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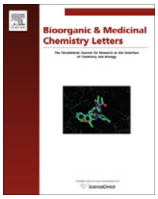

The action of bromoconduritol on ER glucosidase II

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ABSTRACT

Bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene; BCD) has been known as an inhibitor of glucosidase II (G-II), which plays pivotal roles in glycoprotein processing and folding in the ER. Previous works suggested that BCD specifically inhibits the cleavage of the innermost glucose (Glc) among two α1-3 linked Glc residues (cleavage-2). This study addressed the mode of BCD's inhibition toward G-II by using fluorescently labeled substrates. Our analysis clarified that BCD inhibits both cleavage-1 and cleavage-2 activities of G-II. However, the inhibitory activity toward cleavage-2 was 6-fold higher than that toward cleavage-1. Inhibition against both of these activities was retained after dialysis, supporting that BCD exhibits inhibition through irreversible binding to G-II.

A majority of newly generated polypeptides delivered to the endoplasmic reticulum (ER) lumen are modified by N-glycosylation at the asparagine residue of the sequence Asn-Xaa-Ser/Thr. N-Linked glycans are first introduced as a high mannose-type tetradecasaccharide, $Glc_3Man_9GlcNAc_2$ (G3M9).[1] They are then truncated sequentially by glucosidase I (G-I), glucosidase II (G-II) and mannosidases, while incompletely folded glycoproteins are reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (UGGT). Altogether, a combination of these activities gives structurally diverse high mannose-type glycoforms in the ER.[2] In this process, G-I cleaves the outermost α-1,2-linked glucose (Glc) residue to give diglucosylated structure $Glc_2Man_9GlcNAc_2$ (G2M9), which in turn is trimmed by G-II.

G-II is a heterodimeric soluble enzyme, which has a dual activity. It plays a key role in the calnexin (CNX)/calreticulin (CRT) cycle, a folding process specific to N-linked glycoproteins. The cleavage-1 activity of G-II quickly removes the penultimate Glc residue of G2M9 to generate $Glc_1Man_9GlcNAc_2$ (G1M9), which is slowly converted to $Man_9GlcNAc_2$ (M9) by the cleavage-2 activity.[2,3] Since G1M9 serves as an important signal to modulate glycoprotein folding by virtue of its specific binding to lectin chaperones CNX and CRT,[4–6] the activity of G-II regulates both the entry and the departure of glycoproteins in and out from the CNX/CRT cycle.

Bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene; BCD) is an active-site directed irreversible inhibitor of glucosidases.[7–11] Previous studies concluded that BCD inhibits the cleavage-2 activity of G-II, while the cleavage-1 activity is insensitive to BCD. This conclusion relied mainly upon the observation that BCD treatment of influenza virus infected chicken embryo cells gave rise to the accumulation of monoglucosylated glycans $Glc_1Man_{7\text{-}9}GlcNAc_2$ (G1M9-7) of viral glycoproteins.[8] However, the basis of the specificity of BCD toward the latter activity has been unclear.[12–14] Alonso et al. proposed an elegant model, which put forward the existence of two substrate binding sites in G-II. This model hypothesized that the high-affinity substrate binding site (site 1) is responsible for the rapid conversion of G2M9 to G1M9, while G1M9 binds to the low-affinity site (site 2) and is slowly converted to M9.[7,15] In fact, studies using *p*-nitrophenyl α-D-glucopyranoside (*p*NP-Glc) and maltose (Glcα1-4Glc) as substrates estimated the relative efficiency (V_{max}/K_m) of these sites, the former being 20–30 times higher.[7,16]

However, whether these results obtained with these highly artificial substrates reflects the property of G-II as a glycan processing enzyme has not been verified. Our study employed more physiologically relevant high-mannose-type glycans G2M9 and G1M9, which were chemically synthesized and labeled with a fluorophore in order to enhance quantification. In particular, our analyses on the effect of BCD on cleavage-1 and -2 of G-II revealed that BCD inhibits both of these activities, however with different magnitude.

Our study started with the digestion of G1M9- and G2M9-Gly-BODIPY, which were prepared based on a previously reported convergent approach,[17] by rat liver G-II[18] with monitoring by HPLC, confirming that both of them cleaved smoothly by our preparation

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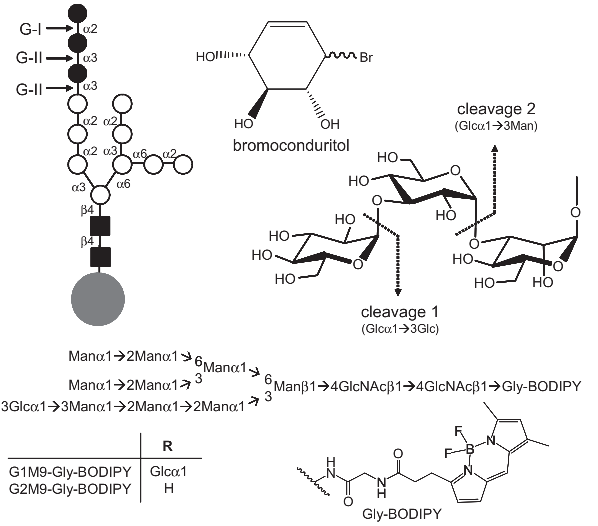

Figure 1. The trimming of high-mannose type glycan by G-II and the synthetic substrates used in this study.

of this enzyme (Fig. 2). Trimming of G1M9-Gly-BODIPY, which reflects the cleavage-2 activity of G-II, gave ~50% conversion within 3 h and reached a plateau after 10 h at 37 °C (Fig. 2a). Incubation of G2M9-Gly-BODIPY under similar conditions (Fig. 2c) showed that the cleavage-1 (G2M9 to G1M9) proceeded quickly, while the cleavage-2 (G1M9 to M9) exhibited markedly slower kinetics, in accordance with previous reports.[12,19–21] Namely, after 1 h at 37 °C, the proportions of G1M9 and M9 were 63% and 8%, respectively, while 29% of G2M9 remained unconsumed. After 10 h, G2M9 was barely detectable, while the amount of M9 steadily increased as the incubation time elongated.

In the presence of 300 μM BCD (Fig. 2b and d), the cleavage-2 was strongly inhibited; the consumption of G1M9 was less than 10% even after 24 h (Fig. 2b). When G2M9 was used as a substrate, the accumulation of G1M9 was observed (Fig. 2d), supporting that BCD preferentially inhibits the cleavage-2 (Fig. 1d).

However, when compared with Figure 2c, BCD's inhibition against the cleavage-1 was obvious. The consumption of G2M9 was ~20% after 1 h, while that in the absence of BCD was ~70%. Meanwhile, the newly generated G1M9 was barely consumed and the yield of M9 was less than 10% even after 24 h. Altogether, these results indicate that BCD inhibits both cleavage-1 and -2 activities of G-II, albeit with different magnitudes.

The difference was quantified by comparing the inhibitory activities under various concentrations of BCD (Fig. 3). For the sake of simplicity, consumption of G2M9 was taken as a measure of the cleavage-1 activity, and the cleavage-2 activity was acquired from the assay using G1M9 as a substrate. In addition, data analysis did not take account of the inhibition caused by the end product (M9).[22] For comparison, activities of other glucosidase inhibitors, deoxynojirimycin (DNJ)[23,24] and castanospermine (CST)[25] were evaluated under the same experimental settings.

The acquired IC_{50} value of BCD against the cleavage-1 was 261 μM, being markedly larger than those of DNJ (8.2 μM) and CST (1.3 μM). On the other hand, the value against the cleavage-2 was 41 μM, being sixfold smaller than that against the cleavage-1. DNJ and CST had IC_{50} values 11.2 and 1.1 μM, respectively, toward the cleavage-2, showing that these compounds inhibit both activities in similar magnitudes (Table 1).

Since BCD is known as an irreversible inhibitor of glucosidases, our study next addressed if the inhibition to G-II is retained after the removal of BCD. Thus, after treatment with BCD (30, 300, or 1000 μM, 37 °C, 3 h), G-II was subjected to dialysis against phosphate buffer (10 mM, pH 7.0) for 12 h at 4 °C (BG-II-S), and its residual activities to catalyze cleavage-1 and -2 were measured

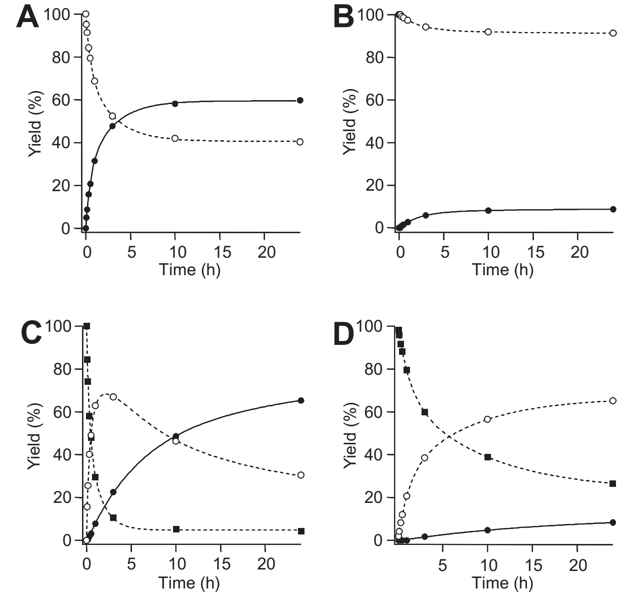

Figure 2. The trimming of G1M9 and G2M9 by rat liver G-II. (A) Time course of the trimming of Glc from G1M9 under standard conditions. (B) Time course of the Glc trimming of G1M9 in the presence of 300 μM BCD (a mixture of isomers). (C) Time course of the trimming of Glc from G2M9 under standard conditions. (D) Time course of the Glc trimming of G2M9 in the presence of 300 μM BCD. ■, G2M9; ○, G1M9; ●, M9. Reaction mixtures contained, in a total volume of 10 μl, 5 μM of G2M9, 0.14 microunit of soluble rat liver G-II, 1 mM deoxymannojirimycin, BCD (0 or 300 μM), 0.05% Triton X-100, 1 mM $CaCl_2$, and 10 mM Hepes (pH 7.4). G-II was preincubated with designated concentrations of BCD. After 30 min at 37 °C, substrate was added and incubated further for 5 min–24 h at 37 °C. After the reactions were stopped by heating at 100 °C for 1 min, the mixtures were analyzed by HPLC under the following conditions: TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH–Et_3N (pH 7.3), linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 ml/min at 40 °C. HPLC was conducted with Waters 2695 (separation module) and Waters 996 (photodiode array detector). BODIPY-labeled glycans were quantified by fluorescence intensities (λ_{ex} = 503 nm, λ_{em} = 513 nm).

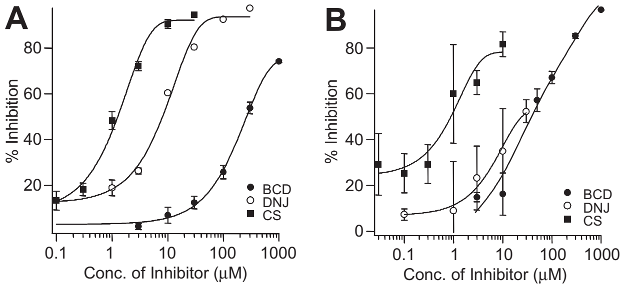

Figure 3. The inhibitory activity of α-glucosidase inhibitors against G-II. (A) Concentration dependence of inhibitory activity of inhibitors against cleavage-1 (A) and cleavage-2 (B) of G-II. ■, castanospermine (CST); ○, deoxynojirimycin (DNJ); ●, bromoconduritol (BCD).

Table 1
IC_{50} values of α-glucosidases against G-II

Compd	Cleavage-1 IC_{50}[a] (μM)	Cleavage-2 IC_{50}[a] (μM)
Bromoconduritol	261.6 (±19.8)	41.0 (±5.5)
Deoxynojirimycin	8.2 (±0.3)	11.2 (±3.1)
Castanospermine	1.3 (±0.2)	1.1 (±0.6)

[a] Values are means of three experiments, standard deviation is given in parentheses.

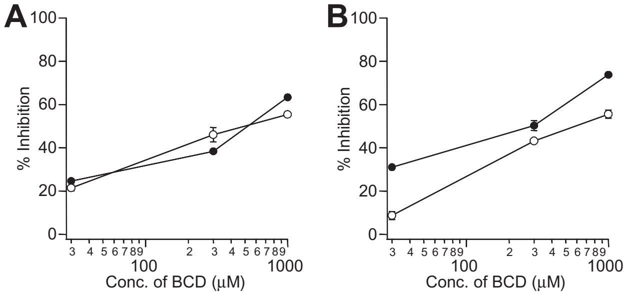

Figure 4. The influence of dialysis on the G-II inhibition. The inhibitory activity of BCD against cleavage-1 (A) and cleavage-2 (B) of G-II. ○, incubated with BCD before dialysis (BG-II-S); ●, incubated with bromoconduritol after dialysis (BG-II-C). G-II was incubated with designated concentrations of BCD (30, 300 or 1000 μM). After 3 h at 37 °C, mixtures were dialyzed against 0.05% Triton X-100 and 10 mM phosphate buffer (pH 7.0) with Slide-A-Lyzer Mini Dialysis Units 10 k MWCO (Pierce, Rockford, USA) for 12 h at 4 °C. These were used as stock solutions of BCD-treated G-II (BG-II-S). Final concentrations of deoxymannojirimycin, Triton X-100, CaCl$_2$, and Hepes (pH 7.4) were 1 mM, 0.05%, 1 mM and 10 mM, respectively. The dialyzed G-II solutions incubated without BCD was used as a control. G-II solutions which were incubated with BCD (30, 300, 1000 μM) after but not before dialysis were also prepared and used for comparison (BG-II-C).

separately using G2M9-Gly-BODIPY and G1M9-Gly-BODIPY, respectively. The activity of G-II that was incubated with BCD after dialysis (BG-II-C) was also measured for comparison (filled circle). Toward the cleavage-1, similar degree of inhibition was observed between BG-II-S and -C (Fig. 4a), indicating that the inhibition was caused by an irreversible binding indeed. Inhibitory activity to the cleavage-2 was also retained after dialysis (Fig. 4b). These results imply that BCD inhibits both activities of G-II through covalent modification. Interestingly, for BG-II-S, reduction of the activity was very similar between two activities, indicating that covalent modification with BCD equally suppressed the cleavage-1 and -2 activity of G-II.

For the cleavage-1, the extent of inhibition increased as the pre-incubation time elongated (Fig. 5). The degree of increment was 1.3% per min and reached 36% for 20 min at 100 μM, while it was 2% per min and reached 68% for 8 min at 1 mM; thus it is likely that the binding of BCD to G-II is an irreversible and slow process. On the other hand, for the cleavage-2, the effect of pre-incubation time was less significant, while the effect of the concentrations of BCD was evident. Taken together, these results suggest that more rapid binding plays a significant role in the inhibition of the cleavage-2. In fact, a difference in the activity between BG-II-S and -C to the cleavage-2 was substantial, the latter exhibiting $\sim$20% higher inhibition.

For several decades, BCD has been considered to inhibit only the cleavage-2 activity of G-II. Our study revealed that BCD inhibits both cleavage-1 and -2 activities of G-II. However, its activity against cleavage-2 was sixfold higher than against cleavage-1. By contrast, other glucosidase inhibitors CST and DNJ exhibited very similar inhibition toward both activities. The irreversible binding of BCD to G-II seems to be responsible for the inhibition of the cleavage-1 as well as cleavage-2, because the inhibition retained after dialysis was similar between these activities.

The difference in responsiveness of two activities toward BCD is in agreement with the hypothesis that G-II has two substrate binding sites. Previous studies[7,16] suggested that BCD binds irreversibly to low affinity binding site, selectively inhibiting the cleavage-2. Our results, on the other hand, indicate that irreversible binding of BCD inhibits two activities to a similar extent, implicating that BCD binds to G-II in a dual manner. The cleavage-2 seems to be sensitive to both reversible and irreversible binding of BCD, while the cleavage-1 is inhibited only by the irreversible binding. Since BCD inhibits both of the two activities of G-II in an irreversible manner, the use of BCD or its derivatives would be valuable for activity-based-profiling and active site mapping of this enzyme.

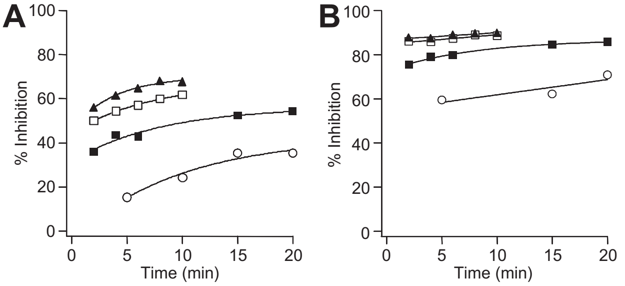

Figure 5. The pre-incubation time dependence of inhibitory activity of BCD. (A) The relative Glc trimming efficiency against cleavage-1 (A) and cleavage-2 (B) of G-II. G-II was preincubated with designated concentrations of BCD (○, 100 μM; ■, 250 μM; □, 500 μM; ▲, 1 mM). After designated time points at 25 °C, substrate was added and incubated further for 20 min at 37 °C. After the reactions were stopped by heating at 100 °C for 1 min, the mixtures were analyzed by HPLC.

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