Zonation of Drug-induced, Hepatic Enzyme Induction: Falsifying Fine Grained Mechanisms within a Validated, Multiscale In Silico Liver

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Abstract

The focus is a previously reported In Silico Liver (ISL) model family, which is an iteratively developed suite of hypotheses about how rat livers function. We implement a medium-grained Enzyme Induction mechanism that is intended to validate against the coarse grained measure of drug fraction in perfusate during single-pass perfusions. It is falsified against a medium grained measure of hepatic zonation. The validation (falsification) of complicated, knowledge-based models requires validation of multiple aspects. Multi-aspect models require methods for integrating distinct aspects. However, such integration may not be straightforward, especially with multiscale, multi-frequency, and/or hierarchical models like ISLs. Falsification (validation) is crucial to the use of ISLs for the iterative process of formulating, testing, and evolving hypotheses about liver mechanisms. Multi-aspect falsification presents the interesting opportunity to falsify a hypothesis in one aspect while simultaneously validating it in another aspect. We provide an example. The ability to scale validation efforts is necessary for effective scientific use of knowledge-based computational models such as ISLs. We demonstrate one such multi-scalar validation/falsification event as part of iterative ISL evolution. We provide a discussion of how the falsification guides the refinement of the hypothesis represented by the simulation.

1. INTRODUCTION

The In Silico Liver (ISL) is part of a proof of principle project demonstrating exploratory experimental methods on synthetic, computational analogs. The project explores concrete hypotheses for potential wet-lab experiments on rat livers, the analog's referent. Liver was chosen as referent because of its complex phenotype, which at some scales and in some aspects, is well behaved and well characterized using population modeling methods like differential equations. At other scales and in other aspects, livers can be complex without any feasible modeling methods. Aspect is defined as the perspective taken when an analogue is observed (including the phenomena on which we focus); one of many functional effects that result and can be observed when an analogue executes.

Agent-based modeling was chosen as the base ISL platform because it is an extensible method that facilitates multi-models across all spectra: homogenous to heterogeneous, continuous to discrete, shallow to deep, regular to chaotic, and across all representation paradigms including equation-based, rule-based, cellular, lattice-free, etc.

Liver is an excellent demonstration referent for hierarchical, multiscale modeling. Hepatic tissue consists of many nearly polyhedral lobules packed together to form secondary units. The lobules, in turn, consist of vascular tubes (sinusoids) lined with endothelial cells, through which blood flows from exterior portal ducts to a central venule. Some solute in blood filters through the endothelial layer into the surrounding Space of Disse, where it contacts and can enter hepatocytes. Various enzymes within hepatocytes bind and metabolize endogenous and exogenous compounds. Liver plays essential roles in the organism and exhibits a broad phenotype at several scales.

2. USE CASE

The target of this study is hepatocytes' ability to regulate (up or down) their enzymes in response to encountered compounds and other physiological signals [1][2][3]. The use case is the experimental protocol for a single-pass, in situ, isolated perfused rat liver. The liver is perfused with an oxygenated fluid. A bolus of compound is added to the perfusate. A fraction collector protocol is used to measure perfusate contents as it flows out of the liver. This use case exercises many of the steady state and fast transient aspects of hepatic function. It was used to study the in situ clearance of cationic drugs [4]. That study's results provide the coarse-grained validation aspect on which we focus: the fraction of compound exiting in relation to the amount of compound in the bolus. Validation of various ISLs against data from the cited experiments is presented in several previous reports [5][6][7][8][9][10].

The output fraction is a cumulative response based on the topological, geometrical, and biochemical attributes of the liver tissue, making it an excellent baseline aspect

against which to validate. The output fraction aspect provides the ISL project with a whole organ regression test to guide the development of refined, falsifiable, mechanistic hypotheses. More refined hypotheses have been validated in the contexts of intrinsic clearance [11] and diseased livers [8][9][10] and falsified in the distribution of enzyme induction [12]. The influential mechanisms reveal themselves in different aspects of ISL phenotype. Consequently, specific measures had to be designed to take observations from ISLs during simulations. For example, in [8][9][10], tracing instrumentation was added to the ISL code to take very specific measurements of compounds (which refer to drug, the extracellular marker sucrose, and metabolite in [4]) at different locations within the ISL lobule at any given time. Obviously, such aspects are easier to measure during simulations than during liver perfusion experiments, if the latter is even feasible. It is important to note that adding a new measure, or changing a measure usually changes the wet-lab use case, but it need not for the analog. This point is made clearly with [11] where intrinsic clearance is measured with an entirely different use case (experimental apparatus and protocol) than in situ clearance.

The aspect and measure investigated here is enzyme count in each zone following each simulation cycle. Because of the way the mechanism was constructed, current enzyme count is a function of past compound and enzyme counts, and the metabolic events in each hepatocyte. Recording these counts leaves the ISL use case the same. However, the hepatic validation data is from in vivo experiments as opposed to in situ experiments. Further, the livers from which this data are taken are entirely different from those that generated the output fraction data. Because the ISL is composed of discrete software objects, it allows (indeed facilitates) individual measures to be similar to those used by the wet-lab experiments and it allows the composition of those measures. The composition of the mechanism produces an articulated structure where the inputs and outputs of each component can be traced and measured in a variety of ways that mimic wet-lab measures. ISL measures are kept programmatically separate and are applied as part of the experimental procedure, again mimicking the experimental wet-lab methods.

Another way to view the protocol is that each component, composite or atomic, presents its phenotype to the other components with which it is composed. So doing allows the hierarchical generation of coarse-grained phenomena from fine-grained phenomena. The structure keeps the hierarchy explicit, which is necessary to mitigate the fact that the validation data is from entirely different experimental subjects, protocols, and use cases. Maintaining, curating, and reasoning over measure and aspect composition, over and above mechanism composition is a primary purpose behind in silico methods.

3. APPROACH

The work described herein is a further iteration of the ISL and the iterative experimental protocol we have been developing. The project context is important because, although falsification is critical to scientific progress, it is perceived more negatively than validation and therefore rare in the literature. When the object of experimentation is the actual biology and the analogs are used to support (hopefully) generalizing a rule into a law, which is often the case when wet-lab experiments, the negative view of falsification and the positive view of validation is warranted. However, as we specialize and begin studying the more useful ISLs directly, as in [8][9][10], to learn how well they may (or not) represent aspects of real livers, the bias toward only publishing validation becomes a problem, because only the end results of expert toil and iteration get published. The knowledge and modeling method required to repeat the unpublished path, from start to finish, are hidden. Mathematics provides a good analogy. Math textbooks are notorious for a dry, theorem-proof presentation format, ignoring all the creative, intuitive, mathematical thinking and understanding that guided the mathematicians toward the formal proofs.

In silico methods enable a research domain to expand so that some research specializes into direct study of the liver in different experimental and intervention contexts, and other research focusing directly on studying the computational construct, the simulation itself. This specialization process leads to an ironic scientific dilemma for computational biology, where the work that most needs documenting, i.e. the methods and mechanisms tried and failed, are often not documented; they lie hidden in laboratory notebooks or computer records, unavailable to those who might learn from them. Further, the new knowledge that some ISL features and mechanisms were falsified is not shared. In [13] we documented several of the methods and mechanisms that were tried but failed to help document their importance. When such information is missing, it becomes increasingly more difficult to learn from the work that is published. Published, validated results must either be reverse engineered to extract the methods used to achieve success or each researcher must rediscover the exploratory process. Both slow overall progress by limiting the ability to build upon the prior work of others. Both the concept of concrete analogs as falsifiable hypotheses and the documentation of falsifications are core to advancing computational biology toward scientifically useful virtual cells, tissues, and organisms [14].

It is noteworthy that a new journal The All Results Journals: Biol (ISSN: 2172-4784) is now publishing biomedical research papers that include or focus on negative wet-lab results.

This enzyme induction (EI) sub-project follows the work in [11][12][15][16]. We focus on common patterns of EI but not specific drug metabolizing enzymes. In

[8][9][10], we take two coarse-grained, validated, aspects and compare observed changes in tracing measures when mechanism parameter values were changed. The results provide not only falsifiable hypotheses for how each generates its (normal or diseased) phenomena, but also hypotheses for the mapping between the (normal vs. diseased) mechanisms. The coarse-grained output profile is refined into measures for finer-grained phenomena exhibited by ISL components. By analogy, this refinement is similar to physical dynamics where a three state model, characterizing position in three dimensions, can be refined into a six or nine state model by considering orientation (roll, pitch, yaw) and the rates of change in position. In the absence of the ability to take multiscale data directly from a particular referent, the parallax between coarse-grained measures of normal and diseased ISLs plus the ability to compose the coarse-grained measures with finer-grained ones, allows us to tighten the set of plausible mechanisms beyond what is facilitated by uniscalar quantitative validation and/or forward-mapped qualitative validation.

The parallax method [17] is more general than the inter-individual example of normal vs. diseased livers. It also works for any aspect of the referent or analog system. Each system component exhibits some number of attributes and behaviors: its phenotype. Further, each aspect targets part of that phenotype. Our understanding of a component becomes more complete and accurate in proportion with the number of aspects of that component we measure and explain. The larger the set of distinct, component aspects exhibited, the more contexts in which that component will "survive" or act robustly. Component autonomy is an important limit or goal that can be approached by using an increasing number of distinct aspects targeted by specified use cases. Autonomous components that are well characterized are easier to validate and translate between contexts.

Because hepatocytes play central liver function roles, establishing the autonomy of hepatocytes used in the ISL is critical. In Silico Hepatocyte Cultures [ISHC] study hepatocytes from an entirely different use case, requiring different aspects and measures. Demonstrating that the same software implementation of hepatocytes analogs can perform robustly in ISHC and ISL contexts helps establish the autonomy and accuracy of the hepatocyte component.

Fine-grained tracing measures, such as those used in [8][9][10] are useful for selecting a subset of plausible, concrete hypothetical mechanisms. However, they are not driven by fine-grained quantitative validation data taken directly from experiments on livers. The 2D liver lobule cross-section in [16] (hereafter, 2D zonation analogue) takes validation data from the literature and constructs a model capable of reproducing them. However, in order to avoid inscription error, an abstract model is developed to help characterize non-local, intralobule enzyme expression patterns to augment the testing framework for the fine-grained model. So doing isolates feedback capable of showing the relationship between periportal and perivenous enzyme expression data, providing a reference model against which to compare more fine-grained mechanisms.

It is important to note that all ISL analogs have so far only modeled endocrine (secretions distributed in the body) and autocrine (secreted by a cell and acting on its surface) signals. However, the mechanistic abstraction enabling zonation within the 2D zonation analogue also allows paracrine, diffusive signals (secreted by a cell and acting on adjacent cells). The 2D zonation analogue also successfully mimics the inherently spatial or zonal nature of EI [2][3] exhibited in the validation data cited in [16].

In [12], we falsified the hypothesis that a uniform input into a heterogeneously composed ISL would produce uniform behavior at a finer scale. SS geometry and lobule network topology transform a uniformly distributed bolus of compound into a heterogeneous distribution of compound and EI. Such falsification demonstrates, in a domain specific context, the complex adaptive systems principle that the forward map from generators to phenomena is surjective (a function whose image is equal to its codomain) but not injective (a function that maps distinct arguments to distinct images), i.e. more than one mechanism can generate the same behavior. Methodologically, it also demonstrates the need for multi-aspect modeling.

The primary distinction between the 2D zonation analogue and the ISL lies in the irregularity of the lobule graph topology. This work strives to achieve a more concrete EI mechanism than that of the 2D zonation analogue in [16] yet a more abstract mechanism than that represented in [8][9][10][12]. It is an attempt to strike a middle ground. It is a hybrid (combined continuous and discrete) mechanism using the discretized continuous equations for R- and B-signals from [16].

4. METHODS

The ISL is described in detail in [10]. Briefly a lobule is represented by a directed graph of sinusoid segments (SSs) flowing from portal vein tracts (PV) to a central vein (CV). There are three zones from PV to CV. The periportal zone I has the most SS nodes (45) and significant intrazone edges (20). Zone II has fewer nodes (20) and fewer intrazone edges (10). The perivenous zone III has three nodes and 0 intrazone edges. Compound is injected into the PV, flows through the SS network and is collected and counted at the CV. Each SS consists of a concentric layering of three cylindrical grids wrapped around a core queue. The core conducts a laminar flow of perfusate (blood) along the length of the SS. The innermost Grid A models more turbulent and viscous flow along the endothelial lining of the sinusoid with a pseudo-random movement biased toward the SS outlet. Grid B is partially populated by endothelial cells into which the smaller compound objects (so-

lute) can partition, but which partially blocks lateral movement of marker solute. Solute that makes it past the endothelial layer enters Grid C, which models the Space of Disse and is partially populated by hepatocyte cells. Both cell types contain binder objects that can sequester compound for some number of simulation cycles. Hepatocytes contain [0-100] enzymes, which metabolize bound compound each iteration if a uniform pseudo-random draw is less than the number of enzymes divided by 100.

Within each hepatoctye, enzymes are induced and eliminated as a function of amounts of compound and enzymes present IQ_{tgt} iterations in the past. Further, enzymes are also eliminated as a function of the hepatocyte's distance from the PV, according to an gradient that increases along the maximal path length from PV to CV. Figure 1 is a visual description of the mechanism. Below is a precise description of how the mechanism works.

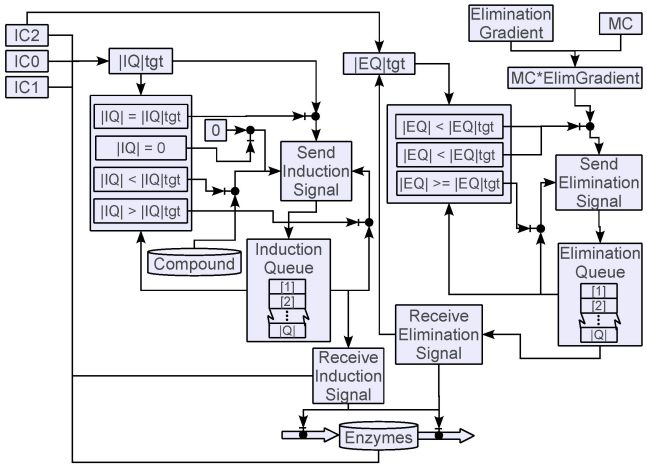

Figure 1. A hybrid diagram of the implemented EI mechanism. IC[0-2] are the induction constant parameters. Arrows indicate influence, positive or negative. Valves are turned on or off by their controller inputs where turning them on allows data to flow. IC[0-3], MC, and 0 are constants. Compound and enzymes are "stocks" that increase or decrease over time (the increase or decrease of compound is not controlled by the EI mechanism). The left side of the diagram represents induction and the right side indicates elimination, as indicated by flow control into and out of the enzyme stock. Walking through the induction sub-graph, IC[0-2] parameterize the calculation of the induction queue ($|IQ|_{tgt}$) size and the elimination queue ($|EQ|_{tgt}$). The target size for the induction queue is used to calculate the value installed on the induction queue. E.g., if the queue is smaller than its target size, compound number is used for the induction signal. If the queue is too large, it simply takes the next value on the queue. If the queue is empty, there is no signal. When the queue has reached its target size, the induction equation is used. Note that the induction signal can be negative, allowing it to raise or lower the stock of enzymes. A similar reading applies for the elimination sub-graph on the right side. Queues advance once within an experimental model cycle, twice within an experiment agent cycle.

4.1. Enzyme induction

$Enzymes^i \equiv$ *number of enzymes for the hepatocyte*

$$0 \leqslant Enzymes^i \leqslant E_{max}$$

$$E_{ratio} = EIC_0 + EIC_1 \cdot e^{zone}$$

$$E_{cycles} = IC_2$$

$$E_{init} = E^0_{induction} \cdot E_{cycles}$$

$$IQ^i_{tgt} = E^i_{induction} \cdot E_{cycles}$$

$$I^i_{signal} = IQ[0] (i.e.\ the\ 1st\ element \in the\ induction\ queue)$$

$$I^i_{schedule} = \begin{cases} push(0), & |IQ|^i = 0 \\ pop(), & |IQ|^i > IQ^i_{tgt} \\ push(Compound^i), & |IQ|^i < IQ^i_{tgt} \\ push(Compound^i), pop(), & |IQ|^i = IQ^i_{tgt} \end{cases}$$

$$E^i_{induction} = IC_0 \cdot I^i_{signal} - IC_1 \cdot Enzymes^i$$

The push() and pop() functions indicate value insertion onto the back and removal from the front of the queue, respectively. For example, when the size of the induction queue is zero, zero is pushed onto the back of the queue. When the queue size is larger than it should be, nothing is added and the value in front is removed.

4.2. Enzyme elimination

$maxpath \equiv$ *max. lobule path length*

$$M^{zone,\,max}_{signal} = (zone + 1)\frac{maxpath}{zones}$$

$$M^{zone,\,min}_{signal} = zone\frac{maxpath}{zones}$$

$$L_{SS} \equiv length\ of\ SS \in grid\ points$$

$$y \equiv position\ along\ the\ length\ of\ the\ SS,\ y < L_{SS}$$

$$M^y_{signal} = y\frac{M^{zone,\,max}_{signal} - M^{zone,\,min}_{signal}}{L_{SS} \cdot (zone + 1)}$$

$$M^u = EQ[0] (i.e.\ the\ 1st\ element \in the\ elimination\ queue)$$

$$MQ^i_{tgt} = M^i \cdot E_{cycles}$$

$$M^i_{schedule} = \begin{cases} push(MC \cdot M^y_{signal}), & |EQ|^i = 0 \\ pop(), & |EQ|^i > MQ^i_{tgt} \\ push(MC \cdot M^y_{signal}), & |EQ|^i < MQ^i_{tgt} \\ push(MC \cdot M^y_{signal}), pop(), & |EQ|^i = MQ^i_{tgt} \end{cases}$$

4.3. Intra-hepatocyte metabolism

$$e_{met} = \frac{(draw < Enzymes_i)}{E_{max}},$$

$$where\ draw \in Uniform^1_0, \forall \{iterate, hepatocyte, binder\}$$

4.4. ISL's multiple scales

ISL's generator-to-phenomena map consists of four integration types: 1) measures, 2) sequencing, 3) frequency, and 4) mechanism. This paper focuses on measure integration across scales. A brief description of the other three types of multiscale composition is warranted. There are three types of sequencing: discrete event (DE), discrete time

(DT), and DT implemented as periodic DE. Intracellular solute is bound stochastically and release is scheduled as a DE. All other DEs are scheduled periodically with a given frequency. All intra-model actions are synchronized to the same frequency. However, the experiment agent and some measures operate at lower frequencies. The EI equations are computed in terms of the current cycle interpreted as discretized continuous time. Mechanisms are composed to span seven scales: objects that map to molecules (binders, enzymes, and solute), cells, intra-sinusoidal spaces, sinusoidal segments (SS) and their connections, the lobule graph, the three model agents, and the experiment agent. While the lobule component of the ISL only spans five scales, the model and experiment agents are required to fully represent the experimental protocol (use case).

5. RESULTS

The coarse-grained outflow profile in Fig. 2 validates the same way as did previous ISL outflow profiles.

Figure 3 shows the middle-grain measure, enzyme count and change in enzyme count per zone over the course of 100 seconds. The final enzyme count in each zone ($2.5x10^6$, $1.1x10^6$, $1.9x10^5$) is proportional to the number of hepatocytes (~35,325, ~18,531, ~2,861) in the three zones. Because the numbers are large, any dynamism in the induction and elimination is lost in the enzyme count measure as it approaches the asymptote. The derived time difference measure shows that dynamism more clearly, especially in zone III where there are fewer hepatocytes.

The first validation target requires that zone III (perivenous) exhibit more induction than zones I or II. That did not occur. The Fig. 3 results clearly falsify the current mechanism. This result is not isolated. Full parameter sweeps were executed over generous ranges for all relevant parameters. All results approached stable enzyme counts with an EI distribution correlated to the distribution of hepatocytes. Some parameter settings caused rapid elimination of all enzymes; some set initial conditions above the asymptote, etc. None showed the classical higher zone III induction exhibited by in vivo experiments.

6. FAILURE ANALYSIS

The above falsification provides new knowledge. Something in the ISL and/or the mechanistic hypothesis is flawed. However, identifying the flaw is complicated by the fact that the model is validated at the coarse grain, albeit with unspecified EI mechanisms, and that a different mechanism implementation validates at the middle grain in [16]. Several potential sources are discussed below.

6.1. Search strategy

ISL phenotype was explored automatically with coarse parameter sweeps in all metabolism and induction parameters. Although the sweeps covered a reasonably large sample of parameter space, the choice of initialization functions (path length based elimination rate, 100 enzymes per hepatocyte, etc.) may have placed the ISL in the same region of the codomain. Although a broader, more computationally expensive search may yield a validating parameterization, the biological significance of such an outcome would be questionable since the functions and parameterization currently in place map to broadly accepted conceptual models of

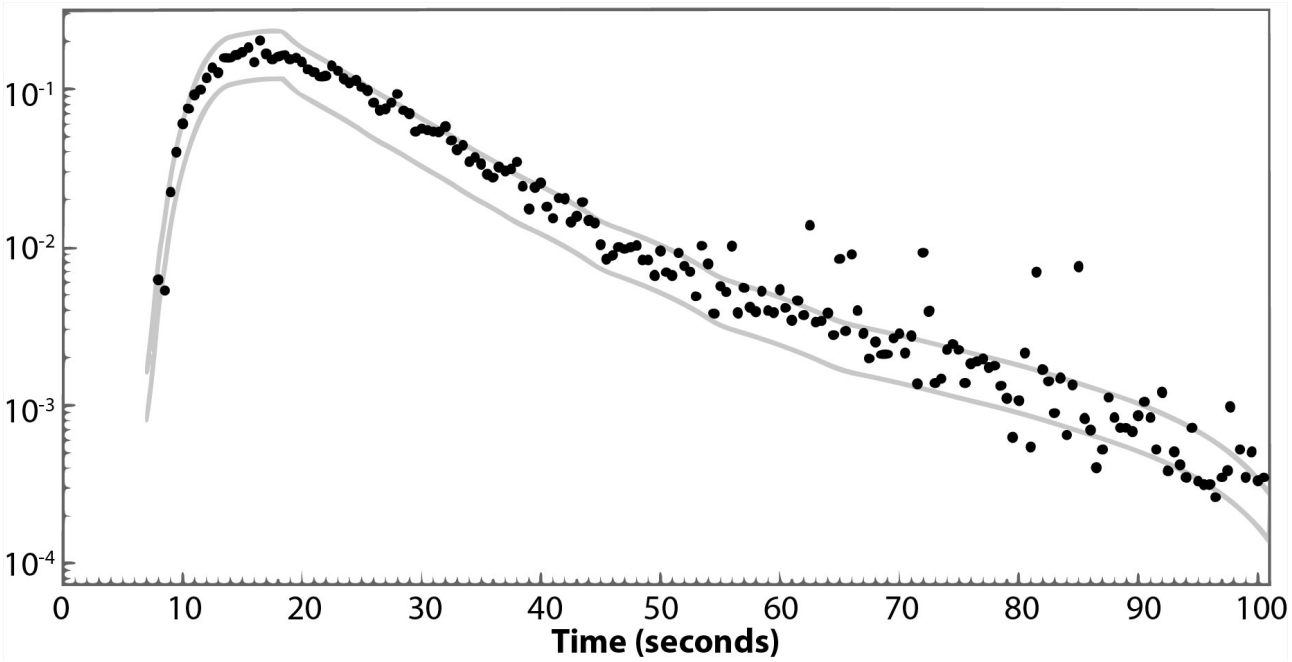

Figure 2. Output fraction over the 100 second execution of the analog. Light gray lines represent a one standard deviation band around the in situ perfused rat liver referent mean for atenolol.

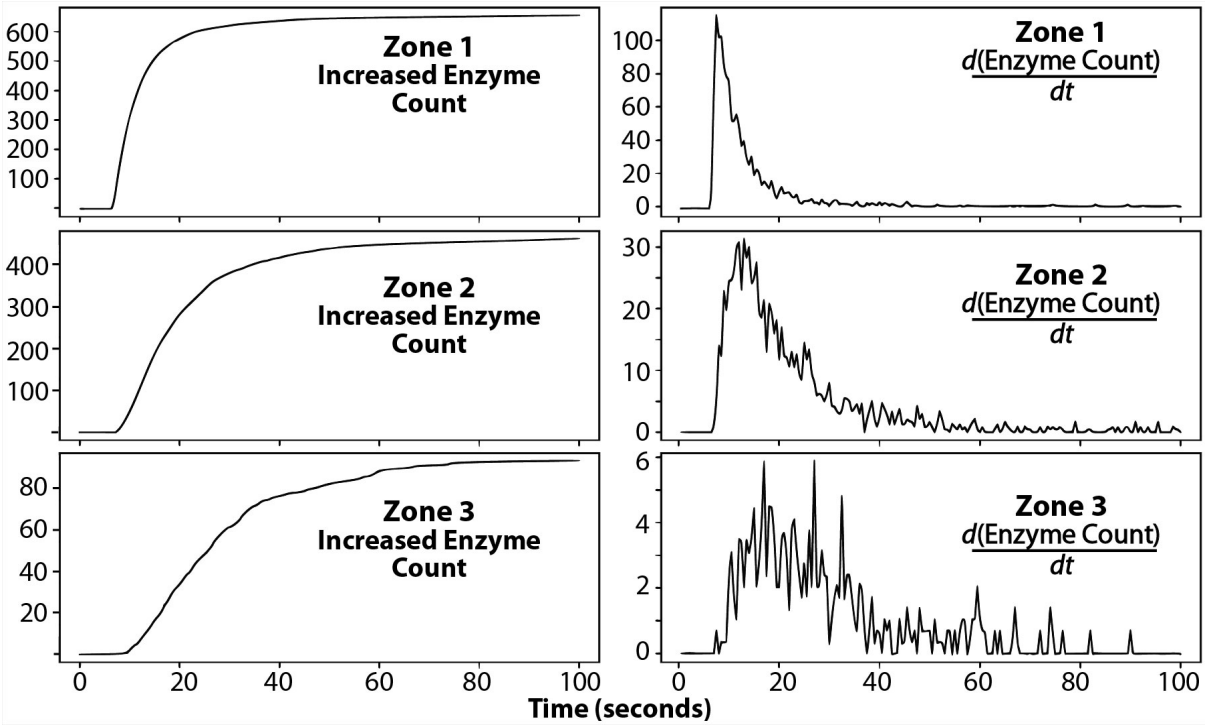

Figure 3. Enzyme count and change over time for Zones I-III.

the referent mechanisms. Hence it makes little sense to simply throw more computing resources at the problem without re-examining those conceptual models along with the coded mechanism.

6.2. Compound attributes

Other efforts [8][9] have focused on compounds with a higher clearance than atenolol. Specific initializations of earlier ISLs have exhibited abiotic artifacts in their outflow profiles measures [5]. It is thus reasonable to speculate that either the falsification of this EI mechanism or the validation against the outflow profile is a function of the inclusiveness of the ISL phenotype. Despite its specificity to the in situ perfused liver experimental protocol and the concreteness of its mechanisms, the ISL is designed to be a generalized model of xenobiotic hepatic clearance that can cover the behavior of many experimental protocols involving compound-liver interactions. If we were confident that this EI mechanism, when integrated with the discrete lobule into a hybrid model, could stand as an accurate model of a more specific set of livers (which we are not), it would be important to explore compounds that are more aggressively cleared by the liver.

6.3. Extended execution

The validation data used for the coarse-grain measure is from an entirely different use case than that from the medium-grain measure. The EI validation data is from a model protocol where rats were sacrificed 12 hours and 3 days after exposure [20][21]. This point is not thought to be a critical because we expect zonation can occur quickly in response to a bolus of an appropriate inducing compound. However, it might be relevant to the transients of the EI mechanism, the environment of the discrete lobule, or the hybrid integration between them.

The buffered and delayed induction and elimination signals may decay after the 200 iterations of the analog. The geometry and topology of the lobule cause a broader distribution of compound in zone I than in zone III, which may indicate a more acute impact of the bolus on the zone III hepatocytes, making the induction buffer and delay longer in zone III. Were the simulation then ended at the proper time, that protocol might show zonation. Even if such a validation were achieved, it would be too protocol specific to be useful. The experimental protocol would have been purposefully chosen to highlight the buffer and delay explicitly inscribed into the EI mechanism. This would amount to an inscription error.

6.4. Recirculation & additional endocrine signals

Related to the above, longer execution, the animal models used for most zonation studies also include fluid (blood) recirculation and multiple up and down regulatory signals for different enzymes. For example, Oinonen and Lindros [18] show the involvement of pituitary and gonadal

hormones. The above EI mechanism relies on compound being detected within an hepatocyte, regardless of a metabolic event. Consequently, having perfusate recirculate over longer execution times should change the dynamics of induction. Because elimination is a delayed function of the enzymes present, it is possible that zonation would arise as concentration of the compound in solution decreased. Adding more regulatory signals to make the mechanism more accurate would increase the ISL control surface and make a) validation easier and b) the model even less falsifiable. However, neither of these results would satisfy the objectives of the project.

6.5. Hybrid method

It is reasonable to question the technological integration of the discretized equation part of the EI mechanism with the discrete event lobule. A prior iteration of the mechanism instantaneously increased and decreased the enzyme count as functions of compound and enzyme counts, respectively. However, this drove the medium-grain measure to saturation very quickly, within 2-3 cycles. The addition of the IC_2 parameter, which specifies how many model iterations it takes to synthesize or destroy an enzyme, plus the delays for induction and elimination, allowed adjustment of the hybrid integration and allowed the analog to show some longer term variation in the EI measures while validating against the output fraction measure. However, there are many more aspects to the interface between the discrete event lobule and the discrete time EI mechanism, including the scales of solute and cells, binding times, resolution of enzyme count (in [0,100]), etc. These represent many productive possibilities for finding an integration method that allows validating against both measures. Migration from a falsified to validated mechanism, including a discrete event to discrete time interface, will likely present lessons regarding hybrid multi-modeling methods as well as concrete multiscale lessons for liver use cases and in silico models.

It is reasonable to speculate that persistent falsification of all local mechanisms could indicate a degree of autonomy between EI and clearance. Perhaps the EI and clearance measures are not as tightly coupled as our assumptions about measure integration require. That would further imply that either metabolism is less important than geometry and topology for the single-pass use case or it is "lost in the noise" for the less cleared compounds.

7. NEXT

Synthetic models are purposefully exploratory. So, the position we are in with this falsified EI mechanism is not merely tolerable, but desirable. It gives us a large array of opportunities for new targets, some of which will involve minor tweaks to EI mechanisms and some significant sampling of largely unexplored parameter space (recirculating, infusion vs. bolus, longer execution, etc.). Others may require major ISL overhauls.

Because hybrid models are interesting and necessary for approaching the ultimate goal of virtual patients, we will redesign the continuous mechanism and its integration with the discrete lobule.

It is reasonable to assume that mechanism failure is largely due to an oversimplification of hepatocyte behavior. Hepatocytes have a very complex phenotype and a more complex mechanism may provide enough intra-hepatic hysteresis to generate effects with local mechanisms, including paracrine signals. The next mechanism iteration will be developed so that an experimental distinction can be made between zonation due to a more complex hepatocyte or due to a hybrid model having improved integration.

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