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PERSPECTIVE

Outside-In Systems Pharmacology Combines Innovative Computational Methods With High-Throughput Whole Vertebrate Studies

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To advance the systems approach in pharmacology, experimental models and computational methods need to be integrated from early drug discovery onward. Here, we propose outside-in model development, a model identification technique to understand and predict the dynamics of a system without requiring prior biological and/or pharmacological knowledge. The advanced data required could be obtained by whole vertebrate, high-throughput, low-resource dose-exposure-effect experimentation with the zebrafish larva. Combinations of these innovative techniques could improve early drug discovery.
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Outside-in model development

Systems biology has developed into a research field elucidating biological pathways and networks in great detail. These efforts are not only fueled by the advanced genetic toolbox in combination with increasingly sensitive and innovative measurement techniques, but especially by combining these experimental approaches with an "inside-out" modeling strategy.[1] Inside-out modeling aims to understand an external behavior (e.g., cellular apoptosis) by describing the internal processes (e.g., signal transduction pathways) in as detailed a way as possible given experimental and mathematical identifiability restraints. The resulting model structures are closely aligned to the underlying processes, whereas the extensive amount of experimental data required serves to estimate the unknown parameters of the model. Similarly, semi mechanistic pharmacometric and systems pharmacology models adhere to this modeling paradigm, shifting focus from target-based to system-based, or phenotypic-based drug development.[2,3] In our current understanding of healthy and diseased organisms as complex systems, a single drug selective for a single target within the network would rarely be fully effective.

"Outside-in" model development, on the other hand, is an approach still mainly used in engineering and there known as black box system identification. It does not require prior knowledge of the system of interest but allows the precise system structure and, thus, the dynamics of the system to be exposed by only observing its response to well-controlled stimuli (**Figure 1a**). More specifically, within such identification approaches, the system of interest is excited with oscillating stimuli of different frequencies in order to measure the time resolved response of the system (**Figure 1b**). For each input frequency, the amplitude of input and output as well as the phase shift between input and output are collected and plotted in a Bode plot (**Figure 1c**). This visual frequency domain representation of the dynamics of the system can be transformed into a formula by fitting a so-called transfer function, which is a representation of a set of linear differential equations that (in matrix notation) contain the state matrix $\boldsymbol{A}$, the input matrix $\boldsymbol{b}$, the output matrix $\boldsymbol{c}^T$, and the feedthrough matrix d (for systems with one input and one output is d scalar). These matrices can then be used to construct the model structure of the identified mathematical model (**Figure 1d**). Thus, the relationship between input and output of a system can be reconstructed if its complete frequency response is known.[4]

One of the advantages of this outside-in approach, the fact that it does not require prior information on the structure or the components of the model, makes this analysis method especially suitable for studying poorly understood (patho)physiological systems, pathways, diseases, or drug effects. Mettetal *et al.*,[5] for example, exposed yeast cells to oscillating NaCl levels of different frequencies and measured a fluorescently labeled protein as biomarker. Application of the outside-in approach led them to identify the dominant dynamics in the osmo-adaptation system in yeast, without considering all known and unknown reactions. This analysis method, albeit well-established in engineering, has many advantages still underappreciated in biology and pharmacology.

In early drug discovery, similarly little may be known about target pathways or drug effects, making this systems identification method of value. Once outside-in modeling uncovers the system structure and its dynamics, new experiments can be designed to inform the system model and its detailed components, which then allow prediction and interspecies translation of drug effects. Outside-in modeling is therefore an opportunity for close collaboration between experimentalist and modeler. Even though meticulous input/output measurements are still required, the outside-in approach does not initially require

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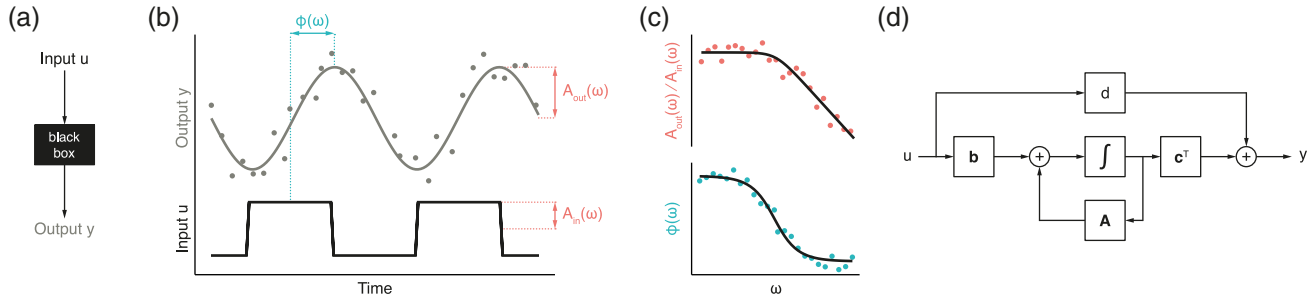

Figure 1 Outside-in model identification in the frequency domain. (**a**) Black box model is excited with input *u* and responds with output *y*. (**b**) Square wave input with amplitude $A_{in}(\omega)$ (black), output measurements (gray dots), and fitted sinusoid with amplitude $A_{out}(\omega)$ and phase shift $\phi(\omega)$ (gray line) are shown. (**c**) Bode plot of amplitude ratio and phase shift measurements (red and blue dots, respectively) vs. the frequency of the oscillations (ω) are used to fit a transfer function (black lines). (**d**) The model structure is derived from the differential equations, which themselves are determined from the transfer function.

exhaustive experimentation typical for inside-out systems biology models, thus enabling a fast turnover in testing drug candidates. This method is furthermore not exclusively tailored to identify model structures from the response of a single target to a single stimulus; it also extends to multiple drugs affecting multiple targets or even the whole organism.

Thus, outside-in modeling can initiate the drug discovery and development learn-and-confirm cycle from the systems perspective.

High-throughput whole vertebrate experiments in zebrafish larvae

To use the outside-in methodology in systems pharmacology, informative data from dedicated input/output experimentation is needed. As complementary pathways and feedback loops may attenuate the effect of a single drug compound, pharmacological treatment targeting multiple pathways seems more promising. This means experimental data should reflect perturbation of the whole organism as a system, with all intended and unintended targets available, rather than merely isolated parts that are taken out of their dynamic physiological context (**Figure 2** right-hand quarters). Additionally, as little time and resources are available in early drug discovery, performing the required stimulations at different frequencies is preferably performed in a high-throughput setup (**Figure 2** upper quarters). These types of high-throughput whole organism experiments are usually performed in invertebrate organisms like yeast (*Saccaromyces cerevisae*), round worms (*Caenorhabditis elegans*), or fruit flies (*Drosophila melanogaster*), but are impractical and unethical for commonly used vertebrate organisms like rodents. However, high-throughput whole vertebrate experiments are now possible in a relatively new model organism; the zebrafish larva (*Danio rerio*).

The zebrafish is an appealing alternative model organism to rodents.[6] They are easily genetically modified, enabling the creation of fluorescent reporter lines, as well as disease models.[7] The zebrafish develops quickly, with all major organ systems like the heart and vasculature, the blood-brain barrier, liver, gastrointestinal tract, and (pronephric) kidney present within 72–120 hours after fertilization. This allows for faster experimentation cycles as compared with traditional vertebrates like rodents. Moreover, the larvae are transparent, making (fluorescence) microscopy highly feasible. Last, their small size of only a few millimeters in the larval stage, and litter sizes of 100–200 eggs per breeding couple every 2 weeks, makes this experimental organism very cost-effective. Therefore, zebrafish larvae are very suitable for experiments required for outside-in system identification in early drug discovery and development.

Innovative experimental methods in the zebrafish have been developed since the turn of the millennium, especially with the zebrafish larvae. Drug exposure is commonly achieved by

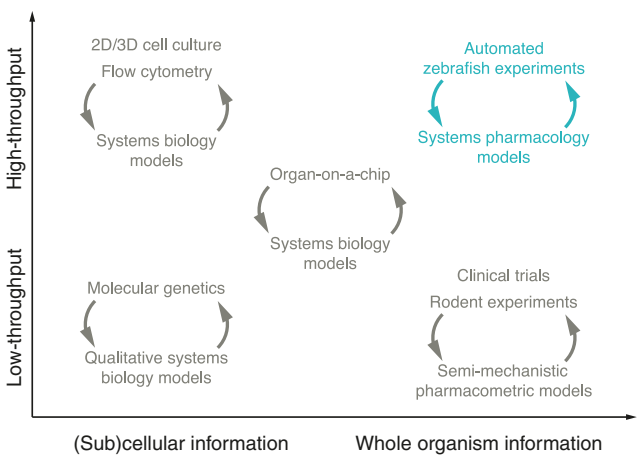

Figure 2 Distribution of current experimental methods and corresponding systems modeling approaches. Models to describe aspects of a physiological system in general can be developed at different levels, from subcellular and cellular to tissue-based, organ-based, and whole organisms. At subcellular and cellular levels, molecular genetics and biomedical experiments have unraveled detailed pathways within cells, and cellular environments in tissues or organs (lower left quarter). Fluorescence labeling, either chemically or genetically, enables high-throughput screening and sorting at cellular level (upper left quarter). Moving toward higher hierarchical levels, organ-on-a-chip informs on organoid processes and interactions (middle). For drug development, the perturbation of the whole organism system by a drug and quantifying the dynamics of the perturbation is very relevant, especially when considering multitarget drugs or combination therapy. Traditionally, from preclinical rodent and clinical patient data, pharmacometric models are used to quantify these effects (lower right quarter). Here, we propose the zebrafish larvae as high-throughput whole vertebrate organism for outside-in model-informed systems pharmacology, to fill the gap of high-throughput studies in whole organisms (upper right quarter).

dissolving the compound of interest in the incubation medium, allowing for uptake through the skin, the gastrointestinal tract after it opens at the fourth day postfertilization, and possibly the gills. Intravenous or intra-yolk administration is, however, also possible, even in a high-throughput setting. Recently, the first pharmacokinetic model in zebrafish larvae has been developed, an important step for this organism toward serving as model organism for systems pharmacology.[8] Additionally, experimental devices are designed to automatically load and position larvae in different chambers under the flow of, for example, drug containing medium. These microfluidic devices enable precisely controlled dynamic flow of exposure solution and wash medium, required for the oscillating stimulus of the outside-in systems identification.[9] Integrating such devices with automated fluorescence microscopy could lead to a closed experimental setup delivering systems pharmacology data in an automated high-throughput fashion.[10]

The availability of these methodologies welcomes experimentalists and modelers to combine forces aimed to tailor experimental data to a systems pharmacology model, and vice versa, continuing the learn-and-confirm iterative approach. Indeed, as the first outside-in data analysis without prior knowledge on the system or target pathways will result in information on important features of the system and identify promising candidates, more detailed experiments, and subsequent analyses can be designed and executed to elucidate underlying physiological mechanisms. When a comprehensive understanding of the studied system and the perturbing drug candidates has been developed, this knowledge should advance in the development pipeline. Knowledge of the system-specific and drug-specific parameters will then enable reliable interspecies translation and extrapolation, first from zebrafish larvae to rodent studies, and finally toward the clinic.

CONCLUSION

Systems pharmacology models inform drug discovery and development decisions. The models should be fit for purpose, combining the best of systems biology's mechanistic understanding and pharmacometrics' quantification of drug perturbations on a system. Although a system can be studied on all biological levels, from subcellular to whole organism, the latter is more in line with the current paradigm of pharmaceutical research. Here, we propose the use of systems pharmacology in drug discovery and development first by outside-in model identification, where the prominent factors between drug input and biological output are modeled without prior knowledge on the pathways or extensive understanding of the involved processes. Indeed, only the relevant rate-limiting elements will be considered. In early drug discovery, less resources and time are available, so experimentation should provide enough information on these elements within a short time frame. In other words, high-throughput experiments are preferred, while retaining information on the drug dose-exposure-response relationship. Such high-throughput experiments with the possibility of well-controlled oscillating stimuli and noninvasive detection of response by fluorescence are possible in zebrafish larvae contained in a microfluidics device. This precisely defined input and output data can in turn be used for black box outside-in model identification, unravelling the most relevant features in a target pathway, and informing on promising candidates affecting these pathways. Our approach is characterized by close interaction between experimentalists and modelers, and constant iteration of experiments and computational analysis, improving each other. When the understanding of the targets, pathways, and system increases with outside-in modeling, new experiments and subsequent computational analyses can be planned. Toward candidate selection, the understanding of the system-specific and drug-specific properties of the developed model can be used to extrapolate drug effects and design rodent studies, continuing the iteration. This cost-effective and fast approach facilitates the learn-and-confirm cycle, potentially improving the development of systems pharmacology models and candidate selection, and eventually possibly drug discovery and development itself.

Conflict of Interest. J.W.T.Y. and P.H.v.d.G. are employees of AstraZeneca and Certara, respectively. As Editor-in-Chief for *CPT: Pharmacometrics & Systems Pharmacology*, P.H.v.d.G. was not involved in the review or decision process for this article.

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