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Covalent modification of phosphatidylethanolamine by benzyl isothiocyanate and the resultant generation of ethanolamine adduct as its metabolite

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Keywords:
Isothiocyanate, BITC, Phosphatidylethanolamine, Adduct, Biomarker

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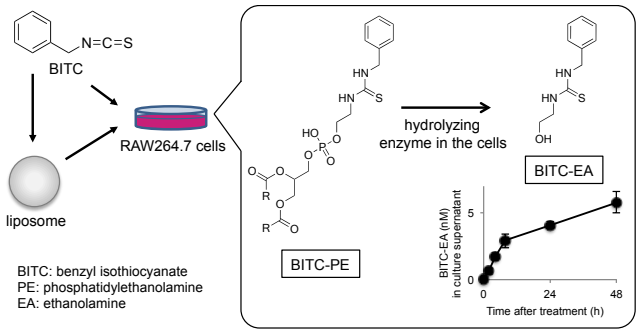

Abstract

Benzyl isothiocyanate (BITC), a dietary isothiocyanate (ITC) derived from cruciferous vegetables, has anticancer properties. It is believed that the ITC moiety (-N=C=S) that reacts predominantly with thiol compounds plays a central role in triggering the activities resulting from these properties. Recent studies have demonstrated that ITCs also covalently modify amino moieties in a protein. In this study, we examined the chemical reaction between BITC and the aminophospholipid, phosphatidylethanolamine (PE), in the cell membrane or lipoprotein particle. To detect the BITC-modified PE, the bond between ethanolamine (EA) and phosphatidic acid in PE was cleaved using phospholipase D to form the BITC–EA adduct, which was then measured. BITC–EA was detected from the BITC-treated unilamellar liposome and low-density lipoprotein even with only a few micromoles of BITC treatment, suggesting that BITC might react with not only a thiol/amino group of a protein but also an amino moiety of an aminophospholipid. Moreover, after incorporating BITC–PE included in the liposomes into the cultured cells or after direct exposure of BITC to the cells, free BITC–EA was excreted and accumulated in the medium in a time-dependent manner. It indicates that an intracellular enzyme catalyzes the cleavage of BITC–PE to produce BITC–EA. Because the ITC–amine adduct is stable, the ITC–EA adduct could be a promising indicator of ITC exposure *in vivo*.

Introduction

Isothiocyanates (ITCs) are naturally occurring molecules that are formed from the glucosinolates of cruciferous vegetables.[1,2] Ingested ITCs are considered to be able to trigger beneficial health effects, such as anticancer properties[1-4], possibly through their reaction with protein thiol groups, even though this reaction is reversible and the ITC–thiol adducts are unstable.[1] On the other hand, the chemical reaction between ITCs and amino moieties has been used in the Edman degradation[5] and protein labeling methods using a fluorescent dye.[6] Even once ITC is conjugated with a thiol, it can still react with lysine, and the ITC–lysine adduct is relatively stable.[7,8] At this point, ITC adduction with a lysine residue is likely to occur in physiological conditions.[9,10] Indeed, the ITC–lysine adduct was identified from human plasma albumin and hemoglobin after consumption of garden cress, watercress, and broccoli as a possible biomarker of ITC exposure.[9] Moreover, a reaction between ITC and amino moieties in a protein could be substantially involved in some biological effects by modulating their functions, such as activation of transient receptor potential ankyrin 1 and inhibition of macrophage migration inhibitory factor.[11-14]

Lipids play various critical roles in several biological actions.[15] Among the lipids, aminophospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine, constitute plasma membranes and lipoproteins. The amino moiety of PE is often modified by nonenzymatic glycation, and the adducts formed are also involved in some biological functions.[16-18] By contrast, conjugates of the products of lipid decomposition with PE or its ethanolamine (EA) metabolites have also been found.[19-22] For example, the reaction of linoleic acid hydroperoxide with PE generates *N*-hexanoyl PE along with *N*-hexanoyl EA as the metabolite.[20,21] To the best of our knowledge, there is no report on the chemical reaction between ITCs and aminophospholipids under physiological conditions. Based on an analogy of their chemical reactivity, ITCs might target PE and generate the ITC–PE adduct along with its metabolite, ITC–EA. The aim of this study was to investigate the reactivity of ITCs with PE and to identify the metabolic fate of the ITC–PE adduct in the cells.

Experimental Procedures

Chemicals

Benzyl ITC (BITC) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) was obtained from NOF CORPORATION (Tokyo, Japan), and egg lecithin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Stable isotopic EA-d_4 (H_2N-CD_2-CD_2-OH) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MI, USA). Human low-density lipoprotein (LDL) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Phospholipase D from *Streptomyces chromofuscus* was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Synthesis of BITC–EA adduct

BITC (10 mM) was mixed with EA (10 mM) or EA-d_4 (10 mM) in borate buffer (0.1 M; pH 9.0) overnight at 37°C. The adducts were purified by reverse-phase high-performance liquid chromatography (HPLC) as follows: BITC-modified EA was isocratically separated using the Develosil Combi-RP column (20 × 100 mm, Nomura Chemical Co., Ltd., Seto, Japan) with 0.1% formic acid/CH_3CN (1/1) as the eluent at a flow rate of 5.0 mL/min. The structures of BITC–EA and its stable isotope-labeled adducts were verified by mass spectrometry.

Preparation of BITC-treated unilamellar liposome and LDL

Egg lecithin and DPPE were dissolved in chloroform and mixed in a glass tube. The solvent was removed by evaporation under a vacuum, and the residue was dispersed in 2-mL phosphate-buffered saline (PBS) with ultrasonic irradiation. The final concentrations of egg lecithin and DPPE were 6.6 and 0.75 mg/mL, respectively. Unilamellar liposome was prepared using the Avanti Mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) according to the manufacturer's recommendations. Briefly, the Mini-extruder was preheated to 60°C, and 1-mL suspension was passed 13 times through a ø19-mm polycarbonate membrane (pore size: 0.1 μm). The resulting unilamellar liposomal suspension was then incubated with 0.5–500 μM of BITC in PBS for 20 h at 37°C. Unreacted BITC was removed from the liposomes by gel filtration using Quantum Prep PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's recommendation.

Another BITC-treated LDL was prepared by combining 1 mg/mL of LDL with 0–100 μM of BITC in PBS for 20 h at 37°C. BITC-modified LDL particles were separated from the unreacted BITC using the PCR Kleen Spin Columns.

Detection of BITC–EA by liquid chromatography with tandem mass spectrometry after treating the liposomes or LDL with phospholipase D

The BITC-treated unilamellar liposomes and LDL were incubated with 250 units of commercial phospholipase D in 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 5.0) with 15 mM of calcium chloride at room temperature for 7 h. The samples were then extracted twice using chloroform/methanol (2/1, *v*/*v*), and the organic phases were concentrated by evaporation under a vacuum. These samples were then dissolved in methanol, and BITC–EA-d_4 (final concentration, 200 nM) was added as the internal standard. The BITC–EA adduct was detected using multiple-reaction monitoring with 211.0/62.0 $[M+H]^+$ and 211.0/91.0 $[M+H]^+$ transitions by conventional HPLC connected to a Xevo TQD (Waters) or API3000 (AB Sciex) triple quadrupole mass spectrometer. HPLC for the Xevo TQD was conducted with a gradient system using solvent A (0.1% formic acid) and solvent B (CH_3CN) with an ACQUITY UPLC BEH C18 (2.1 × 50 mm) column at a flow rate of 0.4 mL/min. The gradient program was as follows: 0 min (A 80%), 0.5 min (A 80%), 3.5 min (A 20%), 3.9 min (A 20%), 4 min (A 80%), and 5 min (A 80%). HPLC for API3000 was also conducted with a gradient system using a Develosil ODS-HG-3 (2.0 × 50 mm) column at a flow rate of 0.2 mL/min with the same solvent combination. The gradient program was as follows: 0 min (A 90%), 5 min (A 50%), 5.1 min (A 90%), and 15 min (A 90%). The peak area of BITC–EA was corrected by the area of the internal standard, BITC-EA-d_4 (215.0/66.0 $[M+H]^+$).

Immunochemical detection of BITC–lysine residues in LDL

BITC–lysine residues were detected using enzyme-linked immunosorbent assay (ELISA) and Western blotting as previously described[7] with some modifications. For ELISA, the BITC-treated LDL (0.01 mg/mL in PBS) was dispensed in wells and incubated overnight at 4°C. After blocking with 1% skim milk, anti-BITC–Lys monoclonal antibody[8] was added and further incubated for 2 h at 37°C. After treatment with horseradish peroxidase–labeled affinity purified antibody to mouse IgG for an additional 1 h, color was developed by adding 100 μL of 3,3',5,5'-tetramethylbenzidine substrate solution (KPL, Gaithersburg MD, USA). The reaction was terminated with 100 μL of 1-M phosphoric acid, and the plate was measured at 450 nm.

For Western blotting, the BITC-treated LDL was applied to 0.6% agarose gel as previously described.[23] After electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-BITC–Lys monoclonal antibody or anti-Apo B antibody for 2 h at room temperature. After treatment

with the corresponding secondary horseradish peroxidase–conjugated antibody for 1 h, the membrane was visualized using Chemi-Lumi One detection reagent (Nacalai Tesque, Japan). The images were captured using the Lumino Image Analyzer LAS-1000plus and analyzed using Image Gauge software (ver. 3.4) (Fujifilm, Tokyo, Japan).

Exposure of BITC-treated unilamellar liposome or BITC to RAW264.7 cells

BITC (500 μM)-treated unilamellar liposomes, prepared as mentioned above, were incubated with 1 μg/mL of Rhodamine PE for 24 h at 4°C. Any Rhodamine PE not absorbed was removed from the liposomes using the Quantum Prep PCR Kleen Spin Columns (Bio-Rad). The 100th dilution of the liposome labeled with Rhodamine PE was added to RAW 264.7 cells at 0, 0.5, 1.0, and 2.0 h in serum-free Dulbecco's modified Eagle's medium (DMEM). After washing three times with PBS, the cells were fixed by Zamboni solution[24] and observed using the Radiance 2000 confocal laser scanning microscope (Bio-Rad).

For metabolic analyses of the adducts, the RAW264.7 cells were exposed to the BITC-treated unilamellar liposome (diluted 10 times) in serum-free DMEM for 2 h. The medium was then refreshed with new serum-free medium. After incubating for 0–48 h, the cell supernatants were collected and 100 nM of BITC–EA-d_4 was added. The samples were filtered using the Mini-UniPrep (GE Healthcare Life Sciences) followed by LC-MS/MS analysis, as previously described. Similarly, the cells were incubated with 0–50 μM BITC for 24 h or 10 μM BITC for 0–48 h, and the supernatants measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS), as described above.

Results

Identification of BITC–PE/EA adduct in the BITC-treated unilamellar liposome

Considering the structural features, the BITC–PE adduct might be highly hydrophobic. Thus, we attempted to confirm the formation of the PE adduct as an EA conjugate dissected from the PE adduct with phospholipase D (Scheme 1). A representative chart for the detection of BITC–EA and its stable isotopes is shown in Figure 1A with their possible fragmentation patterns by LC-MS/MS analysis, and the standard curve of the adduct is shown in Figure 1B. The limitation of detection for the BITC–EA was ~12 fmol. BITC–EA originating from BITC–PE in the BITC liposomes was detected after treating

with commercial bacterial phospholipase D in a BITC dose-dependent manner (Fig. 1C). This indicated that 10–16% of the BITC incubated in the media was bound to PE.

Simultaneous formation of BITC–lysine and BITC–PE adducts in LDL exposed to BITC

Because LDL could be one of the molecular targets in blood plasma for ITCs, human LDL was exposed to BITC under physiological conditions and the BITC adducts that formed were analyzed by Western blotting, ELISA, and LC-MS/MS. As shown in Figure 2A, the BITC–lysine adducts in the apo-B100 protein on the blotted membrane were increased from 25- to 100-μM BITC. The BITC–lysine adduct was more sensitively detected using ELISA from only a few micromoles of BITC in a dose-dependent manner (Fig. 2B). By contrast, LC-MS/MS analyses showed that the BITC–EA adduct was detected as the representative of BITC–PE adducts from LDL exposed to 25–100 μM of BITC (Fig. 2C). The concentration of the detected BITC–EA was <20 nM. These results suggest that BITC reacts with PE and the lysine residues of the apolipoprotein in LDL.

Secretion of the BITC–EA adduct from RAW 264.7 cells preincubated with BITC liposomes

The metabolic fate of the BITC–PE adduct in the cells was examined using unilamellar liposomes as a carrier. First, to determine how liposomes incorporate into cells, the BITC-treated unilamellar liposome containing Rhodamine PE was prepared and then added to the cultured cells. The liposome incorporation was confirmed by fluorescent signals using a confocal microscope. As shown in the Figure 3A, the fluorescent liposomes were incorporated into the cells until 2 h co-incubation in a time-dependent manner. Next, the secretion of BITC–EA was analyzed to confirm the intracellular digestion of the modified PE. Cells were preincubated with BITC-exposed liposomes or control liposimes for 2 h, and the medium was then replaced to remove any free liposomes. The released BITC–EA was detected in the medium after incubating for 2 h and then gradually increased until 48 h (Fig. 3B).

Detection of BITC–EA adduct from RAW 264.7 cells exposed to BITC

The secretion of BITC–EA into the medium was analyzed using the cells treated with BITC. Figure 4A shows that BITC–EA accumulated in a dose-dependent manner in the medium when the cells were exposed to 5–50 μM of BITC for 24 h. The amount BITC–EA adduct accounted for ~0.1% of the BITC that was added to the cells. The release of BITC–EA also increased in a time-dependent manner when the cells were treated with

10-μM BITC (Fig. 4B). The gradual generation of EA conjugate suggests that a hydrolyzing enzyme for BITC–PE is constitutively expressed in RAW 264.7 cells.

Discussion

In this study, we investigated the reaction of ITC with PE along with the metabolic fate of the ITC–PE adducts. To the best of our knowledge, this is the first report on the covalent modification of ITC with PE. The formation of BITC–PE in the BITC-treated liposomes was confirmed by detecting the BITC–EA adduct after treating with commercial phospholipase D (Fig. 1). It is noteworthy that in this model reaction, >10% BITC was conjugated with PE.

It has been reported that allyl ITC (AITC), one of the most common and naturally occurring ITCs, could be rapidly absorbed from the gastrointestinal tract after ingestion of its radioactive isotope. The absorbed AITC is distributed among various organs as metabolites, and then ~50%–80% of the radioactive isotope is excreted in the urine.[25] This indicates that AITC or AITC metabolites circulate in the bloodstream and could thereby interact with certain biomolecules in the organ cells. Indeed, ITCs that had conjugated with lysine residues were found in the albumin and hemoglobin in blood plasma.[9] In plasma, circulating glucose gradually modifies proteins such as lipoproteins, albumin, and hemoglobin through nonenzymatic glycation.[26] Plasma glucose also covalently reacts with PE and generates the amadori–PE adduct.[27] In our study, both the apolipoprotein and PE in the LDL particles were modified by BITC (Fig. 2); therefore, ITCs might target not only the lysine residues of the lipoprotein but also the aminophospholipids in LDL *in vivo*.

The BITC-treated unilamellar liposomes were incorporated into the cells within 2 h, and the BITC–EA adduct was then secreted into the medium in a time-dependent manner (Fig. 3). In addition, BITC–EA was detected 2 h after treating the cultured cells with BITC (Fig. 4). It has been reported that BITC was quickly transferred into the cultured cells within 30 min of treatment, most likely by simple diffusion, and was rapidly secreted.[28] This efflux process is believed to be implemented through the ABC transporters, such as multidrug resistance–associated protein 1 because BITC easily reacts with glutathione (GSH) in the cells.[29] Similar to the efflux of the GSH conjugates, BITC–PE conjugate, which is detected as the corresponding conjugate of EA, was also released within 2 h. These results suggest that (1) aminophospholipids might be one of

the first targets of BITC and (2) the constitutive expression of a hydrolyzing enzyme in the cells might be attributed to the formation of the EA conjugate. The amount of BITC–EA adducts in the medium was gradually increased up to 48 h after BITC treatment (Fig. 4B). This led us to consider the possibility that BITC is transferred from thiols to a polar amine moiety of the cellular membrane phospholipids, as observed in the transfer of AITC from protein thiol to lysine.[7]

There have been no reports on enzyme(s) that degrade the ITC–PE adduct; however, there have been some reports on the metabolism of *N*-acyl PE, which is a precursor of functional *N*-acyl EA, such as anandamide (*N*-arachidonoyl EA).[30] For example, phospholipase A catalyzes the cleavage of *N*-acyl PE to form *N*-acyl lysoPE, and then lysophospholipase D hydrolyzes lysoPE into *N*-acyl EA. Alternatively, *N*-acyl PE–specific phospholipase D, also known as NAPE–PLD, directly catalyzes the release of *N*-acyl EA from *N*-acyl PE.[30,31] Thus, the BITC–PE adduct might be hydrolyzed by either a direct or indirect pathway of some types of phospholipases into BITC–EA (Scheme 2); however, the enzyme for generating ITC–EA has not yet been identified.

In regard to the functions of EA adducts, it is well known that *N*-acyl EAs are involved in some physiological and pathological effects. Anandamide has been identified as an endogenous ligand to the cannabinoid receptor and transient receptor potential vanilloid type 1.[32,33] *N*-palmitoyl EA has anti-inflammatory and analgesic effects,[34,35] and *N*-oleoyl EA has been reported to be a lipid mediator that is involved in the peripheral regulation of food intake.[36] In this study, we found that the ITC–EA adduct formed after ITC was exposed to the cultured cells. Because ingested ITCs have specific biological properties, such as anti-inflammatory and anticancer effects,[1-4] ITC–EA might be involved, at least in part, in some of the health-promoting effects of dietary ITCs. We have performed some experiments to evaluate biological and beneficial functionality of BITC-EA using cultured cells, but there is no notable data currently (unpublished observation).

In conclusion, we have demonstrated that BITC–EA is generated from cultured cells treated with BITC. It is highly likely that the EA adduct might also be formed and present in our body after ingesting cruciferous vegetables. It should be noted that the ITC–EA adduct is a potential stable biomarker to track food-derived ITC ingestion. Although similar to *N*-acyl EAs, ITC–EAs are promising adducts that might contribute to the biological effects of ITCs in humans; however, detection of ITC–EA adducts from biological samples such as blood or urine after ingestion of dietary ITC remains to be achieved.

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Conflict of Interest

The authors declare no competing financial interest.

Abbreviations

BITC, benzyl isothiocyanate; PE, phosphatidylethanolamine; EA, ethanolamine; LDL, low-density lipoprotein; AITC, allyl isothiocyanate; GSH, glutathione.

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Figure legends

Figure 1. (A) Representative charts of BITC–EA adduct and its stable isotopes with their possible fragmentation patterns using LC-MS/MS. (B) Standard curve of the BITC–EA adduct. (C) Detection of BITC–EA from BITC-treated unilamellar liposomes. BITC–EA from the liposomes detected by LC-MS/MS after treatment with commercial phospholipase D. Values are the means ± SD (n = 4).

Figure 2. Immunochemical detection of BITC–lysine residues in BITC-exposed LDL by (A) Western blotting and (B) ELISA. BITC–lysine residues in LDL were detected using anti-BITC–Lys monoclonal antibody. (C) Confirmation of BITC–EA adduct in BITC-exposed LDL using LC-MS/MS. After treatment of BITC–LDL with commercial phospholipase D, the BITC–EA adduct was measured using LC-MS/MS. The peak area of BITC–EA was corrected by the area of BITC–EA-d_4. Values are the means ± SD (n = 2–4).

Figure 3. (A) Incorporation of BITC-treated unilamellar liposomes containing Rhodamine PE in RAW 264.7 cells. Incorporation was confirmed by fluorescent signals using a confocal microscope. (B) Detection of the BITC–EA adduct from the culture supernatant of RAW 264.7 cells. BITC-treated unilamellar liposomes were preincubated with cells for 2 h; the medium was replaced to remove any floating liposomes. BITC–EA in the replaced medium was measured using LC-MS/MS. The peak area of BITC–EA was corrected by the area of BITC–EA-d_4. Values are the means ± SD (n = 4).

Figure 4. (A) Dose-dependent changes and (B) time-dependent changes of the BITC–EA adduct in the supernatant of cultured RAW 264.7 cells after exposure to BITC. BITC–EA in the medium was measured using LC-MS/MS. The peak area of BITC–EA was corrected by the area of BITC–EA-d_4. Values are the means ± SD (n = 4).

Scheme 1. Formation of BITC–PE adduct and its EA metabolite.

Scheme 2. Possible metabolic pathways of BITC–PE to BITC–EA.

Fig. 1

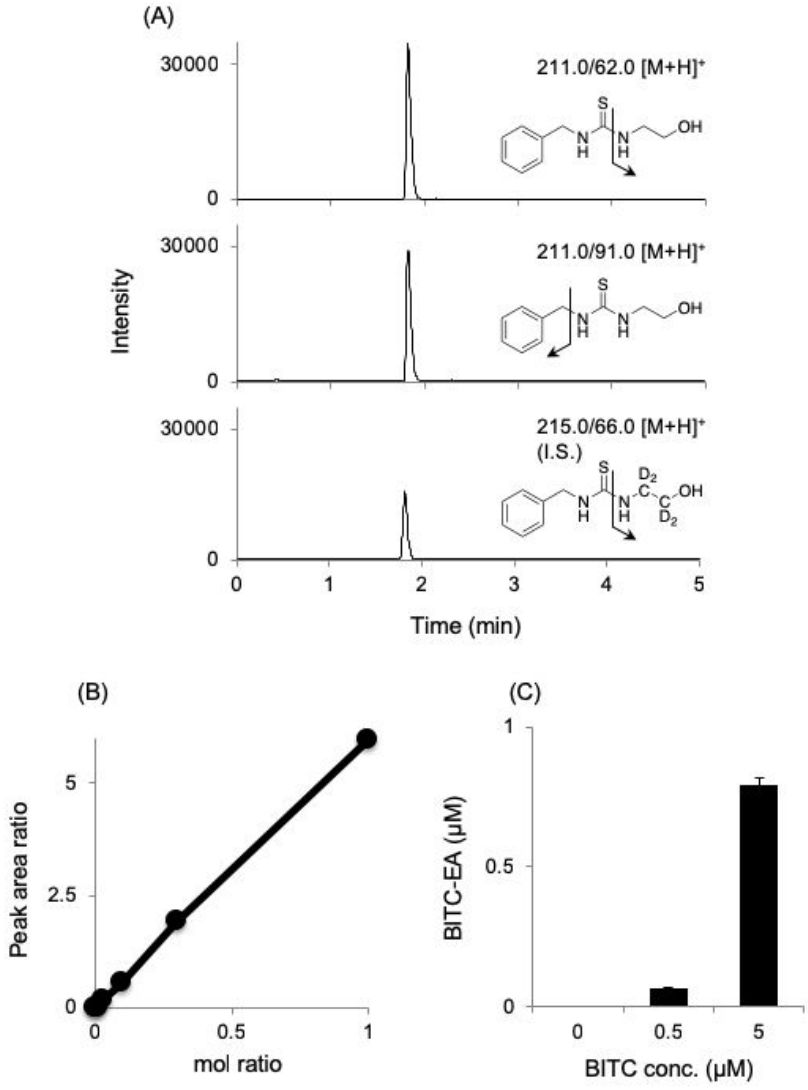

(A)
30000
0
211.0/62.0 [M+H]+
211.0/91.0 [M+H]+
215.0/66.0 [M+H]+
(I.S.)
Intensity
0 1 2 3 4 5
Time (min)
(B)
Peak area ratio
5
2.5
0
0 0.5 1
mol ratio
(C)
BITC-EA (μM)
1
0.5
0
0 0.5 5
BITC conc. (μM)

Fig. 2

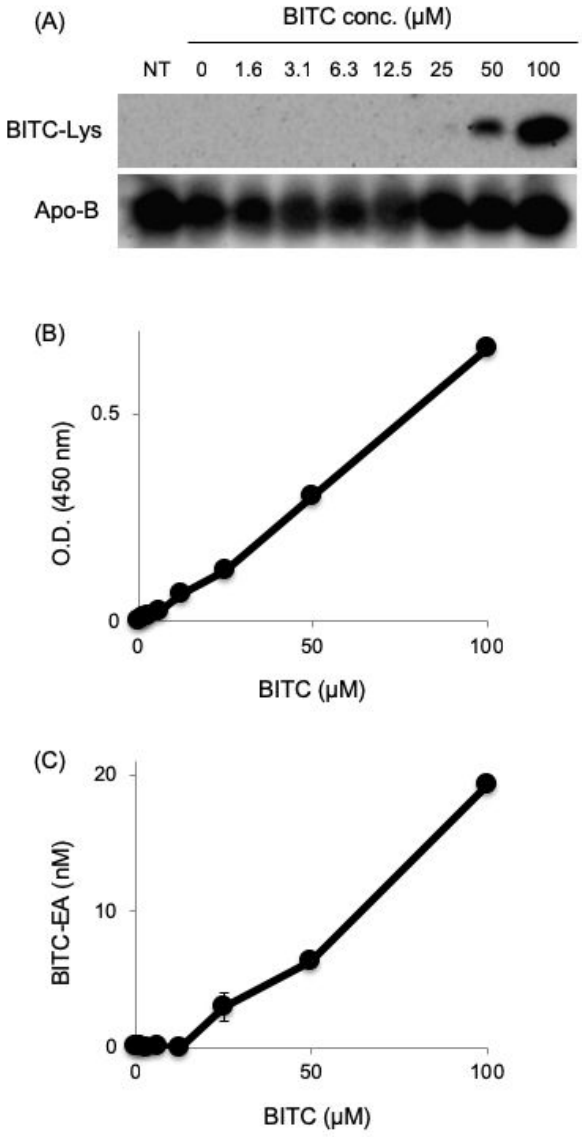

(A)
BITC conc. (μM)
NT 0 1.6 3.1 6.3 12.5 25 50 100
BITC-Lys
Apo-B
(B)
O.D. (450 nm)
0.5
0
0 50 100
BITC (μM)
(C)
BITC-EA (nM)
20
10
0
0 50 100
BITC (μM)

Fig. 3

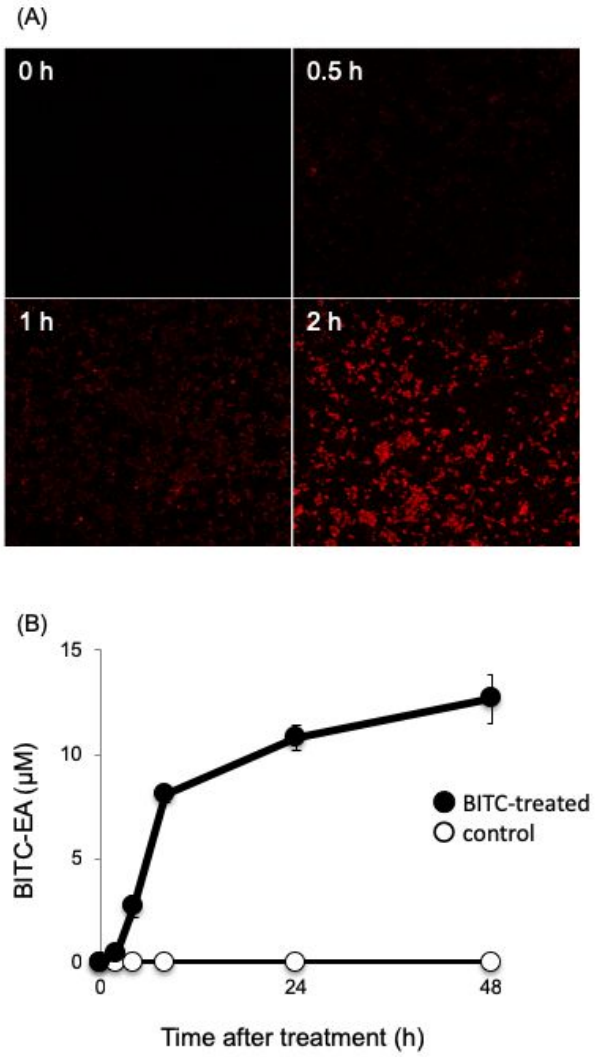
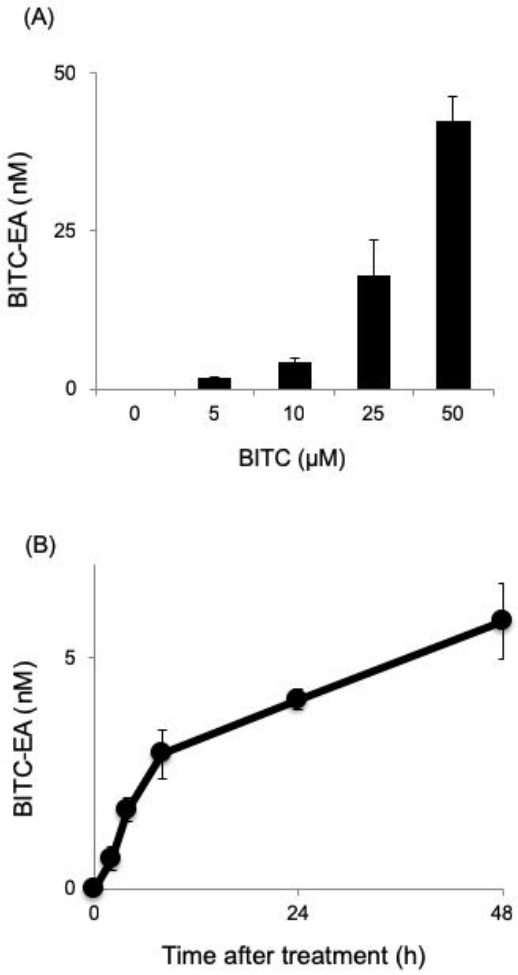
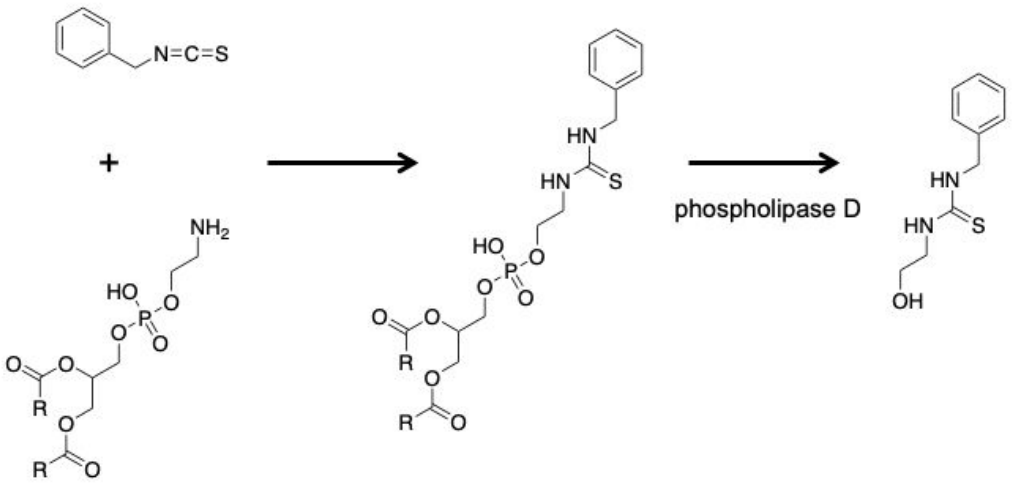

(A)
0 h
0.5 h
1 h
2 h
(B)
BITC-EA (μM)
15
10
5
0
0
24
48
Time after treatment (h)
BITC-treated
control

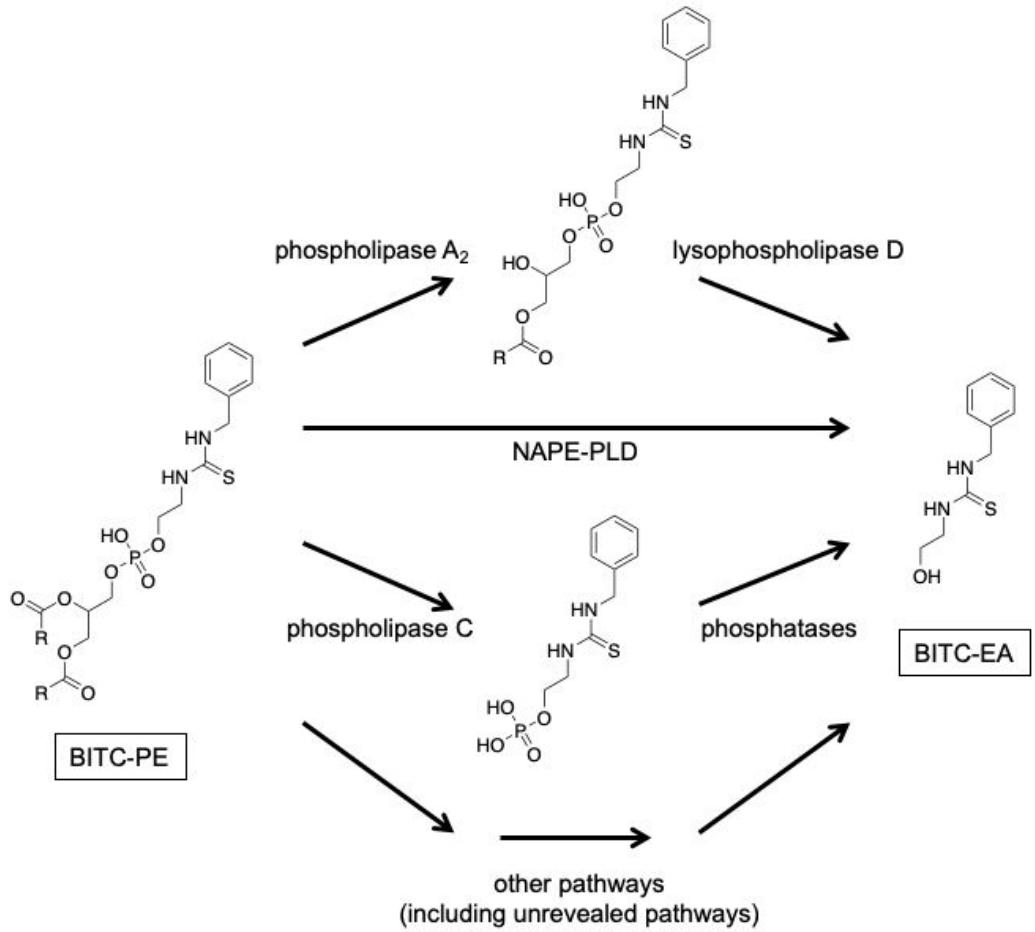
Fig. 4


(A)
BITC-EA (nM)
50
25
0
0
5
10
25
50
BITC (μM)
(B)
BITC-EA (nM)
5
0
0
24
48
Time after treatment (h)

Scheme 1


N=C=S
+
NH2
HO
P
O
O
O
O
R
O
R
O
HN
HN
S
HO
P
O
O
O
O
R
O
R
O
phospholipase D
HN
HN
S
OH

Scheme 2


phospholipase A2
lysophospholipase D
NAPE-PLD
phospholipase C
phosphatases
other pathways
(including unrevealed pathways)
BITC-PE
BITC-EA