In silico and in vitro modelling of hepatocyte drug transport processes: Importance of ABCC2 expression levels in the disposition of carboxydichlorofluoroscein

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Abbreviations: AUC, Area under the concentration-versus-time curve; CDFDA, Carboxydichlorofluorescein Diacetate; CDF, Carboxydichlorofluorescein; Cmax, Maximal concentration achieved.
Abstract

The impact of transport proteins in the disposition of chemicals is becoming increasingly evident. Alteration in disposition can cause altered pharmacokinetic and pharmacodynamic parameters, potentially leading to reduced efficacy or overt toxicity. We have developed a quantitative \textit{in silico} model, based upon literature and experimentally derived data, to model the disposition of carboxydichlorofluorescein (CDF), a substrate for the SLCO1A/B and ABCC sub-families of transporters. Kinetic parameters generated by the \textit{in silico} model closely match both literature and experimentally-derived kinetic values, allowing this model to be used for the examination of transporter action in primary rat hepatocytes. In particular, we show that the \textit{in silico} model is suited to the rapid, accurate, determination of Ki values, using MK571 as a prototypical pan-ABCC inhibitor. \textit{In vitro}-derived data is often used to predict \textit{in vivo} response, and we have examined how differences in protein expression levels between these systems may impact upon chemical disposition. We show that ABCC2 and ABCC3 are over-expressed in sandwich culture hepatocytes by 3.5- and 2.3-fold respectively at the protein level. Correction for this in markedly different disposition of CDF, with the AUC and Cmax of intracellular CDF increasing by 365% and 160% respectively. Finally, using kinetic simulations we show that ABCC2 represents a fragile node within this pathway, with alterations in ABCC2 having the most prominent effects on both the Km and Vmax through the pathway. This is the first demonstration of the utility of modelling approaches to estimate the impact of drug transport processes on chemical disposition.
Introduction

In recent years there has been increasing interest in the role of drug transport proteins and, more specifically, how these proteins may impact upon the disposition, efficacy and toxicity of xenobiotics (Glavinas et al., 2004; Oswald et al., 2007). In parallel with the Phase I and II metabolic enzymes the drug transporter system has evolved to be able to handle the complex chemical set to which humans are exposed (Dean et al., 2001; Hagenbuch and Meier, 2004). In common with drug metabolising enzymes, drug transporter proteins tend to have wide, overlapping substrate specificities and variable, inducible expression in target tissues, allowing the most efficient response to chemical insult; indeed, transcriptional control of drug transporter proteins is under the control of the same nuclear receptors that regulate drug metabolizing enzyme levels (Staudinger et al., 2003; Plant, 2004; Klaassen and Slitt, 2005). This control system means that drug transport proteins tend to be expressed at different levels in different tissues, and that this expression level will alter in response to cellular conditions. Examples of this can be seen in pathological conditions such as cancer, where transporter proteins are often over-expressed in tumours (Glavinas et al., 2004), or in normal tissue following chemical exposure (Nishimura et al., 2006), both of which can result in decreased chemical access and loss of pharmacological efficacy. Another area of interest, but one that is poorly studied at present is the expression of drug transport proteins in in vitro systems, with recent work focussing on the characterization of drug transport levels in vitro for a number of systems (Hilgendorf et al., 2007; Bow et al., 2008; Nishimura and Naito, 2008). However, to date the impact of this on the total transport capacity of cells has not been examined, nor how this altered transporter expression relative to in vivo
levels could contribute to the sometimes poor predictive power for such in vitro screens.

In common with drug metabolizing enzymes, many drug transporters show promiscuity in their substrate binding profile, and this has complicated the development of probe substrates. However, a number of fluorescent probes are now emerging that are diagnostic for the activity of groups of transporters, including rhodamine-123 (SLCO1A, ABCG and ABCC sub-families (Katayama et al., 2007; Perriere et al., 2007)) and carboxydichlorofluorescein (SLCO1A and ABCC subfamilies (Sun et al., 2001; Zamek-Gliszczynski et al., 2003; Lengyel et al., 2008)), allowing the establishment of kinetic parameters of drug transport for a number of model substrates (Zamek-Gliszczynski et al., 2003; Pratt et al., 2006).

Such a data rich environment is thus open to in silico modelling of transport processes, allowing estimations of the biological importance of these processes, and the predictive nature of in vitro systems with respect to compound disposition. Such systems modelling have traditionally followed either a top-down or bottom-up approach; in the former, all data at one (or more) biological level is incorporated into a comprehensive model, whereas bottom-up modelling focuses on a single aspect in detail. Top-down modelling has had considerable success in less complex organisms such as bacteria or yeast (Beste et al., 2007), but models in higher eukaryotes tend to be descriptive rather than quantitative in nature (Oda et al., 2005). To achieve quantitative models of higher organisms bottom-up approaches have been more usually applied, including the examination of membrane transport processes at both the theoretical (Ofer et al., 2006; Bartholome et al., 2007) and practical (Sun and Pang, 2008; Sun et al., 2008) level. Such work demonstrates the potential of this
approach to examine the impact of drug transporter expression on the life cycle of compounds within cells.

Using a bottom-up systems biology approach we have created the first model of a complete transport process in primary rat hepatocytes, including both apical and basolateral transport. Using this model we show that ABCC2 levels are a fragile node controlling the disposition of the tool substrate carboxydichlorofluorescein (CDF), and that the alteration in ABCC2 expression between in vitro and in vivo has a significant impact on CDF flux through hepatocytes.

Materials and Methods

**Primary rat hepatocyte culture:** Freshly isolated rat liver was obtained in ice-cold saline from Han-Wistar rats of approximately 300g in weight; only male rats’ liver was used in order to mitigate the known variation in expression of drug transporter proteins between the sexes (Lu and Klaassen, 2008). Primary hepatocytes were isolated using a modification of the protocol of Seglen (Seglen and Jervell, 1969), followed by enrichment of live cells using Percoll gradient centrifugation.

Primary rat hepatocytes were plated in collagen-coated 6 well plates at a 1.5x10⁶ cells per well, and overlaid with matrigel (BD Biosciences, Oxford, UK) to form a sandwich culture. Cells were culture in Williams E medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.17 μM insulin, 0.03 μM dexamethasone, 50 μg/ml gentamycin and 1 % (v/v) 1 M Hepes

**Carboxydichlorofluorescein Diacetate Assay:** CDFDA is a non-fluorescent chemical that can passively diffuse into cells, with a logP=2.8. Once within cells it is converted by intracellular esterases to the metabolite CDF, which is a fluorescent substrate for ABCC transporters (Zamek-Gliszczynski et al., 2003).
Medium was aspirated from cells and they were washed with HBSS. This wash was repeated and the cells were returned to the incubator for 10 minutes covered in HBSS. After removal of the HBSS, the appropriate working solution of CDFDA was added to the cells, or vehicle control (0.1 % DMSO), and cells incubated at 37 °C as required. Cells were washed three times with HBSS and an image was taken at 10 x magnification using as Axiovert 200 microscope. During imaging cells were exposed to an excitation wavelength of 480 nm using a FITC filter for 2 seconds, followed by quantitation at 530 nm using Axiovert 4.5 software suite.

**Transcript level measurement:** Primers and TAMRA/FAM dual labelled probe specific for ABCC2, ABCC3, SLCO1A1, SLCO1A4, SLCO1B2 and 18s were designed using the Primer Express software (Applied Biosystems, Warrington, UK) and were purchased from MWG (Milton Keynes, UK).

Primary rat hepatocytes were plated in collagen-coated 6 well plates at a concentration of 1.5x10^6 cells per well, and overlayed with matrigel (BD Biosciences, Oxford, UK) to form a sandwich culture. Cells were culture in Williams E medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.17 μM insulin, 0.03 μM dexamethasone, 50 μg/ml gentamycin and 1 % (v/v) 1M Hepes. At specified time points, total RNA was isolated from quadruplicate samples using the RNeasy Mini kit (Qiagen) and was quantified using a Nanodrop Agilent 2100 Bioanalyser.

Total RNA was treated with RNase-free DNase (Promega, Southampton, UK) to remove genomic contamination. Reverse transcription was primed with random hexamers and carried out by Superscript II (Invitrogen) as per the manufacturer’s instructions. To ensure that DNase treated samples were free from genomic contamination an RT- control (lacking enzyme) was carried out for every RNA
sample. cDNA generated from 50ng (all transporters) or 50 pg (18S rRNA) of total RNA was amplified using TaqMan Universal PCR Master Mix with 400 nM primers and 200 nM fluorogenic probe in a total reaction volume of 25 μl. Q-PCR reactions were run on the ABI7000 SDS instrument and quantitation was carried out using the ABI proprietary software against a standard curve generated from human genomic DNA (Promega).

**Protein level measurement:** Primary rat hepatocytes were plated in collagen-coated 6 well plates at a concentration of 1.5x10⁶ cells per well, and overlaid with matrigel (BD Biosciences, Oxford, UK) to form a sandwich culture. Cells were culture in Williams E medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.17 μM insulin, 0.03 μM dexamethasone, 50 μg/ml gentamycin and 1 % (v/v) 1M Hepes. At specified time points total protein was extracted in RIPA buffer (1xPBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1 % SDS, protease inhibitor cocktail), and membrane fraction derived by differential centrifugation.

Total (5 μg per lane) or membrane (5 μg per lane) protein extracts were resolved on 12 % SDS-polyacrylamide gels and then transferred electrophoretically to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Bucks, UK). Membranes were blocked (1 hour) in 5 % fat free dried milk and then probed with primary antibodies against rat ABCC2/ABCC3 (Autogen Bioclear, Calne, Wilts, UK), followed by anti-rabbit IgG (Autogen Bioclear). Bound antibodies were visualised using enhanced chemiluminescence reagents according to the manufactures instructions (Amersham Biosciences). Immunoblots were also probed with an antibody against β-actin to ensure even loading per lane (data not shown)

**In silico model generation and simulation:** In silico models were generated using CellDesigner (v3.5.2; Systems Biology Institute, http://celldesigner.org/index.html), a
graphical front-end for creating process diagrams of biochemical networks in systems Biology Markup Language (SBML; (Hucka et al., 2003)). Each individual chemical or protein is identified as a species (s1.....sn), while interactions between species are identified as reactions (r1....rn). For each reaction a kinetic term can be included, detailing the mathematics underlying the interaction of the species. Derivation of kinetic constants for the reactions studied within the network as presented in table 1, with the total model, as SBML script, being available as supplementary information.

Simulations of created networks are then undertaken using the Jarnac Simulation Service (v2.26b), accessed through the Systems Biology Workbench module (Deckard et al., 2006).

TABLE 1 ABOUT HERE

Results

*Generation of in silico model of CDF transport in primary rat hepatocytes.*

In order to generate a comprehensive model of CDFDA/CDF flux in primary rat hepatocytes it was first necessary to generate a qualitative model of interactions that occurs, or may possibly occur, upon which kinetic parameters can be built. Figure 1 shows the initial model generated from known interactions of CDFDA and CDF with cellular systems.

Following construction of the qualitative model a kinetic equation was added for each reaction, and populated kinetic data from either literature sources or experimentally derived data; these parameters, along with their literature source are provided as supplementary information. The complete SBML-script for the generated model is provided as supplementary information.

Using the Jarnac Simulation Service each individual reaction was tested in isolation to ensure that they produced the inputted kinetic parameters correctly (data
not shown). Following this, the total pathway was simulated under basal conditions, producing an overall $V_{\text{max}} = 98 \pm 3$ pmoles/min/mg and $K_m = 46 \pm 6 \, \mu$M for the pathway. Levels of individual species within the system over time are shown in figure 2a.

**FIGURE 2 ABOUT HERE**

For any generated model to be of any use then it most closely model the *in vitro*, and potentially *in vivo* situation. To assess this we next compared *in silico* generated flux parameters with those derived from sandwich cultures of primary rat hepatocytes. Figure 2b shows efflux of CDF reaches a maximum after four days in sandwich culture, which is concordant with the formation of functioning bile canaliculi within the sandwich cultures (figure 2c). In comparison, monolayer cultured hepatocytes do not form canaliculi correctly, as evidenced by their lack of CDF efflux after four days in culture (figure 2c); indeed, no efflux is seen up to six days in culture (data not shown).

Following construction of the *in silico* model, and its validation against *in vitro* data, we wished to examine if the model could simulate pharmacological modulation of the pathway. To examine this we used MK571, a potent pan-ABCC inhibitor (Gekeler et al., 1995): Exposure of sandwich cultures of primary rat hepatocytes to MK571 results in a derived IC$_{50}$ of $1.9 \pm 3.7 \, \mu$M (Figure 3a), and this was used in subsequent *in silico* simulations, presuming that MK571 had similar characteristics against both ABCC2 and ABCC3. As IC$_{50}$ is dependent on the concentration of substrate, it is often more useful to derive a $K_i$ value for an inhibitor, as this is substrate concentration independent. *In silico* simulation allows a much more rapid determination of $K_i$; the derived Schild plot is presented in figure 3b, showing a right shift characteristic of competitive inhibition, while analysis of the Michaelis-Menton
plot (figure 3b) allows the derivation of $\text{Ki} = 3.5 \pm 0.1 \mu\text{M}$. From these derived data it is possible to predict the effect of inhibitor of all species within the network, as seen in figure 3c. Addition of 10 $\mu\text{M}$ MK571 results in a slowing of flux through the system, resulting in increased levels of intracellular CDF, producing an increase in AUC of s5 (intracellular CDF) of 503 %, with a concomitant increase in $C_{\text{max}}$ by 179 %.

**Drug transport protein levels differ significantly between primary cells in vitro and in vivo**

One potential use of *in silico* modelling is to simulate, and understand, differences between *in vitro* and *in vivo* systems, allowing more accurate extrapolation of data from initial screens to pre-clinical testing. To examine how differences in expression levels of drug transport proteins could affect the disposition of chemicals in these two systems we next measured levels of all influx- (Figure 4a) and efflux- (Figure 4b) transporters implicated in CDF transport within primary rat hepatocytes at the transcript level.

**FIGURE 4 ABOUT HERE**

In general, influx transporter levels appear suppressed in the sandwich culture system, with transcript levels below 100% of levels in the freshly excised liver used to prepare the cultures. However, following four days of culture, when *in vitro* experiments are routinely undertaken, $\text{SLCO1A1}$ and $\text{SLCO1A4}$ transcript levels peak to approximate *in vivo* levels, being 129 % and 119 % respectively, while $\text{SLCO1B2}$ transcript levels are within an order of magnitude of the *in vivo* transcript levels, being 15% (Figure 4a). By comparison, $\text{ABCC2}$ and $\text{ABCC3}$ transcript levels in the sandwich culture system are significantly higher than *in vivo* levels for the first six days of culture, with levels being 8524 % and 1807 % of *in vivo* transcript levels respectively following four days of sandwich culture. As changes in transcript levels
do not always correlate to the protein level, we next assessed the level of these two transporters throughout the culture period via Western blotting, revealing a 4.1- and 2.6-fold variability in ABCC2 and ABCC3 expression levels respectively between three and six days in culture compared to freshly isolated rat liver, which was subsequently used to prepare hepatocytes for the culture experiments (Figure 4b). This variability is significantly larger for ABCC2 than ABCC3, and on day four of culture when in vitro experiments are routinely undertaken ABCC2 protein levels are 3.5-fold higher than those observed in vivo, while ABCC3 protein levels are only 2.3-fold higher. As stated earlier, correct localization of drug transporters is as important as their physical presence and hence it is pertinent to note that these protein levels were derived from membrane fraction and not total protein and hence should reflect active protein. These large deviations in transcript levels compared to in vivo rat liver suggest that they may impact upon the disposition of chemicals transported by these ABCC proteins. To examine this, in silico simulations were undertaken using both the base model, which correlates well with the in vitro sandwich culture, and a model adjusted for the lower levels of ABCC2 and ABCC3 in fresh rat liver.

Kinetic simulations of the network using protein levels from either fresh rat liver or hepatocytes sandwich cultured for four days demonstrated the impact of the alteration of efflux transporter levels described above. Examining the net movement through the pathway (from CDFDA in HBSS to CDF in bile cannaliculi) produces a Vmax of 97.6±3 pmoles/min/mg, which is reduced to 28±0.2 pmoles/min/mg when protein levels are adjusted to the lower transporter levels seen in freshly isolated rat liver. In addition, as the ratio of ABCC2:ABCC3 expression is also altered this impacts on the Km of the network, decreasing it from 47.5±6.5 to 27.6±1 μM; taken together this modelled decrease in Vmax and Km in freshly isolated rat liver relative to in vitro are
suggestive of over prediction of flux through the pathway in the \textit{in vitro} system compared to the \textit{in vivo} situation. In addition to alterations in flux through the entire network, changes in the levels of individual species can be identified between the two simulations (Figure 4c); intracellular levels of CDF (s5) are particularly affected, resulting in a predicted increase in AUC and Cmax by 365\% and 160\% respectively for the \textit{in vivo} simulation compared to baseline \textit{in vitro} simulation.

\textbf{ABCC2 acts as a fragile node in the disposition of CDF within cells}

As demonstrated above, alteration of protein levels within the network can dramatically both alter the total flux through the pathway and the kinetic profile of individual chemical species. However, the impact of altering the levels of each node on flux through the total pathway is variable; robust nodes refer to those proteins where alteration of their expression levels has little impact on either the total flux through the pathway or the kinetic profile of individual chemical species. By comparison, a fragile node refers to a protein within a network for which even small alterations in expression levels can have significant impact on the pathway, altering both the total flux through the pathway and the kinetic profile of individual chemical species. Determination of robust or fragile nodes in a network is an important step in understanding the functioning of the network, including the controlling reactions within that network. We have examined this phenomenon in the generated \textit{in silico} model through sequential alteration of each of the nodes of the network. Figure 4 shows the variability in Vmax and Km of the total pathway when each node is altered one order of magnitude either side of unity. Of the nodes examined altering ABCC2 had the largest impact on total pathway flux, altering Vmax and Km values by 2004\% and 170\% respectively.

\textbf{FIGURE 5 AROUND HERE}
Discussion

Herein we report the generation of an *in silico* model capable of simulating the flux of a compound through a cellular system. A number of attempts have been made to use *in silico* models to examine biological processes, ranging from top-down qualitative models of entire networks (Oda et al., 2005), to theoretical analysis’ of individual biological processes (Schmierer et al., 2008). In the latter category a number of attempts have been made to examine drug transport processes (Ofer et al., 2006; Sun and Pang, 2008; Sun et al., 2008), and the current work adds to this field. The work of Ofer and colleagues examined ABCB1-mediated transport of flavinoids, looking at movement across a single membrane, whereas Sun and colleagues developed a quaternary model to examine movement across both apical and basolateral membranes in a polarised Caco2 cell system (Sun and Pang, 2008; Sun et al., 2008). The approach presented herein has two major differences from these other approaches. First, the use of specialised, but freely available, modelling software removes much of the complication associated with the mathematics of modelling networks. As the model calculates the total flux through the network based upon each individual step, a researcher need only concentrate on deriving data for each step, generally simple enzyme kinetics equations, as opposed to the complete network. In addition, where data is sparse, assumptions can be made or multiple-steps combined to provide the ‘best-case’ scenario: Such an approach is thus much more user-friendly to researcher in general. Second, the modular approach of the system presented herein means that it is relatively easy to alter individual processes, or introduce more processes as they are identified, and study their impact upon the network. This can be of use to enlarge or refine a network when more data is determined, or to examine the role of individual nodes within the network. The latter scenario is of particular
importance with regard to understanding differences between biological systems (e.g. species differences, in vitro versus in vivo or normal versus pathophysiological tissue), an area of intense interest for both the understanding of basic biological processes and the correct prediction of drug action in cells. The data presented herein shows that even altering two nodes within a network (the amounts of ABCC2 and ABCC3) can highlight potentially dramatic effect on the flow through the network, altering both total flux and also the disposition of individual species.

The choice of CDFDA/CDF as a investigative tool has two distinct advantages: First, the conversion between non-fluorescent and fluorescent chemicals produces an ideal tool set for examining multiple steps within the network. As such CDFDA/CDF is ideal for increasing our understanding of the interrelationship between multiple factors (e.g. influx/efflux transporters, metabolism, sequestration) within a cell, which will be applicable for all chemical interactions within cellular networks. Second, the interaction of other novel chemicals with this network can be easily examined through their competition with CDFDA/CDF without the need to develop novel analytical methodologies. In addition, as the generated model is presented through an easy-to-use front-end it is possible to run multiple scenarios to estimate the impact of variability within the parameters, allowing, for example, the extrapolation of data from in vitro to in vivo. An important part of any in silico model is its validation through comparison of the kinetic parameters derived from the simulation with those produced experimentally using sandwich culture of primary rat hepatocytes. Zamek-Gliszczynski and colleagues derived Vmax and Km values of 97±9 pmoles/min/mg and 22±10 mM for CDF transport within sandwich cultures of primary rat hepatocytes (Zamek-Gliszczynski et al., 2003). It is notable that the in vitro- and in silico-derived values for Vmax are within the errors of measurement, suggesting that this component
has been well modelled. In comparison, the Km value is higher in the *in silico* system compared to the *in vitro* measurement of Zamek-Gliszczynski and colleagues. This variance may be explained by two different scenarios. First, it is possible that there is an error in the model, resulting in an overestimation of the ‘negative Km’ for ABCC3-mediated efflux of CDF through the apical membrane. Second, it is possible that temporal differences when transport studies were undertaken might impact upon the overall flux. As can be seen from figure 4, ABCC2 protein levels remain relatively constant, albeit over-expressed, in sandwich cultures from day 3-5; in contrast ABCC3 levels vary significantly. Hence, differences in time of culture between the *in silico* presented herein, and the *in vitro* model of Zamek-Gliszczynski may result in different ABCC3 levels, which would impact upon the Km of the overall pathway. Data on the total flux through the pathway within our system would support the latter hypothesis, as derived Km values are more consistent with the *in silico* model than the Zamek-Gliszczynski values (data not shown).

The utility of the *in silico* system is further shown by its ability to generate inhibition constants. Whereas inhibition is often quoted as an IC50, the value of this is questionable as it is dependent upon not only the inhibitor but also the concentration of the substrate used to measure the IC50. A more generally applicable value is the inhibition constant, Ki, which is substrate concentration independent. However, derivation of a Ki is time consuming and expensive as it requires the generation of IC50 curves over a wide range of substrate concentrations to allow the derivation of a dose ratio, from which the Ki can be derived. From a single piece of experimental data, the IC50 of MK571 at 10μM CDFDA, it is possible to run simulations across a range of both inhibitor and substrate concentrations and derive the Ki. The Ki value for MK571 generated for the *in silico* system (3.5±0.1 μM) agrees very well with
previously published, experimentally derived Ki values for MK571 (Paul et al., 1996). Such an approach has two important advantages: First, derivation of a Ki value \textit{in silico} is rapid, and as demonstrated herein, provides a good estimation of Ki when compared to \textit{in vitro}-derived values. This means that the generated data is robust enough to be used to assess the properties of novel chemicals, and plan in vitro experiments based upon this value, producing a significant increase in workflow efficiency. Second, as the derived Ki is dependent upon multiple simulations across a range of both substrate and inhibitor concentrations its derivation is relatively robust, meaning it can be used to confirm in vitro experiments. From the data shown in Figure 3a it is clear that the derivation of the IC50 is not 100% robust as the data points used are sub-optimal. However, due to the multiple simulations involved in Ki determination these errors are minimised, resulting in a value that closely agrees with an \textit{in vitro}-derived Ki value (Paul et al., 1996). In addition, \textit{in silico} simulation could in fact be used to guide the concentrations used for \textit{in vitro} determination of IC50 values, through the running of multiple simulations spanning a range of potential IC50 values, allowing the determination of what experimental points are based suited to ensure an accurate determination of the IC50 \textit{in vitro}.

As the amount of data concerning drug transporter expression and activity increases it is important to be able to place this in a biological context. For example, much information exists on the expression of drug transporters from both the SLCO and ABC families in a number of different cellular models (Hilgendorf et al., 2007; Bow et al., 2008; Nishimura and Naito, 2008), with such data being used to infer \textit{in vitro-in vivo} correlations in transporter function. However, to increase the utility of such correlations two improvements would be useful. First, the expression data, which is usually captured at the transcript level, must be confirmed in terms of correctly
localized, functional, protein. Second, the impact of altering expression of a single transporter on chemical flux through a pathway must be estimated; it is probable that some transporters have significantly more impact on pathway flux, representing fragile nodes within the system.

One important factor of *in silico* modelling is the ability to derive information on intermediate species within the pathway, and not just the proximate product that is measured within the *in vitro* system. This utility of this can be clearly seen through the simulations of altered protein levels and pharmacological inhibitors, whereby the biggest impact is on the AUC and Cmax of an intermediate species, intracellular CDF. It is easy to envisage where such information could be of importance, as it is often these intermediates that either exhibit pharmacological or toxicological activity. Alterations in the levels of either of these factors could significantly alter the safety/efficacy profile of the compound. A central driver at the discovery/development borderline is the ability to accurately extrapolate data from *in vitro* screens to pre-clinical, and indeed clinical, scenarios. It can be seen that the model herein begins to address the variance seen between *in vitro* and *in vivo* generated data, allowing for correction of differences in protein expression. In addition to the production of correction factors to allow improved extrapolation from *in vitro* to *in vivo* the use of *in silico* modelling allows the testing of nodes within a network to identify those that are fragile; these nodes may represent important points for intervention as they will have the most profound impact on flux through the pathway. One possibility for such intervention is the identification of the proteins whose levels should be altered in vitro to achieve a desired impact on a biological process; this could be the manipulation of cell lines to be more similar to *in vivo*, either normal or a pathological tissue state, or the deliberate compromising of cells so
that they act as more sensitive markers of toxicity. An alternate use of fragile nodes is the identification/refinement of therapeutic targets, whereby only those targets most likely to produce the desired biological effect when manipulated pharmacologically are pursued.

In summary, through the use of both literature-based and experimentally-derived data we have been able to construct an in silico model of the disposition of a chemical through a rat hepatocytes. Further, we have produced the first data to show the utility of such systems in being able to both understand and mimic differences between in vitro and in vivo.
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Footnotes

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Figure Legends

**Figure 1: Generated *in silico* model for CDFDA/CDF transport in cells.** The generated model is based upon known and presumed interactions of CDFDA and CDF with hepatocytes, and was generated using CellDesigner (v3.5.2; Systems Biology Institute, http://celldesigner.org/index.html). Each individual chemical or protein is identified as a species (s1.....sn), while interactions between species are identified as reactions (r1....rn). The kinetic terms and their associated constants that underlie the model are provided in Table 1.

**Figure 2: Bile canaliculi formation reaches an optimum after four days of sandwich culture.** (A) Using the generated *in silico* model, with all parameters as described in tables 1 and 2, CDFDA/CDF levels were simulated for period of three minutes using a starting concentration of 10 μM CDFDA in HBSS. The levels of the major species are shown. (B) Rat hepatocytes were plated on day 0 and sandwich cultured over the following 5 days. On each day cells were washed in HBSS and dosed with 10 μM CDFDA (0.1 % DMSO) before being incubated for 30 minutes at 37 °C, 5 % CO₂ and fluorescent intensity measured. (C) Fluorescent images of sandwich- and monolayer-primary rat hepatocytes after four days in culture and 30 minutes subsequent to 10mM CDFDA challenge. Images are taken at 10x magnification with an Axiovert 100 microscope with the integral digital camera through a FITC (485 nm) filter.
Figure 3: Simulation of competitive inhibition. (A) Rat hepatocytes were sandwich cultured for 4 days post plating (where cells were plated on day 0) and dosed with 10 μM CDFDA and 0 – 30 μM MK571 and incubated for 25 minutes at 37 °C, 5 % CO2. Fluorescent intensity was measured using an Axiovert 100 microscope with FITC filter (485 nm) and supplied software. Data was fitted using non-linear regression analysis (IC50 = 1.9 μM ± 3.7 μM; n=3; error bars = SEM). (B) Using the generated in silico model, with all parameters as described in tables 1 and 2, CDFDA/CDF levels were simulated for period of three minutes using a starting concentration of 0.1 to 15000 μM CDFDA in HBSS. The concentration of inhibitor was increased from 0 μM to 100 μM and the rate of CDF accumulation in the bile canaliculi was measured over 3 minutes. Data was analysed by Schild and Michaelis-Menten kinetics. (C) Kinetic parameters for each of the major CDFDA/CDF species are shown for 10μM CDFDA and 0 μM (left) and 10 μM (right) MK571, and map to those shown in figure 1.

Figure 4: Variation in drug transporter expression during time in culture impacts upon in vitro-in vivo correlation. Rat hepatocytes were cultured for 7 days (where cells were plated on day 0) in either monolayer (▼) or sandwich culture (▲) conformation. Samples of cells were taken each day for analysis by RT-PCR of the expression of the influx transporters SLCO1A1, 1A4 and 1B2 (A) and the efflux transporters ABCC2 and C3 (B). Data is expressed as a percentage of the level in rat liver (100%, —), with RNA isolated from the fresh rat livers subsequently used to prepare hepatocytes cultures. Data was analysed by 2-way ANOVA with Bonferroni post hoc test and show where expression in hepatocytes is significantly different to liver expression (*** = p < 0.001; n=3: error bars = SEM; where no error bars are
observed they are contained within the limits of the data point). Protein levels of ABCC2 and C3 were also measured by Western blot following three, four and five days of culture and compared to the level from fresh rat livers subsequently used to prepare hepatocytes cultures (B). Finally, in silico modelling was used to compare how altered protein levels of ABCC2 and C3 might impact upon disposition of CDFDA/CDF in 4-day sandwich cultures (baseline levels) versus in vivo corrected levels (C). Kinetic parameters for each of the major CDFDA/CDF species are shown and map to those shown in figure 1.

**Figure 5: ABCC2 expression represents a fragile node in CDF disposition within primary rat hepatocytes.** Levels of each node was altered sequentially to 0.1, 0.5, 1, 5, 10 units, relative to baseline model, within the *in silico* model and CDFDA/CDF levels were simulated for period of three minutes using a starting concentration of 0.1 to 2500μM CDFDA in HBSS. The impact of these changes on Vmax and Km are shown.
Table 1: Reaction constants and their derivations for use during in silico modelling

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Kinetic Parameters of Reaction</th>
<th>Source of Data</th>
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</table>
| r1       | **Hydrolysis of CDFDA (s14) to CDF (s5) within the cell**  
Sandwich culture of primary rat hepatocytes: All CDFDA was converted to CDF within 10 seconds. Presume this is 20 half lives, therefore \( t_{1/2} = 0.5 \) seconds.  
For a first order reaction, \( t_{1/2} = \ln 2/k \). Therefore, \( k = \frac{0.693}{0.5} = 1.39 \text{ sec}^{-1} \)  
(Zamek-Gliszczynski et al., 2003) | |
| r2       | **Passive diffusion of CDFDA (s13) into the cell**  
Uptake clearance of 10, 100 and 500 \( \mu \text{M} \) CDFDA into rat hepatocytes = 4.9, 687 and 3374 \( \mu \text{l/min/mg protein} \) respectively.  
Assume to be a reversible first order process, so rate = \( k[s] \). Therefore, \( k \approx 6.8 \text{ min}^{-1} \)  
(Zamek-Gliszczynski et al., 2003) | |
| r3       | **Efflux of CDF (s12) via Abcc2 into the bile canaliculi**  
Abcc2 over-expression in HEK cells: \( K_M = 18.9 \pm 2 \mu \text{M} \) and \( V_{\text{max}} = 95.5 \pm 14.8 \text{ pmol/min/mg protein} \)  
Sandwich culture of primary rat hepatocytes: \( \text{MK571 IC50 at 10} \mu \text{M CDFDA} = 1.9 \pm 3.7 \mu \text{M} \)  
(Pratt et al., 2006) & Experimentally derived |
| r4       | **Uptake of CDFDA (s13) into the cell via active transport**  
As CDFDA is a very lipid soluble molecule, the presence of active transport into the cell will be minimal and transport is unaltered by temperature. **Discounted in the model.**  
(Zamek-Gliszczynski et al., 2003; Wu and Benet, 2005) | |
| r8       | **Binding of CDFDA (s13) to extracellular proteins**  
*In vivo* CDFDA is 22\% protein bound. The model simulates the *in vitro* assay and as hepatocytes were dosed with CDFDA in  
(Zamek-Gliszczynski et al., 2003) | |
<table>
<thead>
<tr>
<th>r10</th>
<th>Conversion of CDFDA (s13) to CDF (s26) outside of the cell</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In PBS $t_{1/2} = 7.6 \pm 0.1$ hours$^1$. For a first order reaction, $t_{1/2} = \ln2/k$. Therefore, $k = \frac{0.693}{456} = 0.00152$ min$^{-1}$</td>
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<tr>
<td></td>
<td>($Zamek$-$Gliszczynski$ et al., 2003)</td>
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<table>
<thead>
<tr>
<th>r11</th>
<th>Uptake of CDF (s26) into the cell via active transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sandwich culture of primary rat hepatocytes: Uptake clearance of CDF into rat hepatocytes = $1.9 \pm 0.1$ μl/min/mg protein and was inhibited by low temperature (10 μM dose).</td>
</tr>
<tr>
<td></td>
<td>Uptake was saturable with and Inhibited by substrates of Oatp’s e.g. taurocholate and rifampicin.</td>
</tr>
<tr>
<td></td>
<td>$K_M$ of $22 \pm 10$ μM and $V_{max} = 97 \pm 9$ pmol/min/mg protein.</td>
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<td></td>
<td>($Zamek$-$Gliszczynski$ et al., 2003)</td>
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<tr>
<th>r12</th>
<th>Passive diffusion of CDF (s26) into the cell.</th>
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<tbody>
<tr>
<td></td>
<td>Sandwich culture of primary rat hepatocytes: Uptake inhibited by low temperature so mainly transporter-mediated. Octanol:Water co-efficient demonstrates no evidence for partition into lipid membranes</td>
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<td></td>
<td>Discounted in the model.</td>
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<tr>
<td></td>
<td>($Zamek$-$Gliszczynski$ et al., 2003) &amp; Experimentally derived</td>
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<tr>
<th>r13</th>
<th>Binding of CDF (s26) to extracellular proteins.</th>
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<tbody>
<tr>
<td></td>
<td>$In vivo$ CDF is 20 % protein bound. The model simulates the $in vitro$ assay and as hepatocytes were cultured in HBSS, containing no extracellular protein, this figure will be zero.</td>
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<tr>
<td></td>
<td>Discounted in the model.</td>
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<tr>
<td></td>
<td>($Zamek$-$Gliszczynski$ et al., 2003)</td>
</tr>
<tr>
<td>Line 14</td>
<td>Efflux of CDF (26) across the sinusoidal membrane via Abcc3.</td>
</tr>
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<td>-------------------</td>
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<tr>
<td>Sandwich culture of primary rat hepatocytes: Rate of efflux is approximately a quarter of efflux via Abcc2</td>
<td>$V_{\text{max}} \approx 24 \text{ pmol/min/mg protein and } K_M \approx 20 \mu\text{M}$</td>
</tr>
<tr>
<td>Sandwich culture of primary rat hepatocytes: MK571 IC50 at $10 \mu\text{M}$ CDFDA = $1.9 \pm 3.7 \mu\text{M}$</td>
<td>(Zamek-Gliszczynski et al., 2003) &amp; Experimentally derived</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

A

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B

C

10 μM CDFDA

10 μM CDFDA + 10 μM MK571