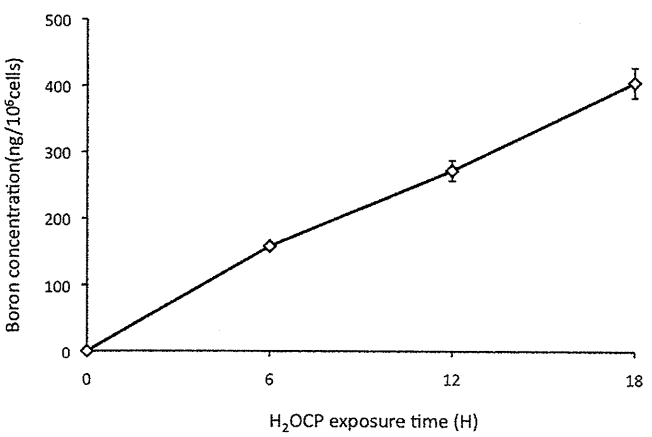

Fig. 1. Cellular uptake of boron (ng B/ml 10^6 cells) after 6, 12, and 18 hours of exposure to 20 μg B/ml H_2OCP.

Colony-Forming Assay

The cytotoxicity of H_2OCP determined by laser irradiation using a colony-forming assay showed that the surviving fraction of cells following exposure to H_2OCP (10 μg B/ml for 18 hours) and laser irradiation was 0.326 ± 0.031, 0.246 ± 0.037, and 0.045 ± 0.001 using 2, 4, and 8 J/cm^2 light dose, respectively. Under the same conditions, the surviving fractions of the laser-only control (without H_2OCP) were 0.861 ± 0.182, 0.776 ± 0.035, and 0.299 ± 0.023, respectively. The most efficient PDT-induced tumoricidal effect was achieved when the cells were irradiated with 8 J/cm^2, 18 hours after exposure to H_2OCP (<0.05) (Fig. 3).

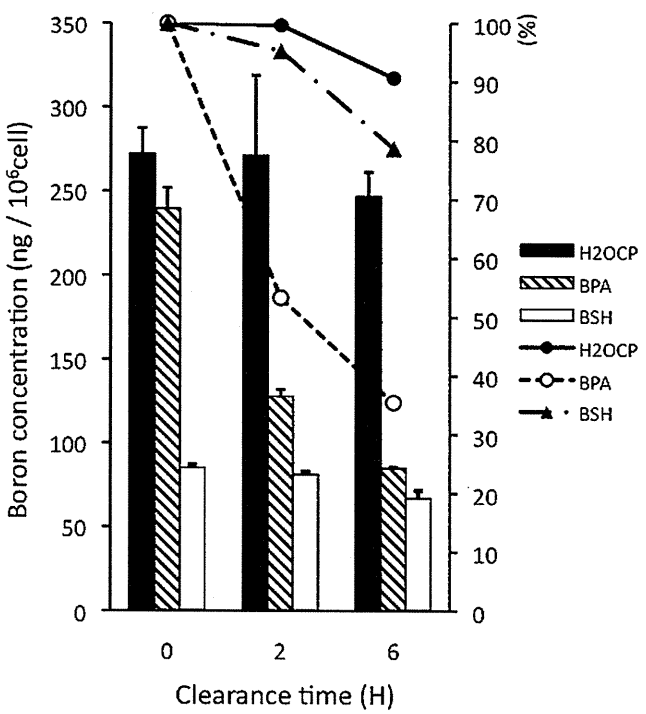

Fig. 2. Comparison of cellular uptake/clearance of boron (ng B/ml 10^6 cells) after exposure to 20 μg B/ml of either H_2OCP, BPA, or BSH under identical conditions. Left and right Y-axes show the measured value (ng B/ml 10^6 cells) and percentage of boron concentration, respectively. The cellular uptake of boron using H_2OCP showed values higher than those for BPA and BSH ($P < 0.05$), and the cellular retention of boron using H_2OCP showed values similar to those obtained using BSH.

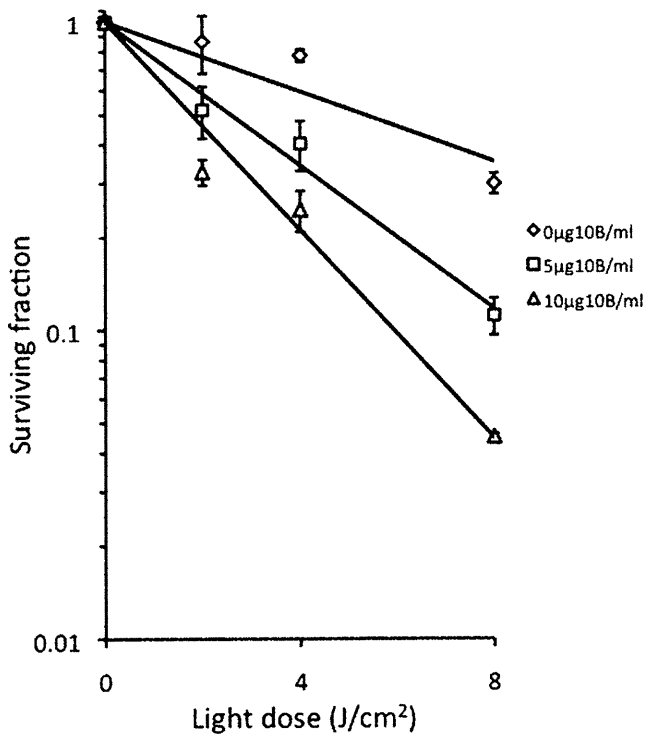

Fig. 3. Colony-forming assay using F98 rat glioma cells exposed to 0 (control), 5, and 10 μg B/ml of H_2OCP and irradiated with light doses of 0, 2, 4, and 8 J/cm^2, respectively. The cell-surviving fraction following laser irradiation (8 J/cm^2, 18 hours after exposure to 10 μg B/ml H_2OCP) was <0.05.

Tumorigenesis of In Vitro Pre-Treated Tumor Cells

The observed tumorigenicity of the implanted pre-treated cells using Kaplan–Meier survival curves revealed median survival times of 12 and 14 days in the untreated and the treated groups, respectively, and mean survival times of 11.8 and 14.6 days after implantation, respectively. In Kaplan–Meier survival curve analysis, these survival times demonstrated a significant difference (log-rank test, $P < 0.05$) (Fig. 4).

Cytotoxicity of H_2OCP in Dark Conditions

The viable cell-counting method revealed the following results. The percentage of cell viability with exposure of 20 μg B/ml H_2OCP was 98.0 ± 1.4% (mean ± SD), while that of cell viability without H_2OCP was 98.0 ± 0.9%. The colony-forming assay showed the following: the surviving fractions with each boron concentration (0, 5, 10, 20, 40 μg B/ml H_2OCP) were 1, 0.99 ± 0.04 (mean ± SD), 0.98 ± 0.05, 0.98 ± 0.01, and 0.98 ± 0.03, respectively. These results showed no significant differences (Welch's t-test, $P > 0.05$).

Fluorescence Microscopy

The fluorescence microscopy showed the intracellular porphyrin fluorescence and images from the co-localization

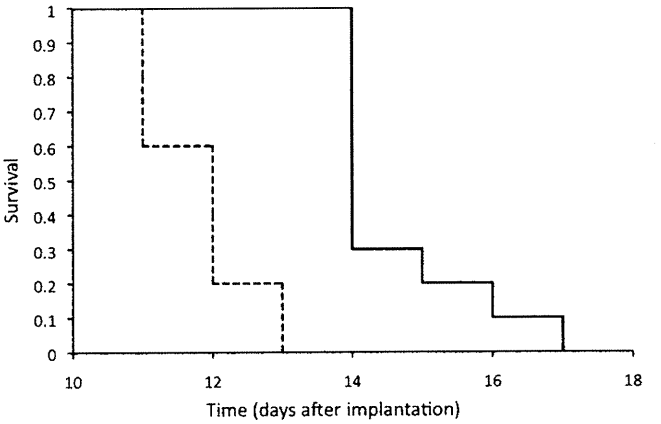

Fig. 4. Kaplan–Meier survival curves following in vitro pre-treated F98 cells using H_2OCP-mediated PDT. Rats were implanted i.c. with F98 cells and were either untreated (dotted line) or treated with PDT (continuous line). Cells were exposed to 20 μg B/ml of H_2OCP for 24 hours at 37°C prior to laser irradiation. After laser irradiation (4 J/cm^2), the tumor cells were implanted into the rats. Cell viability was determined by trypan blue exclusion staining and 10^5 viable cells were implanted stereotactically into the caudate nucleus. The median survival times of the untreated control and the treated group were 12 and 14 days, and the mean survival times were 11.8 and 14.6 days after implantation, respectively ($P < 0.05$).

experiment using the nucleus-specific Hoechst dye. These results showed that H_2OCP was taken up into the cells and also localized in the nuclei (Fig. 5B–D). Although the cells showed evidence of cytotoxic damage, the cytotoxicity of H_2OCP in dark conditions was not found at twice the concentration of H_2OCP used in this fluorescence microscopy experiment. (Fig. 5A).

DISCUSSION

BNCT is a targeted chemo-radiation therapy that significantly increases the therapeutic ratio relative to conventional radiotherapeutic modalities. In BNCT, a ^{10}B-labeled compound delivers therapeutic concentrations of ^{10}B (~30 μg ^{10}B/g tumor) to the target tumor, with high tumor-to-blood and tumor-to-normal-tissue ratios and low cytotoxicity [5,6]. Subsequently the tumor is irradiated with epithermal neutrons that become thermalized at a certain depth within the tissues. The short range (<10 μm) of the α and ^{7}Li high linear energy transfer (high-LET) particles released from the ^{10}B(n, α) ^{7}Li neutron capture reaction makes the tumor microdistribution of ^{10}B critically important in BNCT [21]. Since the high-LET particles are highly cytotoxic, their killing effect depends on the site of generation. These characteristics contribute to the tumor selectivity and strong tumoricidal activity of BNCT, with negligible damage to normal tissue. Therefore, if sufficient quantities of boron can be selectively delivered to tumor tissues, BNCT could be an ideal tumor-selective particle

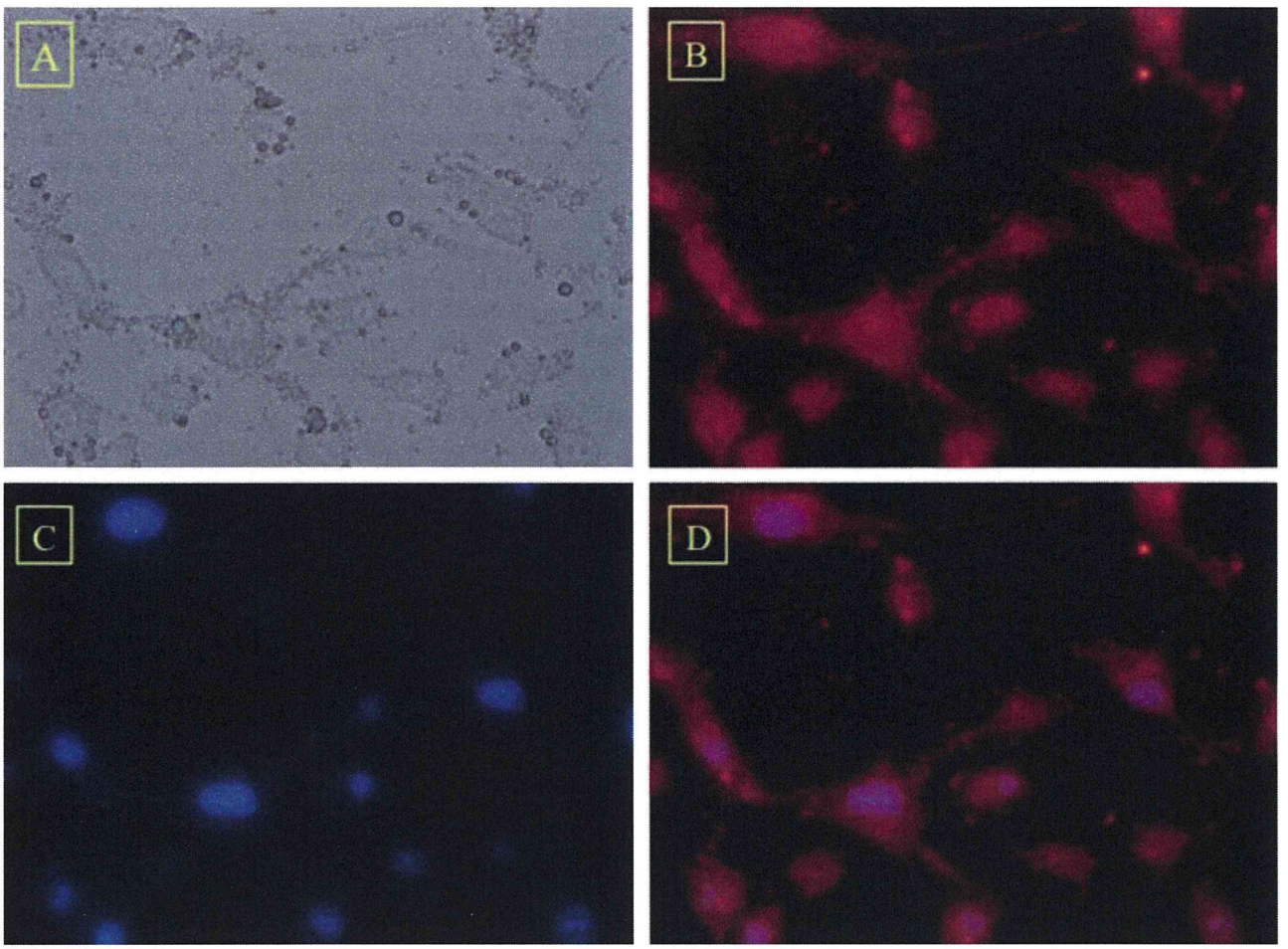

Fig. 5. Images obtained using an inverted fluorescence microscope. **A**: Bright field image. Although the cells showed evidence of cytotoxic damage, the cytotoxicity of H_2OCP in dark conditions was not found at twice the concentration of H_2OCP used in this fluorescence microscopy experiment. **B**: Fluorescence of porphyrin H_2OCP. **C**: Nuclear fluorescence by Hoechst dye (excitation wavelength was 340–380 nm). **D**: Merged image. (Magnification of all images: ×600).

beam irradiation local therapy for malignant gliomas. Clinically, BPA and BSH are currently available for BNCT as boron delivery agents. BPA is a boronated derivative of an essential amino acid (L-phenylalanine) that is actively taken up by tumor cells, presumably via the amino acid transport mechanism [22]. BSH, on the other hand, is believed to preferentially accumulate within tumor tissue via a partially destroyed or leaky blood brain barrier, and is thought to be retained longer than BPA due to its higher hydrophobic character [23]. We have used both of these boron delivery agents in combination in clinical BNCT studies, and have previously been reported on the survival benefit from BNCT for newly diagnosed GB patients [7] as well as for recurrent malignant glioma patients [8]. However, the present results using BPA and BSH are far from satisfactory, and the use of more effective boron delivery agents should provide enhanced clinical outcomes for BNCT. The so-called third-generation of boron delivery agents [6], including boronated porphyrin derivatives, molecular-targeted agents (e.g., to EGFR), and liposome-linked boron delivery agents could potentially greatly increase the efficacy of BNCT in the clinical setting. Among these boron delivery agents, boronated porphyrins are particularly promising because they contain a porphyrin ring with tumor affinity, and they are also excellent photosensitizers for PDT [9]. In a similar fashion to BNCT, PDT is a localized therapy that relies on the specific uptake of a photosensitizer in the tumor relative to the surrounding normal tissue, followed by laser irradiation for activation of the photosensitizer [2,24]. The photoactivation of the sensitizer causes oxidative damage to a variety of cellular targets via the release of singlet oxygen and other reactive oxygen species, with subsequent tumor necrosis. To date, the clinical trials with PDT employed as an adjuvant treatment for human gliomas have used the poorly defined heterogeneous porphyrin mixture hematoporphyrin derivative (HpD) or its more enriched

commercial preparation, Photofrin [2], which does not contain boron. This photosensitizer has been shown to localize preferentially in glioma relative to normal brain tissue. Moreover, reports of PDT as a treatment for animal and human glioma have been encouraging, and a photosensitizer that is more tumor selective than HpD or Photofrin would have great clinical benefits.

In this study, our results showed the positive efficacy of PDT using H_2OCP in a colony-forming assay (Fig. 3) and in tumorigenesis of in vitro pre-treated cells in Fisher rats (Fig. 4). Additionally, H_2OCP accumulated within the glioma cells to a significantly higher extent than BPA or BSH ($P < 0.05$), and was retained inside the cell to approximately the same extent as BSH (Fig. 2). Based on these findings, we postulated that H_2OCP could be applied to both PDT and BNCT for treatment of glioma tumors. In the fluorescence microscopy experiment, although the cells in the bright field image showed evidence of cytotoxic damage (Fig. 5A), the cytotoxicity of H_2OCP in dark conditions was not observed, even at double the concentration of H_2OCP used in the fluorescence microscopy experiment. Therefore, we considered that the damage to the cells in the bright field image was most probably due to technical complications related to irradiation with the laser during imaging, rather than to the cytotoxic effects of H_2OCP itself. Furthermore, H_2OCP was shown to be taken up by F98 rat glioma cells (Fig. 5B–D). Therefore, our results suggest that H_2OCP can be used intraoperatively for photodynamic diagnosis (PDD) and fluorescence-guided resection of brain tumors.

Pre-operative administration of a boronated porphyrin has a number of advantages in the clinical setting. As noted previously, boronated porphyrins are useful in PDD and in fluorescence-guided resection of brain tumors during surgery. Using fluorescence-guided resection of such tumors during surgery, the resection rate can be augmented, with expected further improvements in patient prognosis [25]. In addition, boronated porphyrins can be used with intra-operative PDT and post-operative BNCT. Although the initial results with commonly used photosensitizers for PDT such as Photofrin (or its unpurified form HpD) were very encouraging, treatment failures did occur, mainly due to the limited penetration of light into the brain. In cases with deep lesions, PDT alone may be inadequate to achieve complete tumor treatment, and it would be preferable in such cases to use BNCT as a supplementary treatment, with boron-containing porphyrin as a photosensitizer. Fairchild et al. [26] reported that thermal and epithermal neutrons are transported to a depth of approximately 10 cm in fact, BNCT has been shown to treat deep lesions. Since boronated porphyrins can be effective for BNCT as boron delivery agents while retaining their photosensitizer ability, the limited penetration of light can be overcome using a combination of BNCT and PDT for the treatment of human gliomas.

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REFERENCES

1. Stupp R, Hegi ME, van den Bent MJ, Mason WP, Weller M, Mirimanoff RO, Cairncross JG. Changing paradigms – An update on the multidisciplinary management of malignant glioma. Oncologist 2006;11(2):165–180.
2. Kaye AH, Hill JS. Photodynamic therapy of cerebral tumors. Neurosurg Q 1992;1:233–258.
3. Origitano TC, Caron MJ, Reichman OH. Photodynamic therapy for intracranial neoplasms. Literature review and institutional experience. Mol Chem Neuropathol 1994; 21(2–3):337–352.
4. Kostron H, Obwegeser A, Jakober R. Photodynamic therapy in neurosurgery: A review. J Photochem Photobiol B 1996;36(2): 157–168.
5. Barth RF, Soloway AH, Fairchild RG, Brugger RM. Boron neutron capture therapy for cancer. Realities and prospects. Cancer 1992;70(12):2995–3007.
6. Barth RF, Coderre JA, Vicente MG, Blue TE. Boron neutron capture therapy of cancer: Current status and future prospects. Clin Cancer Res 2005;11(11):3987–4002.
7. Kawabata S, Miyatake S, Kuroiwa T, Yokoyama K, Doi A, Iida K, Miyata S, Nonoguchi N, Michiue H, Takahashi M, Inomata T, Imahori Y, Kirihata M, Sakurai Y, Maruhashi A, Kumada H, Ono K. Boron neutron capture therapy for newly diagnosed glioblastoma. J Radiat Res (Tokyo) 2009;50(1):51–60.
8. Miyatake S, Kawabata S, Yokoyama K, Kuroiwa T, Michiue H, Sakurai Y, Kumada H, Suzuki M, Maruhashi A, Kirihata M, Ono K. Survival benefit of boron neutron capture therapy for recurrent malignant gliomas. J Neurooncol 2009;91(2):199–206.
9. Vicente MGH, Sibrian-Vazquez M. Syntheses of boronated porphyrins and their application in BNCT. In: Kadish KM, Smith KM, Guilard R, eds. The Handbook of Porphyrin Science. Vol. 4. Singapore: World Scientific Publishers; 2010: 191–248.
10. Renner MW, Miura M, Easson MW, Vicente MGH. Recent progress in the syntheses and biological evaluation of boronated porphyrins for boron neutron-capture therapy. Anticancer Agents Med Chem 2006;6(2):145–157.
11. Ceberg CP, Brun A, Kahl SB, Koo MS, Persson BR, Salford LG. A comparative study on the pharmacokinetics and biodistribution of boronated porphyrin (BOPP) and sulfhydryl boron hydride (BSH) in the RG2 rat glioma model. J Neurosurg 1995;83(1):86–92.
12. Ozawa T, Afzal J, Lamborn KR, Bollen AW, Bauer WF, Koo MS, Kahl SB, Deen DF. Toxicity, biodistribution, and convection-enhanced delivery of the boronated porphyrin BOPP in the 9L intracerebral rat glioma model. Int J Radiat Oncol Biol Phys 2005;63(1):247–252.
13. Miura M, Morris GM, Micca PL, Nawrocky MM, Makar MS, Cook SP, Slatkin DN. Synthesis of copper octabromotetracarboranylphenylporphyrin for boron neutron capture therapy and its toxicity and biodistribution in tumour-bearing mice. Br J Radiol 2004;77(919):573–580.
14. Hao E, Sibrian-Vazquez M, Serem W, Garno JC, Fronczek FR, Vicente MG. Synthesis, aggregation and cellular investigations of porphyrin-cobaltacarborane conjugates. Chemistry 2007;13(32):9035–9042.