Translational pharmacokinetics-pharmacodynamics in zebrafish: integration of experimental and computational methods

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Section I. Introduction to high-throughput experimentation in whole vertebrates

Chapter 1

Introduction and scope

1.1 Systems pharmacology in drug development

Drug discovery and development integrates biomedical and pharmacological research at all scales; from elucidation of molecular pathways, through the identification of new chemical entities with the potential of becoming drug compounds, to testing those compounds *in vitro* and *in vivo*, and finally reaching clinical application[1]. Within this process, translation from one experimental context or species to another is important, yet challenging. Reliable translation requires quantitative understanding of underlying pharmacological and (patho)physiological processes.

Quantitative, (semi-)mechanistic pharmacometric modelling is advancing and developed models describe (patho)physiological and pharmacological mechanisms with increasing detail[2]. Quantitatively describing all relevant elements and interactions of a biological system (systems biology) and its interactions with the drug(s) perturbing it (systems pharmacology), improves prediction of drug effects in previously unstudied individuals, (sub)populations, or species. The systems-based approach characterizes the molecular profile of a dimension higher than the reductionistic one of a single molecule and a single target[3]. With that, systems pharmacology contributes to the paradigm shift from chemistry- and biology-based medicine to pathology-directed medicine or systems therapeutics[4].

The increased predictive power of systems pharmacology models comes at a cost of both increased model complexity and the requirement of larger and smarter datasets. It is unlikely that it is possible to gather all required data in these datasets from clinical practice or even mammalian studies as this is often too invasive, cumbersome, or costly. More resource efficient preclinical experiments are therefore necessary to capture all the necessary information on the relevant mechanisms in experimental data to allow for the development of mathematical systems-models that form the basis for the translation to early clinical trials[5]. Known differences in pharmacological or (patho)physiological processes between species can be incorporated to improve the interspecies translation. Starting this learn and confirm cycle with early preclinical data might improve the efficiency and adequacy of the whole developmental process[6]. Therefore, there is a strong need for experimental data and computational methods dedicated to systems pharmacology[7,8].

1.2 Zebrafish larva as vertebrate model organism with high-throughput potential

The zebrafish (*Danio rerio*), especially the zebrafish larva, is increasingly used in drug discovery and early drug development[9]. It forms a link between *in vitro* experiments and *in vivo* studies in mammalian species. The zebrafish is readily genetically modified to develop disease models because of its external fertilization[10], has large litter size, and its embryos and larvae are small and develop rapidly[11], which is ideal for high-throughput experiments. The suitability of this species for high-throughput experiments, with the potential for 1,000-10,000 assays per day[12], allows for the generation of a wealth of relevant data, in a relatively short time and at relatively low costs. But in contrast to *in vitro* or invertebrate experiments at similar rates, the zebrafish larva is a whole vertebrate organism and shows 70% genetic homology with humans[13]. The zebrafish larva is therefore a data and resource efficient model organism in pharmaceutical research[14]. Additionally, it is ethically preferable to perform *in vivo* experiments in the least developed organism, and no ethical approval is necessary for experiments before the larvae start independent feeding[15]. Moreover, zebrafish embryos and larvae in their early life stages are transparent which enables optical imaging of for example fluorescently labelled cells, tissues, or organs[16], or drugs[17]. Because of the non-invasiveness of this technique, repeated longitudinal measurements can be taken, following disease dynamics within an individual. The transparency of the larvae is lost after start of pigmentation, but a transparent adult zebrafish line has been developed to benefit from the possibilities of imaging at later life-stages[18]. Thus, by combining experimental efficiency with the translational potential of a whole vertebrate, the zebrafish larva is a unique model organism for systems pharmacology (Figure 1.1).

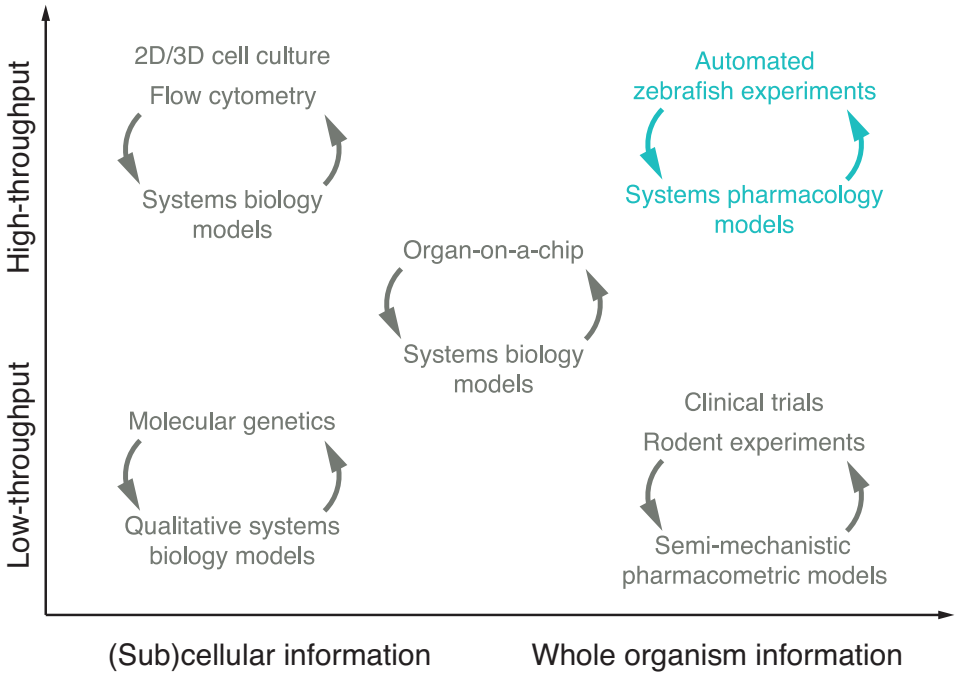

Figure 1.1 Overview of experimental and computational methods relevant for drug development. The horizontal axis specifies the level at which information is gathered, while the vertical axis specifies the speed at which data is collected. From Chapter 3.

1.3 Scope of this thesis

The zebrafish and zebrafish larvae thus bring many advantages to early drug development, which are further described in **Section I**. However, pharmacological experiments with zebrafish and zebrafish larvae are commonly performed by dissolving the drug of interest in the water surrounding the zebrafish. Quantification of the internal drug exposure is often neglected, thereby assuming that the internal and external drug concentrations are the same when interpreting data on the drug efficacy and/or toxicity. This assumption ignores the changes of internal exposure over time, which is driven by pharmacokinetic processes of absorption, distribution, metabolism, and excretion, and this may lead to unreliable interpretation of pharmacological findings (Figure 1.2).

This thesis covers three important elements within the understanding, quantification and subsequent translation of pharmacological findings, that are required for implementation of the zebrafish and zebrafish larva as full member of the preclinical drug development pipeline. It is the scope of our work to develop and integrate experimental and computational methods to quantify these three elements:

1. The internal exposure over time of drugs tested in zebrafish larvae needs to be quantified (**Section II**). Internal exposure is commonly not measured in zebrafish larvae, while it is the concentration at the target site that drives pharmacological effect[19].
2. The internal exposure needs to be linked to disease dynamics (**Section III**). When internal exposure is quantified, it can be related to the observed disease dynamics and changes therein. This will elucidate the onset, intensity, and duration of the drug response on the disease.
3. Differences between species in disease mechanisms need to be quantified, as these are essential for inter-species translation of drug response thereon (**Section IV**). To translate pharmacological findings from zebrafish larvae to higher vertebrates, the differences in pharmacological and (patho) physiological processes between the different species need to be taken into account.

1.4 Introduction to high-throughput experimentation in whole vertebrates

In this introductory Section I, the advantages of zebrafish larvae in drug development are described in detail. In **Chapter 2**, the genetic homology with humans is reviewed with a focus on hepatic metabolism. Using paracetamol (acetaminophen) as paradigm compound, the enzymes responsible for drug oxidation, sulfation, and glucuronidation are identified in the zebrafish. This makes the case that hepatic metabolism studied within the zebrafish, can translate to higher vertebrates. **Chapter 3** gives our perspective on the position of the zebrafish larva within drug development using automation and high-throughput experimentation. Innovative computational methods like outside-in model identification can analyse these large amounts of data obtained in high-throughput experiments in early drug discovery and development to identify the relevant features in a (patho)physiological network upon perturbation thereof. **Chapter 4** gives an example of an experimental set-up that has the potential of high-throughput biomedical data gathering in zebrafish larvae; the Vertebrate Automated Screening Technology (VAST). This bioimager can be used to quantify the volume and surface area of the full zebrafish larva or from fluorescently marked cell-types, tissues, or organs.

1.5 Quantification of internal exposure over time

Quantification of internal exposure over time in zebrafish larvae is challenging because of small sample volumes and low drug amounts in these samples. It requires sensitive bioanalytical techniques in combination with experimental innovations and model-based analysis. In Section II, paracetamol is used as paradigm compound to study internal drug exposure in zebrafish larvae. **Chapter 5** introduces the first pharmacokinetic analysis in zebrafish larvae of 3 days post fertilization after waterborne paracetamol treatment. Two experiments are performed, a constant waterborne treatment experiment and a wash-out experiment. Using internal exposure data gathered from these two experiments, a pharmacokinetic model is developed, which results in a clearance estimate relative to the total volume of the larvae, which is compared to clearance in higher vertebrates. **Chapter 6** expands this line of experimentation to answer the question what the impact of larval age is on pharmacokinetic parameters. In this fast developing organism, a single day can make a difference. Based on this analysis, it is explored at what age pharmacokinetic experiments can be performed best. In **Chapter 7**, the elimination of paracetamol is studied in mechanistic detail. Paracetamol and its two major metabolites are measured, to confirm functionally that metabolism is similar between species, in addition to the genetic homology of metabolising enzymes from Chapter 2. A method to draw nanoscale blood samples is developed resulting in data on blood concentrations, necessary for the pharmacokinetic metabolite model to estimate volume of distribution and absolute clearance, both of which are compared to higher vertebrates.

1.6 Linking internal exposure to disease dynamics

Section III focusses on linking internal exposure to disease dynamics. Repeated measurements of

Figure 1.2 Internal drug exposure over time inside the zebrafish or zebrafish larva needs to be quantified in pharmacological or toxicological studies. It is commonly assumed that internal drug concentration in zebrafish or zebrafish larvae is similar to the external concentration of the waterborne treatment and constant over time (middle). It is however more likely that the concentration inside the zebrafish is lower than outside because of slow absorption or fast elimination (left). Another possibility is drug accumulation inside the zebrafish, where the internal concentration is higher than the external one (right). Quantification of the internal exposure is of importance, because that drives the effect.

biomarkers reflecting a disease during the full time course of treatment improve understanding of the drug response on that disease. The resulting information on the disease dynamics can be used to characterize onset, intensity, and duration of the response of the drug. In **Chapter 8**, tumour size is repeatedly quantified in the neuroblastoma disease model in zebrafish[20] upon waterborne treatment with isotretinoin (13-cis-retinoic acid). The internal exposure over time of this photo-sensitive compound, and of its photo-isomers 9-cis-retinoic acid and all-trans-retinoic acid, is quantified as well. These pharmacodynamic and pharmacokinetic experiments are necessary to link internal exposure to response.

1.7 Mechanistic and quantitative translation of exposure-response from zebrafish to higher vertebrates

Translation of pharmacological findings to higher vertebrates needs to take into account the differences between the different species regarding disease mechanisms and response to pharmacological perturbations. A quantitative understanding of these differences will improve translation of findings for new drugs for a disease. Section IV focusses on that quantitative translation based on tuberculosis, a disease studied extensively in zebrafish[21]. In **Chapter 9**, the natural growth of two strains of *Mycobacterium marinum* are studied over an extensive period of time. The established multistate tuberculosis pharmacometric model[22], developed to quantify natural growth of *Mycobacterium tuberculosis* and drug effect thereon in humans and higher vertebrates, is utilized to quantify the *M. marinum* natural growth data. **Chapter 10** uses one of the *M. marinum* strains to infect zebrafish embryos and subsequently treat them with waterborne isoniazid at increasing doses. Infection is quantified by repeated fluorescence imaging and a quantitative, internal exposure-response relationship is developed. This quantitative relationship is subsequently translated to humans, utilizing translational factors to account for differences in disease mechanisms.

1.8 Discussion, perspectives, and conclusion

The results of the previous sections are discussed in **Chapter 11**, including our recommendations for the inclusion of zebrafish and zebrafish larvae in the drug development pipeline. There is large potential for this model organism to answer pharmacological questions at an early stage in drug development. We envision future perspectives and application in this context, including further extension and improvement of the experimental and computational methodologies that we have developed in this thesis with state-of-the-art methods under development at this point.

1.9 References

1. Lesko LJ, Rowland M, Peck CC, et al. Optimizing the science of drug development: opportunities for better candidate selection and accelerated evaluation in humans. J Clin Pharmacol. 2000;40(8):803-814.
2. Vicini P, Van der Graaf PH. Systems pharmacology for drug discovery and development: paradigm shift or flash in the pan? Clin Pharmacol Ther. 2013;93(5):379-381.
3. Van der Greef J, McBurney RN. Rescuing drug discovery: In vivo systems pathology and systems pharmacology. Nat Rev Drug Discov. 2005;4(12):961-967.
4. Danhof M, Klein K, Stolk P, et al. The future of drug development: The paradigm shift towards systems therapeutics. Drug Discov Today. 2018;23(12):1990-1995.
5. Musuamba F, Manolis E, Holford N, et al. Advanced methods for dose and regimen finding during drug development: summary of the EMA/EFPIA workshop on dose finding (London 4-5 December 2014). CPT Pharmacometrics Syst Pharmacol. 2017;6:418-429.
6. Sheiner LB. Learning versus confirming in clinical drug development. Clin Pharmacol Ther.

1997;61(3):275-291.
7. Nijsen MJ, Wu F, Bansal L, et al. Preclinical QSP modeling in the pharmaceutical industry: an IQ consortium survey examining the current landscape. CPT Pharmacometrics Syst Pharmacol. 2018:Accepted article.
8. Van der Graaf PH. Pharmacometrics and/or systems pharmacology. CPT Pharmacometrics Syst Pharmacol. 2019;8(6):331-332.
9. Rennekamp AJ, Peterson RT. 15 years of zebrafish chemical screening. Curr Opin Chem Biol. 2015;24:58-70.
10. Howe DG, Bradford YM, Eagle A, et al. The zebrafish model organism database: New support for human disease models, mutation details, gene expression phenotypes and searching. Nucleic Acids Res. 2017;45(D1):D758-D768.
11. Kimmel CB, Ballard WW, Kimmel SR, et al. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203:253-310.
12. Ali S, Champagne DL, Spaink HP, Richardson MK. Zebrafish embryos and larvae: A new generation of disease models and drug screens. Birth Defect Res (Part C). 2011;93:115-133.
13. Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496:498-503.
14. Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov. 2005;4(1):35-44.
15. Strähle U, Scholz S, Geisler R, et al. Zebrafish embryos as an alternative to animal experiments — A commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod Toxicol. 2012;33:128-132.
16. Pulak R. Tools for automating the imaging of zebrafish larvae. Methods. 2016;96:118-126.
17. Li Y, Chen T, Miao X, et al. Zebrafish: A promising in vivo model for assessing the delivery of natural products, fluorescence dyes and drugs across the blood-brain barrier. Pharmacol Res. 2017;125(Part B):246-257.
18. White RM, Sessa A, Burke C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell. 2008;2(2):183-189.
19. Morgan P, Van der Graaf PH, Arrowsmith J, et al. Can the flow of medicines be improved? Fundamental pharmacokinetic and pharmacological principles toward improving Phase II survival. Drug Discov Today. 2012;17(9/10):419-424.
20. He S, Mansour MR, Zimmerman MW, et al. Synergy between loss of NF1 and overexpression of MYCN in neuroblastoma is mediated by the GAP-related domain. eLife. 2016;5:e14713.
21. Meijer AH, Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets. 2011;12(7):1000-1017.
22. Clewe O, Aulin L, Hu Y, et al. A multistate tuberculosis pharmacometric model: A framework for studying anti-tubercular drug effects in vitro. J Antimicrob Chemother. 2016;71(4):964-974.CHapter Chapter