Accepted Manuscript

A combined *in vitro/in silico* approach to identifying off-target receptor toxicity

Joseph Leedale, Kieran J. Sharkey, Helen E. Colley, Áine M. Norton, David Peeney, Chantelle L. Mason, Jean G. Sathish, Craig Murdoch, Parveen Sharma, Steven D. Webb

PII: S2589-0042(18)30067-1

DOI: 10.1016/j.isci.2018.05.012

Reference: ISCI 67

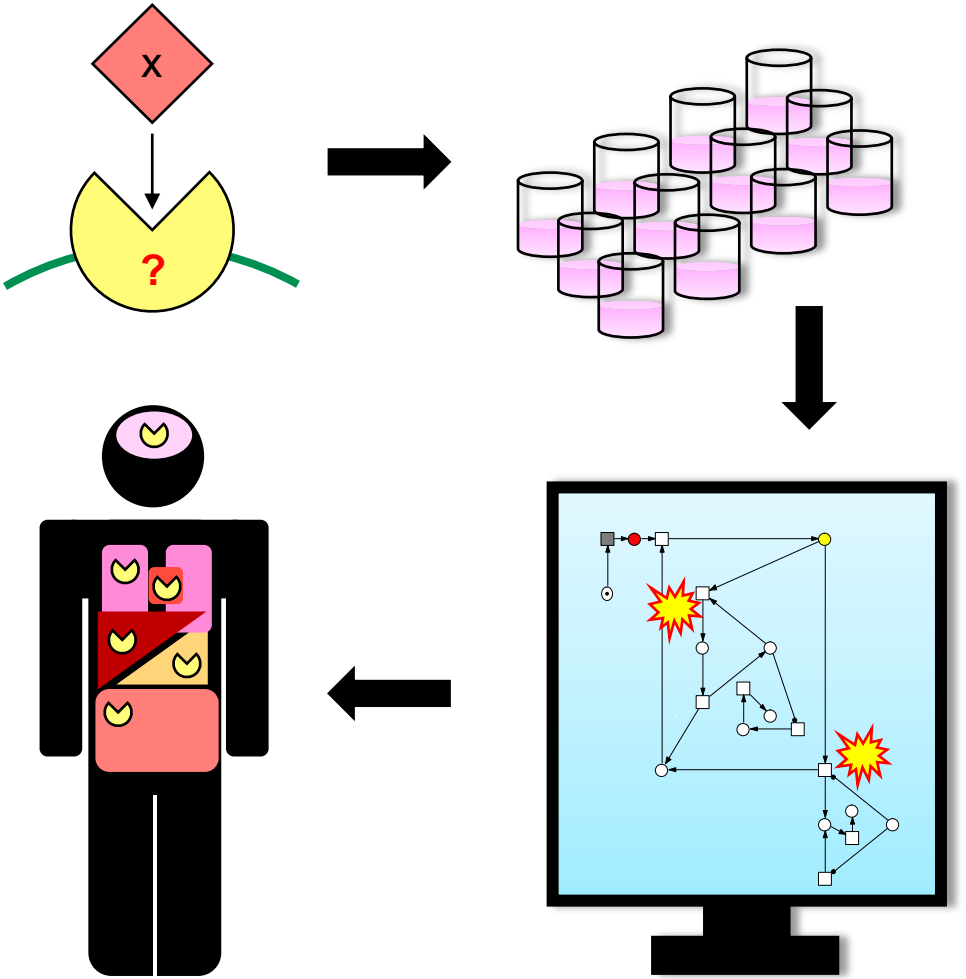
To appear in: *ISCIENCE*

Received Date: 24 November 2017

Revised Date: 19 April 2018

Accepted Date: 15 May 2018

Please cite this article as: Leedale, J., Sharkey, K.J., Colley, H.E., Norton, Á.M., Peeney, D., Mason, C.L., Sathish, J.G., Murdoch, C., Sharma, P., Webb, S.D., A combined *in vitro/in silico* approach to identifying off-target receptor toxicity, *ISCIENCE* (2018), doi: 10.1016/j.isci.2018.05.012.


X
?

A combined *in vitro/in silico* approach to identifying off-target receptor toxicity

Joseph Leedale[1*]†, Kieran J. Sharkey[1], Helen E. Colley[2], Áine M. Norton[3], David Peeney[3], Chantelle L. Mason[4], Jean G. Sathish[3,5], Craig Murdoch[2], Parveen Sharma[3*], Steven D. Webb[4]

[1]EPSRC Liverpool Centre for Mathematics in Healthcare, Dept. of Mathematical Sciences, University of Liverpool, Liverpool, L69 7ZL, UK

[2]School of Clinical Dentistry, University of Sheffield, Sheffield, S10 2TA, UK

[3]Dept. of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, L69 3GE, UK

[4]Dept. of Applied Mathematics, Liverpool John Moores University, Liverpool, L3 3AF, UK

[5]Immuno and Molecular Toxicology, Drug Safety Evaluation, Bristol-Myers Squibb, 1 Squibb Drive, New Brunswick, NJ, 08903, USA

***Correspondence**

For theoretical work:

Joseph Leedale (†lead contact)
EPSRC Liverpool Centre for Mathematics in Healthcare
Dept. of Mathematical Sciences
University of Liverpool
Liverpool L69 7ZL
United Kingdom
Tel: +44 (0)151 794 4049
Email: j.leedale@liverpool.ac.uk

For experimental work:

Parveen Sharma
MRC Centre for Drug Safety Science
Dept. of Molecular and Clinical Pharmacology
University of Liverpool
Liverpool L69 3GE
United Kingdom
Tel: +44 (0)151 795 0149
Email: psharma@liverpool.ac.uk

Summary

Many xenobiotics can bind to off-target receptors and cause toxicity via the dysregulation of downstream transcription factors. Identification of subsequent off-target toxicity in these chemicals has often required extensive chemical testing in animal models. An alternative, integrated *in vitro/in silico* approach for predicting toxic off-target functional responses is presented to refine *in vitro* receptor identification and reduce the burden on *in vivo* testing. As part of the methodology, mathematical modelling is used to mechanistically describe processes that regulate transcriptional activity following receptor-ligand binding informed by transcription factor signalling assays. Critical reactions in the signalling cascade are identified to highlight potential perturbation points in the biochemical network that can guide and optimise additional *in vitro* testing. A physiologically-based pharmacokinetic model provides information on the timing and localisation of different levels of receptor activation informing whole-body toxic potential resulting from off-target binding.

Introduction

Many drugs are designed to interact specifically with cell surface, cytoplasmic or nuclear receptors in order to produce a beneficial therapeutic effect. However, drugs can often bind to and interact with receptors that are not their intended targets and such “off-target” binding may cause what is now often termed a molecular initiating event (MIE); e.g. receptor activation of toxicological relevance that may ultimately lead to an adverse drug reaction (ADR) (Edwards & Aronson, 2000, Guengerich, 2011, Muller & Milton, 2012). In many instances, ADRs can lead to significant morbidity and mortality as well as contributing to high levels of attrition during drug development (Lazarou et al., 1998, Pirmohamed et al., 2004). This can primarily be attributed to an incomplete understanding of the molecular mechanism of action of a given compound and the lack of ability to predict which receptors may be activated unintentionally.

The sole use of *in vitro*-based experimental strategies in the early stages of drug development and chemical testing is important but can lead to an unreliable and incomplete understanding of reactions (Coleman, 2011). Therefore, often considerable numbers of animals are used to screen out chemicals that may cause off-target toxicity with figures for the UK reporting that 306,000 *in vivo* toxicology safety procedures were performed in 2014 (Home Office, 2015). In addition, the chemical industry used almost 345,000 animals in the EU for toxicological or other safety evaluations (European Commission, 2013) and in the USA 3-6 million fish are used annually for whole effluent toxicity testing (Scholz et al., 2013). Furthermore, pharmacokinetics and pharmacodynamics are significantly different between animal models and humans diminishing their effectiveness in detecting toxicity through pre-clinical studies (Lauschke et al., 2016). There is therefore a clear need to develop scientific approaches to identify toxicologically relevant off-target receptor binding in order to reduce the burden of animal use in toxicity testing. The development of a more ethical, non-animal toolkit for initial chemical toxicological assessment using an integrated human-based *in vitro/in silico*

system would enhance current strategies and may even expedite the drug development pipeline.

In intracellular signalling, ligand/receptor interactions lead to the activation of a distinct set of transcription factors, the effects of which tend to be tissue specific. Several companies now offer transcription factor activation profiling platforms and so it is possible to identify and catalogue the transcription factor activation profiles of toxicologically relevant receptors upon binding of their known ligands/drugs. It is assumed that transcription factor profiles generated from off-target receptor activation of any given drug can be matched against known ligand/receptor transcription profiles in order to predict which specific receptor (or class of receptors) has been activated in the initial off-target MIE. However, when testing off-target profiles of new compounds, the resulting transcription profile may not precisely match a known receptor (e.g. partial agonism or the binding of multiple receptors) and therefore a method of refinement is required to narrow the subset of off-target receptors. Our approach aims to refine the *in vitro* receptor identification process for off-target receptors by using information about the changes in receptor-mediated transcription factor activity following the introduction of a given compound and integrating this information with predictive *in silico* models and analysis. This approach allows for the identification of relevant perturbations in the transcription factor signalling pathway that signify the binding of a receptor or smaller range of receptors as well as other points of interest in the transcription factor signalling network that can contribute towards and guide subsequent off-target receptor identification.

Translating the wealth of knowledge on network interactions of cellular components to dynamic models is generally limited by the amount of available quantitative information to accompany these relationships such as molecular amounts and reaction rates. However, qualitative dynamic network modelling can be used to compare with routinely generated semi-quantitative experimental time-course data, where perturbations can provide valuable information about the system. *In silico* modelling of this type then provides a platform for the refinement of more quantitative (parameter based) modelling (Fisher et al., 2013). In such a scenario, the network modelling method of Petri nets provide an effective tool, particularly in the complex, stochastic framework of molecular biological pathways (Chaouiya, 2007, Heiner et al., 2008, Heidary et al., 2015). Petri nets are often used to model multiple species and reactions without defining large quantities of unknown parameters, as modelling emphasis is upon network topology and relative amounts of species rather than specific reaction rates. This emphasis on network structure can then be translated to methods such as flux balance analysis and metabolic control analysis without knowledge of rate constants, as was shown for the switching of the metabolic pathway in *E. Coli* (Edwards et al., 2001, Kitano, 2002).

The identification of off-target receptor binding alone for a given compound is insufficient to predict significant off-target toxicity and so we aim to provide additional information to support and refine the subsequent evaluation of toxic potential. This is achieved by

translating knowledge of receptor binding properties and relative distribution of the receptor throughout the body to a whole-body response to the xenobiotic. This approach utilises a physiologically based pharmacokinetic (PBPK) model adapted specifically for describing receptor activation throughout the body following compound exposure. A PBPK model is a mechanistic, multi-compartment mathematical model that describes the time-course dynamics and overall kinetics of an administered drug dose throughout the organism of interest. PBPK models integrate the physicochemical properties of the substance with the specific physiology of the organism such that the evolution of the ADME (Absorption, Distribution, Metabolism and Excretion) processes can be simulated *in silico*. Drug/substance properties include tissue affinity, membrane permeability, enzymatic stability etc., while the organism/system component include such properties as organ mass/volume and blood flow (Rowland et al., 2011). PBPK modelling is used in this work to couple the pharmacokinetics of a drug to dose-response parameters with the associated off-target receptor in different tissues in order to generate spatio-temporal dynamics of the off-target receptor activation.

Results

Development of the signalling pathway model

As proof of concept, an *in silico* model of the histamine H1 receptor signalling pathway was formulated. This pathway was chosen due to the well understood intracellular signalling interactions involved upon receptor stimulation and the existence of a known off-target partial agonist, lisuride (Bakker et al., 2004). The H1 receptor is a G-protein coupled receptor that, upon activation, leads to dissociation of $G\alpha_{q/11}$ and the $G\beta\gamma$ complex. $G\alpha_{q/11}$ activates phospholipase Cβ (PLCβ) leading to hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) and the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Bakker et al., 2001, Sandal et al., 2013). IP_3 mediates transient intracellular calcium release from the endoplasmic reticulum (Shah et al., 2015) that eventually mediates activation of nuclear factor of activated T-cells (NFAT) (Macian, 2005), cAMP response element-binding protein (CREB) (Johannessen & Moens, 2007) and myocyte enhancer factor-2 (Mef2) transcription factors (Lu et al., 2000). Diacylglycerol simultaneously activates protein kinase C (PKC) and this phosphorylates IκB kinase (IKK), ultimately leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factor activation (La Porta & Comolli, 1997). The $G\beta\gamma$ complex also plays a role in histamine signal transduction; regulating many effectors including adenylate cyclase (AC) (Maruko et al., 2005) and phosphoinositide 3 kinase (PI3K) (Gautam et al., 1998). AC mediates the subsequent activation of protein kinase A via cyclic adenosine monophosphate (cAMP) leading to CREB phosphorylation and transcription factor activation (Mosenden & Taskén, 2011). PI3K mediates the activation of Akt, NF-κB and activating transcription factor 2 (ATF2) (Bence et al., 1997, Breitwieser et al., 2007). To provide semi-quantitative information for the relative transcription factor dynamics as described above, we assayed pathway perturbations using a luciferase reporter-based transcription factor array to calibrate the fold increase expected of key signalling

outputs upon stimulation with an agonist. These transcription factors were identified as NFAT, NF-κB, CREB, Mef2, and ATF2. Incubation of H1 receptor expressing HeLa cells with histamine showed considerable activation of these transcription factors (**Table 1**).

A stochastic Petri net model of the histamine H1 receptor signalling pathway was formulated based on existing knowledge of the pathway and network interactions with the five critical transcription factors determined to be activated following ligand binding. The pathway in this proof of concept provides an illustrative example of what should ultimately form part of a larger cell signalling model that incorporates the complexity of the known toxicological receptors and associated transcription factors in the proposed methodology. The H1 Petri net includes the key dynamic molecular species and appropriate network interactions that are activated during ligand-binding-induced signalling. This pathway is depicted using the modified Edinburgh Pathway Notation (mEPN) format (Freeman et al., 2010) in Figure 1 and directly corresponds to the layout of the Petri net. All rates are equal such that all stochastic transitions are equally likely to fire but are effectively modulated by the concentration of upstream reactants in a mass action process. Time is interpreted qualitatively reflecting the relative order of events. Varying quantities in the mathematical model such as the amount of ligand introduced ("dose") and the total amounts of system species (i.e. moieties of active and inactive states for each protein) modulates the scale of transcriptional activity regulation and as such, these values were optimised to correlate with the experimental signalling assays. This optimisation was carried out by assuming a large-scale continuum approximation of the Petri net to a system of ordinary differential equations (ODEs) and fitting to the corresponding transcription factor output data (Figure 2). It should be noted that the optimal parameter set is non-identifiable for such a large system with relatively few data points to fit. However, this issue was the precise motivation for the combined Petri net/metabolic control analysis approach which is well suited to understanding the relative impact of small perturbations on the transcription factors of interest and prioritise network connectivity information in favour of accurate predictions of parameters and dynamics (Koch et al., 2010). Corresponding pathway reactions, moieties and ODEs can be found in the supplementary material. In addition to providing static information on the network interactions of the signalling pathway and relative changes in steady state activity following receptor activation, Petri nets can also be used to simulate transient temporal dynamics providing further dynamic information on the relative order and scale of transcriptional regulation (Figure 3) following a receptor-ligand binding event. However, it is clear that more data would be required for one to relate this dynamic output to the biological context, and validate any potential predictions about transient dynamics.

Analysis of network perturbations to identify off-target responses

The identification of significant pathway reactions upstream of transcription was achieved using metabolic control analysis (MCA), which is a mathematical technique that tests the sensitivity of a given variable to network perturbations (Kacser & Burns, 1973, Heinrich &

Rapoport, 1974). Specifically, scaled MCA concentration control coefficients provide the ratio between a relative measure of change in the steady state of a system variable as affected by perturbations in network reaction rates. In our illustrative H1 example model, MCA coefficients were calculated for each transcription factor that was experimentally determined to show significant change in activity following binding of the H1 receptor (Figure 4). The rows of the heat map in Figure 4 correspond to the numbered reactions as indicated in the supplementary material. MCA not only points to the direct regulation of gene transcription as critical to H1-associated transcriptional activity (white patches in Figure 4), but to other reactions within the cascade, upstream of the transcription factors and downstream of the target receptor. For example, in this system the transcriptional activity of Mef2 is sensitive to relatively distant biochemical reactions, such as the rate of calcium release from the endoplasmic reticulum (24% of maximum sensitivity provided by perturbation of Mef2 transcription rate). Also, the model suggests that the transcriptional activity of ATF2 is more sensitive to perturbations in PIP2 synthesis than it is to regulation of the BTK:PIP3 complex that directly activates ATF2 by phosphorylation.

The identification of these sensitive perturbation points within the signalling pathway model provide information beyond the transcription factor activity measurements found experimentally, which allows for more optimised, directed experimental designs for receptor identification, if initial screening fails to identify the off-target receptor. For example, for a given compound that was shown to regulate Mef2 transcriptional activity but did not interact with the H1 receptor, this model would inform a proposal to screen for receptors that are known to interact with biochemical reactions identified as being sensitive, such as calcium release, during MCA.

Translation to tissue scales using a PBPK model

Following an *in silico* identification of an off-target receptor, extrapolation to the study of potential *in vivo* toxicity can be performed using a PBPK model. For our illustrative example, receptor binding properties are provided by EC_{50} dose-response curves for the off-target H1 agonist, lisuride (Figure 5A), and measurements of the corresponding binding affinity, K_d (Bakker et al., 2004). The dose-response curves were estimated by fitting the following equation to the dose-response data:

$$Response\% = Min + \frac{(Max - Min)L^n}{EC_{50}^n + L^n}, \quad (1)$$

for ligand concentration L. The optimised parameter values are given in **Table 2**. In order to provide tissue-specific responses we also used Western blot measurements of relative H1 receptor expression in different tissues (Figure 5B-C) and calculated modified tissue-specific EC_{50} values using,

$$EC_{50_i} = \frac{K_d EC_{50}}{R_i(K_d + EC_{50}) - EC_{50}}$$

where i denotes the i^{th} tissue, K_d is the dissociation equilibrium constant for lisuride and R_i is a measure of receptor abundancy in tissue i (see Table 3). For simplicity, this model assumes that the same amount of receptor binding is required to achieve 50% response in each tissue in the absence of any other information, particularly as the response measured is proximal to receptor binding attenuating any potential amplification effects arising from potential signalling cascades in different tissues (Kenakin, 2009). For further information regarding this derivation see the supplementary material.

In order to simulate the pharmacokinetics of lisuride throughout the body, physicochemical properties of the compound were required which were obtained from previously published measurements. These properties include lipophilicity, whether the drug is neutral/acid/base, solubility (obtained from the DrugBank database (Wishart et al., 2006)), molecular weight (O'Neil, 2013), acid dissociation constant (Meloun et al., 2005) and effective permeability (Winiwarter et al., 1998). The time-course dynamics simulated by the PBPK model for drug concentration in each tissue compartment of the body were then coupled to receptor binding properties and relative receptor expression in tissues to provide a predictive temporal response throughout the body. This response can be produced for any dosage regime and various methods of administration such as intravenous, oral and inhalation. The PBPK model was based on the form derived by Peters (2008). The model was optimised for lisuride physicochemical and binding properties and the H1 receptor distribution throughout the different tissues. Example lisuride response kinetics following both intravenous (IV) and oral administrations can be found in Figure 6. The IV dose of 25 μg/mL used in Figure 6 was the same as that used in a previous pharmacokinetic study for relevance (Krause et al., 1991). This experimental data was also the IV data used to optimise the PBPK model to recapitulate the lisuride dynamics in the venous blood compartment and also simulate corresponding oral profiles as per the methodology described by Peters (2008). The oral dose of 0.1 mg chosen for the PBPK model was deemed relevant by matching previous pharmacological studies (Koizumi et al., 1985, Al-Sereiti & Turner, 1989). The dynamic response of the H1 receptor is visualised over time as a solution to equation (1) with tissue-specific EC_{50} values for the pharmacokinetics of lisuride (L) in different parts of the body. Both IV and oral administration simulations are plotted to also highlight the impact of delivery route. This is particularly pertinent in this case where we are studying a receptor which has a relatively high concentration in the gastrointestinal tract. IV administration results in relatively high receptor stimulation in the liver, brain, small intestine and colon at earlier times whereas oral administration results in a more gradual accumulation in these tissues and the receptors in the colon are stimulated at a near maximal level for a relatively long time after oral ingestion. These simulations allow us to compare how the off-target response varies throughout the body over time depending on the pharmacokinetics of the drug coupled with physiologically relevant receptor availability and receptor binding information. Such information is potentially useful to determine whether or not an identified off-target agonist is likely to elicit

an off-target receptor response in an area of high target density based on its physicochemical properties.

Discussion

Adverse drug reactions (ADRs) are a major cause of patient morbidity, mortality and drug attrition during development (Pirmohamed et al., 2004). This can be attributed to a poor understanding of the mechanisms underlying the toxic response and also to a lack of current tools for the prediction of a toxic outcome. Animal models have a limited scope and data obtained using such models may not be ideal for ascertaining toxicity seen in humans. As such, computational systems biology models can be essential tools to improve chemical reaction predictivity (Krewski et al., 2010). In this study, we describe a new *in silico* modelling method that can be used to enhance current knowledge of pathway perturbations in order to provide a new toxicity-testing paradigm based on human biology. In this method, chemical-mediated activation of transcription factors and intracellular signalling pathway molecules were used as readouts to inform and drive a pathway-based *in silico* approach to identify possible upstream receptor(s) engaged by such chemicals. *In vitro* data was then used to inform a PBPK *in silico* modelling platform to understand and rank risk of toxicity at tissue, organ and whole-body levels over time. Key to this integrative approach was the coupling of *in vitro* experimental techniques and advanced *in silico* modelling to create a unique resource that, with further development and parameterisation, could be used to predict the off-target toxicity of compounds that can then inform and direct more focussed *in vivo* experimentation.

Mathematical modelling was used in order to mechanistically describe the processes that lead to regulation of transcriptional activity following the binding of ligand to receptor. This was achieved by designing a signalling pathway model that represented all the relevant processes and biochemical reactions downstream of ligand binding, culminating in the regulation of transcription. We have established a novel *in vitro/in silico* approach using data from assays measuring transcription factor activation and chemically-induced perturbations of intracellular signalling pathways to inform *in silico* pathway modelling. This unbiased pathway-led approach uses computational simulations to identify causality between receptor activation and pathway perturbations to aid identification of the upstream receptor/s engaged by the initial MIE. As proof of concept, an *in silico* Petri net model of the histamine H1 receptor-signalling pathway was formulated with the off-target compound, lisuride. The output of this system provides semi-quantitative temporal dynamics for the entire pathway that can be used to investigate system perturbations, simulate experiments and provide structural pathway predictions. *In vitro* reporter assay data was then used to parameterise and validate the model, and the identification of critical candidate perturbation points was achieved using metabolic control analysis (MCA). Signalling pathway models can be purposely used in this methodology to provide a library of MCA coefficients for a range of transcription factors associated with receptor binding and toxicity, and guide further

experimentation. In the example shown, calcium release from the endoplasmic reticulum and PIP2 synthesis are highlighted as important upstream events for the transcriptional activity of Mef2 and ATF2. If a new compound is shown to induce the activity of these transcription factors but the receptor responsible is not identified via screening for instance, further testing could be guided towards targets that modulate these upstream processes. This illustrates the feasibility of this approach in directing further experimentation towards relevant pathway mechanisms or receptor clusters during the process of receptor identification via focussed *in vitro* assay testing.

In vitro to *in vivo* extrapolations of whole-body consequences of receptor binding was explored using PBPK modelling. The structure of PBPK models typically revolves around the anatomical structure of the organism with different organs and tissues of varying perfusion rates being separated into distinct compartments. These compartments are then coupled through the circulation, whose arterial and venous flow is described to connect the organs in a physiological way. Entrance points (e.g. absorption) of the model depend on the drug administration method (e.g. inhalation, ingestion, injection) while exit points (e.g. excretion) are generally described via the kidneys and intestine. The flow kinetics of the model determine distribution, while metabolism occurs in the liver and intestine. The inherent physiological basis distinguishes true PBPK models from their PK model counterparts that usually simplify the physiology to fewer hypothetical compartments of different flow rates, driven by the data/process of interest, such that they are often more tractable analytically. In contrast, PBPK models are generally more complex but are designed to have a better global representation such that valid extrapolations can be made and disparate experimental data can be integrated during model parameterisation. In this way, PBPK models are less reliant on data-fitting to obtain appropriate values for equation parameters and essentially the same model (with appropriate modifications) can be suitably applied in many different pharmacological scenarios for quantitative risk assessment and therapy optimisation.

PBPK model simulations are increasingly being used in pharmacology, in both academia and industry, in order to provide important predictions of the pharmacokinetic properties and toxic potential of new drugs at an early stage in drug development (Zhao et al., 2011, Jones & Rowland-Yeo, 2013, Tsamandouras et al., 2015). This type of *in silico* testing can offer a quicker, cheaper and more ethical alternative method when compared to traditional *in vivo* experiments performed. Ideally, both experimental and computational methods are used harmoniously to provide a cycle of information and enhanced knowledge iteration as the accuracy of PBPK models inevitably rely on quality experimental data to calibrate rates within the differential equations. In the method reported here, physicochemical properties of the chemical are combined with tissue specific receptor expression and EC_{50} data to predict time-course dynamics of the chemical concentrations in each tissue, as well as tissue level receptor activation responses to that chemical. These predictions can be produced for any dosage regime and various methods of administration. In the example study of the off-target partial agonist of the histamine H1 receptor, lisuride, the combination of lisuride

pharmacokinetics and relative H1 receptor distribution throughout the body allowed us to predict that the dose response would be most significant in the brain, liver and gastrointestinal system. In this case example, these results are supported by prior knowledge of the compound and receptor although the modelling was done agnostic of such prior *in vivo* findings. In particular, receptor response localised to the brain is somewhat expected since lisuride is primarily a psychotherapeutic drug, affecting dopamine and serotonin regulation (Marona-Lewicka et al., 2002). Lisuride is primarily metabolised in the liver, where there is relatively high expression of histamine receptors. There is also high receptor expression in the gastrointestinal tract due to the role of histamine in intestinal secretion and motility (Leurs et al., 1995, Sander et al., 2006). Furthermore, lisuride administration in patients with Parkinson's disease has been associated with gastrointestinal side effects (Ebadi & Pfeiffer, 2004). Although relative response rates have been quantified by the model in different parts of the body at different times, to translate what such a response directly represents in the context of toxicity and clinical relevance is very complicated, and restricted in this methodology, establishing a challenge beyond the scope of this paper. However, these PBPK-based extrapolations do allow us to generate predictive data relevant to risk assessment and further translation to toxicity at the organ and whole-body levels for off-target receptor perturbations. The output provided by this method is intended to identify toxic potential and guide subsequent *in vitro* and *in vivo* experimentation to organs of interest/importance.

The operating parameters of the approach are circumscribed by the extent of current knowledge regarding receptors and their function. This represents a potential limitation of the strategy, although the mathematically-driven signalling pathway model has the potential to identify novel, uncharacterised receptor targets. The challenge of identifying sensitive perturbation points within large-scale networks of receptor signalling pathways required that a semi quantitative network-based approach must be used. This inevitably limits the amount of predictive, dynamic information that can be extrapolated and caution must be exercised such that the utility of mathematical models is preserved by acknowledging the relevant application that stimulated its design. The approach is experimental (with elements of modelling and extrapolation to assess and rank toxicological risk) and does not incorporate prediction of receptor binding based on chemical or receptor structures. The strength of the methodology is predicated on currently available, validated experimental methods as it does not require the development of new, untested technologies and relies on sound criteria-based selection of receptors, and quantifying receptor function and binding using established experimental techniques. Future work requires the development of multiple pathway models based on training chemical data as well as the integration of pathways, which should be optimised and validated with non-training data. Furthermore, the current PBPK framework can be extended to ensure improved predictive potential by incorporating mechanistic tissue models, catering for a wider range of chemicals and capturing population level responses. More work is also needed to translate tissue-level receptor activation responses to measures of toxicity such as relevant biomarkers. Carefully calculated person-to-person variation and

covariances within organism-related parameters would also allow for the prediction of a population response whereby different individuals within a sample population may exhibit different levels of exposure and therefore associated toxicity from the same dosage levels. The combined *in vitro/in silico* approach of this study has shown how the multidisciplinary, iterative process of systems biology can be applied to direct experiments, optimise the utility of generated data and challenge and refine theoretical modelling in order to improve methods for detecting and predicting toxicity caused by compounds that bind to off-target receptors.

Acknowledgments

JL, KJS and SDW acknowledges funding support from the Liverpool Centre for Mathematics in Healthcare (EPSRC grant: EP/N014499/1). All authors acknowledge funding support from the NC3Rs CRACK-IT Challenge 18: Targeting off-targets award.

Author Contributions

JL contributed to the mathematical modelling and wrote the manuscript; KJS and CLM contributed to the mathematical modelling; HEC and CM contributed to the design of the experimental work; AMN and DP performed the experiments; JGS designed the research; PS contributed to, designed and performed the experimental work; SDW contributed to the mathematical modelling and directed the research. All authors read and approved the final manuscript.

Declaration of Interests

The authors declare no competing interests.

References

Al-Sereiti M.R. and Turner P. (1989). The effects of lisuride, terguride and bromocriptine on intraocular pressure (IOP). Br. J. Clin. Pharmacol. 27: 159-163.

Bakker R.A., Schoonus S.B.J., Smit M.J., Timmerman H. and Leurs R. (2001). Histamine H1-receptor activation of nuclear factor-κB: roles for Gβγ-and Gαq/11-subunits in constitutive and agonist-mediated signaling. Mol. Pharmacol. 60: 1133-1142.

Bakker R.A., Weiner D.M., Ter Laak T., Beuming T., Zuiderveld O.P., Edelbroek M., Hacksell U., Timmerman H., Brann M.R. and Leurs R. (2004). 8R-lisuride is a potent stereospecific histamine H1-receptor partial agonist. Mol. Pharmacol. 65: 538-549.

Bence K., Ma W., Kozasa T. and Huang X.-Y. (1997). Direct stimulation of Bruton's tyrosine kinase by Gq-protein α-subunit. Nature 389: 296-299.

Breitwieser W., Lyons S., Flenniken A.M., Ashton G., Bruder G., Willington M., Lacaud G., Kouskoff V. and Jones N. (2007). Feedback regulation of p38 activity via ATF2 is essential for survival of embryonic liver cells. Genes Dev. 21: 2069-2082.

Chaouiya C. (2007). Petri net modelling of biological networks. Briefings in bioinformatics 8: 210-219.

Coleman R.A. (2011). Human tissue in the evaluation of safety and efficacy of new medicines: a viable alternative to animal models? ISRN pharmaceutics 2011.

Ebadi M. and Pfeiffer R.F. (2004). Parkinson's Disease, (CRC Press).

Edwards I.R. and Aronson J.K. (2000). Adverse drug reactions: definitions, diagnosis, and management. The Lancet 356: 1255-1259.

Edwards J.S., Ibarra R.U. and Palsson B.O. (2001). In silico predictions of Escherichia coli metabolic capabilities are consistent with experimental data. Nat. Biotechnol. 19: 125-130.

European Commission (2013). Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union.

444 Fisher C.P., Plant N.J., Moore J.B. and Kierzek A.M. (2013). QSSPN: dynamic simulation of
445 molecular interaction networks describing gene regulation, signalling and whole-cell
446 metabolism in human cells. Bioinformatics 29: 3181-3190.

447
448 Freeman T.C., Raza S., Theocharidis A. and Ghazal P. (2010). The mEPN scheme: an
449 intuitive and flexible graphical system for rendering biological pathways. BMC Syst. Biol. 4:
450 65.

451
452 Gautam N., Downes G.B., Yan K. and Kisselev O. (1998). The G-protein βγ complex. Cell.
453 Signal. 10: 447-455.

454
455 Guengerich F.P. (2011). Mechanisms of drug toxicity and relevance to pharmaceutical
456 development. Drug Metab. Pharmacokinet. 26: 3-14.

457
458 Heidary Z., Ghaisari J., Moein S., Naderi M. and Gheisari Y. (2015). Stochastic Petri Net
459 Modeling of Hypoxia Pathway Predicts a Novel Incoherent Feed-Forward Loop Controlling
460 SDF-1 Expression in Acute Kidney Injury.

461
462 Heiner M., Gilbert D. and Donaldson R. (2008). Petri nets for systems and synthetic biology.
463 Formal methods for computational systems biology, (Springer)**:** 215-264.

464
465 Heinrich R. and Rapoport T.A. (1974). A Linear Steady-State Treatment of Enzymatic
466 Chains. The FEBS Journal 42: 97-105.

467
468 Home Office (2015). Annual Statistics of Scientific Procedures on Living Animals Great
469 Britain 2014.

470
471 Johannessen M. and Moens U. (2007). Multisite phosphorylation of the cAMP response
472 element-binding protein (CREB) by a diversity of protein kinases. Front Biosci 12: e32.

473
474 Jones H.M. and Rowland-Yeo K. (2013). Basic concepts in physiologically based
475 pharmacokinetic modeling in drug discovery and development. CPT: pharmacometrics &
476 systems pharmacology 2: 1-12.

477
478 Kacser H. and Burns J.A. (1973). The Control of Flux. Symp. Soc. Exp. Biol. 27: 65-104.

479
480 Kenakin T. (2009). A pharmacology primer: theory, application and methods, (Academic
481 Press).

Kitano H. (2002). Systems biology: a brief overview. Science 295: 1662-1664.

Koch I., Reisig W. and Schreiber F. (2010). Modeling in systems biology: the Petri net approach, (Springer Science & Business Media).

Koizumi K., Aono T. and Kurachi K. (1985). The effect of lisuride hydrogen maleate on anterior pituitary hormones, oestradiol and cortisol in normal and hyperprolactinaemic women. European Journal of Obstetrics & Gynecology and Reproductive Biology 20: 19-26.

Krause W., Mager T., Kühne G., Duka T. and Voet B. (1991). The pharmacokinetics and pharmacodynamics of lisuride in healthy volunteers after intravenous, intramuscular, and subcutaneous injection. Eur. J. Clin. Pharmacol. 40: 399-403.

Krewski D., Acosta Jr D., Andersen M., Anderson H., Bailar III J.C., Boekelheide K., Brent R., Charnley G., Cheung V.G. and Green Jr S. (2010). Toxicity testing in the 21st century: a vision and a strategy. Journal of Toxicology and Environmental Health, Part B 13: 51-138.

La Porta C.A. and Comolli R. (1997). PKC-dependent modulation of IkB alpha-NFkB pathway in low metastatic B16F1 murine melanoma cells and in highly metastatic BL6 cells. Anticancer Res. 18: 2591-2597.

Lauschke V.M., Hendriks D.F.G., Bell C.C., Andersson T.B. and Ingelman-Sundberg M. (2016). Novel 3D Culture Systems for Studies of Human Liver Function and Assessments of the Hepatotoxicity of Drugs and Drug Candidates. Chem. Res. Toxicol.

Lazarou J., Pomeranz B.H. and Corey P.N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA 279: 1200-1205.

Leurs R., Smit M. and Timmerman H. (1995). Molecular pharmacological aspects of histamine receptors. Pharmacol. Ther. 66: 413-463.

Lu J., McKinsey T.A., Nicol R.L. and Olson E.N. (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proceedings of the National Academy of Sciences 97: 4070-4075.

Macian F. (2005). NFAT proteins: key regulators of T-cell development and function. Nature Reviews Immunology 5: 472-484.

Marona-Lewicka D., Kurrasch-Orbaugh D.M., Selken J.R., Cumbay M.G., Lisnicchia J.G. and Nichols D.E. (2002). Re-evaluation of lisuride pharmacology: 5-hydroxytryptamine1A receptor-mediated behavioral effects overlap its other properties in rats. Psychopharmacology 164: 93-107.

Maruko T., Nakahara T., Sakamoto K., Saito M., Sugimoto N., Takuwa Y. and Ishii K. (2005). Involvement of the βγ subunits of G proteins in the cAMP response induced by stimulation of the histamine H1 receptor. Naunyn-Schmiedeberg's Arch. Pharmacol. 372: 153-159.

Meloun M., Syrovy T. and Vrana A. (2005). The thermodynamic dissociation constants of haemanthamine, lisuride, metergoline and nicergoline by the regression analysis of spectrophotometric data. Anal. Chim. Acta 543: 254-266.

Mosenden R. and Taskén K. (2011). Cyclic AMP-mediated immune regulation—overview of mechanisms of action in T cells. Cell. Signal. 23: 1009-1016.

Muller P.Y. and Milton M.N. (2012). The determination and interpretation of the therapeutic index in drug development. Nature reviews Drug discovery 11: 751-761.

O'Neil M.J. (2013). The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, (Royal Society of Chemistry).

Peters S.A. (2008). Evaluation of a generic physiologically based pharmacokinetic model for lineshape analysis. Clin. Pharmacokinet. 47: 261-275.

Pirmohamed M., James S., Meakin S., Green C., Scott A.K., Walley T.J., Farrar K., Park B.K. and Breckenridge A.M. (2004). Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. BMJ 329: 15-19.

Rowland M., Peck C. and Tucker G. (2011). Physiologically-based pharmacokinetics in drug development and regulatory science. Annu. Rev. Pharmacol. Toxicol. 51: 45-73.

Sandal M., Paltrinieri D., Carloni P., Musiani F. and Giorgetti A. (2013). Structure/function relationships of phospholipases C Beta. Current Protein and Peptide Science 14: 650-657.

Sander L.E., Lorentz A., Sellge G., Coeffier M., Neipp M., Veres T., Frieling T., Meier P.N., Manns M.P. and Bischoff S.C. (2006). Selective expression of histamine receptors H1R, H2R, and H4R, but not H3R, in the human intestinal tract. Gut 55: 498-504.

Scholz S., Sela E., Blaha L., Braunbeck T., Galay-Burgos M., Garcia-Franco M., Guinea J., Kluever N., Schirmer K. and Tanneberger K. (2013). A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regul. Toxicol. Pharmacol. 67: 506-530.

Shah S.Z.A., Zhao D., Khan S.H. and Yang L. (2015). Regulatory mechanisms of endoplasmic reticulum resident IP3 receptors. J. Mol. Neurosci. 56: 938-948.

Tsamandouras N., Rostami-Hodjegan A. and Aarons L. (2015). Combining the 'bottom up'and 'top down'approaches in pharmacokinetic modelling: fitting PBPK models to observed clinical data. Br. J. Clin. Pharmacol. 79: 48-55.

Winiwarter S., Bonham N.M., Ax F., Hallberg A., Lennernäs H. and Karlén A. (1998). Correlation of human jejunal permeability (in vivo) of drugs with experimentally and theoretically derived parameters. A multivariate data analysis approach. J. Med. Chem. 41: 4939-4949.

Wishart D.S., Knox C., Guo A.C., Shrivastava S., Hassanali M., Stothard P., Chang Z. and Woolsey J. (2006). DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res. 34: D668-D672.

Zhao P., Zhang L., Grillo J.A., Liu Q., Bullock J.M., Moon Y.J., Song P., Brar S.S., Madabushi R. and Wu T.C. (2011). Applications of physiologically based pharmacokinetic (PBPK) modeling and simulation during regulatory review. Clin. Pharmacol. Ther. 89: 259-267.

Figure Legends

Figure 1: Schematic representation for the Petri net of the histamine H1 receptor signalling pathway using mEPN notation. The Petri net describes the key relationships between components of the signalling pathway system culminating in the regulation of downstream transcription factor expression stimulated by the binding of a ligand to the histamine H1 receptor.

Figure 2: Optimised transcription factor output. The ligand (histamine) was introduced at t = 0 (Petri net time units) in the model simulation. Prior to t = 0 the model was run to steady state. The model solution was fit to the data via optimisation of the conserved moieties of the signalling pathway. Dotted lines represent the fold increase in transcriptional activity for the relevant transcription factor observed in the transcription assays. Solid lines represent the normalised model solution for the corresponding transcriptional activity as simulated by luciferase dynamics.

Figure 3: Transient dynamic output of the histamine H1 receptor signalling pathway using the stochastic Petri net. This figure illustrates the dynamic output of the stochastic Petri net when a small transient perturbation to the ligand concentration is made at t=200 units, representing the pre-stimulation steady state. Dynamics are shown for model variables that correspond to luciferase signals for transcription factors associated with a receptor stimulation perturbation.

Figure 4: Metabolic Control Analysis (MCA) of the H1 signalling pathway. Scaled concentration control coefficients as a result of MCA are plotted for the activity of five transcription factors modulated by histamine H1 receptor binding. Each row of the heat map numerically corresponds to a reaction term in the signalling pathway model (see supplementary material). Maximum and minimum values in the heat map (white patches) represent maximum sensitivity to perturbation of the reaction terms in the model depicting direct transcriptional regulation rates and luciferase decay rates.

Figure 5: Histamine/lisuride dose response, EC_{50} and kinetic parameters. (A): Ligand (histamine) and partial agonist (lisuride) dose-response assays used to calculate EC_{50} values. (B): Immunoblotting of H1 receptor in murine organs. (C): Relative quantification of immunoblot relative to HeLa cell lysates.

Figure 6: Temporal tissue response predicted by PBPK modelling following doses of lisuride. (A): 25 μg/mL administered intravenously. (B): 0.1 mg administered orally. Tissues are labelled as follows: heart (HE), lungs (LU), kidneys (KI), liver (LI), bone (BO), brain (BR), spleen (SP), small intestine (SI) and colon (CO).

Table Legends

Table 1: Transcription factor changes. Alterations in expression levels of specified genes in the presence of histamine after 6 hours expressed as mean fold changes in relative luciferase units with standard deviation (n=3) as determined by Cignal Reporter Assay.

Table 2: Kinetic parameters of lisuride and the histamine H1 receptor. Receptor activation of the H1-histamine receptor was studied with known agonist (histamine) and off-target agonist (lisuride). Using these assays, each parameter was calculated using GraphPad Prism.

Table 3: Relative amounts of histamine H1 receptor in murine tissue calculated using immunoblot analysis. Values were used to calculate tissue-specific receptor scaling factors for lisuride EC_{50} values when binding to the histamine H1 receptor.

TABLES

Table 1

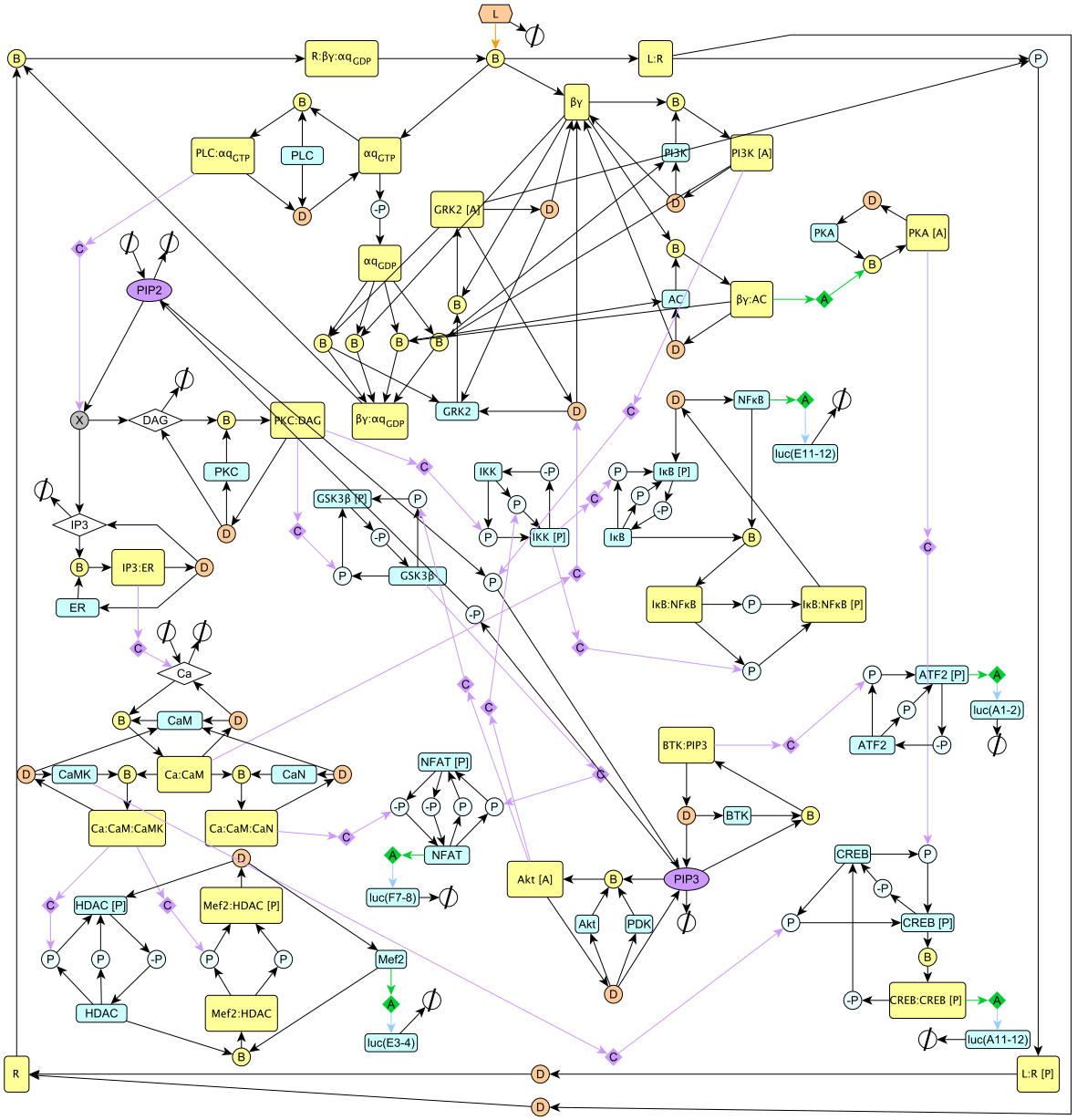
Transcription Factor	Fold change in relative luciferase units
NFAT	1.97 ± 0.063
NFκB	2.18 ± 1.47
CREB	1.54 ± 0.027
MEF2	2.74 ± 1.31
ATF2	1.67 ± 8.99

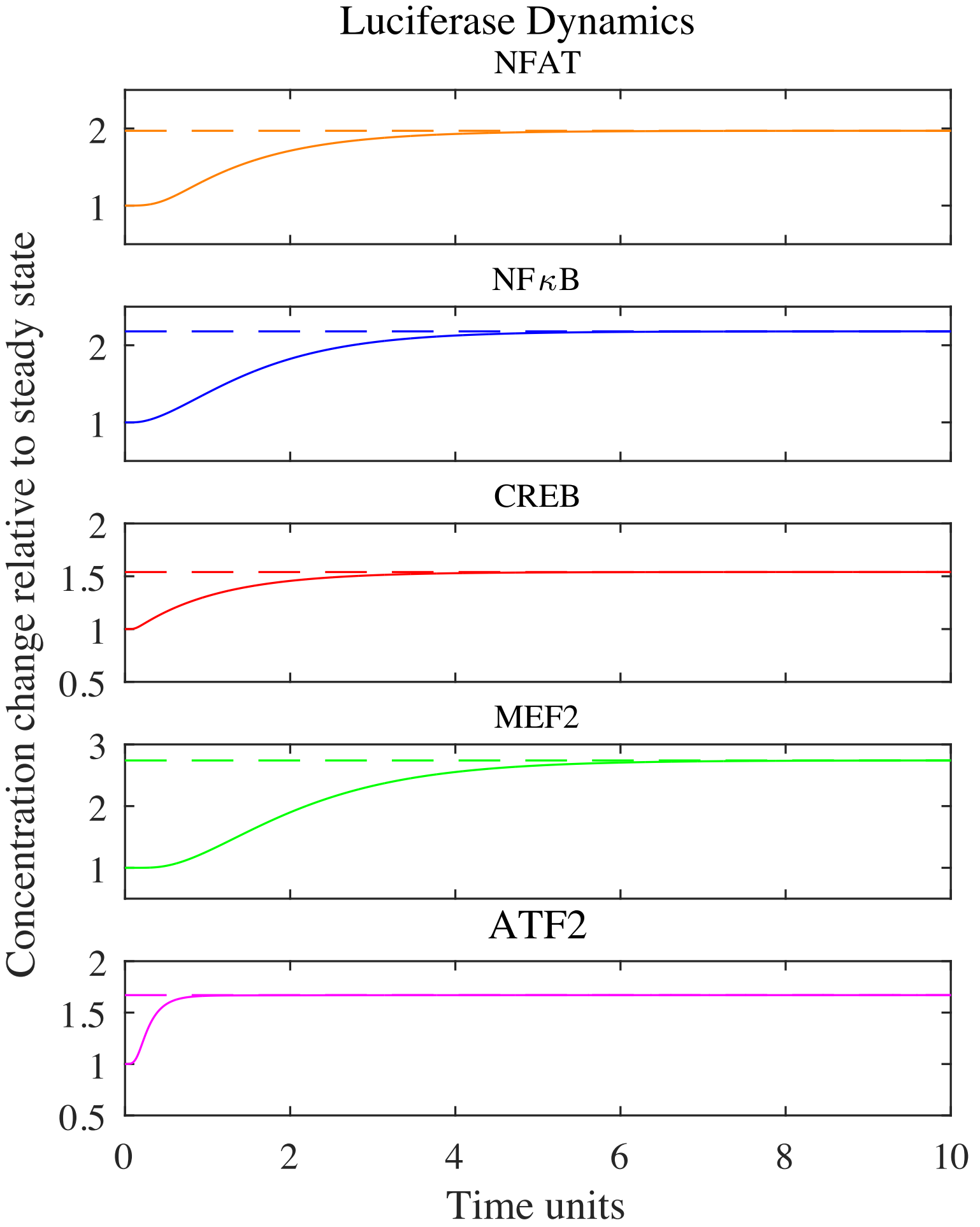
Table 2

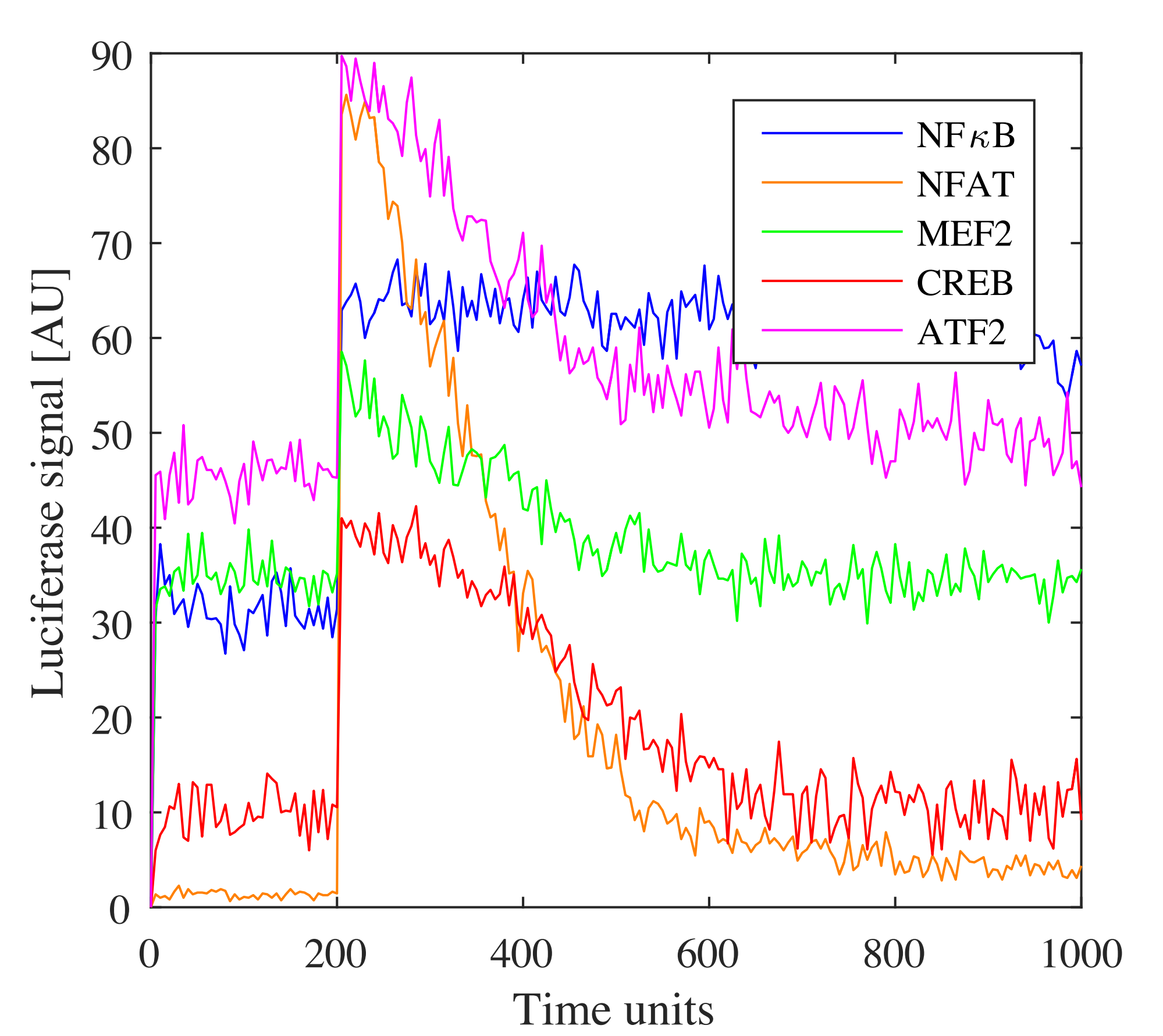
Parameter	Value	Standard Error	Units
Min	7.98 %	1.066	/
Max	36.55 %	0.5863	/
$\log EC_{50}$	-7.968	0.06724	mol/L
n (Hill coefficient)	0.8411	0.1009	/
K_d	8×10^{-9}	0.0577	mol/L

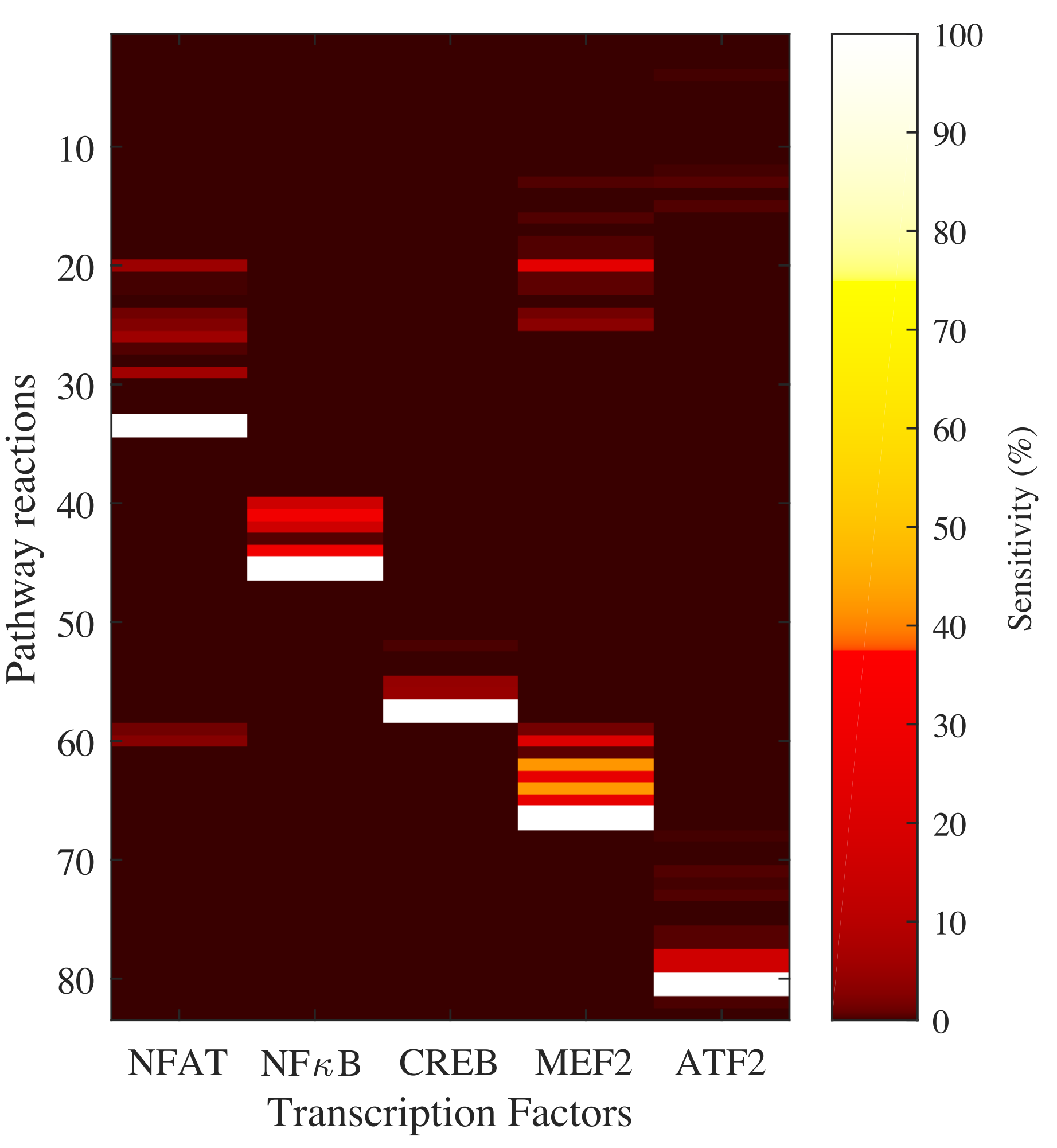
Table 3

Parameter	Value	Tissue
R_{HE}	5.60	Heart
R_{LU}	3.56	Lungs
R_{KI}	6.64	Kidney
R_{LI}	11.63	Liver
R_{BO}	3.88	Skeletal muscle
R_{BR}	5.78	Brain
R_{SP}	5.83	Spleen
R_{SI}	5.56	Small intestine
R_{CO}	25.90	Large intestine

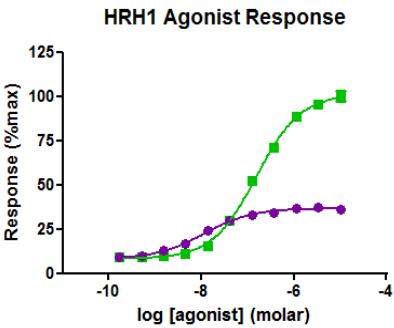

L
R:βγ:αq GDP
L:R
βγ
PLC:αq GTP
PLC
αq GTP
PI3K
PI3K [A]
GRK2 [A]
PKA
PKA [A]
αq GDP
PIP2
AC
βγ:AC
DAG
PKC:DAG
βγ:αq GDP
GRK2
NFκB
luc(E11-12)
PKC
IKK
IκB [P]
IκB
GSK3β [P]
IP3
IKK [P]
IP3:ER
GSK3β
ER
IκB:NFκB
IκB:NFκB [P]
Ca
ATF2 [P]
luc(A1-2)
ATF2
CaM
BTK:PIP3
CaMK
Ca:CaM
CaN
NFAT [P]
BTK
Ca:CaM:CaMK
Ca:CaM:CaN
NFAT
luc(F7-8)
Akt [A]
PIP3
CREB
CREB [P]
HDAC [P]
Mef2:HDAC [P]
Akt
PDK
Mef2
CREB:CREB [P]
HDAC
Mef2:HDAC
luc(E3-4)
luc(A11-12)
R
L:R [P]


Luciferase Dynamics
NFAT
NFκB
CREB
MEF2
ATF2
Concentration change relative to steady state
Time units
0
2
4
6
8
10
0.5
1
1.5
2
3


NFκB
NFAT
MEF2
CREB
ATF2
Luciferase signal [AU]
Time units
0
10
20
30
40
50
60
70
80
90
0
200
400
600
800
1000

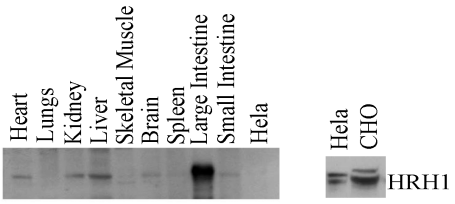

Pathway reactions
10
20
30
40
50
60
70
80
NFAT
NFκB
CREB
MEF2
ATF2
Transcription Factors
100
90
80
70
60
50
40
30
20
10
0
Sensitivity (%)

A

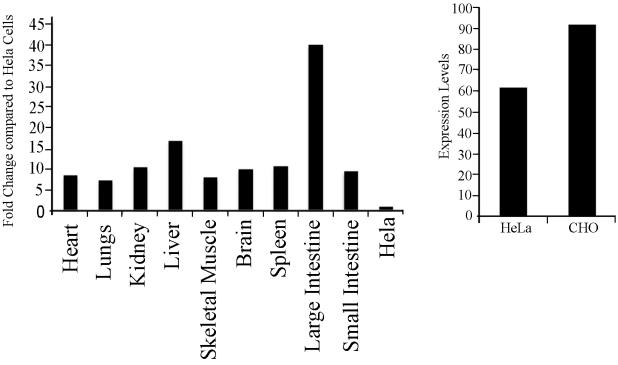


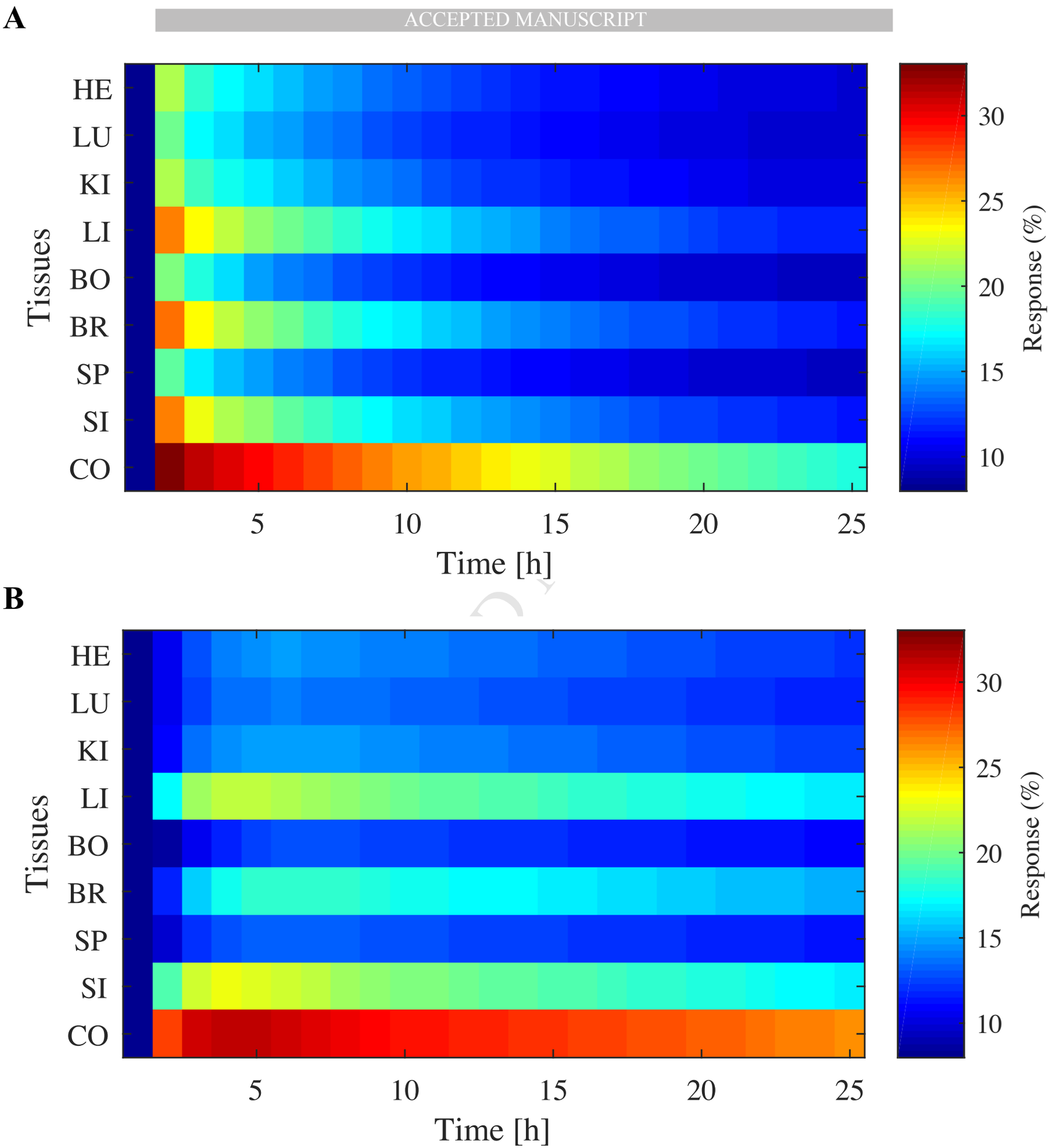
	Histamine	Lisuride
HillSlope	0.9060	0.8411
EC50	1.531e-007	1.076e-008

B



C





A combined *in vitro/in silico* approach to identifying off-target receptor toxicity

Highlights

- Development of *in vitro/in silico* framework for identifying off-target toxicity.
- Mathematical modelling of receptor signalling and related transcriptional activity.
- Identification of key events in the signalling pathway.
- Off-target receptor activation *in vivo* simulated using PBPK modelling.