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ORIGINAL ARTICLE

Screening and evaluation of new inhibitors of hepatic glucose production

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In the course of our screening program for inhibitors of hepatic glucose production in rat hepatoma H4IIE-C3 cells, which were used as model liver cells, five naphtoquinone derivatives—javanicin, solaniol, 9-*O*-methylfusarubin, 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-naphtho[2,3-c]pyran-6,9-dione, 9-*O*-methylbostrycoidin—and vanillin were selected from our natural product library. These naphtoquinone derivatives inhibited hepatic glucose production at IC_{50} values of 3.8–29 μM, but showed cytotoxicity against hepatic cells after incubation for 48 h. However, vanillin showed an IC_{50} value of 32 μM without exhibiting cytotoxicity at 50 μM. Therefore, we examined 12 vanillin derivatives to investigate their inhibitory activities against glucose production. Among these analogs, 4-hydro-3-methoxyacetophenone and 5-nitrosalicylaldehyde exhibited stronger inhibition than the other compounds at IC_{50} values of 25 and 24 μM, respectively, with no cytotoxicity at a concentration of 50 μM. Hence, 4-hydro-3-methoxyacetophenone and 5-nitrosalicylaldehyde may be useful as a lead compound of anti-type 2 diabetic drugs.
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Type-2 diabetes is a severe disease that is prevalent in the developed world, and the incidence of diabetes is increasing even in developing countries. Type-2 diabetes is associated with impaired glucose clearance by the liver with elevated glucose production in the postprandial state and is characterized by insulin resistance.[1] Rigid control of plasma glucose levels is known to decrease the incidence and progression of diabetic complications. The liver maintains blood glucose homeostasis by the absorption of glucose in the postprandial state and by the production of glucose from glycogenolysis and gluconeogenesis in the postabsorptive state. In normal tissues, insulin stimulates glucose uptake by muscles and adipose tissue, and inhibits hepatic glucose production. However, this response to insulin is defective in patients with type-2 diabetes, resulting in long-term hyperglycemia. Therefore, the key to treating diabetes is to reverse or bypass the deficient insulin-signaling pathway in patients with type-2 diabetes. Agents that can inhibit hepatic glucose production through a signaling pathway that is distinct from the insulin-signaling pathway may provide new avenues for inhibiting the elevated glucose production caused by insulin resistance in the case of type-2 diabetes.

Several popular classes of oral therapeutic drugs for diabetes that are available in the market (for example, sulphonylureas and peroxisome proliferator-activated receptor-γ agonists) lower plasma glucose levels by increasing glucose metabolism either through enhanced insulin secretion or improved sensitivity to insulin. Metformin is the only drug that acts primarily by reducing endogenous glucose production without exhibiting a major effect on insulin signaling.[2] Metformin lowers glucose levels without resulting in weight gain. Thus, it is often used to treat patients with type-2 diabetes despite the well-known safety concerns in certain diabetic patient populations and the overall high incidence of gastrointestinal intolerance associated with its use. Hence, agents such as metformin are expected to inhibit hepatic glucose production. We screened inhibitors from our natural product library for their effects on glucose production in the rat liver cell line H4IIE-C3 and selected five naphtoquinone derivatives and vanillin as inhibitors. Further, we also evaluated commercially available and synthesized vanillin derivatives for their inhibitory effects on glucose production.

MATERIALS AND METHODS

General experimental procedures

UV and IR spectra were measured on a DU730 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and an FT-720 spectrophotometer (Horiba, Kyoto, Japan), respectively. High-resolution electrospray ionization-mass spectrometric (HR-ESI-MS) data were recorded on an LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA). NMR spectra were measured on an NMR System 500 NB CL (Varian, Palo Alto, CA, USA) in $CDCl_3$ with the residual solvent

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peak as an internal standard (δ_H 7.24 p.p.m.). Normal-phase medium-pressure liquid chromatography (MPLC) was performed using a Purif-pack SI-60 column (Moritex, Tokyo, Japan). Analytical reverse-phase HPLC was performed on an L-column2 ODS column (4.6 i.d.×150 mm, Chemical Evaluation and Research Institute, Tokyo, Japan) with a 2996 photodiode array detector (Waters) and a 3100 mass detector (Waters). Preparative reverse-phase HPLC was performed on an L-column2 ODS (20 i.d.×150 mm) with an L-2455 photodiode array detector (Hitachi High Technologies, Tokyo, Japan). The reagents and solvents used in our study were of the highest grade available.

Cell culture

Rat hepatoma cell line H4IIE-C3 was used in this study. The cells were grown at 37 °C in an atmosphere containing 5% CO_2 in Minimum Essential Medium Eagle (Sigma, Saint Louis, MO, USA). This medium consisted of 10% fetal bovine serum (Sigma), a 100-μM solution of non-essential amino acid (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate solution (Invitrogen), supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}).

Assay of glucose production in hepatic cells

For performing the screening, H4IIE-C3 cells were inoculated onto a 384-well plate (Cat. No.781182, Greiner Bio-one, Frickenhausen, Germany) at a density of 1.2×10^4 cells per well and incubated overnight in the serum-containing medium. They were then transferred to a serum-free medium and incubated overnight. Next, the medium was replaced with a glucose-free medium—20 μl of Dulbecco's Modified Eagle's Base—which did not contain L-glutamine, glucose, phenol red, sodium pyruvate and sodium bicarbonate (Sigma), supplemented with 3.7 mg l^{-1} sodium bicarbonate and 20 mM pyruvic acid. The samples (0.2 μl) to be screened were then added to the medium by using Multi-dispenser ADS-384–8 (BioTec, Tokyo, Japan) and incubated for 5 h. Subsequently, 10 μl of the medium was transferred to 384-well black plates (Cat. No.784900, Greiner) by using ADS-384–8, and the amount of glucose accumulated in the wells was detected by mixing the contents of the well with 10 μl of Amplex Red Glucose Assay kit solution (Invitrogen).

To confirm the activities of the hit samples, the cells were inoculated into a 96-well plate (Cat. No.353072; BD Biosciences, San Jose, CA, USA) at a density of 6×10^4 cells per 100 μl for each well and incubated as described above. After treatment with the hit samples at various concentrations, 40 μl of the medium was transferred to a 96-well black plate (Cat. No. 3916; Corning Inc., Corning, NY, USA), and the amount of glucose accumulated was determined by mixing 40 μl of the Amplex Red Glucose Assay kit solution.

Cytotoxicity assay

Cytotoxic activities were evaluated by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) colorimetric assay. The 96-well plate was inoculated with H4IIE-C3 cells at a density of 1×10^4 cells per well; these were subsequently treated with compounds at various concentrations and incubated for 48 h. Next, 10 μl of the WST-8 reagent (Cell Counting Kit; Dojindo, Kumamoto, Japan) was added and the cells were incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. Absorbance of the formazan dye formed was measured at 450 nm.

Natural product library

Our in-house natural product library was used as the source of samples for screening. The library contained 40 508 diverse samples of crude metabolites of actinomycetes (20 608 samples), bacteria (6336 samples) other than actinomycetes and fungi (12 672 samples). It also included plant extracts (252 samples) and metabolites isolated from microorganisms (640 samples). All the samples were dissolved in dimethyl sulfoxide.

Compounds

Javanicin[3] (**1**) and solaniol[4] (**2**) were purified from the culture of *Fusarium* sp. no. 351386, which was isolated from a soil sample collected from Tanzawa, Kanagawa Prefecture, Japan, with MPLC and HPLC by activity-guided separation.

9-*O*-Methylfusarubin[5] (**3**), 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-naphtho[2,3-c]pyran-6,9-dione[6] (**4**), 9-*O*-methylbostrycoidin[5] (**5**) and vanillin (**6**) were selected as hit samples from our purified natural product library. These samples were of fungal origin.

Isovanillin (3-hydroxy-4-methoxybenzaldehyde) (**8**), 4-hydroxy-3-methoxyacetophenone (**9**), 4-hydroxy-3,5-dimethoxyacetophenone (**10**), 5-chlorosalicylaldehyde (**12**), 5-nitrosalicylaldehyde (**13**) and 2,4-dihydroxybenzaldehyde (**14**) were purchased from Tokyo Chemical Industry (Tokyo, Japan); vanillic acid (**7**) and 5-bromosalicylaldehyde (**11**) were purchased from Wako Pure Chemical (Osaka, Japan); 2,4,6-trihydroxybenzaldehyde (**15**) was purchased from Aldrich (Saint Louis, MO, USA).

Hydroxy-4,6-dimethoxybenzaldehyde (16)

Oxalyl chloride (16.0 ml, 183 mmol, 2.00 equiv.) in CH_2Cl_2 (44.0 ml) was added dropwise to a solution of *N*,*N*-dimethylformamide (DMF) (18.9 ml, 244 mmol, 2.60 equiv.) in CH_2Cl_2 (90 ml) at room temperature under argon. After the reaction mixture was stirred at the same temperature for 1 h, it was cooled to −15 °C. A solution of 3,5-dimethoxyphenol (14.5 g, 93.8 mmol, 1.00 equiv.) in CH_2Cl_2 (58 ml) was added dropwise to the above reaction mixture for over 45 min. This mixture was stirred at −15 °C for 30 min and then at room temperature for 30 min. Next, 1 M aqueous HCl (130 ml) was added at 0 °C. The mixture was heated to 40 °C after stirring for 2 h at room temperature. After 3 h of stirring at the same temperature, the reaction mixture was added to a mixture of brine and EtOAc at 0 °C. The aqueous layer was extracted with two portions of EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was re-crystallized from CH_2Cl_2-hexane to afford **16** as a white solid.[7]

Thin-layer chromatography, R*f*=0.57 (hexane/ethyl acetate=1/1); ^{1}H NMR ($CDCl_3$) 12.5 (s, 1H), 10.1 (s, 1H), 6.00 (d, *J*=2.2 Hz, 1H), 5.91 (d, *J*=2.2Hz, 1H), 3.84 (s, 3H), 3.83 (s, 3H); ^{13}C NMR ($CDCl_3$) 191.9, 168.2, 166.4, 163.6, 106.1, 93.0, 90.6, 55.8; FT-IR (solid) 2984, 1624, 1498, 1475, 1211, 1157, 1111, 1045 cm^{-1}.

4,6-Bis(benzyloxy)-2-hydroxybenzaldehyde (17)

K_2CO_3 (9.10 g, 65.8 mmol, 2.40 equiv.) was added to a solution of 2,4,6-trihydroxybenzaldehyde (5.30 g, 27.9 mmol, 1.00 equiv.) in DMF (70 ml) at room temperature under argon. The mixture was stirred at the same temperature for 5 min, after which benzyl bromide (6.64 g, 55.8 mmol, 2.00 equiv.) was added to it. After stirring for 5 h, the reaction mixture was added to a mixture of 1 M aqueous HCl and EtOAc at 0 °C. The aqueous layer was extracted with two portions of EtOAc. The combined organic layers were washed with three portions of H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was re-crystallized from CH_2Cl_2-hexane to afford **17** as a white solid.[8]

RESULTS AND DISCUSSION

Screening for inhibitors of glucose production in hepatic cells

We have developed a cell-based assay system for detecting hepatic glucose production by using the rat hepatoma cell line H4IIE-C3, which mimics *in vivo* liver function.[9,10] In this system, after culturing overnight in the serum-free medium, the cells were incubated in a glucose-free medium for 5 h and the amount of glucose accumulated in the medium was detected by glucose oxidase reaction. Glucose oxidase catalyzes the formation of D-gluconolactone and H_2O_2 from D-glucose; and H_2O_2 reacts with the Amplex Red reagent in the presence of horseradish peroxidase to generate a red fluorescent oxidation product, resorufin. Resorufin was quantified using a fluorescence microplate reader (Figure 1a). In this system, H4IIE-C3 cells produced glucose, which was accumulated in the medium at a concentration of 25 μM. When metformin was added into the glucose-free medium as a positive control for inhibition of hepatic glucose production, metformin inhibited the production of glucose in the medium with an IC_{50} value of 1.7 mM without cytotoxicity (IC_{50} >100 mM) (Figure 1b). This result suggests that this system is effective for screening inhibitors of glucose production.

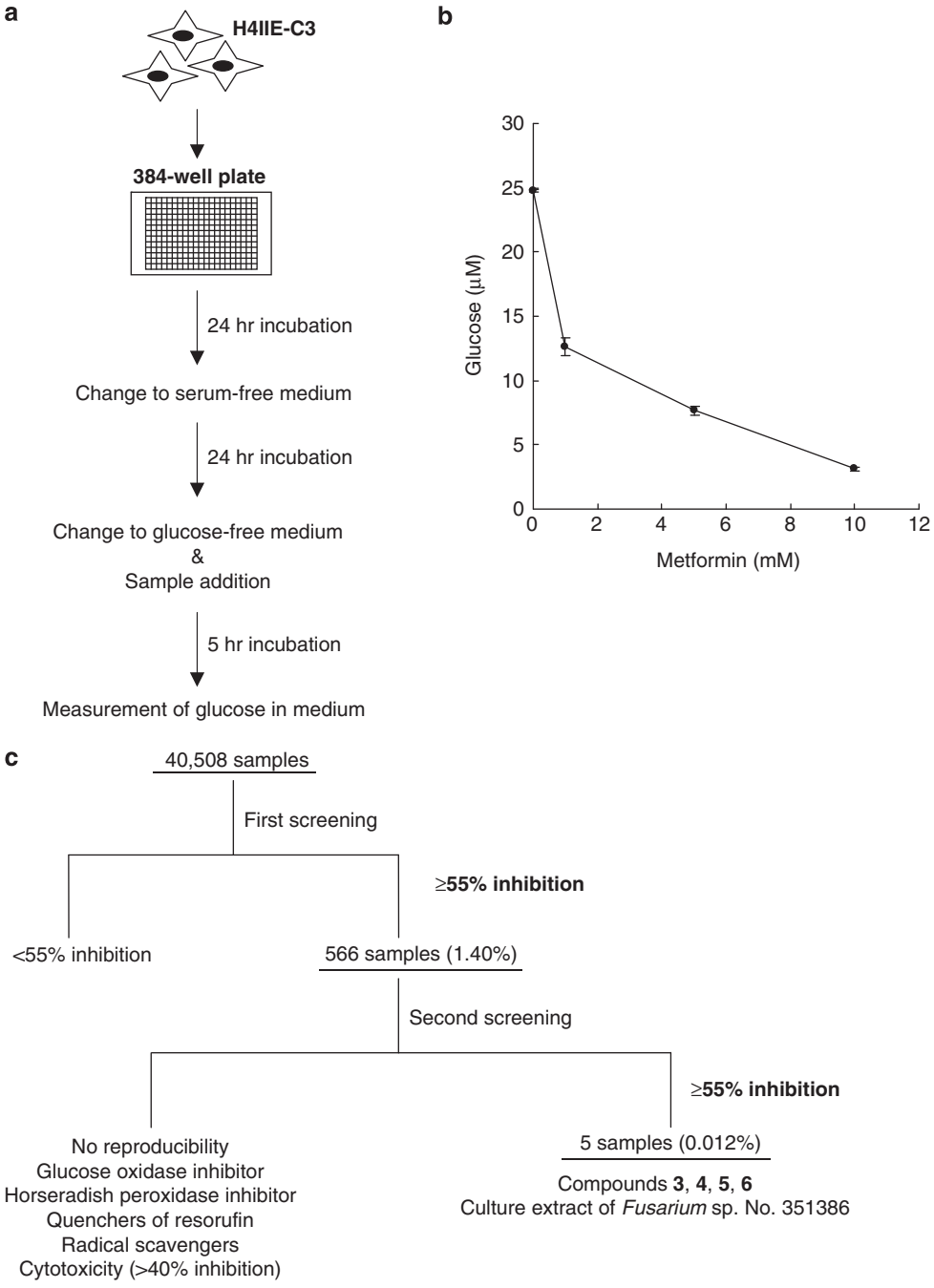


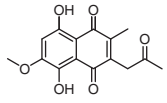
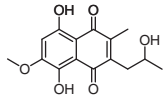
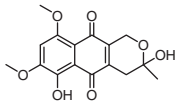
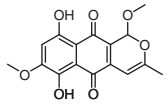
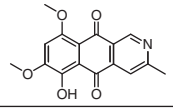
Figure 1 Assay system and the screening of inhibitors of hepatic glucose production. (**a**) Flow chart of the assay system of glucose production in rat liver cells. (**b**) Inhibitory effect of metformin on glucose production. (**c**) Use of this system in screening inhibitors of glucose production from the natural product library.

In the screening process, samples to be screened were added in the glucose-free medium. The samples that showed ⩾55% inhibition of glucose production compared with the glucose production in the medium to which samples were not added were selected as inhibitors of glucose production. A total of 40 508 natural product samples were used for the primary screening. We screened all the samples at a final concentration of 1% in a volume of 20 μl per well using a 384-well format. The first screenings yielded 566 hit samples (1.40%). Among the 566 hit samples, some of them probably behaved as inhibitors of glucose oxidase and horseradish peroxidase in the glucose assay system, some acted as quenchers of resorufin, and some as scavengers of H_2O_2. Hence, we excluded samples that caused a decrease in the fluorescence intensity when 50 μM of glucose solution was separately added into the same assay system (fluorescence intensity, <70%). Furthermore, we excluded samples that showed cytotoxicity during 5 h of incubation (cytotoxicity, >40%). After performing additional reproducible and dose-dependent tests, we finally selected five samples (0.012%) as inhibitors of glucose production. Four of these were **3**[5], **4**[6], **5**[5] and **6**, which were selected from the library of isolated microbial metabolites. The remaining sample was obtained from the culture extract of *Fusarium* sp. no. 351386. Therefore, we cultured this fungus in fermentation media and isolated two active compounds—**1**[3] and **2**[4]—from its culture extract.

Identification of 1 and 2

The structures of the active compounds, **1** and **2**, were determined by HR-ESI-MS, UV and ^{1}H NMR spectra as follows: Javanicin, ^{1}H NMR ($CDCl_3$): δ 13.24. (s, 1H), 12.84 (s, 1H), 6.19 (s, 1H), 3.92 (s, 3H), 3.89 (s, 2H), 2.28 (s, 3H), 2.22 (s, 3H). UV λ_{max} 536, 501, 472, 303; HR-MS *m/z* 291.0868 (calcd for $C_{15}H_{15}O_6$, $[M+H]^+$ 291.0869). Solaniol, ^{1}H NMR δ ($CDCl_3$): 13.31 (s, 1H), 13.04 (s, 1H), 6.19 (s, 1H), 4.11 (m, 1H), 3.91 (s, 3H), 2.93 (d, 2H, *J*=5.5 Hz), 2.33 (s, 3H), 1.30 (d, 3H, *J*=6.0 Hz). UV λ_{max} 540, 504, 475, 307; HR-MS *m/z* 293.1052 (calcd for $C_{15}H_{17}O_6$, $[M+H]^+$ 293.1025).

Table 1 Structures and IC_{50} values for anti-glucose production activity and cytotoxicity of 1–5

Compound	*Structure*	*Glucose production (μM)*	*Cytotoxicity (μM)*
1		3.8	3.3
2		4.4	9.5
3		4.8	2.5
4		6.3	8.8
5		30	>50[a]

[a]**5** showed 30% inhibition of cell growth at a concentration of 50 μM.

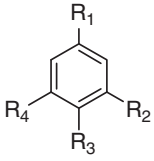
Inhibition of hepatic glucose production by naphthoquinone derivatives

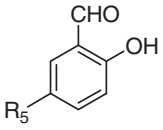
We tested the activities of five naphthoquinone derivatives with regard to inhibition of hepatic glucose production. The structures of **3–5** revealed that they were derivatives of **1** and **2**, as shown in Table 1. All these compounds exhibited inhibitory effects on glucose production. Thus, the naphthoquinone moiety, common to all these compounds, may be essential for their inhibitory activities. The compounds **1–4** showed similar degrees of inhibition (IC_{50}=3.8–6.8 μM); however, the inhibitory activity of **5** (IC_{50}=30 μM) was approximately sixfold lower than the inhibitory activities of **1–4**. Therefore, the pyridine moiety of **5** may have a significant role in inhibiting glucose production. In addition, these compounds, except **5**, exhibited cytotoxic effects (IC_{50}=3.3–8.8 μM) on H4IIE-C3 cells during 48 h of incubation, and **5** resulted in 30% inhibition of cell growth at a concentration of 50 μM. Hence, the IC_{50} values for the cytotoxic activity of these compounds were comparable with those of the inhibitory activity against hepatic glucose production (Table 1). On the basis of these results, it was concluded that these compounds are unsuitable candidates for use in anti-diabetic drugs.

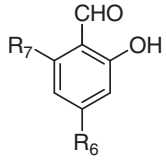
Inhibition of hepatic glucose production by vanillin derivatives

Additional screening showed that **6** from our library was also an inhibitor of glucose production. To examine structure–activity relationship, we compared the inhibitory effects of **6** derivatives **7–17** on glucose production (Table 2). All **6** derivatives inhibited hepatic glucose production and their IC_{50} values (IC_{50}=24–190 μM) were lower than that of metformin (IC_{50}=1.7 mM). In particular, **9** and **13** showed strong inhibitory activities. Moreover, these compounds did not exhibit cytotoxic effects on H4IIE-C3 cells at 50 μM after 48 h of incubation. Thus, **6** derivatives can be considered as suitable

Table 2 Structures of vanillin analogs







R_1	R_2	R_3	R_4	
CHO	OCH_3	OH	H	vanillin (4-hydroxy-3-methoxybenzaldehyde) (**6**)
COOH	OCH_3	OH	H	vanillic acid (4-hydroxy-3-methoxybenzoic acid) (**7**)
CHO	OH	OCH_3	H	isovanillin (3-hydroxy-4-methoxybenzaldehyde) (**8**)
$COCH_3$	OCH_3	OH	H	4-hydroxy-3-methoxyacetophenone (**9**)
$COCH_3$	OCH_3	OH	OCH_3	4-hydroxy-3,5-dimethoxyacetophenone (**10**)

R_5		R_6	R_7	
Br	5-bromosalicylaldehyde (**11**)	OH	H	2,4-dihydroxybenzaldehyde (**14**)
Cl	5-chlorosalicylaldehyde (**12**)	OH	OH	2,4,6-trihydroxybenzaldehyde (**15**)
NO_2	5-nitrosalicylaldehyde (**13**)	OCH_3	OCH_3	2-hydroxy-4,6-dimethoxybenzaldehyde (**16**)
		OBn	OBn	4,6-bis(benzyloxy)-2-hydroxybenzaldehyde (**17**)

Table 3 IC_{50} values for anti-glucose production activity of 6 to 17

Compound	*Glucose production (μM)*
6	32
7	190
8	75
9	25
10	39
11	31
12	49
13	24
14	43
15	61
16	49
17	59

candidates for use in anti-diabetic drugs, as opposed to the naphthoquinone derivatives examined here.

The inhibitory activity of **7** (IC_{50}=190 μM) against glucose production was weaker than that of **6** (IC_{50}=32 μM), **8** (IC_{50}=75 μM), **9** (IC_{50}=25 μM) and **10** (IC_{50}=39 μM) (Table 3). These results suggest that the absence of a carboxyl group in their structure was important for the inhibitory activity. On comparing the inhibitory activities of 5-halogenated salicylaldehydes (**11**, **12**) and 5-nitrosalicylaldehyde (**13**), it was found that **13**, which contains a 5-nitro group, showed the strongest inhibitory activity at an IC_{50} value of 24 μM. The activities of **14**, **15**, **16** and **17** were lower than those of **6**, **11** and **13**. Accordingly, in the case of benzaldehyde, 4-hydroxy-3-methoxy or 2,5-disubstituted moieties may have important roles in the inhibitory activities of these compounds against glucose production.

In this study, we developed a screening system for inhibitors of glucose production. Using the rat hepatoma cell line H4IIE-C3, we screened a total of 40 508 samples and evaluated 12 vanillin derivatives. We found that **9** or **13** inhibits glucose production in hepatic cells without exhibiting cytotoxic effects. In addition to these characteristics, these compounds also satisfy Lipinski's rule of 5[11] for oral drug-like properties as follows: a molecular weight of a compound is <500, hydrogen bond donors are <5, hydrogen bond accepters are <10 and an octanol–water partition co-efficient log *P* is <5. Therefore, **9** or **13** may have the potential to become leading oral anti-diabetic drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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