Published in final edited form as:

Mol Pharm. 2017 August 07; 14(8): 2726–2739. doi:10.1021/acs.molpharmaceut.7b00285.

Incorporation of a Biguanide Scaffold Enhances Drug Uptake by Organic Cation Transporters 1 and 2

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Abstract

Membrane transporters play a significant role in the transport of many endogenous and exogenous compounds. The knowledge of transporter substrate requirements has allowed further development of drugs that utilize them to ensure tissue permeation. In this study, we demonstrate that inclusion of a biguanide functionality can potentiate uptake by the organic cation transporters 1 and 2 (OCT1 and OCT2). We synthesized 18 pairs of structurally diverse compounds, each pair consisting of a parent amino compound and its biguanide analog; and then assessed their cellular uptake in HEK293 cells overexpressing human OCT1 or OCT2. Our results show that addition of the biguanide significantly improved OCT1- and OCT2- mediated transport for the majority of compounds. The biguanides also inhibited the uptake of prototypical substrates of both transporters, 1-methyl-4-phenylpyridinium (MPP⁺) and metformin. We found that molecular weight, molecular volume, Log D (pH 7.4), and accessible surface area were important determinants of OCT2 substrates, but none of these parameters was a significant factor for OCT1. More so, the inhibition of MPP⁺ uptake correlated linearly with that of metformin uptake for the tested biguanides in both cell lines. Taken together, we conclude that the inclusion of the biguanide scaffold in nonsubstrates of OCT1 and OCT2 increase their propensity to become substrates and inhibitors for these transporters.

Keywords

organic cation transporter; metformin; biguanide; substrate; liver

INTRODUCTION

Membrane transporters, such as those of the solute carrier superfamily including organic cation transporters (OCT, SLC22), organic cation/carnitine transporters (OCTN, SLC22),

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.7b00285. Supplementary results for confirmation of OCT1 and OCT2 expression at the protein and mRNA levels in different cell lines; correlation of OCT1 and OCT2 uptake fold increase; uptake results conducted with OCT3-, MATE1-, and MATE2K-overexpressing HEK293 cells; and further analysis for physicochemical properties correlation with uptake and inhibition values (PDF)

The authors declare no competing financial interest.

multidrug and toxin extrusion (MATE, SLC47), and peptide transporters (PEPT, SLC15), play a crucial role in the absorption, distribution, elimination, and toxicity of many drugs.¹⁻⁴ These drug transporters are widely distributed in the body and are particularly expressed in organs of drug disposition, such as intestine, liver, kidney, and brain, where they facilitate the entry or exit of xenobiotics and endogenous substrates.⁵⁻⁷ Drug development has been directed to take advantage of these transporters to improve drug bioavailability especially through the intestine.⁸ For instance, incorporation of the L-carnitine moiety into drug compounds valproyl, ketoprofen, and naproxen has been shown to dramatically improve their uptake by OCTN2.⁹ Valacyclovir, a prodrug of acyclovir, is another successful application of membrane transporter knowledge to improve bioavailability.¹⁰ Acyclovir has an oral bioavailability of 20% while valacyclovir has incorporated in it an ester that aids its uptake *via* PEPT1 and PEPT2, enhancing the bioavailability to 50%. In the same way, the tissue expression pattern of a transporter may be utilized to achieve targeted therapy in order to improve drug exposure while decreasing off-target toxicity.¹¹⁻¹³

The OCTs have been implicated in the transport of endogenous and exogenous cationic compounds, including biguanides, tetraethylammonium, catecholamines, cisplatin, and histamine.^{14,15} This class of transporter proteins are present in humans in three isoforms: OCT1 (SLC22A1), OCT2 (SLC22A2), and OCT3 (SLC22A3). OCT1 is predominantly expressed on the sinusoidal basolateral membrane of liver epithelial cells¹⁶ and OCT2 in the basolateral membrane of renal proximal tubule epithelium cells.¹⁵ OCT3 is expressed in various tissues including brain, testis, and with relatively low levels in the liver and kidney.^{14,17} Known to be polyspecific in nature, this class of membrane transporters has many overlapping substrates (reviewed in detail elsewhere¹⁷⁻¹⁹). Among the substrates, of particular interest is the biguanide class, which includes three previously and currently used antidiabetics, metformin, buformin, and phenformin. This class of compounds is also transported by the MATE transporters,¹⁷ which have high expression in the liver and kidney cells to aid the extrusion of endogenous substrates and xenobiotics.²⁰ In many cases, these MATE transporters work in conjunction with OCTs to mediate the uptake and elimination of substrates.^{17,21} For instance, phenformin and metformin exhibit enhanced bioavailability through their uptake by enterocytes and accumulation in the liver, while being excreted by the kidney through OCTs/MATEs transporters.^{2,23} Thus, the biguanide moiety may allow for efficient uptake of compounds into the liver and kidney, which is mediated by OCTs, and subsequent MATE-mediated biliary and renal excretion. We propose that inclusion of the biguanide moiety to other classes of compounds would allow proper understanding of the structural scope of biguanides tolerated by OCTs.

Although the function and clinical implication emanating from use of OCTs as propagators of uptake into tissues have been well appreciated, there is not much information associated with leveraging this knowledge in further modifying available compounds to attain targeted delivery to certain tissues. This is especially important in cases where the site of action of the compound in question is either liver or kidney.^{24,25} As an excellent OCT substrate, the biguanide-based drug metformin tends to undergo distribution to organs predominantly expressing OCTs.²⁶ For many liver diseases, such as hepatitis, liver cysts, cirrhosis, steatohepatitis, and hepatocellular carcinoma, targeted delivery of drugs to liver is especially important to reduce drug toxicity. On the other hand, efficient elimination from the liver and

kidney is also critical to drug detoxification. If our concept could be proven, new therapeutic agents may be further designed and developed to either target the liver *via* highly expressed OCT1 or have improved elimination because of the efficient organic cation transport system in the liver and kidney.

In this study, we sought to determine whether incorporation of the biguanide group in structurally diverse compounds would improve their uptake by OCT1 and/or OCT2. Metformin, 18 parent amines (including 2-phenylethylamine that is the parent compound of phenformin), and their biguanide analogues were tested in HEK293 cells overexpressing the two uptake transporters. The chosen pairs of compounds were within a molecular volume of 300 Å³, having polar surface area (PSA) ranging from 81 to 173 Å² and consisting largely of compounds with lipophilic side chains due to increased number of drugs which are very lipophilic. We also investigated if the addition of the biguanide scaffold would alter the inhibitory effects of these compounds on OCT1 and OCT2.

EXPERIMENTAL SECTION

Chemical Reagents and Cell Culture.

Trypsin, penicillin-streptomycin solution, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), HEPES, sodium chloride, potassium chloride, D-glucose, potassium phosphate, calcium chloride, magnesium sulfate, and all chemicals used for synthesis (purity >98%) were purchased from Sigma-Aldrich (St. Louis, MO). Poly-D-lysine coated plates and T-175 cell culture flasks were purchased from Corning (Lowell, MA). Acetonitrile and methanol (HPLC grade) were obtained from Sigma-Aldrich.

HEK293 cells obtained from ATCC were stably transfected with OCT2 or empty vector and selected with hygromycin B as was previously described.²⁷ For OCT1 experiments, HEK293 cells were transiently transfected with a functional EGFP-hOCT1 plasmid as previously described²⁸ about 48 h prior to uptake studies. OCT3-, MATE1-, and MATE2K-overexpressing HEK293 cells have been previously described.^{27,29} The cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin solution at 37 °C in a humidified 5% CO₂. Cells at 70–80% confluence were washed with phosphate buffered saline (pH 6.8), and trypsin/EDTA (0.05%) was added for 3 min. The cell suspension was collected in 5 mL of growth medium, and seeded into wells for uptake studies.

Cells seeded in poly-D-lysine coated 24-well plates at a density of 2×10^5 cells/well were used for uptake studies. Cell monolayers were allowed to reach total confluency prior to experiments, about 24 h after seeding. Before each experiment, the Krebs Ringer-Hepes (KRH) uptake buffer (containing 25 mM HEPES, 125 mM sodium chloride, 4.8 mM potassium chloride, 5.6 mM D-glucose, 1.2 mM potassium phosphate, 1.2 mM calcium chloride, 1.2 mM magnesium sulfate) was prepared.

Immunoblot Analysis.

Lysates of HEK293 cells were mixed 1:1 with 2X Laemmli Sample Buffer (Bio-Rad) and resolved on a 4–15% gradient Mini-PROTEAN TGX Gel (Biorad). Western blots were then

performed on a nitrocellulose membrane, blocked with 5% milk in TBST (0.1% Tween 20 in 1X Tris-buffered saline). The membranes were incubated with anti-SLC22A1 or anti-SLC22A2 antibodies (MA5–15730 and PA5–37290, Thermo Fisher Scientific) at 1:1000 or anti- β -actin at 1:5000 (AB8227, Abcam Plc). Chemiluminescence was used to visualize the proteins using Odyssey Fc Imaging System (LI-COR Biosciences).

Real-time PCR.

Total RNA was isolated from HEK293 cells stably or transiently expressing either empty vector or vectors containing OCT transporters using the trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The RNA was reverse transcribed to complementary DNA, and real-time polymerase chain reaction was performed using primers designed with Oligo 5.0 and synthesized by Integrated DNA Technologies. The delta ct method was used to analyze the gene expression and normalized to that of GAPDH.³⁰

Uptake Studies.

The OCT1, OCT2, OCT3, and empty vector cells were washed twice with prewarmed KRH uptake buffer to remove any floating cells. Compounds diluted in uptake buffer were replaced in the wells and incubated for the respective study conditions. For time dependent uptake of metformin and MPP⁺, we incubated the cells with uptake buffer containing 50 μ M metformin or 5 μ M MPP (containing 2.5% ¹⁴C-metformin or ³H-MPP⁺, respectively) for 1, 2, 5, 10, and 30 min. At each time point, cells were washed with cold uptake buffer three times and lysed in 1% Triton X-100 in phosphate-buffered saline for 1 h on a shaker. The lysates were mixed 1:10 with scintillation cocktail (Ultima Gold, PerkinElmer) and the radioactivity was analyzed with a Packard Tri-Carb 2500 TR liquid scintillation counter (PerkinElmer). For screening of compounds at one concentration and one-time point, the cells were incubated with 100 μ M of test compounds to allow detection across the panel of compounds studied. In each experiment, metformin was used as reference positive control. The incubation was stopped by removing the solution and adding 500 μ L of ice-cold uptake buffer (without compounds). The uptake studies with MATE1 and MATE2K overexpressing cell lines were performed as previously described.³¹ Potassium-based buffer (KBB) (containing 10 mM HEPES, 140 mM KCl, 0.4 mM KH₂PO₄, 1.0 mM CaCl₂, 25 mM glucose, 0.8 mM MgSO₄, and 30 mM NH₄Cl) at pH 7.4 was incubated with the cells for 15 min at 37 °C. Subsequently, we washed the cells once with KBB uptake buffer without NH₄Cl and then incubated with 100 μ M of **2f** or **2j** in KBB buffer without NH₄Cl for 10 min. The cells were subsequently washed thrice and after washing, 200 μ L of lysing solution (1:1 methanol in water) containing internal standard (compound k as described below or metformin) was added. The cell plates were shaken with the lysing solution for at least 10 min at room temperature. The cell lysates were then centrifuged at 12000 rpm for 15 min. Aliquots of the supernatant were transferred to new tubes and drug concentration was quantified by LC-MS/MS as described below.

Inhibition Studies.

Similar to the uptake studies, cells were seeded 24 h before the experiments. The compounds were incubated at 50 μ M with or without 5 μ M MPP⁺ or 50 μ M metformin containing 2.5%

^3H -MPP⁺ or ^{14}C -metformin, respectively (Moravek Biochemicals, Brea, CA), in KRH uptake buffer. Following the incubation period, the cells were washed with uptake buffer three times and lysed with 1% Triton X-100. Cell lysates were mixed with scintillation cocktail at 1:10 ratio and the radioactivity was analyzed with a Packard Tri-Carb 2500 TR liquid scintillation counter.

Liquid Chromatography–Mass Spectrometry (LC/MS/MS) Analysis.

The analysis of compound in the supernatant was carried out on Agilent 1200 series UPLC equipment (Agilent Technologies, California) equipped with a hybrid triple quadrupole 4000 QTRAP system (AB SCIEX, Massachusetts). The biguanide samples were quantified using a Cortecs HILIC 2.7 μm 2.1 $\times$ 100 mm column and that of the parent compounds were quantified using a Cortecs C18+ 2.7 μm 2.1 $\times$ 75 mm column. A 5 min gradient elution with 0.4 mL/min flow rate was used for all samples. The gradient is as follows: for biguanides –99% to 1% mobile phase B over 1.5 min, 99% to 1% A over 2 min, and 99% B over 1.5 min; for the parent compounds –99% to 1% A over 1.5 min, 99% to 1% B over 2 min, and 99% A over 1.5 min. Mobile phase A was made up of 0.1% formic acid and 10 mM ammonium acetate in water while B was made up of 0.1% formic acid in acetonitrile. The ion transitions for each of the compounds were monitored in the positive ion electrospray ionization mode and annotated (Table S1). The peak areas obtained for each of the compounds were adjusted by the peak area of the internal standard and protein concentration, estimated using the bicinchoninic acid assay.

Computational Analysis and Compound Physicochemical Properties.

All calculations were done using molecular operating environment (MOE) (Chemical Computing Group Inc., Montreal). First, all 2D structures were converted into 3D structures in mol2 format files and then all possible conformations were generated using the “Conformation Import” application in MOE with default calculation settings. Potential energies were evaluated using the MMFF94x force field.³¹ Three shape descriptors were considered here to describe the shapes of all the biguanide derivatives. This includes accessible surface area (ASA), van der Waals surface area and van der Waals molecular volume using Boltzmann weighted average. LogD and molecular weight were calculated using ACD/PhysChem Suite (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

Data and Statistical Analysis.

Statistical analysis for each sample set was carried out using the Student’s paired *t*-test and a *p*-value less than 0.05 was considered statistically significant. Data presented are an average of at least three determinations and error bars presented in each figure denotes the standard deviation of triplicate samples except otherwise noted.

Total uptake by OCT1-, OCT2-, or empty vector-transfected cells was calculated by dividing the amount of compound quantified in the cell lysate by the amount of total protein present in the well. The standard deviations were calculated using error propagation. Then the uptake fold increase was calculated as noted below:

$$\text{Uptake Fold Increase} = \frac{\text{Uptake}_{\text{HEK-OCT}}}{\text{Uptake}_{\text{Empty vector}}}$$

$$\text{Metformin / MPP}^+\text{Uptake Fold} = \frac{\text{Substrate uptake}_{\text{HEK-OCT with inhibitor}}}{\text{Substrate uptake}_{\text{HEK-OCT without inhibitor}}}$$

Where $\text{Uptake}_{\text{HEK-OCT}}$ is the uptake in cells overexpressing the transporter, $\text{Uptake}_{\text{Empty vector}}$ is the uptake in cells transfected with the empty vector, $\text{Substrate Uptake}_{\text{HEK-OCT without inhibitor}}$ is the uptake of metformin or MPP^+ alone, and $\text{Substrate Uptake}_{\text{HEK-OCT with inhibitor}}$ is the uptake of metformin or MPP^+ with each compound acting as an inhibitor. Concentration dependent uptake data were fitted to the Michaelis–Menten equation and a nonsaturable uptake component described by the equation below:

$$V = \frac{V_{\max} * S}{K_m + S} + P * S$$

In the equation above, V is the uptake rate, $V_{\max}$ represents the maximum uptake rate, K_m is the Michaelis–Menten constant, S is the substrate concentration used, and P is the nonsaturable clearance parameter. For the correlation analysis, Pearson comparison was performed between uptake fold increase values and physicochemical parameters (molecular weight, molecular volume, accessible surface area, and Log D at pH 7.4) to determine their strength of association with the fold increase. Person correlation coefficient (r) values were assessed on the basis of low (± 0.3 to 0.5), moderate (± 0.5 to 0.7), or strong (± 0.7 to 1).

General Experimental Methods for Compound Synthesis.

Starting materials were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. All ^1H NMR and ^{13}C NMR spectra were recorded on spectrometers operating at 400 and 100 MHz, respectively. Chemical shifts (δ) are expressed in ppm, and coupling constants J are given in hertz. Proton chemical shifts are reported relative to residual solvent peak (CDCl_3 at 7.26 ppm, CD_3OD at 3.31 ppm, and $\text{DMSO}-d_6$ at 2.50 ppm). The following abbreviations are used for signal multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, quin = quintet, sept = septet, dt = doublet of triplets. Carbon chemical shifts are reported relative to solvent peaks (CDCl_3 at 77.0 ppm and $\text{DMSO}-d_6$ at 39.51 ppm). Mass spectra were recorded using electrospray as the ionization technique.

Synthesis and Characterization of the Final Products.

To a solution of **1a–q** (1.5 mmol) and **3** (84 mg, 1.0 mmol) in CH_3CN (1.0 mL) was added TMSCl (1.5 mmol, 190.4 μL). The mixture was allowed to stir at 120 °C overnight. After cooling to room temperature, the resulting precipitate was filtered and washed with acetone, or acetone and DCM (**2e** and **2b**) or cold CH_3CN (**2c**) (Figure 1).

The final products consist of pairs of compounds, an amine and its biguanide analogue. The R groups used were made of lipophilic (a–h, j–l, o–q, phenformin) and hydrophilic (i, m, n) chains. As for their physicochemical properties, molecular volume were within 300 Å³; the PSA for the lipophilic chains ranged from 81 to 96 Å², and that of the hydrophilic chains ranged from 121 to 173 Å². Chemical characterizations of the obtained final products are as follow.

1-n-Pentylbiguanide HCl (2a).—White solid. Yield: 21%, 43.5 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.56 (br, 1H), 6.97 (br, 6H), 3.10–3.04 (m, 2H), 1.44–1.41 (m, 2H), 1.26–1.25 (m, 4H), 0.87–0.84 (m, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 159.1, 41.2, 28.9, 22.3, 14.3, 2.3. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₇H₁₇N₅: 172.1557, found: 172.1559.

1-(4-Phenyl-butyl)-biguanide HCl (2b).—Yellow solid. Yield: 21%, 57.4 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.59 (br, 1H), 7.29–7.25 (m, 2H), 7.20–7.14 (m, 3H), 6.95 (br, 5H), 3.11 (br, 2H), 2.59–2.55 (m, 2H), 1.60–1.56 (m, 2H), 1.47–1.45 (m, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 159.1, 157.4, 142.4, 128.7, 126.1, 41.0, 35.2, 28.9, 28.6. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₂H₁₉N₅: 233.3190, found: 234.1722.

1-(3-Phenylpropyl)-biguanide HCl (2c).—White solid. Yield: 22%, 56.7 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.68 (br, 1H), 7.28–7.25 (t, *J* = 6.8 Hz, 2H), 7.22–7.20 (d, *J* = 6.8 Hz, 2H), 7.18–7.15 (t, *J* = 7 Hz, 1H), 6.95 (br, 6H), 3.10–3.07 (m, 2H), 2.62–2.58 (t, *J* = 7.4 Hz, 2H), 1.75–1.71 (m, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 159.2, 142.0, 128.8, 128.7, 126.2, 32.8, 31.2, 2.3. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₁H₁₇N₅: 220.1557, found: 220.1557.

1-(p-Chlorophenethyl)-biguanide HCl (2d).—White solid. Yield: 31%, 74.1 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.46 (br, 1H), 7.35–7.33 (d, *J* = 8 Hz, 2H), 7.28–7.26 (d, *J* = 8.4 Hz, 2H), 6.98–6.94 (br, 4H), 6.72 (s, 2H), 3.33–3.28 (m, 2H), 2.77–2.73 (t, *J* = 7 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.4, 158.9, 138.5, 131.3, 131.1, 128.7, 118.9, 42.6, 34.6. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₀H₁₄ClN₅: 240.1010, found 240.1023.

2-(2,4-Dichlorophenyl)ethyl-biguanide (2e).—White solid. Yield: 1%, 4.0 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.59 (s, 1H), 7.43–7.37 (m, 2H), 6.91 (br, 6H), 3.31–3.29 (m, 2H), 2.89–2.86 (t, *J* = 7 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 158.9, 136.0, 134.5, 132.9, 132.2, 129.0, 127.8, 40.7, 32.7. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₀H₁₃Cl₂N₅: 274.0621, found: 274.0624.

2,2-Diphenylethyl-biguanide (2f).—White solid. Yield: 64%, 180.3 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.30–7.29 (d, *J* = 3.6 Hz, 8H), 7.21–7.17 (m, 2H), 7.09–6.71 (br, 6H), 4.25–4.22 (t, *J* = 7.6 Hz, 1H), 3.79–3.75 (t, *J* = 6.4 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 142.9, 129.0, 128.3, 126.9, 50.4, 45.6. HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₆H₁₉N₅: 282.1713, found: 282.1718.

2-(4-Biphenyl)ethyl-biguanide (2g).—White solid. Yield: 15%, 40.9 mg. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.64–7.62 (d, *J* = 8 Hz, 2H), 7.60–7.58 (d, *J* = 8 Hz, 2H), 7.46–7.43 (t, *J*

= 7.6 Hz, 2H), 7.35–7.33 (m, 3H), 6.95–6.91 (br, 6H), 3.38–3.36 (m, 2H), 2.83–2.79 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 159.0, 140.4, 138.8, 138.6, 129.8, 129.4, 127.7, 127.1, 127.0, 42.8, 35.0. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{16}\text{H}_{19}\text{N}_5$: 282.1713, 282.1722.

1-(2-Phenoxyethyl)-biguanide (2h).—White solid. Yield: 72%, 160.1 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 7.92 (s, 1H), 7.8–7.6.85 (br, 6H), 7.31–7.27 (t, J = 7.8 Hz, 3H), 6.95–6.92 (d, J = 8.8 Hz, 2 H), 4.06–4.03 (t, J = 5.2 Hz, 2H), 3.56–3.52 (m, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 158.5, 157.8, 130.0, 121.4, 115.0, 66.3, 40.8. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}$: 222.1349, found: 180.1250.

1-(p-Methoxybenzyl)-biguanide HCl (2i).—White solid. Yield: 16%, 40.3 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 7.73 (br, 1H), 7.24–7.22 (d, J = 8.8 Hz, 2H), 6.97 (br, 6H), 6.90–6.88 (d, J = 8 Hz, 2H), 4.26–4.25 (d, J = 5.2 Hz, 2H), 3.73 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 160.5, 158.8, 131.0, 129.1, 114.2, 55.5, 44.0. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}$: 222.1349, found: 222.1358.

2,3-Dihydro-1H-inden-2-yl-biguanide HCl (2j).—White solid. Yield: 9%, 23.2 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 7.64 (br, 1H), 7.22–7.21 (m, 2H), 7.16–7.13 (m, 2H), 7.0 (br, 6H), 4.38 (br, 1H), 3.22–3.16, (dd, J_1 = 7.2 Hz, J_2 = 15.6 Hz, 2H), 2.84–2.79 (dd, J_1 = 4.6 Hz, J_2 = 15.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 141.3, 127.0, 124.9, 52.45. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{11}\text{H}_{15}\text{N}_5$: 218.1400, found: 218.1404.

1-(p-Methyl)-biguanide HCl (2k).—White solid. Yield: 5%, 12.3 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 9.60 (s, 1H), 7.22–7.21 (m, 6H), 7.10–7.08 (d, J = 7.6 Hz, 2H), 7.02 (br, 2H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 166.1, 160.7, 141.1, 137.7, 134.3, 126.4, 25.6. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_9\text{H}_{13}\text{N}_5$: 192.1244, found: 192.1252.

1-(p-Chlorophenyl)-biguanide HCl (2l).—White solid. Yield: 30%, 74.2 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 9.93 (s, 1H), 7.42–7.39 (m, 6H), 7.35–7.33 (m, 2H), 7.09 (br, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 166.6, 160.0, 143.0, 133.7, 132.0, 127.3. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_8\text{H}_{10}\text{ClN}_5$: 212.0697, found: 212.0707.

p-(3-Aminoguanidino)-benzoic Acid (2m).—Brown solid. Yield: 47%, 104.4 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 10.22 (br, 1H), 7.86–7.84 (d, J = 8 Hz, 2H), 7.51 (br, 6H), 7.20 (br, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 167.8, 161.9, 1540.8, 130.6, 119.6. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_9\text{H}_{11}\text{N}_5\text{O}_2$: 221.0913, found: 222.0991.

4H-1-Benzopyran-4-one-biguanide HCl (2n).—Brown solid. Yield: 38%, 111.7 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 7.89–7.87 (d, J = 8.8 Hz, 1H), 7.84–7.83 (d, J = 1.6 Hz, 1H), 7.62 (br, 4H), 7.31–7.29 (m, 1H), 7.21 (br, 2H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 176.5, 166.8, 162.2, 157.0, 154.3, 144.6, 125.8, 118.1, 177.4, 110.2, 106.8, 20.4. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_7\text{H}_{17}\text{N}_5$: 160.1142, found: 260.1149.

1-(m-Phenoxyphenyl)-biguanide HCl (2o).—White solid. Yield 27%, 80.9 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 9.89 (s, 1H), 7.39–7.34 (m, 8H), 7.12–7.08 (m, 3H), 6.98–6.95 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 161.6, 157.7, 155.9, 152.5, 134.8, 130.4, 123.4, 123.3, 119.8, 118.3. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{15}\text{N}_5\text{O}$: 270.1349, found: 270.1351.

1-[p-(p-Phenoxy)phenyl]-biguanide HCl (2p).—White solid. Yield: 56%, 189.8 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 9.85 (s, 1H), 7.42–7.38 (m, 4H), 7.33 (br, 4H), 7.09 (br, 2H), 7.02–6.99 (d, J = 8.8 Hz, 2H), 6.98–6.96 (d, J = 8.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 166.4, 161.5, 160.6, 156.8, 140.0, 135.0, 131.8, 128.1, 124.8, 124.6. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{14}\text{ClN}_5\text{O}$: 304.0960, found: 304.0956.

3-Heptylbenzyl-biguanide HCl (2q).—White solid. Yield: 71%, 221.8 mg. ^1H NMR (400 MHz, DMSO- d_6) δ 9.39 (s, 1H), 7.30–7.28 (d, J = 7.6 Hz, 2H), 7.24 (s, 4H), 7.20–7.18 (d, J = 8 Hz, 2H), 7.03 (s, 2H), 1.61–1.58 (m, 2H), 1.34–1.32 (m, 8H), 0.94–0.91 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 161.4, 155.9, 137.9, 136.6, 125.8, 121.6, 34.9, 31.7, 31.5, 29.0, 29.0, 22.5, 14.4. HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{15}\text{H}_{25}\text{N}_5$: 276.2183, found: 276.2182..

RESULTS

Validation of the Overexpression of OCT1 and OCT2 in HEK293 Cells.

Immunoblotting and real-time PCR were used to validate the overexpression of human isoforms of OCT1 (HEK-OCT1) and OCT2 (HEK-OCT2) transgenes in HEK293 (Figure S1). The uptake of prototypical substrates, MPP⁺ and metformin, was used to assess the function of these transporters as described previously.²⁸ The uptake was higher in the HEK-OCT1 and HEK-OCT2 cells than the empty vector controls (HEK-P5) and was linear up to 10 min prior to plateau (Figure 2). Subsequent uptake experiments were then carried out for 10 min, all in room temperature.

Incorporation of the Biguanide Moiety Leads to Improved Uptake by OCT1 and OCT2.

To ascertain if addition of the biguanide moiety led to improvement in transport by OCT1 and OCT2, the uptake of amine parent and their corresponding biguanides was assessed in the transporter overexpressing cells and compared to that of uptake in empty vector overexpressing cells to attain an uptake fold increase ratio (as indicated in the methods). In this study, a fold uptake increase of 1.5 and greater over HEK-P5 (control) was used as the cutoff for defining OCT substrates for both parents and biguanides (Figure 3). Most of the biguanides, 16 (phenformin, **2a–2p**) and 14 (phenformin, **2a–l**, **2o–p**) out of 18 biguanides tested were substrates for OCT1 and OCT2, respectively. For the parent compounds, only **1q**, and three others (**1m**, **1n**, and **1q**) surpassed the substrate threshold in OCT1 and OCT2 cells, respectively. **2m** was the only compound to have a decrease in OCT2-mediated transport when compared to its amino parent. In comparison to the parent compound, addition of the biguanides moiety lead to increase in all, but one compound (q) in OCT1 (Figure 3a). For OCT2 cells, only four compounds (m,n,p,q) had no improvement upon addition of the biguanide moiety (Figure 3b). While there is little or no correlation between

OCT1 and OCT2 uptake observed for the compounds assayed in this uptake experiment (Figure S2), the results suggest that the addition of a biguanide moiety improved transporter-mediated uptake of the majority of the compounds over the parent compounds.

Inhibitory Effects of Parent and Biguanide Analogs on the Activities of OCT1 and OCT2.

Another aspect of our experiment was to determine whether the addition of biguanide moiety would enhance the inhibitory effect of the compounds on prototypical OCT substrates. Parent amines were assayed against MPP⁺ uptake while the biguanides were tested against both MPP⁺ and metformin uptake. Uptake assays were first performed with 5 μ M MPP⁺ in the presence or absence of 50 μ M of the biguanides or their parent compounds (Figure 4a,b). Half of the compounds (phenformin, **2e–f**, **2h**, **2k–l**, and **2n–q**), upon addition of the biguanide moiety, had increased inhibition of OCT1-mediated uptake of MPP⁺. However, the inhibition of OCT2-mediated uptake of MPP⁺ was not enhanced for most of the compounds upon addition of the biguanide moiety. In many cases (phenformin, **2b–e**, **2g**, **2i–j**), this addition instead resulted in less inhibition of OCT2 activity at the concentration tested. Taken together, there seems to be increased inhibition of MPP⁺ by swapping amines for biguanide moieties in some cases, however, there were more than a few instances where biguanides had similar or lower inhibition than the amine parents, opposing a conclusion that biguanide addition causes enhanced inhibition of either OCT1 or OCT2.

We further performed similar inhibition studies with metformin, another prototypical substrate but also a biguanide (Figure 4c and d). All the biguanides, except **2m**, inhibited both OCT1 and OCT2 mediated metformin uptake. **2m** only inhibited OCT1 mediated metformin uptake. In HEK-OCT2 cells, inhibition of metformin uptake was more potent for most of the compounds than for their inhibition of MPP⁺ uptake. In addition, we found that six biguanides (**2c**, **2g–h**, **2j**, and **2p–q**) inhibited metformin transport by 50% or more in HEK-OCT1 cells and 13 biguanides (**2a–f**, **2h**, **2j**, **2n–q**, and phenformin) in HEK-OCT2 cells at the concentration of 50 μ M of metformin. Consistent with the uptake results (Figure 3), these inhibition data overall confirmed that addition of the biguanide scaffold led to an interaction between the compound and OCT transporters.

Uptake and Inhibition Kinetics of **2f** and **2j**.

To determine if incorporation of the biguanide scaffold enhanced uptake by the transporters, we performed concentration-dependent studies with two of the biguanides with relatively good uptake fold increase ratios, **2f** and **2j** (Figure 5a). The transport profile of 0.1–1000 μ M of each of the compounds showed a concentration-dependent increase in uptake, which was fitted to the Michaelis–Menten equation with a non-saturable component (described in methods). The uptake in OCT-overexpressed cells was significantly higher than that of the mock cells. For OCT2, **2f** exhibited a moderately higher transporter affinity ($K_m = 12.4 \mu$ M; $V_{max} = 1.8$ pmol/min/mg protein; $P = 0.6$ fl/min/mg protein) than **2j** ($K_m = 31.7 \mu$ M; $V_{max} = 1.1$ pmol/min/mg protein; $P = 0.1$ fl/min/mg protein) (Table 1), albeit there seemed to be a significant nonsaturable uptake process occurring for the former compound (Figure 5a). The same trend was seen for OCT1-mediated uptake kinetics with the two compounds. For OCT1, however, the two compounds had a similar transporter affinity (**2f**: $K_m = 14.2 \mu$ M, $V_{max} = 2.0$ pmol/min/mg protein, $P = 1.7$ fl/min/mg protein; **2j**: $K_m = 16.9 \mu$ M, $V_{max} = 0.3$

pmol/min/mg protein, $P = 0.6$ fl/min/mg protein). The trend in the above-described uptake ratios of **2f** compared with **2j** (Figure 3) was somewhat dissimilar to their calculated kinetic parameters here, which suggests that the uptake ratios for the various compounds at a single concentration may not necessarily be indicative of their affinity to the transporters due to possible influence of passive transport and other unknown mechanisms that may play a role in overall uptake.

Inhibition kinetics was further carried out for both **2f** and **2j** using similar range of concentrations as in the above uptake kinetic studies in the presence of metformin (50 μM), for both OCT1 and OCT2 (Figure 5b). The inhibition data were fitted to the Log [inhibitor] vs metformin uptake to determine IC_{50} . The obtained values noted in Table 1 show that **2j** ($\text{IC}_{50} = 53.4 \mu\text{M}$) presented a relatively similar inhibitory potency toward OCT2-mediated metformin uptake as **2f** ($\text{IC}_{50} = 52.7 \mu\text{M}$). However, **2j** ($\text{IC}_{50} = 22.1 \mu\text{M}$) was a more potent inhibitor than **2f** ($\text{IC}_{50} = 85.5 \mu\text{M}$) in OCT1 cells. In consideration of the uptake kinetics above (Figure 5a), the data indicate that IC_{50} may not be an accurate predictor of affinity to the transporter for a substrate compound, attesting to the complexity of interaction that may exist between compounds and the membrane transporters either as their substrates or as inhibitors.

Effect of Incorporation of the Biguanide Moiety on Uptake of 2f and 2j in OCT3, MATE2K, and MATE1 Overexpressing Cells.

Because the OCT and MATE transporters have overlap in substrate selectivity,¹⁷ we made another attempt to assess the uptake of the selected compounds from our study, **2f** and **2j**, in HEK293 cells stably overexpressing OCT3, MATE2K, and MATE1 transporters. We have previously validated the functions of these cells in our recent articles.^{27,29} Similar to the design of the uptake studies for OCT1 and OCT2, we performed the experiment with a single concentration of 100 μM for each of the compounds. There was significant increase in uptake fold ratio for **2j**, but not **2f**, in OCT3 cells (Figure S3a). For both compounds, we found that addition of the biguanide moiety significantly improved their uptake more than the parent compounds for both MATE2K and MATE1 (Figure S3b and S3c). The data suggest that addition of the biguanide moiety may also improve uptake by OCT3, MATE1, and MATE2k; albeit, further comprehensive experiments need to be done with other biguanides.

Physicochemical Properties Drive the Uptake Kinetics for Biguanides.

Physicochemical parameters are important descriptors of certain processes in drug disposition. A number of these parameters have been correlated with the uptake properties and inhibitory capabilities of many OCT substrates and inhibitors.^{21,32} Here, we assessed the correlation between uptake fold increase and molecular weight, molecular volume, accessible surface area, and calculated LogD of the tested compounds. We found no significant correlation between the uptakes by OCT1 for the biguanides with their physicochemical parameters (Figure 6). The lack of a rich data set may explain the absence of significant correlation, contrary to previous reports generally for OCT1 substrates.^{33,34} The uptake mediated by OCT2 was moderately correlated with molecular volume, which has been characterized as a key descriptor of uptake by OCT2,³⁴ as increase in this

parameter appears to have an inverse effect on OCT2-mediated uptake. This trend was similarly observed for molecular weight, LogD, and accessible surface area in our study.

In the inhibition studies, molecular volume, molecular weight, and surface accessible area of the compounds examined moderately correlated with the biguanides inhibition of OCT1-mediated uptake of metformin and MPP⁺ (Figures 7 and S4). This was not the case for their inhibition of uptake mediated by OCT2, as the correlation coefficient was very low and insignificant ($p > 0.05$). The data suggest that multiple mechanisms may be involved in the inhibition of OCT2 by biguanides. In addition, Hacker and colleagues³⁵ have recently reported a moderate correlation between inhibition of MPP⁺ uptake and that of metformin mediated by OCT2. We find consistency in this correlation with our OCT2 inhibition data for both substrates ($r = 0.70$; $p = 0.00040$), where increase in the inhibition of metformin uptake was correlated well with the increase in the inhibition of MPP⁺ uptake by the biguanides (Figure S5). We also found a moderate but significant correlation ($r = 0.58$; $p = 0.0055$) for OCT1. Therefore, both reference substrates may be employed interchangeably in inhibition studies to screen compounds, as had been previously concluded. Together, these results suggest that certain physicochemical properties including molecular volume, molecular weight, Log D, and accessible surface area are moderate descriptors for the uptake of biguanides mediated by OCT2 (but not OCT1). Rather, these intrinsic properties of the biguanides were moderate descriptors for their inhibition against OCT1 (but not OCT2) activity.

DISCUSSION

Biguanides have been majorly studied for their beneficial effects as antidiabetics.³⁶ The model biguanide, metformin, which is still the first line treatment for diabetes, has been shown to elicit its effect through its functions in the liver.²⁴ It has been shown that in order to permeate the liver and accumulate at the site of action, OCT1 is expected to shuttle this cationic compound from the extracellular space into hepatocytes. Absence of OCT1 in mice may abrogate the glucose-lowering effect of the compounds in the biguanides class, metformin, phenformin, and buformin.³⁷ Thus, it is likely that the accumulation of biguanides in the liver depends on OCT1 function. We reasoned that since the already known biguanides have been shown *in vivo* and *in vitro* to be efficiently transported by OCTs, attaching the biguanide scaffold to some compounds may cause them to become OCT substrates. Thus, by introducing the biguanide moiety in compounds that are not OCT1 or OCT2 substrate, we demonstrate that this handle can be utilized to improve cellular uptake mediated by OCT1 and OCT2.

We focused on whether incorporating the biguanide moiety into amines would improve their uptake by organic cation transporters. These parent amine compounds were chosen based on size and polarity so the impact of these two variable could be studied. It has been described that uptake by OCT1 and OCT2 drastically decreases for compounds over 500 Å³.³⁴ Therefore, small side chains (R groups) were chosen so the molecular volume of the amine and biguanide forms would not exceed 300 Å³. The R groups were composed of lipophilic (a–h, j–l, o–q, phenformin) and hydrophilic (i, m, n) chains. The PSA of biguanides with lipophilic chains ranged from 81 to 96 Å², and the hydrophilic chains ranged from 121 to

173 Å². A greater number of lipophilic chains than hydrophilic chains were chosen due to our interest in investigating the addition of the biguanide moiety to lipophilic drugs in order to increase their uptake by OCT. With these structurally diverse amines and their biguanide analogues, we also investigated the correlation between the biguanide physicochemical properties and their interaction with the two OCTs.

Consistent with already existing biguanides reported in the literature,³⁷ we found that incorporation of the biguanide scaffold in the parent compounds in this study led to an overall increase in uptake by OCT1 and OCT2. In contrast, the uptake between the empty vector transfected and the transporter overexpressed cells was no different for most of the parent compounds in our study. In a particular case, the addition of the biguanide group did not improve uptake for **1m/2m** in OCT2 cells. For this pair of compounds, the PSA was the highest among the studied compounds, which may have played a role in the lower uptake observed. Besides, because the transport assays were carried out for all compounds using the incubation conditions optimized according to metformin uptake, it is possible that some of the biguanides might actually exhibit different and/or more complex kinetics. We observed that 1 pair (**1/2q**) and 4 pairs (**1/2m**, **1/2n**, **1/2p**, and **1/2q**) had only a meager, but significant increase in transport by OCT1 and OCT2, respectively. While the exact reason remains unclear, this may be due to their structural constituents, relatively higher passive uptake, or the site of addition of the biguanide moiety that causes them to have a lower uptake ratio against the control cells. Overall, this study strongly suggests that having a biguanide included in impermeable compounds will lead to improved OCT-mediated uptake (Figure 8).

The uptake ratios for OCT2 were relatively higher than those for OCT1 but more compounds had an increase in uptake by OCT1 than OCT2 when the biguanide was added to the structure. It is noteworthy to mention that we used different systems to assess the uptakes by the two transporters, transient transfection for OCT1 and stable expression for OCT2. Besides, we did not quantitatively measure the relative expression of OCT2 compared to that of OCT1 in the cells. Nonetheless, it has been reported that the biguanide antidiabetic drugs are more efficiently transported by OCT2 as compared to OCT1.³⁸

In the case where transporter-mediated uptake is the major route of targeted tissue delivery of compounds, deletion of the specific transporters may lead to drastic decrease in the accumulation of their substrates in the tissues.^{14,39} For compounds whose target site is in the specific tissue expressing OCTs transporters, ablation of this entry point may also result in inactivation of key substrate drugs.²⁴ This has become especially important in many disease states that alter the expression of these drug carriers, including cancer and diabetes.^{40,41} As such, the concept established in this study allows for direct delivery of compounds to specific tissues expressing OCT1/2 and lessening side effect in other tissues, with knowledge of the transporter expressions in the tissues either in healthy individuals or in a disease state.

Tissue targeting is among the benefits that incorporating a biguanide moiety confers for certain compounds. However, it is foreseeable that not all addition of the biguanide moiety may be beneficial. Depending on the chemical site of addition, this may alter the effect of a drug positively or negatively. The cytotoxicity of the compounds were not tested in the

current study, but this may vary from compound to compound. In the past, biguanides, such as phenformin and buformin, were discontinued due to their effect of lactic acidosis.⁴² Nevertheless, metformin has been shown to be safe and well tolerated even at very high doses. As a future drug development, compounds may be designed such that they are cleaved to their parent compounds and metformin upon entry into the specific tissues. This allows for maintenance of drug potency, allowing for lower dosage administration and without much concern of toxicity resulting from metformin. When given at lower doses, metformin has little or no effect so uncoupling the compound should only maintain the effect of the parent compound.

Despite sequence similarities between OCT1 and OCT2 and an extensive list of overlapping substrates, some specific drivers for being relatively specific substrates/inhibitors for OCT1 or OCT2 have been identified.^{34,43} The substrates of OCT1 usually are less than 500 Å³ and an increase in molecular weight and volume has a negative effect on transport. On the other hand, OCT2-mediated transport is affected positively by an increase in polar surface area (PSA), lipophilicity, and number of hydrogen bonds.²¹ OCT2 has a higher preference for hydrophilic compounds than OCT1. In our study, we further uncover some key drivers for the uptake of biguanides as well as their inhibitory capability for OCT transporters. Molecular weight, molecular volume, accessible surface area, and Log D (pH 7.4) presented significant correlation with OCT2-mediated uptake. For OCT1, however, we found no strong correlation with any of these physicochemical parameters. We found the optimal size of biguanides for OCT2-mediated uptake to be around 200 and 250 Å³. Log P and Log D values were significantly correlated with OCT2-mediated transport; and hydrophilic biguanides (negative Log P and Log D) were greatly transported compared to more hydrophobic compounds. An accessible surface area (ASA) of around 450 Å² was also correlated to a higher OCT2-mediated transport. ASA is the area available for solvent binding on a molecule surface. We concluded that increased ASA would decrease the likelihood of interaction between the molecule functional groups and the amino acid residues in the transporter. This, in turn, would decrease the likelihood of molecule being transported as observed in Figure 6.

OCT1 inhibitors tend to have high molecular weight, positive net charge and high lipophilicity. For example, *n*-alkylammonium compounds with long chains and hydrophobicity tend to be OCT1 inhibitors.³² Inhibitors of OCT2 usually have high molecular volume. OCT1 and OCT2 substrates have a positively charged functional group, but some neutral compounds can also be transported. Zwitterion compounds do not tend to be OCT1 or OCT2 substrates.³⁴ These structure–transporter–activity relationships for OCT1 and OCT2 have been reported using inhibition data as well as substrate uptake data.^{34,44,45} Molecular weight, accessible surface area, and molecular volume were moderate descriptors for OCT1 inhibition in our study, but not OCT2. The reason for no correlation for OCT2 is unknown and may be due to difference in interaction of the compounds with the two transporters.

Consistent with other reports,⁴³ we found that some of the parent compounds and most of the biguanides assayed in this study were inhibitors of the uptake of MPP⁺ mediated by OCT1 and OCT2 at the concentration tested. However, our hypothesis that the biguanides

might be more potent inhibitors, as compared to the parent compounds, was not consistently observed either in OCT1 or OCT2 cells. We further analyzed the uptake kinetics and that of inhibition against a typical substrate for two (**2j** and **2f**) of the novel biguanide substrates of OCT1 and OCT2 synthesized in this study. The results were somewhat contradictory with our uptake fold increase ratios estimated at the concentration of 100 μ M for the two compounds. **2j** had a higher uptake fold increase in OCT2 cells compared to **2f**, but the reverse was the case for their OCT1-mediated uptake as well as their K_m values in the concentration-dependent studies. These findings suggest that the uptake rate obtained from single concentration incubation may not be the best indicator for how good a substrate the compounds are in this study. In addition, the estimation of their affinity (IC_{50}) by inhibition kinetics for both **2f** and **2j** did not accurately reflect the affinity (K_m) observed in the uptake kinetic studies. Overall, while addition of the biguanide scaffold certainly conferred some inhibitory effects along with being transported by the transporter proteins, further studies need to be carried out to fully understand specific interactions of OCT transporters with the biguanide class.

Current biguanide drugs have been characterized as substrates for additional transporters, such as OCT3, MATE1, and MATE2K.^{25,46} Interestingly, for the two compounds **2f** and **2j** that were further examined in this regard, they were either none or very weak substrates for OCT3, suggesting a possible transporter selectivity and thus tissue targeting for new biguanide compounds. MATE1 and MATE2k, which are expressed primarily in the liver and kidney, work in concert with OCTs to eliminate xenobiotics. Both **2f** and **2j** were substrates of MATE1 and MATE2K in this study. Therefore, while further studies are warranted, we speculate that for other compounds and those tested here, addition of biguanide functionality may be able to provide an efficient excretion route *in vivo* via the MATE expressed in the kidney and liver, as has been shown for other OCT substrates and biguanides.^{47–49}

To conclude, we have demonstrated that addition of a biguanide functional group to structurally diverse transporter-impermeable compounds enhances their OCT-mediated transport (Figure 8). Physicochemical properties, especially accessible surface area, molecular weight, and volume, correlate with the uptake of biguanides by OCT2, but not OCT1. Targeting may be attained in delivery of such compounds to the tissues with significant expression of these OCT transporters, including liver and kidney.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The present study was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award R01GM099742 and by the US Food and Drug Administration (FDA) under Award U01FD004320. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH and FDA.

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Figure 1.

Chemical synthetic scheme and compounds assayed. (a) Structure of metformin, phenformin, and MPP⁺. (b) General scheme for synthesis of biguanides. TMSCl, trimethylsilyl chloride; CH₃CN, acetonitrile. The compounds have either lipophilic (a–h, j–l, o–q, phenformin) or hydrophilic (i, m, n) side-chains.

Figure 2.

Functional validation of OCT1 and OCT2 in HEK293. Time dependent uptake of MPP⁺ in (a) OCT1 and (b) OCT2 cells and metformin in (c) OCT1 and (d) OCT2 cells. The experiments were carried out with 5 μ M MPP⁺ containing 2.5% [³H]-MPP⁺ or 50 μ M Metformin containing 2.5% [¹⁴C]metformin. Data are presented as mean $\pm$ standard deviation ($n = 3$).

Figure 3.

Uptake of biguanides and their parent compounds in HEK293 cells expressing OCT1 and OCT2. Uptake of 100 μ M biguanides or their parent compounds in HEK293 cells expressing (a) OCT1 and (b) OCT2 for 10 min. Metrics for the calculation are outlined in the method section. The data are uptake fold increase adjusted by protein concentration, presented as mean $\pm$ standard deviation ($n = 3$) from a representative experiment. Dotted lines denote the 1.5 cutoff line we used as a threshold for substrates; # denotes compounds that did not have any significantly improved uptake upon addition of the biguanide scaffold. Met, metformin; Phen, phenformin.

Figure 4. Inhibition of $[^3\text{H}]\text{-MPP}^+$ and $[^{14}\text{C}]\text{-Metformin}$ uptake by biguanides and their parent compounds. Uptake of $5\ \mu\text{M}$ MPP^+ (a and b) and $50\ \mu\text{M}$ metformin (c and d) (containing 2.5% $^3\text{H}\text{-MPP}^+$ or $^{14}\text{C}\text{-metformin}$) in the absence (control) or presence of $50\ \mu\text{M}$ biguanides or their parent compounds in HEK293 cells expressing OCT1 and OCT2. Each data represent substrate accumulation fold over control (dotted line) adjusted by total protein concentration, and presented as mean $\pm$ standard deviation ($n = 3$) from a representative experiment. Met, metformin; Phen, phenformin.

Figure 5.

Uptake kinetic and inhibition analysis of **2f** and **2j** in HEK293 cells expressing OCT1 and OCT2. Concentration dependent (a) uptake rate (V) of **2f** and **2j** and their (b) inhibition of metformin uptake were assayed in HEK293 cells expressing OCT1 and OCT2. The cells were incubated for 10 min. (b) A fixed concentration of 50 μ M metformin was used for the inhibition assays; control, uptake without **2f** or **2j**. Data are presented as mean $\pm$ standard deviation ($n = 6$).

Figure 6.

Physicochemical descriptors of OCT1- and OCT2-mediated uptake of biguanides.

Parameters including (a) molecular weight, (b) molecular volume, (c) accessible surface area, and (d) Log D (pH 7.4) were estimated and plotted against the uptake mediated by OCT1 and OCT2 for biguanides in HEK293 cells as shown in Figure 3.

Figure 7.

Correlation analysis of metformin uptake mediated by OCT1 and OCT2 in the presence of various biguanides versus (a) molecular weight, (b) molecular volume (Boltzman average), (c) accessible surface area, and (d) Log D (pH 7.4). The metformin uptake mediated by OCT1 and OCT2 in HEK293 cells in the presence of different biguanides are shown in Figure 4c,d.

Figure 8.
Mechanistic illustration for the use of biguanide moiety as a handle for OCT-mediated transport of compounds.

Table 1.Estimates of Kinetic Parameters for the Uptake of 2f and 2j Mediated by OCT1 and OCT2^a

compound	parameters	units	OCT2	OCT1
2f	$V_{\max}$	pmol/mg/min	1.8 ± 0.2	2.0 ± 0.1
	K_m	μM	12 ± 4.0	14 ± 2.8
	P	fl/min/mg	0.6 ± 0.3	1.7 ± 0.2
	IC_{50}	μM	53 ± 3.9	86 ± 14.1
2j	$V_{\max}$	pmol/mg/min	1.1 ± 0.04	0.3 ± 0.05
	K_m	μM	32 ± 3.2	17 ± 6.6
	P	fl/min/mg	0.1 ± 0.05	0.6 ± 0.08
	IC_{50}	μM	53 ± 4.5	22 ± 4.9

^aThe parameter estimates are derived from fitting kinetics data in Graphpad prism 5 with the equation provided in the data analysis section. Data are presented as estimates $\pm$ standard error ($n = 6$). $V_{\max}$ is the maximum uptake rate, K_m is the Michaelis-Menten constant, P is the non-saturable clearance parameter, and IC_{50} is the half-inhibitory concentration of the compound towards metformin uptake.