Intracellular Boron Accumulation in CHO-K1 Cells Using Amino Acid Transport Control

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Abstract

BPA used in BNCT has a similar structure to some essential amino acids and is transported into tumor cells by amino acid transport systems. Previous study groups have tried various techniques of loading BPA to increase intracellular boron concentration. CHO-K1 cells demonstrate system L (LAT1) activity and are suitable for specifying the transport system of a neutral amino acid. In this study, we examined the intracellular accumulation of boron in CHO-K1 cells by amino

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acid transport control, which involves co-loading with L-type amino acid esters. Intracellular boron accumulation in CHO-K1 cells showed the greatest increased upon co-loading 1.0 mM BPA, with 1.0 mM L-Tyr-O-Et and incubating for 60 min. This increase is caused by activation of a system L amino acid exchanger between BPA and L-Tyr. The amino acid esters are metabolized to amino acids by intracellular hydrolytic enzymes that increase the concentrations of intracellular amino acids and stimulate exchange transportation. We expect that this amino acid transport control will be useful for enhancing intracellular boron accumulation.

Keywords: L-p-boronophenylalanine (BPA); boron neutron capture therapy (BNCT); amino acid transport; Chinese hamster ovary cells

1. Introduction

Intracellular boron accumulation is crucial for improving the clinical outcome of boron neutron capture therapy (BNCT). One of the clinically capable ^{10}B compounds used in BNCT trials is L-*p*-boronophenylalanine (BPA), which has a structure similar to the essential amino acids tyrosine and phenylalanine and is transported into tumor cells by amino acid transport systems [1, 2]. The transport of neutral amino acids relies on system L (LAT1; an amino acid exchanger) activity and Chinese hamster ovary cells (CHO-K1 cells) are reported to be suitable for specifying the transport system of a neutral amino acid [3-5].

Previous studies [5] suggest that the uptake of synthetic neutral amino acids by CHO-K1 cells is increased by co-loading with L-type amino acid esters. In some previous reports on in vitro experiments using BPA [1, 6-10], cell-lines including 9L, C6, SCCVII, V79, and T98 were used for neutron irradiation experiments not associated with amino acid transport control.

In this study, we assumed that BPA, which is also a neutral amino acid, would exhibit a similar increasing effect by co-loading in CHO-K1 cells. The parameters for loading BPA in CHO-

K1 cells were optimized to determine the intracellular accumulation of boron in CHO-K1 cells by amino acid transport through co-loading with L-type amino acid esters.

2. Materials and Methods

2.1 Cell line

We purchased CHO-K1 cells (No. RCB0285) from the RIKEN BioResource Center cell bank (Tsukuba, Japan), plastic tissue culture dishes (No. 150288; diameter, 60 mm) from Falcon (), and plastic culture flasks (No. 156367; surface area, 25 cm^2) from Nalge Nunc International.

CHO-K1 cells were seeded onto 60-mm-diameter dishes containing Dulbecco's modified Eagle's medium (DMEM) (No. D6429; Sigma-Aldrich Japan K.K., Tokyo, Japan) with 10% fetal bovine serum without antibiotics in an atmosphere of 5% CO_2 and 95% air at 37°C (pH 7.4). Subculture was performed every five days using 0.02% EDTA and 0.05% trypsin. After removal of the culture medium, each dish was washed with 5.0 ml of Dulbecco's phosphate-buffered saline (DPBS) (No. D8622) as an incubation medium, which had been pre-incubated for 10 min.

2.2 Optimizing BPA concentration and BPA incubation time for increased intracellular boron accumulation in CHO-K1 cells

There are no previous reports of loading BPA in CHO-K1 cells. Therefore, we tried to optimize the parameters of concentration-dependence and incubation time for loading BPA to increase boron accumulation in CHO-K1 cells. In the BPA concentration-dependence experiment, we loaded CHO-K1 cells (n=4) with 2.0 mL of BPA at concentrations of 0.001 mM (0.21 μg/ml), 0.003 mM (0.63 μg/ml), 0.01 mM (2.09 μg/ml), 0.03 mM (6.27 μg/ml), 0.1 mM (20.90 μg/ml), 0.3 mM (62.70 μg/ml), 1.0 mM (209.01 μg/ml), or 3.0 mM (627.03 μg/ml) for 10 min. In the BPA incubation time experiment, we loaded CHO-K1 cells (n=4) with 2.0 ml of 1.0 mM BPA at incubation times of 5, 10, 15, 30, 60, or 120 min. After removal of the incubation medium, the samples were rinsed twice with ice-cold DPBS. Each sample was then exfoliated with trypsin and placed in test tubes with DPBS. In addition, each sample was suspended in 1.0 ml of 10% nitric acid, placed in sample tubes, and cryopreserved for ashing.

2.3 Screening of effective amino acids esters for increased intracellular boron accumulation

We co-loaded BPA with amino acid esters as a method of amino acid transport control. To identify the amino acid esters that increase boron accumulation in CHO-K1 cells, 1.0 mM of each was co-loaded with 1.0 mM BPA and incubated for 120 min. The following amino acid esters were tested: L-tyrosine ethyl ester (L-Tyr-O-Et), glycine ethyl ester (Gly-O-Et), L-alanine ethyl ester (L-Ala-O-Et), L-serine ethyl ester (L-Ser-O-Et), L-leucine ethyl ester (L-Leu-O-Et), L-phenylalanine ethyl ester (L-Phe-O-Et), L-methionine ethyl ester (L-Met-O-Et), L-valine ethyl ester (L-Val-O-Et), L-lysine ethyl ester (L-Lys-O-Et), and L-arginine ethyl ester (L-Arg-O-Et). Each sample was stored by the same technique described in section 2.2 prior to ashing.

2.4 Measurement of intracellular boron accumulation at different incubation times and concentrations of amino acid esters

In this experiment, we used the amino acid esters that increased intracellular boron accumulation in the method described in section 2.3. To determine an effective incubation time, 1.0 mM L-Tyr-O-Et and 1.0 mM L-Phe-O-Et were co-loaded with 1.0 mM BPA and incubated for 60, 120, and 180 min. Furthermore, to determine the effective amino acid ester concentration, L-Tyr-O-Et at concentrations of 0.1, 0.3, 1.0, 3.0, and 10.0 mM was co-loaded with 1.0 mM BPA and incubated for 120 min. Each sample was stored by the same technique described in method 2.2 prior to ashing.

2.5 Measurement of intracellular boron accumulation in CHO-K1 cells by inductively coupled plasma atomic emission spectroscopy (ICP-AES)

Each sample, prepared using methods 2.3-2.6, was defrosted to room temperature, and 0.8 ml of 60% concentrated nitric acid was added for ashing. Ashing involved subjecting the samples to thermolysis at 115 °C for 120 min using an aluminum-block bath (ALB-221; Scinics Corporation, Tokyo). We then added Milli-Q water to a final volume of 2.0 ml and filtered the mixture through a membrane filter to obtain the liquid for measurement. The boron concentration in each sample was measured by ICP-AES (ICPS-8100; Shimadzu Corporation, Kyoto, Japan). For ICP-AES, we

prepared four standard solutions (0.01, 0.1, 1.0, and 10.0 ppm) by diluting 50 ml portions of a boron standard solution (1000 pm) to volume with 10% nitric acid; we also prepared a blank solution of 10% nitric acid (0 ppm). The detection limit of boron for our ICP-AES system was 0.066 ppm.

2.6 Statistical analysis

Data were collated as means ± standard deviation of four samples and each experiment was performed in duplicate. Results were analyzed using the Student's *t* test and $P<0.01$, or $P<0.05$ were considered statistically significant.

3. Results

In an initial BPA concentration-dependence experiment, the intracellular boron accumulation rate [% of control] was calculated from the intracellular boron concentration on loading 0.001 mM BPA as the control. The intracellular boron accumulation (mean±SD) was 100±11.5% on loading 0.001 mM BPA and gradually increased to 126±20.0% at 3.0 mM BPA. The maximum increase of 16% was recorded between 0.3 and 1.0 mM compared with 5% (0.001-0.3 mM) and 5% (1.0-3.0 mM). In particular, BPA concentrations of 1.0 mM or more increased the intracellular accumulation of boron ($P<0.01$). In an initial investigation of the time course of BPA uptake into CHO-K1 cells by loading 1.0 mM BPA, the control was the intracellular boron concentration at 5 min. Intracellular boron accumulation (mean±SD) was 105±9.5% at 15 min and gently increased to 113±9.2% at 60 min. The intracellular accumulation of boron at 60 min and beyond represented equilibration.

Figure 1 shows the intracellular accumulation of boron on co-loading 1.0 mM BPA with 1.0 mM amino acid esters (L-Tyr-O-Et, Gly-O-Et, L-Ala-O-Et, L-Ser-O-Et, L-Leu-O-Et, L-Phe-O-Et, L-Met-O-Et, L-Val-O-Et, L-Lys-O-Et, and L-Arg-O-Et) in CHO-K1 cells. The intracellular boron accumulation values (mean±SD) were: BPA alone, 100±3.2%; BPA+L-Tyr-O-Et, 115±5.1%; BPA+Gly-O-Et, 93±9.54%; BPA+L-Ala-O-Et, 92±5.0%; BPA+L-Ser-O-Et, 84±11.5%; BPA+L-Leu-O-Et, 93±2.35%; BPA+L-Phe-O-Et, 109±2.9%; BPA+L-Met-O-Et, 107±3.6%; BPA+L-Val-O-

Et, 88±8.47%; BPA+L-Lys-O-Et, 85±6.1%; and BPA+L-Arg-O-Et, 91±6.7%. The Student's *t* test revealed significant differences between L-Tyr-O-Et ($P<0.01$) and L-Phe-O-Et ($P<0.05$). Co-loading BPA and L-Tyr-O-Et increased the intracellular accumulation of boron by 15%, and L-Phe-O-Et increased the amount of accumulation by 9% compared with BPA alone.

Fig. 1

Figure 2 shows the time course of uptake into CHO-K1 cells by loading BPA (1.0 mM) alone, co-loading BPA (1.0 mM) with L-Phe-O-Et (1.0 mM), and co-loading BPA (1.0 mM) with L-Tyr-O-Et (1.0 mM). The intracellular boron accumulation values by BPA alone and BPA+L-Phe-O-Et (mean±SD) for 60 min were 100±0.5% and 93±2.2%, respectively, and the increase in intracellular accumulation at 60 min or beyond represented equilibration. In BPA+L-Tyr-O-Et, intracellular accumulation of boron (mean±SD) was 111±6.1% at 60 min and increased to 125±11.5% at 180 min. Co-loading BPA with L-Tyr-O-Et and incubating for 180 min increased the uptake of boron by 29% compared with BPA alone. On the other hand, co-loading BPA with L-Phe-O-Et did not have a significant effect on BPA uptake compared with BPA alone.

Fig. 2

L-Tyr-O-Et showed concentration-dependency in the accumulation of intracellular boron in CHO-K1 cells (Fig. 3). Intracellular boron accumulation values (means±SD) were: 0.1 mM, 100±8.0%; 0.3 mM, 102±11.2%; 1.0 mM, 121±4.1%; 3.0 mM, 123±3.1%; and 10.0 mM, 119±7.4%. Concentrations of 0.1 and 0.3 mM L-Tyr-O-Et did not affect the intracellular accumulation of boron. However, the rate of intracellular boron accumulation increased to a maximum of 19% with 1.0 mM and to a maximum of 23% with 3.0 mM. Moreover, the intracellular accumulation of boron was steady when the concentration of L-Tyr-O-Et was 1.0-10.0 mM ($P<0.01$).

Fig. 3

4. Discussion

Intracellular boron accumulation was unchanged when BPA concentration was 0.001 or 0.3 mM, while an increase was found when concentrations of 1.0 mM or greater were used, indicating

the influence of trans-stimulation in amino acid transport. By changing the incubation time from 5 to 30 min, the intracellular accumulation of boron gradually increased and reached a plateau at 120 min. Consequent elevation of the intracellular concentration of BPA activated the uptake of amino acids. BPA co-loaded with amino acid esters such as L-Tyr-O-Et and L-Phe-O-Et in CHO-K1 cells resulted in concentration-dependent enhanced intracellular accumulation of boron compared with BPA alone. If the elevation of BPA and the esters is not caused by diffusion through cell membrane permeability but by active transport, our method could avoid competitive inhibition, which potentially minimizes the benefit of enhancing amino acid transport by co-loading.

In this study, the BPA concentration that induced trans-stimulation in CHO-K1 cells was greater than 1.0 mM. Previous reports [1, 2] have suggested that an increase in BPA uptake in response to certain amino acid concentrations inside the cell membrane is caused by co-transport of sodium ion and an amino acid connected to a carrier, or passive transport depending on the concentration gradient between the outside and inside of the cell membrane. In an examination using mouse melanoma cells, Papaspyrou et al. [2] showed that the intracellular accumulation of boron from loading BPA alone is increased at a concentration of 0.5 mM or more. Moreover, loading 1.0 mM BPA after pre-loading with 5.0 mM L-Tyr increased the intracellular accumulation of boron about 1.8- to 2.7-fold. In an examination using C6 glioma cells [1], loading 2.0 mmol BPA alone after pre-loading with 50 μg/g L-DOPA increased the accumulation of intracellular boron about five-fold. The increased intracellular accumulation of boron is presumed to be due to the activation of amino acid transport according to the concentration gradient as a result of pre-loading with L-Tyr and L-DOPA. The similar biological properties that BPA as an amino acid shares with L-Tyr and L-DOPA may have led to activated accumulation in CHO-K1 cells by the same transport mechanisms.

By changing the incubation times of BPA, the intracellular accumulation of boron slowly increased for 0-30 min. Intracellular accumulation reached 13% of the maximum peak at 60 min before reaching a plateau from 60-120 min. Shikano et al. tried to increase the uptake of ^{125}I-3-iodo-

α-methyl-L-tyrosine (4-^{125}I-mIMT) using CHO-K1 cells. 4-^{125}I-mIMT is the neutral amino acid Tyr labeled with ^{125}I. In that experiment, the uptake of 4-^{125}I-mIMT in CHO-K1 cells reached a peak at 10 min [11]. This result may be extrapolated to the intracellular accumulation of boron in BPA, where 10 min incubation is equivalent to that of 60 min or more, because BPA is also a neutral amino acid. However, an incubation time of 10 min may be insufficient to allow the accumulation of enough intracellular boron because the transport activity of Phe is substantially lower than that of Tyr. On the other hand, an examination of mouse melanoma cells indicates that the intracellular accumulation of boron within 30 min is low, and a longer incubation time results in greater accumulation [2]. Moreover, the intracellular accumulation of boron after loading at 2 h in C6 glioma cells is lower by approximately 86% compared with that at 4 h [1]. Furthermore, in clinical BNCT for brain tumors, intracellular boron concentrations reach a maximum peak 7 h after loading BPA [12]. In accordance with our experiment and these previous reports, a long incubation time is preferable for obtaining high intracellular accumulation of boron. Therefore, the incubation time of BPA in CHO-K1 cells should be more than 60 min.

In this experiment, we determined the amino acid esters that increased boron accumulation in CHO-K1 cells and determined their incubation times and concentration-dependency. Co-loading BPA with amino acid esters such as L-Tyr-O-Et and L-Phe-O-Et in CHO-K1 cells resulted in greater accumulation of intracellular boron than that by BPA alone. The maximum increase in accumulation was noted on co-loading BPA with L-Tyr-O-Et (15%). As a result of the incubation time with L-Tyr-O-Et and L-Phe-O-Et, intracellular accumulation with L-Tyr-O-Et was higher by 18-29% than that with BPA alone or with L-Phe-O-Et, and these differences reached a maximum at 180 min. In the concentration-dependence investigation of amino acid esters, the intracellular accumulation of boron largely increased at 1.0 mM, and accumulation remained high at concentrations of 3.0 mM or higher. The main factor affecting the increase when co-loading with L-Tyr-O-Et and L-Phe-O-Et is caused by the structure of BPA. Therefore, BPA is taken into cells through system L because it is a neutral amino acid similar to Phe or Tyr. In tumor cells, most of the

essential amino acids that are neutral with large side-chains, such as branched-chain or aromatic amino acids, enter the cells through system L [10, 13]. A component of system L, 4F2hc protein, was established as an activator of system L by an expression experiment in *Xenopus oogonia*. [14]. Kanai et al. [3] showed that LAT1 expression is enhanced by 4F2hc in human malignant tumor tissue. LAT1 has high affinity to amino groups with large side-chains, and the hydrophobicity of the side-chain is important for substrate recognition of LAT1. Therefore, substances such as Tyr and Phe possessing an alpha amino acid structure are accepted at the substrate binding site. Jara et al. reported that the uptake of L-Tyr increases because the L-substrates, such as L-Tyr or L-Met, are accumulated in the cells beforehand [15]. Both Tyr and Phe are taken into cells through system L (LAT1) but the intracellular accumulation of boron from loading L-Tyr-O-Et was higher than that from L-Phe-O-Et. Our data is similar to a previous report on artificial neutral amino acid (123I-3-iodo-α-methyl-L-Tyrosine: IMT) uptake using CHO-K1 cells [5]. The driving force of system L (LAT1) is related to the amino acid concentration accumulated in cells in advance and the amino acid concentration gradient causes extra- and intracellular transport exchange. To increase intracellular boron accumulation, therefore, it is important that the transport exchange is activated by the preceding intracellular amino acid concentration. Furthermore, it is essential to increase the concentration above a certain level to initiate this transport. In our experiment, we established that the co-loading parameters required for trans-stimulation were an L-Tyr-O-Et concentration of more than 1.0 mM, and an L-Tyr-O-Et incubation period of greater than 60 min.

The method of transport in our experiment was different to the BPA loading techniques of previous reports [1, 2, and 6]. In these reports, BPA was taken into cells through system L (LAT1). However, in our method, BPA was taken into cells by membrane permeability. Therefore, our method did not show characteristic competitive inhibition between BPA and the amino acid esters that occurs in transport exchange through system L (LAT1). The amino acid esters taken into the cells are metabolized to amino acids by intracellular hydrolytic enzymes. The intracellular amino acid level then rises and this phenomenon induces trans-stimulation and increases LAT1 on the cell

membrane surface. Therefore an amino acid gradient for the obligatory exchange of intracellular amino acids with extracellular amino acids occurs. In our study, L-Tyr-O-Et was taken into cells by membrane permeability and L-Tyr was metabolized to amino acids by intracellular hydrolytic enzymes. Transport exchange between extracellular BPA and intracellular L-Tyr through system L was then activated according to the concentration gradient of the intracellular amino acids (Fig. 4). Shikano et al. reported that the uptake of extracellular IMT reaches a maximum from co-loading with L-Tyr-O-Et at 60 min in CHO-K1 cells and is maintained for 180 min [5]. In addition to this study, there are examinations of BPA uptake using co-loading with L-Tyr or L-DOPA [1, 3, and 6], which are taken into cells via system L (LAT1). These reports indicate that intracellular boron accumulation is increased by loading BPA continuously after system L (LAT1) is activated by pre-loading an L-substrate (Fig. 5). Wittig et al. [16] reported that pre-loading with L-DOPA is a useful technique to increase BPA uptake while co-loading with L-DOPA inhibits BPA uptake. Therefore, the L substrate that is left in the cells might inhibit uptake by continuous loading of BPA where L-Tyr and L-DOPA are pre-loaded. That is to say that co-loading with L substrate, which requires an adequately long incubation period, shows inefficient BPA uptake. Furthermore, it is problematic that the intracellular accumulation of boron is increased because co-loading BPA with L-DOPA produces competitive inhibition between BPA and L-DOPA. On the other hand, amino acid transport control using the amino acid ester that we adopted in this study can increase the accumulation of intracellular boron even on co-loading BPA with amino acid esters because our method complicates competitive inhibition between BPA and the amino acid ester.

Fig. 4

Fig. 5

Activation of system L by increasing the intracellular amino acid concentration can potentially be effective for increasing the intracellular accumulation of BPA. Amino acid transport control by co-loading BPA with L-Tyr-O-Et can increase intracellular boron accumulation. In our experiment using CHO-K1 cells, intracellular boron accumulation was increased by modifying the

L-Tyr-O-Et incubation time and concentration to more than 60 min and greater than 1.0 mM, respectively.

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Figure Captions

Fig. 1 Effects of co-loading BPA (1.0 mM) with amino acid esters (1.0 mM). * $P<0.01$ versus uptake by loading BPA alone. ** $P<0.05$ versus uptake by loading BPA alone.

Fig. 2 Time course of uptake into CHO-K1 cells by loading BPA (1.0 mM) alone, co-loading BPA

(1.0 mM) with L-Phe-O-Et (1.0 mM), or co-loading BPA (1.0 mM) with L-Tyr-O-Et (1.0 mM).

Fig. 3 L-Tyr-O-Et concentration-dependency on intracellular boron accumulation in CHO-K1 cells. * $P<0.01$ versus uptake by loading L-Tyr-O-Et (0.001 mM).

Fig. 4 Mechanism of uptake of BPA into CHO-K1 cells by co-loading with an amino acid ester.

Fig. 5 Mechanism of uptake of BPA into cells by co-loading with L system substrates.

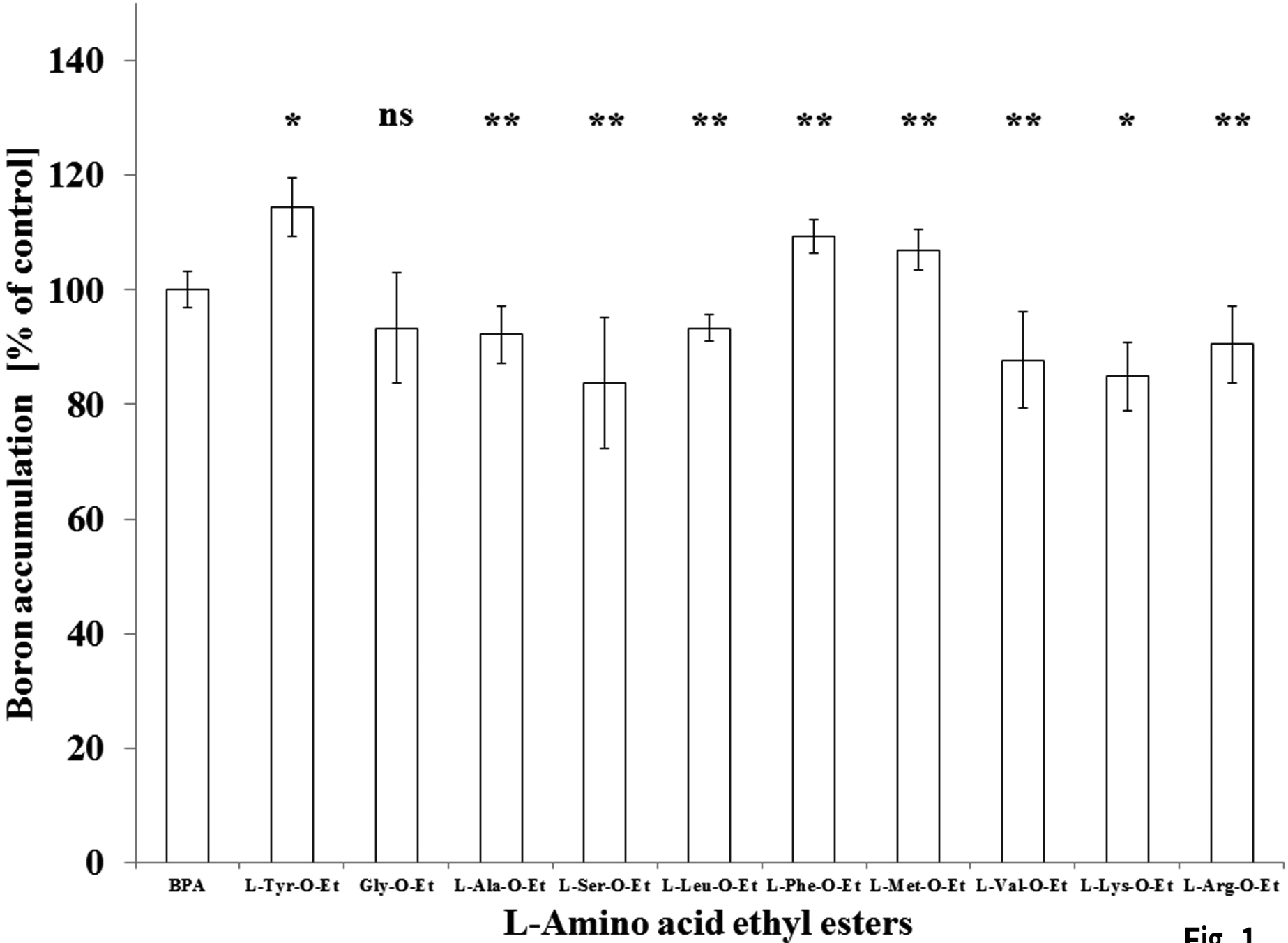
Boron accumulation [% of control]
140
120
100
80
60
40
20
0
* ns ** ** ** ** ** ** * **
BPA
L-Tyr-O-Et
Gly-O-Et
L-Ala-O-Et
L-Ser-O-Et
L-Leu-O-Et
L-Phe-O-Et
L-Met-O-Et
L-Val-O-Et
L-Lys-O-Et
L-Arg-O-Et
L-Amino acid ethyl esters

Fig. 1

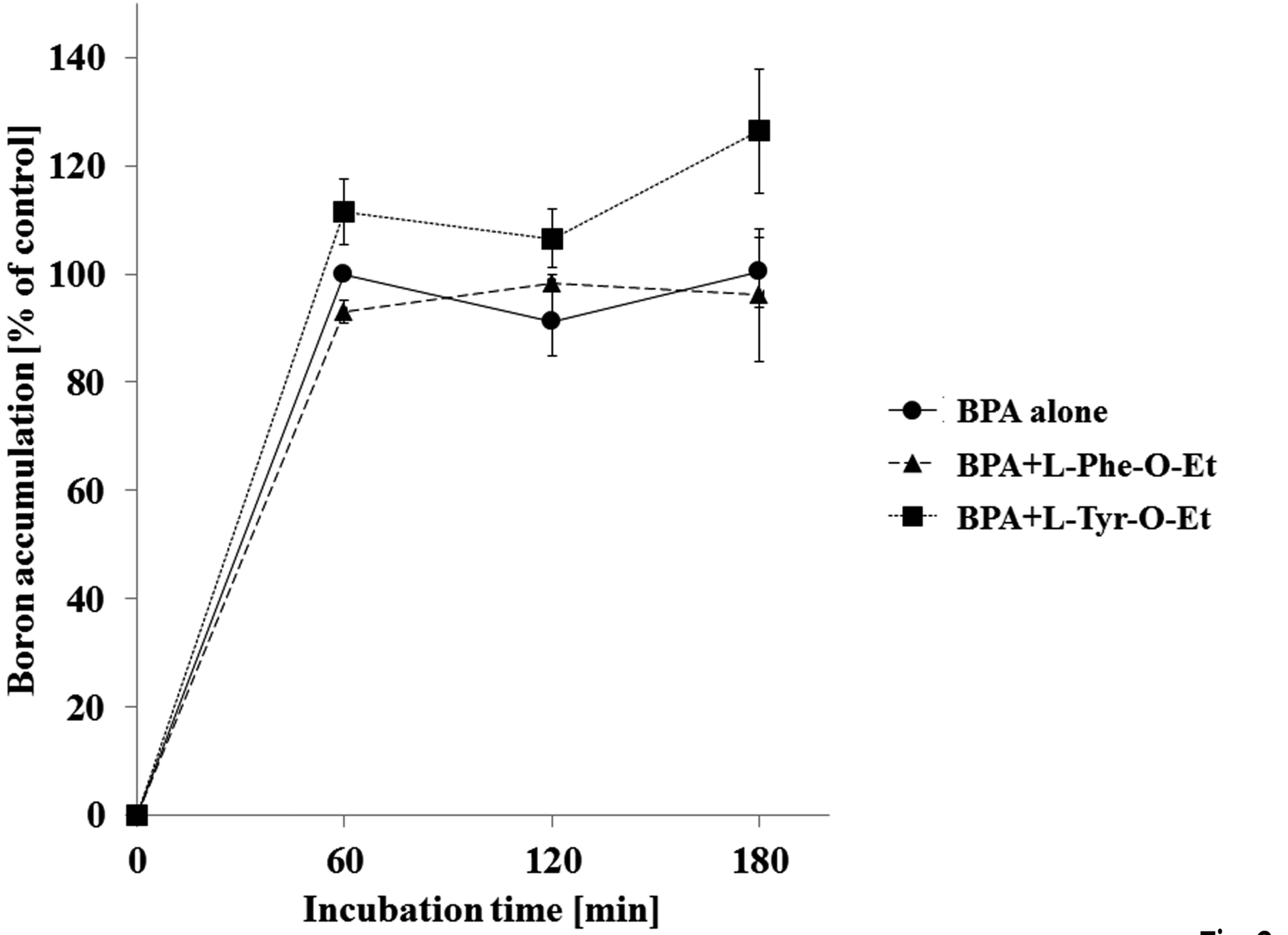
140
120
100
80
60
40
20
0
Boron accumulation [% of control]
0
60
120
180
Incubation time [min]
BPA alone
BPA+L-Phe-O-Et
BPA+L-Tyr-O-Et

Fig. 2

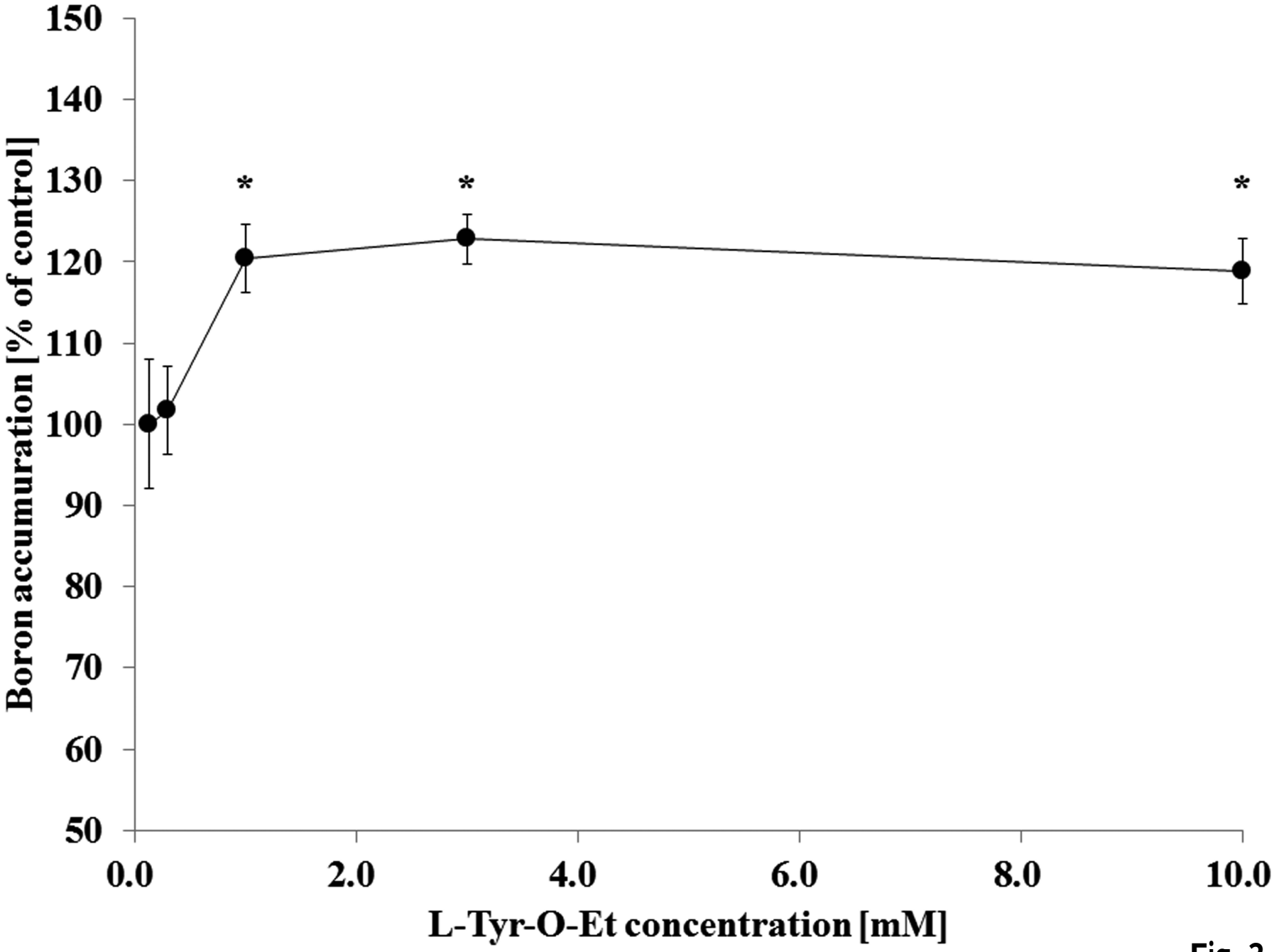
150
140
130
120
110
100
90
80
70
60
50
Boron accumuration [% of control]
*
*
*
0.0
2.0
4.0
6.0
8.0
10.0
L-Tyr-O-Et concentration [mM]

Fig. 3

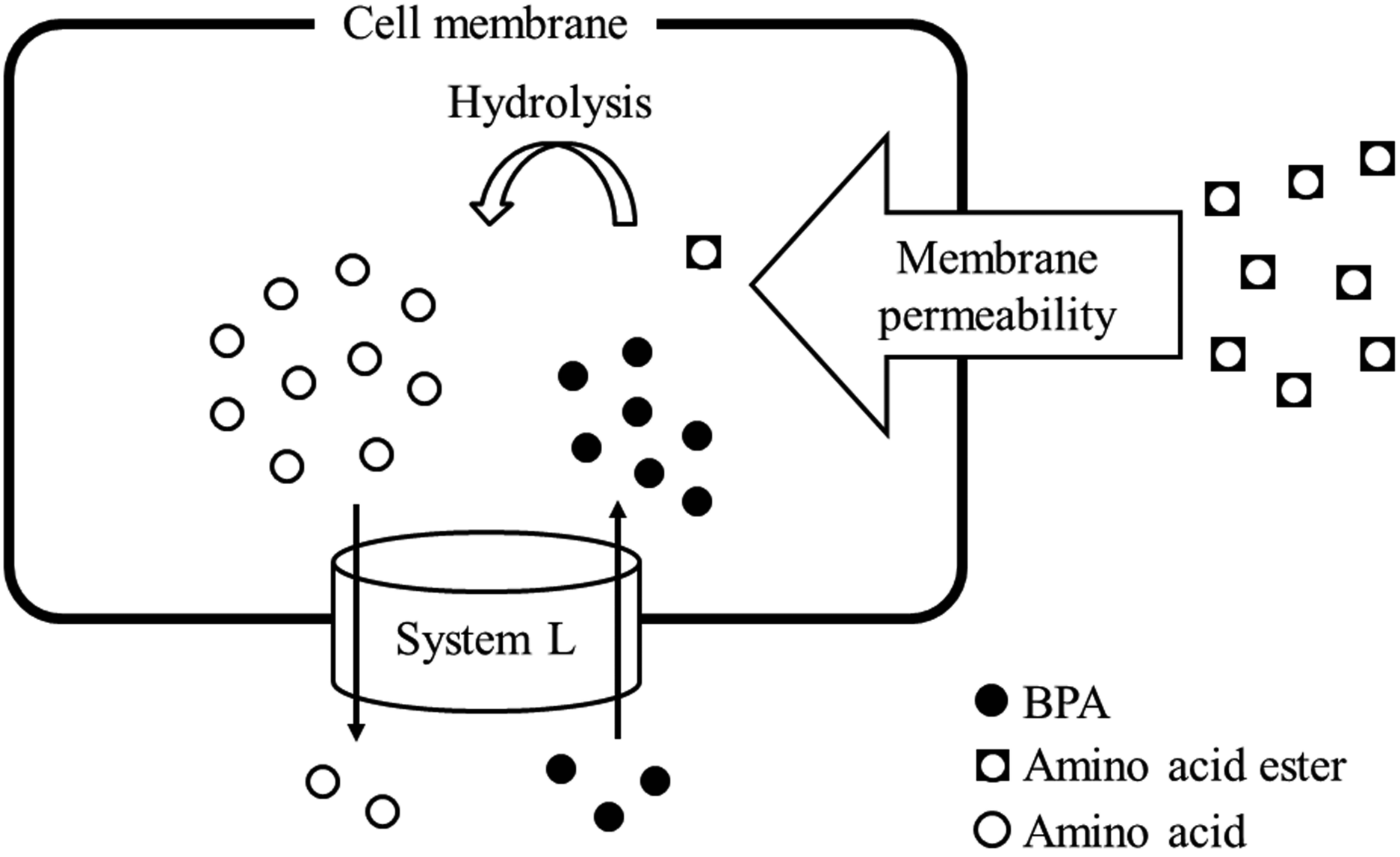
Cell membrane
Hydrolysis
Membrane permeability
System L
BPA
Amino acid ester
Amino acid

Fig. 4

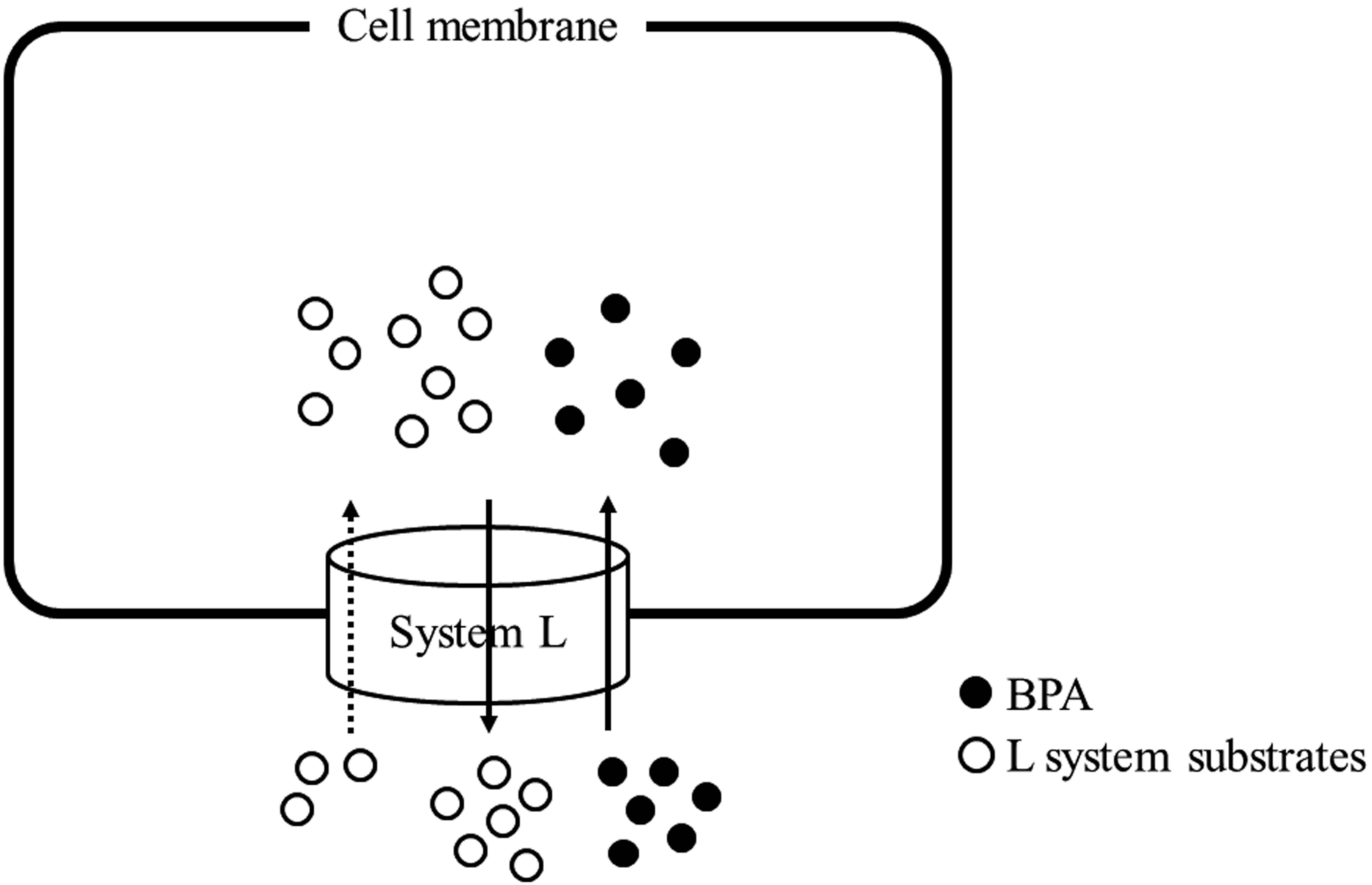

Fig. 5