DAPAGLIFLOZIN is one of several new the SGLT2 (Sodium/glucose cotransporter 2) inhibitors that are currently used as an anti-diabetic oral medication [1]. Dapagliflozin reduces glucose absorption in epithelial cell of the kidney proximal tubule by inhibiting SGLT2 function [1]. Although SGLT2 is highly expressed in kidney, it is ubiquitously expressed in all human and mouse tissues (2, information from GeneCard). Consistent with SGLT2 gene expression profiles, administration of isotope labeled dapagliflozin to rodent demonstrated ubiquitous distribution with highest levels in kidney, liver and colon [2]. On the other hand, dapagliflozin is metabolized by UGT1A9, which is highly expressed in kidney with relatively ubiquitous expression in human and mouse (information from GeneCard) and its metabolized product (glucuronic acid conjugated) dapagliflozin loses its activity as SGLT2 inhibitor [3].

HCT116 is a human colon cancer derived cell line that does not express UGT1A9, whereas colon tumors all express SGLT2 [4, 5]. This cell line therefore provided an opportunity to determine the effect of dapagliflozin in the absence of its metabolic inactivation. Interestingly we have found that treatment of these cells with dapagliflozin resulted in cell death, suggesting that dapagliflozin may provide a novel therapy for the treatment of colon tumors.

Materials and Methods

Reagents

Dapagliflozin and Phlorizin were purchased from Funakoshi. SGLT2 antibody was purchased from Santa Cruz Biotechnology. PARP, phosphor-Erk, Erk, and caspase-3 antibodies were purchased from Cell signaling. The horseradish peroxidase (HRP) conjugated anti rabbit or mouse IgG antibody was obtained from Thermo Scientific. Cell culture medium and reagents were from Life Technologies. All of other chemicals used in this study were purchased from Sigma-Aldrich.

Cell culture

HCT116 cells were purchased from Japanese Collection of Research Bioresources Cell Bank. These
cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The cells were grown to sub-confluent and incubated with either DMSO as vehicle, Phlorizin, or dapagliflozin as indicated concentration in Fig. legend absence or presence of serum for 24 h at 37 °C.

**Immunoblotting**

Scraped frozen cells were rocked for 10 min at 4 °C with NP-40 lysis buffer (25 mM Hepes, pH 7.4, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/mL aprotinin, 1 µg/mL pepstatin, 5 µg/mL leupeptin). Insoluble material was separated from the soluble extract by centrifugation for 10 min at 4 °C and the total protein amount in the supernatant was determined by BCA method and samples were normalized to total protein content. The samples were resuspended in SDS sample buffer and heated at 100 °C for 5 min. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The samples were immunoblotted with specific antibody as indicated in Fig. legends.

**Cell proliferation assay by XTT**

Cell viability was estimated by XTT cell proliferation assay kit [6] following manufacturer’s instruction (Roche Applied Science, Tokyo, Japan). Briefly seeded cells were mixed with prepared 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide (XTT) working solution for 6 h at 37 °C under 5% CO2 and absorbance value was obtained at 490 nm with a reference correction at 630 nm.

**Statistical analysis**

All data are expressed as mean +/- standard deviation in Figs. Data were analyzed using 1-factor ANOVA to compare the means of all the groups. The Turkey-Kramer multiple comparisons procedure was used to determine statistical differences between the means with a p values less than 0.05 evaluated for statistical significance by InStat 2.00 program.

**Results**

**Dapagliflozin induces HCT116 cell death**

Various concentration of dapagliflozin was added in the medium and 24hrs later light microscopic observation was performed. As shown in Fig. 1a, cell number was decreased in a dapagliflozin dose dependent manner with essentially a complete loss of cells at 2µM of dapagliflozin. Quantification by determination of protein levels remaining on the tissue culture plates also demonstrated maximal loss of cells between 1 and 2µM of dapagliflozin (Fig. 1b). The loss of cells primarily resulted from cells floating off the plates, as we observed many floating cells. These observations suggested dapagliflozin decreased adherence and the number of cells attached were decreased before collection of medium (Fig. 1c). In parallel, the protein levels in the medium increased in proportion to the decrease on the plate (Fig. 1d).

**The SGLT2 inhibitor phlorizin has no effect on HCT116 cell number**

To determine whether the cell death induced by dapagliflozin was due to inhibition of SGLT2, we examined the effect of another pharmacological SGLT2 inhibitor, phlorizin. As shown in Fig. 2, there was no significant effect of phlorizin on HCT116 cell attachment at doses known to inhibit SGTL2 [7].

**SGLT2 expression in HCT116 cell**

In order to confirm if HCT116 cells express SGLT2, SGLT2 immunoblotting was performed and demonstrated the presence of SGLT2 (Fig. 3).

**Effect of dapagliflozin on PARP and caspase-3, Erk phosphorylation, and cell viability of HCT116 cell**

In order to examine dapagliflozin affects apoptosis and cell growth, we performed uncleaved PPAR (Poly (ADP-ribose) polymerase) and caspase-3 and phosphor-Erk immunoblottings. As shown in Fig. 4a, dapagliflozin treatment had no effect on the level of uncleaved or cleaved PARP or caspase-3 levels suggesting a non-apoptotic form of cell death. In contrast, dapagliflozin enhanced the phosphorylation of p42 Erk1 and to a smaller extent p44 Erk2. In contrast, phlorizin had no significant effect on Erk phosphorylation compared to control DMSO treated cells. These results were confirmed by XTT cell proliferation assay as shown in Fig. 4b and 4c. Dapagliflozin significantly reduced cell number and cell viability as shown in Fig. 4b and these results were affected in the presence of mitogen-activated protein kinase kinase (MEK) inhibitor [8].
Fig. 1  Effect of dapagliflozin on HCT116 cell
a. Various doses of dapagliflozin was added in culture medium and 24h later HCT 116 cells were observed. Experiments were repeated four times.  b. HCT 116 cells amount remained on the culture wells were estimated by protein assay. Experiments were independently repeated four times as described above.  c. Observation of HCT 116 cells treated with 2µM of dapagliflozin before and after medium change.  d. Estimation of the sediment floating cells from the medium by protein assay. Experiments were independently performed four times.

Fig. 2  Effect of phlorizin on HCT116 cell
Various doses of phlorizin were added in culture medium and 24h later HCT 116 cells were observed. Experiments were repeated four times.
SGLT2 expression on HCT 116 cells was confirmed by SGLT2 immunoblotting. This picture is one of the four results independently repeated.

Effect of phlorizin and dapagliflozin on PARP and Erk

Estimation of apoptosis by uncleaved, cleaved PARP (Poly (ADP-ribose) polymerase) and caspase-3 amount. Experiments were independently performed four times. a, b. Estimation of cell proliferation by phosphor-Erk immunoblottings (a) and XTT cell proliferation assay (b). Experiments were independently performed four times. c. Estimation of cell proliferation by XTT cell proliferation assay in the presence of MEK inhibitor. Experiments were independently performed four times.
Discussion

SGLT2 inhibitors such as dapagliflozin are being increasingly used to treat type 2 diabetic patients. We have made an unexpected observation that dapagliflozin as an apparent cytotoxic effect on human colon HCT116 cells in culture. This effect of dapagliflozin is likely independent of SGLT2 transport inhibition as phlorizin was completely without effect. Although the mechanism of dapagliflozin cellular toxicity is not known, dapagliflozin treatment had no effect on PARP and caspase-3 cleave that is a well-established marker of apoptosis. In contrast, dapagliflozin treatment increased Erk phosphorylation that typically drives cell growth. Erk was reported to inhibit cell adhesion in neural cell [9]. Based on our data including MEK inhibitor experiments, dapagliflozin reduced HCT116 cell adherence concomitant with increase Erk activation is consistent with enhanced cell rounding and loss of adherence junctions that occurs during cell division. In contrast, lack of adherence typically results in cell death, whether this is true in vivo or whether increased proliferation will result in an increase in metastasis remains to be determined.

What accounts for the difference between dapagliflozin and phlorizin? Dapagliflozin is inactivated following glucuronic acid conjugation by UGT1A9. Although it is thought that this is strictly a degradation pathway, it is unknown whether persistent levels of active unmodified dapagliflozin have additional off-target effects. The data presented in manuscript suggests that this in fact the case as HCT116 cells do not display any appreciable UGT1A9 activity and a pharmacologically unrelated SGLT2 inhibitor had no significant effect on cell adherence. Further studies are now needed to determine the potential effects of dapagliflozin in patients with colon cancer that is UGT1A9 negative and the potential effects of other SGLT2 inhibitors on colon cancer cells.

Finally, this manuscript is the first paper to demonstrate the possibility of drug repositioning for dapagliflozin as anti-cancer reagent.

Author Contributions

T.S., E.Y., Y.S., A.O., R.S., J.O., performed experiments and contributed discussion. Y.M. contributed discussion. S.O. wrote manuscript.

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Disclosure Statement

The authors have nothing to declare.

References