In Silico Hepatocyte: Agent-Based Modeling of the Biliary Excretion of Drugs *In Vitro*

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Keywords – biological modeling, in silico, hepatocyte, biliary excretion, systems biology

Abstract

We have used a stochastic agent directed, cellular automata-based synthetic method to instantiate, test, and partially validate simulation models of cultured primary hepatocytes (the primary cell of the liver). Here we focus on hepatocytes grown *in vitro* using a "sandwich" culture method that enables their properties and behaviors to more closely match those observed *in vivo* in intact laboratory rats. The models, referred to as *in silico hepatocytes* (ISH), are currently low resolution. Additional components can be easily added as needed. The mechanisms involve interactions among objects representing the key components: extracellular media, cells, intercellular tight junctions, intercellular lumen, transporters, metabolic enzymes, cytosolic binding factors, and drugs. The interactions take into account the physicochemical properties of the four simulated drugs (salicylate, taurocholate, enkephalin, and methotrexate). We validated the ISH using *in vitro* measures of cellular uptake and biliary clearance of the four compounds. The ISHs are designed for stand-alone experimentation; they can also function as components in hierarchical multi-models of larger systems such a liver within a whole simulated organism.

1. INTRODUCTION

Modeling and simulation of biological systems is done both in continuous and discrete domains. Differential equations have been the tool of choice in the continuous domain. The behaviors and features of a biological system that are referred to collectively as its phenotype are too diverse and complex to be described by even very large sets of differential equations, and the apparent informal, stochastic nature of biological phenomena cannot be easily conveyed. In the discrete domain, *cellular automata* (CA) approaches have been used [1] as tools for modeling complex collections of biological processes. The *lattice gas* models, also known as particle systems, comprise a well-known CA class. Usually driven by random events, these models consist of a discrete grid on which particles move about and interact with each other. When implemented in an object-oriented framework, the objects within the lattice can become independent software agents.

A class of biological models is presented in [2] which is based on the idea of "middle-out" constructive (synthetic) modeling strategy rather than the traditional top-down and bottom-up modeling and simulation approaches. Members of this class are referred to as *biomimetic in silico devices*. They are designed to generate behaviors that are useful analogues of a set of referent behaviors. The analogues are constructed from software components that are designed to map logically to biological components at multiple levels of resolution, which facilitates modeling more complex biological phenomena.

Following the guidelines presented in [2], [3], and [4], and using a CA-based, agent directed approach we propose a biomimetic device called *in silico hepatocyte* (ISH) to simulate attributes of hepatocytes (the parenchymal liver cells) grown in various *in vitro* environments.

A goal of this work is to develop an ISH that is sufficiently flexible to be used as a component in larger simulation models of more complicated *in vitro* and *in vivo* experimental systems such as the perfused rat liver discussed in [2] or the liver of a simulated patient. The design and structure of the current ISH is described in the Methods section. Its performance is demonstrated by using it to simulate the *in vitro* hepatic biliary excretion that can be observed and quantified using the specialized culture conditions described in [5] and [6]: rat hepatocytes that have grown for 5 days in a "sandwich" culture are used to predict the *in vivo* biliary intrinsic clearance of drugs. The cumulative uptake of drugs by hepatocytes is measured under two different conditions: 1) standard, Ca-containing media and 2) media that is free of calcium ion (hereafter, Ca-free) for up to 10 minutes. The biliary excretion and clearance (Cl_B) of each drug are estimated from the difference between the cumulative uptakes in the presence and absence of Ca^{2+}.

2. BIOLOGICAL BACKGROUND

The liver can metabolize and excrete into bile many of the compounds and toxins that find their way into blood.

 ISBN 1-56555-303-9

This is an important step in their use by or elimination from the body. Bile passes into the small intestine and from there, a fraction its content is reabsorbed and some of that may be ultimately eliminated by the kidney. The parenchymal cells of the liver, hepatocytes, excrete bile into intercellular spaces between themselves. These spaces merge to form bile canaliculi (Fig. 1). In humans, the canaliculi merge and deliver their content to the gall bladder. In the *in vitro* sandwich-culture system, however, the bile can be sequestered in spaces (small lumens) that are created by adjacent hepatocytes that have formed tight junctions between themselves, as illustrated in Figure 2A. The tight junctions function as a seal between the luminal contents and the media external to the cells. The hepatocyte sandwich-culture system can be broadly subdivided into three spaces: intracellular (cytosol), canalicular lumen, and the incubation medium. In the system, Ca^{2+} is responsible for maintaining the barrier function of the tight junctions: they form a seal between the canalicular lumen and the incubation buffer. The barrier can be disrupted by depletion of Ca^{2+} from the incubation medium. When such media is used, the solution (biliary secretions from hepatocytes) that had accumulated in the canalicular lumen mixes with the incubation medium. Therefore, the cumulative uptake when the standard media is used represents the amount of substrate in both intracellular (cytosol) and in the canalicular lumen. However, when media that is Ca-free is used, the cumulative uptake represents the amount of substrate in cytosolic compartment only [5]. Thus, the amount of substrate excreted in the canalicular lumen (i.e. *in vitro* counterpart to biliary excretion) can be estimated from the difference between the cumulative uptake in presence and absence of Ca^{2+}. The biliary excretion estimated by this method is consistent with *in vivo* biliary excretion [5].

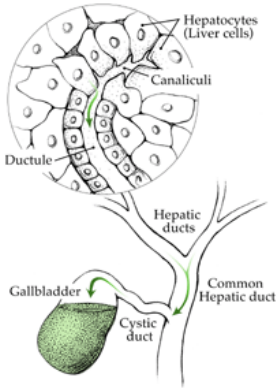

Figure 1. Hepatocytes excrete bile into canalicular spaces *in vivo* [7].

3. METHODS

We use agent-directed programming. We adopt the Functional Unit Representation Method (FURM) [3], [8] and framework, which makes use of three different models: an articulated, functional unit model (ArtModel); an accepted mathematical model—the reference model (RefModel); and an (*in vitro*) experimental data (DatModel) for validating the ArtModel. Within each simulation cycle these three models are executed by an *experiment agent* (ExperAgent). The ExperAgent is responsible for: managing the resources required for each experiment, controlling the models, taking data from the models, progressing from one experimental setup to the next, scoring each model against some performance measure, and acquiring telemetric data from the in silico experiments.

We represent hepatocytes using fixed agents placed in a 2D grid where mobile objects representing solute can interact with them stochastically. To avoid confusion hereafter and clearly distinguish *in vitro* components and features from corresponding in silico components and features, such as a "hepatocyte," a "solute," or "excreted," we use SMALL CAPS when referring to the in silico system. We model the canalicular lumen (center, Fig. 2A) as an object acting as a container inside the simulated HEPATOCYTE into which SOLUTES can be EXCRETED by TRANSPORTERS. Simply eliminating this space simulates Ca^{2+} disruption of tight junctions and mixing of what would have been luminal contents with the extracellular media. A sketch identifying several key features is shown in Figure 2.

- *The Incubation Medium* is represented by a two dimensional square grid in which HEPATOCYTES and SOLUTES can be placed to interact with each other. SOLUTES move about using a Moore neighborhood.

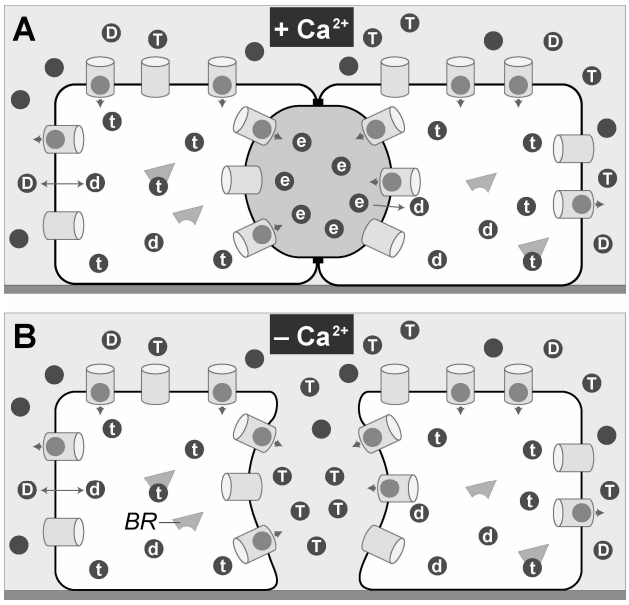

Figure 2. An illustration of the model of hepatocytes in a sandwich culture *in vitro*: the cells are shown growing attached to a solid support. **A**: Two hepatocytes (white), attached by tight junctions, are shown with a canalicular lumen space (shaded) between them; the external culture medium includes Ca^{2+}. **B**: The same system as in **A** is illustrated but with Ca^{2+} depleted from the media (breaks tight junctions). Gray circles are solute objects. A blank circle (no letter) is a SOLUTE in the media space that is not otherwise designated. Empty cylinders are transporter objects. Cylinders with light gray circles represent SOLUTE being transported (arrow indicates direction). *BR*: binder object t: solute object that has been transported into a HEPATOCYTE T: SOLUTE that has been transported out of a HEPATOCYTE into the media d: SOLUTE that has diffused into a HEPATOCYTE D: SOLUTE that has diffused back out e: SOLUTE that has been transported into the lumen space.

- *Drug Compounds* (SOLUTES) are represented as independent, mobile objects that move around stochastically, governed by the flow of the incubation medium. During an experiment the event histories of SOLUTES (and other objects) can be tracked individually or as groups, such as SOLUTE that has been TRANSPORTED out of a CELL, or that has diffused into a CELL.
- *HEPATOCYTES* are represented as shown in Fig. 2. Each is constructed from objects that represent hepatocyte components and the environment: factors that can bind drug, enzymes, transporters, and a space for biliary excretion.
- A *Binder* is an object within HEPATOCYTES that can bind a free SOLUTE and hold onto it for a specified number of binding cycles.
- An *ENZYME* is a specialized form of binder. It can "metabolize" a bound SOLUTE by replacing it, following the binding period, with a metabolite object and destroying the replaced SOLUTE (for more details about these in silico Binders and Enzymes see [9], [10], and [11]).
- *TRANSPORTERS* belong to a subclass of binders. They can bind with free SOLUTE that is either inside or outside, and transport them to the opposite side of the CELL MEMBRANE, independent of the local SOLUTE concentration. When needed, TRANSPORTERS can be subdivided further into specialized forms. The following are three of the important TRANSPORTER parameters:
  - *Transport_in/out_probability* specifies the probability that a TRANSPORTER will bind a given SOLUTE, once that SOLUTE is detected by the TRANSPORTER, and TRANSPORT it in or out of the CELL.
  - *Binding_cycles* specifies the number of simulation cycles a SOLUTE will remain attached to a TRANSPORTER.
  - *Excretion_space* stores excreted SOLUTES until they are removed to an EXTRACELLULAR space. In this work, under standard culture conditions (with Ca^{2+}), they are not removed by outer spaces. They stay within this space simulating the fact they are "sealed" within a luminal space (designated e in Fig. 2A). The average number of SOLUTE objects in this space is determined by the parameter *Excretion_Mean*, which is the mean of an exponential probability distribution. A SOLUTE in this space may diffuse back into the CELL (e-to-d in Fig. 2A), depending on its physicochemical properties.

3.1 In Silico SOLUTE Kinetics

Figure 3 shows the trace of a SOLUTE in the simulation. SOLUTES are initially placed uniformly and randomly in the 2D space external to HEPATOCYTES. At each simulation cycle, a SOLUTE may stay in place or move randomly in one of eight directions (N, NE, E, SE, S, SW, W or NW with a probability of 1/9). A SOLUTE may, depending on its properties, partition into an encountered HEPATOCYTE. There is also a chance that it may be transported (actively imported) into the CELL by TRANSPORTERS.

3.2 Membrane Diffusion

Partitioning of SOLUTE into and out of HEPATOCYTE CYTOSOL is simulated as follows: when a free SOLUTE in the "incubation medium" (the 2D space) encounters a CELL, it may enter the CELL based on the values of two parameters: *Solute_Membrane_Cross-In_Probability* and *Average_ - Cell_Capacity*. The former, which is governed by SOLUTE properties, is the probability that the SOLUTE enters the CELL passively. The latter is the mean of an exponential distribution which determines the number of objects a CELL can accommodate by passive transport. The probability that a SOLUTE partitions into the CELL decreases as the number of intracellular SOLUTE objects increases. Each unbound intracellular SOLUTE may also partition out of the CELL with a probability of *Solute_Membrane_Cross-Out_Probability.*

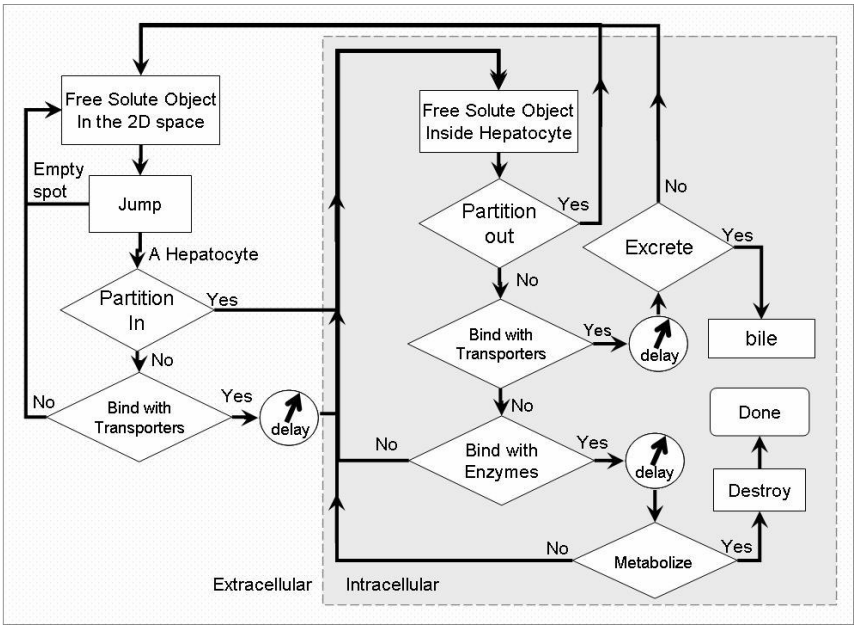

Figure 3. Trace of a SOLUTE object (representing drug) in the model.

3.3 Active Transport

In silico, if a SOLUTE fails to enter the CELL by passive transport, it will be given a chance to bind with TRANSPORTERS that recognize it with probability of *transport-in_probability*. If recognized, it gets transported into the CELL. We assume that there are no spatially explicit arrangements of TRANSPORTERS within a CELL membrane.

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In vivo, biliary excretion is performed by canalicular membrane transporters. In silico, as Fig. 3 illustrates, once an intracellular SOLUTE binds to a TRANSPORTER, there is chance that the SOLUTE will get EXCRETED based on an exponential probability distribution with mean *Excrete_Mean*. If excreted, the object is put in a waiting list to be removed by external spaces (such as some bile canalicular space when HEPATOCYTES are organized within a simulated hepatic lobule). If not removed, the list represent solute "sealed" between HEPATOCYTES. If not excreted, the SOLUTE is placed in the transported-out list. Objects in that list are treated as if they had been transported out by the basolateral membrane TRANSPORTER, and are transferred to the 2D extracellular space (the simulated incubation medium) in the next simulation cycle.

3.4 In Silico Uptake and Efflux Studies

The cell culture media is represented by the 2D space; SOLUTE within HEPATOCYTES maps to substrate in the cytosolic compartment. SOLUTE in the *excretion space* maps to the excreted substrate in the canalicular lumen (bile). When the standard media was simulated, the in silico cumulative uptake was calculated using Eq. 1.

$$Uptake_{in\ silico} = \text{total of (partitioned-in + transported-in + excreted) SOLUTE} \quad (1)$$

When the Ca-free media is being simulated, the average number of excreted objects (*Excrete_Mean*) was set to zero to simulate the effect of Ca^{2+} depletion on the barrier function of tight junctions. Consequently, the in silico cumulative uptake for simulated Ca-free media could also be calculated by Eq. 1. The values of simulated efflux for both standard and Ca-free media were calculated using Eq. 2.

$$Efflux_{in\ silico} = \text{total of (partitioned-out + transported-out) SOLUTE} \quad (2)$$

3.5 Data Analysis

The *in vitro Biliary Excretion Index* is calculated using Eq. 3 [5]. The same equation was used for the corresponding in silico calculation.

$$\text{Biliary Excretion Index} = (Uptake_{standard} - Uptake_{Ca\text{-}free})/Uptake_{standard} \quad (3)$$

Biliary clearance by the sandwich-cultured hepatocytes, $Cl_{B(culture)}$ is calculated according to Eq. 4 [5].

$$Cl_{B(culture)} = (Uptake_{standard} - Uptake_{Ca\text{-}free})/(Time_{incubation} \cdot Concentration_{medium}) \quad (4)$$

Where $Time_{incubation}$ is the incubation time and $Concentration_{medium}$ represents the initial substrate concentration in the incubation medium. In silico, the same equation was used to calculate the biliary clearance; the in silico SOLUTE concentration is defined as:

$$\text{In Silico Concentration} = \text{(total SOLUTE)/(total locations in the 2D space)} \quad (5)$$

3.6 Parameter Estimation

We used an optimization technique to estimate the parameters of this model. After each simulation experiment a similarity measure (SM) algorithm [12] assigns a similarity score to the output of the simulation. This score provides a measure of how similar the current output is to the referent experimental data. The goal is to maximize the SM. The optimization algorithm used the Nelder and Mead Simplex [13] method. The algorithm has been used widely [14-17] for almost 40 years to solve parameter estimation problems. It is still the method of choice for many practitioners because it is straightforward to code and easy to use. The technique belongs to a class of methods, which do not require derivatives and are often claimed to be robust for problems with discontinuities or where function values are noisy. This property makes it a good choice for helping to optimize our ISHs. There are several different versions and extensions. We are using the one described in [18] with minor changes.

The following parameters were used in the search: Excretion_Mean, Average_Cell_Capacity, Solute_Transport-In_Probability, Solute_Transport-Out_Probability, Solute_-Transport_Cycles, Solute_Membrane_Cross-In_Probability, Solute_Membrane_Cross-Out_Probability, Solute_Binding_-Probability and Solute_Binding_Cycles. For each of the four drugs a different set of parameter values was selected. Others, listed in Table 1, such as Space_Size, Hepatocyte_Density, Max/Min_Binders_per_Cell, etc., were fixed for all four drugs. Total_Solute_Particles was calculated according to the *in vitro* concentration of the corresponding drug; see the Appendix for details. Table 2 shows the optimized parameter values for enkephalin and salicylate. In order to simulate the depletion of Ca^{2+} the Excretion_Mean was set to zero to essentially eliminate the excretion space.

4. RESULTS

4.1 In Silico Biliary Excretion

The referent for this model is an *in vitro* system used for studying primary rat hepatocytes [5]. Liu et al [5] show that hepatocytes cultured in a collagen-sandwich configuration for up to five days establish intact canalicular networks, and reestablish polarized excretion of organic anions and

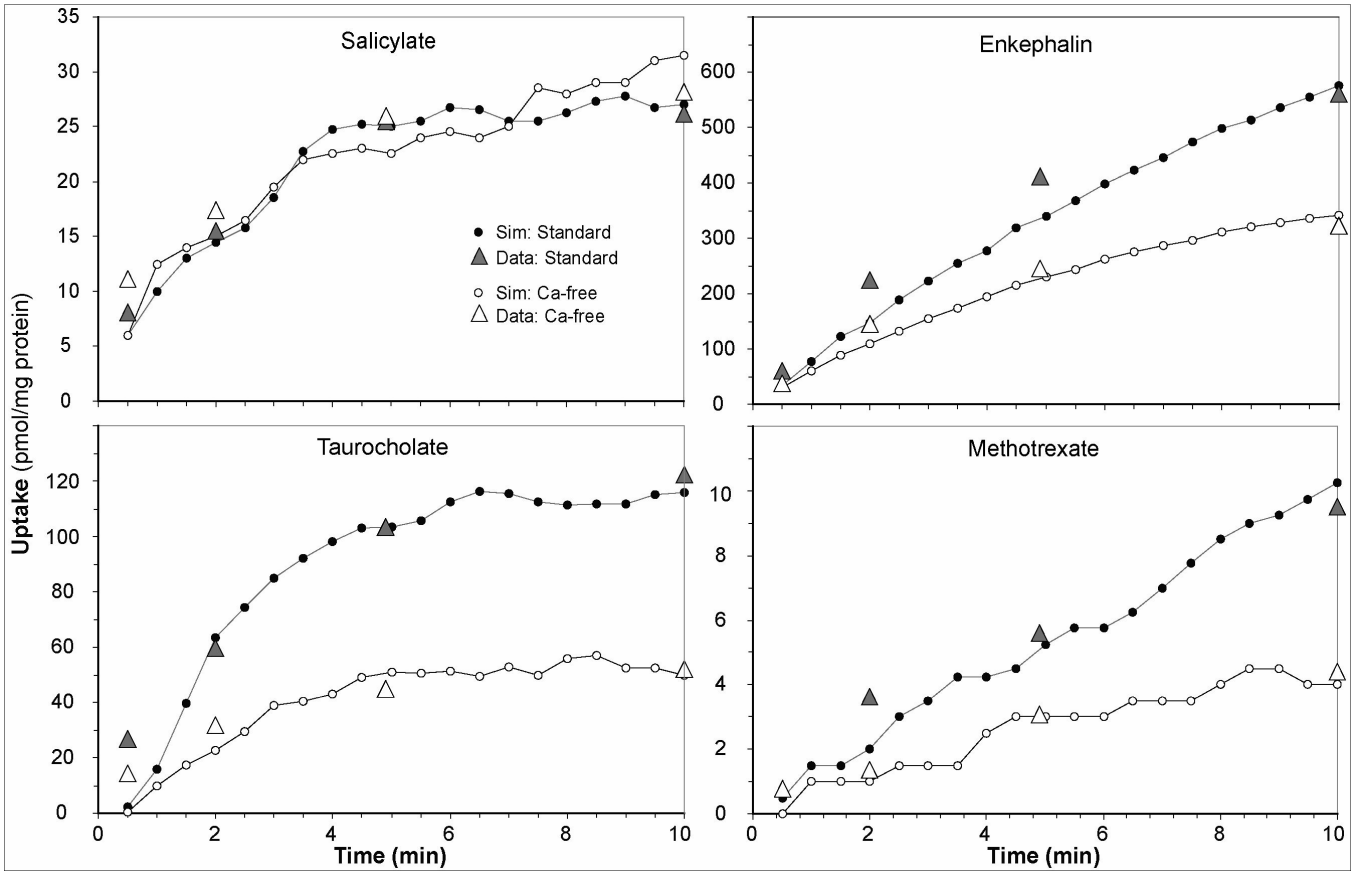

Figure 4. Simulated and *in vitro* outputs are shown for four compounds studied in standard and Ca-free media. The legend within the Salicylate plot frame also applies to the other three plots.

Table 1 - Calculated and fixed parameter values

Parameter		Enkephalin	Salicylate
In vitro	Cell density (cells/ml)	6.67×10^{5}	6.67×10^{5}
	Drug concentration (M)	1.50×10^{-5}	1.00×10^{-6}
α (unit conversion constant)		1.78×10^{11}	1.78×10^{11}
In silico	Space Size	53*54	53*54
	Hepatocyte Dencity	0.2	0.2
	Min_Binders_per_Cell	5	5
	Max_Binders_per_Cell	10	10
	Min_Transporters_per_Cell	5	5
	Max_Transporters_per_Cell	10	10

bile acids. The system is a useful *in vitro* model for investigating the hepatobiliary disposition of compounds. The authors report that after the cells have been maintained sandwich culture for five days, the cumulative uptake of [^{3}H] taurocholate (a common component of bile) by the hepatocytes was significantly higher in standard Ca-containing media, compared with that of Ca-free media. The difference is a consequence of accumulation of taurocholate in canalicular spaces. [^{3}H] Taurocholate efflux from cell pre-loaded with drug for five days was greater in Ca-free compared with standard Ca-containing media.

Table 2 - Optimized Parameter values found for enkephalin and salicylate in standard buffer

Parameter	Enkephalin	Salicylate
Total_Solute_Particles	2290	153
artHepExcretionMean	0.008	0.0046
artCellAverageCapacity	0.16	0.01
artSoluteTransportInProb	0.0046	1×10^{-6}
artSoluteTransportOutProb	0.040	1×10^{-6}
artSoluteTransportCycles	1	2
artSoluteMembraneCrossInProb	0.0078	0.058
artSoluteMembraneCrossOutProb	0.144	0.207
artMetabolizationProb	0	0
artSoluteBindingProb	0.002	0.067
artSoluteBindingCycles	3	3

4.2 Drug Uptake

Figure 4 shows the uptake of four drugs in well-established, sandwich-cultured hepatocytes using both standard and Ca-free media. Also shown are the in silico uptake values using an ISH optimized for each of the four drugs. The values were calculated as follows:

$$Uptake_{in\ silico} = \text{total of (partitioned-in + transported-in + excreted) SOLUTE}$$

$$\text{Biliary Excretion Index} = (Uptake_{standard} - Uptake_{Ca\text{-}free})/Uptake_{standard} \quad (6)$$

$$Cl_{B(culture)} = (Uptake_{standard} - Uptake_{Ca\text{-}free})/(Time_{incubation} \cdot Concentration_{medium}) \quad (7)$$

The duration of the in silico experiments was 20 cycles. Each unit of simulation time was 2 cycles. Figure 5 shows the correlation of in silico and *in vitro* Biliary Excretion Index and Biliary Clearance of compounds. We suggest that the *in vitro* and in silico values in Fig. 4 are experimentally indistinguishable because the in silico values are within the range of variability that is seen for repeated wetlab experiments.

5. CONCLUSION

Using an agent based, constructive approach, we have presented and partially validated a set of simulation models for uptake and biliary secretion of compounds by hepatocytes grown *in vitro*. We have demonstrated how this model can be used to simulate the *in vitro* biliary excretion of drugs by hepatocytes grown in a sandwich culture.

The models are instantiations of the mechanism hypothesized by Liu et al. [5]. Consequently, our in silico experimental results provide direct evidence that, at the low level of resolution used, the mechanism is an accurate representation of the actual *in vitro* events.

Although the parameters do not map directly to measurable biological counterparts, they can be estimated for a new drug using machine-learning tools such as Fuzzy Logic, Neural Networks etc. One of the important, future tasks is to demonstrate how this model can be used to predict the biliary clearance of drugs.

The goal of scientific, biomedical simulation, in contrast to engineering simulation, is to discover plausible mechanisms for how a system might generate specific behavior. In cases where many of the elements of a process are unknown or unclear, we can build families of simulations that circumscribe an in silico behavior space that partially overlaps the behaviors of the referent system. When building such simulation families, many of the parameter values can be taken from or enlightened by data from biological referents. However, many parameters remain artificial or abstract. In the latter case they provide flexibility and allow more control over the search of the model behavior space. When the behaviors of these models and the referent biological system begin to converge, analysis of the artificial parameters is expected to help researchers generate new hypotheses for those parts of the system that are not explicitly available for study in the biological experiments.

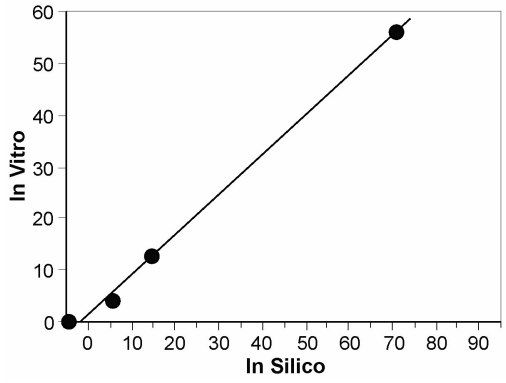

Figure 5. Correlation of in silico and *in vitro* biliary clearance. Circles show the calculated values from simulation (in silico) results vs. in vitro ($R^2 = 0.997$).

ACKNOWLEDGEMENTS

This research was funded in part by the CDH Research Foundation[1] R21-CDH-00101 and CAH. We thank Nasim Sassan for assisting with evaluation of *in vitro* systems and data, Pearl Johnson for assisting with manuscript preparation, and the members of the BioSystems Group for helpful discussion and commentary.

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APPENDIX

Parameter Calculations

The in vitro data of four drugs was obtained from [5]. The incubation conditions are reported to be the same for all four drugs, however the concentration of the drugs varies from 1 µM to 15 µM. In order to be consistent with the *in vitro* experiments, the in silico relative ratio of DRUGS to HEPATOCYTES should be similar.

We define the following:
C_h: *in vitro* concentration of hepatocytes
C_s: *in vitro* concentration of solute
P: number of SOLUTES in the 2D space (or Total-Solute-Particles)
H: number of HEPATOCYTES in the 2D space
S: number of 2D spaces
d_h: density of HEPATOCYTES in the 2D space ($d_h = H/S$)

Obviously, the total number of HEPATOCYTES and SOLUTES should be less than the total number of grid spaces:

$$P + H < S, \text{ also in vitro} \quad \text{(A1)}$$

$$C_s = A_1/V,\ C_h = A_2/V \Rightarrow A_1/A_2 = C_s/C_h$$

where C_s and C_h are the apparent concentrations of solute and hepatocytes respectively, A_1 and A_2 are the amounts of solute and hepatocyte, and V is the system volume.

$$P/H = \alpha \bullet C_s/C_h \quad \text{(A2)}$$

where α is an *in vitro* to *in silico* unit conversion constant. The problem is to choose α such that P and H each satisfy the above constraint for all the four drugs.

Let $k_{max} = max(C_s/C_h)$

$$\Rightarrow P/H < \alpha \bullet k_{max} \quad \text{(A3)}$$

On the other hand,

$$P + H < S \Leftrightarrow P/H < (S/H) - 1 \Leftrightarrow P/H < (1/d_h) - 1$$

Consequently, the above constraint (Eq. A3) will be satisfied if we choose α such that

$$(1/d_h) - 1 < \alpha \bullet k_{max}$$

which implies

$$\alpha > [(1/d_h) - 1]/\max(C_s/C_h) \quad \text{(A4)}$$

In our case the C_h was 6.67×10^5 (cells/ml) for all 4 drugs, and the max C_s was 15 µM. Choosing $d_h = 0.2$ yields $\alpha > 1.78 \times 10^{11}$. Selecting $\alpha = 1.78 \times 10^{11}$*(cells/pmol),* P can be calculated by Eq. A2:
$P = 1.78 \times 10^{11} \bullet d_h \bullet S \bullet C_s(\mu M)/C_h$*(cells/ml).*

 ISBN 1-56555-303-9