Rapid Communication

SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation.

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Running Head: SGLT2 inhibitor on breast cancer cell

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Currently, cancer is one of the major causes of death in patients with type 2 diabetes mellitus. We have previously reported the anti-prostate and anti-breast cancer effect of glucagon-like peptide-1 receptor agonist exendin-4. In the present study, we examined the anti-cancer effect of SGLT2 (sodium-glucose cotransporter 2) inhibitor ipragliflozin using a breast cancer model. In human breast cancer cell line MCF-7 cell, SGLT2 expression was detected using both RT-PCR and immunohistochemistry. 1-50nM ipragliflozin significantly and dose-dependently suppressed the growth curve of MCF-7 cells. BrdU assay revealed that ipragliflozin attenuates the proliferation rate of MCF-7 cell in a dose dependent manner. Because the anti-breast cancer effect of ipragliflozin was completely canceled by knocking down of SGLT2, this effect could be induced by SGLT2 inhibition by ipragliflozin. We next measured membrane potential and whole cell current using the patch clamp technique. When we treated MCF-7 cell with ipragliflozin or glucose free medium, membrane hyperpolarization was observed. In addition, glucose free medium and knock-down of SGLT2 by siRNA suppressed glucose induced whole cell current of MCF-7 cell, suggesting that ipragliflozin inhibits sodium and glucose cotransport through SGLT2. Further, JC-1 fluorescence was significantly increased, suggesting the change of mitochondrial membrane potential. These data suggest that
SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation via membrane hyperpolarization and mitochondrial membrane instability.
INTRODUCTION

Recently, cancer is emerging as a major cause of death in patients with diabetes mellitus [1]. Especially in Japan, cancer is the leading cause of death in patients with T2DM (type 2 diabetes mellitus). Accordingly, the Japan Diabetes Society and Japan Cancer Association have therefore issued a warning regarding increased cancer risk in patients with diabetes mellitus [2]. Notably, T2DM and metabolic syndrome which are caused by obesity have been suggested to be associated with a higher risk of many cancers [3].

Based on these evidences, therapeutic strategy for T2DM which could decrease not only glucose level but also cancer risk and progression should be required. In our previous reports, we have investigated anti-cancer effect of GLP-1 (glucagon-like peptide-1) receptor agonist using prostate cancer [4] and breast cancer models [5]. In addition, further reduction of prostate cancer growth was observed by combined therapy with metformin and GLP-1 receptor agonist [6].

On the other hand, SGLT2 (sodium-glucose cotransporter 2) inhibitor is an anti-diabetic agent currently permitted in clinical application. Because of its unique glucose lowering mechanism and cardiovascular protective effect, much attention have been focused on SGLT2 inhibitor, recently. SGLT2 inhibitor ipragliflozin is made in Japan [7] and was the first SGLT2 inhibitor enter clinical use in Japan. We have previously reported that...
Ipragliflozin increased adiponectin and HDL-cholesterol, and decreased HbA1c, body mass index, serum C-peptide level and blood pressure in Japanese patients with T2DM [8]. In the present study, we examined anti-cancer effect of SGLT2 inhibitor ipragliflozin using breast cancer cell.

MATERIALS AND METHODS

Cell culture and cell proliferation assays

The MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The KPL-1 human breast cancer cell line was kindly provided by Dr. Junichi Kurebayashi, Kawasaki Medical School [9]. All breast cancer cells were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium), and all media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell proliferation assays were performed as described previously [4, 5, 6] with minor modifications. Briefly, cells were seeded in 12-well tissue culture plates and maintained in complete media with or without 1–50 μM ipragliflozin (kindly provided by Astellas Pharma Inc., Tokyo, Japan). Cell proliferation was analyzed after 0–4 days after by cell counting using a hemocytometer.

Immunohistochemistry
Paraffin sections were incubated with anti-SGLT2 (ab37296; Abcam, Cambridge, UK) and subsequently incubated with Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Thermo Fisher Scientific, Rockford, IL, USA). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and visualized with confocal microscopy.

**RT and quantitative real-time PCR**

RT (Reverse Transcription) and quantitative real-time PCR (Polymerase Chain Reaction) were performed as described previously [4, 6]. Total mRNA from breast cancer cells was isolated using RNeasy Mini Kits (Qiagen, Venlo, the Netherlands) and reverse-transcribed into cDNA. PCR reactions were performed using a Light Cycler 2.0 (Roche, Basel, Switzerland) and SYBR Premix Ex Taq™ II (Takara, Otsu, Japan). Each sample was analyzed in triplicate and normalized against TATA-binding protein (TBP) mRNA expression. The primer sequences used were as follows: human TBP, 5'-TGCTGCGGTAATCATGAGGATA-3' (forward), 5'-TGAAGTCCAAGAACTTAGCTGGAA-3' (reverse); human SGLT2, 5'-TGATCTGATTGGCAGTCAC-3' (forward), 5'-TTTTTGGACAGGGGAAAGGC-3' (reverse). PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

**BrdU (Bromodeoxyuridine) assays**
To evaluate cell proliferation in breast cancer cells, the BrdU incorporation assay was performed using Cell Proliferation ELISA kits (1647229; Roche Applied Science, Mannheim, Germany). Briefly, MCF-7 cells were plated at 5000 cells/well in 96-well culture plates in complete media. After attaining 60%–70% confluence, cells were treated with media containing 10% FBS with or without ipragliflozin (1–100 μM) for 24 h. BrdU solution (10 μM) was added during the last 2 h of stimulation. Next, the cells were dried and fixed, and the cellular DNA was denatured with FixDenat solution (Roche Applied Science) for 30 min at room temperature. A peroxidase-conjugated mouse anti-BrdU monoclonal antibody (Roche Applied Science) was added to the culture plates and the cells were incubated for 90 min at room temperature. Finally, tetramethylbenzidine substrate was added and the plates were incubated for 15 min at room temperature, and the absorbance of the samples was measured using a microplate reader at 450–620 nm. Mean data are expressed as a ratio to control (non-treated) cell proliferation.

*Small interfering (si)RNA knockdown of SGLT2 expression and cell proliferation assay*

To knockdown SGLT2, we used SGLT-2 siRNA (sc-106547; Santa Cruz Biotechnology, CA, USA), which was designed to target human SGLT2, and control siRNA sc-37007
were used as a negative control. For transfection, MCF-7 cells were plated at 2 × 10^5 cells/well in 6-well plates and transfected with 10 nmol/l of siRNA targeting SGLT2 or negative control siRNA using MISSION siRNA Transfection Reagent (Sigma-Aldrich). Seventy-two hours after transfection, cells were subjected to the cell proliferation assay. Briefly, cells were detached and re-plated in 24-well tissue culture plates in complete media with or without 10 μM ipragliflozin. 0-4 days after treatment, cells were collected and counted using a hemocytometer. The siRNA knockdown efficiency was confirmed by RT-PCR of SGLT2.

**Patch clamp measurements**

Whole cell patch recording for current and voltage clamps were recorded using the nystatin-performed patch technique in MCF-7 cell at room temperature (22-25°C) with an Axopatch 200B (Molecular Devices, Sunnyvale, CA) patch-clamp amplifier as describe previously [10]. For whole cell recordings, the Na^+^-based bath solutions contained (in mM) 140 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 320 mosmol/kg-H_2O with D-mannitol). The pipette solution contained (in mM) 55K_2SO_4, 20 KCl, 5 MgCl_2, 0.2 EGTA, and 5 HEPES (pH adjusted to 7.4 with KOH and osmolality adjusted to 300 mosmol/kg-H_2O with D-mannitol). For Figure 3B, ramp pulses (-80mV- +60mV, 0.28V/s) were
applied every 10 s from a holding potential of +40mV.

Mitochondrial permeability potential

Mitochondrial membrane ($\Delta \psi_m$) was examined using JC-1 mitochondrial membrane potential detection kit (#10009172; Cayman Chemicals, Ann Arbor, MI) according to the company’s instructions. MCF-7 cells treated with or without 10$\mu$M ipragliflozin were stained with the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria. At low membrane potential, JC-1 continues to exist as a monomer and produces a green fluorescence (emission at 527 nm). At high membrane potential and polarization, JC-1 forms J aggregates (emission at 590nm) and produces a red fluorescence.

Statistical analysis

Unpaired $t$-tests and two-way ANOVA for repeated measures were performed for statistical analysis as appropriate. $P$-values less than 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

RESULTS

SGLT2 is expressed in human breast cancer cells
To detect the SGLT2 expression on human breast cancer cell, we demonstrated immunohistochemistry. As depicted in Figure 1A, SGLT2 was expressed on the breast cancer cell membrane. Using three types of breast cancer cell lines, such as highly estrogen-sensitive MCF-7 cell, estrogen-independent MDA-MB-231 cell and low estrogen-sensitive KPL-1 cell, quantitative PCR confirmed SGLT2 gene expression (Figure 1B). Compared with KPL-1 cell, significantly higher SGLT2 gene expression was detected in MCF-7 cell. Then, we performed following experiments using MCF-7 cell, which is one of the most popular human breast cancer cell line.

**SGLT2 inhibitor attenuated breast cancer cell proliferation**

We next treated MCF-7 cell with 0-50μM ipragliflozin and drew growth curve. As shown in Figure 2A, ipragliflozin decreased cell number of MCF-7 cell in growth curve in a dose dependent manner. If we knocked down SGLT2 expression using siRNA, the attenuation of cell proliferation induced by ipragliflozin was completely cancelled (Figure 2B), suggesting that ipragliflozin attenuated breast cancer cell proliferation through SGLT2 inhibition. Further, BrdU assay revealed that high dose ipragliflozin inhibited DNA synthesis of MCF-7 cell significantly (Figure 2C). The efficacy of knock down by siRNA was confirmed by RT-PCR (data not shown).

**SGLT2 inhibitor ipragliflozin induced hyperpolarization of MCF-7 cell membrane**
Because SGLT2 uptake not only glucose but also sodium into cytoplasm, we next examined membrane potential using patch clamp technique. As shown in Figure 3A, 10mM ipragliflozin induced hyperpolarization of MCF-7 cell membrane similar to treatment with glucose free medium. The measurement of ΔV revealed the significant reduction of membrane potential by glucose free medium and ipragliflozin (Figure 3A), suggesting that the inhibition of glucose uptake through SGLT2 induced hyperpolarization of MCF-7 cell membrane potential. To confirm the pivotal role of SGLT2 in ipragliflozin-induced membrane hyperpolarization, we knocked down SGLT2 using siRNA, and we treated MCF-7 cell with medium with or without glucose. As shown in Figure 3B, the difference of membrane potential induced by deletion of glucose in cultured medium, was abolished by siSGLT2.

*GLT2 inhibitor ipragliflozin induced mitochondrial membrane instability.*

We next examined the effect of ipragliflozin on mitochondrial membrane potential, because mitochondria are one of the most important intracellular organelle deciding cell death and proliferation. JC-1 dye is an indicator of cell viability, measuring mitochondrial potential. Red fluorescence indicates healthy and intact mitochondria, and green fluorescence indicates poor healthy mitochondria and cells going die, necrosis or apoptosis. As depicted in Figure 4A, much more green fluorescence was observed in
MCF-7 cell treated with ipragliflozin. Further, the plotting of area rate of JC-1 fluorescence, red or green, revealed that mitochondrial membrane instability was induced at early phase of ipragliflozin treatment, Figure 4B.

**DISCUSSION**

In the present study, we investigated anti-breast cancer effect of SGLT2 inhibitor ipragliflozin through cell membrane hyperpolarization and mitochondrial membrane instability. SGLT2 inhibitor is newly identified anti-diabetic agent taking much attention for its glucose lowering effect without body weight gain and hypoglycemia and cardiovascular protective effect. On the other hand, current basic experimental reports suggested that anti-cancer effect of SGLT2 inhibitor, such as pancreatic, prostate [11], liver [12] and colon cancers [13]. However, there is no report which examined the effect of SGLT2 inhibitor on breast cancer. Breast cancer is one of the most critical cancer related to T2DM and obesity. Further, recently published data based on National Health Interview Survey in USA, suggested that 65.3% decline of mortality was observed in breast cancer patients with diabetes compared with patients with no diabetes [14]. Accordingly, glycemic control inhibiting breast cancer progression is important for female patients with DM.
In the present study, we investigated SGLT2 expression in human breast cancer cell, and SGLT2 inhibitor ipragliflozin attenuated breast cancer cell proliferation and DNA synthesis (Figure 1). The dose of ipragliflozin which attenuated breast cancer cell proliferation, 1-10\(\mu\)M, was almost similar to pharmacological serum concentrations [14], suggesting that our data are not so far from clinical conditions. In BrdU assay, high dose, 50-100\(\mu\)M, ipragliflozin reduced DNA synthesis (Figure 2D), however, growth curve was suppressed with lower dose of ipragliflozin (Figure 2A). These data suggested that ipragliflozin attenuated breast cancer cell proliferation through not only inhibiting DNA synthesis but also other mechanisms, such as cell death including apoptosis. Further experiments are required. We focused on sodium transport of SGLT2, because sodium uptake is emerging mechanism of cancer biology including breast cancer [15]. As expected, ipragliflozin shut down sodium uptake through SGLT2 and subsequently induced membrane hyperpolarization of MCF-7 cell. In addition, we investigated that ipragliflozin induced instability of mitochondrial membrane potential which may lead to apoptosis and necrosis of host cell. Hopefully, further experiments may reveal other effects of SGLT2 inhibitor on cancer cells.
In conclusion, we investigated that SGLT2 inhibitor ipragliflozin attenuates breast cancer cell proliferation via membrane hyperpolarization and mitochondrial membrane instability.

ACKNOWLEDGEMENTS

S.K. and T.K. performed experiments and data analysis. T.No. wrote the manuscript and conceived the research hypothesis and design. T.Nu. performed patch clam measurement and wrote manuscript. Y.H., C.I., T.H., Y.FT., N.H. R.M., M.T., R.I. and D.K. reviewed the manuscript. T.Y. conceived the research design and reviewed the manuscript.

DISCLOSURE

This study was supported by a research grant from Astella Pharma. T.N. received lecture fees from Eli Lilly Japan, MDS, Nippon Boehringer Ingelheim, Novartis Pharma, Sumitomo Dainippon Pharma, Takeda Pharmaceutical, Mitsubishi Tanabe Pharma and Ono Pharmaceutical and research grants from Astellas Pharma, Eli Lilly Japan, Sumitomo Dainippon Pharma, LifeScan Japan and Terumo. D.S. received lecture fees from MSD and AstraZeneca. T.Y. received research grants from Sumitomo Dainippon Pharma, Astellas Pharma, Eli Lilly Japan, Ono Pharmaceutical and Mitsubishi Tanabe
Pharma and an endowed chair with MSD, Takeda Pharmaceutical and Nippon Boehringer Ingelheim. D.K. received lecture fees from Takeda Pharmaceutical.

REFERENCES


FIGURE LEGENDS

Fig. 1. SGLT2 is expressed in human breast cancer cells

(A) Immunohistochemistry was performed to examine GLP-1R expression in breast cancer cell lines. All samples were counterstained with DAPI (magnification, 400×). (B) Quantitative RT-PCR was performed using a set of primers targeting 94bp coding region of SGLT2. TBP expression was used for normalization. Unpaired t-tests were performed to calculate statistical significance (*P < 0.05 vs. KPL-1 cell) (n = 3).

Fig. 2. SGLT2 inhibitor attenuated breast cancer cell proliferation
MCF-7 cells were maintained in media supplemented with 10% fetal bovine serum (FBS) with saline or ipragliflozin (1–50 μM). After 0, 24, 48, 72 and 96 h, the cells were harvested and cell proliferation was analyzed by cell counting using a hemocytometer. Black circles with solid line, control (non-treated; DMSO); black circle with dotted line, ipragliflozin (1 μM); white squares with solid line, ipragliflozin (10 μM); white squares with dotted line, ipragliflozin (50 μM). Two-way ANOVA were performed to calculate statistical significance (**P < 0.01 vs. control) (n = 3)

MCF-7 cells were transfected with either negative control duplexes or small interfering siRNA targeting SGLT2 and maintained in media with 10% FBS with DMSO or 10 μM Ex-4. After 0, 24, 48, 72 and 96 h, the cells were harvested, and cell proliferation was analyzed by cell counting using a hemocytometer. Black circles with solid line, siControl and DMSO; black cicle with dotted line, siControl and ipragliflozin (10 μM); white squares with solid line, siSGLT2 and ipragliflozin (10 μM); white squares with dotted line,siSGLT2 and ipragliflozin (10 μM). Two-way ANOVA were performed to calculate statistical significance (**P < 0.01 vs. control) (n = 3)

MCF-7 cells were plated at a density of 5000 cells/well in 96-well plates in media supplemented with 10% FBS, and incubated with ipragliflozin (0–100 μM) for 24 h. BrdU solution was added during the last 2 h, and cells were harvested for measurement of DNA synthesis using a microplate reader at 450–620 nm. Mean data are
expressed as a ratio of control (non-treated) cell proliferation. Unpaired t-tests were performed to calculate statistical significance (n = 3).

**Fig. 3. SGLT2 inhibitor ipragliflozin induced hyperpolarization of MCF-7 cell membrane**

(A) Whole cell patch recording for current and voltage clamps were recorded using the nystatin-performed patch technique in MCF-7 cell at room temperature (22-25°C) with an Axopatch 200B (Molecular Devices, Sunnyvale, CA) patch-clamp amplifier. (B) Ramp pulses (-80mV - +60mV, 0.28V/s) were applied every 10 s from a holding potential of +40mV.

**Fig. 4. GLT2 inhibitor ipragliflozin induced mitochondrial membrane instability**

(A) Mitochondrial membrane potential was assessed by JC-1 staining in MCF-7 cells treated with DMSO or 10 μM ipragliflozin (Ipra) for 48 h. Photos at 0, 24 and 48 h treatment are depicted. (B) Area rate of JC-1 fluorescence was plotted whole time of experiments.
Figure 1

A

SGLT2  DAPI  Merge

B

Relative expression of SGLT2 mRNA

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<th>MCF-7</th>
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SGLT2(94bp)

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TBP(170bp)

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**Figure 2**

**A**

Number of viable cells ($\times 10^4$) over time (hr) with different concentrations of Ipragliflozin.

- **Control (DMSO)**
- **Ipragliflozin (1uM)**
- **Ipragliflozin (10uM)**
- **Ipragliflozin (50uM)**

**B**

Number of viable cells ($\times 10^4$) over time (hr) with SGLT2 knockdown.

- **si Control (DMSO)**
- **Si Control (Ipragliflozin 10uM)**
- **Si SGLT2 (DMSO)**
- **Si SGLT2 (Ipragliflozin 10uM)**

**C**

Relative incorporations against Ipragliflozin concentration (μM).
Figure 4

A

Control
DMSO

Ipra
10μM

B

Area rate of JC-1 fluorescence (DMSO)

Area rate of JC-1 fluorescence (Ipra)