Fluorescently labeled inhibitor for profiling cytoplasmic peptide:N-glycanase

Shinya Hagihara\textsuperscript{1,2}, Ayako Miyazaki\textsuperscript{1,3}, Ichiro Matsuo\textsuperscript{1,2}, Atsushi Tatami\textsuperscript{1,2}, Tadashi Suzuki\textsuperscript{2,4}, Yukishige Ito*\textsuperscript{1,2}

\textsuperscript{1}RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198 Japan, \textsuperscript{2}CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332-1102 Japan, \textsuperscript{3}Graduate School of Science and Engineering, Saitama University, Saitama, 338-8570 Japan, \textsuperscript{4}21st COE (Center of Excellence) Program, Osaka University Graduate School of Medicine, 2-2 Yamadaoka Suita, Osaka 565-0871 Japan

Corresponding author: Yukishige Ito, Synthetic Cellular Chemistry Laboratory, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan, Tel: +81-48-467-9430; Fax: +81-48-462-4680; E-Mail: yukito@riken.jp

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Abstract

Cytoplasmic peptide:N-glycanase (PNGase) is an enzyme that removes N-glycans from misfolded glycoproteins. The function of cytoplasmic PNGase plays a significant role in degradation of misfolded glycoproteins, which is critical for cell viability. Recently, we reported that haloacetoamidyl derivatives of high-mannose-type oligosaccharide selectively modify the catalytic cysteine of cytoplasmic PNGase and serve as its specific inhibitor. Interestingly, a drastically simplified chloroacetamidyl chitobiose derivative [(GlcNAc)_2-ClAc] was also reactive to PNGase. In this paper, it was conjugated to a hydrophobic fluorophore in order to render (GlcNAc)_2-ClAc cells permeable. We demonstrated that this compound [BODIPY-(GlcNAc)_2-ClAc] specifically binds to cytoplasmic PNGase from budding yeast (Png1). To date, only Z-VAD-fmk is known as an inhibitor of PNGase. BODIPY-(GlcNAc)_2-ClAc and Z-VAD-fmk share the same binding site on Png1, while BODIPY-(GlcNAc)_2-ClAc has markedly stronger inhibitory activity. The functional analysis of PNGase using Z-VAD-fmk should be carefully interpreted because of its intrinsic property as a caspase inhibitor. In sharp contrast, chloroacetamidyl chitobiose was an inhibitor of reactive to
caspase. In addition, BODIPY-(GlcNAc)2-ClAc did not bind either chitobiose binding lectins or PNGase from other sources. Moreover, fluorescent microscopy clearly showed that BODIPY-(GlcNAc)2-ClAc was efficiently introduced into cells. These results suggest that this compound could be an in vivo inhibitor of cytoplasmic PNGase.
Introduction

Protein quality control is an essential process for maintaining cell activity, which ensures that only correctly folded proteins are produced. Recent studies have revealed the significant roles of glycoprotein glycans in this process. Degradation of misfolded proteins is also an important facet of this quality control system. Cytoplasmic peptide:N-glycanase (PNGase) is an enzyme involved in the glycoprotein degradation process, and it removes asparagine (N)-linked oligosaccharides from misfolded glycoproteins [1, 2].

In the lumen of the endoplasmic reticulum (ER), high-mannose-type oligosaccharides are co-translationally introduced to asparagine residues of nascent peptide chains [3]. These N-linked oligosaccharides are engaged in quality control of glycoproteins [4-6]. For example, mono-glucosylated forms of N-linked oligosaccharides are recognized by lectin-like chaperone calnexin and its soluble homologue, calreticulin [7]. They assist in glycoprotein folding by recruiting protein disulfide isomerase-like protein ERp57 (ER-60) [8, 9]. After repeated attempts at folding, only correctly folded proteins enter the secretory pathway. In contrast,
terminally misfolded proteins are dislocated to cytosol and degraded by the ubiquitin-proteasome system (ER-associated degradation, ERAD) [10].

Cytoplasmic PNGase is considered to remove N-linked oligosaccharides from glycoproteins destined for degradation, prior to their proteasomal degradation. Because the barrel-shaped proteasome has its active site in the interior of the pore, removal of bulky N-glycans from glycoproteins by PNGase is likely to be a prerequisite for efficient insertion of peptide chains into its degrading chamber [11]. It was actually demonstrated that the efficient degradation of glycosylated ricin A chain requires the PNGase [12]. Intriguingly, it was recently reported that cytoplasmic PNGase forms a glycoprotein-degrading complex with Rad23, proteasome, p97, and the autocrine motility factor receptor (AMFR) around the outer surface of the ER [13]. A multiprotein complex composed of these elements may well be functionally significant, providing a highly ordered conduit toward degradation. Therefore, cytoplasmic PNGase is not merely a deglycosylating enzyme, but is even more important as the key enzyme in the glycoprotein degradation machinery.

In order to precisely investigate the biological function of PNGase, development of
its specific inhibitor is desired. It has been reported that carbobenzoxy-Val-Ala-Asp-α-fluoromethylketone (Z-VAD-fmk) inhibits PNGase activity [14, 15]. This compound, which was originally discovered as a general caspase inhibitor, was shown to form a covalent bond with the catalytic cysteine that conducts nucleophilic attack on the β-amide bond of the asparagine side chain (Figure 1a). However, the usefulness of Z-VAD-fmk is compromised as an in vivo inhibitor, because it also inhibits caspase. Recently, we found that high-mannose-type oligosaccharides with a thiol-reactive haloracetoamidyl group, such as (Man)₉(GlcNAc)₂-XAc, strongly inhibited PNGase by covalently modifying its catalytic cysteine residue (Figure 1b) [16]. Interestingly, drastically simplified haloacetamidyl chitobiose derivatives [(GlcNAc)₂-XAc, X = Cl, Br, I] were also reactive to PNGase, although previous reports indicated that glycoproteins or glycopeptides possessing chitobiose were not received as a substrate for PNGase [11, 17]. Our recent work clarified that N-linked chitobiose can be cleaved from certain peptides [18]. This somewhat unexpected result provided us with the foundation to design a substrate-based inhibitor of our target enzyme PNGase. Our research reported here shows the potency of fluorescently labeled chitobiose derivative
1 as a highly selective inhibitor of PNGase, which was revealed to be cell-permeable.

Results

Design of a fluorescently labeled cell permeable inhibitor

We first evaluated the reactivity of a series of haloacetamidyl chitobiose to cytoplasmic PNGase from budding yeast (Png1). Among haloacetamidyl chitobiose derivatives (GlcNAc)$_2$-$\text{XAc}$ ($\text{X} = \text{Cl}$, Br, I), the order of inhibition potency was IAc>BrAc>ClAc ($\text{IC}_{50} = 0.8$, 2.8 and 19 $\mu$M, respectively). However, at the outset of this research, we were anxious about the in vivo stability of the bromo- and the iodoacetyl groups, so we decided to employ chloroacetamidyl chitobiose [(GlcNAc)$_2$-ClAc] as the core structure in our prototypical inhibitor design.

However, the low permeability of free disaccharide to the cell membrane limits their use to in vivo applications. In order to render cells permeable to (GlcNAc)$_2$-ClAc, we planned to conjugate it with a hydrophobic chromophore. In their study on in vivo oligosaccharide synthesis, Esko et al. reported the easy uptake of disaccharide primers when hydrophobic aglycon was introduced and the number of free hydroxyl groups was $\leq 5$ [19]. Based on this precedent, a hydrophobic substituent had to be introduced to
achieve cell permeability of (GlcNAc)_2-ClAc. In order to employ it as a marker to
detect the cellular localization of the inhibitor, we selected 4,4-difluoro-5,7-dimethyl-4-
bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY Fl) as a substituent.

As for the position to be modified with the fluorophore, the following consideration
led us to exploit the 4-OH of outer GlcNAc. Molecular modeling analysis of the
complex between chitobiose and PNGase suggested that this position projects outward
from the complex. In addition, since we observed that chloroacetamidyl chitotetraose
[(GlcNAc)_4-ClAc] efficiently labeled PNGase (Miyazaki, A. et al., unpublished), the
binding between chitobiose and PNGase was expected to be tolerant to a substitution of
this OH. Taking these together, we designed BODIPY-(GlcNAc)_2-ClAc (1) as a
potential inhibitor of PNGase (Figure 1c).

**Synthesis of BODIPY-(GlcNAc)_2-ClAc (1)**

Synthesis of 1 was started with a protected chitobiose block 2 [20-23] (Scheme 1).
Dephthaloylation and acetylation provided di-N-acetylchitobiose derivative 3, which
was alkylated with t-butyl bromoacetate to give t-butyl ester 4. It was reduced to
primary alcohol 5, which was treated with mesyl chloride and then sodium azide to produce azide 6. Simultaneous benzyl group deprotection and azide reduction were conducted under hydrogenation conditions in the presence of (Boc)₂O to produce N'-(Boc)-aminoethyl di-N-acetylchitobiose 7. The obtained reducing sugar 7 was treated with saturated NH₄HCO₃, followed by acylation with chloroacetyl chloride to furnish chloroacetamidyl sugar 8. After removal of Boc protection of 8 under acidic conditions, condensation of the resulting amine with BODIPY FL using O-(7-azabenzotriazol-1-yl)-<i>N</i>,<i>N</i>,<i>N</i>′,<i>N</i>′-tetramethyluronium hexafluorophosphate (HATU) [24] gave the target compound 1.

**Evaluation of the binding specificity of BODIPY-(GlcNAc)₂-ClAc**

In order to evaluate the selectivity of 1, a cell extract of <i>E. coli</i> expressing Png1 was incubated with 1 for 15 min, and the mixture was applied to SDS-PAGE (Figure 2A). In contrast to CBB staining that showed a ladder band pattern, only a single band was observed in the fluorescent image (lane 2), which was identical to purified Png1. This fluorescent band was not observed in lane 1, where Png1 was not expressed. These results indicate that 1 selectively binds to Png1 among all other <i>E. coli</i> proteins. This
fluorescent labeling was completely abolished when Png1 was pre-incubated with Z-VAD-fmk (lane 4).

As depicted in Figure 2B, the fluorescent labeling of Png1 with 1 was nearly completed within 10 min. In contrast, more than 2 h of pre-incubation with a 20-fold excess of Z-VAD-fmk was necessary for complete inhibition of Png1 labeling with 1 (Figure 2B). These results suggest that Z-VAD-fmk and 1 share the same binding site on Png1, where 1 has markedly stronger reactivity. Indeed, 1 inhibited the deglycosylation of RNaseB at a lower concentration than Z-VAD-fmk (Figure 3A); the observed IC_{50} value of 1 was 12 μM, while that of Z-VAD-fmk was more than 1 mM under the given conditions.

To reveal the selectivity of 1 toward Png1, N-glycanases from other sources were also examined (Figure 2C). PNGase F (from flavobacterium), which has an identical activity to Png1, was not labeled with 1. This result reflects the difference in the catalytic mechanism between PNGase F and eukaryotic PNGase [25]. In addition, PNGase A (from almonds) was also resistant to the labeling with 1 both at pH 7.2 and its optimum pH of 5.5 [26]. Although the structure and catalytic mechanism of PNGase
A have not been clarified, this result indicates that the deglycosylating mechanism of PNGase A is also different from Pngl. In fact, compound 1 did not inhibit deglycosylation with PNGase F or PNGase A (Figure 3B).

Furthermore, wheat germ agglutinin (WGA) and Fbs1 [27, 28], which have an affinity to chitobiose, were not labeled with 1 (Figure 2C). All of these data strongly support the idea that covalent modification with 1 is highly specific to our target PNGase. This enzyme is likely to be unique, possessing both chitobiose-binding ability and a cysteine residue at the active site.

**Cellular uptake of BODIPY-(GlcNAc)₂-ClAc**

To explore the possibility of using compound 1 for *in vivo* studies using living cells, cellular uptake of 1 was investigated by fluorescence microscopy. After being incubated with 1 for 30 min and changing media, HeLa cells were observed by fluorescent microscopy. In order to analyze the cellular uptake of 1, endoplasmic reticulum maker, ER-tracker Red was co-introduced into HeLa cell. Localization of fluorescence of 1 and ER-tracker Red clearly showed that 1 permeated the plasma membrane and was stably
retained in cells, even after media change (Figure 2D). Uptake of compound 1 was also observed with yeast (Figure 2E). Replacement of BODIPY FL with 6-(tetramethylrhodamine-5-carboxamide)hexanoic acid (TAMRA) resulted in the loss of cellular uptake while maintaining the reactivity to Png1 in vitro (data not shown). This suggests that the hydrophobic and non-charged characteristics of BODIPY FL are important for the cell permeability of 1.

To examine if 1 can indeed inhibit the enzyme activity in Hela cells, cells were treated with 20 µM of 1 and the PNGase activity was assayed. As expected, the activity was strongly inhibited by 1 and no activity above background was detected, while the control cytosol prepared from Hela cells exhibited PNGase activity using our in vitro assay methods (220 pmol/h/mg prot) (Figure 3C). This result clearly showed that 1 can inhibit enzyme activity in vivo.

Discussion

Our results clearly showed that chitobiose derivative 1 selectively modifies the catalytic cysteine of cytoplasmic PNGase. This compound labeled no other proteins
from *E. coli* extracts. In addition, it can penetrate the cell membrane. These results strongly suggest that 1 can serve as an *in vivo* inhibitor of cytoplasmic PNGase.

The reactivity and selectivity of 1 override those of Z-VAD-fmk, a known inhibitor of PNGase. The functional analysis of PNGase using Z-VAD-fmk would require careful interpretation because it possesses intrinsic activity as a caspase inhibitor. In marked contrast, a haloacetoamidyl derivative of high-mannose-type oligosaccharide did not have any effects on any caspases examined (caspase 2, 3 and 7) [16]. Furthermore, 1 did not modify other chitobiose-binding proteins, such as lectins and PNGase from other sources. These facts also support the selectivity of 1 and the advantage of using a sugar-based inhibitor for cytoplasmic PNGase.

We showed that 1 is also usable as a marker to analyze the expression of cytoplasmic PNGase. The activity of PNGase was directly visualized with 1 in figure 3b. Activity-based protein profiling has attracted recent interest in chemical biology [29]. The reactivity and selectivity of 1 to cytoplasmic PNGase indicate that it is applicable to profiling PNGase activity in complex proteomes. These findings may be a substantial step toward clarifying the role of cytoplasmic PNGase.
**Materials and methods**

**Materials**

Reagents and solvents were purchased from standard suppliers and used without further purification. Reactions were monitored with TLC plates precoated with Merck silica gel 60 F254. Merck silica gel-60 was used for silica gel flash chromatography. $^1$H and $^{13}$C NMR spectra were measured on a JEOL EX-400 spectrometer. MALDI-TOF MS was recorded in the positive ion mode on an AXIMA-CFR (Shimadzu) equipped with a nitrogen laser with an emission wavelength of 337 nm. The detailed synthetic procedure and preparation of Png1 [16] are presented in supplemental data.

**Carbohydrate-Probe Binding Assay**

To assess the binding of 1 to Png1, 2 μl of the enzyme fraction (2.0 mg/ml) (final 5 μM) or *Escherichia coli* extract expressing Png1 were added to 18 μl of sodium phosphate buffer (pH 7.2) containing 10 mM dithiothreitol and 1 (final concentration of 5 μM unless indicated otherwise). The reaction was performed at 37 °C and was
stopped by adding 20 μL of 2X SDS-PAGE sample buffer. Ten μl of each sample were applied to SDS-PAGE, which was visualized using LAS-1000 (Fujifilm) and the bands detected were quantified using Multi Gauge ver 2.2.

**Inhibition assay for PNGase**

PNGase activity was measured using RNase B (Sigma-Aldrich Corp., St. Louis, MO) as a substrate. Typically, the reaction mixture of 20 μl included purified Png1 (0.5 μM), RNase B (1.6 mg/ml), and 20 mM sodium phosphate (pH 7.2), 150 mM NaCl, 5 mM dithiothreitol, and 1 mM EDTA. The reaction was performed for 30 min at 37 °C and was stopped by adding 2 X sample buffer, followed by SDS-PAGE, and quantitated using Multi Gauge ver. 2.2 (Fujifilm Co., Tokyo, Japan).

**Visualization of cellular uptake of 1**

HeLa cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin in a 5% CO₂/95% air atmosphere at 37 °C. Cells were incubated in the presence of 1 (5 μM) for 30 min at 37 °C. After changing
media, the cells were observed by fluorescent microscopy (IX 71, Olympus, Tokyo, Japan).

Yeast proteasome mutant cells, cim5 (CMY763; MATa cim3-1 ura3-52 leu21 his3-200 Ghislain et al., 1993). Cells were grown in 2 ml of YPD media (1% yeast extract (Difco), 2% Tryptone-Peptone (Difco)) until they reached the stationary phase, and 20 microM of compound 1 was added to the sample. Equal amount of DMSO without compound 1 was added to the control sample. Cells were then washed extensively with PBS after 30 min of incubation, and were examined by a confocal microscopy (FV500/BX-61; Olympus).

Assay for PNGase acitivity

The Hela cells with or without 20 μM of BODIPY-(GlcNAc)2-ClAc for 1 hours, medium was removed, and cells were washed once with PBS and were collected. Cytosol from cells were prepared essentially as described previously [30]. The activity of PNGase was assayed using fetuin [14C]asialoglycopeptide I as the substrate [2, 31]. The reaction mixture in a total volume of 10 μl includes 3 μl of cytosol fraction, 6 μl of
200 mM Hepes buffer (pH 7.1) in 10 mM dithiothreitol, and 1 μl of the substrate (15,000 cpm; 210 pmol). Reaction was carried out for 4 hours and the reaction mixtures were subjected to paper chromatography [2, 31]. The reaction product (deglycosylated peptide) was quantitated with a Bio-Imaging Analyzer (Fuji BAS 2500).

Protein amount in the cytosol was quantitated using Coomassie Protein Assay Reagent Kit (Pierce, Rockfold, IL) according to the manufacturer’s protocol (The Micro Protocol) with bovine serum albumin (Pierce) as the standard.

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assess the functions of calnexin and calreticulin in ER protein folding and quality

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Legends to Figures

Figure 1. Proposed mechanism of deglycosylating enzyme PNGase and design of its inhibitor.

(A) Deglycosylation mechanism of Png1. (B) Haloacetamidyl sugar covalently modifies the catalytic Png1. (C) Designed PNGase inhibitor BODIPY-(GlcNAc)_2-ClAc (I).

Figure 2. Evaluation of BODIPY-(GlcNAc)_2-ClAc (I) as a probe for profiling PNGase. (A) Specific labeling of Png1 analyzed by SDS-PAGE. Each sample was treated with 1 for 15 min and applied to 12.5% SDS-PAGE. Obtained gel profiles were visualized by CBB staining (upper) or the fluorescent imager (bottom). Cell extract of E. coli bearing pET28b-PNG1-(His)_6 without (lane 1) or with (lane 2) induction with 1 mM IPTG; lane 3, Png1 (5 μM) purified with Ni-NTA agarose; lane 4, Png1(5 μM) pre-incubated with Z-VAD-fmk (100 μM) for 2 h. (B) Quantifying fluorescent labeling of Png1 with 1. Png1 was treated with or without 1 for the indicated periods and analyzed by SDS PAGE (top). Png1 was treated with 1 after preincubation with Z-VAD-fmk for the indicated periods (bottom). (C) Analysis of the selectivity of 1 to Png1 by SDS-
PAGE. lane 1, Png1; lane 2, PNGase F; lane 3, PNGase A (pH = 5.2); lane 4, PNGase A (pH = 7.2); lane 5, WGA; lane 6, Fbs1. (D, E) Visualization of cellular uptake of 1. Hela cells (D) and yeast cells (E) were incubated with 1 for 30 min. Cells were then washed and analyzed by fluorescent microscopy. Fluorescent images (D and E, top) were compared with ER-tracker (D, bottom) or phase contrast image (E, bottom).

**Figure 3. Inhibitory activities of BODIPY-(GlcNAc)2-ClAc (I).**

(A) Inhibition of Png1 by 1 or Z-VAD-fmk. RNase B (0.2 mg/mL) was treated with Png1(3.2 µg/mL) in 200 mM Mes-NaOH (ph 6.7) containing 10 mM DTT in the presence of 1 (left) or Z-VAD-fmk (right). CHO+: glycosylated RNase B, CHO-: deglycosylated RNase B. (B) RNase B (0.8 mg/mL) was treated with Png 1 (lane 2 to 5), PNGase F (lane 6, 7) or PNGase A (lane 8, 9) for 1h in the absence or presence of 1. (C) Inhibition of PNGase activity in Hela cells. Hela cells were treated with 1 for 1 h, washed with PBS, and collected. Fetuin [14C] asialoglycopeptide I was treated with cytosol fraction of Hela cells for 4 h and subjected to paper chromatography.
Scheme 1. Synthesis of compound 1.

(a) (i) ethylenediamine, n-butanol: (ii) Ac₂O, NaHCO₃, MeOH, 95% (two steps); (b) t-butyldi(methyl)chlorosilane, NaH, DMF, quant; (c) LiAlH₄, THF, 92%; (d) (i) MsCl, i-Pr₂NEt, CH₂Cl₂; (ii) NaN₃, DMF, 94% (two steps); (e) H₂, Pd(OH)₂/C, (Boc)₂O, MeOH, 83%; (f) (i) sat. (NH₄)HCO₃; (ii) ClCH₂COCl, NaHCO₃, dioxane-H₂O, 96% (two steps); (g) (i) HCl; (ii) BODIPY FL, HATU, i-Pr₂NEt, DMF, 79% (two steps).