Novel inhibitory effect of the antidiabetic drug voglibose on melanogenesis

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Abstract: Overproduction of melanin can lead to medical disorders such as postinflammatory melanoderma and melasma. Therefore, developing antimelanogenic agents is important for both medical and cosmetic purposes. In this report, we demonstrated for the first time that the antidiabetic drug voglibose is a potent antimelanogenic agent. Voglibose is a representative antidiabetic drug possessing inhibitory activity towards human α-glucosidase; it blocked the proper N-glycan modification of tyrosinase, resulting in a dramatic reduction of the tyrosinase protein level by altering its stability and subsequently decreasing melanin production. Acarbose, another antihyperglycaemic drug that has a lower inhibitory effect on human intracellular α-glucosidase compared with voglibose, did not cause any changes in either the N-glycan modification of tyrosinase or the tyrosinase protein level, indicating that voglibose was the most efficient antimelanogenic agent among the widely used antihyperglycaemic agents. Considering that voglibose was originally selected from the valiolamine derivatives in a screen for an oral antidiabetic drug with a strong inhibitory activity towards intestinal α-glucosidase and low cell permeability, we propose an alternative strategy for screening compounds from valiolamine derivatives that show high inhibitory activity towards human intracellular α-glucosidases and high cell permeability, with the goal of obtaining antimelanogenic agents that are effective inside the cells.

Introduction

Melanin, which is produced in melanocytes and melanoma cells from the amino acid tyrosine, plays a major role in protecting cells from cytotoxic radiation and determines the skin colour of mammals (1–3). The overproduction of melanin causes medical problems such as postinflammatory melanoderma and melasma. (4–8). Many studies have focused on understanding the precise mechanism of melanin production because developing antimelanogenic agents is a critical goal in the clinical and cosmetic fields. Although there have been some recent trials to apply siRNAs and bioactive oligopeptides in the skin to suppress melanogenesis (9,10); traditionally, researchers who aimed to develop antimelanogenic agents focused on the small molecule inhibitors of tyrosinase, the rate-limiting enzyme in melanin production (11). The strategies for modulating tyrosinase have included altering its glycosylation, inhibiting its catalytic activity and accelerating its degradation (11).

Tyrosinase is a glycoprotein that requires N-glycan for functionality and proper localization (12–14). The N-glycan processing of tyrosinase is performed by intracellular α-glucosidase and α-mannosidases in the endoplasmic reticulum (ER) and Golgi apparatus (12,15). If these enzymes are inhibited, tyrosinase is aberrantly folded and does not become mature, resulting in hypopigmentation (12,14). Among the general inhibitors of glycosylation,

glucosamine and tunicamycin inhibit melanin production without decreasing the tyrosinase protein level (11,16). Two specific inhibitors, deoxynojirimycin (DNJ), an α-glucosidase inhibitor, and deoxymannojirimycin (DMJ), an α-mannosidase inhibitor, are also direct inhibitors of melanin production that do not affect the tyrosinase protein level (11,14).

The α-glucosidase inhibitors reduce the release of small sugars by inhibiting the hydrolysis of α-1,4-glycosidic bonds in complex carbohydrates (17). These inhibitors were initially developed to treat viral infections and diabetes (17,18). The ER and Golgi α-glucosidase inhibitors prevent or delay virus proliferation by inhibiting viral morphogenesis and infectivity because many viral envelope glycoproteins are essential for the virus life cycle (18,19). For example, DNJ and its derivatives have been studied as antiviral agents against the human immunodeficiency virus (HIV) and the hepatitis B virus (HBV) (19,20). Compared with the ER and Golgi α-glucosidase inhibitors, intestinal α-glucosidase inhibitors are widely used in diabetic therapy because the α-glucosidases isomaltase and sucrase in the brush border of the small intestine digest carbohydrates, releasing monosaccharides that are absorbed as an energy source (17). Acarbose, miglitol and voglibose are three major oral antihyperglycaemic drugs being used to treat type 2 diabetes mellitus by delaying the absorption of glucose and other monosaccharides, thereby decreasing the clinical risk

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incurred by the macrovascular complications of diabetes (21,22). These drugs were developed to have low membrane permeability and high stability in the digestive system because the site of drug action is within the small intestine.

In this study, we first tested the antimelanogenic activity of three major oral antihyperglycaemic drugs and determined that voglibose had a strong inhibitory effect on melanin production by blocking the proper N-glycan processing of tyrosinase, resulting in a dramatic reduction of this protein. This effect was not observed to a significant degree in other melanogenic proteins, such as TRP-1 or -2. Another intestinal α-glucosidase inhibitor, acarbose, did not have these effects on tyrosinase, suggesting that not all intestinal α-glucosidase inhibitors alter the N-glycan processing of tyrosinase. Finally, we propose an alternative strategy to identify effective antimelanogenic agents by screening valiolamine derivatives with high cell permeability and strong inhibitory activity towards the intracellular α-glucosidases; this alternate screen consists of modifying the strategy used to obtain voglibose, which was designed to obtain compounds with low cell permeability and strong inhibition of the intestinal α-glucosidases.

Materials and methods

Cell culture and materials

Human melanoma MNT-1 cell line was kindly provided by Dr. Lee, Ai-Young at Dongguk university, Seoul, Korea and was maintained at 37°C in MEM (Gibco, Carlsbad, CA, USA) with 20% FBS, 10% high-glucose DMEM (Lonza, Basel, Switzerland) as high energy sources to grow well, 20 mM HEPES and antibiotics. Human melanoma WM266-4 cells were maintained at 37°C in RPMI 1640 medium (Gibco) with 10% FBS and antibiotics. Moderately pigmented normal human melanocytes (Cascade Biologics, Portland, OR, USA) were maintained in M-254 medium (Cascade) containing human melanocyte growth supplement-2 (Cascade). Acarbose, miglitol and voglibose were purchased from Sigma (Sigma, St. Louis, MO, USA) and dissolved in the culture medium. Endo H and PNGase F were purchased from New England Bio Labs (New England Bio Labs, Hitchin, UK) and used according to the manufacturer's instructions.

Melanin and tyrosinase activity assay

For the melanin assay, the cell pellets were washed with PBS and dissolved in 1 N NaOH for 1 h at 80°C. The melanin levels were determined by measuring the absorbance at 450 nm. For melanin assay using a reconstructed human skin model, Neoderm-ME was purchased and maintained as the manufacturer's instructions (Tego Science, Seoul, Korea). Under the continuous treatment of 2 mM voglibose for 7 days, Neoderm-ME was irradiated with 20 mJ/cm^2 UVB every other day, total three times. Neoderm-ME was dissolved in 1 N NaOH and sonicated. The debris was clarified by centrifugation at 16 000 × g for 1 min, and the absorbance at 450 nm was measured from supernatants. For the tyrosinase activity assay, the cells were lysed with 1% NP-40 in PBS for 10 min at 4°C and clarified by centrifugation for 20 min at 16 000 × g. The supernatants were combined with 2 mg/ml L-DOPA in PBS and incubated for 1 h at 37°C, and then, the absorbance at 450 nm was measured.

Cell growth assay

The cells were seeded at a density of 1×10^4 per well in 6-well plates. On each day of the assay, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed with PBS and then stained with 500 μl of 0.1% crystal violet for 20 min. The stained cells were washed 5 times with PBS, dried for 5 min and lysed with 1 ml of 10% acetic acid. The absorbance at 590 nm was measured to obtain the cell growth curves.

Western blot analysis

The cells were lysed with 1% NP-40 in a solution of 0.05 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.01 M MgCl$_2$ and a protease inhibitor cocktail (Sigma). The supernatants were cleared by centrifugation at 16 000 × g for 20 min. The supernatants were then removed, and the protein content was quantified using the BCA assay. An aliquot of 20 μg of protein was loaded into each well of an SDS-PAGE gel. For immunoblotting, the SDS-PAGE gel was electroblotted to a PVDF membrane. Anti-TRP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TRP-2 antibody (Santa Cruz Biotechnology), anti-GAPDH antibody (Santa Cruz Biotechnology), anti-MART-1 antibody (Thermo Fisher Scientific, CA, USA) and anti-Tyr antibody (Upstate Biotechnology, Lake Placid, NY, USA) were used for protein detection.

Glucosidase/mannosidase activity assay

The p-nitrophenyl glycoside and p-nitrophenyl mannoside reaction substrates and the yeast α-glucosidase and jack bean α-mannosidase enzymes were purchased from Sigma. 50 μl of 0.2 mM each reaction substrate was combined with the equal volume of either MNT-1 cell lysates or 5 mM each enzyme solution in 50 mM phosphate buffer (pH 6.8), and incubated for 30 min at 37°C. Absorbance at 420 nm was measured after addition of a 1 M Na$_2$CO$_3$ stop solution.

Quantitative real-time PCR

A 2 μg sample of total RNA was reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan) and oligo dT. The gene expression analysis was performed using TaqMan® Universal Master Mix and TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction.

Transmission electron microscopy

Confluent flasks of MNT-1 cells were fixed using Karnovsky fixative for 30 min at RT, collected by centrifugation and then embedded in a low-melting-point agarose matrix. After being washed twice with cold PBS, agarose-embedded cells were postfixed in 2% osmium tetroxide and stained with uranyl acetate. Then, the specimens were dehydrated through a graded ethanol series and embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA) at 60°C for 48 h. Ultrathin sections (approximately 70–90 nm) were stained using uranyl acetate and lead citrate and then observed using a transmission electron microscope (TEM; JEM-1200 EX, JEOL). The TEM images were recorded on negative film and transferred into a computer using a scanner (EPSON Perfection V700 PHOTO™) at 1200 dpi.

Statistical analysis

A two-tailed Student's t-test was used to analyse the differences between the two groups.

Results

Voglibose inhibited human intracellular α-glucosidase activity

Acarbose, miglitol and voglibose are three antidiabetic drugs that are widely used to treat type II diabetes by inhibiting intestinal

α-glucosidases (Fig. 1a). Only miglitol, a 1-deoxynojirimycin (1-DNJ) derivative, has been studied to determine its inhibitory effect on intracellular α-glucosidases, and its precursor DNJ was known as a melanogenesis inhibitor (18). To investigate the direct inhibitory effects of acarbose and voglibose on intracellular α-glucosidases and their possible application as antimelanogenic agents, human melanoma MNT-1 cell extracts were treated with these two diabetic drugs, and then, the α-glucosidase and α-mannosidase activities were analysed. Each drug had a significant inhibitory effect on human intracellular α-glucosidases compared with the control treatment (Fig. 1b), and DNJ and voglibose were more effective than acarbose. Acarbose displayed inhibitory activity towards both human intracellular and yeast α-glucosidases, and DNJ had an additional inhibitory effect on α-mannosidase; however, voglibose inhibited only human intracellular α-glucosidase, suggesting that voglibose is more specific than the other agents and that its inhibitory effect is comparable to that of DNJ (Fig. 1b–e). Considering that these antidiabetic drugs can inhibit human intracellular α-glucosidases, we hypothesized that they could also function as antimelanogenic agents by blocking the N-glycan processing of melanogenic enzymes, which is mediated by intracellular α-glucosidases.
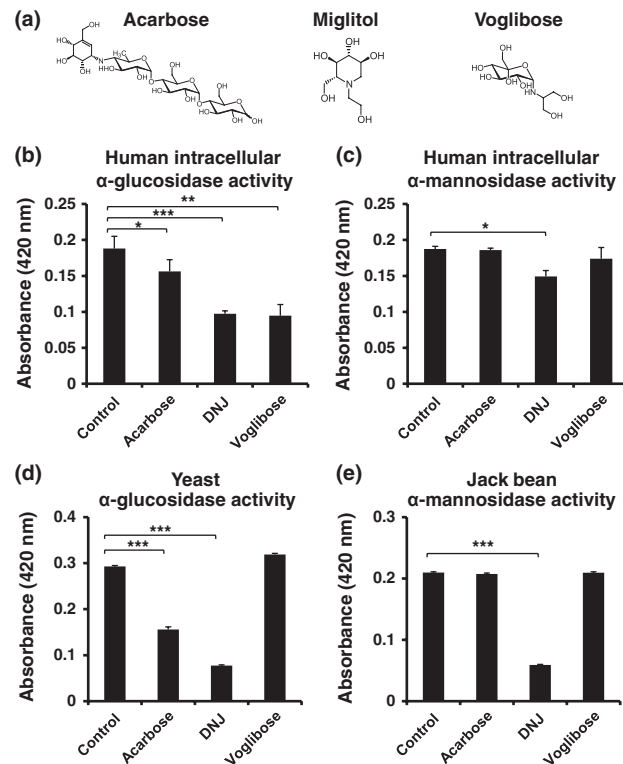

Figure 1. The α-glucosidase and α-mannosidase activity assay using antihyperglycaemic agents. (a) Structures of three major antihyperglycaemic agents widely used in antidiabetic medications. (b) Human intracellular α-glucosidase activity assay. The MNT-1 cell extract was incubated with *p*-nitrophenyl glycoside, and the α-glucosidase activity was spectrophotometrically assayed at 420 nm. (c) Human intracellular α-mannosidase activity assay. The MNT-1 cell extract was incubated with *p*-nitrophenyl mannoside, and the α-mannosidase activity was spectrophotometrically assayed at 420 nm. (d) Yeast α-glucosidase activity assay. (e) Jack bean α-mannosidase activity assay. The data are representative of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$).

Voglibose inhibited melanin production in MNT-1 cells

To test this hypothesis, we first treated MNT-1 human melanoma cells with acarbose, miglitol and voglibose for 7 days and observed the colour of the cell pellets to evaluate the possibility that these agents were antimelanogenic agents (Fig. 2a). Visual evaluation and melanin measurement revealed a remarkable reduction in the melanin content of the voglibose-treated cells compared with that of the cells treated with the other agents, and this effect was dose dependent (Fig. 2a,b), and dosage used for this condition did not affect on cell growth (Fig. 2c). Electron microscopic analysis demonstrated that the non-treated control cells contained melanosomes filled with melanin at various stages of maturity (Fig. 2d, control); however, the voglibose-treated cells contained a number of premature melanosomes without apparent melanin accumulation on protein matrix (Fig. 2d, voglibose), implying functional defects in the melanogenic enzymes rather than structural defects in the melanosomes. We counted the numbers of melanized melanosomes and showed much less numbers in voglibose-treated cells than in control (Fig. 2e).

Voglibose inhibited proper N-glycan processing of tyrosinase and reduced its protein level in MNT-1 cells and normal human melanocytes
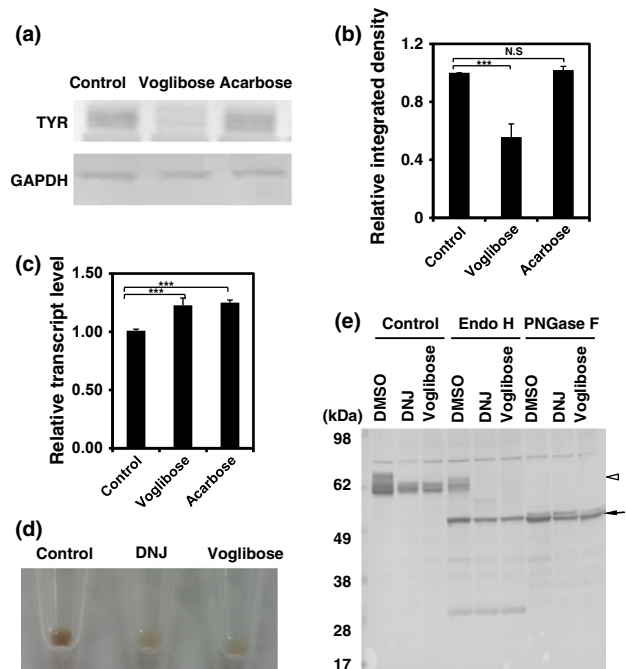
Tyrosinase is the most important melanogenic enzyme involved in the rate-limiting step of melanin synthesis and requires the proper N-glycan processing by intracellular α-glucosidase and α-mannosidases for functionality and proper localization (12–14). We observed that voglibose functions as an inhibitor of human intracellular α-glucosidases (Fig. 1b); therefore, we analysed the N-glycan processing of tyrosinase. After 10 days of voglibose treatment, the tyrosinase content of MNT-1 cells was much less than that of non-treated or acarbose-treated cells (Fig. 3a,b). The decreased level of tyrosinase protein was not due to a reduction in the tyrosinase mRNA because the mRNA level was slightly increased compared with that of the control cells (Fig. 3c), suggesting that voglibose reduced the level of tyrosinase by altering its stability. We also observed that the reduction of tyrosinase activity was dose dependent (data not shown). The inhibitory effects of voglibose in MNT-1 melanoma cells were also observed in normal human melanocytes, which exhibited reduced melanin production compared with control cells (Fig. 3d). We used DNJ as a positive control because it reduces melanin production by inhibiting the N-glycan processing of tyrosinase (11,15). We examined the total protein levels of tyrosinase in normal melanocytes treated with DNJ or voglibose for 7 days. DNJ- or voglibose-treated melanocytes displayed a remarkable reduction in the total tyrosinase compared with that of non-treated cells (Fig. 3e). To verify that voglibose acted as an intracellular α-glucosidase inhibitor by affecting the N-glycan processing of tyrosinase during protein synthesis, we treated cell lysates with Endo H and PNGase F, which deglycosylated the improperly processed N-glycan and all of the N-glycans of tyrosinase, respectively (14,23). Compared with the non-treated control, in which Endo H-resistant tyrosinase bands were present at a relatively high rate, the DNJ- or voglibose-treated cells showed no Endo H-resistant tyrosinase bands (Fig. 3e), suggesting that most of the tyrosinase was not processed properly in the DNJ- or voglibose-treated melanocytes. The observed reduction of tyrosinase in the DNJ-treated cells is inconsistent with previous reports in which DNJ
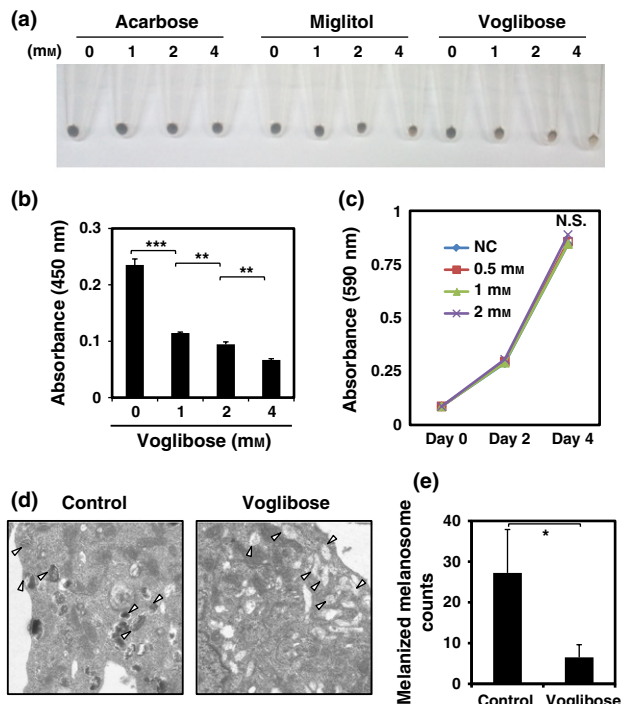
Figure 2. The inhibitory effects of antihyperglycaemic agents on melanin production. (a) After cells were treated with 1 mM solution of acarbose, miglitol or voglibose for 7 days, 5×10^4 cells were collected by centrifugation and monitored. (b) The melanin content was measured at 450 nm. The data are representative of three independent experiments (**$P < 0.01$; ***$P < 0.005$). (c) The cell growth curves after voglibose treatment. Cells were treated with each concentration of voglibose for 4 days, and cell growth assay was performed as described in Materials and methods. (d) Electron microscopy analysis. The arrowheads indicate melanosomes. (e) Melanized melanosome numbers were counted from electron microscopic images. A total of twenty fields from four representative images were counted (*$P < 0.05$).

Figure 3. The effects of voglibose on tyrosinase expression and folding. (a) Protein expression level of tyrosinase in MNT-1 cells. MNT-1 cells were cultured with 1 mM voglibose or 1 mM acarbose for 10 days. (b) Quantification of tyrosinase protein level was performed with ImageJ software (http://rsbweb.nih.gov/ij/download.html) (***$P < 0.005$). (c) mRNA expression level of tyrosinase. The data are representative of three independent experiments (***$P < 0.005$). (d) Normal human melanocytes were cultured with 1 mM deoxynojirimycin (DNJ) or 1 mM voglibose for 7 days. 5×10^4 cells were collected by centrifugation and monitored. (e) Deglycosylation assay. Extracts of normal human melanocytes that had been treated with 1 mM DNJ or voglibose for 7 days were incubated with Endo H or PNGase F. The arrowheads and arrows indicate the Endo H-resistant and Endo H-susceptible proteins, respectively. The Endo H-resistant bands of high molecular weight are the glycosylated forms (arrowhead), whereas the Endo H-susceptible bands of low molecular weight represent the deglycosylated forms (arrow).

was administered for 3 days and did not affect the level of tyrosinase protein (14,24). However, we observed a significant reduction of tyrosinase protein in both MNT-1 human melanoma cells (Fig. 3a) and normal melanocytes after 7 days of treatment (Fig. 3e), suggesting that the tyrosinase decreases in response to DNJ treatment is evident only after long-term treatment. Taken together, we concluded that voglibose reduced melanin production by inhibiting the proper N-glycan processing of tyrosinase mediated by the intracellular α-glucosidases, thereby decreasing tyrosinase protein.

In addition to tyrosinase, several other glycoproteins are also involved in melanin production (13,25). To elucidate the involvement of these melanogenesis-related proteins in voglibose-induced hypopigmentation, immunoblotting analysis was performed. No apparent changes in the melanogenesis-related proteins TRP-2 and MART-1 were detected at various concentrations of voglibose, including 1 mM, at which the total tyrosinase level was significantly reduced (Fig. 3a,d and S1A). Although there was no significant change in the total protein level of another glycoprotein, TRP-1 (Figure S1A), its glycosylation state appeared to be slightly affected by voglibose treatment (Figure S1B). These data suggest that the stability of tyrosinase is more sensitive to its glycosylation state than to that of other glycoproteins, including TRP-1. It is also possible that the changed glycosylation state of TRP-1 could

affect melanin production through altering the interaction with other melanogenic proteins, such as tyrosinase or TRP-2. Acarbose treatment did not affect the total protein levels of TRP-1, TRP-2 or MART-1 or the activity of tyrosinase (Figure S1C,D).

Voglibose inhibited melanin production in a reconstructed human skin model

To observe the inhibitory effect of voglibose on melanin production in human skin, voglibose was applied to the reconstructed human skin for 7 days. Under the continuous treatment of voglibose, the reconstructed human skins were irradiated with 20 mJ/cm^2 UVB every other day, total three times to increase melanin production. The visual and spectrophotometric evaluations revealed the significant reduction of melanin content in voglibose-treated reconstructed human skins compared with control-treated ones (Fig. 4a,b). These results imply the possibility of voglibose as an antimelanogenic agent which is applicable in human skin.

Discussion

Oral antihyperglycaemic agents used to treat type 2 diabetes mellitus were developed to inhibit intestinal α-glucosidases, such as sucrase, isomaltase and maltase, by blocking the cleavage of α-1, 4-glycosidic bonds in carbohydrates (17). Among oral antihyperglycaemic agents, acarbose is a natural product isolated from
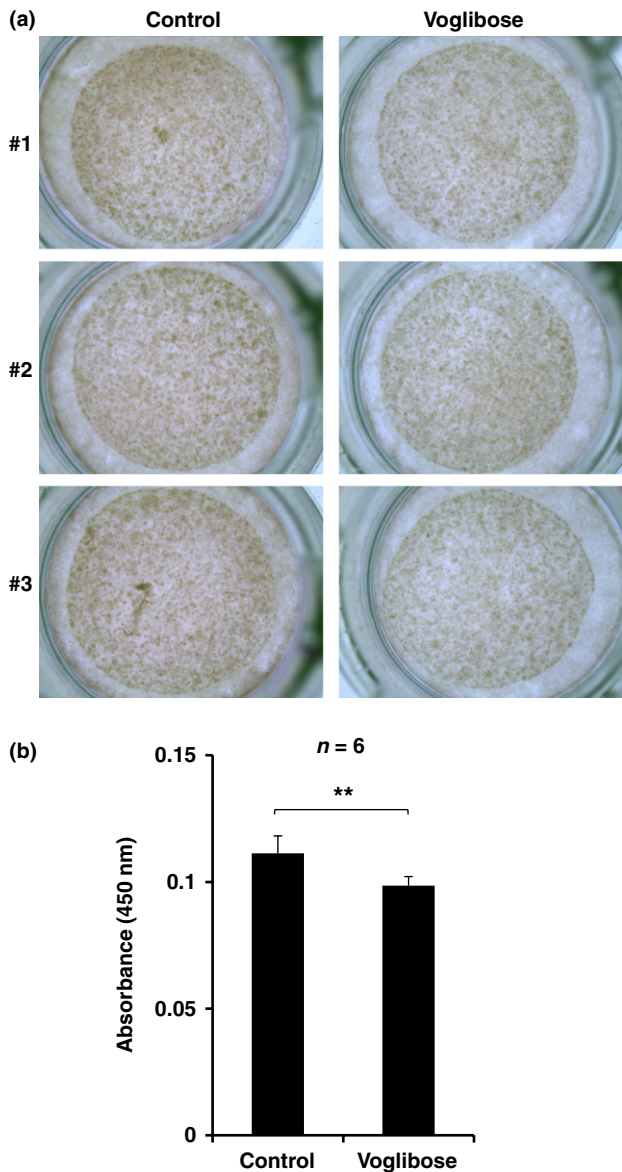
Figure 4. The inhibitory effect of voglibose on melanin production in a reconstructed human skin model. (a) Under the treatment of 2 mM voglibose for 7 days, the reconstructed human skins were irradiated with 20 mJ/cm^2 UVB every other day, totally three times. Three representative images were shown. (b) The melanin content from the lysates was measured at 450 nm after dissolving the reconstructed human skins with 1 N NaOH. Statistical significance is indicated by asterisks ($n = 6$, $**P < 0.01$).

Actinoplanes strains that competitively inhibits intestinal sucrase and isomaltase in the intestinal brush border (26–28). Both miglitol, 1-DNJ derivative, and voglibose, a valiolamine derivative, are research products that have been adapted for use as oral antihyperglycaemic agents with a high stability in the digestive system and a strong inhibitory effect on the intestinal α-glucosidases (29,30). However, the inhibitory effects of acarbose and voglibose on intracellular α-glucosidases have not yet been elucidated. In this study, we first demonstrated that these drugs also have inhibitory effects on intracellular α-glucosidases, but only voglibose shows antimelanogenic activity by blocking the proper N-glycan processing of tyrosinase, suggesting different inhibitory effects on melanin production among this group of antidiabetic drugs (Fig. 2).

Tyrosinase possesses seven N-glycosylation sites, the improper modification of which causes a loss of functional activity (12,15). Type 1 oculocutaneous albinism is caused by mutations in the N-glycosylation sites in tyrosinase (31). Therefore, early attempts to develop antimelanogenic drugs were focused on glycosylation inhibitors, such as glucosamine, tunicamycin, glutathione, ferritin, feldamycin and N-butyl deoxynojirimycin (11). Interestingly, none of those agents induced remarkable changes in the tyrosinase protein expression level (11). However, our data revealed that tyrosinase protein was dramatically reduced after the N-glycan processing was altered by voglibose treatment. Although all of these reagents supposedly inhibit the glycosylation of tyrosinase in the cells, one possible reason for this difference is the time period for treatment. We treated cells with voglibose for 7 days, but the other drugs were administered for relatively shorter time periods, such as 3 days (14,24). Another explanation for the difference might be the different specificity or inhibitory mechanism of each reagent against glycosylation. Among oral antihyperglycaemic agents, voglibose most efficiently blocked melanin production, consistent with its specific inhibitory effect on human intracellular α-glucosidases, which is responsible for N-glycan processing of tyrosinase. Compared with voglibose, acarbose showed a potent inhibitory effect on yeast rather than on human α-glucosidase, and DNJ inhibited the broad spectrum of enzymes, including α-glucosidase or α-mannosidase (Fig. 1). The specific and narrow spectrum of the action of voglibose on human intracellular α-glucosidases might be one reason why voglibose efficiently blocks the intracellular N-glycan processing of tyrosinase.

Voglibose is a derivative of valiolamine (30). N-substituted valiolamine derivatives, including voglibose, were systemically synthesized and tested in a screen for oral antihyperglycaemic drugs that had a strong inhibitory effect on intestinal sucrase and maltase, low cell membrane permeability and high stability in the digestive system, particularly the small intestine (30). In contrast to the oral antihyperglycaemic drugs, for intracellular α-glucosidase inhibitors to have efficient antimelanogenic activities, they must be highly cell permeable, stable within cells or the circulatory system and strongly inhibit human intracellular α-glucosidases. Because each intracellular α-glucosidase inhibitor in medical use induces different cellular responses and produces different side effects (18), the development of more potent agents with fewer side effects is also necessary. Although voglibose has a potent inhibitory effect on melanogenesis by working on the intracellular and intestinal α-glucosidases, valiolamine derivatives can be good candidates to discover the effective antimelanogenic agents by changing the screening scheme to select the agents possessing potent inhibitory activity towards intracellular α-glucosidases, high cell permeability and fewer side effects.

In conclusion, we showed that the antidiabetic drug voglibose could function as an antimelanogenic agent because it causes improper N-glycan processing of tyrosinase by inhibiting intracellular α-glucosidases, thereby decreasing the tyrosinase protein level. Considering that voglibose was originally derived from valiolamine and modified to have low cell permeability, an alternative strategy is possible for screening the high cell-permeable antimela-

nogenic agents among valiolamine derivatives. The development of these antimelanogenic agents would provide a new type of efficient medication (in addition to voglibose) against postinflammatory melanoderma and melasma.

Conflict of interests

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The effects of antihyperglycaemic agents on melanogenesis-related protein expression and folding.