Mechanistic Insight from In Silico Pharmacokinetic Experiments:
Roles of P-glycoprotein, CYP3A4 Enzymes, and Microenvironments

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Abbreviations:
3D: three dimensional; CYP: in silico object that maps to an amount of CYP3A4; HME: in silico hydrophobic microenvironment; ISECC: In Silico Experimental Caco-2 (cell monolayer) Culture; LY: in silico counterpart of LY335779 (zosuquidar trihydrochloride); M7: saquinavir’s first metabolite; M7: in silico object that maps to an amount of M7; M1: in silico metabolite of M7; M&S: modeling and simulation; PCP: physicochemical property; P-gp: p-glycoprotein; PGP: in silico object that maps to an amount of P-gp; PK: pharmacokinetic; PRN: pseudo-random number; SAQ: in silico object that maps to an amount of saquinavir; SM: Similarity Measure; TJ: in silico object that maps to tight junctions.
Abstract

Saquinavir exhibits paradoxical transport across modified Caco-2 cell monolayers [doi: 10.1124/jpet.103.056390] expressing P-glycoprotein and Cyp3A4. The data implicate complicated intracellular transport mechanisms. Drawing on recent discrete event modeling and simulation advances, we built an in silico analogue of the confluent, asymmetric, cell monolayer used in the cited work. We call it In Silico Experimental Caco-2 (cell monolayer) Culture (ISECC). Concrete, working, hypothesized spatial mechanisms were implemented. Validation was achieved when in silico experimental results met Similarity Measure (SM) expectations that targeted 16 wet-lab experimental conditions. Initial mechanistic hypotheses turned out to be necessary parts of a more complicated explanation. We progressed through four stages of an iterative refinement and validation protocol that enabled and facilitated discovery of plausible, new mechanistic details. The process exercised abductive reasoning, a primary means of scientific knowledge creation and creative cognition. The ISECC that survived the most stringent SM challenge produced transport data that was statistically indistinguishable from referent wet-lab observations. It required a 7:1 ratio of apical transporters to metabolizing enzymes, a 97% reduction of efflux activity by an inhibitor, a biased distribution of metabolizing enzymes, heterogeneous intracellular spaces, and restrictions on intracellular drug movement. Experimenting on synthetic analogues like ISECC provides a heretofore-unavailable means of discovering new mechanistic details and testing their plausibility. The approach thus provides a powerful new expansion of the scientific method: an independent, scientific means to challenge, explore, better understand, and improve any inductive mechanism and, importantly, the assumptions on which it rests.
Introduction

Mouly et al. reported paradoxical observations following a vectorial study of saquinavir transport across a monolayer of modified, p-glycoprotein (P-gp) and Cyp3A4-expressing Caco-2 cells: there were higher intracellular levels of saquinavir, yet less metabolite formation after apical compared to basal dosing (Mouly et al., 2004). The data clearly indicated that the intracellular mechanisms during transport were more complicated than anticipated. The authors suggested mechanistic explanations, but no established experimental wet-lab methods were available to test them.

Recent advances in discrete event modeling and simulation (M&S) of complex systems (Hunt et al., 2009) enables implementing in silico methods to test the plausibility of mechanistic hypotheses. We used the synthetic modeling method (Liu and Hunt 2006), (Hunt et al., 2006), (Garmire et al., 2007), (Yan et al., 2008), (Lam et al., 2009a) illustrated in Fig. 1 to implement an in silico analogue of the wet-lab system, and then used it to explore the plausibility of the above explanations. The approach was straightforward: drawing on prior efforts and reusing validated components, we built an in silico analogue of the confluent, asymmetric, cell monolayer system used by Mouly et al. It is designed for experimentation and hypothesis generation and falsification (Hunt et al., 2008), (Lam et al., 2009a). We call it ISECC for In Silico Experimental Caco-2 (cell monolayer) Culture. Concrete, working versions of hypothesized spatial mechanisms were implemented within. In silico experiments used the same design as the referent wet-lab experiments. Simulation following component tuning tested each mechanistic hypothesis. Similarity Measures (SMs) with respect to referent experimental observations were used to set expectations. When results did not meet expectations, the mechanistic hypothesis was falsified; when results did meet expectations, a degree of validation was achieved.

The simple mechanisms hypothesized by Mouly et al. as implemented were falsified: when instantiated in the ISECC they failed to validate. That was unexpected. Although the proposed mechanisms alone were insufficient, it turned out that they were necessary parts of a more complicated explanation. To discover an ISECC mechanism that would validate, we implemented and followed the iterative ISECC
refinement protocol in Fig. 2. It targeted wet-lab results from an expanding subset of 16 experimental conditions. We progressed through four stages. By discovering a set of mechanisms that would validate (achieve the prespecified SMs) for two of the 16 conditions, we achieved Stage 1 (Lam et al., 2008). Stage 4 targeted results of all 16 wet-lab conditions coupled with more stringent SMs. In between were many falsification, re-validation processes. The following illustrates. A specific ISECC mechanism is discovered that validates for six targeted conditions using a prespecified SM. The targeted attribute list is then expanded to include transport results for two additional conditions. The current ISECC is falsified because, for the specified SM, experimental results using it over the eight conditions fail to match referent results. We then readjust the current ISECC and/or add in new components such that the new ISECC validates again. Following the parsimony guideline, validation is achieved by discovering a plausible, marginally more complicated mechanistic hypothesis that survives the experimental challenge. The iterative refinement protocol enabled and facilitated discovery of plausible, new mechanistic details through exercise of abductive scientific reasoning, a primary means of knowledge creation and creative cognition (Hunt et al., 2009). The M&S method also facilitated that discovery by making it relatively easy to implement and explore multiple mechanistic hypotheses.

The ISECC that survived the most stringent SM challenge at Stage 4 produced transport measures that were statistically indistinguishable from referent wet-lab observations. It required a 7:1 ratio of apical transporters to metabolizing enzymes, a 97% reduction of efflux activity by an inhibitor, a biased distribution of metabolizing enzymes, heterogeneous intracellular spaces, and restrictions on drug movement within some of those intracellular spaces.

A purpose of conducting wet-lab experiments like those cited is to gain new knowledge regarding mechanistic details of directional transport and metabolism. As done in the cited work, hypotheses about those details are induced from the data (Rescigno 2001), (Hunt et al., 2008). To date, all hypothesized mechanistic explanations for the data have been conceptual and thus difficult to falsify. Designing and conducting new wet-lab experiments has been the only practicable means to experimentally falsify those hypothesized, conceptual mechanisms. Even when inductive mathematical models have been fit to the
data, the mechanisms remained conceptual. Experimenting on synthetic analogues like ISECC provides a heretofore-unavailable means of discovering new mechanistic details and testing their plausibility. This approach provides a powerful new expansion of the scientific method: an independent, scientific means to challenge, explore, better understand, and improve any inductive mechanism and, importantly, the assumptions on which it rests (Hunt et al., 2009).

Methods

In silico experiments were conducted beginning with a basic structure of the ISECC similar to that detailed in (Liu and Hunt 2006) and (Garmire et al., 2007). The second generation ISECCs used herein map to a monolayer of epithelial cells separating apical from basal media- or fluid-filled compartments. The 3D nature of the in vitro system is simulated using a stack of five 2D toroidal grids. To distinguish clearly in silico components and processes from corresponding in vitro tissue culture counterparts, we use SMALL CAPS when referring to the former; italics denote ISECC parameters, variables and internal states.

In contrast to inductive models, which often focus on precise prediction, the ISECC and the synthetic modeling and simulation (M&S) methods used are designed for discovering and testing plausible, mechanistic explanations (Hunt et al., 2009) of the referent drug transport data (Mouly et al., 2004). The approach is ideal for discovery and understanding of transport phenomena produced by a system of interacting components. A specific ISECC instantiates (is represented by a concrete instance of) a mechanistic hypothesis (Fisher and Henzinger 2007), (Hunt et al., 2008), (Hunt et al., 2009). Execution and comparison of results to referent data tests the hypothesis. A protocol was followed that facilitated generating multiple mechanistic hypotheses. We eliminated the least plausible using in silico experimentation.

Summary of wet-lab methods. Using modified, Cyp3A4 and P-glycoprotein (P-gp) expressing Ca-co-2 monolayers (Schmiedlin-Ren et al., 1997) cultured in Transwell™ devices, Mouly et al. studied the role of Cyp3A4-mediated metabolism (Fitzsimmons et al., 1997), (Eagling et al., 2002), (Parker and Houston 2008) and P-gp-mediated efflux (Kim et al., 1998), (Wacher et al., 1998) in saquinavir metabolism and disposition (Mouly et al., 2004), (Su et al., 2004). In control conditions, saquinavir was added to ei-
ther apical or basal compartment at the start of the experiment to achieve concentrations of 5, 10, 20 or 40 
µM. For the P-gp inhibited conditions, an apical dose of the P-gp inhibitor LY335979 (zosuquidar trihy-
drochloride; simply inhibitor hereafter) (0.5 µM) (Dantzig et al., 1999) was co-administered with saqui-
navir. The cells were incubated at 37° C for up to four hours, at which time the apical and basal media,
along with the cell monolayer were collected and analyzed for saquinavir and its major metabolite M7.

**Objective and approach.** Our objective was to discover a single set of components and microme-
chanisms, which when parameterized would provide a cohesive, concrete, plausible explanation for the
results of sixteen different, experimental conditions presented in Fig. 1of (Mouly et al., 2004), including
the paradoxical observations. We were not seeking a traditional, differential equation explanation of
transport. Rather, we sought an actual, working mechanism—*an analogue*—comprised of quasi-
autonomous biomimetic processes and parts, which when measured during execution would give TRANS-
PORT DATA similar to the referent wet-lab data. There are likely many equally plausible mechanisms that
differ in some details, yet give rise to essentially the same phenomena. For this project, the goal was
simply to discover one. We used the synthetic modeling and simulation method (Hunt et al., 2006), (Lam
et al., 2009a) illustrated in Fig. 1. We started with the ISECC from (Lam et al., 2008), which plugged to-
gether validated, quasi-autonomous software components to form an abstract yet mechanistically realistic
analogue of transport through a monolayer of cells into which we could add, alone or in combination, ob-
jects representing different compounds.

Our experimental approach followed the iterative refinement protocol in Fig. 2: cycles of ISECC
synthesis, testing and evaluation, validation or falsification, assessment, cogitation, and system revision
until one satisfied predetermined similarity criteria. We have used the protocol successfully (Hunt et al.,
2006), (Tang et al., 2007), (Engelberg et al., 2008), (Kim et al., 2009), (Lam et al., 2009a). It strives to
adhere to the guideline of parsimony, which is important when building agent-oriented analogues that are
expected to become increasingly complex. We began by instantiating the conceptual mechanism de-
scribed in (Mouly et al., 2004). Even after multiple rounds of iterative refinement, it failed to mimic wet-
lab results. Failure of that best-at-the-time mechanistic explanation demonstrated that conceptual mecha-
nostic descriptions can be flawed in ways that are not readily apparent. Flaws, when they exist, begin becoming obvious after we implement and begin testing the mechanism synthetically. (Lam et al., 2009a), (Hunt et al., 2009)

**ISECC structure and components.** We specified that ISECC components and their assembly be consistent with a Transwell device having a confluent monolayer of polarized epithelial cells exhibiting the following characteristics. There are at least five distinct spaces: two dosing compartments (apical and basal), and an intracellular space between a pair of asymmetric membrane barriers (apical and basal). Added compounds can move within and between spaces consistent with their physicochemical properties (PCPs). Efflux transporters (P-glycoprotein, etc.) located only on the apical membrane enable facilitated translocation of some compounds across that membrane. An added compound in the intracellular space, upon encountering a subcellular component (a cytochrome P450, for example) can be metabolized; however, those subcellular components are not uniformly distributed within the intracellular space. There are regions of intracellular heterogeneity. Examples include, lysosomes, the nucleus, mitochondria, and endoplasmic reticulum (Khoo et al., 2002), (Vernochet et al., 2005).

Cells are complicated 3-dimensional (3D) structures. For the sake of visualization, it is tempting to use a 3D grid to represent cell spaces and place different objects within that map to subcellular features. However, the available detailed knowledge is insufficient to validate. Even with considerably more knowledge, to complete a 3D representation, we would accumulate a long list of weak assumptions. The approach taken strives to avoid unnecessary assumptions while specifically taking into consideration uncertainty and ignorance.

An ISECC uses five identically sized spaces, S1–S5. The mappings are as follows: S1 → apical compartment; S2 → apical membrane; S3 → intracellular space; S4 → basal membrane; and S5 → basal compartment. S1–S5 are 2D grids. In this study, each space is arbitrarily subdivided using a 50x50 square grid. Using more coarse-grained spaces increases the variance of measures requiring averaging more simulation runs. Using more fine-grained spaces will decrease variances but run times are in-
creased. Objects called elements are placed at each grid location. The S2–S4 elements are containers for cellular components and for compounds moving around within and between spaces. S1 and S5 elements are simply containers for compounds. Each S3 element, for example, maps to small fraction of total intracellular space from apical to basal membranes. A small fraction of S1 and S5 elements map to spaces between cells including tight junctions (TJ) (Liu and Hunt 2006), (Garmire et al., 2007). Elements can have different properties relative to mobile compounds. For example, the solubility of a compound in one element type can be specified to be different than in another element. Elements are given properties so that the compound’s entry and exit from a particular element can be differentiated based on those properties and the compound’s PCPs. For example, a lipid-like element can restrict entry of highly polar or charged compounds, whereas highly hydrophobic compounds can accumulate (Khoo et al., 2002), (Vernochet et al., 2005). An element’s internal logic is a placeholder for more fine-grained mechanistic detail that can be added when that is needed and the information is available to do so. Within S1, S2, S4, and S5, the elements at each grid location are identical.

It was apparent from the original wet-lab data (Mouly et al., 2004) and from our own early work (Yan et al., 2008), (Hunt et al., 2008), (Lam et al., 2009a) that an ISECC would need to simulate some intracellular heterogeneity. We did so by specifying that the space within a S3 element can be heterogeneous. Because an element is the limit of ISECC resolution, the details of within-element heterogeneity are left unresolved. To facilitate conceptualization, we describe an S3 element as being subdivided vertically into subcellular microenvironments called zones (Lam et al., 2008). Technically, we simply specify that objects within an element can be in different states, and behavior is state-dependent. Elements in S1, S2, S4 and S5 behave as if the contents are homogeneous and well mixed. To iteratively improve ISECC validation, it was necessary to increase the number and alter the properties of S3 zones. The following paragraph provides a generalized description applicable to all ISECC described in supplemental material.

The zones of each S3 elements are designated $Z_i$, $i = 0$ to $n$. The parameter $\text{maxZ}$ specifies $n$, and for the various ISECCs studied, $2 \leq n \leq 6$. Zones map to intracellular gradients and microenvironments. A
zone could, for example, map to a compound transport pathway (Weisiger 1996), (Weisiger 2002), (Weisiger 2007), nucleus (Tran et al., 2003), mitochondria (Khoo et al., 2002), a portion of the endoplasmic reticulum, etc. Being below the level of resolution, their shape is indeterminate. Z0 is always adjacent to S2 and Zn is always adjacent to S4. All other zones can be conceptualized as being layered between those two. We encountered a need for some zones, specifically those “close” to a MEMBRANE—Z0 and Zn—to behave as if they were less aqueous, more hydrophobic (Khoo et al., 2002), (Vernochet et al., 2005). Consequently, a parameter (HMspace) controlled fraction of Z0 and Zn zones was designated a HYDROPHOBIC MICROENVIRONMENT (HME). The fraction was tuned for each ISECC. For the final ISECC described herein, Z0 in 15% of elements were specified as being HME. In addition, in one-fifth of the elements containing a HME Z0, their Z6 were also specified as being HME.

**Mobile and stationary objects.** A COMPOUND is a mobile object that maps to an unspecified number of xenobiotic molecules (Liu and Hunt 2007), (Garmire et al., 2007), (Yan et al., 2008), (Lam et al., 2009a). We use three types: SAQ, M7 and M1. They map to saquinavir and its two metabolites (Eagling et al., 2002). Each COMPOUND is assigned a set of PCPs; they map to physicochemical properties of the referent compound. Different, stationery objects are assigned and confined to subsets of S2 and S3 elements. We use two types: CYP and PGP. They are discussed below.

During each simulation cycle, each COMPOUND, selected pseudo-randomly (randomly hereafter), has one opportunity to 1) move laterally to a neighboring element, 2) transit to a neighboring space, and 3) if in S3, to relocate to a different zone in the same element. COMPOUND transition within and between spaces is parameter specified and uses validated algorithms described in (Lam et al., 2008) and the supplement to (Lam et al., 2009a), with one exception: movement into and out of HME zones. Given a lateral movement opportunity, a COMPOUND selects randomly one of its eight neighboring elements and moves there, except special rules apply when the COMPOUND is attempting to move into or out of an element containing HME zone(s). In the final ISECC, the option to move into and out of a HME zone is governed by the value of an in silico distribution constant (value 689.5), which is calculated as described in the Supplement to (Lam et al., 2009a) using the DRUG’s logP, pKa and insilicopH, scaled by relative time-
step duration. Because SAQ has a large $\log P$, once it is in a HME zone, the probability each simulation cycle favors staying there: a SAQ in an HME has $1/(1 + 689.5)$ chance to move from HME Z0 to a neighboring non-HME element.

For a COMPOUND to transit between spaces, it must be sufficiently close to a MEMBRANE interface to transit into that space. The probability of being so located is governed by $\text{closeToInterface}$. Because S2 and S4 elements map to volumes that are very small relative to S1, S3, and S5, and because a simulation cycle maps to minutes of wet-lab time, $\text{closeToInterface}$ for COMPOUNDS in S2 and S4 is always 1. From S3, in addition to being sufficiently close to interface, a COMPOUND must be in Z0 to transit to S2; it must be in $Z_n$ to transit to S4. All COMPOUNDS transitioning from S2 to S3 are placed in Z0. Similarly, all COMPOUNDS transitioning from S4 to S3 are placed in $Z_n$. Depending on PCPs (as in (Garmire et al., 2007)), brief transcellular transport around TJs from S1 to S5 and vice versa, within a single simulation cycle, does occur, but with very low frequency.

COMPOUND probabilistic movement between zones within a S3 element follows rules. Those rules are different for a COMPOUND that is currently in a HME zone and one that is in some other zone. Relocation for a COMPOUND not in a HME zone is controlled by $\text{disperseProb}$ in Table 1; the values are tuned. The number of zones and movements between evolved as part of the iterative refinement process (Fig. 2). There can be up to eight different inter-zone movements, depending on project stage (discussed below) and ISECC specifications. Three general guidelines emerged during ISECC evolution. To enable sufficient COMPOUND to quickly reach S1 or S5, there must be a direct route from $Z_n$ to Z0. There is a bias towards Z0. Relocation between S3 zones in LY-treated-CULTURES (hereafter called LY-CULTURES) is slower than in CONTROL-CULTURES. Additional detailed observations about COMPOUND movement are in the following paragraph.

A COMPOUND in Z0 or $Z_n$ is in one of two states: it either is or is not currently in a HME zone. During a simulation cycle, a COMPOUND that is currently not in a HME zone will be given an opportunity to transition to another zone. In CONTROL CULTURES a COMPOUND may also move to one of four nearest zones: $Z(i+1)$, $Z(i+2)$, $Z(i-1)$, or $Z(i-2)$, where $i+2 \leq n$ and $i-2 \geq 0$. Corresponding COMPOUND
relocation options in ISECC that map to LY-CULTURES are to move to one of two nearest zones: Z(i+1), or Z(i−1), where i+1 ≤ n and i−1 ≥ 0.

An element can contain one (Z0) or two (Z0 and Z6) HME zones. In the first case a COMPOUND currently in the HME zone is not given an option to change zones within that element; it stays in Z0. It may, however, like all COMPOUNDS in non-HME zones, relocate to a corresponding zone in an adjacent element, following the special rules above. In the second case a COMPOUND in either HME zone is given an option switch between HME zones: it moves to Z0 with a probability of 0.8 or to Zn with a probability of 0.2.

We also found it necessary to modulate SAQ mobility between non-HME zones within the same element. This trait could map to nonspecific binding, exceeding a solubility limit, etc. We elected to name the parameter controlling this trait solubility. During each simulation cycle, a SAQ “queries” the total number of SAQS in S3. It will have an opportunity to move to a new zone only if a pseudo-random number (PRN) is less than \[\text{solubility}/(\text{SAQS in } S3 \cdot (1 \pm 0.2 \cdot \text{error})]\], where error is randomly generated and has value between 0 and 1. Solubility (limit) is tuned; the value used for Fig. 3 is 250. This would be the first ISECC property revisited if we were to add a more stringent SM (SM-4) that required a higher degree of ISECC similarity with wet-lab results at the low and high doses.

ENZYMES, TRANSPORTERS. The logic used by each CYP and PGP along with their function verification was reported in (Lam et al., 2008). We have used, reused, verified, and validated several variations of CYP and PGP (Garmire and Hunt 2008), (Lam et al., 2009a). Different model uses and different reference data can call for different capabilities. The ones used here are the simplest. For convenience, the logic diagrams reported in (Lam et al., 2008) for each are provided in Supplement Fig. S2. A stationery CYP maps to an unspecified number of the cytochrome P450 enzymes that metabolize saquinavir and its primary metabolite. A stationery PGP, assigned randomly only to S2 elements, maps to an unspecified number of apical membrane components and processes, including P-gp transporters, that are responsible for facilitated efflux of saquinavir and its metabolite from intracellular space to the apical dosing compartment. Stationary means that the object is assigned randomly to and remains in one element for the duration of the simulation. A PGP can export COMPOUNDS from Z0 (either normal or HME) to S1. Based
on results from Stage 1 ISECC experiments (discussed below), we specified that SAQ can be METABOLIZED by CYP to M7, which in turn can be METABOLIZED by CYP to M1. Further, M7 and M1 are both substrates of CYP and thus are competitive inhibitors of SAQ metabolism. We specified that SAQ, M7 and M1 are substrates of PGP. We also specified that M7 and M1 are less hydrophobic than SAQ. BINDING to and RELEASE from CYP and PGP are governed by \textit{assocProb} and \textit{releaseProb}. CYP has an additional step: METABOLIZE, which is governed by \textit{efficiencyProb} (\textit{efficiencyProb} of PGP is 1). For this study, the specifications and internal logic of CYP and PGP validated in (Lam et al., 2008) were expanded as follows. The CYP and PGP randomly selected their binding neighborhoods at the start of their assigned logic each simulation cycle. CYPs are assigned only to two specific zones. For the finalized Stage 4 ISECC (Fig. 3), the \textit{Z5/Z4} assignment ratio is 3/2.

\textbf{Probabilistic parameters and event scheduling.} Most events, such as binding to CYP, are probabilistic and have probability parameter values in the 0 to 1 range. When an event option arises, a participating component draws a pseudo-random number (PRN) from the designated range. Its value is compared with that of a parameter to decide what action to take. For example, if the PRN is less than the value assigned to \textit{assocProb}, then the event occurs. Otherwise, it does not. By using a uniform distribution with variable frequency parameters throughout the simulation, we added uncertainty that maps to our ignorance about many of the details that are below our level of resolution. Assumptions about frequency values were separated from, and external to the ISECC.

Upon initiation of a simulation, spaces and element objects are created, zones are specified, and all objects (COMPOUND, CYP, and PGP) are assigned to elements and added randomly to a list. During each simulation each object in the order listed is given an opportunity to execute its assigned logic. Execution order is shuffled randomly at the start of each simulation cycle. The number of COMPOUNDS in each space is recorded every five simulation cycles and written to external files for later analysis.

\textbf{Experimental condition.} We simulated 16 different experimental conditions: four treatments at four dosing conditions. The four treatments were: APICAL (S1) SAQ dosing of CONTROL and LY-CULTURES, and BASAL (S5) SAQ dosing of CONTROL and LY-CULTURES. Four SAQ amounts were used:
1,000, 2,000, 4,000 and 8,000. They mapped to 7.5, 15, 30 and 60 nanomoles and 5, 10, 20, 40 µM, respectively. The number of CYP used ranged from 20 to 70, depending on Stage (described below). The number of PGP used for experiments CONTROL CULTURES was one of the following: 250, 400, 500, or 600. The number of PGP used for experiments LY-CULTURES was one of the following: 10, 15, 20, or 25 (early ISECC that used 0 were falsified). To complete one experiment, we averaged results from ten (selected for convenience) repeated simulations. All simulation ran for 120 simulation cycles.

**Groundings.** The units, dimensions, and/or objects to which a variable or model constituent refers establish groundings. Inductive ordinary differential equation models are typically grounded to metric spaces. So doing provides simple, interpretive mappings between output and parameter values and DATA. However, metric grounding creates issues that must be addressed each time one needs to expand the model to include additional phenomena. Adding a term to an equation, for example, requires defining its variables and premises to be quantitatively commensurate with everything else in the model. Such expansions can be challenging and even infeasible when knowledge is limited and uncertainty is high, which is the situation that we faced. Iterative model refinement as described in Fig. 2 becomes slow and complex when the model is grounded to metric space. To discover plausible concrete mechanistic explanations for the targeted data, we needed the ability to simultaneously explore different regions of plausible mechanism space at different levels of detail, and relate results to wet-lab observations. To facilitate that process it must be easy to change mechanistic or component details at any level without having to invest significant time in analogue reengineering. We have discovered that the best way to achieve those objectives is to remove metric grounding from the ISECC and confine it to quantitative feature-to-feature and phenomena-to-phenomena mappings (Lam and Hunt 2009b), and that is what we did.

The mechanisms responsible for generation of the TRANSPORT DATA do not interact according to any external measurement methods. They are independent of any measures used by an outside observer. From that fact, we inferred that the ISECCs needed to achieve our objectives must employ similar internal organization, which in modeling terms, means each component is grounded to other components rather than to a metric imposed by an outside observer, and that is the course we have followed.
During iterative ISECC refinement several different groundings were used. For the final ISECC and the experimental results reported herein, the quantitative groundings were as follows: 1,000 SAQ → 7.5 nanomoles, 75 simulation cycles → three hours (during Stage 1 it was 60 simulation cycles → three hours), and one ISECC experiment → one wet-lab experiment. The SAQ grounding took into consideration the lower limit of analytical detection in referent experiments (Mouly et al., 2004), which was < 0.012 nanomoles, equivalent to 1.6 SAQ objects.

**Similarity measures.** The ultimate goal was to have ISECCs such that simulation results intended to match a specific TRANSPORT experiment would be statistically indistinguishable from a repeat of that targeted wet-lab experiment. Taking into consideration the variability between wet-lab experiments within and between conditions, we specified that our goal would be met by achieving the Stage 4 objectives stated below. The most stringent Similarity Measure (SM), SM-3, was that no more than one of a selected subset of conditions is outside the range of 66.7%–150% of referent values. Meeting SM-3 would imply statistical indistinguishability to wet-lab observation. To enable adhering to the parsimony guideline and progress through many ISECC refinement cycles, two less stringent SM targets were used. SM-1 (least stringent): simulation results of no more than three of the targeted conditions are outside 50%–200% of referent values. SM-2: simulation results of no more than three of the targeted conditions are outside target range of 66.7%–150% of referent value, or no more than one is outside range of 50%–200% of referent values.

**Achieving targeted attributes.** The goal of discovering one ISECC with one set of plausible micromechanisms was approached in stages following the protocol in Fig. 2. Early in the process, results from only two of 16 experimental conditions were targeted using SM-1. At Stage 4 the targeted attribute list was expanded to all 16 experimental conditions using a combination of SM-3 and SM-1.

Robustness to minor parameter change (e.g., ~10% for one parameter and ~5% for 2–4 parameters) was an additional targeted attribute. We illustrate with an example. Having an ISECC that achieved Stage 2, we sought component changes and additions that would enable achieving Stage 3. Several
changes that helped, but failed, we kept. Consider a predecessor to the ISECC that achieved Stage 3. It is possible that there is a small region (maybe more than one) of that ISECC’s component and parameter space that could have achieved Stage 3, but we failed to locate it. However, had we found it, we would have observed that small changes in several parameters caused the ISECC to fall short of the SM target. We encountered several such instances in route to the ISECC in Table 1. We decided that in order to validate, an ISECC must tolerate small changes (in the 5-10% range) in parameters while still achieving targeted SMs. This specification is currently arbitrary and qualitative. However, it can be made more precise and quantitative when there is a need to do so.

Stage 0: meets design specifications
Stage 1: target one dose, under control (no inhibitor) condition only
  Qualitative: paradoxical observation: APICAL dosing produces higher INTRACELLULAR SAQ levels, but less M7 METABOLITE (Mouly et al., 2004)
Stage 2: target the two middle doses, control conditions only
  Qualitative: large difference in receiving compartment after APICAL versus BASAL dosing
  Quantitative: simulation results meet SM-1
Stage 3: target the two middle doses for control and inhibitor treatment
  Qualitative: for inhibitor treatment relative to control: more SAQ in CELLS, more total M7
  Quantitative: results from CONTROL CULTURES meet SM-2; those from LY-CULTURES meet SM-1
Stage 4: target all four doses for control and inhibitor treatment
  Qualitative: evidence of METABOLISM saturation at larger doses (Parker and Houston 2008); similar M7 per INTRACELLULAR SAQ ratio
  Quantitative: results for two middle doses meet SM-3 whereas those from smallest and largest dose meet SM-1

While progressing from early Stage 1 to Stage 4 validation, we followed somewhat standardized parameter sweeping methods to discover a specific ISECC parameterization that would enable achieving the
targeted attributes and SM for that ISECC. We explored four types ISECC characteristic change. 1) Change spatial properties: we changed S3 element properties by subdividing the space within into different numbers and types (HME) of zones. 2) Change mechanistic components: for a given set of elements and properties, we changed the ratios and numbers of CYP and PGP. 3) Change logic used by components. 4) For specific valuations of the three preceding sets of characteristics, we tuned the values of remaining parameters. We began by randomly probing regions of property and parameter space and running experiments. We selected the two or three locations that moved us closest to validation, and randomly sampled nearby parameterizations. That process was repeated until a decision was made to fix the first three ISECC characteristic types. Focus then shifted to tuning just a few of the remaining parameters to improve results even further.

**Software and simulation time.** We reused validated components from previous projects (Liu and Hunt 2006), (Garmire et al., 2007), (Lam et al., 2008), (Lam et al., 2009a). ISECCs were assembled within the Swarm platform using its libraries (http://swarm.org). We coded in Java Swarm. Most experiments used a single processor and ran under Microsoft Windows XP (Redmond, WA) with Java Software Development Kit and Java 2 Runtime Environment installed (Sun Microsystems, Santa Clara, CA). Source code was compiled with Java 2 Software Development Kit SE version 1.4.2_13 and executed with Java 2 Runtime Environment version 1.4.2_13 (www.java.com). Output DATA files were processed, graphed, and analyzed using Microsoft Excel. A complete set of ISECC for all 16 conditions averaged about one hour when using one processor; analysis of results often took longer.

**Results**

**Summary of wet-lab results.** Mouly et al. reported that saquinavir’s apparent permeability is always higher for basal-to-apical transport, compared to that of apical-to-basal (Mouly et al., 2004). Because saquinavir is a P-gp substrate, that result was expected. As predicted, inhibition of P-gp by inhibitor increased apical-to-basal while decreasing measured basal-to-apical permeability. In addition, inhibitor treatment significantly increased the intracellular level of saquinavir, and formation of saquina-
vir metabolite at the end of the three-hour experiments. However, despite the lower intracellular saquinavir level after basal compared to apical dosing, basal dosing consistently, and paradoxically, produced more metabolite M7 in both control and inhibitor-treated cultures, and for the four doses studied. Such a paradox had not been reported previously, and its cause could not be isolated based on experimental observations. The investigators offered two potential explanations: 1) a saquinavir concentration gradient exists intracellularly, and the parent drug presented at a higher concentration at the enzymes’ microenvironment after basal dosing; and 2) secondary metabolism of M7 was greater after apical dosing. Variances in results for repeat experiments were typical of in vitro transport studies. Coefficients of variation of the various measures ranged from 16 to 80% and averaged 35% for experiments on five matched cultures.

**Summary of ISECC experiments.** The ISECC, which validated against the Stage 1 attributes and SM, was described in (Lam et al., 2008). From there, we cycled through the iterative refinement protocol several hundred times before discovering an ISECC that validated by successfully achieving the attributes and SM specified by the Stage 4 criteria. An overview of seventeen ISECC that were thoroughly explored and eventually falsified before achieving the targeted Stage 4 validation criteria is provided as Supplement Fig. S1. All had the same five spaces and all included CYP in S3 and PGP in S2. The results presented in Figs. 4–8 along with Tables 2 and 3 summarize TRANSPORT results for the validated ISECC described in Fig. 3. Figures 4–6 correspond to Fig. 1A–C in (Mouly et al., 2004).

**Plausible explanations for paradoxical results.** The two potential mechanisms offered by Mouly et al. as plausible explanations proved necessary but not sufficient to cause comparable results within the ISECC context. They suggested that an intracellular gradient might exist from dosing to receiving compartment. We implemented that mechanism in a Stage 1 ISECC. We achieved SM-1 for two doses for both control and inhibitor-treated cultures but were unable to achieve SM-2. The accumulation of relatively large amounts of SAQ in the receiving compartment could not be achieved while also keeping the amount of (all) metabolite formed small. The results demonstrated that within the ISECC a gradient effect alone was not sufficient. Thereafter, we discarded the specification that the INTRACELLULAR space
behaves as a well-stirred space. Later experiments demonstrated that allowing S3 to be heterogeneous and contain pockets (HME) having different SAQ affinities was necessary to achieve SM-2 and later SM-3.

Mouly et al. also suggested secondary metabolism (to M1) as an explanatory mechanism, and that formation of M1 from M7 was greater after apical dosing. That mechanism would require M7 disposition to be different following apical and basal dosing. However, within the validated ISECC, secondary METABOLISM caused competitive inhibition of SAQ METABOLISM, and that accounted for why a large fraction of the SAQ dose passed through the CELL while only tiny fraction was present as M7. Furthermore, METABOLISM saturation began influencing results at higher doses.

**P-gp activity, inhibitor-treated cultures, and temporal mapping.** All parameterization decisions were interconnected. We narrowed options by specification early of the number of SAQ to be used per simulation: 1,000–8,000. Exploratory simulations showed that an appropriate number of PGP for that range of SAQ would be 250–600. For simplicity, we simulated inhibition by deactivating all but a few PGP. Results from exploratory simulations indicated that the number of fully active PGP in LY-CULTURES needed to be > 0 and that an appropriate range might be 10–25 PGP. Thereafter, when seeking a parameterization that would validate, experiments of LY-CULTURES used 10, 15, 20, or 25 PGP to see which was best. The validated ISECC in Table 1 used 500 PGP for CONTROL and 15 PGP for LY-CULTURES for the four dose conditions. A quantitative in mapping of the results in Figs. 4–6 to wet-lab means that inhibitor treatment reduced P-gp activity by about 97%.

Each event occurring within a simulation cycle during an ISECC simulation contributes in a small way to overall outcomes. That means that it is possible for a small change in one or a few micro-mechanisms to be offset by other changes, such as changing how a simulation cycle maps to wet-lab time. With that realization in mind, during each iterative refinement cycle alternative ISECC-to-wet-lab temporal mappings were explored to see if an adjustment in that mapping could improve SM outcomes. Once the amounts ranges for dose, PGP, and CYP were fixed, it quickly became apparent that ISECC TRANSPORT results at 60–90 simulation cycles mapped best to the wet-lab three-hour transport data. The choice does not influence any of the ISECC the premises. For the ISECC represented by Table 1, the
ISECC TRANSPORT results at 75 simulation cycles provided the best match to referent data. For that mapping, one simulation cycle maps to ~2.4 minutes.

**Components and features of the ISECC that validated.** Each ISECC for which simulation results matched a prespecified set targeted attributes and a prespecified SM achieved a degree of validation. Improving mapping A in Fig. 1 while also increasing the attributes targeted, increases confidence that there is some validity to mapping B in Fig. 1. The reported measure of transport for each experimental condition is a phenotypic attribute of the in vitro system. If the five-space representation is acceptable for a monolayer of essentially identical cells, then those 16 attributes, taken together, place serious constraints on the space of mechanisms that will validate, especially when the variety and properties of stationary component types allowed is limited to two: CYP and PGP. We argue that an ISECC that validates at Stage 4 should be taken more seriously (as having plausible biological counterparts) than an ISECC that can only validate at Stage 2. Further, any ISECC that validates at or above Stage 1 should be taken more seriously than any descriptive conceptual mechanism. What follows is a description of the features of the ISECC specified by Table 1.

The INTRACELLULAR environment (S3) is subdivided into seven ZONES, with Z0 adjacent to S2, and Z6 adjacent to S4. Z0 in 15% of elements are specified as being HME. In addition, in one-fifth of the elements containing a HME Z0, the Z6 in that element is also specified as being HME. COMPOUND movement into and out of HME is governed by an in silico distribution. SAQ is highly hydrophobic. Once it enters a HME it preferentially stays in HME. The net result is that a large portion of INTRACELLULAR SAQ gets sequestered in HME and is unable to reach METABOLIC ENZYMES. Further, that portion is larger after APICAL dosing. We suggest S3 zones in ISECC map to heterogeneous microenvironment within the cell monolayer.

During INTRACELLULAR COMPOUND movement, there is a bias towards Z0. A COMPOUND within an element may move to the APICAL (Z0) and BASAL (Z6) zones, or to one of the four nearest adjacent zones (Zi + 1, Zi + 2, Zi – 1, or Zi – 2). In LY-CULTURES, COMPOUND movement is slowed (less displacement over time). A COMPOUND within LY-CULTURE S3 element may move to the APICAL (Z0) and BASAL (Z6)
zones, or to one of the two nearest adjacent zones \((Z_i + 1, \text{ or } Z_i - 1)\). These movements, including the effective shortcut from \(Z_0\) to \(Z_6\), may map to any of a variety of yet unidentified micromechanisms. Plausible examples include saquinavir binding (somewhat preferentially) to a saturable component of a transcellular transport system, for example, fatty acid intracellular transport system described by Weisiger (Weisiger 1996), (Weisiger 2002), (Weisiger 2007), or becoming associated (somewhat preferentially) with a nonequilibrium, apically directed intracellular flux of the type described by Kurakin (Kurakin et al., 2009).

Assume that the above saturable, transcellular saquinavir transport hypothesis is valid. In LY-CULTURES, intracellular SAQ movement is slowed. That may map to the inhibitor also competitively inhibiting saquinavir movement. Assume that too is valid. We should then see decreasing apparent permeability (Papp) when the saquinavir dose increases in control cultures, and we should see increasing Papp when saquinavir dose is increased in inhibitor-treated cultures. Both predictions are consistent with wet-lab observations. However, because we had no evidence-based insight into actual mechanisms, the ISECC used abstract SAQ movement rules as placeholders for all plausible, fine-grained mechanisms. By so doing, we preserved ignorance. No component based carrier micromechanism was actually implemented. Consequently, there were no ISECC Papp observations analogous to the above-cited wet-lab data. Exploring that scenario is an option for a future ISECC study.

To achieve Stage 4 validation we found it effective to impose a limit on the amount of SAQ that could be within S3 during a simulation cycle. We did that by specifying SOLUBILITY in S3 to be 250. We suggest that there may be an effective counterpart within the Transwell cells.

The TRANSPORT properties of each explored ISECC type moved closer to validation when CYPs were sequestered within a portion of S3. For the final ISECC, CYPs were assigned 40% to Z4 and 60% to Z5; a 50/50 assignment resulted in failure to achieve the Stage 4 SMs. Those assignments meant that a SAQ was more likely to encounter a CYP when coming from S5 (BASAL dosing) rather than S1 (APICAL dosing). These CYP assignments within the ISECC map to a non-uniform, intracellular distribution of saquinavir metabolizing enzymes. Because movement within S3 was slower in LY-CULTURES, proportion-
ally less SAQ reached Z4 and Z5, and that caused less M7 formation. We suggest that these events may have counterparts within Transwell cells treated with inhibitor.

Because SAQ was assigned a large MW (670) there was very little PARACELLULAR TRANSPORT. If we lower MW, PARACELLULAR TRANSPORT increases. That is because 15% of S1 and S5 elements are marked TJ. The TJ map to all cell-cell attachments and to any spaces between cells. The probability to transit to S1 from S5 when in a TJ element (0.25) was specified to be much greater than probability to transit to S5 from S1 via a TJ element (0.001).

**ISECC robustness to parameter change.** Upon achieving Stage 4 (or an earlier stage) goals, ISECC robustness to parameter change for a variety of parameters was measured. Observing that a small change (5–15%) in one parameter caused the ISECC to invalidate was considered abiotic; a search for a different region of parameter space was initiated. To illustrate the process for the ISECC in Table 1 for both CONTROL and LY-CULTURES, the effects of parameter change on the three TRANSPORT measures are provided in Figs. 7 and 8 for the influential parameter PGP levels and for choice of temporal mapping. The values graphed in both figures are the ratio of ISECC-to-wet-lab transport measures. The number of PGP in Table 1 was 500 for control and 15 for LY-CULTURES. Figure 7 presents TRANSPORT results for 250–600 for control and 10–25 for LY-CULTURES. The temporal mapping for Figs. 4–6 is 75 simulation cycles → three hours. Fig. 8 presents TRANSPORT results for four additional temporal mappings: 30–120 simulation cycles → three hours. Results of these and other robustness explorations (not shown) showed that many other parameter vectors close to the one in Table 1 can also produce ISECC that validate.

**Discussion**

An implication of the ISECC validation evidence is that the mechanisms depicted in Fig. 3 have counterparts during saquinavir transport through Caco-2 cells. The simulation results provide concrete, scientific evidence that interactions with heterogeneous intracellular microenvironments, coupled with intricate, intracellular saquinavir movements, some possibly carrier-mediated, provide a plausible explana-
tion of the cited paradoxical observations. All compounds will encounter such heterogeneity, but the influence may not be evident from traditional assessments of transport data. It seems unlikely that such microenvironment heterogeneity will be confined to Caco-2 cells. Microenvironment differences within and between cell types as a function of health and disease may influence therapeutic availability to target sites. Future therapeutics may exploit heterogeneous intracellular disposition to enhance efficacy and minimize toxicity. The heterogeneities may also contribute to intra- and interindividual variability in disposition and response. Wet-lab technologies and experiments designed to detect the influence of microenvironment heterogeneities are needed to help determine their importance.

The degree of similarity between the ISECC and wet-lab data in Figs. 4–6 gives strength to the hypothesis that the mechanisms depicted in Fig. 3 have counterparts during saquinavir transport through Caco-2 cells. However, as abstract models, ISECC mechanisms are flawed. Nevertheless, we seek to minimize discrepancies by exploring multiple mechanistic explanations using the iterative refinements protocol in Fig. 2, and only exploring those that are not abiotic.

Even though the final ISECC mechanisms are far less complex than what we already know about epithelial cells, a logical initial reaction upon reading the Fig. 3 explanation would be to muse about simpler mechanistic explanations: are there no simpler explanations? There may be, but we have not yet found one. The iterative refinement protocol in Fig. 2, coupled with the parsimony guideline, has proven to be an effective tool in resisting making ISECCs unnecessarily complicated. Following that protocol created a mechanism exploration path (shown in Supplemental Fig. S1) for which the next added mechanistic detail (or complication) depended on predecessor mechanisms. Ideally, we would prefer to explore many branches of many parallel paths. We cannot rule out the existence of alternative paths that lead to somewhat simpler explanations. Some have not been fully explored, in part because our objective was to find one plausible set of micromechanisms to achieve a prespecified SM for all 16 experimental conditions. Below, we present four of several mechanistic exploration paths that may merit future attention, wet-lab as well as in silico.
Path 1: for the path followed, Path 0, we specified that an ISECC represents a monolayer comprised of essentially identical cells. Reality may be more complicated. There may be differences in saquinavir transport within and through different subsets of cells. For example, cells may be classifiable into at least two types for which the intracellular micromechanisms are simpler than those in Fig. 3, but together they achieve the same Stage 4 SMs. We explored this option but failed to come close to the targeted SMs. Wet-lab experiments, possibly using new imaging technologies, could help confirm or rule out such differences and thus indicate the relative importance of exploring this path.

Path 2: for Path 0, we specified that intracellular micromechanisms be invariant. That was not the case in (Lam et al., 2009a). Interactions of cells with saquinavir (and the inhibitor) may initiate time-dependent mechanism changes. We did not explore this path to any appreciable extent, but based on our earlier work, we can be confident that this path could lead to mechanisms that achieve the Stage 4 SMs. However, it would be debatable whether or not successful time-variant mechanisms would be simpler; but they would be different. Again, coupling in silico with wet-lab experiments could indicate the relative importance of exploring this path.

Path 3: Kurakin argues persuasively for abandoning the conventional biological paradigm rooted in classical mechanics and equilibrium thermodynamics in favor of one in which all cell subsystems are viewed as dynamic, adaptive, nonequilibrium systems that are part of their environment (Kurakin 2009). The synthetic M&S method enables building such analogues, but it is not clear that they can be mechanistically simpler than the one in Fig. 3.

Path 4 (not yet explored): given the abstract simplicity of ISECC components, it may be unrealistic to insist on a linear mapping between amount of SAQ used and wet-lab dose.

Because all ISECCs are simplifications of reality, they are mechanistically depleted and thus flawed in specific ways. Nevertheless, some can be very useful. There are many referent system attributes such that when one is added to the targeted attribute list will falsify the Fig. 3 ISECC. We had no evidence indicating that any of the above four mechanistic exploration paths should be preferred over Path 0.
theless, along Path 0 there may be mechanistic branches that did not occur to us yet may contain simpler systems.

One solution to the above mechanism generation and selection issues is, when feasible, to use simulations to identify phenomena that suggest new wet-lab experiments that in turn could rule in or out exploration of a mechanistic branch. Doing so would require timely coordination of wet-lab and simulation experiments at steps 8a and 8b in Fig. 2.

Another strategy for addressing the mechanism generation and selection issues is to seek ensembles of different explanatory analogues spanning an acceptable variety of mechanistic options, and allow them to compete in offering plausible explanations of an increasingly rich set of targeted phenotypic attributes. The cost of implementing such a solution can be kept reasonable by developing automated modeling methods capable of discovering mechanistically different analogues. The information provided in Supplement Fig. S1 documents that we built, tested, and falsified many ISECCs before discovering the one that validated. The process requires extensive human effort and time. The technology can be developed to use software agents within the existing computational framework to manage the process of changing a falsified ISECC by adjusting the parameter vector and/or the mix of components with the objective of achieving a prespecified SM.

There are several features of the ISECC in Fig. 3 that merit discussion. Saquinavir is known to preferentially bind to mitochondria and the nucleus (Khoo et al., 2002). The HME may map in part to these subcellular structures. We suggest that the movement logic used by SAQ to change zones, especially movement between Z0 and Z6, may map to saquinavir binding to mobile components of one or more intracellular transport system, such as the fatty acid transport system (Weisiger 2007). If future evidence supports that mapping, then the ISECC TRANSPORT results can be taken as predictions that the carrier mechanism(s) will be saturated at higher saquinavir doses. The mechanistic changes in LY-CULTURES may map to the inhibitor reducing saquinavir interaction with the carrier system(s), possibly through competitive binding. The preceding conjecture is consistent with wet-lab (but not ISECC) observations that apparent saquinavir permeability is decreased with increasing saquinavir dose in control cultures but
is increased with increasing saquinavir dose in inhibitor-treated cultures. Comparable ISECC results were not observed because, as explained in Results, SAQ movement rules functioned as placeholders for all plausible, fine-grained, componentized mechanisms. Well-designed wet-lab experiments may help clarify the biological implications of this conjecture.

Introducing the idea of a SAQ SOLUBILITY limit within S3 elements enabled improving high and low DOSE ISECC results sufficiently to achieve Stage 4 validation. However, should we set a new Stage 5 goal that includes a more stringent SM for high and low DOSES, the current ISECC would be falsified (invalidated). To achieve the new Stage 5 we would need to explore alternative micromechanistic features including improvements to the current SOLUBILITY algorithm. Given those considerations, it is premature to seek Caco-2 counterparts to which the current SAQ SOLUBILITY micromechanism may map.

Early during the iterative ISECC refinement process, it became clear that the pace of refinement and mechanistic exploration was accelerated by specifying mappings, including temporal mapping (simulation cycles to wet-lab minutes), the final adjustments in the process. So doing seemed counterintuitive initially, because most inductive transport models nail down temporal mappings at the start by equating some parameter of the induced model with some independent wet-lab measure with which it is identified. Doing the same in ISECCs limits one to a small set of refinement paths, while making mechanistic refinement increasingly difficult. We noted that different PGP numbers and temporal mappings were equally acceptable. Because methods of measurement are external to both wet-lab models and ISECCs, we now argue that when feasible, establishing interpretive mappings should be among the last adjustments made within a refinement protocol cycle (Lam and Hunt 2009b). It is noteworthy that moving mapping finalization to the end of the protocol takes advantage of the ignorance- and uncertainty-preserving characteristics of the synthetic method (Hunt et al., 2009).

The extent of P-gp inhibition by the inhibitor amounts used during wet-lab experiments was unknown and so needed to be factored into grounding decisions. Consequently, for every cycle of the protocol, we used four PGP levels in each CONTROL and LY-CULTURE. We then selected the pair (one for each) that moved us closer to validation.
Figure 2B illustrates the following. An ISECC that is marginally just complicated enough to validate will be fragile to changes in targeted attributes (its phenotype): add any one of a number of wet-lab attributes to the targeted set and the ISECC is at risk of being falsified. The advantage of such an ISECC is that the portion of mechanism and parameterization space that enables validation is small. However, by being fragile to attribute change, it is less biomimetic. We argue that a scientifically more interesting ISECC will be one that has some degree of robustness to changes in phenotype, and that requires having a larger mechanism and parameterization space that validates: many somewhat different mechanisms, each having a number of satisfactory parameterization vectors, a process called multi-modeling (Hunt et al., 2009). For multi-modeling to be successful, it must become methodically scientific. Scientific M&S will accelerate scientific progress by facilitating fast-paced cycles of hypothesis (about mechanisms) generation, selection, and falsification. Each cycle requires synthetic M&S coupled with inductive and deductive methods; during such a cycle, abduction drives the creation of mechanistic hypotheses. Those mechanistic hypotheses that meet criteria are selected for in silico experimentation designed to ensure that only those with explanatory, heuristic value survive falsification. The cyclic process exercises, leverages, and enriches the mental models of domain experts in new ways. Multi-modeling that can be semi-automated is the M&S frontier. Nevertheless, during analogue refinement, parsimony remains an important guideline.

A purpose of conducting wet-lab experiments like those cited is to gain new knowledge regarding mechanistic details. Experimenting on synthetic analogues like ISECCs, provides a heretofore unavailable means of discovering new, plausible mechanistic details, especially when relevant wet-lab experiments are highly complicated, costly or impossible. We have demonstrated a new means of achieving deeper insight into the generative mechanisms responsible for phenotype. The approach extends the scientific method to M&S while enabling achieving deeper understandings of pharmacological and therapeutic causal linkages.
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References


Footnotes

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Legends for figures

Figure 1. Relationships between in vitro, transport experiments, conventional induced transport models, and ISECC experiments. A, center: cell monolayers in an experimental context such as a Transwell device are the referent wet-lab systems. During experiments, cellular components interact with transiting drug molecules to cause changes in amount transported and metabolized within specified intervals. Influential mechanistic details are reflected in the collected data. Left: the researcher identifies patterns in the wet-lab transport data. From those and prior transport knowledge, a mechanistic description of what is thought to have occurred is induced, thus establishing an abstract, conceptual mappings from that description to transport mechanisms. In some cases, the researcher goes further and offers a set of transport equations believed capable of describing the data. An algorithmic representation the equations in software is constructed and executed to simulate parameterized equation output. Metrics specify the goodness of fit of the simulated output to the data. Right: the abstract mechanistic description may be different from that on the left side. Software components are designed, specified, coded, verified, and assembled and connected guided by that mechanistic description. The product of the process is a collection of abstract mechanisms rendered in software. A clear mapping—C—is intended to exist between ISECC components and how they plug together, and cell monolayer and intracellular details. Relative similarity is controlled in part by parameterizations. Importantly, mapping C can be concretized iteratively. Compilation and source code execution gives rise to a working analogue. Its dynamics are intended to represent abstractly (mapping B) corresponding dynamics (believed to occur) within the monolayer cultures during an experiment. Mapping B can also be concretized iteratively. Measures of simulated dynamics provide time series DATA that are intended to mimic corresponding measures of wet-lab transport experiments. Achieving increasingly stringent similarity measures enable mapping A to be made concrete. B, Conditions supportive of all three reasoning methods are sketched (see appendix for brief descriptions). Obviously, scientists engaged in drug transport research would like knowledge about all cell
monolayer and subcellular characteristics to be rich and detailed, and for uncertainties to be limited. Such conditions (toward the far right side), which are common non-biological, physical systems, favor developing inductive models that are increasingly precise and predictive. However, for the system and experiments described in (Mouly et al., 2004) we are on the left side, where frequent abduction is needed and synthetic M&S methods can be most useful.

**Figure 2.** The iterative protocol used to refine and improve the ISECC. At the beginning, the set of attributes to be targeted are specified. In this case, that set included results from sixteen different experimental conditions. Similarity goals to be achieved are specified. Abductive reasoning may be required at steps 4-8. Induction and deduction occur during steps 5-7. The final ISECC described herein was preceded by seventeen less complicated ISECC (described in the Supplement). Each in sequence was falsified at step eight. **Bottom:** a graph illustrating the relationship between the parameter space for a specific ISECC that validates and the number of attributes targeted. Indicated is an ISECC that validates by achieving the Stage 3 but not Stage 4 SMs. Its parameter space is small. However, because parameter influences are all networked and the variability in outcomes between simulation runs is non-trivial, there are many similar mechanism and parameter vectors within that space that can produce essentially the same measured simulation phenomena. If the number of attributes targeted is reduced, then for the same ISECC the size of the space that contains parameter vectors that can validate increases. However, if the number of attributes targeted is increased at step eight in the protocol, that same ISECC is falsified because the parameter space that enables validation shrinks to zero. Adding a component or feature to that ISECC makes a new, more complicated ISECC that has its own curve; it may be shifted up so that, again, there is a region of parameter space that contains mechanisms and parameter vectors that can validate. The relationship is shown linear for illustration purposes.

**Figure 3.** Illustration of spatial heterogeneity for the ISECC that achieved Stage 4 validation. Illustrated is the ISECC resulting from Table I parameterizations that gave the DATA in Figs. 4–6. **A:** Five identically sized spaces are used: S1–S5, as described in the text. The element at the interface of two CELLS
(which maps to tight junction spaces between cells, and other cell-cell attachments) is called a TJ. Each of
the 2,500 elements comprising S3 is subdivided into seven zones, Z0–Z6. Because an element is the lowest
level of spatial resolution, the actual shapes and locations of Z0–Z6 are not specified, although aspects of
their relative arrangement are specified. Z0 and Z6 map to large portions of intracellular space adjacent
to each of the membranes. Z1–Z5 are located more centrally, they represent a series of heterogeneous
subcellular microenvironments of indefinite shape and volume; they may map to subcellular structures such as nucleus, endoplasmic reticulum or mitochondria. The Z0 in 15% of S3 elements are
specified to map to subcellular, lipid-like, hydrophobic microenvironments called HME (a SAQ has a high
affinity for these spaces). Some (20%) S3 elements that contain a HME-Z0 also contain a HME-Z6. B: Illustrated are the three types of mobile objects (SAQ, M7, and M1) and the two types of immobile objects
(PGP and CYP). At the initiation of a simulation, objects map to Cyp3A4 enzymes (CYP) and P-gp transporters (PGP) are created and placed randomly in S3 (zones Z4 and Z5 only) and S2 respectively. Objects representing saquinavir (SAQ) are placed either in the APICAL (S1) or BASAL compartment (S5). Those SAQ map to the apical dose and basal dose, respectively. Each simulation cycle, each mobile object gets
an opportunity to move between elements in adjacent spaces, and between elements within the same
space. It can also move between zones within a S3 element. PGP and CYP are confined to assigned ele-
ments. CYP probabilistically METABOLIZE SAQ in Z4 and Z5 to M7, and further METABOLIZE M7 to M1.
PGP is responsible for the active EFFLUX of SAQ and its METABOLITES from Z0 to S1. Other properties of
mobile and immobile objects are as detailed in the text.

Figure 4. Apparent permeabilities. Graphed are apparent permeabilities for saquinavir and SAQ. The
former values correspond to Fig. 1A in (Mouly et al., 2004); the vertical bars show ± 1 SD. The heavy
vertical bars with ISECC DATA show Stage 4 target ranges. For the ISECC experiments, apparent PER-
MEABILITY = (amount of SAQ in receiving compartment after 75 simulation cycles [maps to three hours])
÷ (SAQ dose). The results are means for ten ISECC simulations parameterized using Table 1 values.
**Figure. 5.** In vitro and simulated intracellular saquinavir accumulation. The results are for the same experiments as in Fig. 4. Graphed are the 1) amounts of saquinavir that were intracellular after three hours; they correspond to Fig. 1B in (Mouly et al., 2004), and 2) amounts of SAQ that were INTRACELLULAR (S2 + S3 + S4) after 75 simulation cycles along with target ranges (heavy bars).

**Figure. 6.** In vitro and simulated total metabolite. The results are for the same experiments as in Figs. 4 and 5. Graphed are 1) the dose fraction present as M7 after three hours; the values correspond to Fig. 1C in (Mouly et al., 2004), and 2) the fraction of DOSE present as M7 within all ISECC spaces after 75 simulation cycles along with target ranges (heavy bars). For each dose, the lower limit of M7 detection is indicated.

**Figure. 7.** Robustness of ISECC to changes in PGP numbers. Separate sets of experiments using the ISECC in Table 1 were completed using the indicated number of PGP. Everything else was unchanged. Bar heights are ratios of ISECC-to-wet-lab results of the type in Figs. 4–6 at matched intervals (75 simulation cycles and three hours) after APICAL (A, C) and BASAL (B, D) dosing. A and B: control conditions: the results are for PGP = 250–600; results for 500 and 600 meet SM-3; all results meet SM-2. C and D: Inhibitor treatments: results are for 10–25 active PGP remaining after inhibitor treatment; the DATA for 10 and 15 PGP meet SM-3; all results meet SM-2. Note that coefficients of variation of the wet-lab measures ranged from 16 to 80% and averaged 35%. Comparable adjustments of other parameters and components caused the same gradual change in ISECC TRANSPORT DATA.

**Figure. 8.** Robustness of ISECC to changes in temporal mapping. Temporal mapping is established by selecting the number of simulation cycles that map to three hours of wet-lab time. Y-values are ratios (as in Fig. 7) of ISECC-to-wet-lab results of the type in Figs. 4–6. X-values are TIME in simulation cycles. A and C: results from apical dosing. B and D: results from basal dosing. A and B: control conditions; C and D: Inhibitor treatments. Diamonds ◆: amount of SAQ in RECEIVING COMPARTMENT; squares ■: amount of SAQ in CELL; circles ○: total amount of M7. All results for TIME = 60-120 meet SM-2. The
box shows the target range for SM-3. Results within the box for \( \text{TIME} = 70-90 \) also meet SM-3. Comparable adjustments of other parameters and components caused the same gradual change in ISECC TRANSPORT DATA for temporal mappings.
### Table

**TABLE 1. Parameters** and values for the ISECC in Fig. 3 that validated for Stage 4.

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<tr>
<td><strong>TJsimportance</strong></td>
<td>Fraction of ELEMENTS that are TJ</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td><strong>HMspace</strong></td>
<td>Fraction of ELEMENTS that are HME</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td><strong>numPgps</strong></td>
<td>Number of PGP</td>
<td>500</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>numCyps</strong></td>
<td>Number of CYP</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td><strong>insilicoPH</strong></td>
<td>In silico pH</td>
<td>7.4</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td>In silico molecular weight</td>
<td>670</td>
<td>670</td>
<td></td>
</tr>
<tr>
<td><strong>logP</strong></td>
<td>In silico logP</td>
<td>3.025</td>
<td>3.025</td>
<td></td>
</tr>
<tr>
<td><strong>pKa</strong></td>
<td>In silico pKa closest to in silico pH</td>
<td>7.13</td>
<td>7.13</td>
<td></td>
</tr>
<tr>
<td><strong>solubility</strong></td>
<td>In silico intracellular solubility</td>
<td>250</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><strong>closeToInterface</strong></td>
<td>Fraction sufficiently close to membrane interface</td>
<td>S1</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>transitProb</strong></td>
<td>Trans-membrane transit probability between SPACES</td>
<td>S1→S2</td>
<td>0.218</td>
<td>0.218</td>
</tr>
<tr>
<td></td>
<td>S1→S5</td>
<td>0.002</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S2→S1</td>
<td>0.476</td>
<td>0.476</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S2→S3</td>
<td>0.476</td>
<td>0.476</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3→S2</td>
<td>0.086</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3→S4</td>
<td>0.086</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S4→S3</td>
<td>0.476</td>
<td>0.476</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S4→S5</td>
<td>0.476</td>
<td>0.476</td>
<td></td>
</tr>
</tbody>
</table>
### Interface to transit

<table>
<thead>
<tr>
<th>Path</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5→S1</td>
<td>0.333</td>
</tr>
<tr>
<td>S5→S4</td>
<td>0.019</td>
</tr>
</tbody>
</table>

### Dispersion

<table>
<thead>
<tr>
<th>Path</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zi→Z0</td>
<td>0.235</td>
</tr>
<tr>
<td>Zi→Z(i–2)</td>
<td>0.059</td>
</tr>
<tr>
<td>Zi→Z(i–1)</td>
<td>0.176</td>
</tr>
<tr>
<td>Zi→Z(i+1)</td>
<td>0.176</td>
</tr>
<tr>
<td>Zi→Z(i+2)</td>
<td>0.059</td>
</tr>
<tr>
<td>Zi→Zn</td>
<td>0.118</td>
</tr>
</tbody>
</table>

### disperseProb

- Dispersion probability across intracellular ZONES for DRUGS not in HME
- Dispersed DRUGS in HME present
  - Z0→Zn: 0.2
  - Zn→Z0: 0.8

### isaSubstrate

<table>
<thead>
<tr>
<th>Protein</th>
<th>CYP</th>
<th>PGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>true</td>
<td>true</td>
<td>true</td>
</tr>
</tbody>
</table>

### assocProb

<table>
<thead>
<tr>
<th>Protein</th>
<th>CYP</th>
<th>PGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>0.975</td>
<td>0.975</td>
<td></td>
</tr>
</tbody>
</table>

### releaseProb

<table>
<thead>
<tr>
<th>Protein</th>
<th>CYP</th>
<th>PGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

### sitesN

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Neighbor size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP</td>
<td>30</td>
</tr>
<tr>
<td>PGP</td>
<td>60</td>
</tr>
</tbody>
</table>

### maxSites

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Maximum capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP</td>
<td>1</td>
</tr>
<tr>
<td>PGP</td>
<td>10</td>
</tr>
</tbody>
</table>

### efficiencyProb

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CYP</th>
<th>PGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

### randomizeOrder

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Randomize order of activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>true</td>
<td></td>
</tr>
</tbody>
</table>

### a2bDirection

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dosing compartment: S1 = apical, S5 = basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 or S5</td>
<td></td>
</tr>
</tbody>
</table>

### numSolute

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dose: number of SAQS (*1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 4, 8</td>
<td>1000</td>
</tr>
<tr>
<td>simulationStep</td>
<td>Number of cycles to run</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

1 Parameters for M7 and M1 are the same as those of SAQ except for the following:

Lower \( \log P \), lower \( MW \), lower \( assocProb \) to CYP and PGP, and lower \( releaseProb \) from CYP and PGP

§ Parameter values different between control and inhibitor-treated experiments
Fig. 1

A Conventional Induced Transport Model

Simulation Output
Algorithm Execution

Induced Mechanistic Description

Conceived Equations

Conceptual Mapping

Mechanism: Drug-Cell Component Interaction

In Vitro Transport Experiments

Measured Transport Data
Effects: Systemic Phenomena

Hypothesized Realistic Mapping

ISECC Experiments

Measured ISECC Data

ISECC Events

Hypothesized

Mechanism: Drug Object-ISECC Component Interactions

ISECC Parameter Values

B Conditions More Conducive to and/or Supportive of:

- inductive/deductive reasoning
- abductive reasoning
- synthetic M&S
- grounding to metric spaces
- internal, relational grounding
- precise predictions
- discovering plausible mechanisms

Imprecise Measures/Uncertainty

Precise

High

Low

System Information

Complete

Detailed

Fine-grained, generative knowledge of mechanistic details

Observation/Phenomena

Expected

Predictable

Surprising, Puzzling, New
Objective: Discover mechanisms that enable measures of ISECC \( S_{AQ} \) transport to achieve most stringent SM

1. Stage 1: The initial set of targeted transport data at is a small subset

2. Select granularity level that will enable comparing measures of simulated and targeted attributes

3. For each transport condition, specify a desired level of transport similarity (e.g., 75% of measures within a factor of 2). Approach in stages: begin with relaxed measures of a few target attributes; increase stringency by narrowing target range and/or number of measurements in target range.

4. Posit coarse-grained, discrete, partial mechanisms for which transport measures mimic some key target measures, while requiring as few components (number and variety) as is reasonable

5. Provide specifications. Create logic to be used by each component. Instantiate components and mechanisms

6. Conduct many, simulation experiments. Measure \( S_{AQ} \) transport to establish in silico–in vitro transport similarity, and lack thereof

7. Tune to achieve ISECC similarity specified at step 3. Effort fails: return to step 4. When successful, return to step 3, move to next Stage and increase the stringency of the Similarity Measure

8a. Add one or more referent experimental conditions until the Stage 1 ISECC is falsified. Return to step 2. Strive to match new wet-lab results with as little ISECC reengineering as possible

8b. Alternatively or in conjunction, undertake wet-lab experiments to challenge and possibly falsify the surviving mechanistic theories.

Fig. 2

Achieving Specified Similarity Measure

<table>
<thead>
<tr>
<th>Size of Parameter Space</th>
<th>Number of Attributes Targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Stage 3 ISECC</td>
</tr>
</tbody>
</table>
Fig. 5

Saquinavir in Cells

5 μM = 1000 SAQ
10 μM = 2000 SAQ

Target Range

In Vitro

Fraction of Dose

Apical Basal A2B+L Y B2A/L Y

20 μM = 4000 SAQ
40 μM = 8000 SAQ

ISECC
Fig. 6

Total M7

Fraction of Dose

Limit of Detection (LOD)

ISECC

5 μM = 1000 SAQ

10 μM = 2000 SAQ

50 μM = 8000 SAQ

Apical, Basal, A2B+LY, B2A+LY

Target Range

In Vitro

LOD
Appendix

**Inductive, deductive, and abductive reasoning.** Conditions supportive of all three reasoning methods are sketched in Fig. 1B. **Induction** is arrival at a conjecture (universal conclusion) based on a pattern observed in many particular cases. Induction begins with the measurement of a collection of objects. It is fundamentally and foremost a method for studying phenomena, not mechanism. The data from which a pattern is induced is a statement *solely* about the phenomena for which the measure was designed. Hence, every data set, and subsequently every inductive model, has embedded in it the aspects or usage protocols plus premises commensurate with the measures used to take the data. In general, a model induced from large sets of the same type data will be more precise, more specific, and more suitable for prediction. On the other hand, a model induced from lots of variant data (measures of different phenotypic attributes) will be less precise but more resilient and more general; its predictions will reflect greater uncertainty. In the former context, one has to worry most about over-fitting data, which makes the model too specific to a single data set to be useful. At the same time, one has to worry about inducing a model that is too general and misses crucial patterns in the data.

Often, the modeler is also interested in the generative mechanisms responsible for the data. In those cases, features of idealized, conceptual constructs (a feedback circuit, a two-compartment model, etc.) can provide mechanistic insight. This is particularly true when the mathematical description of an idealized measure of a construct feature (given a set of premises) is known to have a form that is the same (or nearly the same) as the induced mathematical description (a sum of exponentials, for example). How well the parameterized features of the conceptual construct map to components and features of the referent, along with the acceptability of the premises, are separate issues outside the current scope. The initially induced mathematical description is prosaically expanded to include the conceptual features, but remains a hypothesis about patterns in the data.
Deduction is automatic and/or mechanical transformation of a set of statements. It is the purely mechanical (syntactic) transformation of the premises to a conclusion. As such, no meaning (semantics) need exist for deductive systems. All executing computer programs (absent human or real-world interactions) are deductive systems. Likewise, mathematical transformations are also deductive systems. The most fundamental element of deduction is the engine that actually makes the transformation. In the case of a computer program, the instruction pointer provides the impetus for transformation. In mathematics, the engine is the human manipulating the symbols. A simulation is an operating, deductive system designed to mimic the behavior of some referent. Its alphabet and grammar are specified in part by the language in which the program is written and in part by the constructs the programmer creates. The premises are statements about the initial conditions of the program. The conclusions are statements about the final conditions of the program. The conclusions (of interest) become the outputs or “behaviors” of the simulation, but no new knowledge can be created. Any meaning applied to the premises, grammar, or conclusions are inferred by those examining the program and simulation, and so remain conceptual and hypothetical.

Abduction is arrival at a conjecture based on a pattern observed in one or a few particular cases. Abduction is conceptualizing (multiple) mechanisms: explanations that, if true, could account for or generate a similar anomalous, interesting, or surprising observation. Abductive inference involves hypothesis generation and selection, and is an important occurrence during wet-lab research. Abduction is most likely and appropriate for ambiguous systems (the left side of Fig. 1B). The task of resolving the ambiguity is best approached through multiple aspects and with multiple mechanistic hypotheses measured with multiple measures.

To illustrate abductive inference, consider the following situation: measurements of an experimental treatment group exhibit unexpected values when compared to those of control groups and data from past experiments. Further, the results do not fit well with known categories of similar phenomena. In such a situation, researchers offer many speculative, candidate explanations: were this condition or circumstance true, it could explain the anomalous or new observations. Some explanations may focus on material used
in the experiment (a possible bad batch of reagent, etc.). Other explanations may focus on the conduct of the experiment (the samples may have been mishandled, etc). Others are ideas about mechanistic explanations. Generation of varied explanatory hypotheses, some highly speculative, following the observation of the anomalous behavior is part of abduction. The next phase involves a process that narrows the competing ideas to those deemed most plausible. Following abduction, the consequences of these hypotheses are logically or experimentally deduced, and then evaluated using induction. After testing, when the set of plausible hypotheses is dramatically reduced, those remaining represent the current best explanation(s) until some new observation falsifies one or more of them. At that stage, the entire scientific reasoning cycle may repeat itself.

The above cycle (Fig. 2) occurs frequently when engineering and refining a synthetic analogue, like ISECCs. The behaviors of the first implementation often fall short, frequently far short of expectations, even though it is the modeler’s best hypothesis about how components should plug together to obtain the targeted phenomena. That shortfall falsifies the best hypothesis. The modeler has learned that the selected region of mechanism space is too abiotic. The modeler must rethink plausible micro-mechanisms. The solution is to jump to another region of mechanism space and experiment to determine if the new micro-mechanisms produce phenomena that are more similar to targeted phenomena. So doing exercises creativity. When improvements are seen, the modeler can conjecture that the new micro-mechanisms are more biotic. Each failed cycle exercises thinking creatively about plausible mechanisms. Each improvement in the similarity of the analogue’s behavior to that of the referent adds new knowledge and improves insight into referent mechanisms. Both failed and fruitful cycles are often characterized by abductive reasoning.

Like induction, abduction starts with a measure selected by the researcher. As such, the hypothetical mechanisms inferred (current beliefs) are inherently and irrevocably dependent upon the measure through which the phenomena are defined and revealed. They are aspect and perspective dependent. Change aspect and/or perspective and the hypothetical mechanisms inferred may change. Unlike induction, however, abduction does not necessarily produce (overly) precise or (overly) general mechanistic explanations.
More often, as with ISECCs, they are specific to the observation or experiment and its context. That is because the focus of abduction is on the current few interesting cases. The hypotheses (explanatory models) must be elaborated through deduction followed by validation through induction in order to learn how precise or general each hypothetical mechanism actually is. For that reason, abduction preserves ignorance, in contrast to the truth preservation of deduction. The researcher is just as ignorant after abducting an explanation as before.

Figure S1. Overview of eighteen ISECCs that were thoroughly explored and eventually falsified before achieving the targeted Stage 4 validation criteria. A series of 18 ISECCs were assembled and tested. Analogues meeting the specification of the Stages are noted. At each step, the parameter space was extensively explored until an observable improvement in similarity was achieved. Key parameter adjustments, rule changes and/or component added are noted for each steps. Branching occurred when changes initially improved similarity but later failed to validate with additional modifications.
Figure S2. Internal logic used each cycle by PGPS (A) and CYPS (B). The internal logic of PGP and CYP is similar. First, each decides whether it can bind a COMPOUND within its local neighborhood (neighborhood is randomly selected and its size is specified by siteN). 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 or when active site is full. A: A PGP 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 < 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 < efficiencyProb it is METABOLIZED. If PRN < releaseProb, the bound object may be released.