Ipragliflozin, a selective sodium glucose cotransporter 2 (SGLT2) inhibitor, is used for the treatment of type 2 diabetes mellitus. To date, the only known in vitro pharmacological characteristic of ipragliflozin is its selectivity for SGLT2 over SGLT1, which was previously reported by our group. Therefore, in this study, we investigated other in vitro pharmacological characteristics of ipragliflozin and compared them with those of phlorizin, a naturally occurring SGLT inhibitor. Selectivity of ipragliflozin and phlorizin for human (h) SGLT2 over hSGLT3, hSGLT4, hSGLT5, hSGLT6 and hSodium/myo-inositol (MI) cotransporter 1 (hSMIT1) was examined in Chinese hamster ovary (CHO) cells overexpressing each transporter using specific radio-ligands. Ipragliflozin had higher selectivity for hSGLT2 than other hSGLTs. Phlorizin showed lower selectivity for hSGLT2 compared to hSGLT1. Studies using CHO cells overexpressing hSGLT2 demonstrated that both ipragliflozin and phlorizin competitively inhibited SGLT2-mediated methyl-α-L-glucopyranoside (AMG) uptake with an inhibitory constant (K_i) of 2.28 and 20.2 nM, respectively. Ipragliflozin, but not phlorizin, inhibited hSGLT2 in a wash-resistant manner, suggesting that binding of ipragliflozin to hSGLT2 was persistent. These data demonstrate that ipragliflozin is a competitive inhibitor of SGLT2, has high selectivity for SGLT2 over not only SGLT1 but also other SGLT family members, and binds persistently to hSGLT2.

Key words: ipragliflozin; sodium glucose cotransporter 2 inhibitor; anti-diabetic drug; phlorizin

INTRODUCTION

Sodium glucose co-transporters (SGLTs) are a class of transporters expressed on the membranes of cells in various tissues. Two SGLTs in particular, SGLT1 and SGLT2, play important roles in glucose transport in the small intestine and kidney. SGLT1, a high affinity, low capacity Na+/glucose cotransporter, is abundant in the small intestine and is localized to the S3 segment of the renal proximal tubules. In contrast, SGLT2, a low affinity, high capacity Na+/glucose cotransporter, is mainly localized to the S1 and S2 segments of the renal proximal tubules and plays a central role in the reabsorption of filtered glucose via the glomeruli. Given that upregulation of the expression of SGLT2 in type 2 diabetes mellitus (T2DM) increases the maximum reabsorption/transport capacity for glucose, inhibition of SGLT2 represents a novel strategy for the treatment of patients with T2DM.

Currently, several SGLT2 inhibitors are used for the treatment of T2DM. The majority of SGLT2 inhibitors are derivatives of phlorizin, a natural O-glucoside compound. The principal pharmacological action of phlorizin is to induce renal glucosuria and block intestinal glucose absorption through inhibition of SGLT1 and SGLT2. However, phlorizin is unsuitable as an antihyperglycemic medication due to several critical factors, including its low selectivity for SGLT2, low oral bioavailability and undesirable metabolite production. Consequently, many C-glucoside derivatives have been synthesized and developed to overcome these limitations. Ipragliflozin is one such compound. It selectively binds to human (h) SGLT2 and is orally available. We previously conducted extensive in vivo pharmacological profiling of ipragliflozin and reported that it increased urinary glucose excretion, exhibited antihyperglycemic activity, preserved pancreatic β-cells, and prevented diabetic macrovascular and microvascular complications in T2DM mouse and rat models. In contrast, the sole in vitro pharmacological study of ipragliflozin has only reported its selectivity for SGLT2 over SGLT1.

Here, we investigated the selectivity of ipragliflozin for other hSGLTs (SGLT3, 4, 5, and 6) and sodium/myo-inositol (MI) cotransporter 1 (SMIT1), and its mode of inhibition against hSGLT2 in Chinese hamster ovary (CHO) cells overexpressing hSGLT2. In addition, we conducted a washout study to examine whether ipragliflozin persistently inhibits hSGLT2. Phlorizin, a naturally occurring non-selective SGLT inhibitor, was used as a reference compound.

MATERIALS AND METHODS

**Drugs and Materials** Ipragliflozin L-proline (Fig. 1A) was synthesized at Astellas Pharma Inc. (Ibaraki, Japan). Phlorizin (Fig. 1B), methyl-α-D-glucopyranoside (AMG), myo-inositol (MI), and fructose were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). [3H]-AMG and myo-[3H]-inositol ([3H]-MI) were obtained from PerkinElmer, Inc. (Boston, MA, U.S.A.), and [14C]-fructose was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). All drugs were initially dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentration using the appropriate buffer for each assay.

**Inhibitory Activity against Human SGLT Subtypes** Full-length cDNA sequences of hSGLT1, hSGLT2, hSGLT3, hSGLT4, hSGLT5, hSGLT6 and hSMIT1 were cloned and transfected into CHO cells using standard techniques as described previously, and stable cell lines expressing each transporter were generated. Each hSGLT-expressing cell line...
was used in SGLT inhibition assays within six passages. SGLT inhibition assays were performed using a modified version of the methods described by Suzuki et al., Grempler et al., and our previous study. Briefly, cells expressing each hSGLT transporter were pre-incubated in Ham’s F12 medium containing 10% fetal bovine serum at 37°C for 24 h. The medium was replaced with choline assay buffer (140 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 5 mM Tris–HCl, pH 7.4) and cells were incubated at 37°C for 20 min. The choline assay buffer was subsequently replaced with 25 µL of sodium assay buffer, in which choline chloride was replaced with the same concentration of NaCl, containing various concentrations of the drug and 55 µM of a specific ligand mixture (radio-labeled and unlabeled ligand). The cells were incubated at 37°C for 2 to 4 h. After the incubation, the cells were washed twice with ice-cold choline assay buffer containing 10 mM of the respective specific ligand, and solubilized in 25 µL of 0.5% sodium dodecyl sulfate (SDS) solution. The cell lysate was mixed with 75 µL of MicroScint MS-40 (Packard Instrument Co., Meriden, CT, U.S.A.), and the radioactivity was measured using a Top Count Microplate Scintillation Counter (Packard Instrument Co.). IC₅₀ values were calculated using a regression analysis. [¹⁴C]-AMG, [¹⁴C]-fructose and [³H]-MI were used as the specific radio-labeled ligands for the hSGLT3 and 4, hSGLT5, and hSGLT6 and SMIT1 assays, respectively. Incorporation of the specific radio-labeled ligands into cells incubated in choline assay buffer was used as a negative control.

**Mode of Inhibition against hSGLT2**
The mode of inhibition of ipragliflozin and phlorizin against SGLT2 activity was analyzed using Lineweaver–Burk plots (Fig. 2). Cells overexpressing SGLT2 were incubated in 200 µL of sodium assay buffer containing various concentrations of an AMG mixture ([¹⁴C]-AMG and unlabeled AMG) and 1, 3 or 10 nM of ipragliflozin; 10, 30 or 100 nM of phlorizin; or DMSO at 37°C for 2 h. After the incubation, the cells were washed twice with 200 µL of ice-cold choline assay buffer. The cells were subsequently solubilized, and the radioactivity of the cell lysate was measured as described above. The inhibitory constant (Kᵢ) values for SGLT2 were calculated by fitting a Michaelis–Menten equation to the data. The data were analyzed using GraphPad Prism 7.03 (GraphPad Software, Inc., CA, U.S.A.).

**Inhibitory Activity against hSGLT2 Following Removal of Drugs**
We also examined the inhibitory activity of ipragliflozin and phlorizin against hSGLT2 after removing these drugs. The experimental procedure is shown in Fig. 3A. CHO cells overexpressing hSGLT2 were seeded and cultured as described above. The medium was replaced with 25 µL of sodium assay buffer containing 360 nM of ipragliflozin or 4700 nM of phlorizin (approximately equivalent to the respective predetermined 99% maximal inhibition concentration (IC₉₀) for AMG uptake) and the cells were cultured at 37°C for 2 h. The cells were subsequently washed once with drug-free sodium assay buffer and further incubated in 25 µL of drug-free sodium assay buffer containing 2.5 µL of [¹⁴C]-AMG (Wash condition) at 37°C for 1 h. As a control, incubation in the presence of the drugs was continued at 37°C for 2 h, before 2.5 µL of [¹⁴C]-AMG was added and the cells were further incubated at 37°C for 1 h (No wash condition). After the incubation, the cells were washed once with ice-cold choline assay buffer, solubilized and the radioactivity of the cell lysate was measured as described above.

**Table 1. SGLT2 IC₅₀ Values and the SGLT2 Selectivity of Ipragliflozin and Phlorizin**

| Inhibitor | IC₅₀ nmol/L (95% CI) (fold) |
|-----------|-----------------------------|
| hSGLT2    | hSGLT1 [AMG] | hSGLT3 [AMG] | hSGLT4 [AMG] | hSGLT5 [fructose] | hSGLT6 [myo-inositol] | hSMIT1 [myo-inositol] |
| Ipragliflozin | 7.38* (6.75–8.07)* | 1876* (1570–2242)* | 30400 (23500–39300)* | 15900 (9630–26200) | 459 (386–546) | 10400 (8520–12600) |
| Phlorizin | 34.6* (24.1–49.7)* | 210* (176–251)* | 40400 (17400–94000) | 9530 (3430–26500) | 11400 (897–1450) | 14800 (7160–30700) |
|           |               |               |               |               |               |                   |

*Results show the compounds’ IC₅₀ and 95% confidence interval of the geometric mean (CI) for SGLT2, hSGLT1, hSGLT3, hSGLT4, hSGLT5, hSGLT6 and hSMIT1, and selectivity for SGLT2. [¹⁴C]-AMG was used as a substrate for hSGLT1, hSGLT2, hSGLT3 and hSGLT4; [¹⁴C]-fructose as a substrate for hSGLT5; and [³H]-myo-inositol as a substrate for hSGLT6 and hSMIT1. Inhibition of SGLT activity was defined as the concentration of ipragliflozin or phlorizin required to prevent 50% Na⁺-dependent AMG, fructose or myo-inositol uptake during the 2–4 h incubation. IC₅₀ values represent the geometric mean (95% CI) from three separate experiments. The ratio of the IC₅₀ of a compound for hSGLT1, hSGLT3, hSGLT4, hSGLT5, hSGLT6, or hSMIT1 to the IC₅₀ for hSGLT2 is expressed as fold increase. ND: not detected.
**Statistical Analysis**  
Results are expressed as the geometric mean (95% confidence interval of geometric mean) or mean ± standard error of the mean (S.E.M.). The IC<sub>50</sub> was calculated using regression analysis. All data analyses were conducted using SAS statistical software (version 9.4, SAS Institute Japan, Ltd., Tokyo, Japan). For the washout study, comparison of drug effects versus control effects was analyzed using Dunnett’s multiple comparisons test.

**RESULTS**

**Selective Inhibition against SGLT2**  
Table 1 shows the inhibitory activity of ipragliflozin and phlorizin against the seven hSGLTs (hSGLT1, hSGLT2, hSGLT3, hSGLT4, hSGLT5, hSGLT6, and hSMIT1). The IC<sub>50</sub> values of ipragliflozin and phlorizin for hSGLT2 were 7.38 and 34.6 nM, respectively, which were smaller than those of other hSGLT family members. In addition, selectivity of ipragliflozin for SGLT2 over SGLT1, according to the ratio of the IC<sub>50</sub> for hSGLT1 to that for hSGLT2, was higher than that of phlorizin.

**Mode of Inhibition against SGLT2**  
The mode of inhibition of ipragliflozin and phlorizin against hSGLT2 was analyzed using Lineweaver–Burk plots. The plots showed that both compounds inhibited AMG uptake in a competi-
tive manner (Fig. 2). The $K_i$ values of ipragliflozin (Fig. 2A) and phlorizin (Fig. 2B) for hSGLT2 were 2.28 nM (95% CI: 1.08–4.83 nM) and 20.2 nM (95% CI: 15.0–27.3 nM), respectively. Ipragliflozin showed almost 9 times more potent affinity for hSGLT2 than phlorizin did for hSGLT2.

Inhibitory Activity against hSGLT2 in the Presence of, and Following Removal of the Drugs We examined $[^{14}C]$-AMG uptake mediated by hSGLT2 in the presence of, and following removal of ipragliflozin and phlorizin. Due to the differences in $IC_{50}$ and $K_i$ values (ipragliflozin: $IC_{50} = 7.38$ nM, $K_i = 2.28$ nM; phlorizin: $IC_{50} = 34.6$ nM, $K_i = 20.2$ nM) (Table 1 and Fig. 2), the $IC_{99}$ of phlorizin used in this study was approximately 13 times higher than that of ipragliflozin. As shown in Fig. 3B, both drugs completely inhibited $[^{14}C]$-AMG uptake. While removal of phlorizin resulted in full recovery of $[^{14}C]$-AMG uptake (Fig. 3B), removal of ipragliflozin only resulted in partial recovery of uptake (Fig. 3C).

DISCUSSION

While studies have reported the selectivity of ipragliflozin for SGLT2 over SGLT1,9 no studies have reported on the drug’s other in vitro pharmacology characteristics. In this study, we further assessed the pharmacological properties of ipragliflozin in vitro and compared them with those of phlorizin, a naturally occurring SGLT inhibitor. Among seven hSGLT subtypes, ipragliflozin selectively inhibited hSGLT2, and its selectivity for hSGLT2 was much higher than that of phlorizin. Lineweaver–Burk analysis demonstrated that ipragliflozin competitively inhibited SGLT2-mediated AMG uptake with a $K_i$ value of 2.28 nM. In addition, the inhibitory effect of ipragliflozin on $[^{14}C]$-AMG uptake persisted even after the removal of ipragliflozin.

The $IC_{99}$ values of ipragliflozin and phlorizin for hSGLT subtypes determined in the present study were consistent with those reported by other groups.19,20 Together, these previous and present results demonstrate that ipragliflozin shows higher selectivity for hSGLT2 than other hSGLTs, and that the inhibitory activity of ipragliflozin against SGLT2 is approximately 4 to 6 times more potent than that of phlorizin.9,19,20 Lineweaver–Burk analysis showed that ipragliflozin competitively inhibited SGLT2-mediated AMG uptake, similar to phlorizin; however, the $K_i$ value of ipragliflozin was approximately 9 times higher than that of phlorizin. Previous reports revealed that the $K_i$ values of luseogliflozin, tofogliflozin and canagliflozin were 1.1,21 2.99 and 4.0 nM, respectively. This suggests that the binding affinity of these SGLT2 inhibitors to hSGLT2 is comparable to that of ipragliflozin.

We demonstrated that the inhibitory effect of ipragliflozin on $[^{14}C]$-AMG uptake was wash resistant, suggesting that the binding of ipragliflozin to hSGLT2 is persistent. In contrast, phlorizin did not show any inhibitory effect on $[^{14}C]$-AMG uptake after wash out. Hummel et al. performed wash out experiments using an electrophysiological technique in hSGLT2-expressing human embryonic kidney 293T (HEK293T) cells and found that phlorizin rapidly dissociated from SGLT2 (half-time off rate $t_{1/2, off} = 30$ s), while dapagliflozin dissociated from hSGLT2 at a 10-fold slower rate ($t_{1/2, off} = 300$ s).22 Although their experimental method differed from that used in the present study, both studies showed that phlorizin dissociates easily from hSGLT2 compared to ipragliflozin and dapagliflozin. Other SGLT2 inhibitors, namely empagliflozin and luseogliflozin, also show slow dissociation from hSGLT2.20,21 While all of these SGLT2 inhibitors displayed slow dissociation kinetics from hSGLT2, the molecular mechanism governing the interaction between these SGLT2 inhibitors and their target transporter remains unclear. Their slow dissociation from hSGLT2 might contribute to longer elimination $t_{1/2}$ in the kidney than in plasma, which was observed in our previous study in rats.23 Future studies should elucidate the details of the interaction between SGLT2 inhibitors and hSGLT2.

In conclusion, we demonstrated that ipragliflozin is a competitive inhibitor of SGLT2, has high selectivity for SGLT2 over not only SGLT1 but also other SGLT family members, and binds persistently to hSGLT2.

Conflict of Interest Toshiyuki Takasu, Masanori Yokono, Atsuo Tahara, and Shoji Takakura are employees of Astellas Pharma Inc. The study was funded by Astellas Pharma Inc.

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