SUPPLEMENTAL MATERIAL

Discovering Plausible Mechanistic Details of Hepatic Drug Interactions

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The following merits repeating. We have not tried to describe how the specific, detailed events responsible, at multiple levels, for the PK data in Fig. 3 likely occurred within those perfused livers. That cannot be done: the precise knowledge needed to do so is simply not available. What we did is diagrammed in Fig. 1. We built an abstract, analogue systems in software, RISLs, using autonomous components, with the expectation that measures of their behaviors, following the referent experimental protocols, would be sufficiently similar to the data in Fig. 3 so that RISL mechanisms could stand as a plausible hypothesis for what may have occurred during those experiments. Individual RISL mechanisms were not intended to be 1:1 physical or chemical descriptions of referent events (again, we lack the detailed knowledge). Because the RISL is abstract, so must be its mechanisms. Rather, our intent has been that RISL mechanisms map logically and intuitively to referent events. For example, if we know that a drug had to cross at least one biological barrier to access metabolic enzymes in order to undergo metabolism, then we specified that the same must be true within the RISL. Logically, we also wanted to draw as extensively as possible from accepted knowledge and theory in building mechanisms, and we wanted our constructed objects to behave consistent with current knowledge and theory. For example, if it is known that a referent compound in its ionized state cannot cross biological barriers at physiological pH, then we expected the same behavior from a RISL COMPONENT. Initial component verification (the components works the way it has been designed to work) and face validation (component function based on performance during simulation is judged reasonable by domain experts) therefore consisted of providing evidence that the RISL component (and its mechanism) exhibit behavior consistent with current information and knowledge. The efforts below built on earlier verification and validation (against wet-lab data) experiments (Garmire et al., 2007), and we incorporate those results by reference. Compared to earlier, referenced analogue systems, a goal for the RISL has been to make all internal events relativistic (scale free). That requires that a separate scaling mapping is needed to relate RISL events to referent events. Doing so required the additional verification experiments below.

Methods: Additional Detail

The following additional information, along with that under a corresponding Results section below, contains more detail on what was done and how the RISL functioned. It is provided to facilitate repeating all or part of this work. When there is a counterpart, the section subheadings are the same as those used on the body of the paper.

Objective and Approach. How simple or complex must a device be to represent the liver during recirculating perfusion experiments, when sampling times are several minutes apart? The answer depends on intended use and especially the referent data being simulated. Hunt et al. (Hunt et al., 2006) and Yan et al. (Yan et al. 2008a), (Yan et al., 2008b) describe an In Silico Liver comprised of similarly constructed LOBULES. Each LOBULE is comprised of several multi-level agents called Sinusoid Segments arranged into a network that forms three zones. That level of detail (system granularity) was supported by the referent wet-lab data against which their simulations validated. That data came from single pass liver perfusion studies: outflow was collected and analyzed every few seconds. However, for experimental designs similar to the recalculating system used to obtain the data in Fig. 1 (Lau et al., 2004), more abstract, simplified representations have sufficed (Booth et al., 1996), (Booth et al., 1998), (Tirona and Pang, 1999), (Matsuura et al., 2001), because rates of drug level change within lobular spaces are slowed when perfusate recalculates compared to single pass perfusions. We therefore inferred that neither
zation nor different Sinusoid Segment types would be needed to achieve our objective. Specifically, we inferred that a LOBULE could be represented by one Sinusoid Segment. We began there. Results of subsequent in silico experiments supported that inference. To meet the needs of this study, we added a bile space, S5, and then tailored the other four spaces to meet study needs. The resulting analogue has several features in common with the In Silico Transwell Device (Garmire et al., 2007). The In Silico Liver cited above was designed to exhibit ten capabilities. The following five are especially relevant:

- It must be easy to reconfigure and reuse an RISL to represent different histological, physiological, or experimental conditions.
- In order to represent particular specifics of different experiments, it must be relatively simple to change RISL usage and assumptions, and increase or decrease detail, without requiring significant re-engineering.
- To facilitate the two preceding capabilities, it must be easy to join, disconnect, and replace RISL components: the components articulate easily.
- It must be straightforward to separately validate components, and that is facilitated by making simulation details visualizable and measurable. Consequently, the RISL must be transparent.
- The RISL must be usable for simulating the disposition, clearance, and metabolic properties of a wide variety of compounds, separately or in the same experiment.

**Discretization.** The potential of discretization decisions and specifications to create artifacts that damage the model’s ability to mimic the referent were taken into consideration during design and final specification of RISL components. The following are illustrative. The extracellular, intra-sinusoidal region is an infinitely divisible, continuous space. The path of a compound within would appear smooth at any achievable level of resolution. Within S1, that space is represented using 2500 elements: from one simulation cycle to the next, a COMPOUND’s location is fixed. The number of grid spaces and the time interval to which a simulation cycle maps needed to be specified so that the discretized space and movement have been sufficiently smoothed to avoid artifacts; such discretization decisions can impact the ability of the analogue to mimic the referent. Processes that are very fast (relative to the sampling interval), such partitioning and binding are treated as events, and so discretization of that process into an instantaneous event has no impact on the ability of the model to mimic the referent. METABOLISM and TRANSPORT are somewhat slower and are believed to involve several steps. However, relative to the key events being simulated—the change in perfusate levels of COMPOUND and METABOLITE at intervals of minutes, they too can be treated as events, for which the detailed chemistry is ignored. Such discretization does not create any artifacts that damage the RISL’s ability to mimic the referent.

**Selecting the number of Monte-Carlo simulations.** Each Monte Carlo run (RISL execution) is an experiment. Early in the simulation exercise, we decided on the number needed to represent the mean RISL behavior for a given parameterization. Here, we show the data supporting our decision to use at least six Monte-Carlo experiments.

Using an early RISL similar to the one used for Fig. S7, we ran 50 repeat experiments. For each, we made measurements of PERFUSATE DIGOXIN and METABOLITE levels at simulation cycles 5, 10, and 20. We then calculated their cumulative average. Results are presented in Fig. S1. We see that the cumulative averages do not change significantly after n = 6.
Tunable Parameter Values. By drawing on the body of knowledge available for each event, we can select a likely initial range for each parameter value. For example, if a drug is known to have an above average affinity for CYP3A4, then we can infer the following. The probability of binding for any drug molecule that is in close proximity to CYP3A4 within a 10 second interval will likely be in the range 0.6–1.0. Absent any other information, we can start with a value of 0.8, for example, and then adjust it to improve the degree to which simulated and referent observables match. The width of the initially considered range contracts or expands based on prior knowledge or lack thereof. Because of the networked nature of the many independent, probabilistic events (the outcome at the end of each simulation cycle emerges as a property of them all), precise values of each parameter are not as critical as is often required with traditional, inductive, equation based models. A 5% change in the value of the key RISL probabilistic parameters did not typically produce a statistically significant change in a measured system level behavior, especially when the number of replicate simulations is kept small, six in this case (examples are provided in Fig S6).

If uncertainty about the parameter’s value is complete, we can simply assign a new random value for each event. So doing marginally increases simulation variance, but it also provides an objective means of dealing with uncertainties, which is an advantage of this modeling and simulation approach. By drawing on prior experience in validating similar analogues, several of our initial parameter estimates proved adequate to achieve our objective. Because the reference data were unique, we elected not to undertake parameter fine-tuning.

Logic used by TRANSPORTERS, BINDERS, and CYP.

Each simulation cycle, each PGP, OAT, MT, CYP, and COMPOUND follows specified logic to select the action or actions to take based on its local environment. We have validated and used CYP and PGP agents in different contexts (Liu and Hunt, 2006), (Sheikh-Bahaei and Hunt, 2006), (Garmire et al., 2007), (Yan et al., 2008b). CYPs can have multiple active and regulatory sites. However, for simplicity, the RISL does not use the latter. Each CYP scans each COMPOUND in its neighborhood to determine which have non-zero values of assocProb; for those that do, there is an opportunity during that same cycle for binding. A CYP can bind any COMPOUND that has a non-zero assocProb, whether it is a substrate or not. However, only substrates are METABOLIZED. Each CYP may bind multiple COMPOUNDS; the maximum number is controlled by the parameter maxSites. Following that, each CYP has an opportunity to METABOLIZE a bound COMPOUND.
The binding sites within a CYP are identical and independent. However, details of CYP operations are below the level of mechanistic resolution and so do not map to biochemical counterparts. CYP parameter values are listed in Tables 2, 4 and 5, along with those that follow. At the start of a simulation, all CYPS are assigned randomly to elements within S3. Each simulation cycle, each CYP steps through its assigned logic to determine what action to take. The logic (diagrammed in Fig. S2B) and its use can be summarized as follows. A CYP examines its adjacent neighborhood. Neighborhood size is controlled by a parameter (sitesN). The probability that a CYP-DRUG binding event will occur for any DRUG within a CYP’S neighborhood is governed by assocProb (Table 4), the value of which maps to affinity.

The logic used by PGP and OAT, diagrammed in Fig. S2A, can be summarized as follows. Each cycle, they first scan for COMPOUNDS in their neighborhood. Binding is next. A PGP or OAT, like CYP, can bind anything that has a non-zero assocProb. A PRN is drawn from a uniform [0-1] distribution. If its value is less than assocProb (Table 4), the COMPOUND is bound. That process repeats for the next unoccupied site, and so on. The maximum number of bound COMPOUND is specified by the parameter maxSites. Then, they determine if each COMPOUND bound earlier will be released. That probability is controlled by the parameter releaseProb. For an inhibitor, that probability is small. When it is decided to release the bound COMPOUND, the PGP (or OAT) can release it into the space of origin, such as S3 (S1 for OAT), or into the destination adjacent space, such as S5 (S3 for OAT). However, only substrates are TRANSPORTED (RELEASED) to the destination space. The probability of releasing SUBSTRATE into the space of origin is controlled by (TRANSPORT) efficiencyProb.

**Figure S2.** Internal logic used each cycle by TRANSPORTERS (A), ENZYMES (B), BINDERS (C) and COMPOUND (D). The internal logic of TRANSPORTERS, ENZYMES and BINDERS is similar. First, each decides whether it can bind a COMPOUND within its local neighborhood (neighborhood size is specified by sitesN). BINDING is probabilistic. The value of assocProb maps to the compound’s affinity for its binding partner. When a PRN < assocProb binding occurs. When multiple COMPOUNDS are within a neighborhood, the process continues until all COMPOUNDS have had one opportunity to bind. A: A TRANSPORTER first decides whether or not to release the bound COMPOUND. If PRN < releaseProb, its selects a location to place the COMPOUND. When the COMPOUND is a
SUBSTRATE and \( PRN < \text{efficiencyProb} \) the COMPOUND is transported to a destination (across the barrier). Otherwise, the COMPOUND is released to the space from which it was bound.

**B**: Each CYP first decides whether or not to METABOLIZE a bound COMPOUND. When \( PRN < \text{efficiencyProb} \) it is METABOLIZED. If \( PRN < \text{releaseProb} \), the bound object may be released. **C**: A binder’s internal logic is similar to that of a CYP, except there is no METABOLISM step. **D**: The logic used by mobile objects to move about is divided into two phases: 1) lateral movement within the same space, which simulates dispersion, and 2) transition to a neighboring space, representing passive permeation. A COMPOUND will use different transit rules depending on the space in which it is located. A COMPOUND bound to stationery objects is not given an opportunity to relocate during that cycle (even when it gets released during that cycle). COMPOUNDS that transition to BILE (S5) are not given an opportunity to return; they are REMOVED.

**Probability scaling**

Consider a probabilistic event \( X \). The probability of its occurrence during a simulation cycle is \( P_{\text{old}}(X) = 0.3 \). Assume that one simulation cycle (the “old” cycle) maps to 1 second. Suppose we desire to span more wet-lab time with one simulation cycle. We want one new simulation cycle to map to 4 seconds; the process is to be scaled up by a factor \( (SF) \) of 4. What is the new probability \( P_{\text{scaled}}(X) \) for the same event within the new simulation cycle?

Increasing \( P_{\text{old}}(X) \) by \( SF \), yields an incorrect result.

\[
P_{\text{scaled}}(X) = 4 \times P_{\text{old}}(X) = 1.2 > 1
\]

Instead, we allow four trials of the probabilistic event within one, new cycle. If the new simulation cycle maps to an event interval that is four times longer than with the original simulation cycle, then the original event is now being given four options to occur. After four options, the probability of \( P_{\text{scaled}}(\text{not } X) \) is therefore

\[
P_{\text{scaled}}(\sim X) = (1 - P_{\text{old}}(X))^4
\]

So,

\[
P_{\text{scaled}}(X) = 1 - P_{\text{scaled}}(\sim X) = 1 - (1 - P_{\text{old}}(X))^4 = 1 - (1 - 0.3)^4 = 0.76
\]

It is easiest to understand when \( SF \) is a positive integer. The same can be generalized to any positive number of scaling factor \( (SF) \).

\[
P_{\text{scaled}}(X) = 1 - (1 - P_{\text{old}}(X))^{SF}
\]

For \( SF > 1 \), \( P_{\text{scaled}}(X) > P_{\text{old}}(X) \), so the process is sped up; for \( 0 < SF < 1 \), the \( P_{\text{scaled}}(X) < P_{\text{old}}(X) \), so the process is slowed down.

The relationship is used to scale probabilities relative to the time to which a simulation cycle maps and other COMPOUND-PROTEIN interactions. The parameter \( \text{timestepfactor} \) is the factor used to scale all transit probability initial estimates.

The same relationship was used for time-variant parameters when we required that the probabilistic event of interest needed to “speed up” or “slow down.” The time-variant parameters \( \text{closeToInterface} \), which specifies fraction transitional and CYP’S \( \text{efficiencyProb} \) are both time-dependent. Fig. 4 shows them being scaled down from their initial value using the above relationship, with the value of \( SF \) decreasing.

**Passive dispersion**

Each COMPOUND had a chance during each simulation cycle to disperse passively within its current space. In order to simplify implementation, all ELEMENTS within the same space were treated as being identical, and having no particular shape, volume or absolute location. We used toroidal spaces to eliminate edge effects: a COMPOUND going out of a space to the right (or top) reappeared on the left (or bottom) and vice versa. We specified an intuitive, relative location of ELEMENTS to each other, and identified each with a \((x, y)\) coordinate. Each ELEMENT had eight neighboring ELEMENTS. During that simulation cycle, a COMPOUND in an ELEMENT has equal
chance to moving to any one of its eight neighboring ELEMENTS, or to staying within its current ELEMENT. Doing so has enabled us to track specific COMPOUNDS, should that be desired for later verification or face validation purposes.

<table>
<thead>
<tr>
<th>Coordinate</th>
<th>x - 1</th>
<th>x</th>
<th>x + 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>y - 1</td>
<td>PRN = 1</td>
<td>PRN = 2</td>
<td>PRN = 3</td>
</tr>
<tr>
<td>y</td>
<td>PRN = 4</td>
<td>PRN = 5</td>
<td>PRN = 6</td>
</tr>
<tr>
<td>y + 1</td>
<td>PRN = 7</td>
<td>PRN = 8</td>
<td>PRN = 9</td>
</tr>
</tbody>
</table>

During a simulation cycle, each COMPOUND generates a PRN that is an integer between 1-9, inclusive. A COMPOUND currently in ELEMENT (x, y) will move to the new location in the diagram above.

An alternative implementation would have been to relocate each COMPOUND randomly during each simulation cycle. The end-result is the same. The disadvantage of the latter is that it would be more difficult to track particular COMPOUNDS.

COMPOUND relocations are independent events. Each ELEMENT can hold any number of COMPOUNDS. When moving within a RISL space, a COMPOUND does not consider the number of COMPOUNDS in the target ELEMENT. Because COMPOUNDS are placed randomly within S1 at the start of an experiment, there is no need for that additional complication.

ELEMENTS can be made different from others within the same space, should the need arise. ELEMENTS can have different internal properties (e.g., simulating a more lipophilic or acidic region), enabling, for example, different COMPOUND solubilities. In the current RISL, all ELEMENTS within a space are identical.

**Passive transition**

Each COMPOUND gets a chance to PARTITION passively into a MEMBRANOUS space (S2 or S4) during each simulation cycle. Although numerous models are available to characterize in vitro passive permeability, none have been validated in a complex in vivo system such as the recirculating isolated perfused rat liver. Absent reliable values, and given the relativistic RISL design, we needed a method to arrive logically at initial rates of passive transition. We formulated the following approach by adapting established physicochemical models. The following is a list of specifications made to enable arriving at initial estimates, which may or may not need to be adjusted subsequently during RISL tuning.

1. In the referent system, a compound must be sufficiently close to the aqueous-membranous interface such that within the time interval corresponding to a simulation cycle (10 seconds in the RISL) the compound can reach the membrane (or aqueous space). In RISLs, we labeled each COMPOUND with a state variable transitional, and used a parameter closeToInterface to specify whether the compound was sufficiently close to an interface.
2. Compound exists in multiple physicochemical states in aqueous media, some of which have greater intrinsic permeabilities. Being charged compromises permeability. When estimating initial transit probabilities for RISLs, we take into account the referent compound’s expected differential permeability.
3. Chemical structure (functional groups), together with composition and properties of the resident medium, such as pH, ionic strength etc, affects the relative abundance of physicochemical states, thus influencing overall permeability. In RISLs, we used parameter insilicoPH for the spaces as an analogous measure of the tendency of compound being less permeable (ionized) in that particular space. Each compound also has in silico
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4. Compound size also influences rates of permeation: the larger the compound, the less permeable. We used the referent compound’s molecular weight as a surrogate measure of size in affecting permeation in the RISL.

5. Within 10 seconds, compound within a small volume on either side of a permeable cell barrier will have equilibrated, all other factors being held constant. As a result, the ratio of concentrations within the two adjacent spaces relates directly to the probability of finding a molecule in each space by the end of the next simulation cycle, which in turn gives the probability of transit during the cycle.

We use terms and methods such as the Henderson-Hasselbalch equation in order to draw as often as possible from established knowledge and accepted methods. We fully appreciate that applicability of these models have not been validated within complex biological systems (such as recirculating isolated rat liver) and that many of the measures (such as pH within of the intracellular matrix) are not well-defined and cannot be measured or validated during a perfusion experiment. Using these models and methods to make initial parameter estimates may handicap the RISL with unverifiable assumptions. However, we accepted that risk because we believe that we can limit risk by drawing on established theory as the logical first step. To date, the resulting initial estimates of transit probabilities have proven to be adequate for achieving our goal of implementing plausible mechanisms. Multiple approaches are feasible, and as part of future projects, we intend to explore alternatives. See (Garmire et al., 2007) for an alternate approach, one that was designed not to be fully relativistic, yet it works well in the context of the model used. The models and methods used in estimating the transit probabilities are independent of the rest of the model and can be separately validated and modified as needed.

Initial estimates of transit probabilities

The following is the physicochemical model used to obtain initial estimates of transit probabilities. These transit probabilities govern the likelihood of passive transition from one space to another when the COMPOUND is in the transitional state (specified as being close to interface). Consider a multi-protic acidic compound A (H\textsubscript{x}A) in space S, having pH = pH\textsubscript{S}. Define

\[
R_S = \frac{\text{fraction ionized}}{\text{fraction unionized}} = \frac{[A^{x-}] + [HA^{(x-1)^-}] + \ldots + [H_{(x-1)]A^{(-x)}]}{[H_x A]} = \sum_{k=0}^{x-1} \left[ \frac{H_x A^{(x-k)^-}}{[H_x A]} \right]
\]

From the Henderson-Hasselbalch equation,

\[
[H_x A]^{x^-} = 10^{pK_{a_1} - pH_S} \quad \text{and} \quad [A^{x^-}] = 10^{pK_{a_1} + pK_{a_2} + \ldots + pK_{a_{(x-1)}} - (x-1)pH_S}
\]

So,

\[
R_S = 10^{(pK_{a_1} - pH_S)} + 10^{(pK_{a_1} + pK_{a_2} - 2pH_S)} + \ldots + 10^{(pK_{a_1} + pK_{a_2} + \ldots + pK_{a_{(x-1)}} - (x-1)pH_S)}
\]

\[
R_S = \sum_{m=1}^{x-1} 10^{\left( \sum_{k=1}^{m} pK_{a_k} \right) - mpH_S}
\]

Fraction unionized = \frac{1}{R_S + 1} \quad \text{and} \quad \text{fraction ionized} = \frac{R_S}{R_S + 1}

Let P\textsubscript{S} be the partition coefficient of the neutral or most permeable specie, H\textsubscript{xA}.

Define the distribution coefficient D\textsubscript{S} between a space containing a cell membrane M and aqueous space S,
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\[ D_S = \frac{\text{amount of all species of drug A in membrane space M}}{\text{amount of all species of drug A in aqueous space S}} \]

Assuming the partition coefficient of all ionized species to be \( k_{PN} \), \( k < 1 \).

\[ D_S = \frac{P_N \left( \frac{1}{\sqrt[1]{R_{S}+1}} \right) + k_{PN} \left( \frac{R_{S}}{\sqrt[1]{R_{S}+1}} \right)}{1 + \frac{R_{S}}{\sqrt[1]{R_{S}+1}}} = P_N \left( \frac{1}{\sqrt[1]{R_{S}+1}} \right) + k_{PN} \left( \frac{R_{S}}{\sqrt[1]{R_{S}+1}} \right) \]

Analogously, for COMPOUND A, the in silico analogue of real drug A, we have analogous in silico parameters, \( \log P \) (a measure of \( P_{SN} \)), \( pKa \), and \( \text{insilicoPh} \). Using these in silico parameters reflecting physicochemical properties, we calculate an in silico \( D_S \) for COMPOUND A. In the RISL, we used \( k = 0.001 \).

Define transit probability (transitProb) from an aqueous space S (such as S1, S3, and S5 in Fig. 2) to a space M containing a membrane (such as S2 and S4 in Fig. 2), \( p(S \rightarrow M) \), be the probability that COMPOUND A transitions from S to M within the current simulation cycle.

Specify that the interval during which the referent transition would have actually occurred is smaller than a simulation cycle. Also specify that the in silico effective size of COMPOUND A is inversely proportional to the square-root of the value of the molecular weight property assigned to COMPOUND A (\( MW_A \)).

\[ p(S \rightarrow M) = \frac{D_S}{D_S + 1} \times \frac{1}{\sqrt{MW_A}} \]

For COMPOUND A in M flanked by two aqueous spaces S and S',

\[ p(M \rightarrow S) = \frac{D_S'}{D_S + D_S' + D_S D_S'} \times \frac{1}{\sqrt{MW_A}}, \text{ and} \]

\[ p(M \rightarrow S') = \frac{D_S}{D_S + D_S' + D_S D_S'} \times \frac{1}{\sqrt{MW_A}} \]

These initial transit probability values may be adjusted (scaled) for use with simulation cycles that map to different wet-lab times by the parameter \( \text{timestepfactor} \), using the probability scaling method described above. Within the RISL, \( \text{timestepfactor} = 2 \).

\[ p_{scaled} = 1 - \left( (1 - p_{old})^{\text{timestepfactor}} \right) \]

Relationship between COMPOUND (physicochemical) properties and initial transit probability estimates

We investigated the consequences of changing COMPOUND (physicochemical) property values on initial measures transit to verify that transit probability estimates behaved consistent with theory. We used DIGOXIN, RIFAMPICIN, QUINIDINE and DIGOXIN METABOLITE. We adjusted one of their RISL properties (for example, \( MW \)) over a range of values, while holding the others (\( \log P \), and \( \text{insilicoPh} \)) constant. When conducting the experiments, we removed all TRANSPORTERS and time-variant processes. We measured initial permeability across a single barrier: the number of COMPOUNDS that reached S3 from S1 after 20 simulation cycles. The results in Fig. S3 are consistent with the above physicochemical models used to derive the initial transit probability estimates.
**Figure S3.** Relationship COMPOUND (physicochemical) properties and initial transit probability estimates. Each symbol is one RISL experiment. Y-axis: number of COMPOUNDS in S3 after 20 simulation cycles when the initial dose is 1,000. **A:** Graphed are relationships between MW (x-axis) and RISL measures of initial permeability. Symbols: QUINIDINE (light blue), METABOLITE (yellow), DIGOXIN (red), RIFAMPICIN (purple). The curve is a fit of an inverse power relationship to the pooled data. **B:** Graphed are relationships between logP (x-axis) and initial permeability (y-axis), as specified in A. Symbol colors are the same as in A. The curve is a parabola fit to the pooled data; the peak occurs at about logP = 3.3. **C:** Graphed are relationships between insilicoPH and initial permeability. Symbols: DIGOXIN (red crosses), METABOLITE (yellow pluses), QUINIDINE (light blue diamonds), RIFAMPICIN (purple diamonds). The two trend lines are for only the QUINIDINE and RIFAMPICIN data. QUINIDINE behaves as would a weak basic having pKa = 8.6: PERMEABILITY increases with increasing insilicoPH. RIFAMPICIN behaves as would an amphoteric compound having a weak acid group (pKa = 1.7) and a weak basic group (pKa = 7.9): PERMEABILITY decreases with increasing PH. DIGOXIN and its METABOLITE behave as would compounds for which the fraction ionized does not change over the insilicoPH range.

**Movements of COMPOUNDS.**

What is the probability that during a 10 second interval within a perfused liver that a compound will be close enough to an interface to transition (partition) passively from one space to another? An answer can only be an approximation. In the RISL, that probability is specified by the parameter closestToInterface. If close, the probability of actually transiting is specified by transitProb. After making several assumptions about microarchitectural details of cell monolayers, Garmire et al. (Garmire et al., 2007) used estimates of the relative size of cellular spaces and the known properties of compounds to estimate a compound’s relative intracellular location and likelihood to partition. Doing so required making several assumptions. We elected not to make those assumptions for two
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reasons: we lacked knowledge about specific lobular microarchitectural details and we wanted to make all RISL mechanisms relativistic. in addition, we have conflated numerous lobular microarchitectural features (that cannot be known with precision) and represented them using three abstract spaces. *Closest Interface* and *transitProb* are tunable parameters, the values of which are constrained by the referent compound’s listed properties. Initial estimates were obtained for each referent compound using values of molecular weight, logP, and pKₐ as described above, analogous to those used by Garmire et al. Thereafter, these parameters were tuned, along with the others, to improve SM values. Tuned values are listed in tables 3, S1, and S2.

The sequence in which each mobile object’s transit decision was made followed the same pattern for each space. The process is described first for a compound in barrier space S4 and then for a compound in cell interior space S3 that is close to an interface. p[S4→S3] is the value of *transitProb* during a cycle for the transition from S4 to S3. First, a PRN is drawn from [0–1). If PRN < p[S4→S3]/(a(p[S4→S5] + p[S4→S3])), then the compound transits from S4 to S5; else, if PRN < 1/a (a ≥ 1), it transits from S4 to S3; else, it stays in S4. Because the relative properties of S1, S2, and S3 in the current RISL, as are the relative properties of S2 and S4, the logic is the same for S2. Following exploration, we specified a = 1.2; that value is arbitrary; using a value larger than 1.0 helped insure that a compound would not still be found in S2 or S4 after more than three cycles. Once it is determined that a compound in S3 is close to an interface (we do not specify which one), a PRN is generated: if PRN < p[S3→S4]/(1 + (p[S3→S4] + p[S3→S2])), the compound transits from S3 to S4; else, if PRN < (p[S3→S4] + p[S3→S2])/(1 + (p[S3→S4] + p[S3→S2])), it transits from S3 to S2; else, it stays in S3. The logic would be similar for a compound in S5 transiting to S4; however, for this study, that option was precluded.

Simulated Perfusion Flow. We considered several options to simulate flow in Fig. 2 as described in the text. One option would be to simulate flow within the space (e.g., S1) representing perfusate, analogous to how it was done in (Hunt et al., 2006). S1 could be made circular and much larger than the hepatic spaces. compounds could move around the circular space simulating flow. Transitioning into hepatic spaces would occur only in the portion of S1 that overlaps with the extracellular hepatic space. If we had simultaneous wet-lab measures of concentrations entering and exiting the liver against which to validate extraction, such a design would be merited, but we do not. In fact, perfusate flow was sufficiently fast to make reliable measures of that type difficult. Having a circular S1 containing circulating compounds does match the reality of the referent system, but it is not an essential feature for inclusion in an abstract analogue.

Another option would be to layer three spaces above S2. Call them S1.1, S1.2, and S1.3, with S1.3 being adjacent to S2. Only compound in S1.3 would have the option to transition to S2 within a simulation cycle. Compound in S1.1 would map to compound that is in perfusate external to the liver. Compound in S1.2 would map to compound that is internal, within rapidly equilibrating extracellular spaces. Adjusting the exchange rate between S1.1 and S1.2 would map to perfusate flow. For simplicity, we elected to confine those three spaces: we represented the merged set using just one space, S1.

**Transporter-mediated transition and CYP-mediated metabolism**

In Figs. S4A,B we present verification evidence demonstrating the linear relationship between transporter/metabolic activity and number of transporters/CYPs and the value of the substrate’s *assocProb* (affinity). For transporters, we present data in Fig. S4C,D that show the consequences active transport with concurrent passive transition. Finally, in Fig. S4E, the graphs show measures of CYP function are consistent with Michaelis-Menten kinetics.

A  

B
Figure S4. TRANSPORTER and CYP verification and consistency with simple Michaelis-Menten kinetics. **A:** The data show a linear relationship between permeability as fraction of dose (y-axis) and the number of TRANSPORTERS. **B:** The data show a linear relationship between permeability (y-axis) and the value of the COMPOUND’s assocProb (affinity; x-axis). **C:** The graph shows the combined effects of active transport and passive transition on the permeation of COMPOUNDS that are substrates for the TRANSPORTER and can also undergo passive transition. The graph shows the permeability after 2000 simulation.
cycles. Being a TRANSPORTER substrate has a greater effect on less permeable (lower logP) COMPOUNDS. Also, when TRANSPORTER assocProb is high, TRANSPORTER-mediated permeation dominates. D: The graph shows the permeation time-profile (x-axis: simulation cycles) of the simulations in C. The coloring scheme is the same. E: The graphs show the rate of METABOLISM (y-axis: number of METABOLITES formed after 10 simulation cycles) when starting with different numbers of COMPOUNDS (x-axis). The duration of each experiment was 10 simulation cycles. All components were confined to S3; 50 CYP were used. Graphs from top to bottom: 1) no inhibition; 2) competitive inhibition using 250 additional COMPOUNDS that are INHIBITORS; 3) competitive inhibition using 1,000 COMPOUNDS that are INHIBITORS; 4) noncompetitive inhibition caused by reducing efficiencyProb to 40% of it original value. The data are consistent with simple Michalis-Menten kinetics.

Fraction of administered dose calculations
In wet-lab experiments, the investigator selects specific experiment features to measure because observing all potentially important aspects of the experiment is infeasible. In the wet-lab experiments, the measure of the time course of relative abundance of administered drug was concentration, because only a tiny perfusate aliquot would be consumed. It is not uncommon for a small portion of administered dose to be lost and not recovered in later measurements.

An obvious advantage of RISL experiments is that the system is completely transparent. We can “see” where everything is and what each component is doing at the end of any simulation cycle. We can measure the exact relative abundance of administered COMPOUNDS, as fraction of total administered DOSE.

In order to compare referent and RISL PK profiles, we needed to either transform wet-lab concentration measurements into fraction of administered dose in perfusate, or transform RISL measurements to concentrations. The latter would have been complicated. We elected the former because it required only specifying a common denominator, in units of nanomolar, for all concentration measurement.

There are several options in selecting that common denominator:
1) It was reported that 10 µg of digoxin was used, and that the volume of perfusate used was 110 mL. That gave 116 nM.
2) It was also reported that digoxin, dissolved in sufficient diluent, was added to the perfusate to give an initial concentration of about 110 nM.
3) Finally, it was reported that the initial, perfusate concentration without the rat liver was about 121.5 nM.

We elected to use 121.5 nM as the common denominator because it required making no assumptions about the actual volume being used. Using a different common denominator would shift the values to which we are comparing our simulation results and, in turn, change the assessment of whether or not a given set of RISL results meets a SM.

Inhibitor time-course profiles
It was reported that each inhibitor was added (separately) to the perfusate of an already-established, perfused liver 10 minutes prior to addition of digoxin. Figure 3 in the (Lau et al., 2004) shows the reported PK profiles for each inhibitor. Presented in Fig. S5 are separate time-course profiles for the two INHIBITORS during a typical simulation experiment. They are qualitatively and quantitatively similar to the reported PK profiles. We elected not to fine-tune these profiles further and not to include them in the set of targeted attributes, because doing so would not have provided additional insight into the referent data in Fig. 3. We assert that the observed qualitative and quantitative similarities between the data in Fig. S5 and the referent data are adequate for the specified objectives.
Figure S5. RISL PK profiles for RIFAMPICIN and QUINIDINE. X-axis: time after addition of INHIBITOR; y-axis: fraction of administered dose. A: RIFAMPICIN, the uptake inhibitor; B: QUINIDINE, the efflux inhibitor. Each profile is similar to its referent in Fig. 3 of (Lau et al., 2004). DIGOXIN was administered 10 minutes after the INHIBITOR.

Parameter sensitivities

A classical sensitivity analysis studies of how output variation of a mathematical model can be apportioned, qualitatively or quantitatively, to different sources of variation in the input of a model. Such an analysis investigates model robustness when the study includes some form of mathematical modeling. The RISL is not a classical mathematical model. The goals and objectives are different. Nevertheless, we did not want to include components that were not needed to achieve one or more attributes. To that end, as part of verification exercises, we did observe the consequences of inactivating components, which are presented later in this supplement. During the many tuning experiments, we also observed the consequences of changing the values of most of the parameters. In Fig. S6, we present data demonstrating the effect of a minor alteration of each of two key parameters: transitProb(S1→S2) and DIGOXIN’s assocProb to CYP. We ran these experiments using the same conditions and parameter values used for Fig. 3, except that the value of each of the two parameters was increased 5%. The results demonstrate that changes in RISL parameter values of a few percent do not significantly alter RISL PK profiles.
Mechanisms of hepatic drug interactions: Supplement

Figure S6. Influence of small changes in two of the more important parameters. X-axis: time after adding DIGOXIN to PERFUSATE; y-axis: fraction of dose; closed symbols: results from Fig. 3; open symbol: results from increasing parameter value 5%; left: DIGOXIN in PERFUSATE; right: METABOLITE in PERFUSATE. A: Graphed are the results showing the effect of 5% increase in transitProb(S1→S2). B: Graphed are results showing the effect of 5% increase in DIGOXIN’s assocProb for CYP.

Results: Additional Detail

A Simple Hypothesis Fails

Figure S7. Comparison of wet-lab experimental data to simulated results using the simple, linear RISL described in the text. All graphed values are averages of six simulation experiments. All RISL mechanistic events were time invariant and linear relative to amounts. Key parameter values are listed in Table S1. Those not shown are the same as in the Text. A: Solid symbols are values reported in (Lau et al., 2004) converted to fraction of digoxin dose (0.4 to 1.0) remaining in the perfusate for each of the three treatments. The three open symbols at 60 min., along with the gray curves, are mean results for each of the three indicated treatments, simulated using the simple RISL. The simulated values at 60 min. (but not at earlier times) achieved the first Similarity Measure (SM-1). B: The closed symbols and treatments are the same as in A, but the values are the digoxin metabolite levels in perfusate, as fraction of reported dose (0.0 to 0.4). As in A, the three open symbols at 60 min., along with the gray curves, are mean results for each of the three treatments simulated using the simple RISL. The simulated values at 60 min. (but not at earlier times) achieved SM-1. However, as described in the text, no time-invariant parameterization of this RISL was found that enabled achieving SM-2

Metabolism in the simple time-invariant RISL

To achieve SM-2, we speculated that some level of metabolite may have been present in perfusate shortly after digoxin was added (t = 0). One plausible cause was that hepatocytes were injured during earlier manipulations to establish the system, resulting in metabolic enzymes being released from hepatocytes into perfusate prior to the time digoxin was administered. This mechanism was later implemented. Other plausible causes are: 1) presence of analytically indistinguishable impurities; 2) digoxin hydrolysis produced measurable amounts of primary metabolite (formation of the metabolite from hydrolysis of digoxin is acid catalyzed); 3) a liver derived contaminant co-elutes (HPLC) with the metabolite during assay. In Fig. S7, we specified that 5% of the administered dose was metabolite at t = 0. Consistent with that hypothesis, we decreased the initial concentration denominator from 121.5 nM to 115 nM. That change explains why wet-lab values in Figs. S7, S8 and 3 differ slightly. Figure S7 is the evidence that the preceding hypothesis helps improve similarity sufficiently so that SM-1 is achieved. We noted, however, that the shapes of the profiles (RISL and wet-lab) were dissimilar, and so we began to explore time-variant mechanisms.
Table S1. Key parameter values for the simple time-invariant RISL in Fig. S7

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Remarks</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>numPgps</td>
<td>Number of PGP</td>
<td>25</td>
</tr>
<tr>
<td>numOats</td>
<td>Number of OAT</td>
<td>50</td>
</tr>
<tr>
<td>numCyps</td>
<td>Number of CYP (ENZYMES)</td>
<td>15</td>
</tr>
<tr>
<td>numBinders</td>
<td>Number of BINDERS</td>
<td>25</td>
</tr>
<tr>
<td>timestepfactor</td>
<td>Scaling factor for passive permeation</td>
<td>10</td>
</tr>
<tr>
<td>assocProb</td>
<td>DIGOXIN’S association probability to</td>
<td></td>
</tr>
<tr>
<td>releaseProb</td>
<td>DIGOXIN’S dissociation probability from</td>
<td></td>
</tr>
<tr>
<td>closeToInterface</td>
<td>Fraction sufficiently close to interface such that it is transitional for DIGOXIN</td>
<td></td>
</tr>
<tr>
<td>maxSites</td>
<td>Number of substrate binding sites per stationary object</td>
<td>4</td>
</tr>
<tr>
<td>efficiencyProb</td>
<td>Efficiency parameter for stationary objects</td>
<td>1</td>
</tr>
<tr>
<td>sitesN</td>
<td>Size of neighborhood (number of ELEMENTS) scanned each cycle by a stationary object</td>
<td>4</td>
</tr>
</tbody>
</table>

Implementing Mechanistic Deterioration Improved Similarity.

Figure S8. Simulated results that achieved SM-2, but not SM-3, using RISLs with the time-variant mechanisms graphed in Fig. S9. The symbols are the mean wet-lab data. The curves are mean ($n = 6$) simulated values. Time-variant RISLs differ in four ways from the time-invariant RISLs used to generate the simulation results in Fig. S7. A METABOLITE-specific TRANSPORTER was added to S2 to increase the rate of return at early times of METABOLITE to PERFUSATE. The accessibility of S2 to mobile objects in the PERFUSATE decreased with time as specified in the text and Tables S2–S4, decreasing the rate of both passive and active HEPATIC permeation by all mobile objects (the three DRUGS). The efficiency of CYP decreased with time as graphed in Fig. S9B. Loss of efficiency suggests loss of viability or diminished perfusion or some combination. In addition, a portion of those immobile objects became inactive (Fig. S9C) each cycle. A: Shown is the mean DIGOXIN PERFUSATE levels for each of the three indicated treatments. In all three cases, simulated values achieved SM-2; most were within 10% of referent values. For the QUINIDINE PREDOSE case, DIGOXIN PERFUSATE levels, as fraction of DOSE, reached a minimum of about 0.4 at 30 minutes, and then began increasing slightly. That was caused by DIGOXIN returning primarily from S3 because of eroding TRANSPORT and
METABOLIC functions. **B:** Shown are the mean METABOLITE PERFUSATE levels corresponding to the three treatments in **A.** We achieved an early decline in DIGOXIN levels along with rapid rise in METABOLITE level, both of which were not achieved using the time-invariant RISL (Fig. S7). Even though METABOLITE levels for both the RIFAMPICIN and QUINIDINE PREDOSE cases are far from target values, the shapes of the METABOLITE time course profiles were similar to the shapes of the referent profiles. Collectively, the six sets of simulated data in **A** and **B** achieved SM-2. Further improvement was infeasible without making the time-variant mechanism more complicated, which was done to achieve the RISL results in text Fig. 3. A change in parameterization that would bring the RIFAMPICIN-PREDOSE curve closer to referent values (open diamonds) would cause the each of the other five profiles (and possibly the RIFAMPICIN alone and the QUINIDINE alone profiles) to also shift. The net result of such an effort can be failure to achieve SM-2.

**Table S2.** Compound specific parameters values for the RISL data in Fig. S8

<table>
<thead>
<tr>
<th>COMPOUND Parameters</th>
<th>Remarks</th>
<th>DIGOXIN</th>
<th>RIFAMPICIN</th>
<th>QUINIDINE</th>
<th>METABOLITE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MW</strong>^c^</td>
<td>molecular weight of the solute</td>
<td>781</td>
<td>822</td>
<td>324</td>
<td>601</td>
</tr>
<tr>
<td><strong>logP</strong>^c^</td>
<td>logP - common logarithm of octanol/water partition coefficient</td>
<td>1.14</td>
<td>3.60</td>
<td>2.53</td>
<td>1.60</td>
</tr>
<tr>
<td><strong>pKa</strong>^c^</td>
<td>pKa closest to insilicoPH</td>
<td>13.5</td>
<td>7.9</td>
<td>8.6</td>
<td>13.5</td>
</tr>
<tr>
<td><strong>inhibitorType</strong></td>
<td>uptake inhibitor? True/False</td>
<td>n/a</td>
<td>TRUE</td>
<td>FALSE</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>closeToInterface</th>
<th>Fraction sufficiently close to interface such that it is transitional^d^</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S1</strong></td>
<td>0.750</td>
</tr>
<tr>
<td><strong>S3</strong></td>
<td>0.125</td>
</tr>
<tr>
<td><strong>S5</strong></td>
<td>0.100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>transitProb</strong></th>
<th>Initial estimates of trans-membrane transit probabilities, after scaling by timestepfactor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S1→S2</strong></td>
<td>0.11</td>
</tr>
<tr>
<td><strong>S2→S1</strong></td>
<td>0.19</td>
</tr>
<tr>
<td><strong>S2→S3</strong></td>
<td>0.19</td>
</tr>
<tr>
<td><strong>S3→S2</strong></td>
<td>0.11</td>
</tr>
<tr>
<td><strong>S3→S4</strong></td>
<td>0.11</td>
</tr>
<tr>
<td><strong>S4→S3</strong></td>
<td>0.19</td>
</tr>
<tr>
<td><strong>S4→S5</strong></td>
<td>0.19</td>
</tr>
<tr>
<td><strong>S5→S4</strong></td>
<td>0.11</td>
</tr>
</tbody>
</table>

^a^ Physicochemical properties reflect those of the referent drug.
^b^ Physicochemical properties reflect those of digoxigenin bis-digitoxoside.
^c^ Molecular weight, logP, and pKa are used in calculating the initial, passive transit probabilities, as described earlier. For pKa, only the value closest to 7.4 is listed.
^d^ The closeToInterface value for all solutes in S2 and S4 was always 1. This reflects the fact that the referent barriers are sufficiently thin so that all solutes within them at the start of a 10 second interval (a simulation cycle) will have had an opportunity to transition by the end of that interval.

**Table S3.** Parameters for COMPOUND-PROTEIN interactions for the RISL data in Fig. S8

<table>
<thead>
<tr>
<th>COMPOUND-PROTEIN Interaction Parameters</th>
<th>DIGOXIN</th>
<th>RIFAMPICIN</th>
<th>QUINIDINE</th>
<th>METABOLITE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>isaSubstrate</strong></td>
<td>MT</td>
<td>FALSE</td>
<td>FALSE</td>
<td>FALSE</td>
</tr>
<tr>
<td><strong>substrate of ?</strong></td>
<td>PGP</td>
<td>TRUE</td>
<td>FALSE</td>
<td>FALSE</td>
</tr>
<tr>
<td><strong>TRUE / FALSE</strong></td>
<td>OAT</td>
<td>TRUE</td>
<td>TRUE</td>
<td>FALSE</td>
</tr>
</tbody>
</table>
Table S4. Time-variant parameters and their values, corresponding to Fig. S9

<table>
<thead>
<tr>
<th>Time-variant parameter</th>
<th>Initial value</th>
<th>Terminal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective SURFACE AREA changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sitesN (neighborhood)</td>
<td>CYP 15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PGP 9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>OAT 20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MT 35</td>
<td>4</td>
</tr>
<tr>
<td>METABOLIC and TRANSPORT activity changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>efficiencyProb</td>
<td>CYP 0.99</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>PGP 1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OAT 0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>MT 1</td>
<td>1</td>
</tr>
<tr>
<td>defunctProb</td>
<td>CYP 0.0025</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>PGP 0.00028</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>OAT 0.0035</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>MT 0.00028</td>
<td>0.0026</td>
</tr>
</tbody>
</table>

Fraction transitional in S1 0.35 0.096

d The method for changing fraction transitional and efficiencyProb (Fig. S9A and S9B) is detailed earlier, under Parameter scaling.

Figure S9. Values of time-variant parameters for the RISL data in Fig. S8. Each graph shows time-dependent parameter values used to generate the experimental results shown in Fig. S8. A: The graph shows the time-variant values of the fraction of COMPOUNDS in...
Consequences of Selectively Turning Off Mechanistic Features

We focus on four conditions, corresponding to the answer to four questions. E) Enzymes: are ENZYMES in S1 beginning at $t = 0$? S) Surface area: are COMPOUND accessible HEPATIC surface areas and access to CYP, PGP, and OAT shrinking as in Fig. 4 A and B? D) Dysfunction: are ENZYMES and TRANSPORTERS becoming less efficient and defunct as in Fig. 4C and D? B) Binders: are BINDERS present? The key function of BINDERS is to retain METABOLITE within CELLS (within S3), a process that is inhibited by RIFAMPICIN in the RISL.

In Figs. S10 and S11, the consequences of eight of 16 possible combinations are shown. The combinations are coded as follows. When the answer to all four questions in the above order (ESDB) is yes, then that RISL is identified as 1111. That corresponds to the RISL used for Fig. 3. The “healthy” RISL in Fig. 5 is identified as 0001. All RISLs in Figs. S10 and S11 should be compared to these extremes as well as to each other.
Figure S10. Perfusate profiles for four different RISLs. A: ESDB = 0111. Shown are the consequences of not having ENZYMES in S1. There was always too much parent DRUG, and too little METABOLITE. ENZYMES in S1 convert some DIGOXIN to METABOLITE early on. B: ESDB = 1101. Shown are the consequences of keeping S1 and S3 ENZYMES active for the duration of the experiment. METABOLITE formation does not level off, because ENZYMES are not dying out. However, their neighborhood (siteN) still shrinks. C: ESDB = 1011. Shown are the consequences of ENZYMES and TRANSPORTERS becoming less efficient and defunct, but their neighborhoods do not shrink. The initial drop of DIGOXIN become much less drastic, and correspondingly fewer METABOLITE return to perfusate at early time D: ESDB = 1110. Shown are the consequences of having no BINDERS. DIGOXIN PERFUSATE levels were very much the same, but without BINDERS, more METABOLITE entered PERFUSATE in DIGOXIN-only and QUINIDINE-only groups.
Figure S11. Perfusate profiles for four different RISLs. A: ESDB = 1001. The most dramatic change was when ENZYMES and TRANSPORTERS stayed active and their access along with accessible HEPATIC surface areas does not shrink. Note that BINDERS were present, so the RIFAMPICIN treatment group had the highest PERFUSATE METABOLITE levels. B: ESDB = 0101. Shown are the consequences of keeping ENZYME and TRANSPORTER activities and numbers constant. C: ESDB = 0011. Shown are the consequences of keeping constant COMPOUND accessible HEPATIC surface areas and access to CYP, PGP, and OAT. D: ESDB = 0110. Shown are the consequences of removing the S1 enzymes and BINDERS.