INTRODUCTION

Acute drug-induced liver injury causes significant morbidity and mortality and has halted development of dozens of promising pharmaceuticals. Acetaminophen (APAP) induced liver injury (AILI) in mice is the accepted model for xenobiotic induced liver injury in humans. A precondition to developing strategies that anticipate and possibly prevent such injury is a reliable mechanistic explanation of how, when, and where key temporal features of APAP toxicity emerge. Yet, even after three decades of intense investigation, an explanation remains elusive. The weight of evidence supports this hypothesis (Core Mechanism): location dependent differences in reactive metabolite (NAPQI) formation within hepatic lobules (zonation) are necessary and sufficient to account for necrosis occurring first adjacent to the lobule's central vein (CV). However, challenging that hypothesis directly in mice is currently infeasible because doing so would require sequential intralobular measurements within the same mouse.

We challenged and falsified Core Mechanism using virtual experiments on virtual mice containing a concretized liver analog comprised of biomimetic lobules in which autonomous hepatocyte agent counterparts utilized a parsimonious version of Core Mechanism. The virtual mouse model, called a Mouse Analog (MoAn), contains a body and a liver lobule. Also, We use the Iterative Refinement (IR) protocol, which is a combination of the scientific method and software engineering, to perform these virtual experiments. As a prerequisite, we achieved multiple prespecified qualitative and quantitative validation targets [refs]. Virtual hepatocyte analogs (HepAns) use a portal vein to central vein (PV-to-CV) gradient to customize responses to APAP and damage based on location (functional zonation). Parameter space searches failed to identify parameterizations able to cause simulated necrosis to occur first adjacent to CV. We posited that at least one additional mechanistic feature must exhibit zonation. We instantiated competing hypotheses. For Mechanism 1, the likelihood of GSH being depleted below a critical level (through reaction with NAPQI) increases PV-to-CV. For competing Mechanism 2, each hepatocyte's ability to repair NAPQI-induced mitochondrial damage diminishes PV-to-CV. We hypothesized that one or both mechanisms would be sufficient to achieve the validation target. Both mechanisms individually were falsified. Thus, an even more complicated yet still parsimonious mechanistic explanation was required. We combined the two mechanisms (Mechanism 3) and identified parameterizations that achieved the validation target. Mechanism 3 during execution is the first concrete explanation of how spatially hererogeneous temporal features, which are measurably similar to features of APAP hepatotoxicity in mice, can be generated.

METHODS

We perform virtual experiments on concrete, biomimetic analogs, which are composed of interacting software agents and objects. The analog's behaviors (i.e. phenotypes) are advanced through time by discrete-event simulation. Also, we use Monte Carlo (MC) sampling to represent the inherent variability and uncertainty in

complex biological systems. Finally, our methodology, called the Iterative Refinement (IR) protocol, combines the scientific method and good software engineering practices to validate or falsify our analogs by comparing in silico measurements to wet-lab measurements. Essentially, the IR protocol is a way to "evolve" our analogs in a virtual environment.

VE occurs within a virtual reality in which a variety of software enitities exist. These entities, which we call analogs, are composed of models that are analogous to the referent biological and experimental system of interest in three important ways: 1) they are concrete (i.e. exist and not abstract), 2) their components and interactions mimic biology, or biomimetic, and 3) they are multiscale in construction and behavior. The difference between an analog and a software "program" is this property of concreteness because a program is functionally abstract (i.e. it processes data in some way), whereas an analog can just exist in its virtual environment and, theoretically, not do anything. We strive for our analogs to be biomimetic because we are trying to understand biology; therefore, we construct the analog's components and interactions to resemble the corresponding biological components and interactions as much as knowledge provides. Biological systems are multiscale in space, from molecular to ecosystems, and in observable phenomena, such as protein transport to organismal development. Therefore, according to biomimicry, analogs exhibit multiple levels of both spatial organization and temporal evolution of behavior.

Salient characteristics of wet-lab experiments include pervasive uncertainty, sparse system information, and considerable variability, which make distinguishing causes from effects difficult. Agent-based methods provide the flexibility, extensibility, and generality needed to assemble software mechanisms that become increasingly biomimetic during execution. Agents are quasi-autonomous software objects with state information (e.g. how many molecules of a certain type) and rules of behavior (if this situation, then do this action). Many biological processes are analogized as logical statements; for instance, if protein A binds to protein B; then protein B is activated. Our agents implement similar rule-based behavior when mediating interacting components. We advance are mechanisms mediated by agents throught time so they can exhibit their behaviors using discrete-event simulation, which is essentially a queque in which events are added to be executed in a certain order.

The analog of typically the most interest in a VE is the one containing models of biology (i.e. a member of the living counterparts category of VEs). In this chapter the referent biological system of interest is acetominophen induced liver injury (AILI) within a whole mouse. Therefore, the object of VE is the Mouse Analog (MoAn), a discrete event, object- and agent-oriented multiscale model comprised of spaces and objects that map to required mouse counterparts. A MoAn is composed, in the simplest form for this use case, of a body and liver model. The body model is just a spatial compartment which can be dosed with objects that map to acetominophen (or other compounds) and its contents sampled. The liver model, called an in silico liver (ISL), is more complicated, containing multiple components and analogs, especially the hepatocyte analog (HepAn),

which contains the mechanistic model for metabolism. In the course of an experiment, to simulate blood flow, a fraction of the contents of the body, including the dosed drug, are transfered to the portal vein (PV) of the ISL, moved through the Lobule Analog (LobuAn) by a biased random walk, exited through the central vein (CV), and then back to the body compartment. An objective is that measurements of phenomena taken during execution correlate directly with measurements of characteristic AILI phenomena in mice.

A LobuAn (Figure #) is represented by a directed graph with nodes called sinusoid segments (SSs), or vascular tubes, containing blood flowing from the PV to the CV. Lobules are physiologically divided into three zones, one near the PV and CV and one in between these. Zone 1 near the PV has the most SS nodes (45) and significant intrazone edges (20). Zone 2 has fewer nodes (20) and fewer intrazone edges (10). Zone 3 near the CV has three nodes and 0 intrazone edges. Acetominophen (APAP), a type of Solute (i.e. chemical objects) are injected into the body, a fraction arrive at the PV, flow through the SS network, then exit through the CV into the body, in which they are collected and counted. Each SS, an agent, consists of a concentric layering of three cylindrical grids wrapped around a center queue. The center conducts a laminar flow of perfusate (maps to blood) along the length of the SS. The innermost Grid A models more turbulent and viscous flow along the endothelial lining of the sinusoid by using a pseudo-random movement biased toward the SS outlet. Grid B is partially populated by Endothelial Cell agents into which Solute (but not Marker) objects can partition, and which partially blocks lateral Marker movement. Solute that makes it past the Endothelial layer enters Grid C, which models the Space of Disse and is partially populated by HepAns. Both Cell types contain Binder objects that can sequester compound for some number of simulation cycles (SCycs). Currently 1 SCyc maps to 1 second. HepAns contain Binders called Enzymes, which may metabolize bound compounds according to a probabilistic distribution over space from PV-to-CV. What concerns us in this work is intra-hepatocyte mechanisms (see below), specifically what happens after NAPQI formation.

Figure # caption?. Although illustrated, Bile Network does not influence AILI. Rather than abstract away considerable uncertainty and variability, we strive to represent it within and across scales as follows. No two LobuAn executions are the same because 1) there are Monte Carlo (MC) variations in Sinusoid Segment (SS) dimensions, graph composition, event management modules, and parameterizations; 2) most events are probabilistic; and 3) some events are networked. Because measurements of phenomena during a single execution can be very “noisy,” a typical MoAn experiment uses 24 MC LobuAn variants, each with a unique combination of flow networks and components. All flow paths follow a 3-zone interconnected graph. A SS located at each graph node maps to aspects of tissue micro-architecture and function. SS sizes are MC determined within constraints; 45, 20, and 3 are used in zones 1, 2, 3, respectively; one LobuAn uses 1 flow network + 68 SS. Each SS has a Core and concentric toroidal spaces. APAP and other mobile objects 1) enter and exit an SS via Core and the Blood-Cell Interface; and 2) percolate through spaces influenced by flow, and those that

survive eventually exit the CV. Cell objects are agents. They regulate compound entry and exit. ECAn agents occupy most of Endothelial Cell Space; the only event occurring currently within an ECAn is non-specific APAP “binding.” ~14,000 HepAn agents occupy most of Hepatocyte Space. Between Hepatocyte and Endothelial Cell Spaces is a Cell-free Space of Dissé (not shown). A PV-CV gradient (not illustrated) maps to measures of one or more common blood attributes, such as pO2 . HepAns use the local gradient value to parameterize their own location-dependent, parameters. Contained within each HepAn are three previously validated event management modules; they map to 1) all material removal tasks; 2) all xenobiotic metabolic events plus non-specific binding; and 3) regulation of metabolic capabilities. Simultaneous, small changes (e.g., 5-10%) in several parameters can offset each other and may produce no detectable change in measured events. Thus, linear sensitivity studies are less informative and meaningful than complete location changes in analog parameter space. We use batch parameter space sampling to identify small subsets of parameters (such as number of SS in one zone) that are most influential for particular AILI attributes.

Because all wet-lab experiments are concrete, our approach involves building, experimenting on, and refining concrete biomimetic analog mechanisms but constructed using software components. That concreteness helps distinguish our approach from more abstract techniques common in mathematical MSM. During execution, analog mechanisms generate phenomena which, when measured, may be qualitatively and/or quantitatively similar to corresponding wet-lab measured phenomena. When there are many analog-to-wet-lab phenomenal and mechanistic similarities, we have a concretely testable theory for how the biological mechanism may actually function in a particular wet-lab setting. Analog mechanisms thus stand as the current best mechanistic explanation of that phenomenon. When falsified because it cannot also adequately mimic a new phenomenon, the concreteness makes it easy to determine the failure’s cause and hypothesize corrective revisions.

Complex biological systems have many sources of variability and uncertainty that must be accounted for in our simulations. Variability is what changes and uncertainty is by how much. We use Monte Carlo (MC) sampling to introduce both variability and uncertainty. For example, wet-lab experiments exhibit variabilities across samples; analogously, we vary the spatial architecture of our analogs pseudo-randomly, repeat simulations and average results. Many biomolecular processes are inherently probabilistic (e.g. biochemical reactions); therefore, we use probabilistic parameters for these uncertainties. In addition, some probabilistic parameters are not scalar but vary over space. During analog execution, agent-mediated Monte Carlo sampling of probability distributions over space and time determine the location and timing of mechanistic events.

Our Iterative Refinement (IR) protocol approach to virtual experimentation combines the scientific method and good software engineering practices. First, a referent “targeted attribute” (TA) is selected from a list of TAs that we eventually wish to explain. A TA can be either qualitative, such as a description, or quantitative, such as

data collected from wet-lab experiments. Second, a hypothesis is formulated as a virtual mechanism. The analog's mechanisms produce the analog's behaviors or phenomena. Third, we refactor and add to extant mechanism code (from already studied analogs) to create the specified mechanisms. A series of simulation experiments are performed. Measurements, such as component numbers, event location, and timing, are measured and recorded. Fourth, simulation and referent results are compared using a "similarity criterion" (SC). If the SC is achieved, the analog has achieved a degree of validation. Finally, the validated analog's mechanism granularity is either increased parsimoniously or additional TAs are specified, and the process is repeated with the objective of falsifying (or not) the implemented mechanism. When the analog mechanisms with their embedded knowledge survive the challenge, the analog can stand as a plausible, concrete, valid explanation of the targeted phenomena. For the above approach, mechanism (i.e. explanation) falsification is just as important as validation. The validation process involves systematically sampling analog phenotypes. This is accomplished by sampling parameterizations. Our general approach is to perform virtual experiments by iterating through the IR protocol, which is a combination of the scientific method and software engineering (see Online Methods). We parsimoniously implement an agent-mediated mechanism as a hypothesis of a phenomenon of interest, which is represented either as an qualitative observation or a quantitative measurement called a target attribute (TA). We test the hypothesis by either varying the parameterization of the mechanism or increasing the granularity of the mechanism until the mechanistic hypothesis is validated, which means the phenotype of the mechanism (i.e. behavior, output) matches the TA according to a pre-specified similarity criterion (SC). If validated, then we can conclude that the implemented mechanism is a plausible explanation for the phenomenon; however, if falsified (i.e. no match according to SC), then we can conclude that the implemented mechanism in not an explanation of the phenomenon (Popper,phisci?). Each iteration through the IR protocol is either for increasing the stringency of the SC (i.e. the "closeness" of the match) or for explaining addition TAs. Through multiple iterations our multiscale, knowledg-embedded, agent-based model, called analogs, becoming increasing explantory across many different phenomena, which increases model confidence in accurate predictions. In summary, we followed a five stage plan: 1) Assume an hypothesis is true. 2) Specify characteristic AILI phenomena for a 300 mg/kg APAP dose to serve as qualitative and quantitative validation targets (VTs). A VT is the combination of a TA and a SC that must be achieved, such as being within ± 1SD of a wet-lab measurement. 3) Instantiate a biomimetic MoAn mechanism consistent with the hypothesis. When implementing MoAn mechanisms we follow a strong parsimony guideline, which is avoid making them any more fine grain than is needed to achieve current VTs, and then 4) iteratively refine and revise the mechanism until VTs are achieved.

Verification (i.e. making sure the analog does what its supposed to do), validation, and falsification are the processes by which experimenters evolve and select among mechanisms to be used in different contexts to achieve an expanding set of

validation targets. The process is somewhat analogous to the method of directed evolution used in protein engineering. Mutations are introduced into a protein; it is then selected (or not) among other variants for a particular desired property using a screen. We implement analog mechanism changes motivated by current knowledge and/or inferences drawn from wet-lab observations. Survival of an analog mechanism is determined by whether validation targets are achieved.

For virtual experimentation an analog contains mechanisms, which are hypotheses on the generation of phenonmena. A mechanism is a series of events or a causal cascade mediated by software agents, components, and entities within a virtual space. A set of parameters (i.e. parameterization) define the structure and execution of the mechanism. Within a MoAn many mechanistic events occur generating different AILI phenomena; however, the ones of most concern are APAP metabolism, GSH depletion, mitochondrial damage production, repair of damage, and cell death. Several mechanisms were implemented and tested following the IR Protocol. Hepatocytes analogs (HepAns) within MoAns are agents that mediate many of these mechanisms (Figure 3), which are transport across the plasma membrane (Figure 3a), APAP binding (Figure 3b), APAP metabolism (Figure 3c & d), glutathione (GSH) depletion by reactive metabolite NAPQI (Figure 3e), non-mitochondrial (nonMD) and mitochondrial (mitoD) production by NAPQI (Figure 3f), amplification of mitoD from ROS and RHS produced my dysfunctional mitochondria (Figure 3g), repair events to both nonMD and mitoD (e.g. DNA repair and mitochondrial autophagy) (Figure 3h), triggering cell death after mitoD reachs a threshold (Figure 3i), and the amount of time from when cell death is triggered and the cell actually dies (Figure 3j). In MoAns and HepAns, parameters controlling most mechanisms are probabilities of event occurrence per SCyc, and are designated p(·), but some are fractions or simple scalars (Figure 4). Location-dependent (i.e. where between PV and CV) probabilistic values of the three key APAP metabolism parameters are plotted in Figure 4 (a, b, & c). Enzyme objects within Hepatocytes use those parameterizations to specify the probability of metabolism/SCyc. At PV the probability of forming G (maps to glucuronidation), S (maps to sulfation), or N (maps to NAPQI) is the same; however, near the CV less G and S but more N is generated. Once formed, p(G, S removal from Cell) = 0.5. N cannot exit the Cell back into Blood but can enter the biliary canal, and nonMD and mitoD cannot exit the Cell. Drawing on accepted conceptual mechanisms from available knowledge, we specified that N reacts in two ways: 1) it depletes Hepatocyte GSH. GSH and N combine stoichiometrically, eliminating N and depleting the Cell's GSH pool; and 2) it binds to cell components, predominantly protein adducts, which are damage objects (i.e. nonMD and mitoD), and itself is eliminated. There is experimental evidence that normal GSH levels decrease PV-to-CV; therefore, we specified a location-dependent GSH depletion threshold (Figure 4d). Prior to reaching the threshold, p(N removal) = 0.9. Each N removal reduces the threshold value by one. Once the threshold is reached, GSH is "depleted"; thereafter, p(N → nonMD/mitoD) = 0.5 and is location-independent (i.e. constant along the PV-to-CV distance). MitoD amplication is that when a mitoD is produced a further amount of mitoD, chosen from a gaussian distribution, are produced. We introduced a location-

dependent repair mechanism with the objective of achieving the validation targets as we cycle through the IR protocol. An analog Repair event corresponds to removing either nonMD or mitoD object and replacing it with an R object with probability p(nonMD/mitoD → R). From the literature some damage is repaired easily whereas some damage is more difficult and likely requires more energy. A parsimonious solution was to "split" Damage into nonMD and mitoD, and that p(nonMD → R) and p(mitoD → R) have different location dependences. Because near CV pO2 is chronically low, which increases the risk of oxidative damage, the literature consensus is that some repair functions are normally elevated in zone 3 relative to zones 1 and 2; therefore, p(nonMD → R) maps to those repair processes. In addition, we conjectured that some mitochondrial damage might be less effectively repaired as pO2 decreases and because of the energy requirement for mitochondria autophagy; therefore, p(mitoD → R) maps to those repair processes. We achieved validation using a decreasing sigmoid function from PV-to-CV for p(mitoD → R). Mitochondria, through the mitochondrial permeability transistion (MPT) and JNK, play a central role in mediating hepatocyte death or necrosis. The necrosis trigger mechanism was a simple threshold: if number (mitoD) > threshold value, then the cell will die. With those mechanisms implemented, we performed simulations using 24 Monte Carlo analog variants. Amounts of generated objects (i.e., G, S, N, nonMD, mitoD, & R) along with location/timing of cell death events were measured, and the results averaged.

Figure # caption? The HepAn mechanisms (from top to bottom) include movement of compounds within spaces and into/out of cells, binding, metabolism, GSH depletion, non-mitochondrial and mitochondrial damage production, mitochondrial damage amplification, repair of damage, cell death triggered, cell death observation after a delay, and cell death inhibition.

RESULTS

The wet-lab experiments we endeavor to mimic are the observations of AILI within ip-dosed mice. From a previously validated in silico liver [summersim'14], we added a "body" compartment to create an intial MoAn, called MoAn1. An objective is that measurements of phenomena taken during execution correlate directly with measurements of characteristic AILI phenomena in mice. Hereafter, when describing a MoAn feature that has a direct wet-lab counterpart, such as a plasma profile, the MoAn feature is capitalized (e.g. Plasma Profile).

The two main TAs of interest that MoAn1 must exhibit as a phenotype are APAP clearance measured in plasma and the temporal profile of necrosis. The mechanisms of APAP metabolism, damage/repair production, glutathione (GSH) depletion, and cell death (see Methods) were unchange from the pre-validated liver; however, because of the body, some parameters controlling adsorption and dispersion were changed to validate APAP clearance in plasma. Most parameterizations were falsified (i.e. did not match to wet-lab values within one standard deviation, see Supplemental Material?) but one that validated is shown in Figure 1A. Also, this APAP clearance profile has a similar

qualitative shape to those obtained in many other wet-lab experiments in the literature [refs]. The dose dependence of APAP clearance has also been validated qualitatively (see Supplemental Material). The second TA of interest is the amount of necrosis, or cell death, that accumulates over time. From our wet-lab collaborators we obtained the minimum and maximum percentages of necrosis after a certain amount of time over a 24 hour period (Figure 1B). Taking these two observations, a rough average and standard deviation can be calculated. Also, performing a MoAn experiment corresponding to 24 hours would take a prohibitive amount of real-world time; therefore, the time axis has been normalized for a 6 hour experiment. For MoAns the cell death mechanism is composed of both a "tipping point" above which mitochondrial damage causes the hepatocyte cell to die and a "death delay" which is the amount of time between triggering cell death (i.e. pass beyond the tipping point) and the cell actually dying (see Methods). In MoAn1, this death delay was essentially immediate (either 1 or 2 seconds or simulation cycles, SCyc), which, from the literature, is not biomimetic because each cell varies in the detailed cascade of events beyond triggering cell death and to eventual necrosis, such as the amount of damage, capability of repair, available energy, etc.; therefore, these series of events take some time to execute. In addition, this temporal variability is stochastic leading to a probability distribution over death delays. Because of this non-biomimicry the death delay distribution of MoAn1 was falsified (Figure 1B); therefore, other distriubutions were required. Many parameterized probability distributions were proposed in which some were falsified others validated (see Supplemental Material), of the ones validated we chose the simplest (i.e. Occam's Razor) which is a uniform distribution between a minimum and maximum value (Figure1B), subsequently called MoAn2.

At this point after many iterations through the IR protocol we have evolved our MoAn to exhibit the measureable phenotypes of APAP plasma clearance profile and temporal necrosis profile, which all achieve validation with wet-lab observations and measurements. Furthermore, including the ones mentioned above, five validation targets (VT = TA + SC) were attempted by experimentation, some of these involved lobule zonation, in which physiological changes are along a spatial axis from portal vein (PV) to central vein (CV). These five are: 1) MoAn's APAP Plasma Profile following IP dosing falls within ± 1 SD of wet-lab values. 2) Hepatic Extraction ratio is in the range 0.5-0.75. 3) APAP Metabolism in-creases from PV-to-CV. 4) the reactive metabolite, NAPQI, as fraction of Metabolites formed, increases PV-to-CV, and G & S, as fraction of metabolites formed, decreases PV-to-CV . 5) Together, MoAn objects G & S (map to APAP-glucuronide & -sulfate) account for > 50% of APAP Metabolites. We parameterized the MoAn mechanisms so that VTs 1–5 were achieved (the black lines in panels a, b, & c of Fig. 4 in Online Methods, for now, ignore the red curves; they are discussed later), in which the results are shown in Figure 3 panels a, b, and c.

The above five MoAn VTs were preconditions for testing a key hypothesis, the Core Mechanism. This null hypothesis is that hepatic zonation of formation and reaction of NAPQI are necessary and sufficient conditions for emergence of characteristic AILI phenomena. However, it cannot be tested directly in mice because doing so requires

simultaneous multiscale hepatic measurements, which currently are infeasible. We tested a variant of that hypothesis using virtual experimentation: zonation of NAPQI formation and reaction within concrete HepAns are necessary and sufficient conditions for the generation of biomimetic phenomena similar to characteristic AILI phenomena (ref 2 in U01). These AILI phenomena were represented by the following VTs: 6) HepAn Death events (maps to necrosis) occur first close to CV then progresses toward PV, 7) At 15 min after the APAP dose there is no HepAn Death, 8) Few HepAn Death events occur in zone 1, adjacent to PV, 9) Amounts of NAPQI increase PV-to-CV and are greatest in zone 3 HepAns, 10) By 30 min, NAPQI amounts are approximately twice that at 15 min, and 11) The maximum rate of HepAn Death occurs at about 12 h.

Our MoAn, with key results presented in Figure 3, provides the foundation for moving forward on these experiments. Its coarse grain mechanisms during execution stand as plausible, concrete, observable and challengeable causal cascade explanations of how all eleven AILI VTs are generated in mice. The following is an abridged description of our MoAn features and the causal cascade (see Methods). A typical experiment starts with an APAP dose of 125K objects. First order absorption into MoAn simulates APAP absorption following an IP dose. Any of dozens of events may occur during each simulation cycle (SCyc). Mobile objects within MoAn may enter a lobule analog (LobuAn); other objects may exit. Objects within LobuAn may percolate within and between Lobular spaces. All critical MoAn115 events occur within hepatocyte analogs (HepAns) contained within LobuAns. Any of the events illustrated in Fig. 3 may occur within HepAns. Each HepAn is a quasi-autonomous agent (it can be “isolated,” experimented upon, analogous to in vitro experiments on isolated hepatocytes, and returned to MoAn (ref 6 of U01). Each HepAn uses the local value of a PV-to-CV gradient to specify all of its Lobule location-sensitive parameters. The gradient maps to one or more blood signals, such as pO2. NAPQI first depletes GSH, thereafter NAPQI => nonMD (maps to a conflation of non-mitochondrial damage products resulting from a NAPQI reactions elsewhere within in the cell) or mitoD (mitoD maps to a conflation of mitochondrial damage products resulting from NAPQI reactions) with equal probability, independent of location (i.e. constant along PV-to_CV). However, the ability of a HepAn to repair or remove damage, nonMD or mitoD, is location-dependent; note that its PV-to-CV change is sigmoidal. Also, mitoD can be amplified (mitoD => 2 mitoD) but there is a limit of six such events per Cell location. Death is triggered in an individual HepAn when mitoD exceeds a threshold value (a parsimonious specification of being constant and location independent), but there is a lag-time before Necrosis becomes detectable

Despite extensive searching, the implemented zonated mechanisms of APAP metabolism and NAPQI production were inadequate to generate signficantly more damage near CV in order to achieve VTs 6–11. Because MoAn mechanisms are concrete, that failure falsified the null hypothesis. Therefore, to account for these phenomena, a finer grained (i.e. more complicated) mechanistic explanations were required. Following several cycles through the IR Protocol, we discovered an expanded set of MoAn mechanisms and parameterizations that did achieve the VTs. Furthermore, zonation of three new mechanisms (parameterizations shown in panels d, f, & g of

Figure 4 in Online Methods) of GSH depletion and repair of damage proved necessary and essential. The importance of those additional mechanistic features to establishing a plausible causal cascade was not previously recognized. The results shown in Figure 3d demonstrate that VTs 6-8 were achieved. The profiles of NAPQI and mitoD (panels c & g) are similar as expected. The profiles in panels c, e, & g seem counterintuitive because CV values peak sooner than midway values. However, the explanation is that the probability of an APAP metabolic event is greater near CV. The profile in panel f may also seem counterintuitive because more glutathione (GSH) depletion events occur midway. That is a consequence primarily of two features: there is less GSH to be depleted near CV; there is much less NAPQI to deplete GSH near PV.

To challenge the null hypothesis, we created four different MoAns, C, M, G, and N. Analog C has both GSH depletion (hereafter, simply GSH) and mitoD Repair zonation, Analog M has GSH zonation but no mitoD Repair zonation, Analog G has no GSH zonation but mitoD Repair zonation, and Analog N has neither GSH nor mitoD Repair zonation. We measured when and where a Cell Death event occurs and cumulate these over time. Figure 4A shows that eliminating either zonation decreases cumulative Necrotic events. It is noteworthy that the decrease for analogs M (which has GSH but not mitoD Repair zonation) and N (neither zonation) is about the same: elimination of GSH zonation has no additional effect on the decrease in Necrotic events. However, Panel B shows that GSH zonation has a profound effect on location of early Necrotic events. Total events at 83 minutes in Panel A span the range 11,800 to 13,600. Figure 4B shows when and where Necrotic events occurred. Distance from CV-to-PV is measured in grid points (Cell locations). Because of interconnections primarily in Zone 1, a few paths can be quite long. About 50% of Cell locations are > 20 grid points from CV. Zone 3 extends out to about 8 grid points from CV. The values plotted are 100-event moving averages. A more vulnerable Cell is one in which a smaller amount of NAPQI will trigger Necrosis. Relative to analog C, eliminating GSH zonation increases the number of vulnerable Cells at locations further from CV. However, because of Metabolism zonation, most vulnerable Cells in Zones 1 and 2 are exposed to relatively smaller amounts of NAPQI. Consequently, eliminating GSH zonation dramatically shifts early necrotic events away from CV. Eliminating mitoD Repair dramatically shifts Necrotic event locations even further from CV. Relative to analog M, additionally eliminating GSH zonation shifts locations of earliest Necrotic events even further from CV. In the latter case, cross-sections of analog C Lobules would show very few Necrotic Cells—little evidence of tissue Toxicity—even though eliminating both zonal influences reduces actual Toxicity by only about 15% (however, that Toxicity may still be evident in Plasma marker levels). In conclusion, these results falsified the null hypothesis, and zonation of GSH and mitochondrial damage repair is required for hepatic necrosis.

DISCUSSION

There are three main results of this work: 1) We have developed a multiscale agent-based model that simulates many AILI phenomena. 2) The IR protocol is a

thorough and systematic procedure for virtual experimentation. 3) Virtual experimentation, most easily and efficiently executed with agent-based models, is a viable way to explain complex biological phenomena, such as AILI. Our in silico mouse analogs exhibit many different phenotypes or behaviors that can be measured and compared to real-word measurements and data; most importantly for this work is the spatial and temporal pattern of APAP-induced liver necrosis. Other phenotypes are APAP clearance, the production of APAP metabolites, distribution of solutes through the body and liver, the accumulation of non-mitochondrial and mitochonrial damage, GSH depletion, and the repair of this damage. Although the phenotypes are generally visualized as temporal plots (i.e. the amount of something vs time), we can also visualized phenotypes spatially for a better comparison to microscopic data measured on the referent real-world system. Moreover, all these phenotypes of our analogs arise through hypothesized mechanisms implemented into one agent-based model. These mechanisms can be measure and challenged directly with experimental evidence from the wet-lab. This synthetic constructed analog embodies the knowledge we have gained from real-world experiments and possible knowledge from falsification through virtual experiments. The IR protocol is the method to obtain this knowledge by using software engineering to evolve an analog (i.e. agent-based model) to better match the real-world though the scientific method. The IR protocol will eventually be automated allowing us to accelerate the evolution of our analogs through “Experimental agents”, which will map to real-world experimenters. The flexibility, adaptability, and specificity of agent-based models enable virtual experimentation to explain complex biological phenomenon. Virtual experimentation is a way to disentangle complex webs of cause and effect relationships which plague biomedical research.

There are other mathematical ODE-based models that exist to explain AILI and liver necrosis (DILISim). Conceptually, these models can be imagined as objects for use in virtual experimentation because of the search for a set of equations and parameters that “fit” data from wet-lab measurements or find solutions that “solve” the problem of matching the model's output to the referent's phenomena. Basically, as perscribed by the IR protocol and inscribed by the scientific method, the procedure of virtual experimentation is to start with a description of the phenomenon (targeted attribute, phenomenon of interest), propose an hypothesis (mechanism), search the relevant space (change parameters, perform experiments), and select the models that match the referent phenomenon (validation/falsification based on similarity criteria, draw conclusion on whether the hypothesis is true/false). However, there are some important differences between agent-based models and mathematical models, such as ODE/PDE PKPD models (cite Tutorial, specifically Table 1), that make agent-based models superior for efficiently cycling through the IR protocol in performing virtual experimentation. Analogs (i.e. our biomimetic agent-based models) are concrete and modular; wherease mathematical models are conceptual with different models tightly coupled. Analogs exist in a virutal reality and embody our knowledge of how its phenotypes, and its referent phenomena, are generated. Analog components, spaces, and mechanisms can easily be dissected, removed, and/or replaced to change the

analog for different experiments. Mathematical models are built for a specific experimental context and can not easily be modified for different experimental contexts. Furthermore, analogs separate models of pattern generation (generating different phenotypes) and models that compare these patterns to the referent system; analogs are relationally grounded and loosely coupled and mathematical models are abolutely grounded and tightly coupled (ref). Essentially, as like model structure above, analogs are flexible with interpretations based on the execution of the mechanism and the observation of its behavior; mathematical modesl are inflexible built for a specific context with interpretation based on the link between parameters and the referent (absolute grounding). Executing many interations of the IR protocol easily and efficiently require model flexibility. In addition, the best performance of the IR protocol and virtual experimentation requires separation of model structure and execution from the referent system. Analogs exist in their own virtual world with the requirement that they mimic, as best they can, in both structure and function the referent system. The IR protocol "evolves" the analogs in their world. Mathematical models are inducted from the referent to describe its phenomena but they are not separated.

We think virtual experimentation will be a valuable tool for biological and biomedical research. Many questions and problems in these fields are highly complex with many different and intertwined cause and effect relationships. Many wet-lab experiments contain many sources of uncertainty and variability that do not allow definitive conclusions. Also, many desirable experiments are currently infeasible. Virtual experimentation with agent-based models are an effective way to tackle these challenges. Many steps of the IR protocol, if not the entirety, can be automated with agent-based models allowing analogs to evolve much more rapidly. With this powerful improvement the eventual goal can be realized: a virtual world with virtual patients having virtual organs in which knowledge learned and predictions drawn from there can be immediately applied to the real world.