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Imino Sugars are Potent Agonists of the Human Glucose Sensor SGLT3*

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Running title: Imino Sugars Potently Activate a Human Glucose Sensor

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Abbreviations: hSGLT3, human sodium/glucose cotransporter type 3; hSGLT1, human sodium/glucose cotransporter type 1; GLUT, facilitative Na^+-independent glucose transporter; $K_{0.5}$, apparent affinity; K_i, inhibition constant; E_m, membrane potential; $\Delta E_{m(max)}$, maximum change in membrane potential; I_{max}, maximal current; αMDG, α-Methyl-D-glucose; glucose, D-glucose; galactose, D-galactose; 1DOglc, 1,5-anhydro-D-glucitol; 2DOglc, 2-deoxy-D-glucose; 3DOglc, 3-deoxy-D-glucose; 4DOglc, 4-deoxy-D-glucose; 6DOglc, 6-deoxy-D-glucose; 4D4Fglc, 4-deoxy-4-fluoro-D-glucose; 4D4Fgal, 4-deoxy-4-fluoro-D-galactose; DNJ, 1-deoxynojirimycin; DGJ, 1-deoxygalactonojirimycin; 1SO-DNJ, 1-deoxynojirimycin-1-sulfonic acid; NE-DNJ, N-ethyl-1-deoxynojirimycin; NEOH-DNJ, N-β-hydroxyethyl-1-deoxynojirmycin; NB-DNJ, N-butyl-1-deoxynojirimycin; NB-DGJ, N-butyl-1-deoxygalactonojirimycin

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Abstract:

Imino sugars are used to treat type 2 diabetes mellitus (Glyset®; miglitol) and lysosomal storage disorders (Zavesca®; miglustat) based on the inhibition of α-glucosidases and glucosyltranferases. In this substrate specificity study, we examined the interactions of imino sugars with a novel human glucose sensor, sodium/glucose cotransporter type 3 (hSGLT3), using expression in *Xenopus laevis* oocytes and electrophysiology. The results for hSGLT3 are compared to those for α-glucosidases and human SGLT type 1 (hSGLT1), a well-characterized sodium/glucose cotransporter of the SGLT gene family. Generally, substrates have lower apparent affinities ($K_{0.5}$) for hSGLT3 than hSGLT1 (D-glucose, α-methyl-D-glucose, 1-deoxy-D-glucose, and 4-deoxy-4-fluoro-D-glucose exhibit $K_{0.5}$ values of 19, 21, 43 and 17 mM for hSGLT3; and 0.5, 0.7, 10 and 0.07 mM for hSGLT1). However, specificity of hSGLT3 binding is greater (D-galactose and 4-deoxy-4-fluoro-D-galactose are not hSGLT3 substrates, but have hSGLT1 $K_{0.5}$ values of 0.6 and 1.3 mM). An important deviation from this trend is potent hSGLT3 activation by the imino sugars 1-deoxynojirimycin (DNJ), N-hydroxylethyl-1-deoxynojirimycin (miglitol), N-butyl-1-deoxynojirimycin (miglustat), N-ethyl-1-deoxynojirimycin and 1-deoxynojirimycin-1-sulfonic acid, with $K_{0.5}$ values of 0.5 – 9 microM. The diastereomer 1-deoxygalactonojirimycin activates hSGT3 with a $K_{0.5}$ value of 11 mM, a 3000-fold less potent interaction than is observed for DNJ (4 microM). These imino sugar binding characteristics are similar to those for α-glucosidases, but there are no interactions with hSGLT1. This work provides insights into hSGLT3 & 1 substrate binding interactions, establishes a pharmacological profile to

study endogenous hSGLT3, and may have important ramifications for the clinical application of imino sugars.

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Introduction:

Efforts to curb the emerging epidemics of diabetes and obesity are focusing on dietary supplements for prevention and treatment. Important therapeutics emerging from these efforts are imino sugars. For example, N-hydroxylethyl-1-deoxynojirimycin (Glyset®; miglitol) is used to treat type 2 diabetes mellitus based on α-glucosidase inhibition [meta-analysis: (van de Laar *et al.*, 2005)]. In the gastrointestinal (GI) tract, α-glucosidases convert disaccharides to monosaccharides, which is necessary for absorption. Inhibition of α-glucosidases by miglitol hence reduces intestinal glucose absorption and subsequently, blood glucose levels. Beyond the GI tract, the imino sugar N-butyl-1-deoxynojirimycin (Zavesca®; miglustat) is used to treat lysosomal storage disorders based on glucosyltranferase inhibition (Butters *et al.*, 2005). Our attention was drawn to imino sugars based on their GI activity, as we recently identified a novel human glucose sensor, the sodium/glucose cotransporter type 3 (hSGLT3) expressed in the GI tract and neuromuscular junction of skeletal muscle (Diez-Sampedro *et al.*, 2003;Wright *et al.*, 2006).

hSGLT3 is one of eleven members of the functionally diverse human SLC5 gene family (Wright *et al.*, 2004;Wright *et al.*, 2006). In the presence of D-glucose (glucose), hSGLT3 depolarizes the membrane potential due to an uncoupled inward Na^+ current at pH 7.5 (Diez-Sampedro *et al.*, 2003), mediated by a transporter and/or channel mechanism. This glucose-activated inward Na^+ current is not accompanied by glucose transport, unlike other members of the SLC5 gene family. For example, hSGLT1, the best characterized member of this family, actively cotransports Na^+ and glucose in a tight 2:1 stoichiometry (Mackenzie *et al.*, 1998;Parent *et al.*, 1992a;Parent *et al.*, 1992b).

Supporting the glucose-sensing role for hSGLT3 is its expression in the intestinal autonomic nervous system and neuroendocrine cells of the GI tract where there is evidence that hSGLT3 substrates stimulate peptide and 5-hydroxytryptamine secretion (Freeman *et al.*, 2006;Wright *et al.*, 2006).

In this study, we examine hSGLT3 substrate specificity, including interactions with imino sugars, and compare the results to those for hSGLT1 and α-glucosidases. The experiments were performed using electrophysiological assays on *X. laevis* oocytes expressing hSGLT3 and hSGLT1. Two of the imino sugars examined are miglitol and miglustat. The results are discussed in terms of the molecular architecture of the substrate binding site. We find that imino sugars are potent and specific agonists of hSGLT3, but not hSGLT1. In contrast, hSGLT3 exhibits substrate specificity similar to α-glucosidases, suggesting an analogous substrate binding site. Hence, a crystal structure of a α-glucosidase with bound imino sugar is used to provide clues into the architecture of the hSGLT3 binding site. Potential physiological ramifications of clinically used imino sugars targeting hSGLT3 glucose sensors are discussed.

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Materials and Methods:

Materials – Substrates were purchased from Sigma (St. Louis, MO), Toronto Research Chemicals (North York, Ontario, Canada) or CMS Chemicals (Oxfordshire, United Kingdom). All other chemicals were purchased from Sigma (St. Louis, MO) or Fisher Chemicals (Fairlawn, NJ).

Expression of human SGLT3 & 1 in Xenopus laevis oocytes – Stage V-VI oocytes were extracted from *X. laevis* (NASCO, Fort Atkinson, WI) ovaries as approved by the University of California Chancellor's Committee on Animal Research (Ikeda *et al.*, 1989;Parent *et al.*, 1992a). Oocytes were isolated from ovaries using 3 mg/ml collagenase type 1 in Barth's solution (Parent *et al.*, 1992a) for 1 hr and defolliculated with 0.02 g/ml bovine serum albumin, 120 mM KH_2PO_4 for 30 min. Oocytes were then injected with 50 ng human SGLT3 (Diez-Sampedro *et al.*, 2003) or human SGLT1 (Hediger *et al.*, 1989) cRNA and maintained at 18°C for 3-6 days before use in Barth's solution with 5 mg/ml gentamycin, 5.75 mg/ml ciprofloxacin (Bayer, West Haven, CT) and 100 units/ml penicillin G sodium - 100 mg/l streptomycin sulfate (Gibco, Invitogen Corporation, Carlsbad, CA) .

Electrophysiology – cRNA-injected and control non-injected oocytes were impaled with two micro-electrodes, each filled with 3 M KCl and having a resistance of 0.5 megaOhms, in a rapid perfusion chamber (Loo *et al.*, 1993;Parent *et al.*, 1992a). Oocytes were superfused at 22°C and exposed to sugars dissolved in a Na^+ buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes/Tris and pH 7.4). Na^+-free conditions were achieved by substituting choline for Na^+. Steady-state changes in membrane potential and current were measured under open-circuit conditions and

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voltage-clamp at -50 mV, respectively. Voltage-clamp experiments were controlled with CLAMPEX in pCLAMP (Axon Instruments, Foster City, CA) and continuous recordings of membrane potential and current were acquired with a chart recorder.

Under open circuit conditions, apparent affinities ($K_{0.5}$) and maximal substrate-induced membrane potential changes ($\Delta E_{m(max)}$) were calculated with the equation $\Delta E_m / \Delta E_{m(max)} = [S]/(K_{0.5} + [S])$, with [S] = sugar concentration (Equation #1). Similarly, under voltage-clamp conditions, apparent affinities ($K_{0.5}$) and maximal substrate-induced currents (I_{max}) are calculated using $I/I_{max} = [S]/(K_{0.5} + [S])$. Student's t-tests and estimations of kinetic parameters are presented as mean values ± standard deviation (n = number of observations), and curve-fittings using the non-linear and linear fitting methods were derived with SIGMAPLOT (Systat Software, Point Richmond, CA).

Inhibition of hSGLT3 by phlorizin was assessed via Dixon plots, with inhibition of sugar-induced depolarizations measured at 10, 20 and 40 mM α-methyl-D-glucose (αMDG). For each αMDG concentration, the reciprocal of the inhibited depolarization induced by phlorizin was analyzed by linear regression. The inhibition constant (K_i) was the negative inhibition concentration where the regression lines intersected.

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Results:

The proposed physiological role of hSGLT3 is depolarization of the resting membrane potential in response to external glucose. Thus, membrane depolarizations measured under open-circuit conditions were used predominately to monitor hSGLT3 responses to various agonists or antagonists (see Fig. 1 for representative structures). α-Methyl-D-glucose (αMDG) was chosen as the model substrate as it is specific for SGLTs relative to the GLUT facilitative sugar transporters (Diez-Sampedro *et al.*, 2001;Wright *et al.*, 1980) and is not metabolized. The maximum depolarizations observed for αMDG ranged between 5 – 20mV, depending on the hSGLT3 expression level. To account for variable expression levels, the kinetics presented in this study were normalized with respect to the maximal αMDG-induced depolarization measured in the same oocyte.

hSGLT3 kinetics for glucose and αMDG

The time course of the resting membrane potential (E_m) of an oocyte injected with hSGLT3-cRNA and bathed in a Na^+ buffer is shown in Fig 2A. Addition of 20 mM αMDG resulted in a 5 mV depolarization that was fully reversible. Subsequent exposure to 20 mM glucose also induced a reversible 5 mV depolarization. Fig. 2B illustrates that the hSGLT3 responses to αMDG and glucose in this oocyte had similar apparent affinities ($K_{0.5}$) and maximum depolarizations ($\Delta E_{m(max)}$). Experiments with additional oocytes demonstrated that the $K_{0.5}$ values for αMDG and glucose of 21 ± 6 mM (n = 42) and 19 ± 6 mM (n = 3) were not significantly different ($p > 0.5$). The kinetics of hSGLT3 activation by αMDG were also measured under voltage-clamp (-50 mV). The $K_{0.5}$ value

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was 22 ± 3 mM (n = 3), which was not significantly different from the aforementioned $K_{0.5}$ derived under open-circuit conditions ($p > 0.5$).

Sugar Selectivity

For hSGLT3, the role of the glucose hydroxyl at carbon number 1 (C1-OH) was assessed by measuring the depolarizations induced by 0 - 100 mM 1,5-anhydro-D-glucitol (1-deoxy-D-glucose, 1DOglc). This resulted in a $K_{0.5}$ = 43 ± 10 mM and $\Delta E_{m(max)}$ relative to 100 mM αMDG of 170 ± 20%, n = 4 (Fig. 3). The same approach was used to assess the C2- and C3-OH moieties. Exposure to 2-deoxy-D-glucose (2DOglc) or 3-deoxy-D-glucose (3DOglc) induced no observable changes in E_m at concentrations up to 100 mM. Neither 2DOglc (50 mM) nor 3DOglc (20 mM) inhibited depolarizations induced by 5 or 10 mM αMDG. Analysis of the C4-OH with 4-deoxy-D-glucose (4DOglc) resulted in a $K_{0.5}$ > 50 mM and $\Delta E_{m(max)}$ that was not determinable at concentrations up to 100 mM. For the C6-OH, exposure to 0 - 100 mM 6-deoxy-D-glucose (6DOglc) resulted in small depolarizations that precluded an accurate analysis. We estimated the $K_{0.5}$ > 50 mM. To extend this study to hitherto untested aspects of hSGLT1, we exposed oocytes expressing hSGLT1 and voltage-clamped at -50 mV to 10 mM 3DOglc and observed no inhibitory effect on the steady-state currents induced by 1 mM αMDG. Exposure to 0 – 5 mM 4DOglc (n = 3) under voltage-clamp (-50 mV) resulted in activation with a $K_{0.5}$ = 0.4 ± 0.05 mM and I_{max} = 100 ± 3% of αMDG.

Since hSGLT3 discriminates between glucose and D-galactose (galactose) (Diez-Sampedro *et al.*, 2003), diastereomers at the C4-OH, this hydroxyl group was further examined. To verify that galactose does not interact with hSGLT3, we tested for inhibition and observed that 20 mM galactose had no detectable effect on

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depolarizations induced by 5mM αMDG. The interaction of the C4-OH with hSGTL3 was also assessed with fluorine substitution. Exposing hSGLT3 to 4-deoxy-4-fluoro-D-glucose (4D4Fglc) resulted in activation with a $K_{0.5}$ = 17 ± 1 mM and $\Delta E_{m(max)}$ = 100 ± 4% of αMDG (n = 3), neither of which were statistically different from αMDG (p > 0.2, Fig. 3). In contrast, 50 mM 4-deoxy-4-fluoro-D-galactose (4D4Fgal) had no effect on the E_m and 20 mM 4D4Fgal failed to inhibit depolarizations induced by 5 mM αMDG. In extending these fluorine substitution studies to hitherto untested aspects of hSGLT1, exposure to 4D4Fgal (n = 3) resulted in a $K_{0.5}$ = 1.3 ± 0.1 mM and I_{max} = 110 ± 2% of αMDG.

Inhibition of hSGLT3

Phlorizin (Fig. 1), a high affinity hSGLT1 competitive inhibitor also inhibited hSGLT3. The inhibition constant (K_i) was determined by measuring the inhibitory effect of 0.010 – 1.0 mM phlorizin on depolarizations induced by 10 - 40 mM αMDG. Plotting the reciprocal of these responses as a function of phlorizin concentration (Dixon plot) in three experiments yields a K_i = 0.12 ± 0.05 mM (Fig. 4). In the absence of an activating sugar, 1 mM phlorizin induced a hyperpolarization of ~2 mV, depending on the hSGLT3 expression level. In control non-injected oocytes, 1 mM phlorizin did not induce a detectable response. This suggests phlorizin blocks a sugar-independent inward current mediated by hSGLT3, as occurs with hSGLT1 (Parent *et al.*, 1992a;Parent *et al.*, 1992b).

Effects of imino sugars

The contribution of the pyranose oxygen was examined by measuring the responses to a series of imino sugars based on 1-deoxynojirimyin (DNJ, Fig. 1).

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Exposure of hSGLT3 to DNJ revealed potent activation with a $K_{0.5}$ = 4 ± 1 μM (Fig. 5A & B). To test for stereospecificity in the imino sugars as observed with glucose and galactose, hSGLT3 was exposed to 1-deoxygalactonojirimycin (DGJ) (Fig. 1), which resulted in a substantially less potent activation relative to DNJ, with a $K_{0.5}$ = 11 ± 4 mM (Fig. 5A). To examine the affect of N-alkylation and substitution at C1 of DNJ, hSGLT3 was exposed to N-ethyl-1-deoxynojirimycin (NE-DNJ) and 1-deoxynojirimycin-1-sulfonic acid (1SO-DNJ), which also resulted in potent activation with $K_{0.5}$ values = 3 ± 1 and 9 ± 4 μM (Fig 5B). The effect of N-alkylation was further tested with the clinically administered N-β-hydroxyethyl-1-deoxynojirmycin (miglitol, NEOH-DNJ) and N-butyl-1-deoxynojirimycin (miglustat, NB-DNJ), which resulted in activation with $K_{0.5}$ values = 3 ± 1 and 0.5 ± 0.1 μM (Fig 5C).

In addition, greater maximal responses relative to αMDG were observed for DGJ, DNJ and NEOH-DNJ with $\Delta E_{m(max)}$ values = 440 ± 74, 260 ± 36 and 200 ± 22% that of αMDG, respectively (Fig 5A & C). The $\Delta E_{m(max)}$ values for NE-DNJ, 1SO-DNJ and NB-DNJ of 130 ± 36, 100 ± 30 and 88 ± 11% of αMDG were not significantly different than αMDG ($p > 0.2$, Fig 5B & C). The activation of hSGLT3 by imino sugars was also studied under voltage clamp. At -50mV, the maximal inward current induced by DNJ (250 μM) was 55 nA, compared to the 19 nA induced by αMDG (100 mM) in the same oocyte. Thus, the ratio of the maximal current induced by DN and αMDG was similar (290%) to the membrane depolarizations induced by the sugars.

The depolarizations induced by hSGLT3 in response to imino sugars were tested for phlorizin sensitivity. In the presence of 1 mM phloridzin, the depolarizations induced by 2 μM DNJ, NEOH-DNJ, NE-DNJ and 1SO-DNJ were inhibited by 80, 80, 70 and

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90%; and 0.5 μM NB-DNJ was inhibited by 80%. The effect of imino sugars on hSGLT3 was Na^+-dependent, as in the absence of Na^+, 25 μM DNJ and 20 μM NEOH-DNJ induced no observable change in E_m (Data not shown).

For hSGLT1, in contrast, no observable responses were induced by exposure to 20 mM DNJ, 5 mM DGJ; 50 μM NEOH-DNJ, NE-DNJ and 1SO-DNJ; and 20 μM NB-DNJ. Similarly, no observable responses were detected in control non-injected oocytes.

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Discussion:

This study identifies specific agonists needed to elucidate the physiological role of the putative glucose sensor hSGLT3 (Diez-Sampedro *et al.*, 2003). We employ *X. laevis* oocyte expression and an electrophysiological assay to compare the substrate specificity of hSGLT3 relative to hSGLT1. The affinity and efficacy of deoxy- and imino-sugars are measured relative to those for a non-metabolized model substrate, αMDG. In general, hSGLT3 substrate binding occurs with lower affinity but greater specificity compared to hSGLT1 (Table 1). For example, the hSGLT3 apparent affinity for glucose is 40-fold lower that of hSGLT1; and hSGLT3 does not recognize galactose, whereas hSGLT1 indiscriminately transports both glucose and galactose. A striking exception to this pattern is a very high hSGLT3 affinity for imino sugars (glucose $K_{0.5}$ / NB-DNJ $K_{0.5}$ = 40,000), which are not recognized by hSGLT1 (Table 1). This indicates imino sugars are excellent substrates to determine the physiological role of hSGLT3. To the best of our knowledge, this is the first report revealing that imino sugars target hSGLT3, which may have important ramifications for the clinical application of imino sugars.

Comparison with α-glucosidases

Several aspects of hSGLT3 substrate selectivity (Table 1) are similar to those identified for α-glucosidases (Asano, 2003;Platt and Butters, 2000) for example; 1) the affinity range for imino sugars - maltase exhibits IC_{50} values of 0.36 μM for DNJ to 2.1 μM for NB-DNJ to infinity for DGJ (Godin *et al.*, 2004;Kato *et al.*, 2005); 2) at least a 1000-fold higher affinity for imino sugars than endogenous sugars; 3) N-alkylation of imino sugars is well tolerated; 4) the glucose C2-, 3-, 4-, and 6-OH groups are required for high affinity binding; and 5) the C1-OH is not required for binding. Some differences

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also exist, reflected by the higher hSGLT3 apparent affinity for NB-DNJ than DNJ, which is reversed with maltase (Kato *et al.*, 2005).

The commonalities in substrate specificity suggest similar binding site architectures in hSGLT3 and α-glucosidases. This led us to examine high-resolution crystal structures of α-glucosidases, such as the glycoamylase-DNJ complex (Harris *et al.*, 1993). Primary interactions involve the DNJ C2-, 3-, 4-, and 6-OH groups at contact distances of 2.57 - 3.32 Å from Arg-305, carbonyl-177, Arg-54 and Asp-55, respectively. The nitrogen of DNJ is within 2.82 Å of water molecules 500 and 582. Examination of the free enzyme reveals that DNJ displaces 6 of 7 well-ordered water molecules from the binding site, approximately corresponding to the DNJ C2-, 3-, 4-, and 6-OH moieties. These features mirror the interactions of carbohydrates with binding proteins and transporters (Abramson *et al.*, 2003;Quiocho, 1989;Taroni *et al.*, 2000). The essential nature of the C2-, 3-, 4-, and 6-OH groups in the glycoamylase-DNJ complex and in this analysis of hSGLT3 is consistent with a similar substrate binding site.

Primary sequence analysis of hSGLT3 and hSGLT1

Significant differences exist between hSGLT3 and hSGLT1 with respect to substrate binding; for example, in hSGLT1: 1) imino sugars do not interact; 2) the C4- or 6-OH moieties are not required for binding; and 3) orientation of the C4-OH is not important (kinetics of glucose ≈ galactose). The primary sequences of hSGLT3 & 1 were examined to explain these differences. In the C-terminal sugar binding domain of 9 SGLT1 transporters, 31 polar and aromatic side chains are conserved (Wright *et al.*, 2004;Wright *et al.*, 2006). In hSGLT3, 30 of these 31 residues are conserved, with only E457 varying from Q457 in hSGLT1. Since studies demonstrated that Q457 in hSGLT1

interacts with the pyranose oxygen (Diez-Sampedro *et al.*, 2001), we hypothesized that high affinity binding of imino sugars to hSGLT3 is due in part to a specific interaction with E457. However, a Q457E hSGLT1 mutant does not interact with DNJ and in the glucoamylase-DNJ complex, the nitrogen of DNJ interacts with only water (Harris *et al.*, 1993). A tertiary structure will likely provide important insights into the high affinity interaction between imino sugars and hSGLT3.

Glucose and imino sugar C4-OH interaction with hSGLT3 and hSGLT1

The ability of hSGLT3 to discriminate between glucose and galactose (diastereomers at C4) extends to imino sugars, as DGJ $K_{0.5}$ / DNJ $K_{0.5}$ = 2750 (Table 1). Substitution of the C4-OH with -F at the equatorial and axial positions did not significantly influence the interaction with hSGLT3, since the kinetics of 4D4Fglc ≈ glucose, and neither 4D4Fgal nor galactose interact (Table 1). The ability of -F to mimic the -OH at C4 may be the result of hydrogen-bonding; however, recent evidence suggests organic -F is unlikely to participate in hydrogen-bonds (Biffinger *et al.*, 2004;Dunitz, 2004;Hoffmann and Rychlewski, 2001;Park *et al.*, 2001). A possibility is the displacement of water molecules interacting with hSGLT3, which -F would do equally well as -OH. In the unbound state, a hydrated binding site may stabilize hSGLT3 and need to be disrupted in order for conformational changes induced by substrate binding to proceed efficiently.

For hSGLT1, less specificity with respect to the C4-OH orientation is observed, the $K_{0.5}$ of glucose ≈ galactose (Table 1). Additionally, the hSGLT1 kinetics for 4DOglc ≈ glucose, suggesting the C4-OH does not contribute significantly. However, an equatorial -F substitution increased the apparent affinity (glucose $K_{0.5}$ / 4D4Fglc $K_{0.5}$ =

7) for hSGLT1 (Table 1). This may occur through a static dipole-dipole or charge-dipole interaction (Biffinger *et al.*, 2004), for which a -OH group would have relatively less interaction.

Phlorizin Inhibition

This study reveals that phlorizin inhibits hSGLT3, albeit with lower affinity than hSGLT1 (Hirayama *et al.*, 2001). A variation between hSGLT3 & 1 is observed in the potency of phlorizin binding relative to αMDG. For hSGLT1, the αMDG $K_{0.5}$ / phlorizin K_i = 2500. Whereas, in hSGLT3, the αMDG $K_{0.5}$ / phlorizin K_i = 175 (Table 1). This may arise from structurally different hydrophobic patches (Hirayama *et al.*, 2001) adjacent to the substrate binding site. This assessment is a critical component of establishing a pharmacological profile, providing a concentration range for which phlorizin can discriminate between hSGLT3 & 1.

Variation in maximum hSGLT3 responses (efficacy)

An important aspect in identifying pharmacological probes for hSGLT3 is efficacy, the maximal response. For imino sugars, the range of $\Delta E_{m(max)}$ was 88 - 440% of that observed for αMDG. Similar results were obtained for maximum currents recorded under voltage clamp, as the I_{max} values for DNJ were 290% of those recorded for αMDG in the same hSGLT3 oocyte. N-alkylation of imino sugars appears to influence this, as the maximum depolarization for NB-DNJ ≈ αMDG and for NE-DNJ is 2-fold less than DNJ. The C1-OH also appears to be a factor, as 1DOglc exhibits a 1.7-fold greater efficacy than αMDG and substitution at C1 of DNJ results in a reduction of efficacy, 1SO-DNJ ≈ αMDG. Finally, the ability of DGJ to induce the largest hSGLT3

response suggests yet another mechanism, independent of N-alkylation or the C1-OH, influences the maximal response.

Assuming a transporter-like function, the variation in turnover number implies multiple hSGLT3 conformations between agonist binding and membrane depolarization. A similar dependence of turnover number on substrate has been observed with hSGLT1 (Diez-Sampedro *et al.*, 2000). For hSGLT3, this may result from conformational transitions proceeding at different rates depending on the agonist bound, mediated by an induced-fit mechanism (Klingenberg, 2005). For example, the butyl moiety of NB-DNJ may interact with a hydrophobic pocket in hSGLT3 and reduce the rate of a conformational change relative to DNJ. Future experiments modeling hSGLT3, such as concurrent electrophysiology and fluorescent studies (Loo *et al.*, 2005), may help in understanding this variation in efficacy.

Implications of hSGLT3 activation by imino sugars

Clinical trials employing DNJ-based therapeutics may provide important clues regarding hSGLT3 function. Recent clinical trials with NB-DNJ (miglustat) at a target plasma concentration of 10 μM indicate that patients suffered peripheral neuropathies, dose-dependent tremors, paresthesias and cognitive dysfunction (Cox *et al.*, 2000;Elstein *et al.*, 2004;Pastores and Barnett, 2003). Furthermore, in similar clinical trials (Andersson *et al.*, 2004), adverse gastrointestinal effects of NB-DNJ are not observed with the galactose isomer, N-butyl-1-deoxygalactonojirimycin (NB-DGJ). The etiology of these adverse effects is not understood (Pastores and Barnett, 2003).

This study reveals that the concentration of NB-DNJ used in the clinical test (target of 10 μM) is saturating for hSGLT3 ($K_{0.5}$ = 0.5 μM). Since hSGLT3 is expressed at the human neuromuscular junction (Diez-Sampedro *et al.*, 2003), its activation may underlie the peripheral neuropathies and tremors associated with NB-DNJ. Similarly, the hSGLT3 substrate specificity presented here may explain the adverse enteric effects attributable to NB-DNJ but not NB-DGJ. This correlation is supported by reports indicating SGLT-mediated (Gribble *et al.*, 2003;Kim *et al.*, 2001;Raybould *et al.*, 2004) and more specifically SGLT3-mediated (Freeman *et al.*, 2006) glucose sensing in studies of enteric regulation and nutrient sensing. The substrate specificities identified here, particularly the potent and specific activation by imino sugars will serve as a powerful tool to examine the contribution of hSGLT3 to these adverse effects and explore the physiological role of hSGLT3.

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Legends for Figures:

Figure 1. Chemical structures of glucose, 1-deoxynojirimycin (DNJ), 1-deoxynojirigalactomycin (DGJ) and phlorizin.

Figure 2. **hSGLT3 in *Xenopus laevis* oocytes.** A, a time course of the resting membrane potential from an oocyte expressing hSGLT3 illustrating analogous 5 mV membrane depolarizations (upward deflections) induced by 20 mM αMDG and glucose. The oocyte was under constant perfusion and the sugars were dissolved in Na^+ buffer (see *Materials and Methods*). The resting membrane potential was -42 mV. B, from the same hSGLT3 oocyte, a representative plot of the membrane depolarizations induced by 0 - 100 mM αMDG and glucose. The dose-response relations were fitted with Michaelis-Menten kinetics (see Materials and Methods) and the $K_{0.5}$ values presented are the mean ± sd from 42 oocytes for αMDG and 3 oocytes for glucose.

Figure 3. **Activation of hSGLT3 by deoxy-glucose analogs.** Representative dose-response curves of hSGLT3 activation by 0 – 100 mM 1-deoxy-D-glucose (1DOglc) and 4-deoxy-4-fluoro-D-glucose (4D4Fglc). The curves were normalized to the maximum depolarization observed for αMDG (100 mM) from the same oocyte. The $K_{0.5}$ values presented are the average values obtained from 4 oocytes for 1DOglc and 3 oocytes for 4D4Fglc.

Figure 4. **Inhibition of hSGLT3 by phlorizin.** A Dixon plot illustrating the ability of phlorizin (0.010 – 1.0 mM) to inhibit hSGLT3 responses (membrane depolarizations) to

20 and 40 mM αMDG. The reciprocal of the depolarizations measured for each αMDG concentration were plotted as a function of phlorizin concentration. A linear regression of the responses from each αMDG concentration was generated and the inhibition constant (K_i) was derived from the points of intersection (see Material and Methods). The K_i value presented is the average value obtained from 3 different experiments.

Figure 5. **Activation of hSGLT3 by imino sugars.** The typical membrane depolarizations induced by hSGLT3 in response to: A, 0.5 – 20 μM DNJ and 200 – 30000 μM 1-deoxygalactonojirimycin (DGJ), a logarithmic scale was used for the large concentration range; B, 0 – 20 μM 1-deoxynojirimycin (DNJ), N-ethyl-1-deoxynojirimycin (NE-DNJ) and 1-deoxynojirimycin-1-sulfonic acid (1SO-DNJ); and C, 0 – 20 μM N-hydroxyethyl-1-deoxynojirimycin (NEOH-DNJ, miglitol) and 0 – 10 mM N-butyl-1-deoxynojirimycin (NB-DNJ, miglustat). Each response was normalized to the maximum depolarization observed for αMDG (100 mM) from the same oocyte. The $K_{0.5}$ values presented for each substrate are the average values obtained from 3 oocytes.

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Table 1. **A summary of the hSGLT3 and hSGLT1 kinetics**.

Activation parameters ± standard deviations were obtained from 3 - 42 different oocytes and the phlorizin K_i ± standard deviation was obtained from three different αMDG concentrations. *Not determined* indicates the small membrane depolarizations precluded an accurate kinetic assessment.

Substrate	**hSGLT3**		**hSGLT1**	
	$K_{0.5}$ (mM)	**$\Delta E_{m(max)}$ (% αMDG)**	**$K_{0.5}$ (mM)**	**$\Delta E_{m(max)}$ (% αMDG)**
D-Glucose	19 ± 6	108 ± 3	0.5 ± 0.02[a]	83 ± 5[a]
α-Methyl-D-Glucose	21 ± 6	100	0.7 ± 0.04[a]	100
1-Deoxy-D-Glucose	43 ± 10	170 ± 20	10 ± 1[a]	83 ± 5[a]
2-Deoxy-D-Glucose	Not Detected		>100[a]	83 ± 5[a]
3-Deoxy-D-Glucose	Not Detected		Not Detected	
4-Deoxy-D-Glucose	> 50 mM	Not determined	0.4 ± 0.05	100 ± 3
4-Deoxy-4-Fluoro-D-Glucose	17 ± 1	100 ± 4	0.07 ± 0.01[a]	83 ± 5[a]
D-Galactose	Not Detected		0.6 ± 0.02[a]	83 ± 5[a]
4-Deoxy-4-Fluoro-D-Galactose	Not Detected		1.3 ± 0.09	110 ± 2
6-Deoxy-D-Glucose	> 50 mM	Not determined	3 ± 0.5[a]	83 ± 5[a]
1-Deoxynojirimycin	0.004 ± 0.001	260 ± 36	Not Detected	
1-Deoxygalactonojirimycin	11 ± 4	440 ± 74	Not Detected	
N-Ethyl-1-Deoxynojirimycin	0.003 ± 0.001	130 ± 36	Not Detected	
N-Hydroxyethyl-1-	0.003 ± 0.001	200 ± 22	Not Detected	

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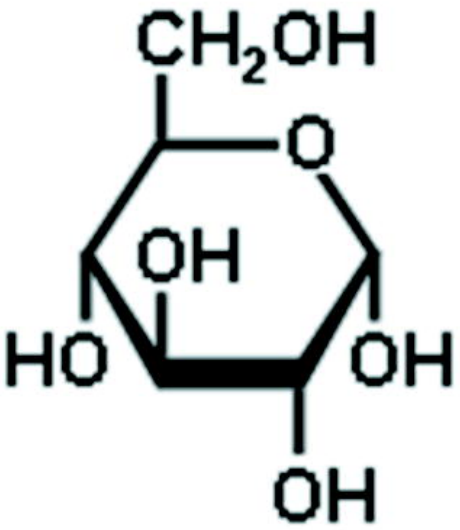
Deoxynojirmycin (miglitol)				
N-Butyl-1-Deoxynojirimycin (miglustat)	0.0005 ± 0.0001	88 ± 11	Not Detected	
1-Deoxynojirimycin-1-Sulfonic Acid	0.009 ± 0.004	100 ± 30	Not Detected	
Inhibitor	**K_i (mM)**	**% Inhibition**	**K_i (mM)**	**% Inhibition**
Phlorizin	0.12 ± 0.05	100	0.0002 ± 0.00001[b]	100[b]

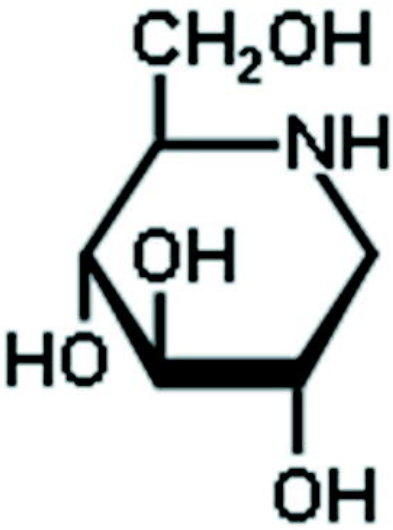
[a] (Diez-Sampedro *et al.*, 2001)

[b] (Hirayama *et al.*, 2001)

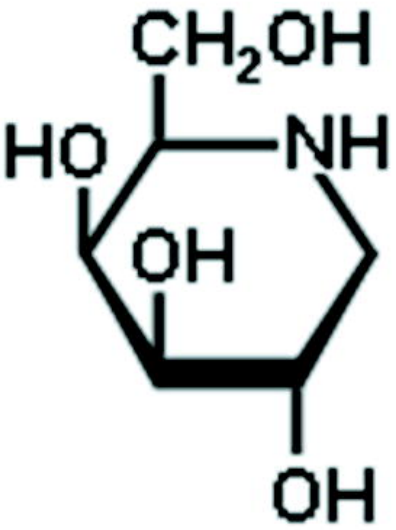
Figure 1



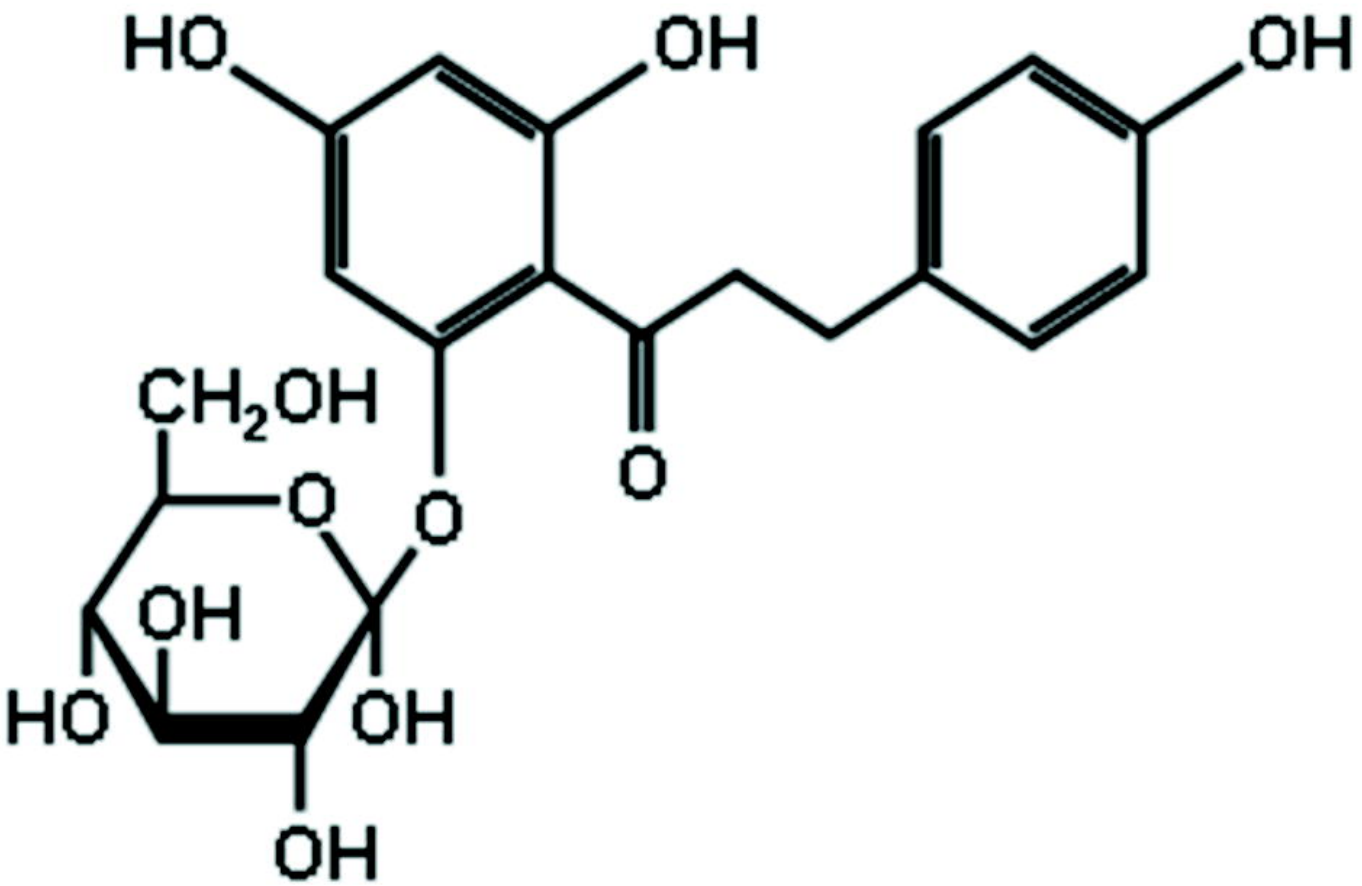
D-Glucose



DNJ



DGJ



Phlorizin

Figure 2

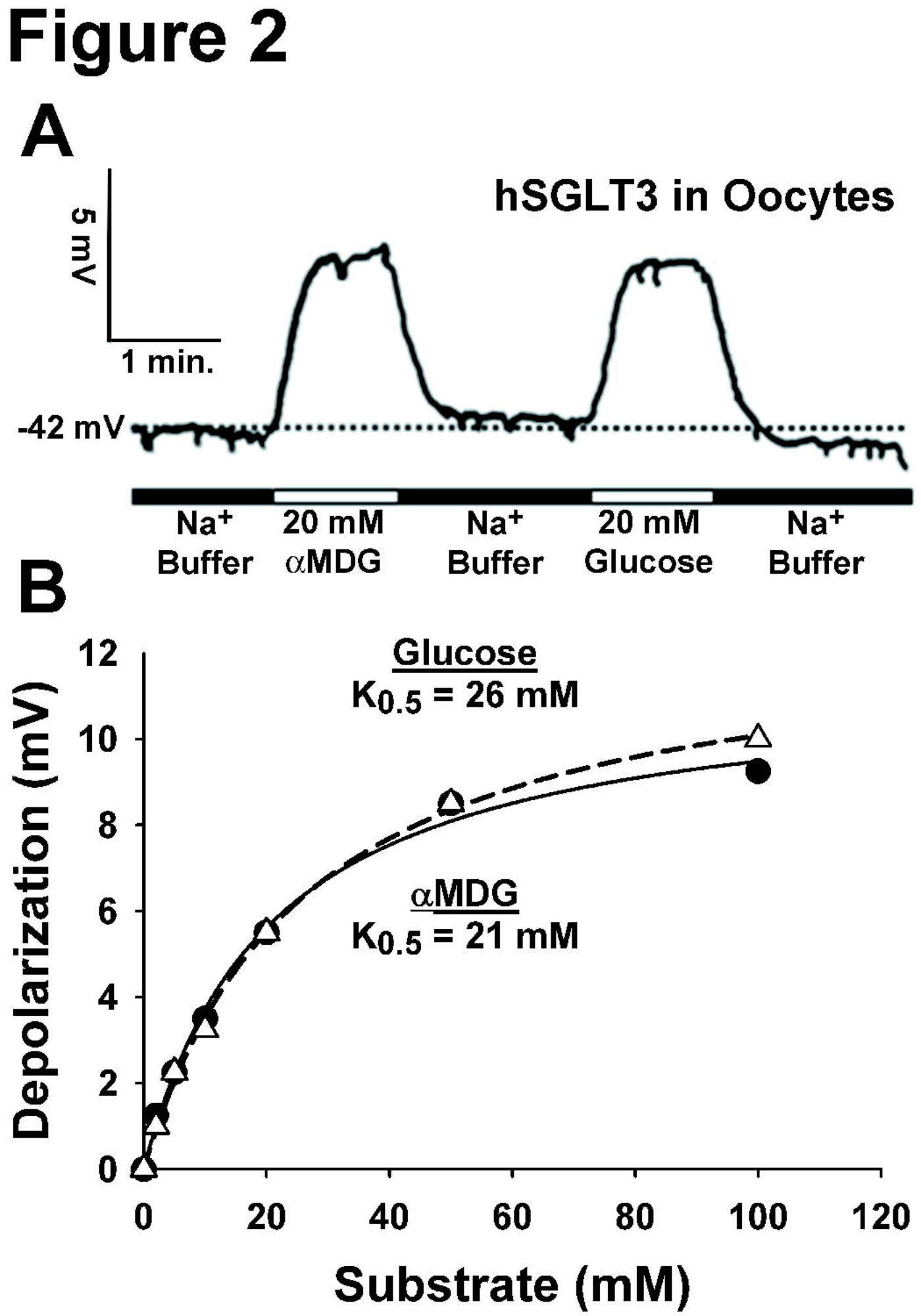

A
hSGLT3 in Oocytes
5 mV
1 min.
-42 mV
Na+ Buffer
20 mM αMDG
Na+ Buffer
20 mM Glucose
Na+ Buffer
B
Glucose
K0.5 = 26 mM
αMDG
K0.5 = 21 mM
Depolarization (mV)
Substrate (mM)
0
2
4
6
8
10
12
0
20
40
60
80
100
120

Figure 3

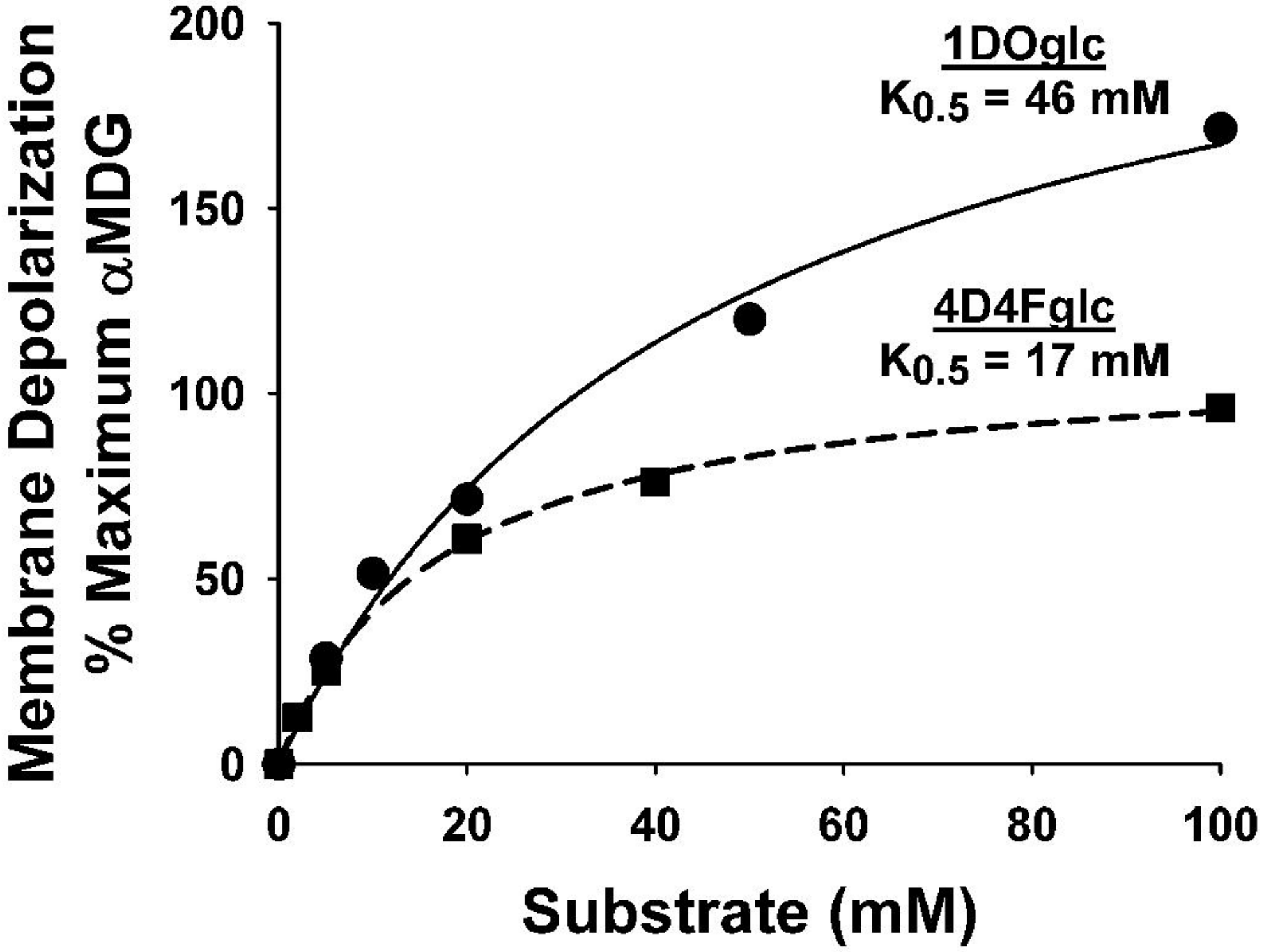

Membrane Depolarization
% Maximum αMDG
200
150
100
50
0
0
20
40
60
80
100
Substrate (mM)
1DOglc
K0.5 = 46 mM
4D4Fglc
K0.5 = 17 mM

Figure 4

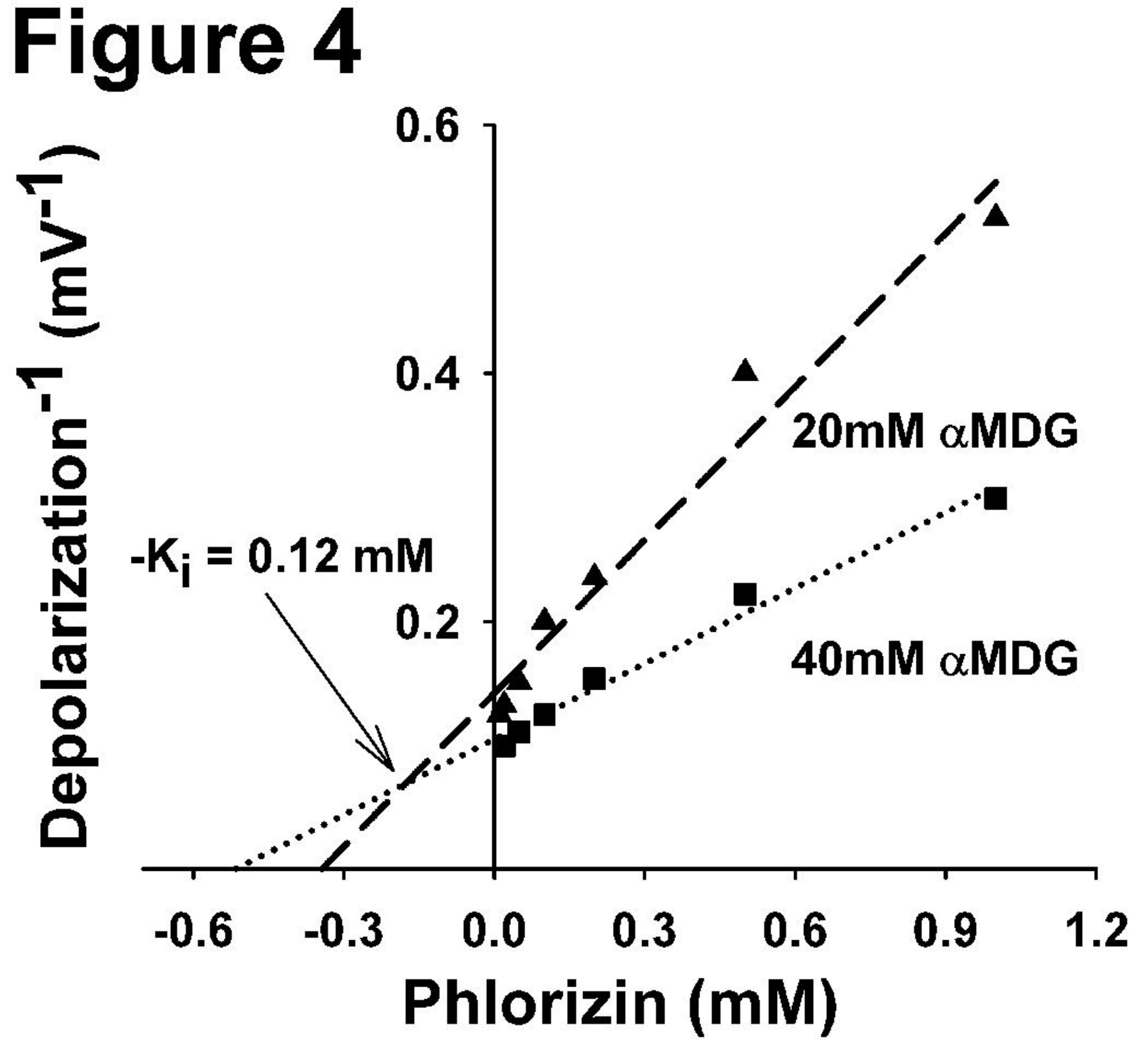
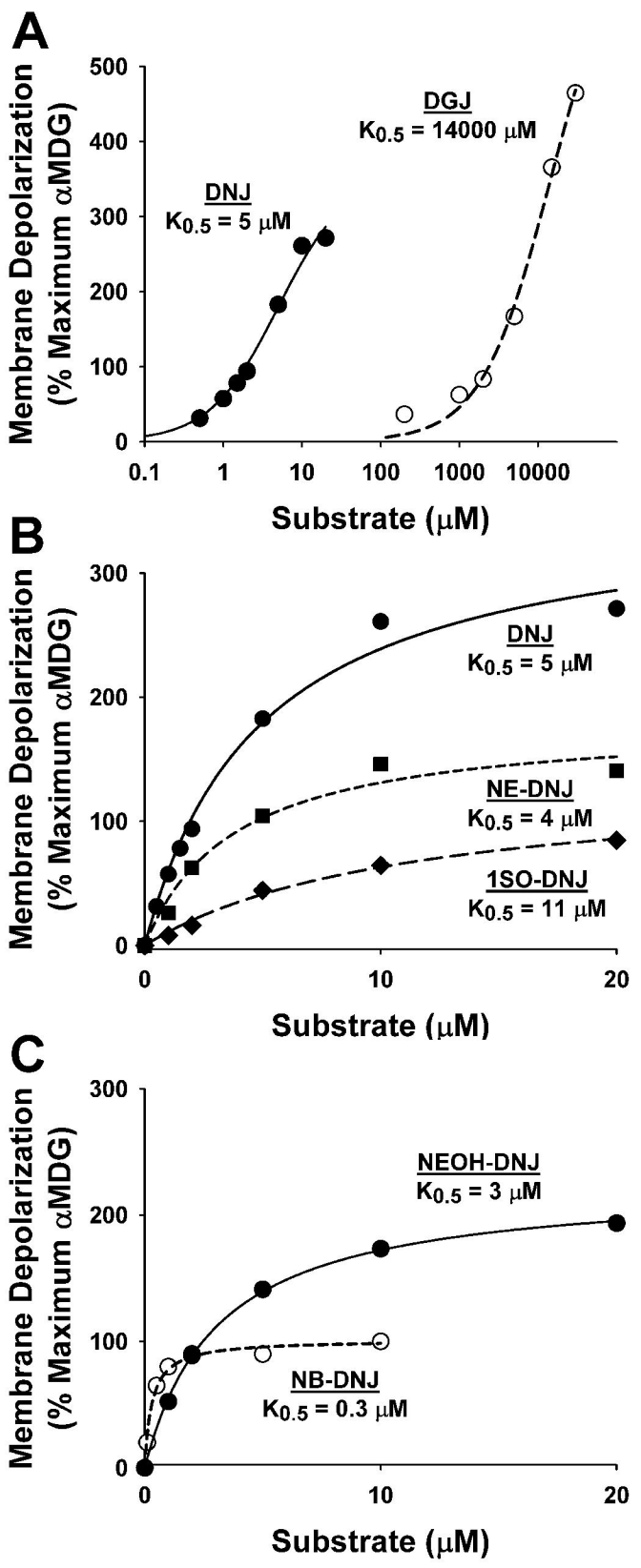

0.6
0.4
0.2
Depolarization^{-1} (mV^{-1})
20mM αMDG
40mM αMDG
-K_i = 0.12 mM
-0.6
-0.3
0.0
0.3
0.6
0.9
1.2
Phlorizin (mM)

Figure 5


A
Membrane Depolarization (% Maximum αMDG)
500
400
300
200
100
0
DGJ
K0.5 = 14000 μM
DNJ
K0.5 = 5 μM
0.1
1
10
100
1000
10000
Substrate (μM)
B
Membrane Depolarization (% Maximum αMDG)
300
200
100
0
DNJ
K0.5 = 5 μM
NE-DNJ
K0.5 = 4 μM
1SO-DNJ
K0.5 = 11 μM
0
10
20
Substrate (μM)
C
Membrane Depolarization (% Maximum αMDG)
300
200
100
0
NEOH-DNJ
K0.5 = 3 μM
NB-DNJ
K0.5 = 0.3 μM
0
10
20
Substrate (μM)