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BRIEF REPORT

SGLT6 - A pharmacological target for the treatment of obesity?

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ABSTRACT

Despite increased knowledge of nutrient intake regulation and energy homeostasis, treatment options for obesity remain limited. Food reward consists of two branches: gustatory and post-ingestive nutritive information. Drosophila lacking dSLC5A11 (sodium/glucose cotransporter 6-SGLT6) prefer L-glucose over D-glucose independently of their state of satiety. Human SGLT6 is an active transporter of myo-inositol and D-glucose. We investigated expression of SGLT6 in human tissue and found a significant expression in the small intestine and brain. The preference between a metabolizable and a non-metabolizable sugar was tested in 3 mouse models with a selective and potent SGLT6 inhibitor. No influence on sugar preference was seen with SGLT6 inhibition. These studies suggest that SGLT6 does not play a significant role in nutrient sensing in mammals.

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Introduction

Continuing the trend from the last half of the 20th century, increases in the prevalence of obesity across the world represent a growing health crisis.[1,2] Obesity substantially increases an individual's risk of cardiovascular disease, stroke, peripheral vascular disease, renal failure, cancer, osteoarthritis, and Type 2 diabetes mellitus.[3] Weight loss achieved by normal diet and exercise for the majority of patients is small and has a high rate of recidivism.[4] Despite a greater understanding of the regulatory mechanisms that control body weight and clear therapeutic need, pharmacological options for weight reduction remain limited.

Eating is usually a pleasurable and rewarding activity sharing brain circuitry with other pleasurable activities. High caloric content and sweet taste increase the rewarding value of food. Food reward has at least two components: gustatory and post-ingestive nutrient. Palatable food is primarily detected by external chemosensory taste receptors, but a preference for sugar can develop in animals lacking taste receptors based upon the nutritional value of the sugar.[5] Wild-type drosophila flies show a preference for D-glucose when hungry, but shift their preference for the sweeter, non-caloric mirror image L-glucose when fed. Flies lacking the transporter dSLC5A11 (SGLT6) prefer L-glucose over D-glucose independently of their state of satiety.[6] This suggested gustatory reward alone is sufficient to modulate behavior. We hypothesized that inhibition of the post-nutrient reward system could assist with diet maintenance in humans. Using mice as a model system we were unable to provide evidence that SGLT6 plays a role in nutrient sensing in mammals.

Results and discussion

Several previously described characteristics of SGLT family members and SCL5A11 specifically were congruent with the possibility that it could play a role in nutrient sensing. First, SGLT1 had been shown to be present on hypothalamic neurons, and ligands for the transporter could induce signaling.[7] Additionally, this signaling could be blocked pharmacologically.[7] Secondly, previously SCL5A11 had been shown to transport myo-inositol and to a lesser degree D-glucose in a Na^+ dependent manner.[8] Furthermore, this transport could be inhibited by phlorizin indicating that SGLT6 was pharmacologically tractable. Lastly, analogous with in-house expression data (not shown), SGLT6 was previously shown to be expressed in the small intestine and brain.[9] Strong expression in the hypothalamus and substantia nigra, brain areas linked to control of food intake and reward processing, was suggestive of a brain-gut axis that may play a role in recognizing nutrients and integrating the reward associated with their ingestion.

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Supplementary data for this article can be accessed here.

Confirmation of the mRNA expression data was performed with immunohistochemistry. It was used to determine the cellular expression of SGLT6 in un-diseased brain and small intestine samples of mouse, rat and human origin. A similar staining pattern was observed among all three species. In the brain, SGLT6 was broadly expressed and found on a cellular level intracytoplasmic of neuronal cells with involvement of the axonal and dendritic processes (Figure 1(a-f)). In the small intestine, epithelial cells of the intestinal villi, cells of the lamina propria as well as cells of the myenteric plexus were positive for SGLT6 (Figure 1(g-i)). No staining was achieved by replacing the SGLT6 primary antibody with a suitable isotype control antibody (Supplementary Figure 1(a-f): for the brain and Supplementary Figure 1(g-i) for the small intestine). Based upon this expression profile, mouse was chosen as our model species.

Our next goal was to identify a potent and selective SGLT-6 inhibitor with CNS exposure. A key priority was to achieve selectivity vs. SGLT1 as lack of this transporter is associated with malaise and gastrointestinal distress.[10,11] This side-effect would skew any behavioral phenotype. Selectivity vs. sodium-myoinositol cotransporter-1 (SMIT1- SLC5A3) was desired to further reduce potential side effects. While selectivity vs. SGLT2 would be desired from establishing proof of mechanism for SGLT6 inhibition, based upon positive effects of SGLT2 inhibition in treating diabetes, and associated cardiovascular mortality a dual inhibitor would not be considered unattractive.[12] Via a small screening campaign, followed by structure optimization, we identified the SGLT-6 inhibitor "Cpd B", combining good potency and good selectivity (Table 1).

In vitro, Cpd B is characterized by a low solubility and a high plasma protein binding (1% unbound) (Table 1). The apical to basolateral permeability in Caco-2 cells, which is an in vitro indicator for drug absorption, is high. The pharmacokinetics after intravenous administration is characterized by a low clearance and a moderate volume of distribution indicative for a good tissue distribution (Table 1). The concentration-time profile after oral administration of 4.6 mg/kg and 30 mg/kg as suspension is depicted in

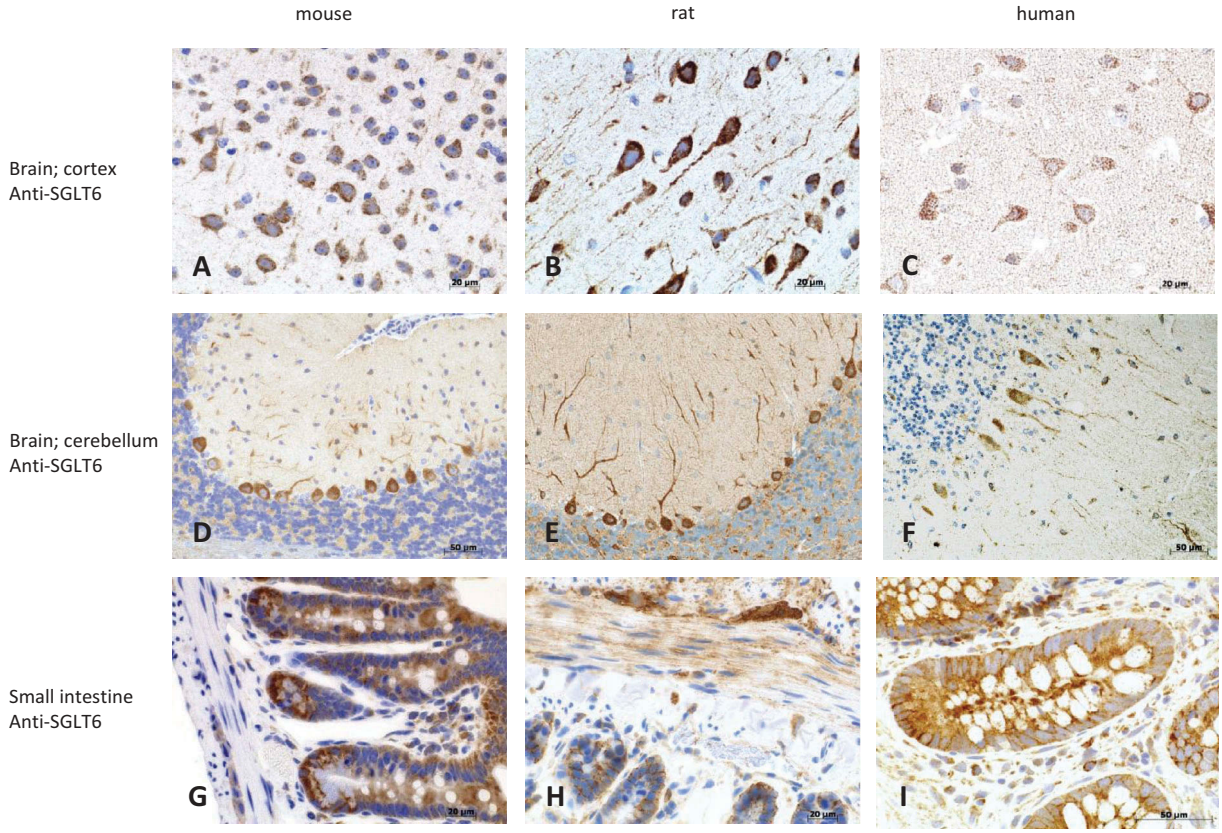

Figure 1. SGLT6 immunohistochemistry. Strong cytoplasmic SGLT6 staining is present in neuronal cells of mouse (a), rat (b) and human (c) cortical brain areas and includes the neuronal processes, also. Similar staining pattern is shown by the cerebellar purkinje cells (d-f). In the small intestine (g-i), SGLT6 is found intracytoplasmic in epithelial cells of the villi, cells of the lamina propria and myenteric ganglion cells. Isotype controls of the respective tissues are negative (Supplementary Figure 1). SGLT6 staining is indicated in brown (DAB), nuclei are counterstained in blue with Hematoxylin.

Table 1. Tool compound characteristics.

Parameters	Cpd-B		
in vitro potency			
Targeted transporters: **SGLT6**/SGLT2 IC_{50} [nM]	2	~ 54	
Adverse transporters: SGLT1/SMIT1 IC_{50} [nM]	> 2700	> 6600	
in vitro PK characteristics			
Solubility pH 2.2/4.5/6.8 [µg/mL]	< 1	< 1	< 1
Caco-2: $P_{app,a-b}$ [10^{-6} cm/s]/$P_{app,b-a}$/$P_{app,a-b}$	9.2	0.6	
MDCK: $P_{app,a-b}$ [10^{-6} cm/s]/$P_{app,b-a}$/$P_{app,a-b}$	8.8	3.5	
Plasma protein binding [% unbound]	1.0		
in vivo intravenous PK properties in mice*			
Clearance [mL/min/kg]	6.2 ± 0.3		
Volume of distribution [L/kg]	2.5 ± 0.2		
MRT_{disp} [h]	6.9 ± 0.2		
$T_{1/2}$ [h]	5.0 ± 0.2		

MRT_{disp} = Mean residence time after intravascular administration, $T_{1/2}$ = terminal half-life,

$P_{app,a-b}$ = apparent permeability in apical to basolateral direction, $P_{app,b-a}$ = apparent permeability in basolateral to apical direction * Data are mean ± SD, n = 3

Supplementary Figure 2. Despite the low solubility, we observed a fast absorption with maximum plasma concentrations at 1–2 hours and a high oral bioavailability of 64% and 71% respectively.

As the target is located in the brain we were also interested into the brain penetration of our compounds. Madin-Carby Canine Kidney (MDCK) cells overexpressing the human efflux transporter P-glycoprotein (P-gp) on their apical membrane are frequently used to predict blood-brain-barrier penetrations of drugs.[13] P-gp substrates typically show a higher apparent permeability in basolateral to apical direction than in apical to basolateral direction. This in-vitro efflux often translates into an in-vivo efflux. When incubated at a concentration of 10 µM in this assay we observed an in-vitro Efflux of 3.5 (Table 1), indicative for a likely moderate in vivo efflux at the blood-brain barrier. The central exposure in mice was investigated by measuring plasma, brain and muscle concentrations after oral administration of the compounds. The Plasma, brain and muscle concentration at 1 hour after administration of 4.6 mg/kg of the compound were 2520 ± 244 nM, 908 ± 131 nM and 4140 ± 1050 nM. Thus, a substantial brain exposure was reached. The muscle is, in contrast to the brain, a tissue without P-gp expression. We use the concentration ratio between muscle and brain as measure for P-gp mediated in vivo efflux. The calculated in vivo efflux of 4.51 ± 0.54 is comparable to that measured in MDCK cells. The calculated unbound brain concentrations ($C_{u,BR}$) in this experiment were 5.6 nM.

With substantial brain exposure reached with the SGLT6 inhibitors, we began a series of behavioral tests in mice to characterize the compounds. The compounds were well tolerated and an Irwin Test did not indicate physiological or neurological changes as a result of compound administration. Fluid and food intake (slightly) was increased (Supplementary Figure 3). We attributed the increased intake to the SGLT2 component of the molecule as it is known that increased food intake (to replace lost glucose) and water intake (to compensate for diuresis) occurs with SGLT2 inhibition.[10,11]

Having selected an appropriate species, the mouse, and characterized a selective and potent SGLT6 inhibitor (Cpd B) we set out to test if an inhibition of SGLT6 could alter the preference between a metabolizable and a non-metabolizable sugar in two different preference models as described in the method section.

In the first model the concentrations of sucrose as metabolizable sugar and sucralose as non-metabolizable sugar were chosen to achieve a clear preference in vehicle treated animals for sucrose. We hypothesized that an inhibition of SGLT6 would lead to a reduced post-ingestive sensing of the metabolizable sugar shifting therefore the preference away from the sucrose solution. Treatment with the SGLT6 inhibitor, Cpd B, dosed at 30 mg/kg twice a day started 2 d before the preference test to achieve steady state plasma levels. The control group was dosed in a similar fashion only using vehicle. Mice were exposed to the sugars for two days and bottles were shifted between days to avoid side bias. At study end plasma samples were taken to measure exposure levels. In Figure 2(a) the licks per sugar solution are reported as percent of total licks. Animals treated with vehicle showed an 84% preference for sucrose over sucralose. Treatment with Cpd B did not result in a preference shift towards the non-metabolizable sucralose resulting in a similar preference for sucrose of 85% (Figure 3(a)). To look at the data set in a more detailed fashion Figure2(b) (Vehicle) and Figure 2(c) (Cpd B) show the absolute licks per hour for both sugars. Overall the same profile was seen between vehicle and Cpd B treated animals regarding the licks per sugar. However, a slight increase in total licks for both sugar solutions was seen for the Cpd B treated group reflecting most likely a general increased intake of liquid due to the SGLT2 inhibitory part of the compound. Measurement of plasma levels at study end revealed exposure of 5111 nM at trough was reached during the experiment. The calculated free brain concentrations of 11.4 nM correspond to 5.7 fold the in vitro IC_{50}. Therefore, we conclude that SGLT6 was potently inhibited during the experiment which did not result in a significant change on sugar preference in this model.

In the second preference model the concentrations of sucrose as metabolizable sugar and a combination of sucralose and saccharine as non-metabolizable sugar were chosen to achieve nearly equal preference at the start of the experiment. Earlier reports suggest that the solutions are equally sweet and therefore, that the two sugar solutions are closely matched in palatability. Treatment with Cpd B dosed at 30 mg/kg twice a day was started one day before mice received the choice between the two sugar solutions. Figure 3(a) represents

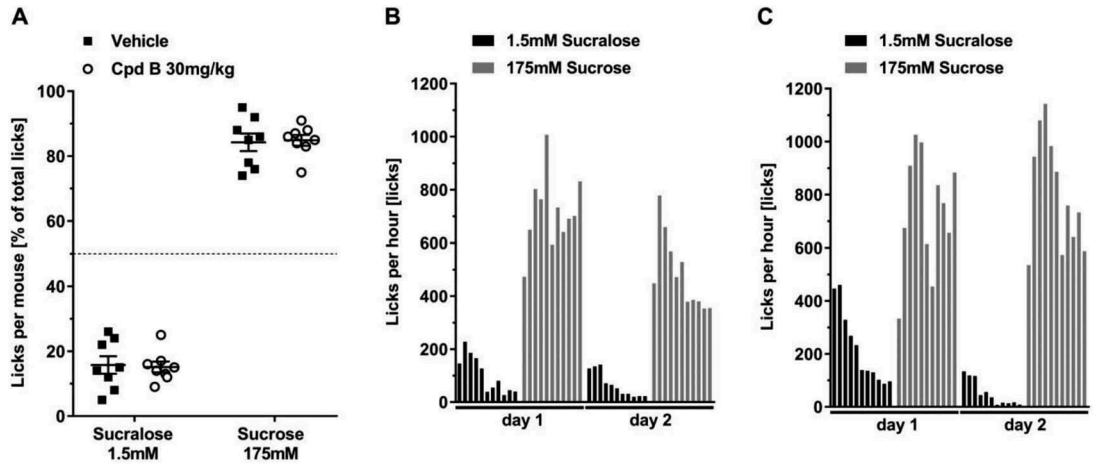

Figure 2. Effect of an SGLT6 inhibitor on sucrose vs. sucralose preference. Single housed mice (n = 8/group) treated either with Cpd B (30 mg/kg, BID) or vehicle received a choice between 175 mM sucrose and 1.5 mM sucralose for 2 consecutive days. Preference was assessed using a lickometer system recording licks per mouse. Data is presented as percent of total licks of 2 d for sucrose or sucralose (a) or absolute licks per hour for vehicle (b) or Cpd B (c). Data is given as Mean ± SEM (a) or as Mean (b, c). Data (a) were compared by 2-way ANOVA followed by Bonferroni multiple comparison test.

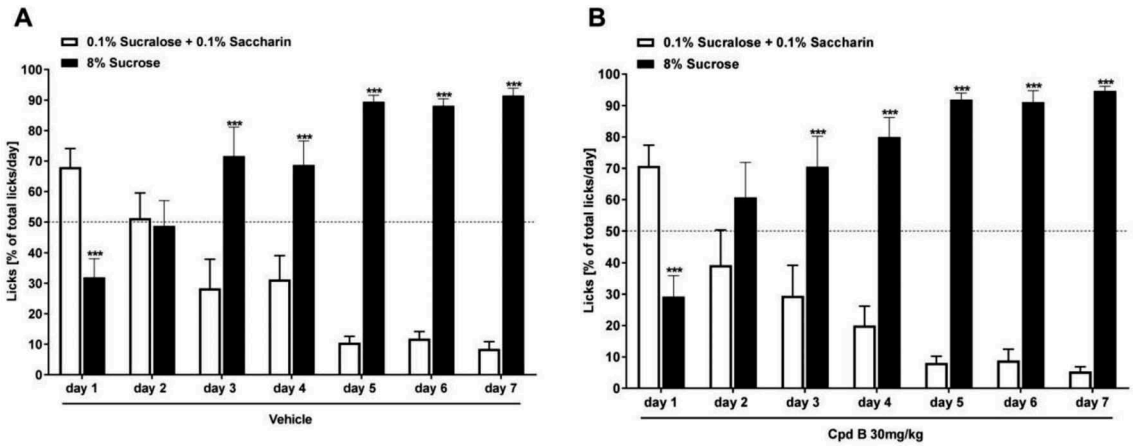

Figure 3. Effect of an SGLT6 inhibitor on preference shift between sucrose vs. sucralose/saccharine. Single housed mice (n = 8/group) treated either with Cpd B (30 mg/kg, BID) or vehicle received a choice between a mix of 0.1% sucralose/0.1% saccharine and 8% sucrose for 7 consecutive days. Preference was assessed using a lickometer system recording licks per mouse. Data is presented as percent of total licks per day for sucrose or sucralose/saccharine. Data is given as Mean ± SEM. Data per graph were compared by 2-way ANOVA followed by Bonferroni multiple comparison, ***p < 0.001 vs. sucralose/saccharine.

mice treated with vehicle and reports again licks per sugar as percent of total licks. On day 1 mice showed a slight increase in preference towards the non-metabolizable sugar solution which was equal in preference compared to the sucrose solution on day 2. From day 3 onwards mice developed consistently more preference towards the sucrose solution indicative for a possible sensing of the post-ingestive sensing of the caloric value. Mice treated with Cpd B showed similar to the vehicle mice a slight preference towards the sucralose/saccharine solution on day 1 with no significant preference on day 2 between both sugar solutions (Figure 3(b)). From day 3 onwards a similar increase in sucrose preference was seen compared to vehicle group again indicating that inhibition of SGLT6 had no impact on the preference shift in this model. Trough plasma exposure levels of 4927 nM documented a sufficient exposure level.

As it has been suggested that the energy state of mice impact their preference behavior we set out to conduct a final experiment with mice in an energy deficient state. Therefore, mice were food restricted to achieve a body weight loss of approximately 10% (Figure 4(a)). Again treatment with the SGLT6 inhibitor, Cpd B, dosed at 30 mg/kg twice a day started 2 d before the preference test. This time we chose the sugar concentrations based on a clear preference towards the non-metabolizable sugar

solution. This was tested beforehand on mice with ad libitum access to food (data not shown). We expected that the mice will be more sensitive towards the additional caloric value of the metabolizable sugar solution when their body weight had been significantly reduced. Unexpectantly mice in an energy deficient state and treated only with vehicle showed no response on their sugar preference as depicted in Figure 4(a). Treatment with Cpd B had similar no effect on the initial preference. In conclusion we did not see any preferences shift based on the energy state of mice and also not upon inhibition of SGLT6.

To summarize the result reported here we could show that maintaining unbound brain concentrations of a specific SGLT6 inhibitor, corresponding to at least 5 fold IC_{50} has no impact on the preference between a metabolizable and a non-metabolizable sugar in three different preference models. We conclude that SGLT6 in the mouse does not play a significant role in altering the post-ingestive nutrient sensing.

Materials and methods

In vitro potency

Affinity of ligands for the various SLC transporters was determined essentially as described previously.[14] Briefly, HEK293 cells stably over-expressing hSGLT6, hSGLT2 or hSGLT1 and MDCK cells (endogenously expressing SMIT1) were used for the sodium-dependent monosaccharide transport inhibition assay.[14] Cells were pre-incubated in uptake buffer (10 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 100 µM Glucose, 0.1% BSA) at 37°C with test compound added at different concentrations 20 minutes before the initiation of the uptake experiment. The uptake reaction was started by the addition of labelled monosaccharide. After incubation for 4 hours at 37°C, the cells were washed two times with 300 µl PBS and then lysed in 0.1 N NaOH with intermittent shaking for 5 minutes. The lysate was mixed with 200 µl MicroScint 20 and shaken for 60 minutes and counted for radioactivity in the TopCount NXT.

Plasma protein binding was determined by equilibrium dialysis as described.[15] Briefly, compound was spiked into plasma to get a final plasma concentration of 10 µM. Equilibrium dialysis against 100 mM potassium phosphate buffer, pH 7.4 was carried out for 2 hours at 37°C under rotation. At the end of equilibrium dialysis, plasma and buffer samples were collected and compound concentrations were measured after sample preparation by HPLC-MS/MS. Percent compound unbound was calculated as:

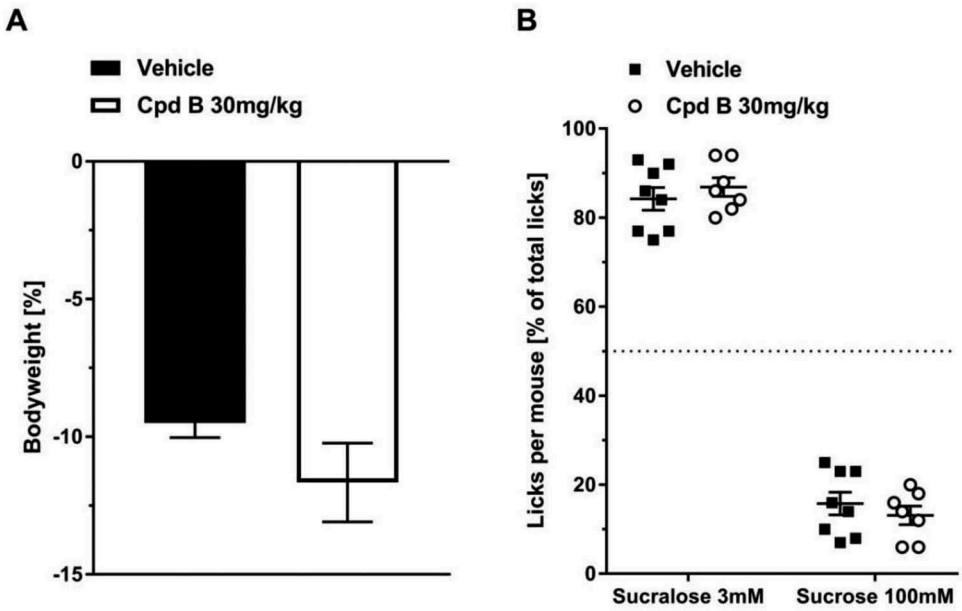

Figure 4. Effect of an SGLT6 inhibitor on sucrose vs. sucralose preference in caloric restricted mice. Single housed mice were caloric restricted to decrease bodyweight by approx. 10% (n = 7–8/group) (a). Mice with reduced bodyweight were treated either with Cpd B (30 mg/kg, BID) or vehicle and received a choice between 100 mM sucrose and 3 mM sucralose for 2 consecutive days. Preference was assessed using a lickometer system recording licks per mouse. Data is presented as percent of total licks of 2 d for sucrose or sucralose (b). Data is given as Mean ± SEM (a, b). Data (a) were compared by Student t-test, data (b) were compared by 2-way ANOVA followed by Bonferroni multiple comparison test.

$$\%\ unbound = 100 - \left(\frac{plasma\ concentration - buffer\ concentration}{plasma\ concentration} \times 100\right).$$

The fraction unbound in plasma ($f_{u,PL}$) was calculated as: (% unbound)/100.

In vitro permeability was investigated in Caco-2 cells as model for intestinal absorption and in P-gp overexpressing MDCK cells as model for blood brain barrier function. The Caco-2 assay was performed as described by Sieger et al.[16] Shortly, confluent monolayers of Caco-2 cells grown on semipermeable membranes were spiked on the apical or basolateral side with 10 µM final drug concentration and serial buffer samples taken from the retrograde side of the filter plate. Drug concentrations were measured by means of HPLC-MS and apparent permeability (P_{app}) was calculated for the apical to basolateral (a-b) and basolateral to apical (b-a) direction. The efflux ratio was calculated as $P_{app,b\text{-}a}/P_{app,a\text{-}b}$. The assay with P-gp overexpressing MDCK cells was done accordingly.

The in vivo Pharmacokinetics was investigated in male C57BL6 mice upon intravenous and oral administration of the test compounds. For the intravenous groups the compounds were dissolved in 6.4% Hydroxypropyl-beta-Cyclodextrin/water and administered as bolus via the tail vein (1 µmol/kg @ 5 mL/kg) to 3 animals per compound. For oral dosing compound was dissolved in 0.015% Polysorbat 80/0.5% methylcellulose in water and administered by gavage (10 mL/kg) at final doses of 4.6 mg/kg and 30 mg/kg. Serial sampling was performed via puncture of the saphenous vein into K3-EDTDA coated vials. A maximum of 20 µL blood was taken per sampling time. Upon protein precipitation with acetonitrile, addition of internal standard, compound concentrations were determined by means of high performance liquid chromatography coupled with tandem mass spectrometry in the positive ionization mode.

The compound concentration-time data were analyzed by non-compartmental Pharmacokinetic (PK) data analysis (ToxKin®, Version 3, Boehringer Ingelheim, Germany). The PK parameters were calculated as described elsewhere.[17]

For tissue distribution, oral dosing was performed as described above. Animals were sacrificed at the time points indicated in the results section, plasma, a piece of leg muscle and brain were taken. Tissue was homogenized as described[15] and concentrations were determined by means of HPLC-MS/MS. Tissue concentration ratios were calculated per animal and descriptive statistics calculated. The unbound brain concentrations ($C_{u,BR}$) were calculated as:

$C_{u,BR} = \frac{C_{PL} \times f_{u,PL}}{In\ vivo\ Efflux}$, where C_{PL} is the total plasma concentration, $f_{u,PL}$ is the fraction unbound in plasma, and In vivo Efflux is the concentration ration of muscle and brain.

PK experiments performed at BioDuro, China, were approved by IACUC at BioDuro, in accordance with the Guide for the care and use of laboratory animals, China National Experimental Animal Quality Management Guidelines, the Office of Laboratory Animal Welfare (OLAW) and BioDuro IACUC policies and SOPs.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde prior to paraffin embedding (FFPE). Three-micron tissue sections were de-waxed with xylene, rehydrated in a graded ethanol series and blocked with 3% hydrogen peroxide. For an immunohistochemical analysis of SGLT6, Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) for 20 min. Sections were incubated with rabbit anti-SGLT6 (ABIN2433855; antikörper-online.de) 1:1000 diluted with Leica Primary Antibody Diluent (AR9352; Leica Biosystems, Nussloch, Germany) for 1 hr at room temperature. An anti-rabbit HRP-conjugated antibody followed by DAB chromogen was used for detection, followed by Hematoxylin counterstaining (Bond™ Polymer Refine Detection, Cat# 37072). For Isotype controls, a rabbit IgG, 1 mg/mL (dianova, DLN-13124) was used instead of the primary anti-SGLT6 antibody. Staining was performed on the automated Leica IHC Bond-Max™ platform (Leica Biosystems, Nussloch, Germany). Microscopy was performed with a Zeiss AxioImager M2 microscope and images were created using AxioVision software (Zeiss, Oberkochen, Germany).

Preference models

Apparatus

All preference experiments were performed in a Noldus behavioral chamber (PhenoTyper, Noldus Information Technology, Wageningen, Netherlands) equipped with two water bottles connected to two contact lickometers. When the mouse makes contact with the spout the lickometer measures the change in capacitance between the spout and the metal plate attached to the electronic box. There occurs no current flow through the animal. Each individual contact with the spout as well as the length of the contact is recorded. Licks were recorded using the EthoVision© XT software from Noldus.

Animals

Male C57BL/6J (B6) mice from Charles River Germany were used for all experiments in an age range of 10 to 11 weeks. Animal number per study group is given for

each individual experiment. During the experiment mice were single housed in the behavioral chambers enriched with bedding material, a mouse house and a wooden gnawing stick. Mice had ad libitum access to food and liquid throughout the experiment and were held at 20–22°C, relative humidity of 55 ± 10% and a 12:12 h light–dark cycle. All animal experiments were conducted in accordance with internationally accepted animal welfare guidelines and were approved by the committee for animal research in Germany.

Preference test model 1

Two day choice preferences was based on a report of Domingos et al.[5,18] For the test naive mice were adapted to the test cages for two days. On day 3 treatment with either vehicle (n = 8) or Cpd B at a dose of 30 mg/kg (n = 8) was started. Test compound was dosed twice a day on a 12:12 h schedule starting 30 minutes before the dark phase. Mice were continuously dosed for the rest of the experiment. On day 5 the mice were given the choice of 1.5 mM sucralose and 175 mM sucrose for two days. The left–right position of the solution bottles were switch on the second day to avoid side bias. The concentrations of the sucrose and sucralose solutions were based on the results of dose-response curves tested vs water and chosen among the plateau values. For sucrose and sucralose the lowest dose still in the plateau was selected. The different molarities reflect additionally also the different ligand binding affinities for either sucrose or sucralose to the taste receptor.

To test the two day preferences in mice in an energy deficient state mice were caloric restricted to achieve a maximal body weight drop of 10%. For the test naive mice were adapted to the test cages for two days. On day 3 treatment with either vehicle (n = 8) or compound B at a dose of 30 mg/kg (n = 8) was started. Test compound was dosed twice a day on a 12:12 h schedule starting 30 minutes before the dark phase. Mice were continuously dosed for the rest of the experiment. In addition starting with day 3 mice were caloric restricted to achieve an energy deficient state. Caloric restricted was maintained until study end and mice were monitored for a maximal weight loss of 10%. On day 5 the mice were given the choice of 3 mM sucralose and 100 mM sucrose for two days. The left–right position of the solution bottles were switch on the second day to avoid side bias. The concentrations of the sucrose and sucralose solutions were based on experiments testing each sweetener vs. water and selected to show a clear preference for sucralose in vehicle treated animals.

Preference test model 2

The prefence shift model was based on a report of Sclafani et al.[19] Sclafani et al showed that a 0.1% sucralose + saccharin solution is very palatable and has no or minimal postoral inhibitory actions in B6 mice.[19] Furthermore, they reported that a 0.8% sucralose solution was equally preferred to a mixture of 0.1% sucralose + saccharin suggesting that the solutions are equally sweet and the two sweetener solutions are closely matched in palatability. For the test naive mice were adapted to the test cages for one day. On day 2 treatment with either vehicle (n = 8) or compound B at a dose of 30 mg/kg (n = 8) was started. Test compound was dosed twice a day on a 12:12 h schedule starting 30 minutes before the dark phase. Mice were continuously dosed for the rest of the experiment. On day 3 mice were presented with a choice of 0.8% sucralose vs. 0.1% sucralose + saccharin solution for 7 consecutive days. The left–right position of the different solution bottles was switched every day to avoid side bias. Data from the lickometer was recorded during the night phase.

Data calculation

All data from the preference models is shown as % of total licks for each sweetener. Data for the preference model one summarize the two test days mice were exposed to the sweetener solutions. Data for preference model 2 shows each test day separately.

Plasma sampling for exposure measurements

Blood was taken from the vena facialis 12 h after the last dose and 50 µl EDTA plasma was prepared for the analysis of drug levels.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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