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

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Section V. Summary, discussion, and conclusion

Chapter 11

Summary, discussion, and conclusion

11.1 The zebrafish in systems pharmacology

The development of new efficacious and safe drugs is one of the most challenging endeavours in biomedical research. The process includes research on all biomedical scales, from molecular and (sub)cellular scales to whole organisms including different animal species, before reaching the clinical phase. The right target, compound, and dose for treatment of a disease are selected based on prior knowledge and experimental results, for which reliable translation between different experimental contexts and species is essential. Quantitative pharmacology is key in steering that translation into the right direction[1]. Pharmacokinetic parameters – the absorption rate constant, distribution volume, and clearance – describing the dose-exposure relationship over time, can be translated or scaled between species[2]. The same is true for pharmacodynamic parameters that describe the exposure-response relationship, like turnover rates, or drug potency (EC_{50}) or efficacy (E_{max})[3]. The more mechanistic a quantitative pharmacological model is, the more reliable the translation can be expected to be. Integrating quantitative pharmacology with the mechanistic discipline of systems biology – defined as systems pharmacology – therefore instils more confidence in the translation of exposure-response relationships[4]. Systems pharmacology is part of the new systems therapeutics paradigm, which considers all relevant actors in a (patho)physiological system instead of a single interaction between drug and target[5].

Systems pharmacology has the potential to become the standard in drug development to address mechanistic questions on disease and drug pharmacology[6]. It does lean heavily on preclinical data to identify and characterize (patho)physiological processes. However, a lack of dedicated experimental data may hinder successful implementation of systems pharmacology[7]. This is especially the case for data obtained in the full biological context of a whole organism, rather than from isolated *in vitro* assays. Moreover, because of constraints in both resources and time, high-throughput experimentation is preferable. It is unlikely that acquisition of these types and these amounts of experimental data will be feasible in clinical practice or even preclinical mammalian experiments. Conventional *in vitro* experiments are performed at this scale and speed, but lack information on the biological context. Innovative organ-on-a-chip systems, consisting of human cell cultures grown under physiological conditions on microfluidic devices to represent living and functioning organ tissues[8,9], is an improvement in that respect. However, drug targets in different tissue, off-target effects, or physiological signaling between tissues are still not included[10]. Experiments at *in vitro* scale and throughput but within the proper biological context are therefore needed.

The zebrafish (*Danio rerio*) has become an established vertebrate model organism in biomedical research[11]. Especially in its larval stage it is often used, because of its many advantages including optical transparency for non-invasive observations of cells, tissues, or organs. Of all human genes, 71% has an orthologue in the zebrafish, of the human genes involved in disease this is 82%[12]. For comparison, the mouse, a conventional preclinical mammalian species, has an orthologue of 83% of all human genes[12]. However, experimental studies including development of disease models using mice are longer in duration and more expensive than in zebrafish. Because of its external fertilization, genetically modification in zebrafish is much easier compared to higher vertebrates, to create models for human disease[13]. To create a model for human disease for example in the mouse, genetic modification of murine embryos is required which subsequently need to be implanted in a carrier mouse and carried until term[14]. Moreover, the advantages of small size, large litter size, and fast development, make the zebrafish larva an ideal model organism for high-throughput experimentation[15]. It is also ethically preferable to perform *in vivo* experiments in the least developed organism and no ethical approval is required for experimentation on the larval stage of the zebrafish (i.e. first 5 days post fertilization)[16]. The zebrafish larva therefore combines the experimental efficiency of high-throughput potential with the translational value of result from the biological context of a whole vertebrate organism.

Pharmacological or toxicological experiments with zebrafish and zebrafish larvae are commonly performed by dissolving drugs into the water in which the zebrafish swim. Drug effects, or lack thereof,

are interpreted based on the assumption that the external drug concentration reflects the internal concentration inside the zebrafish or zebrafish larva. This assumption ignores basic pharmacokinetics; absorption, distribution, metabolism, and excretion of drugs by an organism, that causes the internal drug exposure to change over time and potentially reach internal steady state concentrations different from the external concentration. This is important because it is the internal exposure at the target site that drives the drug effect[17]. Therefore, ignoring this concentration, and its changes over time, hinders interpretation of drug effects. For translation of drug effects to higher vertebrates, including humans, it is also important to link the internal exposure (pharmacokinetics) to measures of the drug response (pharmacodynamics), to quantify the exposure-response, or pharmacokinetic-pharmacodynamic relationship. To quantify the drug response, the disease dynamics, and changes therein upon treatment, need to be measured, as well as between-species differences in the underlying (patho)physiological and pharmacological processes. A model-based approach can then correct for these differences with translational factors, for inter-species translation of drug effects.

In this thesis, we have therefore developed and integrated innovative experimental and computational methods for the quantification of:

I) internal drug exposure over time in zebrafish larvae after waterborne drug treatment;
II) disease dynamics and drug-induced changes therein;
III) between-species differences in disease mechanisms and drug effects thereon.

Only with a quantitative understanding of these three elements can pharmacological findings be reliably translated and can the zebrafish become a full member of the preclinical drug development pipeline.

11.2 Introduction to high-throughput experimentation in whole vertebrate

The advantages of pharmacological experiments in zebrafish were introduced in **Section I**. Firstly, the need for quantification of internal exposure over time was introduced. It is important to have a mechanistic and quantitative understanding of drug metabolism by the zebrafish. Drug metabolites resulting from biotransformation in for example the liver, can be pharmacologically active or toxic. We therefore reviewed hepatic metabolism of the zebrafish in the context of systems pharmacology in **Chapter 2**. The metabolising enzymes in zebrafish were compared to their human orthologues, with a focus on oxidative and conjugative metabolism involved in the metabolism of our paradigm compound paracetamol (acetaminophen). Genes related to the most important cytochrome P450 (CYP) enzyme isoforms in humans catalysing oxidative metabolism, CYP3A4 and CYP2E1, are expressed in zebrafish as well. The same holds for sulfotransferases, which catalyse conjugation of drugs with sulfate-groups. The enzymes responsible for glucuronidation in humans, the UDP-glucuronosyltransferases, have paralogues in zebrafish, enzymes with comparable metabolic function. The genetic comparison from this chapter, showing similar enzymes for drug metabolism between zebrafish and humans, suggested that this species could yield meaningful results regarding effects from potential drug metabolites. It is important to quantify the internal drug exposure, including their metabolites, to confirm this suggestion. Internal exposure could subsequently be linked to measures of hepatic dysfunction, like biomarkers or organ size.

An important advantage of the zebrafish for pharmacological experiments is its potential for high-throughput experimentation. In **Chapter 3** we introduced the possibilities of high-throughput zebrafish experiments to support systems pharmacology model development. Systems pharmacology models require large datasets that inform on the behaviour of the (patho)physiological system upon pharmacological perturbation. These datasets should include relevant information on all intended and unintended targets and pathways. Because of constraints in both time and resources, experimental data should be gathered as efficiently as possible. This is possible with high-throughput experimentation. In contrast to conventionally used organisms for high-throughput experiments, like yeast (*Saccharomyces*

cerevisiae), round worms (*Caenorhabditis elegans*), or fruit flies (*Drosophila melanogaster*), the zebrafish is a vertebrate organism with larger anticipated translational power to higher vertebrates used in drug development, than these invertebrate species (**Figure 3.2**). In this chapter, we suggested an innovative analysis method for early drug discovery and development. Outside-in model identification is a technique that uses oscillating stimuli at different frequencies to construct a model structure between input and output without prior knowledge on the system driving the output. Using microfluidic devices specifically designed for zebrafish larvae, waterborne stimuli like drug exposure could be tested at different frequencies and observations of for example fluorescence markers from the zebrafish could subsequently be analysed by outside-in modelling. This approach, characterized by a requirement for close collaboration between experimental and computational researchers, would lead to an understanding of rate-limiting steps in the relevant (patho)physiological pathways early in drug development process.

Zebrafish larvae are transparent which is ideal for optical imaging. A microscopy set-up with high-throughput potential that takes advantage of this, is the Vertebrate Automated Screening Technology (VAST) BioImager. The VAST BioImager consists of a capillary in which zebrafish larvae are automatically loaded from a sampling tube or well plate. The capillary is located under a microscope and rotates to capture images of the larva from all angles. **Chapter 4** introduced the application of this method to zebrafish larvae with a fluorescent marker expressed in the liver. By taking multiple images from a total of 25 different angles, a three-dimensional reconstruction of the full larva and of the liver of the larva can be made. Subsequent quantification of surface area and volume of the whole organism, and the liver therein, results in much more information than two-dimensional imaging. The proof of concept in this chapter utilized only a limited number of larvae to develop the imaging architecture and reconstruction algorithm. This method could in the future be applied to quantify shape and volume of different organs during larval development, to constitute a reference database that can for example be used for physiology-based modelling. Additionally, high-throughput screening could be performed of for example impact of hepatotoxic drugs on liver volume and shape, or other application in which organ size is a measure of efficacy or toxicity. With such automated objective and quantitative measurements of drug effects over time, an exposure-response relationship of the drug can be determined, necessary for translation of drug effects to higher vertebrates.

11.2.1 Methodological innovation

- We developed an imaging method to enable high-throughput data acquisition on fluorescently labelled tissues or organs in zebrafish larvae upon pharmacological perturbation.

11.2.2 Key messages

- Internal exposure of a drug over time is essential for proper interpretation and translation of drug effects and side effects.

- Genetic homology in metabolic enzymes between zebrafish and humans suggests experiments in zebrafish to yield meaningful results regarding metabolite formation.

- Zebrafish larvae are ideal whole-vertebrate experimental subjects for high-throughput experiments in systems pharmacology.

- Close collaboration between experimental and computational scientists is important in systems pharmacology.

11.3 Quantification of internal exposure over time

The concentration of a drug at the target site drives drug effects[17]. It is therefore essential to quantify the internal exposure in zebrafish larvae after waterborne drug treatment when assessing drug effects. **Section II** focussed on the development of experimental, bioanalytical, and computational methods to quantify internal drug exposure over time and characterize the pharmacokinetic processes of absorption, distribution, metabolism, and excretion. Paracetamol was used as paradigm compound. Because of the small larval size of only several hundred nanolitres – quantified previously using the VAST BioImager[18] – and low drug amounts in the larvae, it is recognized to be challenging to measure internal drug exposure[19].

As a proof of concept, in **Chapter 5** the internal exposure over time of paracetamol and its two major metabolites was quantified in zebrafish larvae of 3 days post fertilization (dpf). Two types of experiments were performed. In the first, zebrafish larvae were exposed to a constant concentration of waterborne paracetamol for 180 minutes, while in the second the larvae were transferred to drug-free medium after 60 minutes of waterborne paracetamol treatment, to study elimination for an additional 240 minutes. A liquid chromatography-mass spectrometry (LC-MS/MS) method was developed to quantify the paradigm compound paracetamol and its major metabolites paracetamol-glucuronide and paracetamol-sulfate in homogenate samples. Steady state values of paracetamol amounts were reached within 120 minutes of waterborne treatment. In the elimination experiment, paracetamol amounts showed a mono-exponential decline, while internal amounts of its metabolites increased at first, before declining as well after 120 minutes. The major metabolite of paracetamol in the zebrafish larva at 3 dpf was paracetamol-sulfate, which was more abundant than paracetamol-glucuronide. This is in contrast to the metabolic profile of adult humans, in which paracetamol-glucuronide has been reported to be the major metabolite, accounting for 50% of the dose or more[20–22]. However, studies in human neonates and infants have shown that because of maturation of metabolic pathways, paracetamol-sulfate is more abundant than paracetamol-glucuronide[23–26]. Because zebrafish larvae have also not yet reached maturity, a similar metabolic maturation could explain the ratio between paracetamol-glucuronide and paracetamol-sulfate.

Paracetamol amounts over time from zebrafish homogenates were analysed by non-linear mixed effects modelling. The pharmacokinetic model quantified the absorption rate constant and the elimination rate constant. Distribution of paracetamol was assumed to be homogenous throughout the total larval volume. This assumption was necessary to calculate absolute clearance values from the elimination rate constant, as blood concentrations of paracetamol were lacking. Absolute clearance is of importance because it is required for the inter-species scaling of this important pharmacokinetic parameter. To assess if scaling parameters quantified in zebrafish to higher vertebrates was reliable, absolute paracetamol clearance was compared to reported values in 12 higher vertebrates from literature. This scaling is based on the established allometric theory, which states that bodyweight is a predictor of drug clearance between species[27,28]. Inter-species scaling is always based on assumptions, like those in allometric theory, but also here on paracetamol distribution, or the lack of impact of maturation or temperature on the quantification of the parameter of interest. The strength of our quantitative model-based approach, is that these assumptions can be tested and corrected, which instils confidence in our model-based interspecies translation of pharmacological parameters from zebrafish to higher vertebrates.

The zebrafish larva at 3 dpf showed a metabolic profile of paracetamol similar to neonates and infants, suggesting an impact of immaturity on metabolic clearance, and possibly on other pharmacokinetic parameters as well. Zebrafish development is rapid[29] and adulthood is reached within 3 months[30]. It is therefore of interest to study the impact of age on the pharmacokinetics of paracetamol in zebrafish larvae. In **Chapter 6**, we therefore repeated the experiments from Chapter 5 with zebrafish larvae of 4 and 5 dpf. Internal paracetamol amounts showed an increase in plateau values between 3 and 4

dpf, but not between 4 and 5 dpf. The mono-exponential decline of paracetamol amounts observed in the elimination experiment became steeper with age. However, no change in the ratio between paracetamol-glucuronide and paracetamol-sulfate was found (data not shown). It was concluded that an impact of maturation was clear on absorption and elimination. However, the maturation in both the sulfation and glucuronidation pathways were not observed to impact the metabolite ratio between these two metabolites within the three days studied here.

The data on paracetamol amounts from the two experiments in zebrafish larvae of 3, 4, and 5 dpf were combined and analysed using a pharmacokinetic model with age as covariate on absorption and elimination. The relationship between age and absorption rate was discrete, resulting in an increase of the absorption rate constant of 106% between 3 and 4 dpf. This was attributed to the opening of the gastro-intestinal (GI) tract, which completes at 4 dpf[16,31]. When the GI-tract is fully open, oral absorption, in addition to transdermal absorption, contributes to the internal exposure over time. A similar effect has been reported for the antihistamine diphenhydramine[32]. Age was related to paracetamol elimination following a power function, where the elimination rate constant increased 17.5% per dpf. This increase in elimination as a result of metabolism and excretion, might result from growth of the responsible organs, as well as from maturation of enzyme systems and/or transporters[33]. Absolute clearance was calculated as previously described and showed an increase with age and a shift towards the allometric relationship of paracetamol clearance from the 12 higher vertebrates (**Figure 6.4**). It was clear that age had an impact on the internal exposure over time, at least for our paradigm compound paracetamol. This means that a difference of a single day can influence the internal drug exposure over time and corresponding outcome measures. Because both drugs and their metabolites can be pharmacologically active, we advise to perform short-term drug treatment experiments in zebrafish larvae at 5 dpf, optimizing exposure both to the parent drug, because of increased absorption in comparison to 3 dpf, and to drug metabolites, because of increased anticipated metabolism.

The zebrafish was shown to express the metabolic enzymes responsible for oxidation and conjugation of our paradigm compound paracetamol (Chapter 2). The major metabolites paracetamol-glucuronide and paracetamol-sulfate were observed after waterborne treatment with paracetamol (Chapter 5). In **Chapter 7**, this metabolism was studied in more mechanistic and quantitative detail, using a pharmacokinetic metabolite model. The paracetamol internal exposure data at 5 dpf (Chapter 6) were combined with internal exposure data in these larvae of the paracetamol-glucuronide and paracetamol-sulfate, and amounts of parent and metabolites excreted into the treatment medium. A quantification of the pharmacokinetic processes of distribution and absolute clearance is essential for reliable inter-species extrapolation of these parameters. Quantification thereof requires blood concentrations in addition to total amounts. Although methods had been developed to sample blood from adult zebrafish[34–36], this was not yet possible for zebrafish larvae. We have therefore developed a method to draw blood samples from zebrafish larvae of 5 dpf, based on puncture of the posterior cardinal vein[37] by a needle pulled from a capillary with an original diameter of 0.75 mm. The resulting nanolitre-scale blood samples were pooled together to reach quantifiable levels. In these blood samples, both paracetamol and its major metabolites were measured. Paracetamol concentrations were only 10% of external concentrations, which was also reported elsewhere[38]. The data on blood concentrations, combined with the total paracetamol and metabolite amounts from homogenates and amounts excreted to the treatment medium, were simultaneously analysed using the metabolite model we developed. Both absolute paracetamol clearance and volume of distribution obtained with this method correlated well with those reported in higher vertebrates (**Figure 7.5**, **Figure 7.6**).

The formation, distribution, and excretion of the major metabolites of paracetamol were quantified. It was especially of interest to be able to quantify volume of the distribution for the two metabolites. Normally, assumptions on volume of distribution are needed for a metabolite model to remain mathematically identifiable without data on total metabolite amounts[26,39]. Here however, with data on

both total amounts and blood concentrations for these metabolites available, the distribution volume could be quantified by the model. Biotransformation of paracetamol to paracetamol-sulfate was found to be time-dependent. Within the studied time period, sulfation decreased to a minimum, which could be explained by depletion of the sulfate-group donor necessary for this biotransformation[40–42]. It would be interesting to extend the time period of the experiments to be able to include the production of the sulfate-group donor by the zebrafish larva[43], or measure it directly[44].

We have developed and integrated experimental, bioanalytical and computational methods to quantify internal exposure over time in zebrafish larvae after waterborne drug treatment. Model-based quantification of the pharmacokinetic processes enabled a quantitative understanding of the changes of internal exposure over time and subsequent inter-species extrapolation of drug clearance and volume of distribution. When in conventional fish studies internal exposure is of interest, the bioconcentration factor (BCF) is applied[45]. This ratio of internal to external concentration is more useful than just the external concentration, but ignores the kinetics of concentrations changing over time, and is therefore highly variable and dependent on experimental design[46]. Recently, internal exposure over time was studied in zebrafish for other molecules (metals[47–50] or microplastics[51]) or in other disciplines (environmental sciences[52,53] or toxicology[54–58]), but not analysed to the extent of the quantitative translational model-based approach of this section. It was our focus to quantitatively understand internal exposure over time and to enable translation of pharmacokinetic parameters between species, essential for the role of the zebrafish in drug development.

11.3.1 Methodological innovations

- We developed a nanoscale blood sampling method in zebrafish larvae to enable the quantification of drug concentrations in blood, in addition to drug amounts in homogenates.
- We developed LC-MS/MS methods sensitive enough for zebrafish samples of small volumes, to quantify drug and metabolites internal exposure over time.
- We developed non-linear mixed effects models to quantify distribution volume and absolute drug clearance in zebrafish larvae for the first time, essential for the translation of drug pharmacokinetics to higher vertebrates.

11.3.2 Key messages

- For the first time, the translational potential for scaling pharmacokinetics from zebrafish larvae to higher vertebrates has been substantiated.
- Short-term drug treatment experiments in zebrafish larvae are best performed at 5 dpf
- The immaturity of zebrafish larvae has implications for both pharmacokinetics, and the interpretation of drug effects and inter-species translation thereof.

11.4 Linking internal exposure to disease dynamics

A disease is not static but shows changes over time, as the result of disease progression or drug treatment[59]. It is therefore important to have repeated measures of disease dynamics and to link these to internal drug exposure to quantify the drug effect thereon, which was the focus of **Section III**. As disease model, the recently developed zebrafish model for neuroblastoma was studied. This transgenic

zebrafish line spontaneously develops a neuroblastoma tumour after three weeks, which also expresses a fluorescence marker[60]. By fluorescence microscopy tumour size could be quantified and it was reported that isotretinoin (13-cis-retinoic acid) had an effect on neuroblastoma development after 7 days of waterborne treatment[60]. In **Chapter 8**, we quantified tumour development in juvenile zebrafish by multiple measures of tumour size over the 7 days of treatment with 0, 1, 1.5, and 2 µM of waterborne isotretinoin, and linked these to quantified internal exposure of isotretinoin.

Quantification of isotretinoin proved to be challenging because it is a photo-sensitive compound[61–63]. Exposure to ultraviolet (UV) light results in isomerization of isotretinoin into 9-cis-retinoic acid and all-trans-retinoic acid. A sensitive LC-MS/MS method was developed to distinguish the isomers. Additionally, the UV-exposure was minimized by performing experiments after sunset or in laboratories without windows. This resulted in less than 10% of isomerization in the treatment medium and on average 16.4% within the (transparent) juvenile zebrafish.

It was anticipated that internal exposure would reach a plateau after waterborne treatment for a prolonged period of time, similar to that of paracetamol in the previous section. However, after start of treatment and the two treatment medium refreshments, a peak of isotretinoin amounts was followed by a 100-fold drop in internal exposure. Subsequent quantification of corresponding treatment medium samples showed a concentration lower than the nominal dose at the onset of the experiment and this external concentration also declined over the dosing intervals. Model-based analysis of both the internal exposure in the zebrafish and the external concentration simultaneously, characterized these profiles and the absorption from and excretion into the treatment medium by the juvenile zebrafish.

Every 24 hours, zebrafish were fixated to quantify tumour area by two-dimensional fluorescence microscopy. Unfortunately, no statistically significant difference was observed between the different dose groups and control, so no drug effect on the tumour size could be quantified. The lack of drug effect in our experiments could be explained by the internal exposure profile. The internal exposure of isotretinoin was because of the fast decline upon drug treatment, subtherapeutic for the majority of the dosing interval. This result underlines the importance of quantification of internal drug exposure over time, to enable reliable interpretation of observed drug effects, or lack thereof.

Another reason for the lack of a significant drug effects might be the high variability in tumour size per individual juvenile zebrafish. Because we took single measurements per individual, we could not correct for this inter-individual variability. This was in contrast to previous reports, where measurements before and after treatment within the same zebrafish were taken to quantify drug effect[60]. Tumour size measurements could be more precise when instead of its two-dimensional area, its three-dimensional surface area and volume was quantified. Optical projection tomography (OPT) is a three-dimensional microscopy set-up similar to the VAST BioImager but suitable for samples larger than a capillary, like the juvenile zebrafish[64,65]. The advantage of the transparent zebrafish, in contrast to more opaque OPT samples like murine heart tissue, is no sample clearing is necessary to reveal the fluorescent signal of the tumour[66]. A feasibility study with a representative zebrafish with fluorescent neuroblastoma was performed, which yielded promising results (**Figure 8.6**). Three-dimensionally, the tumour was better distinguishable and not partly hidden by the melanocyte-umbrella[67]. Another improvement to quantify tumour dynamics over time could be the repeated measurements of biomarkers as measure of (patho) physiological changes[68,69].

Alternatively, protein binding of isotretinoin[70] might be of influence as it prevents target binding. However, a major drug binding plasma protein, albumin, is absent in zebrafish and no other proteins of importance to drug binding were reported in plasma[71,72]. Another consideration could be the fact that the mechanism of action of isotretinoin was not fully elucidated and its isomers might be responsible for (part of) the drug effects[63,73]. In that case the observed effects from previous studies might have been

diminished as a result of our endeavours to minimize photo-isomerisation. Application of the integrative experimental and computational methods we developed here could be used to test this hypothesis of active isomers and contribute to the elucidation of the mechanism of action of isotretinoin.

11.4.1 Methodological innovation

- We developed a three-dimensional microscopy method to quantify tumour size in juvenile zebrafish more accurately and precisely in comparison to conventional two-dimensional microscopy.

11.4.2 Key messages

- The common assumption that external and internal drug concentrations are constant upon waterborne drug treatment of zebrafish should always be tested by quantitative measurements of both zebrafish and treatment medium over time.

- Pharmacokinetic and pharmacodynamic experiments, quantifying both internal drug exposure over time, and changes in disease dynamics over time upon drug exposure, should both be performed to interpret and quantify drug effects or lack thereof.

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

An exposure-response relationship, linking internal drug exposure to the disease dynamics, is the fundament of inter-species translation of drug effects. Another important element for this inter-species translation is taking into account between-species differences in disease mechanisms. The (patho) physiological processes might differ between vertebrates, for which a quantitative model-based approach can correct by utilizing translational factors. **Section IV** focussed on quantification of between-species differences in (patho)physiological processes to the benefit of mechanistic and quantitative translation of exposure-response from zebrafish to higher vertebrates. Specific focus was on tuberculosis (TB), of which the pathology and treatment has been studied extensively in the zebrafish[74–79]. Zebrafish larvae infected with the aquatic pathogen *Mycobacterium marinum*, a close relative of the human pathogen *Mycobacterium tuberculosis*, served as disease model[80].

In **Chapter 9**, the natural growth of *M. marinum* was studied and compared to that of *M. tuberculosis*. Two strains, a poikilotherm-derived strain E11[81] and a human-derived strain M^{USA} [82], were grown undisturbed for more than 200 days and viability assessed as colony forming units (CFU) was determined at different timepoints. An established TB-model, the multistate tuberculosis pharmacometric (MTP) model[83,84], was utilized to quantitatively characterize this natural growth. The MTP model distinguishes three states of mycobacteria, a fast-multiplying, a slow-multiplying, and a non-multiplying state. The natural growth of *M. tuberculosis* is characterized in this model by the quantified growth rates and transfer rates between the states. Distinction of mycobacteria in the different multiplying states is of importance, because antibiotic effects on the mycobacteria could differ depending on their growth behaviour. The MTP model was successfully applied to quantify drug treatment of *M. tuberculosis in vitro*[83,85], in mice[86,87], and in patients[88,89]. It was here applied to the natural growth data of the two strains of *M. marinum*, to be able to compare their natural growth to that of *M. tuberculosis*. The E11 strain showed a more latent growth behaviour most similar to that of *M. tuberculosis*, while M^{USA} growth was more aggressive (**Figure 9.1**). These findings were in line with previous results[81]. Because growth rates of E11 and *M. tuberculosis* were also most similar, we suggested that the use of E11 was most preferable when studying TB in zebrafish.

Quantification of the internal exposure-response relationship in the zebrafish infected with *M. marinum* as disease model for TB was the focus of **Chapter 10**, with the aim to assess how reliable a translation of the drug effect to humans would be based on such a quantitative internal exposure-response relationship. The antibiotic isoniazid, which shows largest bactericidal activity of the standard of care drugs currently used against TB in the first days of treatment[90], was chosen as paradigm compound. Zebrafish larvae were infected with E11, per the recommendation of Chapter 9, at 28 hours post fertilization. Waterborne treatment with increasing doses of isoniazid at 0.25-10x minimum inhibitory concentration (MIC) started after the infection had 2 days to establish. Internal exposure was quantified in homogenates and blood samples. Fluorescent *M. marinum* were utilized to enable repeated measurements of bacterial burden within individual larvae by non-invasive fluorescence imaging. Automated fluorescent image analysis[91,92] was applied to these images to quantify bacterial burden based on fluorescent pixel count. Simultaneous pharmacokinetic-pharmacodynamic modelling was performed on the combined data of total drug amounts in homogenates, drug concentrations in blood samples, and bacterial burden from fluorescence imaging. Internal exposure reached steady state within 12 hours, but steady state levels increased with each day post fertilization. An impact of age was quantified on the absorption of isoniazid, similar to Chapter 5. Age was also expected to impact elimination, but because the majority of data was obtained when internal amounts were at steady state when rate of absorption and rate of elimination are equal, a distinctive impact on both absorption and elimination was not mathematically identifiable based on the current data. The age effect on absorption should therefore be interpreted as a net increase with age of absorption relative to that of elimination. Blood concentration of isoniazid was only 20% of external concentrations. The bacterial burden showed exponential growth and a decrease in growth with increasing isoniazid exposure. A dose of 5x MIC in the incubation medium resulted in bacteriostasis, which was expected when taking into account the relationship between internal and external concentrations.

As proof of concept for translation, the quantitative exposure-response relationship of isoniazid was translated to humans. Isoniazid concentration over time in humans was simulated for the therapeutic dose of 300 mg, based on a previously reported pharmacokinetic model[93]. Two translational factors were included for the translation, to correct for between-species differences in disease mechanisms and pharmacological response[89]. First, the difference in sensitivity to isoniazid of *M. marinum* and *M. tuberculosis*, as reported by their respective MICs, was taken into account. Second, the difference in stage of infection was taken into account. The fresh infection in zebrafish showed growth in the logarithmic stage, while clinical infections were expected to be in the stationary stage. As described above, drug effect on mycobacteria will differ between different growth behaviour. The MTP model was previously applied to quantify isoniazid effect on *M. tuberculosis* on the different states[85]. The ratio of maximal kill rate on the different states was assumed to reflect the difference in drug effect between the logarithmic stage and stationary stage of infection. With these translational factors, the translation of the exposure-response relationship of isoniazid quantified in zebrafish, correlated reasonably with observations from literature in humans[94–96] (**Figure 10.7**). This was especially the case for the first two days of treatment, the same time period of our experiments. After two days, the isoniazid drug effect was overestimated, which corresponded to reports that the first two days of isoniazid treatment showed a more rapid decline in bacterial burden than after the first two days[90,97]. This decreased drug effect after two days was not quantifiable in the short experiment within the zebrafish larva.

Based on our results from Chapter 9 and Chapter 10, we are confident that the zebrafish is a reliable addition to the preclinical TB drug development workflow using the MTP model. The MTP model has been used previously to quantify drug effects of the current standard of care against TB *in vitro*[83,85], in mice[86,87], and in humans[88,89]. Its application to new anti-TB drugs is the next step for preclinical drug development. The zebrafish is an attractive model organism to test new drugs after *in vitro* experiments, but before mammalian studies. This will result in more information on drug candidates earlier in anti-TB drug development, and with the predictive power of this mechanistic and quantitative model-based approach, a more reliable translation of anti-TB drug effects from the zebrafish to higher vertebrates.

11.5.1 Methodological innovations

- We applied an established mechanistic TB model to characterize natural growth of *M. marinum* for the first time and quantitatively compare it to natural growth of *M. tuberculosis*.

- We developed a method to quantify individual bacterial burden over time in zebrafish larvae upon treatment, based on repeated measures by non-invasive fluorescence imaging and population modelling.

11.5.2 Key messages

- A quantitative model-based approach can translate drug effects between two species which are seemingly quite different, by utilizing a quantitative exposure-response relationship and translational factors that take into account the between-species differences.

- Antibiotic drug effects quantified in the zebrafish could be translated to humans.

11.6 Remaining challenges of the zebrafish in drug development

In the previous sections, we have developed and integrated experimental, bioanalytical, and computational methods to quantify internal drug exposure to be able to quantitatively link it to disease dynamics with the purpose to translate pharmacological findings to higher vertebrates. Quantification of internal drug exposure after waterborne treatment is a major challenge for the zebrafish in drug discovery and development, but not the only one. We discuss three other current challenges. First, the zebrafish is a lower vertebrate than mammalian experimental animals, and not all diseases relevant in higher vertebrates can be examined. Some mammalian organ systems are different (e.g. no cardiac septation) or absent (e.g. respiratory and reproductive organs)[98]. Because the zebrafish is a poikilothermic animal, for example fever cannot be studied and the lower experimental temperatures might impact growth of pathogens or tumours[98,99]. Second, experiments are preferably performed in the embryonic and larval state, when the zebrafish are small and suitable for multi well plates and high-throughput screens. The corresponding immaturity might impact the translation of pharmacological findings[100], as we have also seen in Chapter 6. Another caveat in this respect is the lack of adaptive immune system in embryos and larvae, which only develops after four weeks[99,101]. Third, experiments in zebrafish remain to be standardized and validated[102]. Zebrafish husbandry differs between laboratories[103–109] and treatment conditions like light, temperature, and water composition vary and are often not (completely) reported[110]. Standardized experiments subsequently must be validated with known positive and negative compounds from experiments in higher vertebrates[98,111].

11.7 Future perspectives

In this thesis, we have developed and integrated experimental, bioanalytical, and computational methods for the zebrafish to live up to its potential in early drug discovery and development. Many more opportunities and promising techniques will become available in the near future to answer pharmacological questions and to improve translation from this model organism to higher vertebrates, including humans.

One of the largest advantages of the zebrafish for pharmacological experiments is its versatility and almost endless experimental possibilities. An important example is the role the zebrafish can play in unravelling the importance of the microbiome in pharmacology. The microbiome is expected to impact

health and disease, and to modulate pharmacokinetics and pharmacodynamics[112]. It is difficult to study the microbiome in mammals, as an intervention of the gut-bacteria requires sterilisation and re-colonisation[113]. Because of the zebrafish' external fertilization and the assumption that the zygote is sterile, zebrafish eggs can be sterilized much easier, and colonization of sterile embryos and larvae can simply be done via the medium[114,115]. We have performed a pilot experiment similar to those in Section II in sterile zebrafish to ensure observed metabolites were not the result of bacteria in the gut of the zebrafish. This can be expanded to quantify the impact of the microbiome on pharmacokinetics and on the pharmacokinetic-pharmacodynamic relationship of drugs.

The experimental toolbox in zebrafish will be further expanded. Methods for oral dosing have been developed for adult zebrafish to overcome both translational and drug delivery challenges, for example to treat zebrafish with drugs that are insoluble in water, drugs that are not absorbed from waterborne treatment, or drugs that adhere to the skin of the zebrafish[116,117]. A similar method for oral dosing in zebrafish larvae based on three-dimensional microprinting a syringe gavage tip is currently under development. Another promising addition to the experimental toolbox is the use of microfluidic devices specifically designed to house and position zebrafish larvae[118–121]. The larvae can then be treated with different drug concentrations or frequencies, by changing the flow rate of the treatment medium and washing medium. Integration of these experimental techniques with automated fluorescence imaging (Chapter 4) will result in a high-throughput workflow with many possibilities for measurement of drug effects. This is further improved by the use of robotic injections. Automated robotic injection at a speed of 2,000 injected embryos per hour has been developed[15,78,122] and deep learning has increased efficiency of injections[123]. Currently, a method is under development using the same set-up to automate blood sampling, which will increase throughput of quantification of internal exposure substantially.

One inherent challenge of the zebrafish larva is its small size and subsequent low drug amounts in samples for bioanalytical quantification. Sensitive LC-MS/MS methods have been developed here to quantify drug and metabolites amounts and concentrations in samples which consisted of pooled homogenates or blood samples. Detection limits for drug and metabolites are decreasing to femtomolar levels[124], which would allow measurements of drugs and metabolites amounts or concentrations in individual larvae. Blood samples currently need to be pooled to reach detectable levels, but novel methods are available to measure drug concentrations in single blood samples. One example is backloading the needle with ionization solution and subsequent direct injection into the MS, using nano-electrospray ionization mass spectrometry for improved throughput and efficiency[125]. It has already been applied to quantify drug concentrations in individual cancer cells[126]. Accurate and precise quantification of drug concentrations in blood samples is essential to quantify internal drug exposure, which linked to measurements of the disease dynamics, yields an exposure-response relationship. Reliable quantification of the internal exposure, and exposure-response, are essential for inter-species translation of drug effects. When MS techniques become sensitive enough, minimal blood sample volumes that are required for quantification of drug concentrations will decrease. As a result, repeated blood samples could be drawn from individual larva at different timepoints, enabling the distinction between biological and experimental variability in blood concentrations and reducing noise within the concentration-time data.

The use of zebrafish in early drug discovery and development should always be seen within the context of translating pharmacological findings towards the clinic. The best example of this thus far is the prostaglandin E2 derivative ProHema (16,16-dimethyl-prostaglandin E2), discovered in a phenotypic screen in zebrafish on haematopoietic stem cell formation[127]. It is now in Phase II clinical trials as part of new treatment for graft-versus-host disease in patients receiving allogeneic hematopoietic cell transplantation[128]. Another example is the otoprotective portfolio of urea-thiophene carboxamides[129]. These compounds are the result from a phenotypic screen aimed to find compounds protective of aminoglycoside-induced hearing loss[130]. Zebrafish larvae expressing fluorescent hair cells were used in that screen to mimic loss of hair cells responsible for hearing in humans. The lead compound is currently

in Phase I clinical trials[131]. Similar to these examples, 4 more compounds discovered in zebrafish are in clinical trials and 4 more underway[128]. We envision these are just the first of many drugs reaching clinical development, that were discovered in pharmacological experiments with zebrafish.

11.8 Conclusions

The zebrafish is a promising vertebrate model organism in early drug discovery and development. Translation of pharmacological findings to higher vertebrates requires quantification of the underlying pharmacological and (patho)physiological processes. In this thesis, we therefore developed and integrated innovative experimental and computational methods for the successful quantification of 1) the internal exposure over time after waterborne drug treatment, 2) disease dynamics and drug-induced changes therein, and 3) between-species differences in disease mechanisms. The state-of-the-art methods that we developed included nanoscale blood sampling, sensitive LC-MS/MS methods for drugs and their isomers and metabolites, and three-dimensional microscopy, integrated with non-linear mixed effects modelling to quantify the pharmacological processes in this small vertebrate. This multidisciplinarity enabled quantification of internal drug exposure-response relationships, contributed to positioning the zebrafish in the preclinical drug development pipeline, and inspired continuous collaborations between experimental and computational scientists.

11.9 References

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