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SHORT REPORT

Cellular uptake evaluation of pentagamaboronon-0 (PGB-0) for boron neutron capture therapy (BNCT) against breast cancer cells

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Summary

Pentagamaboronon-0 (PGB-0), a curcumin analog compound, has been synthesized as a candidate of boron-carrier pharmaceutical (BCP) for boron neutron capture therapy (BNCT); however, this compound is poorly soluble in water. To improve its solubility, aqueous formulations of PGB-0 with a monosaccharide, fructose or sorbitol, were successfully synthesized, namely PGB-0-F and PGB-0-So, respectively. The cytotoxicity study showed that PGB-0-F and PGB-0-So exerted low cytotoxicity against MCF-7 and MDA-MB 231 breast cancer cells. The cellular uptake study using inductively coupled plasma optical emission spectrometry (ICP-OES) and DAHMI live-cell imaging indicated that these compounds were accumulated and distributed within the cytoplasm and cell nuclei. The cellular uptake mechanism was also evaluated to clarify the contribution of the glucose transporter, and the results demonstrated that these compounds entered through active transport into MCF-7 cells but through passive diffusion into MDA-MB 231 cells. In conclusion, the sugar formulations of PGB-0 only improved PGB-0 solubility but had no role in its cellular uptake.

Keywords PGB-0 · Breast cancer · BNCT · Cellular uptake · MCF-7 · MDA-MB 231

Introduction

Breast cancer is a heterogeneous disease and a leading cause of mortality among women [1]. According to its heterogeneity, breast cancer can be classified on a molecular basis into at least the following five subtypes: luminal A, luminal B, HER2-positive, basal-like, and normal breast cancer [2, 3]. In Indonesia, the two most common breast cancer subtypes are luminal A (41%) and triple-negative breast cancer (26%) [4]. The luminal A subtype is characterized by a high expression of estrogen and progesterone receptors (ER^+, $PR^{\pm}/^-$, $HER2^-$) but a low expression of proliferation markers (Ki67) and commonly exhibits a positive response to hormone therapy and chemotherapy [2, 5]. However, triple-negative breast cancer, which does not show the expression of the three recognized therapeutic targets (ER^-, PR^-, $HER2^-$), is difficult to treat [2]. In addition, classical chemotherapy encounters few major obstacles for achieving successful treatment, such as cancer chemoresistance, relapse, and triggering of cancer invasion [6, 7]. Therefore, it is necessary to develop new approaches for treating luminal A and triple-negative breast cancer subtypes.

One potential cancer eradication therapy is boron neutron capture therapy (BNCT). Boron-carrier pharmaceuticals (BCPs) comprise important modalities for the successful treatment by BNCT. The first generation of developed boron delivery agents were sodium borocaptate (BSH) and boronphenylalanine (BPA), which have been attempted against various types of cancer cells [8, 9]. Along with the optimization of these two compounds, the development of other boron-containing pharmaceuticals was inclining.

2,5-*Bis*(4-boronic acid)benzylidine cyclopentanone, known by its codename pentagamaboronon-0 (PGB-0) (Fig. 1), is a newly synthesized BCP and a novel curcumin analog based on benzylidene cyclopentanone with boron atom

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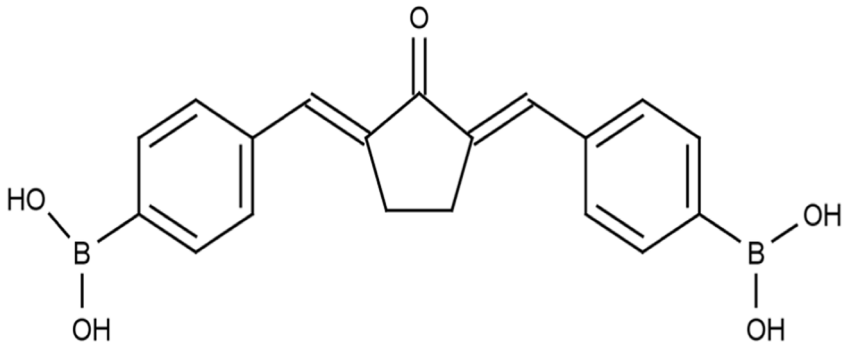

Fig. 1 Chemical structure of pentagamaboronon (PGB-0), 2,5-bis(4-boronic acid benzylidene) cyclopentanone

substitution developed by the Faculty of Pharmacy, Universitas Gadjah Mada. We had earlier developed two anticancer agents, benzylidene cyclopentanone-based curcumin analogs, known as pentagamavunon-0 (PGV-0) and pentagamavunon-1 (PGV-1) [10] that reportedly exhibited anticancer activities against breast cancer [11–13]. Furthermore, PGB-0 exerted cytotoxicity toward HER2-positive breast cancer and decreased the HER2 expression [14]. PGB-0 also exhibited antimetastatic activity toward triple-negative breast cancer cells (unpublished data). However, similar to curcumin, PGB-0 is less soluble in water. Therefore, we developed two sugar formulations of PGB-0, namely PGB-0-Sorbitol (PGB-0-So) and PGB-0-Fructose (PGB-0-F).

In this study, we found that the formulations prepared using the sugar fructose and sorbitol increased the compound solubility. Microdistribution and accumulation examinations of PGB-0-F and PGB-0-So against several cancerous cells revealed that these compounds were uptaken by the cells. In addition, the mechanisms underlying the transport process are described in this article.

Materials and methods

Cell culture

MCF-7 and MDA-MB 231 cells were cultured in a CO_2 incubator (37 °C) in DMEM (Gibco, Invitrogen, USA) supplemented with 10% FBS (Sigma, St. Louis, CA, USA), 150 μg/mL penicillin, and 150 μg/mL streptomycin (Gibco, Invitrogen, USA). The cells were subcultured using trypsin-EDTA (Gibco, Invitrogen, USA) for cell detachment.

Chemicals

PGB-0 was synthesized as previously described (unpublished data). Sugar formulations of PGB-0 were prepared using fructose (PGB-0-F) and sorbitol (PGB-0-So) as described earlier [14]. ρ-Borono-Phenylalanin (BPA) was purchased from Stella pharma (Osaka, Japan).

Cytotoxicity assay

Cytotoxicity was assessed using the water-soluble tetrazolium (WST) assay (Wako pure chemicals, Osaka, Japan) according to the manufacturer's instructions. The MCF-7 and MDA-MB 231 cells (1×10^4 cells) were seeded into separate wells of a 96-well plate. The next day, the cells were treated with PGB-0-F or PGB-0-So at various concentrations for 24 h. Then, 100 μL of WST reagent was added and incubated for 2–3 h. The absorbance was measured on a microplate reader (Bio-Rad) according to the manufacturer's instructions. Percentage cell viability was calculated from the absorbance data.

Analysis of intracellular boron incorporation

Cells were seeded (1.0×10^6 cells/dish) and grown for 24 h. The medium was replaced with an equivalent medium containing 0.3 mM BPA and 0.15 mM PGB-0-F and PGB-0-So, followed by incubation for 24 h. The cells were washed three times with PBS, harvested by trypsinization, and then counted. A mixture of $HClO_4$ (60%, 0.3 mL) and H_2O_2 (31%, 0.6 mL) was added to the cells, followed by heating at 75 °C for 1 h. The mixture was filtered through a membrane filter (Millipore, 0.45 μm), and Milli-Q water was added to make the volume up to 5 mL. The boron concentration was measured by ICP-OES (Shimadzu) [15].

Boron uptake study

Cells were seeded (2×10^5 cells/well) in a 24-well plate and grown for 24 h. The medium was replaced with a sodium-free Hank's Balanced Salt Solution (HBSS) buffer containing 0.15 mM PGB-0-F (the concentration was determined previously by measuring the *Kd* value of PGB-0 by competitive indirect ELISA against anti-BPA mAb, 2B10), followed by incubation for 0, 0.5, 1, 2, 5, 10, and 20 min. The uptake of boron was arrested by adding cold HBSS buffer. Cells were then lysed with 0.5% Tween. Protein content was evaluated using bicinchoninic acid (BCA) as according to the manufacturer's instructions. The boron concentration was determined

by the indirect competitive ELISA against anti-BPA mAb [16, 17].

Analysis of boron microdistribution

Cells were seeded (1.0×10^5 cells) in 35-mm glass-bottomed dishes and grown for 24 h. The medium was replaced with an equivalent medium containing 0.1 mM PGB-0-F or PGB-0-So, followed by incubation for 24 h. The next day, DRAQ5 solution was added to the cells (the final concentration was 5 μM in DMEM), followed by 30-min incubation. After washing once with DMEM, DAHMI solution was added to the cells (the final concentration was 0.1 mM in DMEM), followed by 20-min incubation. The distribution of boron compound within the cells was analyzed under excitation at 405 nm using an LMS 700 confocal scanning laser microscope equipped with a 20× objective [9].

Glucose transporter inhibition study

Cells were seeded (2×10^5 cells/well) in a 24-well plate and grown for 24 h. The medium was replaced with a sodium-free HBSS buffer containing 0.15 mM PGB-0-F and a series of glucose concentrations (0.15, 1.5, 15, and 50 mM), followed by incubation for 20 min. The uptake was stopped by adding cold HBSS buffer. Cells were then lysed with 0.5% Tween. Protein content was calculated using BCA as per the manufacturer's instructions. The boron concentration was determined by the indirect competitive ELISA against anti-BPA mAb [16, 17].

Boron efflux study

Cells were seeded (2×10^5 cells/well) in a 24-well plate and grown for 24 h. The medium was replaced with a medium containing 0.15 mM PGB-0-F, followed by incubation for 24 h. Cells were lysed with 0.5% Tween within 1, 2, and 3 h after medium exchange. Protein content was evaluated using BCA according to the manufacturer's instructions. The concentration of boron was determined by the indirect competitive ELISA against anti-BPA mAb [16, 17].

Results and discussion

Cytotoxicity of PGB-0 against MCF-7 and MDA-MB 231 cells

PGB-0 is a potential BCP for BNCT. To increase the solubility of PGB-0, we developed two sugar formulations of PGB-0 using fructose and sorbitol. No cytotoxicity of PGB-0 in both fructose and sorbitol formulations was observed against MCF-7 and MDA-MB 231 cells up to a concentration of 0.25 mM (Fig. 2). However, we observed precipitation of PGB-0 at a concentration beyond 0.25 mM.

Boron incorporation and microdistribution

Next, we measured the boron concentrations in MCF-7 and MDA-MB 231 cells by inductively coupled plasma optical emission spectrometry (ICP-OES) (Fig. 3). In general, the uptake of BPA, PGB-0-F, and PGB-0-So in MCF-7 cells is higher than the uptake in MDA-MB 231 cells. Both PGB-0-F and PGB-0-So delivered lower intracellular boron amount compared with BPA.

To observe the microdistribution of boron, we performed boron staining using DAHMI boron sensor and DRAQ5 nuclear staining (Fig. 3). In MCF-7 cells, boron accumulation was observed in the cytoplasm, whereas in MDA-MB 231 cells, boron accumulated in the nucleus.

Boron uptake and efflux assay

The uptake mechanism of boron was evaluated by measuring the intracellular boron concentration using the indirect competitive ELISA after PGB-0-F treatment, with or without glucose. The uptake of boron in MCF-7 and MDA-MB 231 cells exhibited a different pattern (Fig. 4). In MCF-7 cells, the boron concentration showed a linear increase with the incubation time (20 min). On the other hand, the boron uptake in MDA-MB 231 cells showed an initial burst for about 2 min, slightly increased, and then exhibited linear accumulation.

To investigate whether the uptake of boron is mediated by glucose transporter, we incubated cells with PGB-0-F in combination with a series of glucose concentrations (Fig. 4). In both MCF-7 and MDA-MB 231 cells, the boron uptake showed no significant difference when compared between glucose-free and glucose-supplemented treatments, which thus indicated that the uptake of PGB-0-F does not correlate with the glucose transporter.

We next evaluated the efflux of boron to measure its release from the cells (Fig. 4). Cells were incubated with PGB-0-F for 24 h, followed by medium change and cell lysis at several time periods. In MCF-7 cells, the intracellular boron concentration remained stable after 3 h of medium change, whereas in MDA-MB 231 cells, the concentration of intracellular boron decreased after 3 h.

We also evaluated the potency of PGB-0, a curcumin analog and a BCP for BNCT, against breast cancer. PGB-0 exhibits low solubility as the parent compound curcumin. To overcome the low bioavailability and stability, we developed sugar formulations using fructose (PGB-0-F) and sorbitol (PGB-0-So), which exhibited no cytotoxicity against MCF-7 and MDA-MB 231 cells up to a concentration of 0.25 mM. However, we observed precipitation of PGB-0 at a concentration beyond 0.25 mM. The precipitation of PGB-0 during the

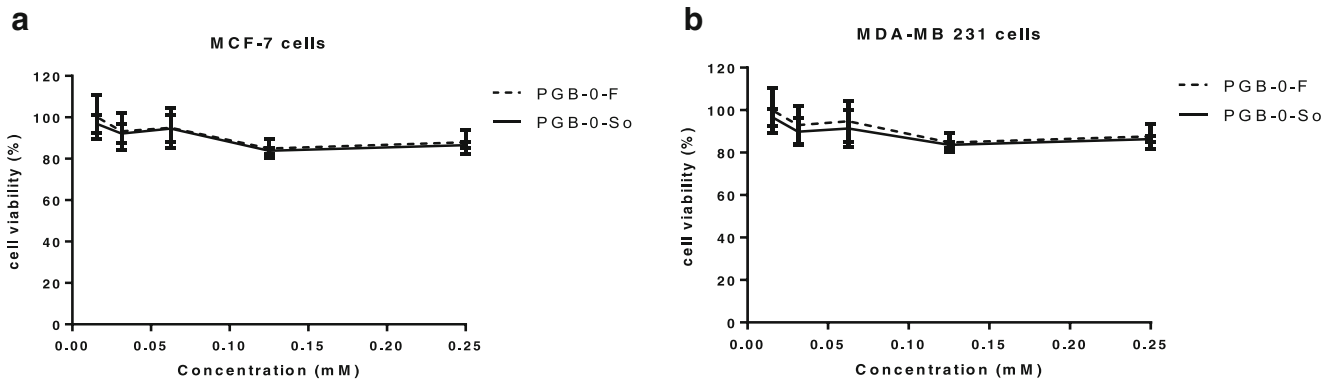

Fig. 2 Cytotoxicity of PGB-0-F and PGB-0-So against MCF-7 (a) and MDA-MB 231 breast cancer cells (b). Cells were treated with serial concentrations of PGB-0-F and PGB-0-So for 24 h and then analyzed by WST assay. Results represent mean ± SD ($n = 5$)

in vitro experiment was probably because the PGB-0 sugar complexes are not completely transported and hence the sugar formulation is dissociated in the cell membrane. However, this proposed mechanism must be confirmed by further studies.

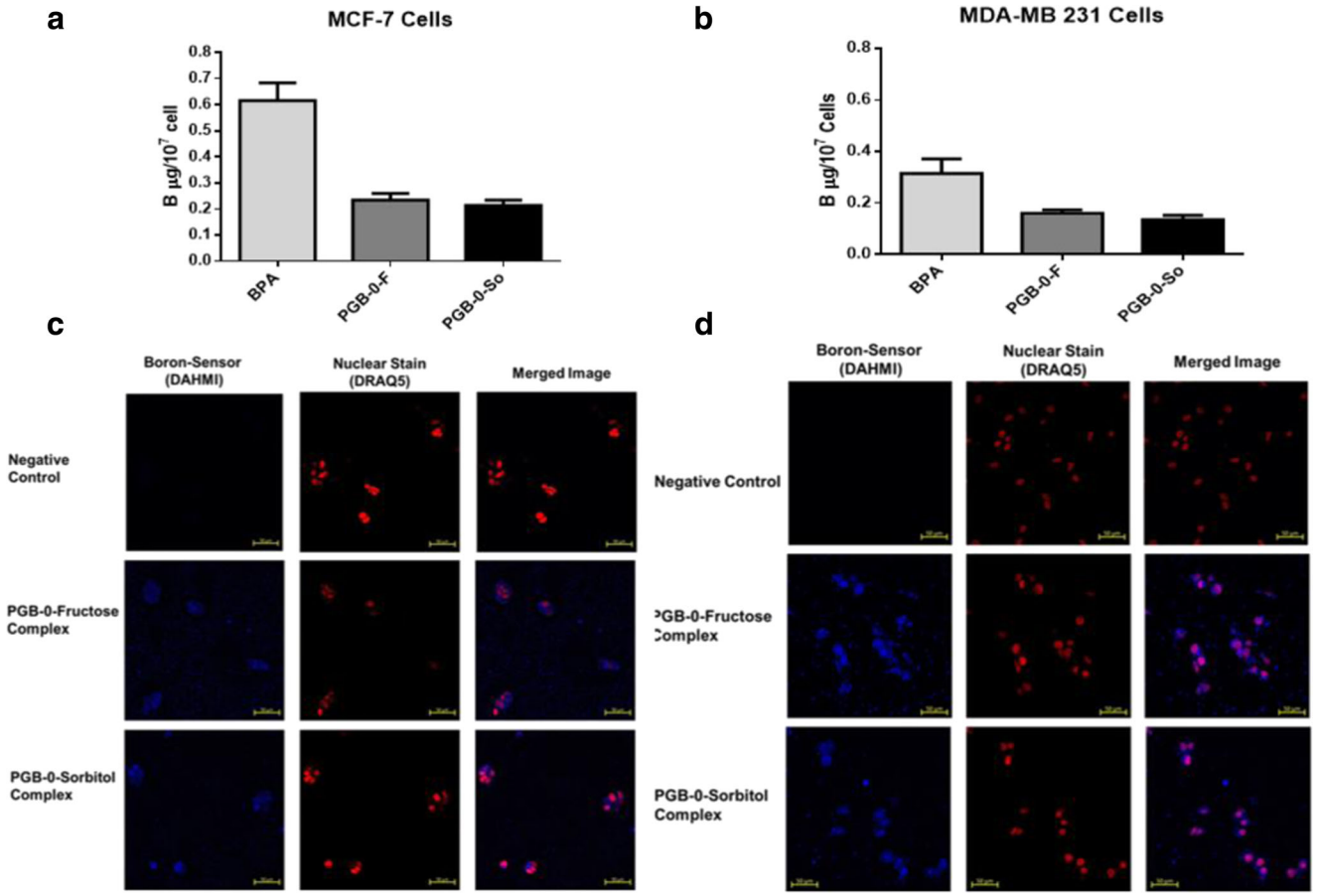

Fig. 3 Boron incorporation upon treatment of BPA, PGB-0-F, and PGB-0-So in MCF-7 (a) and MDA-MB 231 breast cancer cells (b). Cells were treated with 0.3 mM BPA and 0.15 mM PGB-0-F and PGB-0-So for 24 h. The boron concentrations in MCF-7 and MDA-MB 231 cells were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Results represent mean ± SD ($n = 3$). The microdistribution of boron after treatment with BPA, PGB-0-F, and PGB-0-So in MCF-7 (**c**) and MDA-MB 231 breast cancer cells (**d**). Cells were treated with 0.3 mM BPA and 0.15 mM PGB-0-F and PGB-0-So for 24 h. Boron was stained using DAHMI, whereas the nucleus was stained with DRAQ5. The microdistribution were observed by an LMS 700 confocal scannning laser microscope

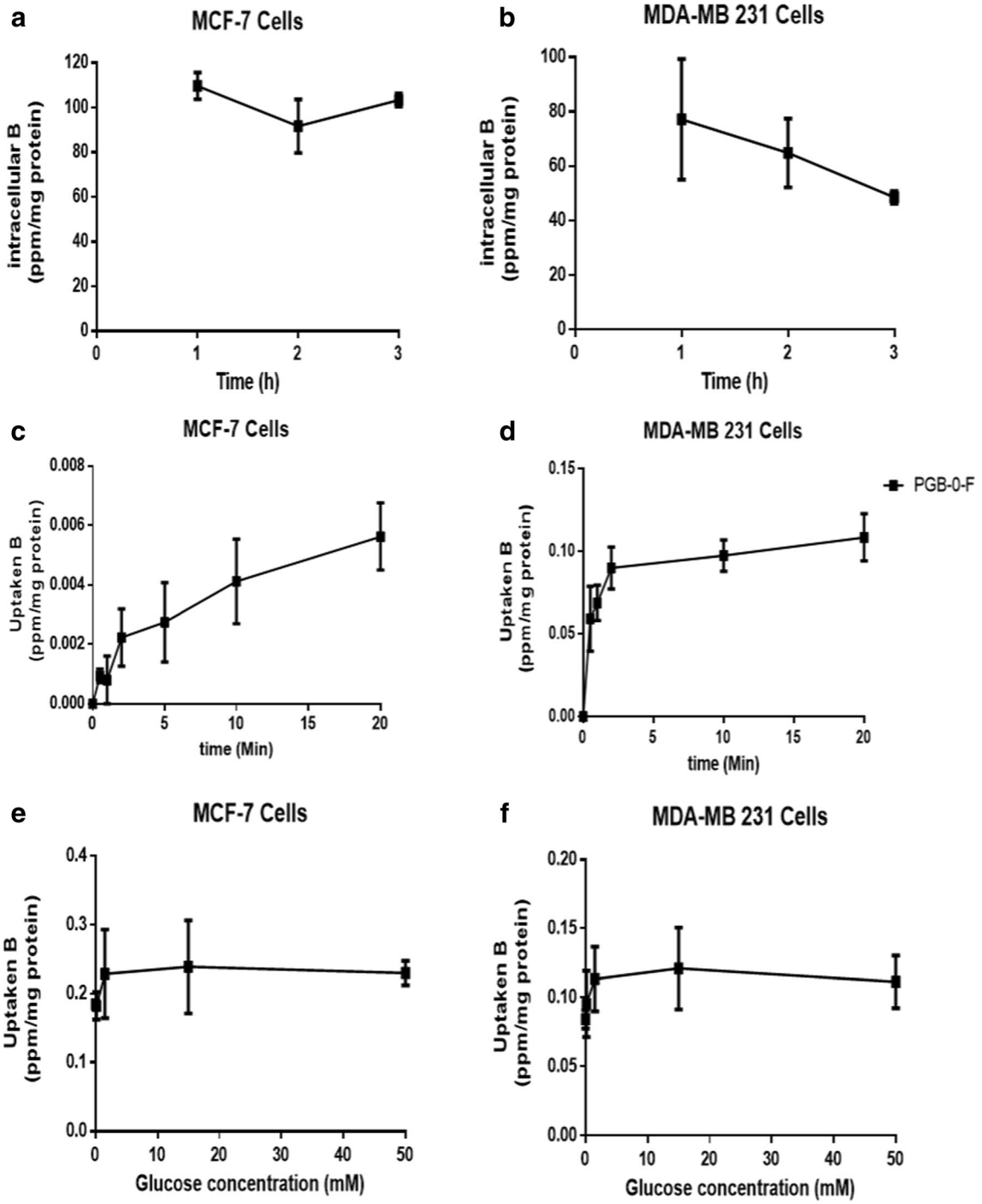

Fig. 4 The uptake of boron in MCF-7 (a) and MDA-MB 231 cells (b). Cells were treated with a sodium-free HBSS buffer containing 0.15 mM PGB-0-F, followed by incubation for 0.5, 1, 2, 5, 10, and 20 min. The uptake of boron in MCF-7 (**c**) and MDA-MB 231 cells (**d**) with or without glucose. Cells were treated with a sodium-free HBSS buffer containing 0.15 mM PGB-0-F and serial concentrations of glucose (0.15, 1.5, 15, and 50 mM), followed by incubation for 20 min. Efflux study of PGB-0 in MCF-7 (**c**) and MDA-231 cells (**d**). Cells were treated with medium containing 0.15 mM PGB-0-F, followed by incubation for 24 h. Cells were lysed with 0.5% Tween within 1, 2, and 3 h after medium exchange. Cells were then lysed with 0.5% Tween. Protein content was evaluated using BCA as per the manufacturer's instructions. Boron concentration was determined by the indirect competitive ELISA against anti-BPA mAb. Results represent mean ± SD ($n = 4$)

A compound for use in BNCT should be nontoxic toward cancer cells and normal cells [18]. Low cytotoxicity is an important parameter for BNCT to maximize the accumulation of boron within cancer cells [19]. To achieve

successful BNCT, BCP must be selectively delivered and accumulated in the tumor cells with ^{10}B concentration at 15–30 ppm (~10^9 atoms/cell) [20, 21]. In the present study, PGB-0 displayed low solubility even in the sugar formulation; therefore, further study is needed to increase its solubility.

We used a sodium-free HBSS buffer in this study because fructose and sorbitol are transported by GLUT5 that is a sodium-independent transporter [22]. Analysis of the intracellular accumulation of boron revealed that the uptake of PGB-0 in MCF-7 cells was better than that in MDA-MB 231 cells. The doubling time of MCF-7 cells (30.2 ± 0.7 h) is longer than that of MDA-MB 231 cells (28.1 ± 1.2 h) [23]; however, the mean cell diameter of MCF-7 cells is 25 μm, and that of MDA-MB 231 cells is 18 μm [24], which thus provide more cellular components that are responsible for boron uptake.

The uptake of PGB-0-F by MDA-MB 231 cells (0.1 ppm/mg at 20 min) was approximately 20 times greater than that by MCF-7 cells (0.005 ppm/mg). In general, the uptake rate via active transport is greater than that via passive diffusion, and membrane transport by passive diffusion would not change extensively depending on the type of cells. In addition, the initial uptake rate (in the first 2 min) was much greater in MDA-MB231 cells than in MCF-7 cells. However, the time course of PGB-0-F in MCF-7 and MDA-MB 231 cells exhibited the pattern of active transport and passive diffusion, respectively.

The uptake study of PGB-0 revealed that boron was uptaken into MCF-7 cells via active transport, as shown by the linear time course of boron concentration, which is similar to the active transport profile of the sex hormone [25]. The possible mechanism of PGB-0 uptake in MCF-7 cells is via receptor-mediated endocytosis as PGB-0 may interact with estrogen receptor that is highly expressed on MCF-7 cells. Active transport against a gradient concentration is often mediated by the coupling of ions or molecule transfer through an energy-supplying process [26]. The time course concentration of PGB-0 in MCF-7 cells is similar to the time course concentration of BPA in 9 L cells, which is mediated by active transport [27]. The amount of uptaken PGB-0 in MDA-MB 231 cells, which are more malignant, is higher than that in MCF-7 cells. This phenomenon is similar to the uptake of BPA in 9 L cells, which are malignant, and higher than that in V79 normal cells [27]. A previous in silico study of the curcumin analogs PGV-0, PGV-1, HGV-0, and HGV-1 reported interaction between these compounds and the estrogen receptor alpha, thus indicating their potential as selective estrogen receptor modulators (SERMS) [28]. An in silico study revealed interaction between PGB-0 and the ATP-binding site of EGFR, showing its potential to inhibit EGF signaling pathways [14]. Probably, there exist interactions between PGB-0 and regulatory proteins that are highly expressed on MCF-7 cells, e.g., estrogen receptor and progesterone receptor. This possible mechanism must be further explored by binding studies between estrogen receptor and PGB-0.

The time course of boron uptake via passive diffusion is not linear [29], and accordingly, the results of the present study in MDA-MB 231 cells indicated that boron uptake was mediated by passive diffusion. The time course of PGB-0 concentration in MDA-MB 231 cells is similar to the time course concentration of boric acid in 9 L cells, which is considered to be transported into the cells by diffusion [27]. The glucose transporter inhibition study revealed that there was no significant difference in boron uptake in the presence of glucose, indicating that the uptake is independent of glucose transporter. However, further study using a specific glucose transporter inhibitor is needed.

The efflux study demonstrated that intracellular boron concentration remained stable after 3 h of medium change in MCF-7 cells. This phenomenon is supported by the DAHMI staining that revealed accumulation of PGB-0 in the cytoplasm. Further binding studies are required to confirm the interaction between PGB-0 and receptors that are highly expressed on MCF-7 cells, e.g., estrogen receptor and progesterone receptor. In MDA-MB 231 cells, the boron concentration decreased after 3 h of medium change, indicating that PGB-0 did not bind to the specific protein and thus easily released from MDA-MB 231 cells.

In the present study, we observed that up to 3 h, PGB-0 exhibited low efflux in MCF-7 cells and therefore could be targeted to luminal A breast cancer cells. Moreover, PGB-0 could target the triple-negative MDA-MB 231 breast cancer cells. A previous study of BPA efflux (incubated at 37 °C in MEM) in 9 L cells demonstrated that after 2 h, there was hardly any boron remaining in the cells [27].

BPA is widely used as a BCP in BNCT for various types of cancers such as head and neck cancer [30–32] and melanoma [33]. However, only one study has demonstrated the use of BPA for BNCT in breast cancer treatment [34]. The present study showed that PGB-0 is a good candidate as a BCP in BNCT of breast cancer based on the good uptake, microdistribution, and low efflux. Therefore, the sugar formulations developed in this study could improve PGB-0 solubility and be a good candidate as a BCP against breast cancer.

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Compliance with ethical standards

Conflict of interest All the authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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