Abstract

Notwithstanding that high rates of glucose uptake and glycolysis are common in neoplasia, pharmacological efforts to inhibit glucose utilization for cancer treatment have not been successful. Recent evidence suggests that in addition to classical glucose transporters, sodium-glucose transporters (SGLTs) are expressed by cancers. We therefore investigated the possibility that SGLT inhibitors, which are used in treatment of type 2 diabetes, may exert antineoplastic activity by limiting glucose uptake. We show that the SGLT2 inhibitor canagliflozin inhibits proliferation of breast cancer cells. Surprisingly, the antiproliferative effects of canagliflozin are not affected by glucose availability nor by the level of expression of SGLT2. Canagliflozin reduces oxygen consumption and glutamine metabolism through the citric acid cycle. The antiproliferative effects of canagliflozin are linked to inhibition of glutamine metabolism that fuels respiration, which represents a previously unanticipated mechanism of its potential antineoplastic action.

Keywords: Canagliflozin, Dapagliflozin, Breast cancer, Glutamine, Tumor metabolism

Introduction

Inhibitors of sodium-coupled glucose co-transporter 2 (SGLT2; SLC5A2) including dapagliflozin, canagliflozin, and empagliflozin are widely used in the treatment of type 2 diabetes [1]. They inhibit reabsorption of glucose in the kidney, which results in increased glucose excretion and reduction in glucose blood levels [1]. These drugs are related to the natural product phlorizin and have similar effects on glucose reabsorption in the kidney, but have different potencies and structural features [2].

In addition to their clinically established role as antidiabetic agents, SGLT2 inhibitors display anticancer properties in experimental models [3]. Canagliflozin impairs cellular proliferation of prostate [4], lung [4], and hepatocellular cancer cells [5-7] in vivo, while dapagliflozin suppresses renal cancer cell proliferation [8]. In vivo, canagliflozin reduces tumor growth in pancreatic [9], lung [10] and liver [5] cancers, while dapagliflozin exhibits...
antineoplastic effects in renal [8], breast [11], and colon [11] cancer models. However, there is no consensus regarding the underlying mechanism of SGLT2 anticancer activity. While there is evidence that some cancers express SGLT family transporters [3, 9, 12], and imaging with $\alpha$-methyl-4-deoxy-4,4-$^{18}$F]fluoro-D-glucopyranoside suggests that SGLT transporters may be functional in certain cancers [9], genetic evidence for a role of SGLT family members in glucose transport by cancers is lacking [13]. Importantly, several groups argue that the antineoplastic effects of SGLT2 inhibitors are related to reduced glucose transport through SGLT2 [5, 8-10]. However, others have proposed additional mechanisms in cancer, such as inhibition of the electron transport chain [4] or reduction in insulinemia secondary to reduced glucose [11]. Thus, studies regarding the mechanisms underpinning putative antiproliferative effects of SGLT2 inhibitors are of interest, in part because the documentation of substantial glucose transport into cancer cells by transporters other than those of the GLUT family would have major implications for cancer metabolism and potential therapeutic options.

Herein, we report that canagliflozin, and to a lesser extent dapagliflozin, inhibit proliferation of breast cancer cells independently of glucose availability. Furthermore, we show that SGLT2 depletion does not attenuate cancer cell proliferation in glucose replete conditions. Finally, we show that canagliflozin impacts glutamine metabolism and mitochondrial respiration. Collectively, these data suggest that the antineoplastic effects of canagliflozin are largely independent of their effects on glucose uptake.

Materials and methods

Cell lines and reagents

SKBR3, BT-474, and MCF7 cell lines were purchased from ATCC. SKBR3 cells were grown in RPMI media (11 mM glucose, 2 mM glutamine) supplemented with 10% FBS and 1% gentamycin, while BT-474 and MCF7 cells were grown in DMEM media (25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate) supplemented with 10% FBS and 1% penicillin/streptomycin. NT2196 and NT2197 cells are immortalized NMuMG mouse mammary epithelial cells that were transformed with an activated variant of Neu (rat form of ErbB2), which has been previously described in [14] and grown in DMEM media (25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate) supplemented with 10% FBS, 1% penicillin/streptomycin, 10 μg/mL insulin, 20 mM HEPES, pH 7.5, and 2 mM glutamine, to obtain a final glucose concentration of 6 mM. Canagliflozin and dapagliflozin were purchased from ADOQQ Bioscience. All other reagents were purchased from Sigma unless otherwise stated.

Cell proliferation

Cell proliferation was quantified using an automated TC10 cell counter (Bio-Rad) and viability was determined using trypan blue exclusion assay. See Supplemental Methods for more information.

Western blotting

Protein samples were prepared using lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate,) with protease and phosphatase inhibitors. Immunobots were incubated in primary antibodies: SLC5A2 (Abcam; ab37296), GLUD1 (Abcam; ab166618) or β-Acin (Cell Signaling; #84571) and visualized using Bio-Rad Clarity Western ECL (Bio-Rad; 170-5061).

siRNA transfection

In Fig. 1F, SKBR3 cells were transfected upon seeding (30,000 cells at day 0) with 200nM SLC5A2 siRNA (ThermoFisher Scientific; AM16708) or Silencer Negative Control No.1 (ThermoFisher Scientific; AM4611) using HiPerFect reagent (Qiagen) according to the manufacturer’s instructions. Cells were transfected for 24 h, then treated with canagliflozin or DMSO (control) for 48 h. In Fig. S1D-E, SKBR3 cells were transfected upon seeding (30,000 cells at day 0) with 50nM SLC5A2 siRNA (ThermoFisher Scientific; 4392420, s12956), 50nM GLUD1 siRNA (ThermoFisher Scientific; 4390824 s14) or Silencer Negative Control No.2 (ThermoFisher Scientific; AM4613) using Lipofectamine RNAiMAX (ThermoFisher Scientific). Cells were transfected for 24 h, then treated with canagliflozin or DMSO (control) for 72 h. The use of 50nM siRNA in Fig. S1D-E was to accommodate the high sensitivity of GLUD1 siRNA on SKBR3 cells.

Seahorse respirometry

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using Seahorse XF technology (Agilent Technologies, CA, USA) according to the manufacturer’s protocol. Briefly, cells were seeded 50,000 cells per well in 250 μL of culture media and incubated overnight at 37 °C. Cells were washed twice and incubated in 525 μL of XF media, containing 11 mM glucose, 2 mM glutamine for 1 h at 37 °C in a CO2-free incubator. Measurements for OCR and ECAR were conducted in an XF24 Seahorse instrument and values were normalized to cell counts. Cellular mitochondrial and glycolytic ATP production was quantified according to algorithms introduced in Mookerjee et al [15].

Mitochondrial isolation and respirometry

Mitochondrial suspensions were derived from skeletal muscle as previously described [16]. The RCR (respiratory control ratio) was used to evaluate the integrity of mitochondrial preparations, in which only samples with RCR values of at least 3 in controls were used [16]. Respiration of mitochondrial suspensions were performed using a Digital Model 10 Clark-type Electrode (Rank Brothers, Cambridge, UK) and incubated in KHEB assay buffer as previously described [16].

Metabolic assays

Glucose and glutamine consumption, as well as lactate and glutamate production were measured from extracellular media using the Nova BioProfile Analyzer 400 (Nova Biomedical) and normalized to cell number as previously described [16]. Galactose consumption was measured using an assay kit according to the manufacturer’s instructions (Sigma; MAK012). Briefly, 30,000 SKBR3 cells were plated and grown in glucose-free, galactose-supplemented media for 48 h. On the day of extraction, supernatants were harvested and processed in 96-well dishes. Samples were incubated with the reaction mix for 1 h and absorbance was measured at 570 nm. A standard curve was used to measure the concentration of galactose in samples. Glutamate dehydrogenase enzymatic activity was assessed using an assay kit according to the manufacturer’s instructions (Sigma; MAK099). μLh

Stable isotope tracing experiments and metabolite quantification

All metabolomics extractions and analyses were performed as previously described [17]. See Supplemental Methods for more information.

Results

Canagliflozin inhibits proliferation in the absence of glucose

We used 2 human HER2+ breast cancer cell lines, SKBR3 and BT-474, and 2 murine cell lines expressing an activated form of ErbB2 (NT2196...
and NT2197). These cell lines were selected as they exhibit high rates of aerobic glycolysis and glucose uptake [18-20] and are thus expected to exhibit high sensitivity to glucose uptake inhibitors. In glucose replete media, canagliflozin, and to a lesser extent dapagliflozin, inhibited the proliferation of both SKBR3 and BT-474 cells (Fig. 1A, B). In NT2197 cells, suppression of proliferation by dapagliflozin was modest, while that of canagliflozin was similar to that seen in the 2 human cell lines (Fig. 1C). Canagliflozin also exerted stronger antiproliferative effects than dapagliflozin on MCF7 cells, a model of luminal A breast cancer (Fig. S1A). To determine whether canagliflozin or dapagliflozin act by inhibiting glucose transport, we compared their effects on proliferation in media containing either glucose or galactose. Cells cultured in the presence of galactose are dependent on mitochondrial metabolism for ATP production and proliferation [16] [21].

In the presence of glucose, 50 μM canagliflozin significantly reduced SKBR3 cell proliferation, comparable to the impact of 5 mM metformin (Fig. 1D). The impact of dapagliflozin was modest (Fig. 1D). Unexpectedly, the degree of inhibition of proliferation by canagliflozin or dapagliflozin was similar in the presence or absence of glucose, suggesting that the antiproliferative effects of canagliflozin are independent of glucose transport (Fig. 1D and Fig. S1B).

In galactose-substituted, glucose-free media, the proliferation of SKBR3 cells was reduced relative to glucose replete media (Fig. 1D and Fig. S1B). In agreement with the increased dependence on mitochondrial metabolism of cells grown in galactose media, the proliferation and viability of cancer cells cultured in galactose media was more strongly suppressed by metformin, an antidiabetic drug that blocks mitochondrial complex I [16, 22] and that is being investigated for usage in cancer treatment [23], than in glucose replete media (Fig. 1D-E). Strikingly, canagliflozin, and to a lesser extent dapagliflozin, potentiated the antiproliferative effects of metformin under these conditions and canagliflozin further reduced cell viability, suggesting that canagliflozin and metformin impact metabolism by distinct mechanisms (Fig. 1D-E).

It has been reported that SGLT transporters can also import galactose [24]. We observed no significant difference in the concentration of galactose
in the media of SKBR3 cells in the presence or absence of canagliflozin (Fig. S1C). Indeed, the antiproliferative response of canagliflozin was similar in cells deprived of glucose, but supplemented with galactose, compared to cells deprived of glucose without galactose supplementation (Fig. S1B). Taken together, the experiments on the proliferation of cancer cells in glucose and galactose media indicate that the antiproliferative effects of canagliflozin are unrelated to inhibition of glucose or galactose transport.

To directly test whether the antiproliferative effects of canagliflozin on breast cancer cells are dependent on SGLT2, we reduced its expression using RNA interference. SGLT2 depletion did not influence the basal proliferation of SKBR3 cells (Fig. 1F and Fig. S1D), nor did it affect the antiproliferative effects of canagliflozin across different concentrations (Fig. 1F and Fig. S1E). These data suggest that antineoplastic effects of canagliflozin are largely independent of SGLT2 inhibition.

Canagliflozin perturbs mitochondrial respiration

Considering the prior report that canagliflozin inhibits respiratory complex I in cancer cells [4], we first examined the effects of dapagliflozin and canagliflozin on cellular oxygen consumption and ATP production. Cellular respiration was significantly inhibited by canagliflozin, but not dapagliflozin, at low micromolar concentrations (Fig. 2A-D). This is reminiscent of the
inhibition of mitochondrial respiration by metformin and other biguanides, which are known inhibitors of complex I of the electron transport chain [16, 22]. We next measured the effects of canagliflozin on bioenergetic capacity, by determining ATP production from oxidative phosphorylation (J ATP ox) relative to ATP production from glycolysis (J ATP glyc) [15]. Canagliflozin, and to a much lesser extent dapagliflozin, decreased J ATP ox while exerting an increase in J ATP glyc (Fig. 2B-D) under basal conditions. They also resulted in a decrease in global bioenergetic capacity compared to controls (Fig. 2C). These data show that canagliflozin is a potent inhibitor of mitochondrial respiration and total ATP production.

To determine whether SGLT2 inhibitors can directly act on mitochondria, we tested the impact of canagliflozin and dapagliflozin on isolated mitochondria derived from murine tissue as previously described and based on the evolutionary conservation of electron transport chain components [16]. To determine the effect of drugs on the electron transport chain, mitochondrial suspensions were incubated with complex I substrates (malate, pyruvate) or complex II substrate (succinate, in the presence of the complex I inhibitor rotenone). Canagliflozin and dapagliflozin failed to suppress complex I-dependent respiration, as they had no effect on basal respiration (state 2), ADP-stimulated respiration (state 3), uncoupled respiration (state 4), or the maximal respiratory capacity (following FCCP treatment) of mitochondria respiring on complex I substrates (Fig. 2E-H, Fig. S2A). This was in contrast with rotenone and metformin, known inhibitors of complex I, that were used as controls (Fig. 2E-H). Furthermore, canagliflozin and dapagliflozin did not perturb basal respiration (state 2) or basal uncoupled respiration (state 4) of mitochondrial suspensions respiring...
Fig. 4. Canagliflozin inhibits glutamine metabolism. (A) Basal respiration of SKBR3 cells treated with canagliflozin (50 μM), dapagliflozin (50 μM), or DMSO (control) in the presence or absence of glutamine (2 mM) (n = 4). (B) Cell proliferation of SKBR3 cells treated with canagliflozin or DMSO (control) and grown in media containing glutamine (2 mM) or no glutamine after 48 h (n = 3). (C) Extracellular metabolite levels in media containing SKBR3 cells treated with canagliflozin (50 μM), dapagliflozin (50 μM), or DMSO (control, dotted line) after 24 h (n = 5). (D) Glutamate dehydrogenase activity assay in SKBR3 cells treated with canagliflozin (50 μM), dapagliflozin (50 μM), or DMSO (control) for 24 h (n = 3). (E-F) Cell proliferation of SKBR3 cells treated with canagliflozin (50 μM) or DMSO (control) and supplemented with either 1 mM dimethyl-oxoglutarate (DMG) or DMSO (control) for 72 h (n = 3). All data are presented as means ± SEM, *P < 0.05, (A-B) Two-way ANOVA, Tukey’s posthoc test; (C-D) One-way ANOVA, Dunnett’s posthoc test with untreated cells as control; (E) One-way ANOVA, Tukey’s posthoc test; (F) Student’s paired t test.

Canagliflozin decreases glutamine-mediated anaplerosis

Metabolic profiling of SKBR3 cells revealed that canagliflozin reduced several citric acid cycle intermediates (citrate, alpha-ketoglutarate and succinate), while dapagliflozin only modestly affected succinate and fumarate levels (Fig. 3A). Canagliflozin also increased the levels of glutamine, histidine and leucine and decreased the amounts of alanine, aspartate, and proline. In contrast, dapagliflozin had little effect on amino acid levels. Considering that HER2+ breast cancer cells exhibit increased glutamine dependency for growth [17], we hypothesized that canagliflozin may interfere with glutamine-mediated anaplerosis. To test this, we employed stable isotope tracer analysis to follow the incorporation of labeled glutamine into the citric acid cycle. Treatment of SKBR3 cells with canagliflozin decreased the fraction of labeled alpha-ketoglutarate m+5, succinate m+4, fumarate m+4, malate m+4, and citrate m+4, thereby confirming that canagliflozin perturbs the metabolism of glutamine through the citric acid cycle (Fig. 3B). Unlike canagliflozin, the glutamine tracing upon dapagliflozin treatment was comparable to controls. Together, these results demonstrate that canagliflozin

on complex II substrates. However, canagliflozin, but not dapagliflozin, significantly reduced state 3 and the maximal respiratory capacity of mitochondria (Fig. 2I-L, Fig. S2B). These actions of canagliflozin are similar to those of low dose (100 μM) of malonate, a known inhibitor of complex II, and differ from those of complex I inhibitors [25]. Overall, these data demonstrate that canagliflozin may act on complex II-dependent mitochondrial respiration when mitochondria elicit high flux through the electron transport chain (state 3 respiration and FCCP mediated respiration).

To elucidate whether modulation of complex II contributes to the antiproliferative effects of canagliflozin, NMuMG and NT2196 cells were treated with doses of canagliflozin or malonate that produce comparable inhibition of complex II-dependent respiration. Strikingly, low dose (100 μM) malonate did not impact cell proliferation compared to control conditions (Fig. S3). On the other hand, canagliflozin caused a strong antiproliferative response in both cell lines (Fig. S3). Thus, the antiproliferative effects of canagliflozin are unlikely due to complex II inhibition.
perturbs the citric acid cycle, alters amino acids levels and inhibits glutamine anaplerosis. Canagliflozin mediates antiproliferative response through inhibition of glutamine metabolism

Canagliflozin decreased the respiration of cells in the presence of glutamine, but not in its absence (Fig. 4A). This suggests that canagliflozin perturbs cellular respiration by impairing glutamine utilization. Consistent with these findings, canagliflozin reduced cell proliferation in the presence of glutamine, but was unable to further suppress proliferation in glutamine-deprived cells (Fig. 4B). In the first step of glutaminolysis, glutamine is converted to glutamate, which can act as a source of carbon to fuel the citric acid cycle. Glutamate dehydrogenase (GDH; GLUD1) is the enzyme that directly links glutamate to the citric acid cycle intermediate alpha-ketoglutarate, and is shown to support breast cancer cell proliferation and tumorigenesis through the recycling of ammonia [26]. In support of this point, GLUD1 depletion greatly diminished breast cancer cell proliferation (Fig. 5D). Canagliflozin treatment enhanced glutamine uptake, while dramatically increasing the concentration of glutamate in the extracellular media (Fig. 4C), although intracellular alpha-ketoglutarate levels were decreased (Fig. 3A-B). This suggests an impairment of glutamine utilization by cells. Given the large impact of canagliflozin on glutamate export by cells, we carried out a cell-free assay to determine whether canagliflozin inhibits the activity of glutamate dehydrogenase. Canagliflozin, but not dapagliflozin, decreased the activity of glutamate dehydrogenase by nearly 50% (Fig. 4D). Furthermore, we demonstrate that the antiproliferative effect of canagliflozin is partially attenuated through supplementation with dimethyl-oxoglutarate, a cell-permeable reagent that bypasses glutamate dehydrogenase and restores intracellular alpha-ketoglutarate levels (Fig. 4E-F). Overall, these data highlight perturbation of glutamine metabolism as a mechanism underlying the antiproliferative response of canagliflozin on breast cancer cells.

Discussion

We report metabolic changes, which are associated with antiproliferative effects of canagliflozin on breast cancer cells. The pattern of alterations included accumulation of glutamate in the media, which was paralleled by decreased activity of glutamate dehydrogenase. Although we cannot completely exclude the possibility that in some contexts SGLT2 inhibitors exert antineoplastic effects via inhibition of SGLT2-mediated glucose transport as reported by others [5], our results point to SGLT2-independent actions of canagliflozin. While our manuscript was being prepared, another laboratory investigating the basis for the nephrotoxicity occasionally encountered with canagliflozin therapy also reported inhibition of glutamate dehydrogenase in a human renal proximal tubule model system [27]. Collectively with our findings, there is thus mounting evidence for an effect of canagliflozin on glutamine metabolism.

Prior studies have explored targeting glutamine utilization in the clinic [28-30], particularly for the subset of neoplasms that exhibit 'glutamine addiction' [31, 32]. Glutaminase inhibitors are for instance in clinical trials (e.g., ClinicalTrials.gov identifier: NCT020718620). However, there has been no attempt to therapeutically target glutamate dehydrogenase, and previously described inhibitors of this enzyme lack specificity and/or do not exhibit optimal drug–like properties [33].

High expression of glutamate dehydrogenase has been identified as a marker of poor cancer prognosis [34] and its activity is associated with adaptation of neoplastic cells to metabolic stress [35, 36]. Furthermore, this enzyme has been shown to regulate redox homeostasis in a manner that favors neoplastic proliferation [37]. Our results may have particular implications for cancers that have mutations in isocitrate dehydrogenase (which converts isocitrate to alpha-ketoglutarate in the citric acid cycle). The mutated enzyme produces the oncometabolite D-2-hydroxyglutarate instead of alpha-ketoglutarate, and may create an exploitable dependency on glutamate dehydrogenase to provide alpha-ketoglutarate [38-40].

Apart from its glutamate dehydrogenase inhibitory activity, prior reports [4,27] concluded that canagliflozin inhibits oxidative phosphorylation by interfering with complex I functions. Our data confirm inhibition of oxidative phosphorylation, but by interfering with complex II functions and only when there is very high flux through the electron transport chain (state 3 and in the presence of FCCP), that do not necessarily occur frequently in vivo. In fact, breast cancer cells at baseline exhibit a mitochondrial state that is closer to that of state 4, as they often exhibit greater uncoupled respiration compared to nontransformed cells [16]. As canagliflozin does not significantly impact mitochondrial respiration under basal conditions (states 2 and 4), canagliflozin would not be expected to perturb complex II functions in cancer cells at baseline, as evidenced by the unaltered levels of succinate (Fig. 3A), a metabolite which is known to accumulate upon complex II inhibition [41]. Hence, perturbation of glutamine metabolism by canagliflozin may be more physiologically relevant, as our findings were observed under normal growth conditions.

It is of interest in the context of our observation that canagliflozin is active at ~50 μM in vivo. Chronic dosing in humans of 100 mg/d and 300 mg/d achieves serum levels of 2.5 and 7.6 μM, respectively (Product monograph INOKANA, Jansen Inc). If the relationship between administered oral dose and serum level is linear (an untested assumption, as there may be a limit to the absorption of the drug following oral administration of large doses), a crude estimate would be that a dose of 2000 mg/d would be required to achieve serum levels in the 50 μM range. As the drug was developed for diabetes, doses higher than those achieving maximal renal glucose excretion have not been studied clinically, so safety data for higher doses are unavailable. In view of our results, and prior reports [7] of in vivo antineoplastic activity of SGLT2 inhibitors, further preclinical studies to determine antineoplastic activity of canagliflozin in a broad range of in vivo models are justified.

Taken together, our study uncovered that the SGLT2 inhibitor canagliflozin has antineoplastic properties, which are linked to the rewiring of cellular metabolism. Canagliflozin perturbs the citric acid cycle through the inhibition of glutamine metabolism, and this is accompanied by impaired cellular respiration and reduced proliferation. The antineoplastic effects of canagliflozin occur in the absence of glucose and upon knockdown of SGLT2, suggesting that canagliflozin has SGLT2-independent metabolic actions in neoplasia.

Data availability

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution

DP, OU, RP NC, LH performed and analyzed experiments. IT, JS-P, and MP supervised the study. DP, IT, JS-P, and MP conceived and designed the study. DP IT, JS-P, and MP wrote the manuscript with input from all authors who have approved the final manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.02.003.
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