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4-Acetoxyscirpendiol of *Paecilomyces tenuipes* Inhibits Na^+/D-Glucose Cotransporter Expressed in *Xenopus laevis* Oocytes

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Cordyceps, an entomopathogenic fungus, contains many health-promoting ingredients. Recent reports indicate that the consumption of cordyceps helps reduce blood-sugar content in diabetics. However, the mechanism underlying this reduction in circulatory sugar content is not fully understood. Methanolic extracts were prepared from the fruiting bodies of *Paecilomyces tenuipes*, and 4-beta acetoxyscirpendiol (4-ASD) was eventually isolated and purified. Na^+/Glucose transporter-1 (SGLT-1) was expressed in *Xenopus* oocytes, and the effect of 4-ASD on SGLT-1 was analyzed utilizing a voltage clamp and by performing 2-deoxy-D-glucose (2-DOG) uptake studies. 4-ASD was shown to significantly inhibit SGLT-1 activity compared to the non-treated control in a dose-dependent manner. In the presence of the derivatives of 4-ASD (diacetoxyscirpenol or 15-acetoxyscirpendiol), SGLT-1 activity was greatly inhibited in an 4-ASD-like manner. Of these derivatives, 15-acetoxyscirepenol inhibited SGLT-1 as well as 4-ASD, whereas diacetoxyscirpenol was slightly less effective. Taken together, these results strongly indicate that 4-ASD in *P. tenuipes* may lower blood sugar levels in the circulatory system. We conclude that 4-ASD and its derivatives are effective SGLT-1 inhibitors.

Keywords: Acetoxyscirpendiol, Cordyceps, Diabetes, SGLT-1, *Xenopus* oocytes, Voltage clamp

Introduction

The entomopathogenic fungus Cordyceps is traditionally believed to contain many ingredients that promote health, well-being, and fitness, and some cordyceps species have been used as tonics and antitussives from ancient times in Asia. In particular, in China cordyceps is highly regarded as one of the most effective traditional medicines with anti-aging and general curative effects (Konno *et al*., 2001; Nam *et al*., 2001; Kiho *et al*., 2002; Kikuchi *et al*., 2004). Of the numerous effects exerted by cordyceps species like *Paecilomyces tenuipes*, the lowering of blood sugar content is probably considered to be the most interesting (Kiho *et al*., 1999; Talpur *et al*., 2002; Kikuchi *et al*., 2004). Many phytochemists have searched for chemical components associated with this hypoglycemic effect. However, the physiological mechanism whereby cordyceps consumption reduces blood sugar levels in the circulatory system is not fully understood. Moreover, the key to understanding this mechanism requires an analysis of functional glucose transport in the presence of chemical components purified from cordyceps.

There are two classes of specific glucose transport (Baldwin, 1993): (1) facilitative Na^+-independent sugar transporters (GLUT family; gene name SLC2A), and (2) the Na^+-dependent glucose co-transporters (SGLT, members of a larger family of Na^+-dependent transporters; gene name SLC5A) (Wood *et al*., 2003). Glucose transporters are integral membrane proteins that mediate the transport of glucose and structurally related substances across membranes from blood to cells (Doege *et al*., 2000). At least thirteen different glucose transporters have been identified and assigned to the facilitated-diffusion glucose-transporter family (GLUT family), also known as "uniporters," and the Na^+-dependent glucose-transporter family (SGLT family), also known as "ion-coupled cotransporters" or "symporters" (Kirwan *et al*., 2003). The GLUT gene encodes a protein that is involved in the active transport of glucose and galactose into eukaryotic and some prokaryotic cells, whilst SGLT-1 works to drive sugar across the kidney membrane against the concentration gradient (Rumsey *et al*., 2003). This Na^+-dependent transport of D-glucose is prompted by an inner negative membrane potential and acidity, and is inhibited by phloridzin, a specific inhibitor of SGLT. Of the two glucose-transport systems, blood-glucose

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levels largely depend on SGLT since it allows glucose to be rapidly transported against concentration gradients, and thus, final blood glucose concentrations are predominantly governed by SGLTs.

In the present study, methanol extraction was used to isolate the components responsible for reducing blood sugar content from the fruiting bodies of *P. tenuipes*. Of the numerous candidates, 4-beta-acetoxyscirpene-3 alpha, 15-diol (4-ASD) was subsequently purified from methanolic extracts. An analysis of its chemical structure revealed that 4-ASD belongs to a class of trichothecenes. According to Nam *et al*. (2001), 4-ASD induced significant levels of apoptosis in various human cancer cell lines. Thus, since the inhibition of small intestinal Na^+/glucose co-transporter (SGLT-1) may help reduce blood-glucose concentrations, we investigated the effect of 4-ASD on its activity.

The *Xenopus* oocyte expression system has been employed to study the functional and structural characteristics of glucose transporters. *Xenopus* oocyte systems have been chosen by researchers for membrane channel protein studies because they express functionally viable membrane proteins when microinjected with appropriate species (Lee *et al*., 1995; Lee, 1998; Malden *et al*., 2003). Following the heterologous expression of human SGLT-1 in *Xenopus laevis* oocytes, the expressed SGLT-1 was subjected to electrophysiological measurements and D-glucose influx studies in the presence of 4-ASD or its analogs, and 4-ASD was confirmed as a functional ingredient with an inhibitory effect. Other chemical analogues of ASD also showed comparable inhibitory effects on SGLT-1 activity.

Materials and Methods

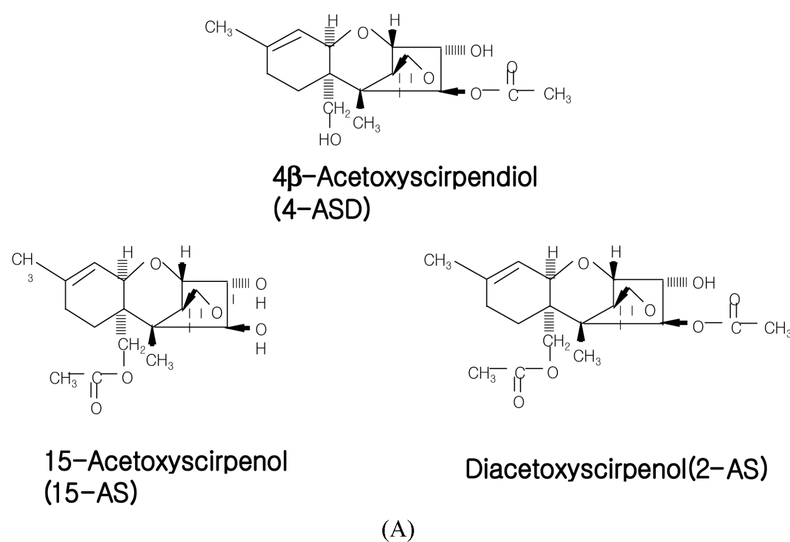
Microinjection of SGLT-1 cRNA into oocytes The cRNAs of human SGLT-1 (hSGLT-1) were synthesized from pSP6-hSGLT-1 harboring cloned cDNA using Sp6 polymerase according to the manufacturer's protocol (Promega, Madison, USA). An ovary was manually excised from an adult *Xenopus* and defolliculated oocytes were injected with hSGLT-1 cRNA, as described by Lee *et al.* (1995, 1998). Before microinjection, the oocytes were copiously washed in Barth Solution [5-mM KOH, 100-mM NaOH, 0.5-mM $CaCl_2$, 2-mM $MgCl_2$, 100-mM methanesulfonic acid, and 10-mM HEPES (pH 7.4)] and stage 4 or 5 oocytes were injected with 50 nl of the SGLT-1 expression injection mixture containing 50 ng cRNA. Following injection, oocytes were incubated in Barth Solution at 14°C for 24 h before uptake and electrophysiological assays. Natural 4-ASD was isolated according to Nam *et al*. (2001). Briefly, artificially cultivated conidophores of *P. tenuipes* were first extracted in methanol, and the extracts obtained were the concentrated approximately 10-fold at 45°C under reduced pressure. The concentrated samples were then suspended in distilled water and the aqueous layer was extracted with the same volume of ethyl acetate to obtain 4-ASD. Analogs of 4-ASD and phloridzin, a known SGLT-1 blocker, were purchased from Sigma Chemical Co. (St. Louis, USA) (Fig. 1): 15-acetoxyscirpendiol (Natural Mycotoxin Registry Number: 2623-22-5) was named 15-AS, and diacetoxyscirpenol (Natural Mycotoxin Registry Number: 2270-40-8) as 2-AS. Phloridzin (Fig. 1B), a glucoside of the flavonoid-like polyphenol phloretin, is a known specific nontransportable inhibitor of SGLT-1.

Analysis of hSGLT-1 expression Following microinjection and incubation, SGLT-1 was extracted in phosphate-buffered saline (PBS) containing 0.2% mercaptoethanol using a Dounce homogenizer. The transmembranal feature of SGLT-1 was assayed by surface biotinylation as described by Lee *et al* (1998). Injected oocytes biotinylated with 1.0 mg/ml EZ-link-sulfo-NHS-LC-biotin (Pierce, Rockford, USA), and subsequently precipitated using Neutravidin-conjugated beads. Precipitated proteins were electrophoresed and detected by Western blotting using antisera against hSGLT-1 (Acris Antibodies, Hiddenhausen, Germany). In addition, hSGLT-1 expression was functionally analyzed by monitoring the entry of α-^{3}H-2-deoxy-D-glucose (2-DOG) into oocytes according to the procedure described in the following section.

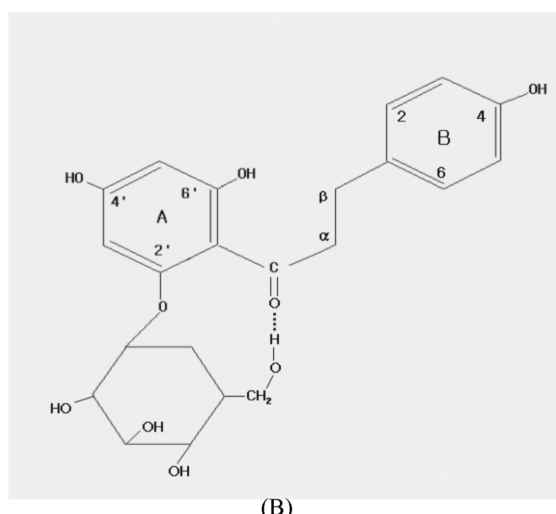
α-^{3}H-2-deoxy-D-glucose (2-DOG) uptake comparison and inhibition by 4-ASD SGLT-1 transport was measured using ^{3}H-labeled α-2-deoxy -D-glucose (2-DOG), a non-metabolized model substrate. 4-ASD inhibition of 2-deoxy-D-glucose (2-DOG) uptake was assayed by incubating 5 oocytes in 2 mM ^{3}H-2-DOG containing 4-ASD at concentrations ranging from 0 to 5mM in 1 mL of Barth solution. After a 10-minute incubation, oocytes were thoroughly washed with cold Barth solution. Glucose entry was analyzed using 3H-2-DOG (2 mCi/0.5 mL) after a 30-min influx period. 2-DOG influx was initiated by incubating five oocytes in 1 mL of Barth's solution containing 2.5 mCi of ^{3}H 2-DOG and cold 2-DOG at concentrations from 1 to 50 mM and at a constant osmolarity of 179.1 mOsm/L, which was achieved by adding a 1-M sucrose solution. Oocytes were then placed in a scintillation vial containing 0.5 mL of Barth's solution for 2 min. They were then transferred to another scintillation vial and 500 ul of 0.1% SDS was added to both of these vials and vortex mixed. NEN scintillation cocktail (DuPont NEN, Boston, USA, 5 mL) was added before counting.

Electrophysiological experiments Oocytes were subjected to electrophysiological experiments at 22-25°C using the two-microelectrode voltage-clamp method in a rapid perfusion chamber with a OC-725 voltage clamp amplifier (Warner Instrument, Hamden, USA). Oocytes were perfused in a solution containing (in mM) 88 NaCl, 2 KCl, 1.8 $CaCl_2$, and 10 HEPES-NaOH, pH 7.4. In the Na^+-free solution, the Na^+ was replaced with choline, and the pH was adjusted by adding a 1-M KOH to eqilibrium in equilibrating buffer. Electrodes were filled with 3-M KCl and the membrane potential was normally maintained at a holding potential of −50 mV. 4-ASD inhibition of the oocyte-expressed SGLT-1 was assayed after incubating oocytes in 4-ASD at concentrations ranging from 0 to 1 mM. Membrane voltage control and data collection were performed using pCLAMP 6 software (Axon Instruments).

Kinetic analysis and inhibition by 4-ASD and its derivatives To obtain Michaelis-Menten kinetics, oocytes were incubated with five different 2-DOG concentrations (5, 15, 30, 60, and 120 mM) for 10



(A)



(B)

Fig. 1. Structures of 4-ASD, 2-AS, 15-AS, and Phloridzin. (A) 4-ASD has five functional groups: R_1 (OH), R_2 (OAc), R_3 (H), R_4 (H) and R_5 (H). 2-AS has an additional -OAc at R_5 instead of (H). In 15-AS, the R_2 and R_5 positions are reversed as compared to 4-beta acetoxyscirpendiol; and thus, its R_2 and R_5 positions are -H and -OAc, respectively. (B) Phloridzin is the best known inhibitor of SGLT-1.

h. They were then subjected to zero trans and equilibrium assays (Due *et al*., 1995). Zero trans influx was analyzed using 2-DOG (2 mCi/0.5 ml) and a 30-min influx period. The influx of 2-DOG was initiated by incubating five oocytes in 1 mL of Barth's solution containing 2.5 mCi of ^{3}H-2-DOG and cold 2-DOG at concentrations of 1 to 50 mM, at a constant osmolarity of 179.1 mOsm/L, which was achieved by adding a 1-M sucrose solution. The oocytes were then transferred to a scintillation vial containing 0.5 mL of Barth's solution. Control experiments were undertaken on oocytes injected with water under identical conditions, and control transport rates were subtracted from the transport rates of oocytes expressing SGLT-1. Equilibrium-exchange influx of 3-OMG into *X. laevis* oocytes was performed after an overnight incubation at 18°C in 1 mL of Barth's solution containing cold 2-DOG at concentrations of 1 to 50 mM. Osmolarity was also maintained at 179.1 mOsm/L by adding 1-M sucrose. K_m and V_{max} were calculated using GRAPHPAD PRISM™ software (GraphPad Software, San Diego, USA).

Results

This study assayed whether 4-ASD, isolated from *P. tenuipes*, and its derivatives, 2-AS and 15-AS, serve as the functional blood-sugar content lowering components. The extent of glucose transport inhibition by 4-ASD and its derivatives was measured in the *X. laevis* oocyte system expressing significant amounts of membrane-bound SGLT-1. Following the injection of the cRNA of human SGLT-1, expressed proteins were initially precipitated by avidin-conjugated agarose and detected by immunoblotting using anti-hSGLT1, and hSGLT-1 was detected in the pool of biotinylated proteins (Fig. 2A). The molecular size detected corresponded to the known size of SGLT-1. No hSGLT-1 was detected in oocytes injected with water. Fig. 2B shows that oocytes expressing SGLT-1 showed a significant increase in the uptake of 2-DOG. In terms of the amount of mRNA injected, degrees of uptake by oocytes were

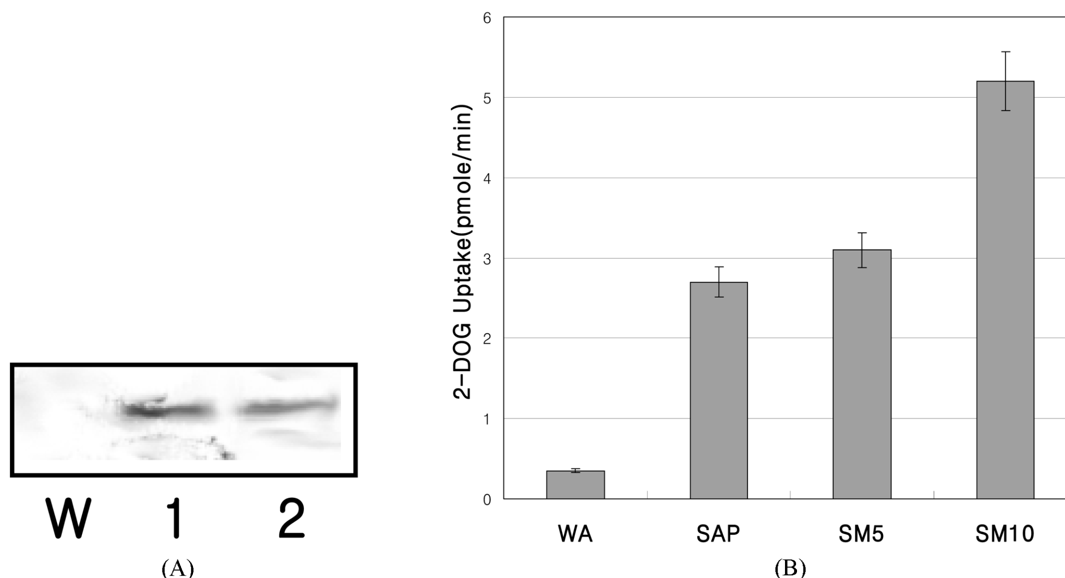


Fig. 2. Functional expression of SGLT-1 in *Xenopus* oocyte. (A) Expression of SGLT-1 in *Xenopus* oocytes. Membrane fractions were prepared from oocytes injected with the cRNA of human SGLT-1. Proteins were precipitated and detected by immunoblotting for hSGLT-1. Lane W represents the result of a water injected oocyte. Lane 1 shows proteins precipitated by avidin-conjugated agarose, whereas lane 2 shows proteins immunoprecipitated by anti-hSGLT1 antisera. (B) Comparison of 2-DOG oocyte uptakes. 2-DOG uptake efficiency was compared for oocytes treated by injecting with water (WA) or with SGLT-1 mRNA (SM). SM5 and SM10 refer to oocytes injected with 5 ng or 10 ng of SGLT-1 mRNA, respectively. Following injection, oocytes were incubated with 5 mM 2-DOG for 6 hours. 2-DOG entry into oocytes was measured after copiously washing oocytes.

evidently different. Oocytes injected with 10 ng of SGLT-1 mRNA showed higher uptake activity than those injected with 5 ng of SGLT-1 mRNA. These results are consistent with those of previous studies, i.e., that the amount of mRNA injected into oocytes is correlated with glucose-transporter protein expression and the transport rates of SGLT-1 (Mandal *et al*., 2003).

Electrophysiological experiments were performed to determine whether 4-ASD affects the activity of SGLT-1 expressed in oocytes. Fig. 3 shows that hSGLT-1 expressing *Xenopus* oocytes show substantial Na^+-driven current increases after the addition of 100 mM D-glucose. However, when pre-incubated with 5 mM phloridzin, a known SGLT blocker, oocytes did not respond to the addition of glucose (Fig. 3A). Moreover, the addition of 0.5 mM 4-ASD, significantly attenuated the Na^+ inward current, as was observed for phloridzin (Fig. 3B). However, when 4-ASD was removed by washing oocytes with perfusion media currents were restored to the 4-ASD-free level, which indicates that the inhibitory effect of 4-ASD is reversible.

The effect of 4-ASD on SGLT-1 was measured in the presence of 0.5-mM 2-DOG (Fig. 4). At the fixed concentration of 2-DOG, SGLT-1 activity was significantly reduced, and the fractional uptake rate (v/v_o = inhibited/non-inhibited uptake rate) was found to have reduced in a dose-dependent manner. In this case, as a non-inhibited control we used SGLT-1-expressing oocytes that had not been treated with 4-ASD. The

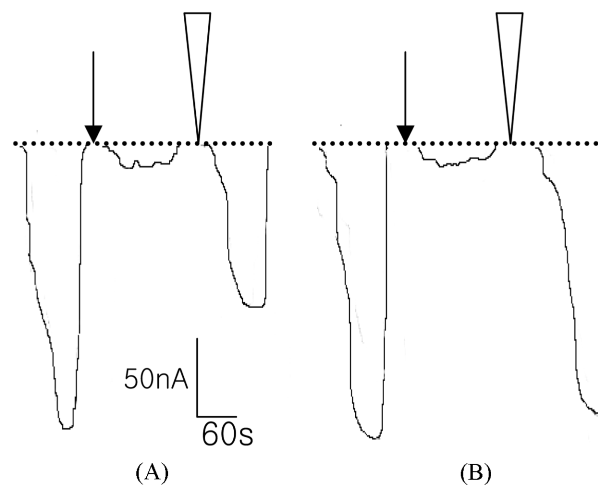


Fig. 3. Inhibition of glucose-induced current by 4-ASD in a single oocyte expressing hSGLT-1. An oocyte expressing hSGLT-1 examined electrophysiologically at a membrane potential of −50 mV. (A) After adding 100 mM glucose to the bath solution (arrow), oocytes showed a Na^+-inward current of −150 nA. However, treatment with 0.5 mM phloridzin for 1 min, inhibited oocyte current even after adding 100 mM of glucose to the bath solution. (B) When incubated with 0.5 mM 4-ASD, the oocytes showed major reduction in Na^+-induced current similarly as (A). When the oocyte was washed with perfusion media (downward wedge), oocyte current returned to its previous level (~120 nA).

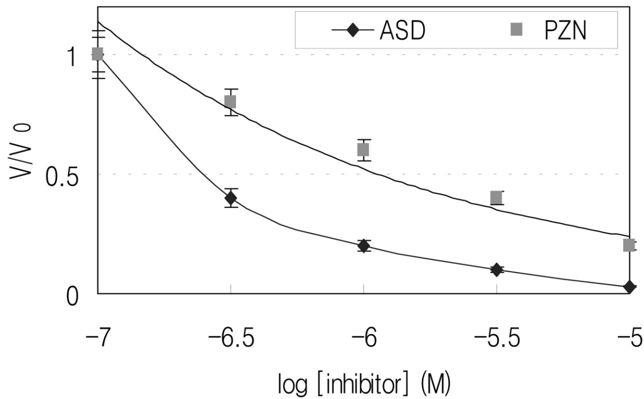


Fig. 4. Effect of 4-ASD on 2-DOG uptake by SGLT-1. The effects of 4-ASD analogues on SGLT-1 were measured in the presence of 0.5 mM 2-DOG. *Xenopus* oocytes expressing SGLT-1 were incubated with 4-ASD or phloridzin (PZN). V/V_0 values were calculated as fractional value of inhibited and non-inhibited uptake rates. Error bars refer to S.E. (n = 5) Ed- rephrase

transport of 2-DOG into oocytes expressing SGLT-1 was found to be consistently reduced to the levels surpassing that of phosphoridzin in the presence of 4-ASD ranging 1-10 µM.

The inhibitory effect of 4-ASD on SGLT-1 was further analyzed. The functional characteristics of 4-ASD inhibition on SGLT-1 activity were studied by analyzing the effect of 4-ASD on the kinetics of glucose inward transport under two conditions. Fig. 5A shows that the apparent half-saturation rate constant and the maximal velocity of zero-trans 2-DOG uptake decreased in the presence of 4-ASD. A reduction in the half-saturation-rate constant by 4-ASD was further supported by the results of an equilibrium-exchange experiment using 3-OMG as a glucose analog (Fig. 5B). Unlike 2-DOG, 3-OMG transported through SGLT-1 is not phosphorylated following influx into the cell. Due to severe inhibition of the SGLT-1's catalytic activity by 4-ASD, accumulation of 3-OMG at a 100 mM 3-OMG equilibrium concentration becomes significantly attenuated versus the 4-ASD-free control. Considering the Michaelis-Menten constants of SGLT-1, which are consistent with previous reports (Murata *et al.*, 2002), the values of K_m and V_{max} obtained by equilibrium exchange and zero-trans influx experiments appear genuine.

We also investigated whether 4-ASD analogs affect SGLT-1 activity like 4-ASD (Fig. 5). SGLT-1 activity appeared to be affected by all the 4-ASD analogs tested, though 15-AS showed the highest level of inhibition. This result suggests that futher chemical library screening be conducted to identify potentially useful inhibitory agents.

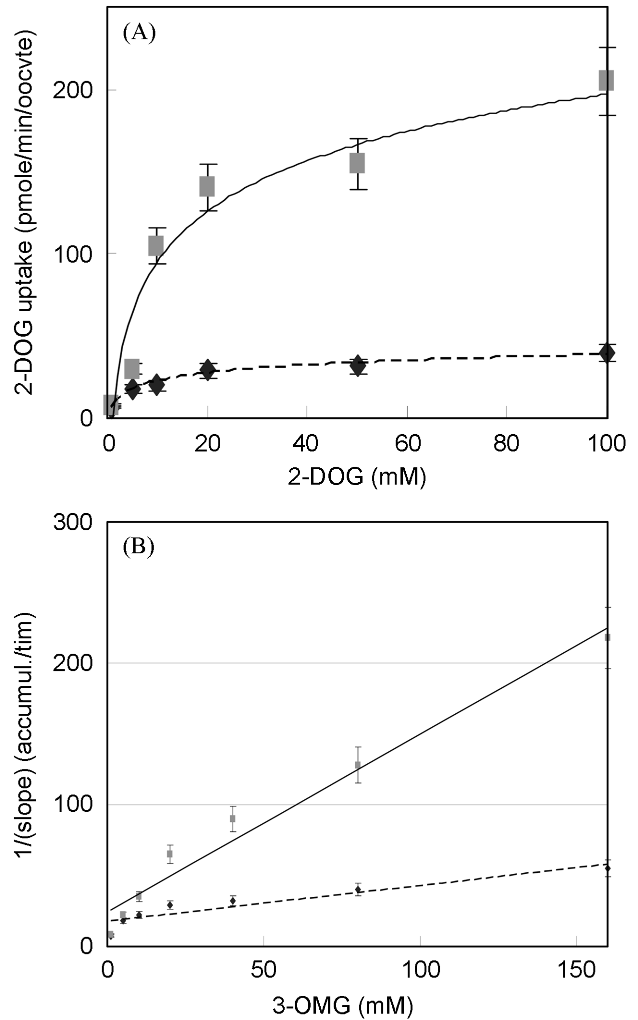


Fig. 5. Kinetic analysis of 4-ASD inhibition on 2-DOG or 3-OMG uptake. (A) Zero-trans influx of 2-DOG at the indicated concentrations was determined in oocytes expressing SGLT-1 in the absence or presence of 5 µM 4-ASD (Michaelis-Menten graphs). Closed squares (■) refer to the 4-ASD-free treatment while closed diamonds (◆) refer to 4-ASD treatment. Values were standardized by subtracting values of non-injected oocytes. Each value represents the mean value of 10 oocytes and is corrected for 2-DOG uptake in non-injected *Xenopus* oocytes (4-ASD treatment: K_m = 2.9 mM V_{max} = 24 pmole/min/oocyte; ASD-free treatment: K_m = 8.5 mM V_{max} = 157 pmole/min/oocyte). (B) Equilibrium exchange influx kinetic values were determined at the 3-OMG equilibrium concentrations indicated. As in Fig. 5A, closed squares (■) refer to 4-ASD-free treatment and closed diamonds (◆) to 4-ASD treatment. 3-OMG accumulation was monitored for 1 h and is expressed on a logarithmic scale. 10 oocytes were employed per group at each time point. Negative reciprocals of the slopes were plotted against 3-OMG concentrations to obtain the Hanes plot (Due *et al.*, 1995). On the y-axis, 1/(slope) refers to the reciprocal of each absolute value (4-ASD-free treatment: K_m = 22.0 mM V_{max} = 198+13; 4-ASD-treatment: K_m = 3.8+0.9 V_{max} = 39+5). Error bars refer to S.E. (n = 5).

Discussion

In Eastern Asia, cordyceps is traditionally administered to prevent or cure diabetes mellitus. However, to date, the

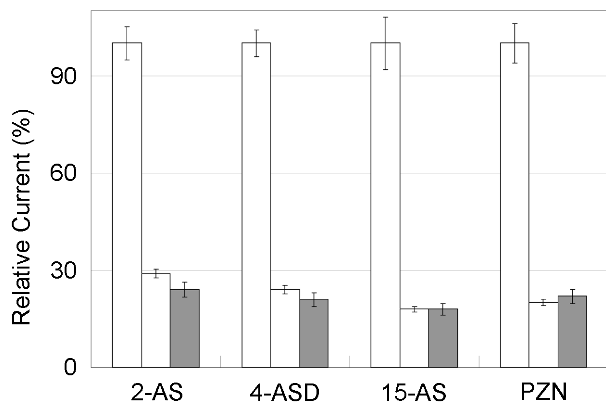


Fig. 6. Effects of 4-ASD analogs on SGLT-1. The effects of 4-ASD analogs and phloridzin (PZN) on SGLT-1 were assayed by voltage clamping. The effects of 4-ASD analogs on SGLT-1 were measured in the presence of 50 mM glucose. Measurements are expressed as relative values taking the 4-ASD-free value to be 100%. Each column represents mean+S.E. (n = 5). Light bar refers to the non-treated control. Striated and dark bars refer to the treatment with 0.2 mM and 1 mM of each inhibitor, respectively.

cordyceps components responsible for this hypoglycemic effect have not been identified. In the present study, the *Xenopus-laevis*-oocyte expression system was utilized to assay the transport of the glucose analogs 2-deoxy-D-glucose and 3-OMG to characterize electrophysiologically the glucose-transport properties of SGLT-1 in the presence of ASD. The results demonstrate that 4-ASD significantly inhibits the activity of SGLT-1. Moreover, the hypoglycemic effect of cordyceps, can be explained by a direct interaction between 4-ASD and SGLT-1. In addition, *P. tenuipes* may serve as a promising natural source of biologically active substances in addition to 4-ASD.

SGLT-1 expressing *Xenopus laevis* oocytes demonstrated that 4-ASD significantly reduces 2-DOG inward transport in a dose-dependent manner. This result suggests that 4-ASD and its derivatives act as SGLT inhibitors. When treating diabetes, the important alternative routes of glucose disposal are dependent of the activities of SGLT inhibitors. *Xenopus laevis* oocytes showed significant reductions in transport across *Xenopus*-oocyte membranes expressing high amounts of SGLT-1.

Kinetic analysis indicated that 4-ASD treatment reduces K_m and V_{max}. This demonstrates that its mode of inhibition may be comparable to that of phloridzin, a known SGLT-1 inhibitor. According to the results of kinetic analysis, normalized V_{max} in the presence of 4-ASD and phloridzin was significantly lower than that in the presence of SGLT-1 under 4-ASD-free conditions. This reduction in transport activity was probably due to different binding affinities to SGLT-1 between the two inhibitors, because K_m values were significantly affected. Thus, it is likely that 4-ASD affects one or more steps in the transport mechanism at the immediate stage of substrate binding. Moreover, 4-ASD inhibition of 3-OMG uptake showed more than a 6-fold reduction in apparent affinity. This reduced affinity was reflected by a change in the K_m of 3-OMG. Taken together, the results of the kinetic study indicate that 4-ASD is involved even in the equilibrium binding of glucose to glucose transporter. This result strongly indicates that inhibition of SGLT-1 by 4-ASD is directly targeted at SGLT-1 rather than at signaling pathways leading to glucose uptake and metabolism.

4-ASD structural derivatives inhibited glucose influx via hSGLT-1 in a manner comparable to that of 4-ASD. Of the 4-ASD analogs examined 15-AS showed the highest degree of inhibition. Moreover, these analogs are easily synthesized in quantity, and could potentially be used as drug to treat postprandial glucose surge. To date, the specific nontransportable inhibitors of SGLT1 are the glucosides of flavonoid-like polyphenols, like phloridzin. The present study shows that 4-ASD and its analogs are potential inhibitors of SGLT-1. As the inhibitory steps involving glucosides are well understood (Oulianova *et al.*, 2001), inhibition by 4-ASD, despite the chemical structural differences of the 4-ASD analogues, strongly implies that additional inhibitory pathways reduce the overall performance of SGLTs. Further *in vivo* study of SGLT family gene expression and of the biochemical pathways affecting by the presence of 4-ASD may be necessary, since the present study used *Xenopus* oocytes which heterologously express SGLT-1.

4-ASD has five functional groups of R_1 (OH), R_2 (OAc), R_3 (H), R_4 (H) and R_5 (H). The 2-AS has an additional -OAc at R5 instead of (H). In 15-AS the 4-ASD R_2 and R_5 positions are switched; i.e., R2 and R5 are -H and -OAc. As shown in Fig. 5, 15-ASD had the highest inhibitory effect. Moreover, 4-ASD and 15-AS share similar structural features except for the -H and -OAc exchange. This structural alteration could explain the observed inhibitory effect difference but further study, using various analogs, should be carried out to finalize identify the case of the difference.

In conclusion, the results of the present study show that 4-ASD can prevent insulin-stimulated glucose uptake in *Xenopus* oocytes expressing SGLT-1. This inhibitory effect is not due to a non-specific effect on insulin-signaling pathways; rather, it is due to the direct inhibition of glucose transporters. In addition, 4-ASD does not participate in glucose metabolism, but rather it interferes with the translocation of glucose transporters. The results of the present study suggest that 4-ASD, also called trichothecene mycotoxin, which is present as an active principle in *Paecilomyces tenuipes*, should be considered as a potential treatment for diabetes.

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