COVER PAGE FOR PROTOCOL AND STATISTICAL ANALYSIS PLAN

Official Study Title: Paradoxical Stimulation of Hepatic Glucose Production with Dapagliflozin

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Paradoxical Stimulation of Hepatic Glucose Production with Dapagliflozin (P2).

Subjects: 48 T2DM subjects (age = 18-70 years; BMI = 21-45 kg/m^2; males or females) according to ADA criteria. Subjects taking drugs known to affect glucose metabolism (other than metformin and sulfonylurea) will be excluded. Other than diabetes, subjects must be in good general health as determined by physical exam, medical history, blood chemistries, CBC, TSH, T4, EKG and urinanalysis. Only subjects whose body weight has been stable (± 3 lbs) over the preceding three months and who do not participate in an excessively heavy exercise program will be included. Individuals with evidence of proliferative diabetic retinopathy, plasma creatinine >1.4 females or >1.5 males, or 24-hour urine albumin excretion > 300 mg will be excluded. Diabetic subjects will be recruited from the South Texas Veterans Health Care System and Texas Diabetes Institute (TDI) in San Antonio (Deputy Director = Ralph A. DeFronzo, MD). TDI is the largest institute in the United States that provides comprehensive care for ~ 10,000 unduplicated T2DM patients. Therefore, we do not anticipate any problem in recruiting the required number of patients for this study.

Study Design: After screening, eligible subjects will receive three 5-hour measurements of endogenous glucose production (EGP), which is the biosynthesis of new glucose, with administration of study drug after a 3-hour tracer equilibration period. Hepatic glucose production (HGP), which is the net release of glucose from the liver, will be measured for 5 hours after drug administration to allow sufficient time for a significant increase in HGP above baseline after dapagliflozin administration (10). In study 1, HGP will be measured for 5 hours after dapagliflozin (10 mg) or placebo administration. **This is the control study. We expect to observe the "paradoxical" rise in EGP following dapagliflozin as described in the data section.** Study 2 will be performed under glucose clamp conditions (i.e. maintaining the plasma glucose concentration stable at each subject's fasting level). **This study will define whether the decline in plasma glucose concentration is the trigger to stimulate EGP.** Study 3 will be performed under pancreatic clamp conditions (maintaining the plasma glucagon and insulin concentrations constant at the basal level). **This study will define whether the increase in plasma glucagon and/or the decrease in plasma insulin are the trigger to stimulate EGP.** Subjects will be randomized in a 2:1 ratio; 32 subjects will receive dapagliflozin and 16 subjects will receive placebo. Each study will be performed on a separate day, after a 10-12 hour overnight fast within 1-2 week period. Following studies 1-3, subjects will return for a renal (kidney) MRI-measurement to record kidney size.

Screening Visit. Medical history is obtained and physical exam performed. Blood is drawn for FPG, blood chemistries, lipid profile, HbA1c, and thyroid function. Urine analysis, EKG and pregnancy test are performed.

Study 1: Effect of Dapagliflozin on EGP: EGP will be measured with 3-^{3}H-glucose infusion. The 3-^{3}H-glucose infusion will be started at 6 AM and arterialized blood samples will be drawn from a catheter placed retrogradely in a vein on the dorsum of the hand to measure the basal rate of EGP. The hand will be placed in a box heated to 50-60°C (122-140°F) to ensure arterialization of the venous blood. After a 3 hour tracer equilibration period (at 9 AM) subjects will receive dapagliflozin (10 mg) or placebo, and blood samples will be drawn every 20 minutes for 5 hours (until 2 PM) for the measurement of plasma glucose, insulin, C-peptide, glucagon, cortisol, catecholamine concentrations and [3-^{3}H]-glucose specific activity. The total amount of blood taken during this time is 266 ml or 18 tablespoons. Urine will be collected from 6 to 9 AM and from 9 AM to 2 PM. Urinary volume and glucose conc will be measured and the rate of urinary glucose excretion quantitated. The study will end at 2 PM and subjects will be allowed to return home.

Study 2: Effect of Dapagliflozin on EGP With Glucose Clamp: EGP will be measured with 3-^{3}H-glucose infusion as described in Study 1 above. The 3-^{3}H-glucose infusion will be started at 6 AM to measure the basal rate of EGP. After a 3 hour tracer equilibration period (at 9 AM) subjects will receive dapagliflozin (10 mg) or placebo, and the plasma glucose conc will be measured every 5 minutes for 5 hours (from 9AM to 2 PM). A variable infusion of 20% dextrose will be adjusted to clamp the plasma glucose conc at the fasting level (±5%) until the end of the study (5 PM). Blood samples will be drawn every 20 minutes for 5 hours (2 PM) for the measurement of plasma glucose, insulin, C-peptide, glucagon, cortisol, catecholamine concentrations and [3-^{3}H]-glucose specific activity. The total amount of blood taken during this time is about 296 ml or 20 tablespoons. Urine will be collected from 6 to 9 AM and from 9 AM to 2 PM. Urinary volume and glucose concentration will be measured and the rate of urinary glucose excretion quantitated. The study will end at 2 PM and subjects will be allowed to return home. Subjects will be asked to collect a 24- Hour Urine Collection to determine creatinine excretion.

Study 3: Effect of Dapagliflozin on EGP with Pancreatic Clamp: In this study, EGP will be measured as described in Study 1 above and plasma insulin and glucagon concentrations will be clamped at the basal level using the pancreatic clamp technique (17,62), while the plasma glucose concentration will be allowed to

decrease spontaneously after dapagliflozin and placebo administration. Somatostatin (750 µg/h) infusion will be started at 5:45 AM (15 minutes before the start of [3-^{3}H]-glucose infusion [6 AM]) along with basal infusions of glucagon (0.3 ng/kg.min) and insulin (0.1 mU/kg.min) to replace basal plasma glucagon and insulin concentrations. The somatostatin, glucagon, and insulin infusions will be continued for the entire duration of the study (until 2 PM). At 6 AM a prime (0.4 uCi x FPG) – continuous (0.4 uCi/min) infusion of [3-^{3}H]-glucose will be started and continued to 2 PM. At 9AM, subjects will ingest dapagliflozin (10 mg) or placebo (2:1 randomization ratio) and blood samples will be drawn at -30, -20, -10, 5 and 0 minutes before and every 20 minutes after drug administration (until 2 PM) for the measurement of plasma glucose, insulin, C-peptide, glucagon, cortisol, catecholamine concentrations, and plasma [3-^{3}H]-glucose specific activity. The total amount of blood taken during this time is about 266 ml or 18 tablespoons. Urine will be collected from 6 to 9 AM and from 9 AM to 2 PM. Urinary volume and glucose concentration will be measured and the rate of urinary glucose excretion will be calculated. The study will end at 2 PM.

Study 4: Renal (Kidney) MRI: A renal MRI will be performed on subjects for the measurement of kidney size. Before entering the MRI scanner, the subject will be surveyed for magnetic articles on his/her person or clothing and briefed regarding the safety requirements of the MRI study.

Data Analysis and Statistical Methods: Under steady-state postabsorptive conditions, the basal rate of endogenous glucose appearance (Ra = bEGP) equals the 3-^{3}H-glucose infusion rate divided by steady state plasma tritiated glucose specific activity. After drug administration, non-steady conditions for 3-^{3}H-glucose specific activity prevail and the rate whole body of glucose appearance (Ra) is calculated from Steele's equation (57). During the glucose clamp study, the rate of EGP after drug administration equals Ra minus glucose infusion rate, while, during the pancreatic clamp, Ra = EGP since no cold glucose is infused. The difference in EGP during the last hour of the study (240-300 minute) between subjects receiving dapagliflzin and subjects receiving placebo represents the increase in EGP caused by dapagliflozin (glucosuria-stimulated increase in EGP). The glucosuria-stimulated increase in EGP will be compared among the three studies with ANOVA. Post hoc testing will be performed with a Bonferroni correction for multiple comparisons. Only subjects who complete all three protocols will be analyzed. **The difference in glucosuria-stimulated increase in EGP between study 2 and study 1 represents the contribution of the decrease in plasma glucose conc to the increase in EGP, while the difference between study 3 and study 1 represents the contribution of change in islet hormones (insulin/glucagon) to the increase in EGP.**

Anticipated Results and Potential Limitations. We believe that the increase in plasma glucagon conc and/or decrease in plasma insulin conc in response to glucosuria is (are) important signal(s) responsible, at least in part, for the increase in EGP, which we anticipate will be derived primarily from the liver. Insulin and glucagon are powerful regulators of HGP. Therefore, we anticipate that, at least in part, an increase in HGP secondary to the rise in plasma glucagon concentration and decrease in plasma insulin concentration in response to dapagliflozin-induced glucosuria will account for the majority of increase in EGP in both NGT and T2DM subjects (to be explored in Protocol HSC20160596H).. Because the decrease in FPG conc in our published study (10) was comparable in dapgliflozin-treated and placebo-treated individuals, while the increase in plasma glucagon conc occurred only in dapagliflozin-treated subjects (Figure 2), we believe that the decrease in the FPG brought about by dapagliflozin plays only a minor (if any) role in triggering the increase in glucagon secretion and EGP. Moreover, previous studies with dapagliflozin in NGT subjects have reported no change in FPG following 2-weeks of treatment with dapagliflozin (55,56). Although EGP was not measured in these studies (58,59), glucose production had to increase following dapagliflozin to match the 72 grams of glucosuria and maintain the plasma glucose conc constant at the basal level. Based upon these observations, we believe that a decrease in peripheral plasma glucose conc plays very little direct or indirect (via stimulation of glucagon secretion and decrease in insulin secretion) role in the glucosuria-induced increase in EGP. Measurement of glucose production under glucose clamp conditions (Study 2) will provide a definitive answer to this question. Thus, we anticipate that the increase in plasma glucagon, decrease in plasma insulin, and increase in EGP will take place despite clamping plasma glucose conc at the fasting level in T2DM subjects.

We anticipate that clamping the pancreatic hormones (glucagon and insulin) with somatostatin in T2DM (Study 3) will prevent, in part, the increase in EGP/HGP. We believe that, under these pancreatic clamp conditions, the decrease in plasma glucose conc produced by dapagliflozin will be greater than the decrease observed without the pancreatic clamp (study 1). If this scenario is correct, the clinical implications would be enormous. If the rise in EGP following dapagliflozin-induced glucosuria can be prevented by inhibition of

glucagon and stimulation of insulin secretion, the decrease in plasma glucose conc and HbA1c could be greatly amplified by concomitant administration of a GLP-1 receptor agonist to inhibit the increase in EGP.

Recently, SGLT2 receptors have been identified on the alpha cell and addition of a SGLT2 inhibitor to cultured alpha cells resulted in an increase in glucagon secretion (55). Although this, in part, could explain the increase in plasma glucagon secretion, as discussed below, we believe that other mechanisms also must contribute to the stimulation of glucagon secretion.

The mechanism by which glucosuria stimulates glucagon secretion is not clear. Although it is possible that the decrease in fasting plasma glucose conc results in inhibition of insulin secretion and stimulation of glucagon secretion, we believe that this is an unlikely scenario. Another possible mechanism responsible for the increase in glucagon secretion is a neural reflex which is activated by glucosuria and involves the CNS. Thus, the glucosuria produced by inhibiting SGLT2 with dapagliflozin results in a decreased return of glucose from the tubular lumen to the renal vein. This will result in a widening of the glucose A-V difference between renal artery and renal vein. Since dapagliflozin inhibits the reuptake of ~50% of the filtered glucose (47), the plasma glucose conc in the renal vein will be at least ~10% lower than that in the renal artery. In PROTOCOL 11038 (HSC20160596H), we will measure the effect of dapagliflozin on the renal A-V glucose conc difference. A widening of the A-V glucose conc difference could provide a neural stimulus to the brain (independent of any change in plasma glucose conc) and (i) stimulate glucagon secretion, (ii) inhibit insulin secretion, and/or (iii) directly stimulate HGP, independent of changes in plasma glucagon or insulin, to compensate for the urinary glucose loss. A temporal relationship between the magnitude of renal glucose A-V difference and the increase in plasma glucagon/decrease in plasma insulin conc or increase in EGP (measured in PROTOCOL 11038) will support this hypothesis. Although the existence of such an A-V glucose signal across the kidney never has been demonstrated, a similar A-V glucose conc difference between the hepatic artery and portal vein has been shown to activate a neural reflex that plays an important role in the regulation of hepatic glucose production and uptake (6,60,61). **If the pancreatic clamp study prevents the paradoxical rise in EGP, this will provide strong evidence for an important role of glucagon (increased) and/or insulin (decreased) secretion in the stimulation of EGP. The specific contribution of change in plasma concentration of each hormone (increase in glucagon versus decrease in insulin) to the increase in EGP will be examined in a separate set of studies by clamping each hormone (glucagon alone and insulin alone) individually.**

Sample Size Calculation: The mean difference in EGP during the last hour of EGP measurement in dapagliflozin-treated and placebo-treated subjects was 0.70±0.34 (mean±SD) (10). To detect a 50% decrease in this difference at alpha <0.05 during the glucose clamp (Study 2) or during the pancreatic clamp (Study 3) with 90% power and alpha =0.05, we computed that 48 subjects, 32 dapagliflozin-treated and 16 placebo treated are required at 2:1 randomization. Therefore, we have set the sample size at 50 to account for drop outs since subjects must complete all three studies to be included in the data analysis.

Risk/Benefit

Potential risks include the following:

a) Blood withdrawal. All studies involve the withdrawal of blood. Total blood drawn during the study will not exceed 500 ml, or one pint of blood over a 4 week period. We do not believe that this amount of blood loss will pose any risk to the subject's health. Subjects will be questioned about their history of blood donation and subjects who have donated blood in the previous two months will not be studied. The subjects will be told that they should not donate blood for two months after the study. Blood hematocrit will be measured in every subject during the screening visit and any subject with a hematocrit of less than 34% will not be studied.

b) IV lines. Catheters will be placed in an anticubital vein and a hand vein for the measurement of glucose production. Local hematomas occur in about 1% of catheterization. Infection is possible (<1%), but we have not experienced this complications. One instance of thrombophlebitis has been observed (<0.1%). The hand with the catheter will be placed in a warm (65°C) transparent plastic box to arterialize venous blood. We have observed one instance of skin burning (2nd degree) using the heated box (<0.01%). Subjects will be informed to tell us if their hand feels excessively warm or uncomfortable.

c) Tritiated glucose is given during these studies. The radiation exposure from tritiated glucose is well within guidelines (Shreve WW et al. Proc 2nd International Conference on Peaceful Uses of

Atomic Energy, Geneva, 1958; U.S. Department of Commerce National Bureau of Standards Handbook 69, 1969). The cumulative radiation exposure also is well within the dose range (1,000 µCi) approved by the University of Texas Health Science Center Radiation Committee. The Radiation Safety Committee will approve these studies before anyone is enrolled. At these low exposures, risk is minimal.

d) The procedures for minimizing risk are listed above in context with the specific risks.

e) Information learned about all subjects will be kept confidential and handled in strict accordance with HIPAA guidelines. Subjects will not be identified in any way in any publication. With the precautions outlined above, the risk/benefit ratio in the study is very low.

f) Serious hypersensitivity reactions to dapagliflozin have been described. Anyone with a history of hypersensitivity reaction to dapagliflozin will be excluded.

g) Diabetic subjects with severe renal impairment (eGFR < 45 ml/min), end-stage renal disease, or dialysis will be excluded.

h) Subjects with uncontrolled hypertension (BP > 160/100 mmHg) will be excluded; subjects with orthostatic hypotension (decrease in BP > 15/10 mmHg) when standing will be excluded.

i) Ketoacidosis

The U.S. Food and Drug Administration (FDA) has warned that dapagliflozin may lead to ketoacidosis, a serious condition where the body produces high levels of blood acids called ketones that may require hospitalization.

Patients will be asked to pay close attention for any signs of ketoacidosis and call us immediately if they experience symptoms such as difficulty breathing, nausea, vomiting, abdominal pain, confusion, and unusual fatigue or sleepiness. We will evaluate for the presence of acidosis, including ketoacidosis in patients experiencing these signs or symptoms and discontinue dapagliflozin if acidosis is confirmed and take appropriate measures to correct the acidosis and monitor sugar levels.

j) Urosepsis and Pyelonephritis

Rarely, dapagliflozin treatment has been associated with bladder and kidney infection. If you experience pain or burning urination, pass cloudy or blood-tinged urine, or experience fever and chills, please call us immediately so that we can examine your urine and obtain a urine culture.

We also identified 19 cases of life-threatening blood infections (urosepsis) and kidney infections (pyelonephritis) that started as urinary tract infections with the SGLT2 inhibitors reported to FAERS from March 2013 through October 2014. All 19 patients were hospitalized, and a few required admission to an intensive care unit or dialysis in order to treat kidney failure.

k) Hypoglycemia may occur with dapagliflozin, especially when dapagliflozin is given in combination with a sulfonylurea or insulin. During the study we will measure your blood glucose level frequently to prevent this complication.

l) Genital mycotic (fungal) infections may occur in individuals who take dapagliflozin. They occur in about 6-8% of females and 1-2% of males. If you have any genital discomfort (itching, burning, discharge), please let us know so that we can give you something to treat the infection.

m) Dapagliflozin can cause an increase in the LDL cholesterol. We will check your LDL cholesterol and if it is greater than 120 mg/dl you will not be able to take part in the present study.

n) If you have a history of bladder cancer or currently have bladder cancer, please let us know,

since individuals with bladder cancer are excluded from the study.

Based upon the above considerations, the benefit (knowledge to be gained) to risk ratio is very favorable.

Safety

Definition of adverse events

An adverse event is the development of an undesirable medical condition or the deterioration of a pre-existing medical condition following or during exposure to a pharmaceutical product, whether or not considered causally related to the product.
The term AE is used to include both serious and non-serious AEs.

Definitions of serious adverse event

A serious adverse event (SAE) is an AE occurring during any study phase (i.e., run-in, treatment, washout, follow-up), that fulfils one or more of the following criteria:
Results in death

Is immediately life-threatening

Requires in-patient hospitalization or prolongation of existing hospitalization

Results in persistent or significant disability/incapacity or substantial disruption of the ability to conduct normal life functions

Is a congenital abnormality or birth defect

Is an important medical event that may jeopardize the subject or may require medical intervention to prevent one of the outcomes listed above

The causality of SAEs (their relationship to all study treatment) will be assessed by the investigator(s) and communicated to AstraZeneca (AZ).

Recording of adverse events

Follow-up of unresolved adverse events
Any AEs that are unresolved at the patient's last AE assessment at the end of the study are followed up by the investigator for as long as medically indicated, but without further recording in the CRF. AstraZeneca retains the right to request additional information for any subject with ongoing AE(s)/SAE(s) at the end of the study, if judged necessary.

The following variables will be collected for each AE:
AE (verbatim)

The date and time when the AE started and stopped

Whether the AE is serious or not

Investigator causality rating against the Investigational Product (yes or no)

Action taken with regard to investigational product*:* (AE caused subject's withdrawal from study *(*yes or no*)*

Outcome

In addition, the following variables will be collected for SAEs:
Date AE met criteria for serious AE

Date Investigator became aware of serious AE

Date of hospitalization

Date of discharge

Probable cause of death

Date of death

Autopsy performed

Causality assessment in relation to Study drug(s)

Causality assessment in relation to Other medication

Causality assessment in relation to Additional Study Drugs

Description of AE.

Causality assessment:

The Investigator will assess causal relationship between Investigational Product and each Adverse Event, and answer 'yes' or 'no' to the question 'Do you consider that there is a reasonable possibility that the event may have been caused by the investigational product?'

For SAEs, a causal relationship will also be assessed for other concomitant medications, study procedures, and comparator study drugs. Note that for SAEs that could be associated with any study procedure the causal relationship is implied as 'yes'.

Reporting of serious adverse events

Investigators will inform the IRB of the UTHSCSA within 24 hours of any adverse events. Investigators and other site personnel will inform the FDA, via a MedWatch/AdEERs form, of any serious or unexpected adverse events that occur in accordance with the reporting obligations and will concurrently forward all such reports to AZ. A copy of the MedWatch/AdEERs report will be faxed to AZ at the time the event is reported to the FDA. It is the responsibility of the investigator to compile all necessary information and ensure that the FDA receives a report according to the FDA reporting requirement timelines and to ensure that these reports are also submitted to AZ at the same time.
When reporting to AZ, a cover page will accompany the MedWatch/AdEERs form indicating the following:
Investigator Sponsored Study (ISS)

The investigator's name and address

The trial name/title and AZ ISS reference number

Investigative site will also indicate, either in the SAE report or in the cover page, the causality of events in relation to all study medications and if the SAE is related to disease progression, as determined by the (PI).
The SAE report and accompanying cover page will be sent by way of fax to AZ's designated fax line: 1-302-886-4114 or via email: AEMailboxClinicalTrialTCS@astrazeneca.com.

Serious adverse events that do not require expedited reporting to the FDA will be reported to AZ using the MedDRA coding language for serious adverse events.
In the case of blinded trials, AZ will request that the Sponsor either provide a copy of the randomization code/code break information or unblind those SAEs which require expedited reporting.
All SAEs will be reported to AZ, whether or not considered causally related to the investigational product. All SAEs will be documented. The investigator will be responsible for informing the WCMC-Institutional Review Board (IRB) of the SAE.

Safety assessments

Safety assessments will consist of monitoring and recording all TEAEs, SAEs, AEs leading to discontinuation/withdrawal from study, laboratory evaluation for hematology, blood chemistry, and urine values; pregnancy testing; measurement of vital signs and ECGs; and performance of physical examinations.

AstraZeneca is the manufacturer of the study drug/medication.
Label, storage and distribution are a sponsor's responsibility.

Investigational products

Description or Identity of investigational product(s)

Investigational product	Dosage form and strength	Manufacturer
Dapagliflozin 10 mg	Green, plain, diamond shaped, film coated 10 mg tablet	AstraZeneca
Matching placebo for dapagliflozin 10 mg	Green, plain, diamond shaped, film coated tablet	AstraZeneca

The tablets contain lactose, which may cause discomfort in lactose-intolerant individuals.
Dapagliflozin and its matching placebo will be supplied in bottles.

Labeling
The sponsors will label the study medication in accordance with Good Manufacturing Practice (GMP) and local regulatory guidelines for labeling (the labels will fulfill GMP Annex 13 requirements for labeling).

Storage
All study drugs should be kept in a secure place under appropriate storage conditions. The investigational product label on the bottle specifies the appropriate storage.

P2 Flow Sheet

Time (min)	Glucose	3-H-glucose	Insulin	C-peptide	Glucagon	Cortisol/NE/Epi
-180	X	X				
-30	X	X				
-20	X	X	X	X	X	X
-10	X	X	X	X	X	X
-5	X	X				
0	X	X	X	X	X	X
	Administer	Drug	Dapa or	Placebo		
10	X	X	X	X	X	
20	X	X	X	X	X	X
30	X	X	X	X	X	
40	X	X	X	X	X	X
50	X	X	X	X	X	
60	X	X	X	X	X	X
75	X	X	X	X	X	
90	X	X	X	X	X	X
105	X	X	X	X	X	
120	X	X	X	X	X	X
140	X	X	X	X	X	
160	X	X	X	X	X	
180	X	X	X	X	X	X
200	X	X	X	X	X	
220	X	X	X	X	X	
240	X	X	X	X	X	X
260	X	X	X	X	X	
280	X	X	X	X	X	
285	X	X				
290	X	X	X	X	X	
295	X	X				
300	X	X	X	X	X	X
Number	28	28	23	23	2311	
Volume (ml)	14	56	46	46		44

Total blood loss = 252 ml