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Antiviral Activity of Methylelaiophylin, an α-Glucosidase Inhibitor

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Methylelaiophylin isolated from *Streptomyces melanosporofaciens* was selected as an α-glucosidase inhibitor with an IC_{50} value of 10 μM. It showed mixed-type inhibition of α-glucosidase with a K_i value of 5.94 μM. In addition, methylelaiophylin inhibited the intracellular trafficking of hemagglutinin-neuramidase (HN), a glycoprotein of Newcastle disease virus (NDV), in baby hamster kidney (BHK) cells. Methylelaiophylin inhibited the cell surface expression of NDV-HN glycoprotein without significantly affecting HN glycoprotein synthesis in NDV-infected BHK cells.

Keywords: α-Glucosidase inhibitor, methylelaiophylin, trafficking inhibitor

The oligosaccharide moiety of many glycoproteins performs pivotal functions in a wide range of cell physiologies. Glucosidase, which affects the pathway of oligosaccharide synthesis, is involved in the biosynthesis of N-linked oligosaccharide chains in glycoproteins to be transported on the cell surface [6]. Functional studies of glucosidases in the cell have been investigated with several inhibitors. Glucosidase inhibitions in the cell bring about an alteration on cell-to-cell signaling and virus recognition to the cell. For this reason, the extensive application of glucosidase inhibitors will be helpful in the treatment of viral infection, cancer, and genetic disorders [2, 4, 13, 22]. For these reasons, glycosidase inhibitors are important tools for studying glycosylation in cells and could be useful as therapeutic agents [3].

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Interestingly, nojirimycin, *N*-butyldeoxynojirimycin, nectrisine, and castanospermine, known as α-glucosidase inhibitors, block HIV-mediated syncytium formation and suppress HIV replication during HIV infection [5, 9, 14, 24, 28]. Generally, HIV-1 envelope glycoprotein (Env) is synthesized as the precursor gp160, which is proteolytically cleaved into gp120 (surface protein) and gp41 (transmembrane protein). Both subunits remain noncovalently associated within oligomeric structures during transportation to the cell surface [7]. After virus budding, HIV entry into cells begins with gp120 binding to CD4+ receptors on lymphocytes through interaction with the cell surface antigen CD4 [24]. Gp41 receptors on T helper cells bind to other lymphocytes to fuse the cell membrane of several T helper cells (syncytium formation) [25]. Deoxynojirimycin and its analogs also mimic saccharides, thus inhibiting α-glucosidase activity in cells. Accordingly, they block the trimming of the Env glycan precursor (*i.e.*, the cleavage of the three Glc residues from the $Glc_3Man_9GlcNAc_2$ precursor glycan) within the endoplasmic reticulum. These inhibitors seem to affect the processing of N-linked oligosaccharides attached on the specific region (PNGS) of external gp120. This change in glycosylation by α-glucosidase inhibitors is likely to explain the inhibition in the cleaving of the gp160 precursor into gp41 and gp120, seen in HIV-infected cells. Moreover, this inhibition results in a suppression of HIV replication and syncytium formation *in vitro* [9, 14, 24].

Several inhibitors of α-glucosidase also inhibited syncytium formation in baby hamster kidney (BHK) cells infected with Newcastle disease virus (NDV) [11]. Syncytium formation and hemolytic activity in the cell are suppressed when NDV-infected BHK cells are treated with nectrisine or castanospermine. However, the inhibitor does not inhibit on the synthesis and cell-surface expression of the hemagglutinin-neuraminidase (HN) viral glycoprotein [28].

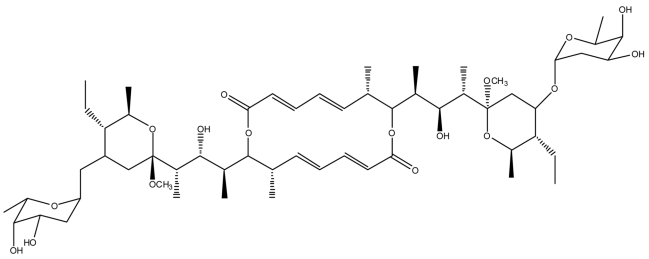

Fig. 1. Structure of methylelaiophylin.

A major mechanism of action of α-glucosidase inhibitors in the inhibition of HIV replication is the hindrance of viral entry at post-CD4 binding, due to an effect on viral envelope components [9].

During screening for antibiotics, methylelaiophylin, a derivative of elaiophylin, was isolated as an active principle from *Streptomyces melanosporofaciens* [17]. Elaiophylin, a 16-numbered macrolide antibiotic isolated from cultures of *Streptomyces melanosporus*, was first reported by Arcamone *et al*. [1]. Elaiophylin is a macrolide antibiotic that inhibits testosterone 5-reductase and plasma membrane-proton ATPase (P-ATPase) [20]. It has also been found to have antifungal, antiprotozoal, antitumor, antihelmintic, and immunosuppressive activities [8, 10].

Recently, we discovered the antiviral effect of methylelaiophylin while screening for α-glucosidase inhibition. To evaluate whether methylelaiophylin inhibits various commercially available glucosidases, an assay was performed as described previously [16]. In brief, α-glucosidase and the other glucosidases (obtained from Sigma Chemical Co., St. Louis, MO, USA) were assayed using 50 mM phosphate buffer at pH 6.7, and the appropriate *p*-nitrophenol (PNP) glycosides (at 1 mM) were used as substrates. The concentrations of the enzymes are specified in each experiment. A sample at the designated concentration was added to the enzyme solution in the buffer and incubated at 30°C for 1 h, and the substrate was then added to initiate the enzyme reaction. When pretreatment was not specified, mixtures of substrate and sample at a given concentration were prepared beforehand and added to the enzyme solution. The enzyme reaction was carried out at 30°C for 30 min. The reaction was stopped by the addition of 3 volumes of 1 M Na_2CO_3, and the release of PNP was monitored at 405 nm. One unit of activity was defined as the amount of enzyme liberating 1.0 μmol of PNP per minute under the assay conditions.

As shown in Fig. 2, α-glucosidase (from yeast) was the most sensitive to methylelaiophylin, and the concentration required for 50% inhibition (IC_{50}) was 10 μM. The IC_{50} value of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), which was used as a positive control α-glucosidase, was 5 μM. At higher concentrations, methylelaiophylin inhibited the activity of α-mannosidase (from jack beans), β-mannosidase

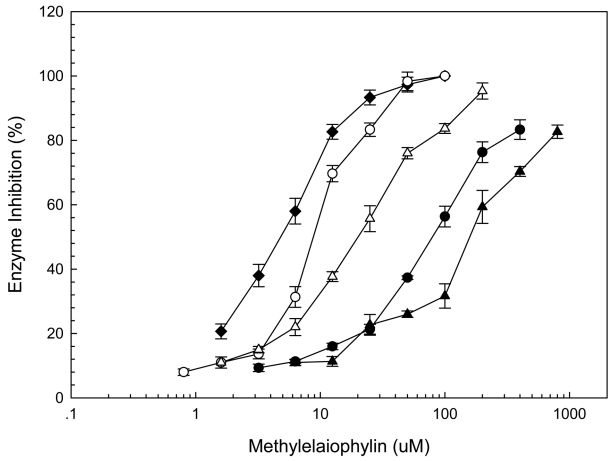

Fig. 2. Inhibition by methylelaiophylin against various glucosidases.
Enzyme solutions were treated with designated concentrations of methylelaiophylin. The amount of enzymes were as follows: 0.5 U/ml α-glucosidase (○), 0.5 U/ml β-glucosidase (●), 0.5 U/ml α-mannosidase (△), and 0.5 U/ml β-mannosidase (▲). 2,2-Diphenyl-1-picrylhydrazyl hydrate was used as a positive control (◆). The mixtures of enzyme and methylelaiophylin were kept at room temperature for 1 h.

(from snail acetone powder), and β-glucosidase (from almond) with sensitivities decreasing in that order. The IC_{50} values were 18, 70, and 105 μM, respectively. These results show that the activity of α-glucosidase is reduced by methylelaiophylin in a dose-dependent manner.

To study the kinetics of enzyme inhibition, the reaction was performed under the above conditions with inhibitors at various concentrations. Inhibition types were determined by Dixon plot and replot of slope versus the reciprocal of the substrate concentration [27]. Lineweaver–Burk plots of α-glucosidase kinetics with methylelaiophylin are shown in Fig. 3. The value of l/V increased when the concentration

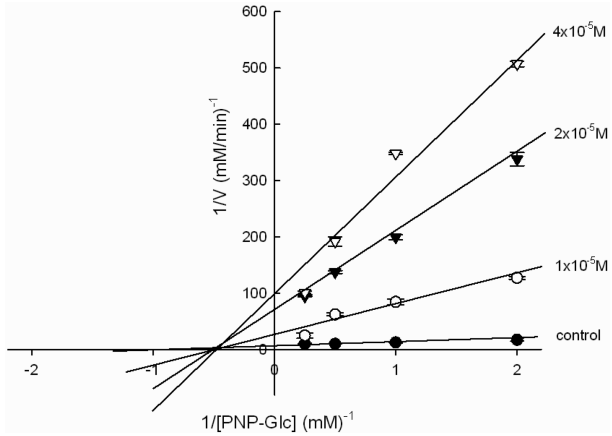

Fig. 3. Lineweaver–Burk plots of the inhibition kinetics of yeast α-glucosidase by methylelaiophylin.
α-Glucosidase (50 μl, 10 U/ml) that was treated first with methylelaiophylin for 1 h at 30°C was treated with a mixture of 50 μl of each designed concentration of PNP-α-glucopyranoside to initiate the enzyme reaction.

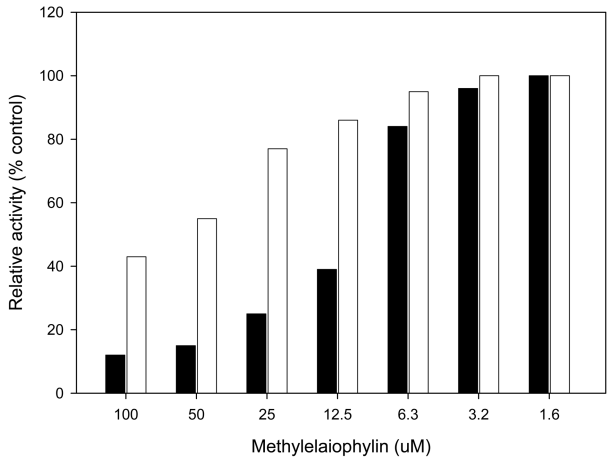

Fig. 4. Blockade of the cell surface expression of NDV-HN glycoprotein by methylelaiophylin with no significant effects on its synthesis.

Monolayer cultures of BHK cells in 6-well or microtiter plates were infected with NDV, and indicated concentrations of methylelaiophylin were added to the cultures hours after infection. Percentages of HAU (white) or HAD (gray) were determined at 14 h of infection. NDV-HN protein synthesis was quantified by determining HAU in whole lysates of infected cultures in microtiter plates, and cell surface expression quantified by measuring the amounts of chicken red blood cells adsorbed to intact infected cells in 6-well plates. Results are expressed as a percentage of the control value.

of the inhibitor increased, but K_m was constant. This result suggests that the binding of methylelaiophylin to α-glucosidase resulted in mixed-type inhibition. The K_i value in this assay was 5.94 μM, which was calculated using the values of V_{max} obtained at 0 and 40 μM methylelaiophylin. These data suggest that methylelaiophylin could be used to study the mechanism of glycosylation in viral disease. These inhibition studies also provide useful knowledge for the design of potent inhibitors to glycosylation of glycoproteins.

Glycoproteins play important roles in various physiological responses in mammalian cells. They are synthesized and secreted into cell membranes through the Golgi complex, and are transported between membrane-bound organelles by repeated cycles of budding and fusion of secretory vesicles [12, 18]. To identify the numerous components of the machinery involved in transport, a number of biochemical and genetic approaches to the study of trafficking have been employed [21, 23]. Previously, an *in vitro* intra-Golgi transport assay was utilized to purify a cytosolic transport factor [26]. The cell surface expression of viral glycoprotein was blocked by brefeldin A [15]. The use of chemicals involved in intracellular trafficking will play an important role in the study of glycoprotein secretory pathways to discover the underlying mechanism of viral infections, including cancer and other degenerative diseases. However, the glycoprotein trafficking mechanism has not been well defined. To study the inhibitory activity of methylelaiophylin at the cellular level, the degree of inhibition of synthesis

Table 1. Effect of methylelaiophylin on syncytium formation.

Concentration (μM)	0.625	1.25	2.5	5	10	20	40
Syncytium formation	-	-	-	-	-	+	++

Confluent monolayer cultures of BHK cells in 96-well titer plates were infected with NDV. Methylelaiophylin was added at 1 h after infection and the cells were incubated for a further 18 h. The syncytium formation in NDV-infected cells was determined under an optical microscope The degrees of syncytium formation are expressed as follows: -, none; +, moderate; ++, severe.

and trafficking of NDV-HN glycoprotein expressed on the surface of BHK cells was measured, because syncytium formation of NDV in BHK cells is similar to that of HIV.

To measure the effect of methylelaiophylin on syncytium formation, confluent monolayer cultures of BHK cells in 96-well microtiter plates were infected with NDV at a concentration of five plaque-forming units (PFU) per cell, and then the designated concentrations of methylelaiophylin were added. Cells were incubated at 37°C for 16 h in a humidified 5% CO_2–95% air incubator. Thereafter, the degree of syncytium formation in NDV-infected cells was determined under an optical microscope [19]. Methylelaiophylin suppressed syncytium formation with an MIC of 10 μM (Table 1). This value is somewhat similar to the IC_{50} value obtained above for the inhibition of glucosidase (Fig. 2).

Synthesis of NDV-HN glycoprotein was quantified by determining hemagglutination units (HAU) in whole lysates of NDV-infected cells [19]. Cell surface expression of NDV-HN glycoprotein was determined by hemadsorption (HAD) as follows. Confluent cultures of BHK cells in 6-well plates (Falcon) were infected with 2 ml of NDV at a concentration of 1 HAU/ml and incubated for 14 h at 37°C in a humidified 5% CO_2–95% air incubator. The medium was removed by aspiration, and then 2 ml of 1% (v/v) chicken red blood cells in cold saline was added to each well and the plate held at 4°C for 30 min with occasional gentle stirring. Unadsorbed red blood cells were removed, and the cell layers were rinsed three times with 2 ml each of cold saline. The adsorbed red blood cells were swollen in distilled water containing 1% ammonia and quantified by measuring the absorption at 550 nm.

To measure the total amount of NDV-HN synthesized, whole NDV-infected cultures were disrupted by brief sonication, and then chicken red blood cells were added to determine the hemagglutination activity in the lysates. The number of HAUs was not significantly decreased at any concentration up to 12.5 μM methylelaiophylin (Fig. 2, white). However, the binding of extracellularly added chicken red blood cells to the surfaces of intact NDV-infected cells (expressed as % HAD) decreased depending on the sample concentration (Fig. 2, black), indicating that the extracts blocked the cell surface expression of NDV-HN glycoprotein in a dose-dependent manner. These results collectively indicate that methylelaiophylin inhibits the

cell surface expression of NDV-HN, but has no significant effect on its synthesis. Elucidation of the inhibition mechanisms for both α-glucosidase and trafficking requires further investigation for the possibility of identifying candidate anti-HIV therapeutic agents.

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