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Mechanistic Simulations Explain Paradoxical Saquinavir Metabolism During In Vitro Vectorial Transport Study

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***Abstract*—We were confronted by an unexpected observation: in a Transwell study of confluent, Cyp3A4 and P-gp-expressing Caco-2 cells, *higher* intracellular saquinavir levels, yet *less* first metabolite (M7) formation were observed following apical dosing, compared to basal dosing [1]. To test two seemingly plausible hypothesized explanations, we constructed an in silico working analogue using the synthetic method. Neither mechanism alone was sufficient, but when combined and tuned within the analogue, the results generated were a semi-quantitative match to the experimental data. After 60 cycles, *more* of the simulated dose was present within analogue cells as parent drug after apical dosing. Furthermore, *less* M7 was present after apical dose. The paradox disappeared by having simulated drugs equilibrate among separate intracellular zones. Building, studying, and exploring mechanistic explanations for complex wet-lab phenomena using the new methods improved insight into the referent system, while providing a straightforward, scientific means of testing the plausibility of mechanistic hypotheses.**

I. Introduction

To enhance our understanding and interpretation of laboratory data, we need improved insight and knowledge of mechanistic details from a system perspective. In support of achieving that goal, we constructed an in silico analogue of a Transwell system to facilitate discovery and verification of plausible, spatiotemporal mechanistic explanations for data collected during in vitro vectorial transport studies. When an objective is improved understanding of mechanisms underlying pharmacokinetic (PK) phenomena, or testing hypotheses about proposed mechanistic explanations, the synthetic method of modeling and simulation offers advantages over traditional methods [2]—[8]. Traditional PK modeling methods induce models that enable one to test hypotheses about the data and its time course. Addressing the problem described below called for application of a new class of mechanistic, discrete, in silico biological system analogues [2]—[8]. The new approach enabled constructing a working analogue of hypothesized mechanisms. Independent, autonomous components were plugged together to construct an analogue that, for the level of abstraction specified, was consistent with current knowledge. Each component in its spatial context mapped to specific biological components. Measurable attributes of these mechanistically realistic analogues can overlap attributes of their referent systems. Each working analogue that has a different parameterization is an implementation of the hypothesized mechanism; executing the analogue is the mechanism in action. In this report, we used these analogues to test the plausibility of mechanistic hypotheses.

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II. Purpose

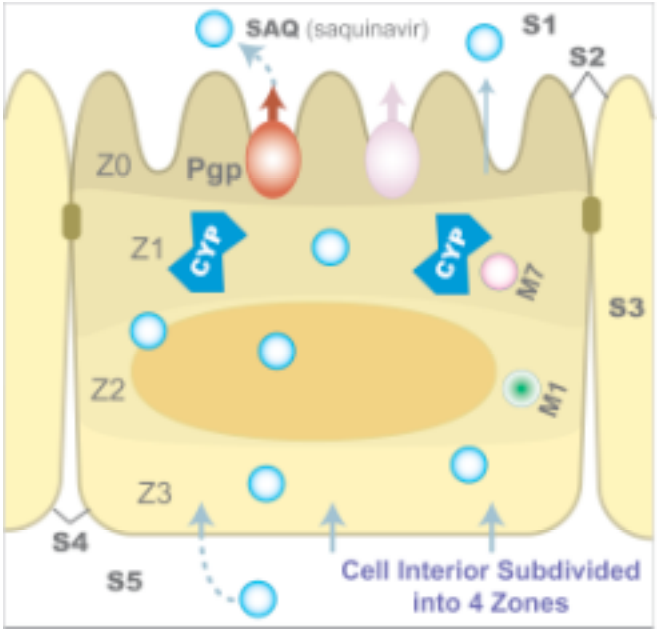
Mouly *et al*., [1] reported unexpected, paradoxical observations in their vectorial transport study with saquinavir: apical dosing produced higher saquinavir intracellular levels, yet lower formation of first metabolite (M7) compared to basal dosing. They offered two scenarios: 1) after basal dosing, an intracellular gradient caused a higher local saquinavir concentration within the enzyme's microenvironment and 2) secondary metabolism of M7 to M1 [9], [10], which was not assayed. To date it has been problematic to determine the plausibility of such mechanistic explanations. We used the analogues to challenge those hypotheses, and thereby achieve greater insight into the referent biological system. By implementing an analogue reflecting their hypothesis and executing an in silico experiment (running a simulation) we could compare measures of simulated dynamics to corresponding wet-lab data, and thereby test the plausibility of a mechanistic hypothesis [2]—[8].

III. Methods

A. Basic Setup

To clearly distinguish in silico components and processes from their biological counterparts, we use SMALL CAPS to denote the former. We built in silico analogues of Transwell devices containing confluent monolayers of Cyp3A4 and P-gp-expressing Caco-2 cells. We began by discretizing time and physical space. Continuous time in the referent is divided into discrete intervals: simulation cycles. The mapping between a simulation cycle and referent time was selected (specified) after satisfaction was achieved with all aspects of analogue dynamics. That mapping is one simulation cycle corresponds to three minutes. Continuous physical space in the referent maps to SPACES and ELEMENTS in the analogue. As illustrated in Fig. 1, each analogue consists of five SPACES: apical COMPARTMENT (S1), apical MEMBRANE (S2), CELL INTERIOR (S3), basal MEMBRANE (S4), and basal COMPARTMENT (S5). Each SPACE contains 2,500 identical ELEMENTS, representing portions of respective medium or cellular content. To represent Cyp3A4 enzymes and P-gp efflux transporters, 20 ENZYMES (CYPS) and 100 TRANSPORTERS (PGPS), were placed randomly in S3 and S2 ELEMENTS, respectively. Each has been validated in similar contexts [2], [3], [5]. CYP and PGP, diagrammed in Fig 2, are autonomous agents, each following their own internal logic. Briefly, CYP examines its neighboring ELEMENTS. The probability that a CYP-DRUG binding event will occur for any DRUG within a CYP'S neighborhood is governed by a parameter, *assocProb* (Table 1). Following

that, each CYP has an opportunity to METABOLIZE a bound DRUG. The frequency at which METABOLISM occurs is controlled by the tunable parameter (METABOLIC) *efficiencyProb*. Only substrates are METABOLIZED. After that, each CYP examines each bound DRUG; each then has an opportunity to be RELEASED within that same cycle. The latter process is governed by another parameter, *releaseProb*. In most cases when the DRUG is a substrate, the released object is the respective metabolite. PGP has a very similar internal logic, having the same binding and release steps, but no metabolism step (Fig. 2). Only substrates are TRANSPORTED. For simplicity, PGP always TRANSPORT bound substrate from origin SPACE to destination SPACE; its (TRANSPORT) *efficiencyProb* is 1.0. At the start of a simulation, 1,000 DRUG objects representing saquinavir (SAQ) were added to one or the other EXTRACELLULAR compartment (S1 or S5). DRUGS are autonomous agents. Each follows the same three steps during a simulation cycle: 1) lateral relocation into neighboring ELEMENTS, 2) vertical dispersion across ZONES (below) if the DRUG is INTRACELLULAR, and 3) passive transition between SPACES. From the dosing compartment, they enter the CELL INTERIOR using a validated [2], [3], [5] passive transition mechanism: the probability of transiting from one space to another is first estimated from physicochemical models and then tuned. They are listed in Table 1. The basic analogue representing the Transwell device was then complete.



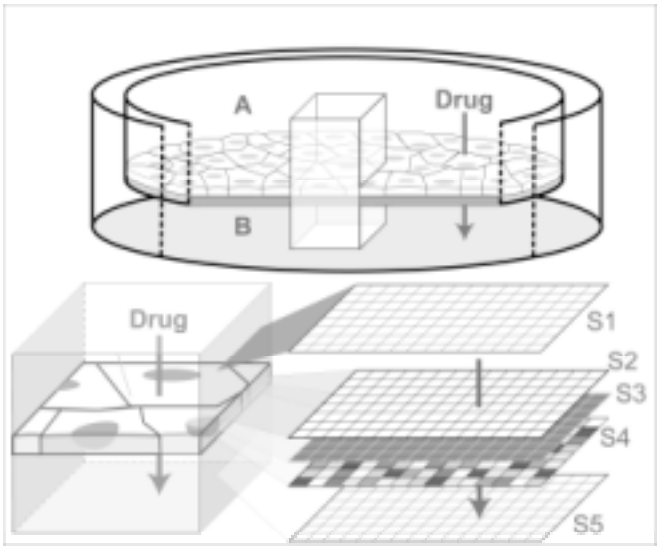


Fig 1: **Top**: Schematic of a Cyp3A4 and P-gp-expressing Caco-2 cell within the Transwell devices illustrated in the lower diagram. The relative locations of the simulated intracellular zones (Z0–Z3) are shown. Placing drug on one side or the other of the cells (chamber A, simulated by Space S1, or B, simulated by Space S5) enables study of vectorial transport *in vitro*; the same is done during an in silico experiment. The **bottom** schematic illustration shows both a Transwell device and the analogue system. The analogue is consisted of five spaces, each representing the indicated portions of confluent Caco-2 cells within a Transwell device. The differ-

Fig 2. Internal logic diagram for PGP (**A**) and CYP (**B**).

B. *Implementing Hypotheses*

To implement the proposed hypotheses, we added additional components to the above, basic analogue. To represent secondary metabolism, first metabolite of saquinavir (M7) is also designated to be a substrate of CYP, and is further METABOLIZED to M1 by CYP. To represent the gradient effect, the following two implementations are needed. First, the CELL INTERIOR is further divided into four INTRACELLULAR ZONES, Z0_Z3, ordered apical to basal side. DRUGS disperse across ZONES during each simulation cycle, according to probabilities listed in Table 1. Second, PGP and CYP may only act upon DRUGS located only within their respective ZONES: PGP only EXPORT DRUGS in Z0 to S1; CYP only METABOLIZE DRUGS (SAQ or M7) in Z1. The capability of modeling, and observing, the zonal spatiotemporal details required for this mechanistic explanation is a clear advantage of our modeling approach over traditional equation based modeling.

TABLE I
PARAMETERS OF THE MODEL

Parameters	Remarks		Values
systemSize	Size of SPACE S1–S5		50x50
numSolute	Dose: number of SAQS		1,000
numPgps[a]	Number of PGP		100
numCyps[a]	Number of CYP		20
transitProb[a]	Trans-membrane transit probability between SPACES	S1→S2 [b]	0.015
		S2→S1	0.417
		S2→S3	0.417
		S3→S2 [b]	0.007
		S3→S4 [b]	0.007
		S4→S3	0.417
		S4→S5	0.417
		S5→S4 [b]	0.007
disperseProb	Dispersion probability across ZONES	Z0→Z1	0.222
		Z0→Z2	0.111
		Z0→Z3	0.0
		Z1→Z2	0.222
		Z1→Z3	0.111
		Z1→Z0	0.333
		Z2→Z3	0.333
		Z2→Z0	0.111
		Z2→Z1	0.222
		Z3→Z0	0.0
		Z3→Z1	0.111
		Z3→Z2	0.222
assocProb[a]	reflects binding affinity to protein	CYP	0.2
		PGP	0.7
releaseProb[a]	reflects dissociation from protein	CYP	0.1
		PGP	1.0
efficiencyProb[a]	efficiency parameter for CYP and PGP	CYP	0.9
		PGP	1.0

[a]These parameters are tuned to produce results shown in Fig 3.
[b]The aqueous to membrane *transitProb* are much smaller than membrane to aqueous *transitProb* because we assumed that only a small fraction of DRUG in aqueous space is sufficiently close to membrane interface to partition into the membrane within the simulation cycle.

C. Target Attributes

Parameters listed in Table 1 were tuned until all three target attributes were met. The target attributes were: 1) results showed a qualitative trend consistent with reported saquinavir data: paradoxically, *more* intracellular SAQ, but *lower* M7 formation, after apical dosing, 2) simulated results were within 50-200% of reported data, and 3) simulated data eventually showed disappearance of paradoxical observations, consistent with observations in other studies. Meeting all three target attributes signified an acceptable level of similarity between simulated results and experimental data, and allowed us to posit that our analogue is a mechanistic realistic representative of the referent system.

D. Software

We coded in Java Swam and used the Swarm platform and libraries (http://swarm.org). Because it is relatively easy to join, replace, modify and disconnect components independent of each other, and that the components rely only on local component interactions, we were able to reuse many of the validated components and modules used in previous studies in [2], [3] and [5]. Most experiments used a single processor and ran under Microsoft Windows XP with Java SDK and J2RE installed. Output data files were processed, graphed and analyzed with Microsoft Excel. All pseudo-random number generation used Swarm's Mersenne Twister algorithm; the initial seed was extracted from the machine's clock. All pseudo-random numbers (PRN) were drawn from uniform distributions. Results are reported as arithmetic mean values of seven, repeat simulation experiments. We assumed that the central limit theorem held for all observations. Simulations ran for 120 cycles (6 HRS); SAQ, M7, and M1 were measured in each SPACE every 20 cycles.

IV. RESULTS

Both hypotheses were plausible. Neither hypothesis alone appeared sufficient to account for the observations from separate simulations (data not shown.) However, when combined, the results (Fig. 3) were a semi-quantitative match to the experimental data: together they provided a plausible explanation for the unexpected observations. After 60 cycles, more of the DOSE was present in S3 as SAQ after apical dosing, compared to basal dosing. Yet, M7 accounted for less of the DOSE after apical, compared to basal dosing.

There was *more* parent saquinavir, but *less* first metabolite after apical dosing: exactly as Mouly *et al.* reported. Importantly, because of the transparency of simulation, we discovered that the gradient effect was causing the paradox – at times before 60 simulation cycles, we observed a higher zonal concentration from the side of dosing to a lower zonal concentration to the side of receiving. The gradient diminished as DRUGS equilibrated among INTRACELLULAR ZONES, and thus the paradox disappeared: after 120 cycles, apical

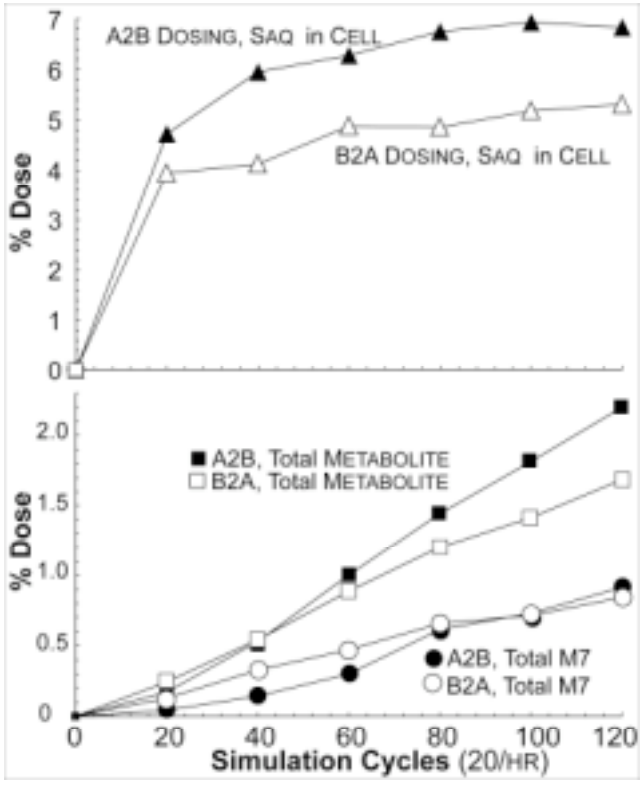


Fig 3. Simulated data showing the paradox (60 cycles) and its disappearance (120 cycles). Time course for (**A**) percent of DOSE present as SAQ INTRACELLULARLY (in S2 + S3 + S4), and (**B**) Percent of DOSE present as METABOLITE in all SPACES combined.

dosing produced a higher INTRACELLULAR SAQ level, as well as higher M7 and total METABOLITE, compared to basal dosing. Secondary metabolism also contributed to the paradox: at 60 cycles, apical dosing produced more total METABOLITES despite lower formation of M7. It is also important to note that after 120 simulation cycles, our results were qualitatively similar to two other similar vectorial studies involving indinavir [11] and cyclosporine [12].

V. DISCUSSION

In this study we demonstrated the ability of our analogues to challenge the mechanistic explanations proposed in [1]: we implemented the hypotheses, tested them by executing the simulation, compared to experimental data, and concluded that the hypotheses were plausible explanations to the observed paradox. This important capability was the motivation for using this class of models, rather than drawing on traditional inductive modeling methods.

We also demonstrated new capabilities offered by our synthetic modeling methods:

- Reusability: we reused components that were validated previously, and reconfigured them for the present study without significant re-engineering. This is possible because the components articulate and use only local mechanism.
- Transparency: simulation details, such as zonal concentrations, are measurable and visualizable. This enables us to witness whether a particular mechanistic intervention is causing a particular observation.
- Spatiotemporal details: we are able to represent spatial and temporal heterogeneity without significantly increasing complicacy or requiring higher-resolution observations.
- Informative: when simulated observations are acceptably similar to experimental data, the analogue provides insights to the biological referent; components and mechanisms in the analogue may have their biological counterparts.

The analogue used is an example of what has been referred to as executable biology [13]. We refer to it as the synthetic method. The method has limitations [4], [14]. For example, compared to inducing equations from observed data, using this method of building and testing in silico mechanisms requires more time, resources, and detailed knowledge about the referent system. It also requires more in silico experimentation to tune parameters so that the analogue produces acceptably similar observations. As the analogue becomes more complicated, acceptable mechanisms are not unique: two or more mechanisms may be found to be acceptable. Importantly, in this report, we do not conclude definitively that the proposed mechanisms caused the paradox. Rather, we showed that they were plausible mechanisms. There may be additional explanations that prove plausible. Further, for a given mechanism, acceptable parameterizations are neither unique nor precise.

Taken together, our modeling and simulation approach complements existing equation-based strategies and offers important, novel capabilities [4], [13].

VI. CONCLUSION

We tested the hypotheses proposed by Mouly *et al*. [1] and found both working together are plausible causes of the paradoxical observation. Achieving the three, targeted attributes have enabled us to assert that the mechanisms implemented in our analogue have biological counterparts in the referent Caco-2 Transwell system. Building, studying, and exploring mechanistic explanations for complex wet-lab phenomena using the above new methods improved insight into the referent system, while providing a straightforward, scientific means of testing the plausibility of mechanistic hypotheses.

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