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Computational Modelling of Paracellular Diffusion and OCT3 Mediated Transport of Metformin in the Perfused Human Placenta

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ABSTRACT

Metformin is an antidiabetic drug, increasingly prescribed in pregnancy and has been shown to cross the human placenta. The mechanisms underlying placental metformin transfer remain unclear. This study investigated the roles of drug transporters and paracellular diffusion in the bidirectional transfer of metformin across the human placental syncytiotrophoblast using placental perfusion experiments and computational modelling. ^{14}C-metformin transfer was observed in the maternal to fetal and fetal to maternal directions and was not competitively inhibited by 5 mM unlabelled metformin. Computational modelling of the data was consistent with overall placental transfer via paracellular diffusion. Interestingly, the model also predicted a transient peak in fetal ^{14}C-metformin release due to trans-stimulation of OCT3 by unlabelled metformin at the basal membrane. To test this hypothesis a second experiment was designed. OCT3 substrates (5 mM metformin, 5 mM verapamil and 10 mM decynium-22) added to the fetal artery trans-stimulated release of ^{14}C-metformin from the placenta into the fetal circulation, while 5 mM corticosterone did not. This study demonstrated activity of OCT3 transporters on the basal membrane of the human syncytiotrophoblast. However, we did not detect a contribution of either OCT3 or apical membrane transporters to overall materno-fetal transfer, which could be represented adequately by paracellular diffusion in our system.

Introduction

The placenta is the interface between the mother and fetus and is thought to provide a barrier to many pharmaceutical drugs crossing to the fetus. However, this barrier has limitations and the transfer of such substances across the placenta needs to be better understood. Understanding the transport mechanisms and pharmacokinetics of drugs crossing the human placenta is essential if we are to make informed choices about the use of medications in pregnancy.

Optimising maternal insulin sensitivity in pregnant women with type 2 or gestational diabetes may improve neonatal outcomes.[1] One medication used to improve insulin resistance is metformin, a positively charged hydrophilic dimethylbiguanide.[2] Metformin crosses the human placenta, with metformin levels in umbilical cord serum equivalent to those detected in maternal blood.[1,3,4] Metformin is not associated with major fetal congenital abnormalities, but has been associated with an increased risk of small for gestational age births.[5]

The human placental syncytiotrophoblast expresses a variety of transport proteins on the apical microvillous membrane (MVM) and the basal membrane (BM), which can mediate the transfer of organic cations such as metformin.[6,7] Basal membrane transport of metformin is thought to be mediated by the organic cation transporter 3 (OCT3, *SLC22A3*),[6] which is the only member of the organic cation transporter family expressed in the human placenta.[8,9] OCT3 can mediate bidirectional transport, but is electrogenic and favours transfer into the cell down the prevailing electrochemical potential gradient.[10]

In mice, knockout studies provide clear evidence for the role of Oct3 in maternal-to-fetal transfer of metformin.[6] In the rat, studies have demonstrated both maternal-to-fetal and fetal-to-maternal metformin transfer, with a concentration dependent clearance of metformin attributed to multidrug and toxin extrusion protein 1 (Mate1, *Slc47a1*) on the MVM and Oct3 on the BM working in conjunction.[11] However, in the human placenta MATE transporter expression appears to be absent[8] and the identity of the transporters that would mediate efflux of metformin across the MVM is not clear.[6] The MVM efflux transporters P-glycoprotein (P-gp, *ABCB1*) and the breast cancer resistance protein (BCRP, *ABCG2*) have been previously

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implicated,[12] but they are not thought to transfer metformin.[13,14] Uptake of metformin on the MVM could potentially be mediated by the serotonin reuptake transporter (SERT, *SLC6A4*).[15] In addition, the organic cation transporter novel type 2 (OCTN2, *SLC22A5*) and norepinephrine transporter (NET, *SLC6A2*) may be involved.[6,7]

Human *ex-vivo* placental perfusions studies demonstrated that metformin transferred readily from the maternal to fetal circulation,[16,17] but present conflicting evidence as to the mechanisms involved. While no transport against a gradient occurred, maternal-to-fetal transfer was found to be dose dependent, consistent with transporter saturation, which was attributed to OCTs.[18] However, placental perfusion studies by Tertti et al. showed that metformin transport in either direction could not be inhibited by cimetidine, an OCT inhibitor, and concluded that OCT-dependent transport systems were unlikely to contribute significantly to metformin transfer across the human placenta.[2] Diffusion of metformin across cell membranes is thought to be minimal due to its hydrophilic nature.[18,19] In the absence of other potential metformin transporters, this may indicate a role for paracellular diffusion.[20] Paracellular diffusion is bi-directional and may be mediated by regions of syncytial damage,[21] recently visualised trans-syncytial nanopores,[22] or a combination of the two.

The existing literature therefore poses questions about placental metformin transfer. Rodent studies suggest a clear role for transporters while more limited human studies are inconclusive. It is important to understand how metformin is transported in the human especially if it is different to rodent models. Therefore, this study uses mathematical modelling of placental perfusion data to determine the contribution of drug transporters and paracellular diffusion to the bidirectional transfer of metformin across the human placenta.

Methods

All placentas used in this study were obtained from uncomplicated pregnancies delivered at term by caesarean section. Placentas were collected with written informed consent from women delivering at the Princess Anne Hospital in Southampton with approval from the Southampton and Southwest Hampshire Local Ethics Committee (11/SC/0529).

Placentas were perfused using the isolated perfused placental cotyledon methodology of Schneider et al.[23] as adapted in our laboratory.[24] Both the maternal and fetal circulation were operated in open-loop configuration (non-recirculating). Placentas were perfused with Earle's Bicarbonate Buffer (EBB: 5 mM glucose, 1.8 mM $CaCl_2$, 0.4 mM $MgSO_4$, 116.4 mM NaCl, 5.4 mM KCl, 26.2 mM $NaHCO_3$, 0.9 mM NaH_2PO_4), gassed with 5% CO_2 and 95% O_2. EBB with 1 g/l BSA was used for the maternal and fetal buffer during the initial setup phase, but BSA was excluded during the actual measurement phase of the experiment to avoid potential effects of protein binding (minimal for metformin). Perfusion via the fetal catheter into a chorionic plate fetal artery was performed at 6 ml/min, while the maternal-side arterial perfusion with EBB was established 15 min later via five maternal catheters at 14 ml/min in total using roller pumps. Throughout the experiments, fetal and maternal placental circulation pressures were recorded using Chart v4.2. Approximately 1.5 ml of venous exudate was collected from maternal and fetal venous outflows at regular intervals. At the end of each experiment, the perfused region of placental cotyledon (the white region cleared of blood) was obtained by trimming off the non-perfused tissue, then blotted and weighed.

Bidirectional Perfusion Experiments

^{14}C-metformin (American Radiolabeled Chemicals, Missouri USA) was perfused into the maternal circulation in EBB at a concentration of 40 nM for 60 min (30 min with ^{14}C-metformin alone and 30 min with ^{14}C-metformin and unlabelled 'cold' metformin (5 mM)). The maternal circulation was then switched to perfusion of EBB alone and the fetal circulation was perfused with ^{14}C-metformin in EBB at a concentration of 40 nM for 60 min (30 minutes with ^{14}C-metformin alone and 30 min with ^{14}C-metformin and unlabelled 'cold' metformin (5 mM)). For each 30 min block, venous exudate was collected from maternal and fetal venous outflows every 5 min. ^{14}C-metformin levels in the fetal and maternal effluent were analysed via liquid scintillation counting (Packard-Perkin Elmer, Massachusetts USA).

OCT3 Experiments

^{14}C-metformin (40 nM) and creatinine (1.8 mM) in EBB were perfused into the maternal circulation. After a 40 min equilibration period, potential OCT3 exchange boli, consisting of 3 ml metformin (5 mM), corticosterone (5 mM), verapamil (5 mM) or decynium 22 (10 mM) (Merck, UK), were injected into the fetal circulation at $t = 76$, 108, 133 and 158 min respectively. Following bolus injection, venous exudate was collected from the maternal and fetal venous outflow at 1, 2, 3, 4, 5, 10, 14, 18, 22 and 24 min. ^{14}C-metformin levels in the fetal and maternal effluent were analysed via liquid scintillation counting (Packard-Perkin Elmer, Massachusetts USA).

Placental Villous Fragment Uptake Studies

Villous fragments (10 mg) were dissected from human term placentas within 30 minutes of collection and allowed to equilibrate in Tyrode's buffer [135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2.H_2O$, 10 mM HEPES, and 5.6 mM glucose (pH 7.4)] for 30 min at 37°C. Fragments were then cultured for 1, 5, 10 or 60 min in 500 μl Tyrode's containing 320 nM ^{14}C-metformin (3.7 GBq, ARC1738; American Radiolabeled Chemicals, Missouri, USA) with or without 10 mM metformin, 20 μM paroxetine or following a 30 min pre-incubation with 15 mM sodium azide, to inhibit transporter-mediated uptake and efflux of metformin. For each placenta, each experimental condition was performed in triplicate, using three Eppendorf tubes containing three fragments each. Uptake was stopped by washing the fragments in cold Tyrode's buffer before homogenizing the fragments in NaOH (50 mM). Fragment uptake of ^{14}C-metformin was determined by liquid scintillation counting (PerkinElmer).

Statistics

To evaluate steady state transfer in the bidirectional experiment, the last two data points of each experimental phase were pooled and analysed using two-way ANOVA. For the OCT3 experiment the steady state transfer of metformin and creatinine was compared using a paired t-test, using matched samples before the first bolus at 70 and 75 min. Significance was assumed at $p \leq 0.05$. All data are reported as mean $\pm$ standard deviation.

For placental villous fragment experiments, two-way ANOVA was conducted to determine whether ^{14}C-metformin uptake was affected by time and treatment with uptake and efflux inhibitors.

Computational Model

Metformin transfer was described using a compartmental model for placental transfer described previously.[25,26] Separate maternal, syncytiotrophoblast and fetal compartments were distinguished. A general modelling framework was implemented based on Fig. 1 including both transporters on the MVM and BM and paracellular diffusion to evaluate their potential role in explaining the experimental results. The rates of change in

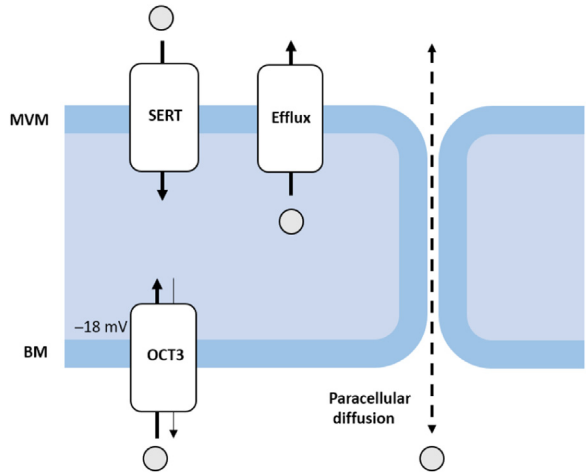


Fig. 1. Schematic of potential transporter mediated and diffusive mechanisms involved in the transfer of metformin across the syncytiotrophoblast. Maternal facing microvillous membrane (MVM) and fetal facing basal membrane (BM). Note, this schematic does not include other placental transporters for organic cations that may be involved such as OCTN2 and NET on the MVM.

concentration due to the fluxes between compartments were assumed to be governed by Eqs. 1–3.

$$\frac{d[c_i]^m}{dt} = \frac{1}{V_m}\left(J^i_{flowm} - J^i_{para} - J^i_{MVMuptake} + J^i_{MVMefflux}\right) \quad (1)$$

$$\frac{d[c_i]^s}{dt} = \frac{1}{V_s}\left(J^i_{MVMuptake} - J^i_{MVMefflux} - J^i_{BM}\right) \quad (2)$$

$$\frac{d[c_i]^f}{dt} = \frac{1}{V_f}\left(J^i_{flowf} + J^i_{para} + J^i_{BM}\right) \quad (3)$$

Here $[c_i]$ (mol/L) are the concentrations of substrate i in the maternal, syncytiotrophoblast and fetal compartments, indicated by superscripts m, s, and f, respectively, while V (L) are the corresponding compartment volumes. J_{flowm} and J_{flowf} are the net flux (mol min^{-1}) due to the perfusion in- and outflow in the respective compartments. J_{para} is the net flux due to paracellular diffusion, $J_{MVMuptake}$ and $J_{MVMefflux}$ are the fluxes due to transporter mediated uptake and efflux across the apical MVM and J_{BM} represents transporter mediated bidirectional transfer across the BM (mol min^{-1}). Based on its low protein binding, the free fraction of metformin was assumed equal to 1.[27]

For simplicity, the measured cotyledon weight in (kg) was equated to the total volume in (L). The volume V of the individual compartments was then calculated based on relative volume fractions of 34%, 15% and 7.4% for the maternal, syncytiotrophoblast and fetal compartments, respectively, as in our previous work.[26,28]

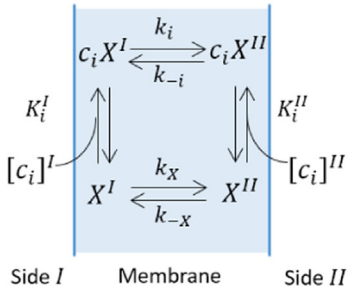
Maternal and Fetal Compartment in- and Outflow

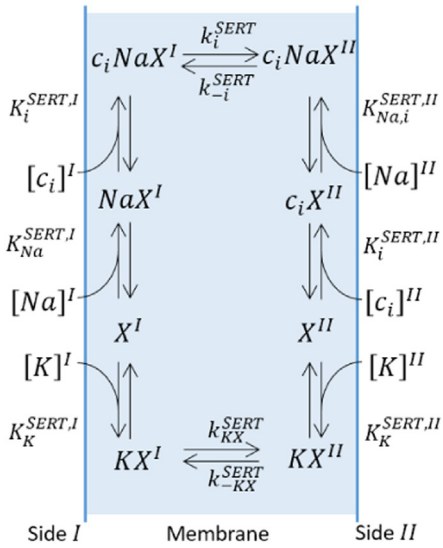
The net fluxes due to in- and outflow in the maternal and fetal compartments are given by Eqs. 4 and 5. Because of the low permeability of metformin, an additional bulk flow term was included to correct for any imperfect fetal flow recovery, based on the assumption that due to the higher fetal pressure any fluid lost from the fetal compartment ends up in the maternal compartment.[21]

$$J^i_{flowm} = Q_{inm}[c_i]^{inm} - (Q_{inm} + \Delta Q_f)[c_i]^m + \Delta Q_f[c_i]^f \quad (4)$$

$$J^i_{flowf} = Q_{inf}[c_i]^{inf} - (Q_{outf} + \Delta Q_f)[c_i]^f \quad (5)$$

Here $[c_i]^{inm}$ and $[c_i]^{inf}$ are the maternal and fetal inlet concentrations (mol/L). Q_{inm} and Q_{inf} (L/min) are the perfusion flow rates for the maternal and fetal circulation, with values of 14 and 6 mL/min





(A) (B)

Fig. 2. Carrier models for OCT3 and SERT. (A) Schematic of OCT3 carrier mediated bidirectional transport model, distinguishing the relevant loaded and unloaded transporter conformational states for each side of the plasma membrane. **(B)** SERT model schematic, incorporating the effect of sodium and potassium gradients in driving cellular uptake. Concentrations of substrate, sodium and potassium are indicated by $[c]$, $[Na]$ and $[K]$, respectively, while X denotes the transporter. Translocation rate constants are indicated by k and dissociation constants by K, see main text for details.

respectively. Q_{outf} is the measured fetal outflow rate during the experiment and $\Delta Q_f = Q_{inf} - Q_{outf}$ is the difference between the fetal in- and outflow.

Paracellular Diffusion

Eq. 6 describes paracellular diffusion directly from the maternal to the fetal compartment, bypassing the syncytiotrophoblast compartment.

$$J^i_{para} = PS_{para}\left([c_i]^m - [c_i]^f\right) \tag{6}$$

PS_{para} (L/min) is the overall cotyledon permeability surface area product. Based on its hydrophilic nature any diffusion of metformin across the apical MVM and BM was considered negligible.[18]

Basal Membrane Transporter Model

A carrier based model was implemented to describe the bidirectional, sodium independent, electrogenic transfer of metformin across the BM via OCT3,[10] similar to the conceptual model proposed for OCT2.[29] Governing equations for the general carrier model were derived as outlined previously,[30(pp161-164),31] based on the model schematic in Fig. 2A. The net flux of substrate i across the plasma membrane is equivalent to the flux J_{c_iX} of the substrate-transporter complex given by:

$$J_{c_iX} = k_i[c_iX]^I - k_{-i}[c_iX]^{II} \tag{7}$$

where $[c_iX]^I$ and $[c_iX]^{II}$ are the substrate-transporter complex concentrations for substrate i on side I and II, while k_i and k_{-i} are the forward and backward rate constants, respectively. Similarly the flux J_X of the unloaded transporter is given by:

$$J_X = k_X[X]^I - k_{-X}[X]^{II} \tag{8}$$

where $[X]^I$ and $[X]^{II}$ are the unbound transporter concentrations on each side of the membrane and k_X and k_{-X} the forward and backward rate constants of the unloaded transporter. Rapid binding equilibrium on each side of the membrane was assumed,[30(p161)] described by:

$$K_i^I = \frac{[c_i]^I[X]^I}{[c_iX]^I}; \quad K_i^{II} = \frac{[c_i]^{II}[X]^{II}}{[c_iX]^{II}} \tag{9}$$

where K_i^I and K_i^{II} are the dissociation constants for substrate i on side I and II of the plasma membrane. Quasi-steady state transporter cycling is assumed,[30(p161)] which implies that the sum of the unbound and bound transporter fluxes for all substrates $i = 1, \ldots, n$ must be zero:

$$J_X + \sum_{i=1}^{n} J_{c_iX} = 0 \tag{10}$$

Finally, conservation of transporters applies:

$$[X]^I + [X]^{II} + \sum_{i=1}^{n}\left([c_iX]^I + [c_iX]^{II}\right) = x_T^{OCT3} \tag{11}$$

where x_T^{OCT3} is the total transporter density.

Translocation of the substrate-transporter complex between the outwards and inwards facing state was assumed to be electrogenic, with rate constants given by[25]:

$$k_i = k_{i0}e^{\left(\frac{\beta zF\Delta\psi}{RT}\right)}; k_{-i} = k_{-i0}e^{\left(\frac{(\beta-1)zF\Delta\psi}{RT}\right)} \tag{12}$$

while translocation of the unloaded transporter was assumed not to be electrogenic. k_{i0} and k_{-i0} were the forwards and backwards rate constants in absence of a membrane potential difference, z is the substrate charge (+1 for metformin[27]), F is Faraday's constant (9.6485×10^4 Cmol^{-1}), R the gas constant (8.314 JK^{-1}mol^{-1}) and T the absolute temperature (310 K). Without further data, the bias parameter β was set to 0.5 assuming a symmetric barrier. $\Delta\psi = \psi^I - \psi^{II}$ is the electrical potential difference of side I with respect to side II. Thus a negative membrane potential favours uptake of positively charged substrates from side II to I, with the maximum accumulation ratio determined by the electrochemical potentials given by[30(p15)]:

$$\frac{[c_i]^{II}}{[c_i]^I} = e^{\left(\frac{zF\Delta\psi}{RT}\right)} \tag{13}$$

Note that when applied to the basal membrane in the model described by Eq. 2 and 3, side I corresponds to the syncytiotrophoblast and side II to the fetal compartment. A membrane potential $\Delta\psi = -18$ mV was used for the BM, based on an MVM potential of −21 mV at term and a potential difference across the syncytiotrophoblast of −3 mV.[32] From Eq. 13, this gives a fetal to syncytiotrophoblast concentration ratio $[c_i]^{II}/[c_i]^I = 0.51$, or in other words a maximum two-fold intracellular accumulation in the syncytiotrophoblast.

Three substrates were distinguished in the model application with $i = 1, 2, 3$ corresponding to radiolabelled metformin, unlabelled metformin and a competitive inhibitor/exchange substrate, respectively. The system of equations Eqs. 7–11 was solved using the Matlab Symbolic Math Toolbox (v6.0) to obtain analytical functions for J_{c_iX} for each substrate, which were then applied to describe BM transport in the model in Eqs. 2 and 3 using $J^i_{BM} = J_{c_iX}$.

To minimise the number of parameters, $k_{i0} = k_{-i0}$ and $k_X = k_{-X}$ were used and it was initially assumed that $k_{i0}/k_X = 10$, favouring translocation of the loaded over the unloaded transporter. $k_X = 1$ min^{-1} was used as the overall transport rate only depends uniquely on the products of the translocation rates k and transporter density parameter x_T. Without further data, the dissociation constants were assumed to be the same on both sides of the plasma membrane: $K_i^I = K_i^{II}$ and initially the same value of 2.6 mM was used for all substrates based on the K_m for OCT3 and metformin.[15] However, note that the dissociation constants would only be the same as K_m in the case of equal translocation rate constants.[31]

Apical Microvillous Membrane Influx Transporter Model

A model for the serotonin transporter SERT by Rudnick and Sandtner was adopted to described the transporter mediated uptake of metformin from the maternal circulation, which was assumed to be electroneutral, driven by the sodium and potassium gradients.[33] Extension of the model to multiple substrates and derivation to enable the generation of analytical functions was carried out following the same general procedure as described previously,[30(pp161-164),31] based on the model schematic in Fig. 2B: The net flux of substrate i is given by the flux J_{c_iNaX} of the substrate-sodium bound transporter complex:

$$J_{c_iNaX} = k_i^{SERT}[c_iNaX]^I - k_{-i}^{SERT}[c_iNaX]^{II} \tag{14}$$

where $[c_iNaX]^I$ and $[c_iNaX]^{II}$ are the substrate-sodium-transporter complex concentrations for substrate i on side I and II, while k_i^{SERT} and k_{-i}^{SERT} are the forward and backward rate constants, respectively. Similarly the flux J_{KX} of the unloaded potassium bound transporter is given by:

$$J_{KX} = k_{KX}^{SERT}[KX]^I - k_{-KX}^{SERT}[KX]^{II} \tag{15}$$

where $[KX]^I$ and $[KX]^{II}$ are the unloaded potassium bound transporter concentrations on each side of the membrane and k_{KX}^{SERT} and k_{-KX}^{SERT} the forwards and backwards rate constants. Rapid binding equilibrium on each side of the membrane was assumed[30(p161)]:

$$K_i^{SERT,I} = \frac{[c_i]^I[NaX]^I}{[c_iNaX]^I}; K_i^{SERT,II} = \frac{[c_i]^{II}[X]^{II}}{[c_iX]^{II}} \tag{16}$$

$$K_{Na}^{SERT,I} = \frac{[Na]^I[X]^I}{[NaX]^I}; K_{Na,i}^{SERT,II} = \frac{[Na]^{II}[c_iX]^{II}}{[c_iNaX]^{II}} \quad (17)$$

$$K_K^{SERT,I} = \frac{[K]^I[X]^I}{[KX]^I}; K_K^{SERT,II} = \frac{[K]^{II}[X]^{II}}{[KX]^{II}} \quad (18)$$

where $K^{SERT,I}$ and $K^{SERT,II}$ are the dissociation constants for substrate i, sodium and potassium on side I and II of the plasma membrane. The net zero sum of the forwards and backwards transporter fluxes is:

$$J_{KX} + \sum_{i=1}^{n} J_{c_iNaX} = 0 \quad (19)$$

Conservation of transporters, with x_T^{SERT} the total transporter density is given by:

$$[X]^I + [X]^{II} + [KX]^I + [KX]^{II} + [NaX]^I + \sum_{i=1}^{n} \left([c_iNaX]^I + [c_iNaX]^{II} + [c_iX]^{II} \right) = x_T^{SERT} \quad (20)$$

The SERT model (Eqs. 14–20) was solved using the Matlab Symbolic Math Toolbox (v8.1) to generate analytical functions for the flux of two substrates $i = 1, 2$ corresponding to radiolabelled metformin and unlabelled metformin, respectively, which were then applied to describe apical MVM uptake in Eqs. 1 and 2 using $J_{MVMuptake}^i = J_{c_iNaX}$.

To reduce the number of parameters, the dissociation constants for labelled and unlabelled metformin were assumed equal on both sides of the plasma membrane $K_i^{SERT,I} = K_i^{SERT,II}$ and initially set to 4.0 mM, using the value for the K_m for SERT and metformin.[15] The remaining parameters were adopted from the SERT model,[34] with the sodium and potassium dissociation constants calculated from the ratio of the reverse to forward rate constants: $k_i^{SERT} = k_{-i}^{SERT} = 6000$ min^{-1}, $k_{KX}^{SERT} = k_{-KX}^{SERT} = 180$ min^{-1}, $K_K^{SERT,I} = K_K^{SERT,II} = 10$ μM, and $K_{Na}^{SERT,I} = K_{Na,i}^{SERT,II} = 1500$ μM. The extracellular sodium and potassium concentrations used were $[Na]^I = 143.5$ mM, and $[K]^I = 5.4$ mM, and the intracellular concentrations $[Na]^{II} = 13$ mM, and $[K]^{II} = 149$ mM.[35]

Apical Microvillous Membrane Efflux Transporter Model

The effect of transporters mediating the efflux of metformin across the apical MVM was represented using a Michaelis-Menten model:

$$J_{MVMefflux}^i = \frac{V_{efflux}[c_i]^s}{K_{efflux} + [c_{tot}]^s} \quad (21)$$

where substrates $i = 1, 2$ correspond to radiolabelled and unlabelled metformin respectively and $[c_{tot}]^s$ is the sum of concentrations of labelled and unlabelled metformin in the syncytiotrophoblast compartment. V_{efflux} is the maximum transport rate (mol/min) and K_{efflux} the concentration at which the half-maximum rate occurs (mol/L). $K_{efflux} = 100$ nM was used, based on the value reported for P-gp and metformin.[12]

Creatinine Transfer Model

Creatinine transfer was modelled based on Eqs. 1 and 3, including only paracellular diffusion directly from the maternal to the fetal compartment (i.e. with $J_{MVMuptake}^{creatine} = J_{MVMefflux}^{creatine} = J_{BM}^{creatine} = 0$).

Model Implementation

The model was implemented in Matlab (R2018a, The Mathworks, Natick, MA, USA). Eqs. 1–3 where solved numerically using the 'ode45' function (Runge-Kutta (4, 5) method), incorporating the analytical functions for the transporter fluxes derived using the Symbolic Math Toolbox. For the OCT3 experiments, the input concentration for the 5 mM metformin bolus was based on a separately measured concentration-time profile after ^{14}C metformin tracer injection without a placenta. For comparison, the 5 mM verapamil and 10 mM decynium 22 boli were both modelled as an injection of a third solute, using the same parameters as for metformin. The corticosterone bolus was not modelled.

Parameter Estimation

Three distinct scenarios were evaluated for parameter estimation:

1) Paracellular diffusion only, baseline scenario: fitting PS_{para} (with x_T^{SERT}, V_{efflux} and x_T^{OCT3} set to zero).
2) Paracellular diffusion, combined with OCT3 on the BM: fitting PS_{para} and x_T^{OCT3} (with x_T^{SERT} and V_{efflux} set to zero).
3) Paracellular diffusion with all transporters, including SERT and efflux transporters on the MVM and OCT3 on the BM: fitting PS_{para}, x_T^{SERT}, V_{efflux} and x_T^{OCT3}.

The relevant parameters were fitted independently for each scenario and type of experiment (bidirectional and OCT3 experiment). Note that the estimated transporter parameters represent the effective parameters on a cotyledon basis, i.e. incorporating the surface area.

The model were fitted to the experimental data based on the sum of the normalised least square errors for the maternal and fetal concentrations, using the error criterion E_{tot} defined in Eq. 22.

$$E_{tot} = \frac{\sum \left([c_1]_j^m - [c_1]_j^{m\ exp} \right)^2}{\sum \left([c_1]_j^{m\ exp} \right)^2} + \frac{\sum \left([c_1]_j^f - [c_1]_j^{f\ exp} \right)^2}{\sum \left([c_1]_j^{f\ exp} \right)^2} \quad (22)$$

$[c_1]_j^{m\ exp}$ and $[c_1]_j^{f\ exp}$ are the experimental maternal and fetal metformin tracer concentrations measured and $[c_1]_j^m$ and $[c_1]_j^f$ the computational values at time points j. For the bidirectional experiment, Eq. 4 was applied to calculate the error for all time points j corresponding to each transfer direction separately, with the total error consisting of the sum of the values for both directions. For the OCT3 experiment Eq. 4 was applied to all time points j up to and including 132 minutes, i.e. excluding the verapamil and decynium 22 boluses. Parameter estimation was implemented using the Matlab 'globalsearch' function, running the complete model within the parameter estimation loop. Initial parameter estimates were varied to verify the uniqueness of the solution and a sensitivity analysis was conducted.

Results

Bidirectional Experiments

The average placental cotyledon weight for these experiments was 35 ± 6 g and the fetal flow recovery was (93 ± 2.5)% (n = 3 placentas). For maternal tracer infusion, the steady state fetal vein ^{14}C-metformin concentrations, as percentage of stock, were 6.1 ± 1.5% without, and 7.0 ± 1.0% with 5 mM unlabelled metformin. For fetal tracer infusion, the steady state maternal vein ^{14}C-metformin concentrations, were 7.8 ± 4.6% without, and 7.7 ± 7.0% with 5 mM unlabelled metformin. Two-way ANOVA showed no differences for addition of 5 mM unlabelled metformin (p = 0.80), nor the direction of tracer infusion (p = 0.48).

Fig. 3 shows the comparison between the experimental data and computational model predictions. Overall, the steady state experimental data could be represented adequately by the model including

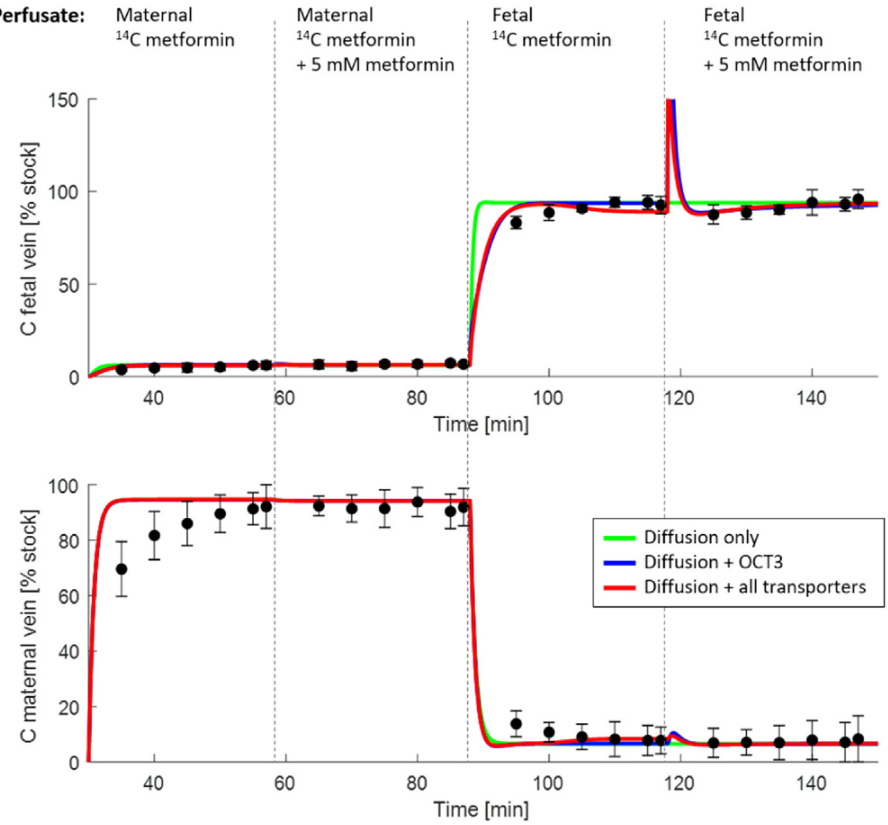


Fig. 3. Bidirectional perfusion experiments, results and model predictions. ^{14}C-metformin tracer infusion in the maternal circulation followed by infusion in the fetal circulation, with or without 5 mM unlabelled metformin. Solid lines represent model fits based on: 1) paracellular diffusion only, 2) diffusion combined with OCT3, and 3) diffusion and all transporters. Experimental results presented as mean ± SD (n = 3).

paracellular diffusion only, with an estimated cotyledon permeability surface area product $PS_{para} = 4.1 \pm 1.4 \times 10^{-4}$ L/min based on individual placenta fits. Inclusion of OCT3 in the model led to a predicted peak in fetal vein ^{14}C-metformin concentration upon addition of 5 mM metformin to the fetal circulation, due to the exchange of accumulated tracer out of the syncytiotrophoblast. However, this could not be confirmed by the data for this experiment due to the sampling interval. A similar, peak was predicted for the model based on diffusion with all transporters. As in the experiment, there was no discernible inhibition of ^{14}C-metformin transfer upon addition of 5 mM metformin to the maternal circulation.

The estimated parameters based on fitting the averaged experimental data for the paracellular diffusion with OCT3 model were: $PS_{para} = 4.3 \times 10^{-4}$ L/min, and $x_T^{OCT3} = 1.2 \times 10^{-5}$ mol. The estimated parameters for the diffusion with all transporters model were: $PS_{para} = 4.3 \times 10^{-4}$ L/min, $x_T^{OCT3} = 4.2 \times 10^{-5}$ mol, $x_T^{SERT} = 4.0 \times 10^{-15}$ mol, and $V_{efflux} = 1.6 \times 10^{-10}$ mol/min.

OCT3 Experiments

The average placental cotyledon weight for these experiments was 41 ± 6 g and the fetal flow recovery was 97 ± 4.7% (n = 4 placentas). ^{14}C-metformin transfer from the placenta into the fetal circulation was trans-stimulated by fetal boli of 5 mM metformin, 5 mM verapamil and 10 mM decynium 22, but not by 5 mM corticosterone. It was verified separately that adding a metformin bolus to the perfusion setup without a placenta did not lead to ^{14}C-metformin tracer release from either circulation. Steady state fetal vein concentrations as a percentage of stock before bolus addition were 9.6 ± 1.6% for ^{14}C-metformin and 7.0 ± 2.9% for creatinine, which was statistically different (paired t-test, p = 0.0095).

The experimental data and model predictions are shown in Fig. 4. The model including only paracellular diffusion could represent the baseline steady state transfer (with $PS_{para} = 7.8 \pm 1.9 \times 10^{-4}$ L/min, based on individual placenta fits), but not the peaks following bolus injection. The model based on paracellular diffusion in combination with OCT3 could represent the baseline transfer and occurrence of peaks following bolus injection. The estimated parameters from fitting the averaged experimental data for the paracellular diffusion with OCT3 model were: $PS_{para} = 7.8 \times 10^{-4}$ L/min, and $x_T^{OCT3} = 8.5 \times 10^{-7}$ mol. Based on the lack of apparent inhibition of materno-fetal transfer in the bidirectional experiment and to avoid overfitting the data, the diffusion with all transporters model was omitted here.

The creatinine data could be represented using the paracellular diffusion only model (Fig. 5), with an estimated $PS_{para}^{creatinine} = 4.7 \pm 2.1 \times 10^{-4}$ L/min based on individual placenta fits.

Model Flux Estimates

The relative fluxes through each route of transfer (Fig. 1) were evaluated for the model with diffusion and all transporters, as applied to the bidirectional perfusion experiment. For the maternal tracer infusion, the magnitude of the ^{14}C-metformin tracer flux in fetal to maternal direction via both OCT3 and MVM efflux transporters at the end of each experimental phase was 6% of diffusion without and 0% with 5 mM metformin. For the fetal tracer infusion, the magnitude of the ^{14}C-metformin tracer flux in fetal to maternal direction via both OCT3 and MVM efflux transporters at the end of each experimental

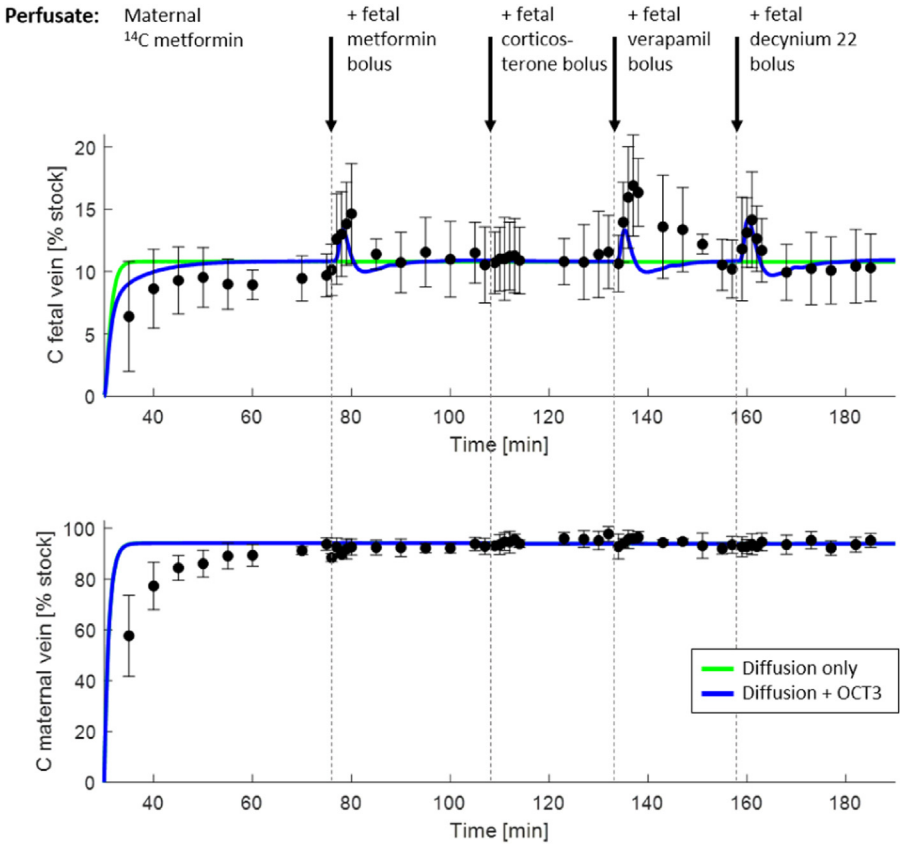


Fig. 4. OCT3 perfusion experiments, results and model predictions. ^{14}C-metformin tracer infusion in the maternal circulation, with the addition of fetal boli of unlabelled metformin, corticosterone, verapamil and decynium 22. Solid lines represent model fits based on: 1) paracellular diffusion only, and 2) paracellular diffusion combined with OCT3. Experimental results presented as mean ± SD (n = 4).

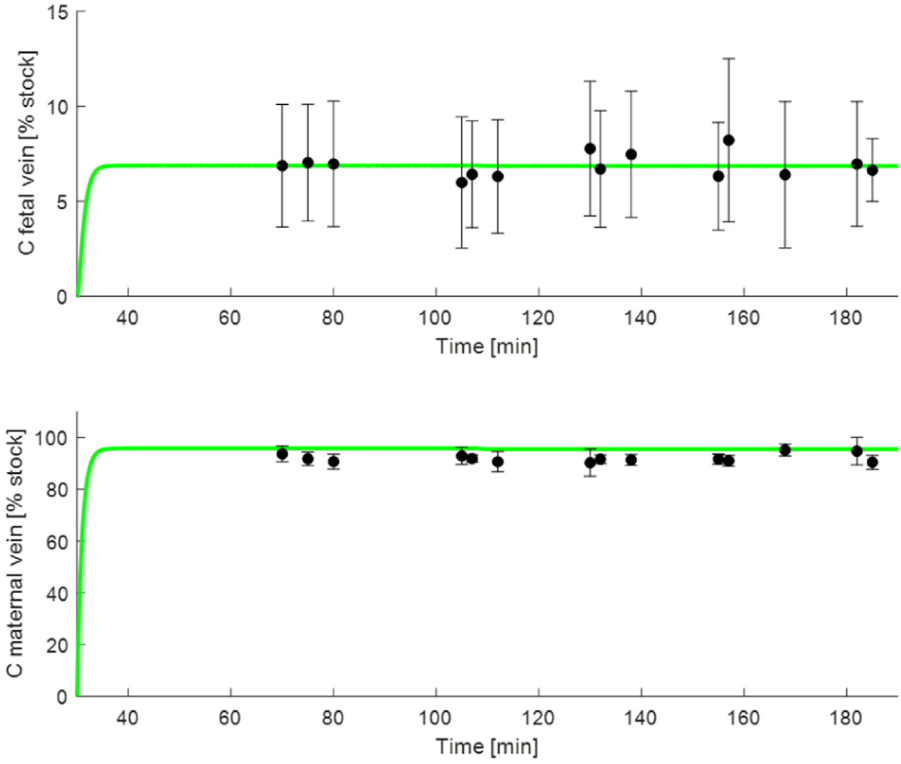


Fig. 5. Creatinine results and model predictions, corresponding to the OCT 3 perfusion experiments. The solid line represent the model fit based on paracellular diffusion only. Experimental results presented as mean ± SD (n = 4).

phase was 89% of diffusion without metformin, while with 5 mM metformin these values were 6% for OCT3 and 0% for MVM efflux respectively. The estimated flux via SERT at the MVM was 0% of diffusion throughout the experiment.

Any lack of full fetal flow recovery was accounted for in the model by a fetal to maternal bulk flow corresponding to the difference between the fetal artery and vein (see Methods). For the bidirectional experiment during the maternal tracer infusion, the magnitude of this bulk flow related ^{14}C-metformin tracer flux in fetal to maternal direction was 7-8% of diffusion, while for fetal tracer infusion this was around 160% due to the high fetal concentrations in this experimental phase and relatively poor fetal flow recovery in these experiments. In contrast, for the OCT3 experiment with higher recovery and only maternal tracer infusion the magnitude of this bulk flow related fetal to maternal flux was 3-4% of diffusion.

Parameter Estimation and Sensitivity

The most important parameter affecting the error in fitting the bidirectional experiment was the diffusive parameter PS_{para}. The fitted value for the MVM uptake parameter (x_T^{SERT}) was small and the fitting error was relatively insensitive to x_T^{SERT}, as long as the associated fluxes were negligible. As reported in the previous section, relatively high fluxes were estimated for the MVM efflux transporter during the fetal tracer infusion phase of the experiment without metformin. This was due to the relative fitting criterion used, where increasing V_{efflux} introduced a mismatch in absolute fetal concentrations, but this was traded off against improving the relative fit for the average maternal concentrations, despite their lower value and greater variation. This can be seen in comparison with the case where the MVM transport parameters were set to zero in the diffusion with OCT3 model (Fig. 3). Note that if paracellular diffusion was set to zero the data could not be fitted successfully based on the combination of all MVM and BM transporters alone (224% increase in error, results not shown). Relatively high values were fitted for the BM transporter parameter x_T^{OCT3}, which controls the transient peak in fetal ^{14}C-metformin, but the fitting error was not very sensitive to its exact value as there was no direct experimental data at the time resolution required for corroboration.

Thus, the model sensitivity was investigated further for the paracellular diffusion model with OCT3, as fitted to the data from the OCT3 experiments (Fig. 4). Baseline steady state transfer to the fetal circulation was directly governed by the paracellular diffusion parameter PS_{para}. Doubling PS_{para} led to a 73% higher baseline transfer, while halving PS_{para} resulted in a 46% lower baseline.

The effect of the OCT3 transporter parameters in controlling peak height is shown in Fig. 6. Increasing transport capacity x_T^{OCT3} initially increased peak height due to trans-stimulation, before decreasing again at higher values of x_T^{OCT3}as higher levels of unlabelled metformin taken up start to inhibit tracer efflux.

Varying k_{i0} changes the ratio between the translocation rate constants of the loaded and unloaded transporter (Fig. 2A), with higher ratios corresponding more closely to an obligatory exchanger, resulting in higher peak heights. Note that reducing k_{i0} by a factor 10 compared to the reference configuration, corresponds to equal loaded and unloaded transporter rate constants ($k_{i0} = 1,\ k_X = 1$ min^{-1}), which largely eliminated the peak (Fig. 6). Equally, increasing the unloaded rate constant by a factor 10 to be equal to the loaded rate constant ($k_{i0} = 10,\ k_X = 10$ min^{-1}) similarly eliminated the peak (results not shown).

Peak height also depended strongly on K, the dissociation constant for metformin and OCT3, with lower values corresponding to higher affinity and larger peaks. The effect of reducing the BM potential difference $\Delta\psi$ was limited as placental reference values were relatively low, but would increase substantially for higher potential differences in the wider physiological range.

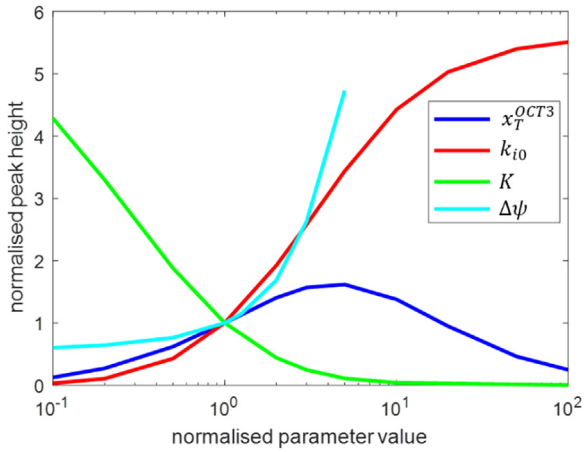


Fig. 6. Sensitivity analysis OCT3 transporter model, as applied to the OCT3 perfusion experiment. Coloured lines represent the effect of parameter variation on the predicted peak in fetal ^{14}C-metformin following addition of the unlabelled metformin bolus to the fetal circulation (Fig. 4). The peak height above baseline and parameters were normalised by the corresponding values for the fitted reference case.

Placental Villous Fragment Uptake Studies

^{14}C-metformin uptake in placental villous fragments is shown in Fig. 7 (n = 3 placentas). Uptake was minimal (< 2%), did not increase linearly with time and was unaffected by cold metformin, the SERT inhibitor paroxetine and pre-treatment with sodium azide to inhibit ATP dependent efflux transporters. Statistical analysis showed a small but statistically significant effect of time (two-way ANOVA, p = 0.0019) but no effect of inhibitors (p = 0.36), nor any interaction (p = 0.97).

Discussion

This study investigated how metformin crosses the human placenta using experimental and computational modelling approaches. The data suggest that, in contrast to rodents, diffusion played a key role in the placental transfer of metformin in these human perfusion experiments. This study highlights both the potential role of paracellular diffusion in mediating fetal exposure to hydrophilic pharmaceuticals and the need to consider the advantages and limitations of particular model systems.

The observation that transfer of ^{14}C-metformin tracer in both maternal-to-fetal or fetal-to-maternal directions was not competitively inhibited by 5 mM unlabelled metformin initially suggested that metformin was not crossing the placenta via a transporter mediated route. However, we did subsequently observe activity of OCT3 confirming that we can detect activity of this transporter in the perfusion model. The computational model based on diffusion alone was able to adequately represent the steady state concentration data without a bias for transfer in either direction. When included, the model predicted the contribution of MVM uptake transporters to be negligible. As metformin is hydrophilic and charged, it is not likely to diffuse across membranes, so diffusion via a paracellular route is the most likely pathway. The maternal to fetal transfer of creatinine (molecular weight 113 g/mol) was similar to that of metformin (129 g/mol), although there was a statistical difference with metformin transfer being slightly higher. Creatinine was used as a paracellular marker for

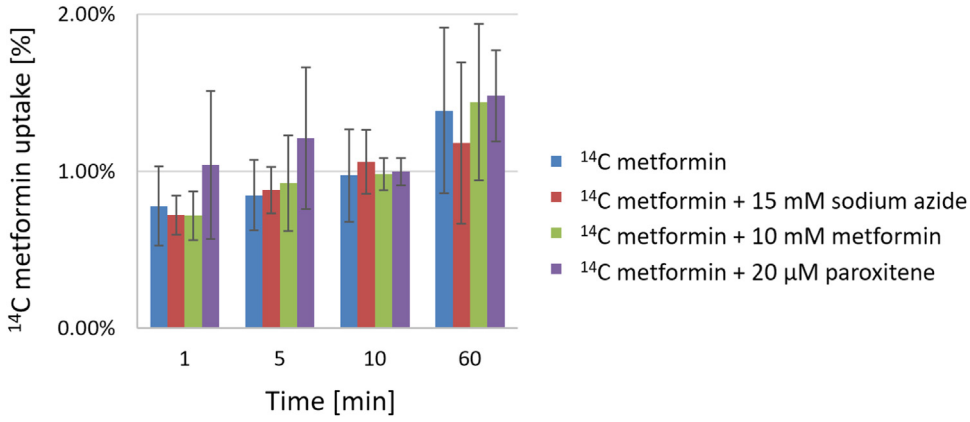


Fig. 7. Placental villous fragments experiments. Uptake of ^{14}C-metformin alone, and with the addition of unlabelled metformin, the SERT inhibitor paroxetine and preincubation with azide to inhibit ATP dependent efflux transporters. Results reported as percentage of ^{14}C metformin added to buffer (mean ± SD, n = 3).

comparison, as in previous studies,[21,36] although it has been reported to be an OCT substrate as well and similar to metformin, any contribution to transfer would require vectorial transport across both MVM and BM.

The computational model predicted that the presence of OCT3 on the BM would lead to a peak in fetal vein ^{14}C-metformin concentrations upon addition of 5 mM unlabelled metformin in the fetal circulation. The reason for this is that the tracer accumulated in the syncytiotrophoblast is exchanged out due to the high inwards directed concentration gradient of unlabelled metformin, similar as has been observed for amino acids.[26,31,37] Since there was no experimental evidence for such a peak in the initial experiments due to the large sampling interval, a second experiment was designed, specifically focussed on the trans-stimulation of BM exchange. Higher resolution sampling confirmed the occurrence of the model predicted peaks in fetal ^{14}C-metformin concentrations after addition of OCT3 substrate boli of unlabelled metformin, verapamil and decynium 22, but not for corticosterone. The higher peak for verapamil may reflect high affinity for OCT3 as indicated by its potency as a competitive inhibitor of OCT3 mediated metformin uptake (K_i = 3.6 μM).[38] It is not clear why corticosterone had not effect, as it is reported to be an OCT3 substrate, and in absence of BSA albumin binding could not play a role.

While evidence was presented for the role of BM transporter such as OCT3 in the intracellular uptake of metformin, the data presented in this study did not provide positive evidence for the role of transporters in MVM uptake or steady state placental transfer, in line with previous reports.[2] However, it cannot be excluded that apical MVM transporters such as SERT also contributed to intracellular uptake in the syncytiotrophoblast, albeit at a relatively low rate as indicated by the lack of inhibition observed in the perfusions and fragments, which may require more sensitive experiments to determine. In terms of the location of placental accumulation, metformin uptake in fetal capillary endothelial cells via OCT3 could potentially also contribute to the peaks observed.[6,38]

The occurrence of paracellular diffusion in the placental is a well-known phenomenon[20] and has been associated with areas of damage to the syncytiotrophoblast,[21] or the presence of water-filled channels or pores.[39-41] Recently, the existence of such trans-syncytial nanopores has been confirmed in human placenta using 3D serial block face scanning electron microscopy.[22] The existence of these nanopores, alongside the observation of diffusion of metformin across the placenta, means that paracellular diffusion should be considered for the transplacental transfer of hydrophilic pharmaceuticals across the placenta, in addition to transport depending on the specific compound.

Calculating permeability surface area products provides a basis for comparison of bidirectional transfer, not affected by differences in compartmental concentrations and flow rates, which can complicate interpretation of clearances.[2] For a diffusive process with low permeability, the lack of difference between the maternal and fetal vein output concentrations in the bidirectional experiment would perhaps be surprising given the difference in flow rate, as this would result in different absolute transfer rates (mol/min) while gradients where similar. However, using the model it was shown that this can be explained fully by accounting for feto-maternal bulk flow due to the incomplete fetal flow recovery in these experiments. Note that while this served to clearly highlight the issue, such bulk flow would make a non-negligible contribution to maternal concentrations even for much higher fetal flow recovery, given the relatively low permeability of metformin. As shown in this study and observed previously,[42] incomplete fetal flow recovery only has a minor effect on maternal to fetal transfer but can have a large impact on the apparent fetal to maternal transfer and such bulk flow should not be interpreted as active transport. Careful measurement of fetal recovery in the perfusion system is therefore essential for accurate interpretation of this data.

In a previous modelling study the placental transfer of metformin was assumed to be primarily mediated by a combination of uptake and efflux transporters on both MVM and BM.[43] However, if the effective transport rates in both directions are similar, such as model would be hard to distinguish from a diffusive process. Their finding that transfer data could not be represented in absence of MVM transporters, may be related to the fact that the model did not include a paracellular route, while metformin only has a very low diffusive plasma membrane permeability. It is not possible to uniquely identify the contributions of two parallel transfer processes, nor the resistances posed by two membranes in series, unless additional information is available from different experimental conditions, such as different saturating concentrations or transporter inhibitors. Metformin transfer across both MVM and BM could be insensitive to inhibition of specific transporters on one membrane if the other membrane is rate limiting.[43] However, inhibition with unlabelled metformin as in the present study would affect both MVM and BM transporters. Therefore, the apparent lack of transporter inhibition in the bidirectional experiment during maternal tracer infusion was key in identifying the contribution of diffusion in the model.

Due to the well-mixed assumption, the compartmental model used in the present study was limited in its ability to capture transient behaviour up to steady state and the same was true for the exact shape and timing of the peak after bolus injection. This means that separate dedicated uptake and exchange experiments e.g. using cells, will be required to fully characterise and precisely determine the OCT3 model parameters for metformin. A previous model for metformin uptake via organic cation transporters incorporated the effect of the electrochemical potential as a driving force in the renal uptake of metformin via OCT1 and 2.[27] However, this would not be able to account for trans-stimulation as observed in the current study. Therefore, the carrier based model used for OCT3 here could be equally useful to describe bidirectional transport and inhibition in different organs, depending on the application.

In conclusion, this study demonstrated that basal membrane transporters could contribute to placental tissue uptake of metformin, but that overall steady state transfer in these human perfusion experiments could be represented adequately based on diffusion alone. Future work should focus on extending the computational-experimental approach presented to a range of drugs and physiological substrates to establish a comprehensive overview of the relative contribution of transporters and paracellular diffusion to placental transfer.

Author contributions

E.L. – Investigation, – Data curation. B.S. – Investigation, – Software. B.S., R.L. and J.C. – Conceptualisation, – Supervision. E.L., B.G., J.C. and R.L. – Writing – review & editing.

Data availability

The Matlab files and experimental data used in this study are available at https://doi.org/10.5258/SOTON/D2561.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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