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Systems pharmacology of hepatic metabolism in zebrafish larvae

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Interspecies translation of pharmacological processes needs to improve to reduce attrition in drug development. Systems pharmacology integrates systems biology and pharmacometrics to characterise and quantify system-specific behaviour upon exposure to drugs in different species. The zebrafish is a suitable vertebrate model organism for systems pharmacology, combining high-throughput potential with high genetic homology to higher vertebrates. Zebrafish larvae have been increasingly used for drug screens, but the influence of internal drug and metabolite exposure is hardly studied. Quantifying this internal exposure is essential for establishing both exposure-response and dose-exposure relationships, needed for translation. The zebrafish may also serve as a suitable model species for translational studies on the occurrence of hepatotoxicity and the influence of hepatic dysfunction on drug metabolism.

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Introduction

Drug development is a complex and costly process with high attrition. Of the terminated drug candidates, the majority fails because of lack of efficacy and safety [1,2]. Efficacy and safety are tested in preclinical experiments, but to improve success rates, interspecies translation needs to move from an empirical to a mechanistic approach [3]. Systems pharmacology is such an approach, combining the strengths of systems biology and pharmacometrics [4]. Understanding the systems of species and their differences helps improving interspecies translation of efficacy and safety data. In this review, we will focus on systems pharmacology of hepatic function and dysfunction, and the importance of understanding the drug exposure over time in a biological system. The unique position of zebrafish larvae as vertebrate model organism for systems pharmacology with high-throughput potential will be discussed.

Systems pharmacology: integrating pharmacometrics and systems biology

Systems pharmacology is a method to contribute to translational medicine by integrating modelling and simulation

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with data from both preclinical and clinical experiments in a 'systems level' mechanistic way, improving interspecies translation of relevant biological processes [5]. Systems pharmacology originates from two established fields, pharmacometrics and systems biology, and aims to quantify the pharmacological perturbations of the biological system of an organism to improve our understanding of the interaction between a drug and a particular biological system.

Pharmacometrics aims to predict drug effects using mathematical models to quantify interactions between organisms and pharmaceutical compounds [6]. This results in pharmacokinetic-pharmacodynamic (PK-PD) models integrating drug pharmacokinetics, which describes drug exposure as concentration versus time, and drug pharmacodynamics, which describes effects versus drug concentration. A schematic of a PK-PD model can be seen in Fig. 1. Observed outcome measures (i.e. concentrations and effects) are described by mathematical equations, from which the underlying primary model parameters are derived. After evaluation of the predictive performance of a model, the model can be used for predictions and to improve interspecies translation of drug pharmacokinetics and pharmacodynamics and to design treatment regimens in both preclinical and clinical studies [7].

Systems biology studies the structure and dynamics of integrated biological systems to understand processes that are too complex to intuitively comprehend by studying its isolated elements only. Like pharmacometrics, this requires quantitative data as well as advanced computational modelling [8]. Systems biology as holistic approach has the advantage of placing part of a system in the biological context of a complete organism. Characterising individual parts of the system, like gene or protein function, from *in vitro* experiments is an important first step [8]. The next step is to elucidate the interaction of these parts in the network of the whole system. This is relevant in for example disease models, as most diseases are not – as previously believed – caused by a single target, for which a single drug can be designed [9]. In contrast to *in vitro* experiments with human cells only, an *in vivo* whole organism experiment can identify compounds able to treat or cure such a disease [10]. Using these complementary experimental data to inform the systems biology model, the understanding of the biological processes in the organism improves, and with it the understanding of how systems differ between species. This may improve interspecies translation.

Zebrafish larvae as vertebrate model organism in drug development

Systems biology models have been developed in invertebrate organisms, such as yeast (*Saccharomyces cerevisiae*), roundworms (*Caenorhabditis elegans*) and fruit flies (*Drosophila*) [11]. These small organisms are easily genetically modified and allow for high-throughput measurements [11]. However in pharmacological studies, a vertebrate species is believed to have improved translational potential due to its increased genetic homology to mammals. The zebrafish (*Danio rerio*) is such a vertebrate model organism that is increasingly used as a model for biomedical studies [12]. Most genes coding for essential proteins such as those in xenobiotic metabolism are evolutionary conserved. Of human and murine genes, 70% and 71% respectively have a zebrafish orthologue [13]. For comparison, 83% of human genes have a murine orthologue [13]. When considering genes for which defects can cause

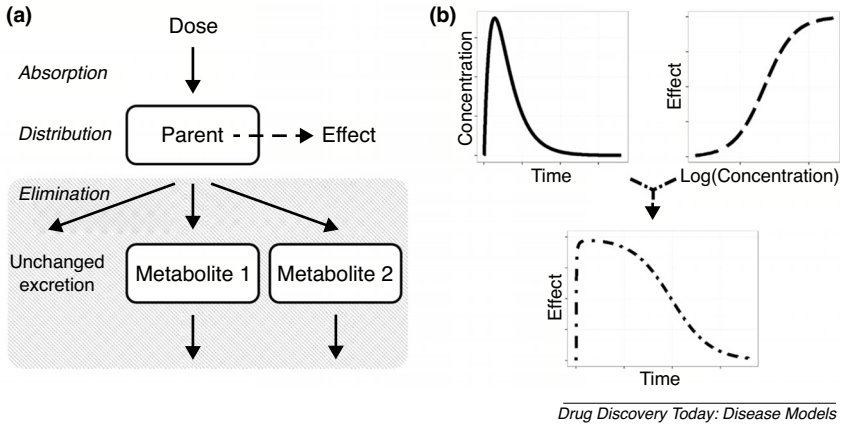

Figure 1. Pharmacology of a parent compound described by a compartmental pharmacokinetic model and a sigmoidal pharmacodynamic model. Panel A. Schematic representation of a compartmental model with absorption, distribution, and elimination by means of metabolism and unchanged excretion, describing the pharmacokinetics of the compound (PK, solid lines in panel B). The concentration of the parent compound drives the effect, in a sigmoidal pharmacodynamic relationship (PD, dashed line in panel B). The PK model becomes more mechanistic when including information on different elimination pathways for example metabolite formation and excretion (grey box); these system-specific properties improve translation of clearance between species. Integrating pharmacokinetics and pharmacodynamics in panel B yields the effect over time profile.

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diseases, 82% of human genes have a zebrafish orthologue [14]. Human disease models in zebrafish larvae have been validated for hyperlipidaemia, liver steatosis, cancer, and mycobacterium infections, among others [15–17].

The use of zebrafish larvae in drug development is increasing because of its many advantages [12]. The four most relevant advantages for drug development include high fecundity, fast development, optical transparency, and easy genetic modification.

Zebrafish have a high reproduction rate. One pair of adult fish yields 100–200 fertilised eggs per mating, reaching up to 10,000 eggs per year. Adults are small (3–5 cm) and are housed in groups of on average 5–13 fish per litre [18]. This combination of fecundity and size results in large numbers of larvae and fish at limited costs [19].

After external fertilisation, the embryo develops in its chorion until hatching between 48–72 h post fertilisation (hpf), reaching the larval stage. At that time, the development of most organs is nearly complete, except for the organs in the gastro-intestinal (GI) tract [20]. After 76 hpf, the liver, pancreas, and gut are fully developed, and at 96 hpf, the GI tract is completely open [21]. Experiments are generally performed in the larval phase when the fish are largely developed but small enough (3–5 mm) to fit in multi well plates up to 384 well format.

Zebrafish are optically transparent during early embryonic and larval stages, enabling non-invasive *in vivo* optical imaging of anatomical and certain (patho)physiological developments. Because it is not required to sacrifice the fish, effects can be observed by microscopy over time in a single subject. An example is the phenotypic assay that has been developed to screen for hepatotoxicity by imaging of liver size, yolk size, and liver degeneration [22]. If automated, these assays have the potential to reach throughput rates of 1000–10,000 assays per day [19].

Forward and reverse genetic modification of the zebrafish is especially easy because the external fertilisation allows injections of the single cell zygote. Genetic modification enables studying gene mutations, as well as mechanisms of action of compounds [11]. It is also possible to humanise zebrafish with human enzymes [23]. Transgenic lines have been developed, expressing fluorescent reporter proteins under control of a wide variety of promoter sequences specific for particular cell types [24]. Due to the transparency of the larvae, both gene expression and function can be examined spatially and over time using fluorescence microscopy [25].

In short, large numbers of fast developing subjects with relevant genetic modifications can be achieved with the potential of automated high-throughput screening in numbers suitable for meaningful statistical analysis [26,27]. Moreover, it is from an ethical perspective best to perform animal experiments – if at all necessary – in the available model organism that is least developed [21]. The zebrafish larvae model organism thus combines ethical and practical advantages with the increased homology to higher vertebrates, compared to invertebrates.

Drug exposure drives effects

For translation of drug effects between species it is not just systems biology and homology of drug targets that are important. The internal exposure of an organism to a drug and its metabolites over time is what drives the drug effects. Quantifying internal exposure is necessary to prevent false positives and negatives [28]. Deriving exposure-response relationships for both desired effects and adverse effects is therefore absolutely essential for interspecies translation of drug pharmacology and toxicology of the parent compound and metabolites. Unfortunately, this is almost always overlooked in pharmacological and toxicological screens with zebrafish larvae. In addition to deriving exposure-response relationships, information on the internal exposure over time in these larvae provides valuable knowledge needed for translation of drug pharmacokinetics from this small vertebrate to higher vertebrates like rodents, monkeys, and even humans, with all their physiological differences. As drug pharmacokinetics drive the dose-exposure relationship, translating it can significantly improve (pre)clinical experimental design by informing dosing rationale.

Internal exposure is quantified by describing the pharmacokinetic processes absorption, distribution, metabolism, and excretion (ADME) of drugs. Drug elimination by metabolism and excretion is quantified as clearance, which is the most important determinant of drug exposure. Metabolism of xenobiotics, including drugs, increases hydrophilicity to improve renal excretion and mostly occurs in the liver. Phase I metabolism is of catabolic nature. Xenobiotics are oxidised, reduced, or hydrolysed. Cytochrome P450 (CYP) enzymes are the most important enzymes in catalysing phase I reactions. Phase II metabolism, also known as conjugation, is anabolic and includes transfer of a hydrophilic moiety from a donor to the xenobiotic. Examples of enzymes catalysing conjugating reactions are sulphotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), amongst others. Drug metabolism can result in reactive metabolites, which may cause toxicity in the metabolising organ [29].

Hepatic metabolism in the zebrafish

A mechanistic understanding of drug metabolism by a preclinical species in relation to human metabolism requires comparison of both amino acid sequence and function of the responsible enzymes. Table 1 shows the relationship of a selection of metabolising enzymes in zebrafish with their corresponding enzymes in humans. These relationships are orthologous, descending from a common ancestral sequence, unless otherwise specified. Specific focus is on the enzymes responsible for paracetamol (Box 1) metabolism.

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Table 1. A selection of metabolising enzymes of zebrafish and their human homologues. Enzymes involved in paracetamol metabolism are printed in italics.

Enzyme family	Zebrafish enzyme	Human enzyme	Ref.
CYP1	CYP1A[a]	CYP1A1/1A2	[30]
	CYP1B1[b]	CYP1B1	[30]
	CYP1D1	CYP1D1P[c]	[30]
	CYP1C1,2	–	[30]
CYP2	CYP2AD2,3,6, CYP2N13, CYP2P1-6, CYP2V1	CYP2J2	[30]
	CYP2K1-8	CYP2W1	[30]
	CYP2R1	CYP2R1	[30]
	CYP2U1	CYP2U1	[30]
	CYP2Y3,4	*CYP2A6,13/B6/F1/S1*	[30]
	CYP2Y3[b], *CYP2P6*[b]	*CYP2E1*	[52]
	CYP2AA1-12	–	[30]
	CYP2AE1,2	–	[30]
	CYP2X1-10	–	[30]
CYP3	CYP3A65	CYP3A4	[30,31]
	CYP3C1[d]	CYP3A4	[53]
	CYP3C1-4	CYP3A-se1[c], -se2[c]	[30]
CYP4	CYP4F43	CYP4Vs	[30]
	CYP4T8	–	[30]
SULT1	*SULT1ST2*[d]	*SULT1A1*	[34]
	SULT1ST5[b]	SULT1B1	[54,55]
	SULT1ST6[b]	SULT1E1	[54,55]
	SULT1ST9[b], *SULT3ST1*[d]	*SULT1A3*	[36,56]
SULT4	SULT4A1[b]	SULT4A1	[57]
UGT1	*UGT1*[e]	*UGT1*	[37,58]
UGT2	UGT2[e]	UGT2	[37,58]
	UGT5	–	[37]

[a] Similar exon structure.
[b] Similar gene structure.
[c] Pseudogene.
[d] Based on function or substrate specificity.
[e] Paralogous relationship.

In humans, the most important CYP enzyme isoforms in drug metabolism are CYP3A4 and CYP2E1. CYP3A4 has at least one orthologue in zebrafish, namely CYP3A65 which is 54% identical in amino acid sequence as well as being identical in function [30,31]. Zebrafish CYP2Y3 and CYP2P6 are both 43% identical to human CYP2E1 in amino acid sequence [32].

Several human SULTs have orthologues in zebrafish, mainly from the SULT1 family [33]. Zebrafish SULT1ST2 shows similar xenobiotic sulphation as human SULT1A1, although no gene orthology has been established [34]. Human SULT1A3 is 49% identical in amino acid sequence to zebrafish SULT1ST9 [35]. Although no clear homology has been found between zebrafish SULT3ST1 and human SULT1A3, this enzyme is responsible for xenobiotic sulphation similar to SULT1A3 [36].

For human and zebrafish UGT enzymes, no orthologous relationships have been found. Instead, these enzymes are so called paralogues, of which the common ancestral gene has been duplicated with different genes in zebrafish and mammals as a result [37]. Despite less genetic overlap than orthologues, paralogues can still have comparable metabolic function. For paracetamol (Box 1) the glucuronide-metabolite that is abundantly formed in humans, has been observed in zebrafish larvae as well [38].

Quantifying metabolising function of the liver

To quantify the metabolising function of hepatic enzymes, pharmacometrics uses for instance non-linear mixed effects modelling to develop empirical compartmental models that quantify pharmacokinetics of drugs in blood, based on concentration-time data. This is the biggest challenge of the zebrafish larvae as model organism for systems pharmacology-based translation of drug pharmacology, as quantifying internal drug and metabolite exposure over time in such small organisms is difficult [12,39]. Currently the external drug concentration in the medium surrounding the larvae is most often used as predictor of the internal drug concentration. It has been tried to predict internal exposure or toxicity based on physicochemical properties of compounds such as hydrophilicity, but without success [40–42]. Only recently, our group developed a sensitive liquid chromatography-mass spectrometry (LC/MS) method to quantify internal exposure of paracetamol (Box 1) as a model compound, which resulted

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Box 1. Model drug paracetamol

Paracetamol (Fig. 2), also known as acetaminophen, is a widely used analgesic [59]. Paracetamol is metabolised in the liver by both phase I and phase II enzymes, and to a limited amount excreted unchanged (<5%). Sulphation by sulphotransferases SULT1A1 and SULT1A3 and glucuronidation by urine 5'-diphospho-glucuronosyltransferase UGT1A6 are responsible for 85% of its metabolism in human adults. The remaining parent compound is oxidised, mainly by cytochrome P450 CYP2E1 to N-acetyl-p-benzoquinone imine (NAPQI), and to a lesser extent by CYP2A6 to methoxy-paracetamol. NAPQI is a toxic metabolite, which reacts with antioxidant glutathione (GSH) to form the nontoxic metabolites cysteine- and mercapturate-paracetamol, among others. At supratherapeutic doses, GSH reserves are depleted and oxidative stress results in hepatotoxicity [60].

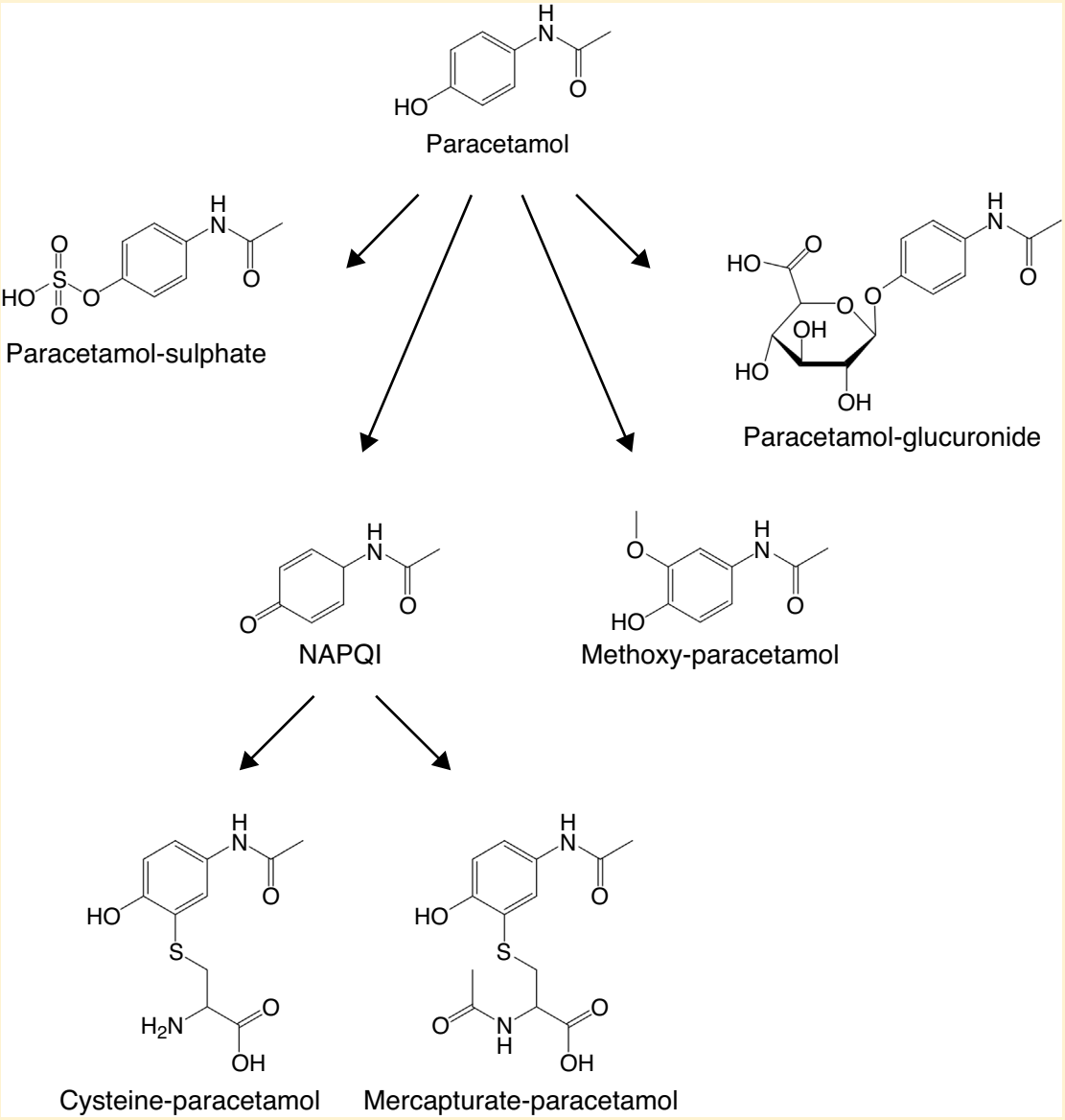

Figure 2. Chemical structure of paracetamol and its major metabolites.

in the first pharmacokinetic model in zebrafish larvae [38]. This model quantified exposure over time of the parent compound, which can be linked to efficacy data. The estimated parameter clearance was scaled between the zebrafish larvae and 12 higher vertebrate species, including rodents, monkeys, and humans, showing reasonable comparability. The model can be extended to include paracetamol major metabolites and their formation rates catalysed by phase I and II enzymes.

Empirical interspecies scaling of paracetamol clearance to higher vertebrates, including humans, was found to be reasonable [38]. When developing new drugs, the clearance in zebrafish might therefore also be used to scale to higher vertebrates and optimise preclinical experimental designs.

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An improvement over this empirical interspecies pharmacokinetic translation, is the systems approach, where mechanistic details on the metabolism can be included into the model, such as the type of enzymes involved in metabolism of a compound of interest. When the differences between the enzymes of the species of interest are known, the mechanistic model can inform the translation of the pharmacokinetics, in a systems pharmacology approach. Additionally, pharmacologically active metabolites can cause off-target (adverse) effects. It is therefore important to establish if the same metabolite species are formed in different vertebrates. If that is the case, it is essential to quantify their exposure over time to translate the exposure-response relationship of these adverse effects.

Quantifying liver dysfunction

Hepatotoxicity is an important adverse effect of drugs. One aim of drug screens in zebrafish larvae is to detect potential toxicity issues. Hepatotoxicity assays in zebrafish have been widely published [22,31,43]. This includes assessment of histopathology and transcriptome profiling in zebrafish larvae [44]. Internal drug and metabolite exposure causing this toxicity needs to be quantified for proper interpretation and translation. Moreover, hepatic dysfunction resulting from this toxicity may impact drug clearance. This impact, and its effect on the exposure-time profile, can be quantified using a similar approach as described in *Quantifying metabolising function of the liver*. The observed adverse effect is then linked to the toxic compound or metabolite (Fig. 1). This provides mechanistic insight in the influence of hepatic dysfunction on the metabolism of both endogenous and exogenous compounds, including drugs. Informed by the relevant system-specific properties, a systems pharmacology model can translate these findings to mammalian model organisms, and humans.

The validity of a pharmaco- or toxicodynamic model increases with the use of mechanistic biomarkers that describe the disease state, in this case hepatic dysfunction [7]. Mechanistic models describe (patho)physiological processes that are important between drug administration and its intended or adverse effect. Biomarkers can be used to characterise and quantify these processes [45]. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are for example widely used hepatotoxicity markers in the clinic. A useful biomarker is both specific and sensitive. ALT is however not liver specific, being expressed in cardiac, renal, and muscle tissue in addition to hepatic tissue [46]. Moreover, it has a delayed response to liver injury, and may not always correlate with clinical symptoms [47,48]. The novel biomarker miRNA-122 has been shown to be a more accurate and time-sensitive alternative to indicate hepatotoxicity in both preclinical and clinical settings [49]. This biomarker has also been studied in zebrafish [50].

Finally, there lies an opportunity for the objective quantification of organ size in zebrafish larvae. Transgenic zebrafish lines with fluorescent organs are available, enabling studying many organs and their development. A reproducible and automated method is the Vertebrate Automated Screening Technology (VAST). In short, complete larvae are withdrawn from a well plate or tube and flow through a capillary linked to a microscope, which captures images of the larvae from different angles in an automated manner. These images can then be processed using 3D silhouette modelling to calculate the volume of the larva [51]. Using this method, we have not only determined the volume of the zebrafish larva at different hpf, but also the volume of its liver [unpublished results]. This combination of techniques can be used to observe toxic effects on organ size. Reversely, it can be used to create a database of organ properties and their development. These system specific properties can then be linked to pharmacokinetic parameters to inform systems pharmacology or physiology-based pharmacokinetic (PBPK) models.

Conclusions

Systems pharmacology, integrating pharmacometrics with systems biology, has the potential to improve interspecies translation of pharmacological findings, and thereby drug development. The zebrafish larva is a promising pre-clinical model organism in systems pharmacology, combining high-throughput potential within a vertebrate species. Drug metabolising enzymes are comparable and metabolic rates can be derived by combining sensitive LC/MS methods and mathematical modelling. Comparable to quantifying liver function, other (patho)physiological processes, for example liver dysfunction, can also be quantified. Pivotal for proper interpretation of these experimental pharmacological findings is characterising the internal drug exposure, which can then be linked to the observed response in an exposure-response relationship needed for interspecies scaling. More emphasis on the pharmacokinetics is therefore required for this species to change the course of drug development in the future.

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