Mathematical modelling and statistical analysis of indocyanine green and other biomarkers of hepatic function and drug-induced liver injury

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Abstract

The diagnosis and evaluation of drug-induced liver injury is a complex problem that often relies on the measurements of biomarkers of hepatic function. The utility and translatability of these biomarkers is dependent on many factors such as practicality, invasiveness, cost, relevance and ease of use. All of this is underlined by assumptions regarding the validity of a given biomarker as a metric that directly relates to hepatic function and consequently, liver injury and even necrosis. To understand biomarker validity it is important to understand the system mechanisms that influence the measurements of these biomarkers and how they are affected by hepatotoxic drug doses. Mathematical modelling allows for the explicit representation of biomarker mechanisms that influence blood dynamics and is therefore a useful tool in enhancing the understanding of the impact of liver injury upon biomarker dynamics. Indocyanine green (ICG) is used as a biomarker of hepatic function due to properties such as exclusive hepatic clearance. Additionally, the biomarker can be used as a visual tool for non-invasive imaging techniques such as multi-spectral optoacoustic tomography (MSOT). A combined systems toxicology framework includes a pharmacokinetic model of ICG clearance that represents underlying biological processes. The framework enhances mechanistic understanding of the relationship between drug dose, ICG kinetics and liver injury over time. Statistical analysis techniques whereby ICG is combined and compared with other established biomarkers of DILI are able to further assess the pre-clinical potential, relevance and translatability of ICG clearance measured by photoacoustic imaging as a metric of liver function.

**Keywords:** Systems toxicology; *in silico*; DILI; ICG; liver toxicity.

# Introduction

Indocyanine green (ICG) is a water-soluble dye that binds to plasmatic proteins when injected intravenously and is exclusively cleared by the liver (Ott, 1998). Without any metabolic change, ICG is excreted via the bile canaliculi and eliminated from the body without enterohepatic circulation (Ott, 1998). ICG clearance is principally dependent on blood flow and hepatocyte integrity and can be used as a metric of liver function (Halle et al., 2014, Levesque et al., 2016). ICG measurements obtained via multi-spectral optoacoustic tomography (MSOT) have been used to monitor liver function impairment in mice after paracetamol (APAP)-induced liver injury (APAP-ILI) (Brillant et al., 2017). The observed delay of ICG clearance (increase in ICG half-life) correlated with elevations in novel and established APAP-ILI biomarkers and histopathological scores for acute liver damage. ICG clearance was determined by assuming a simple first order decay of the signal intensity, fitting to the experimental data and calculating the corresponding half-life. This is a useful metric but it is limited as it omits key features of the underlying biology and as such, it is difficult to elucidate the mechanistic effects of hepatocyte death, varying hepatic flow rate, reduction in hepatic ATP, etc.

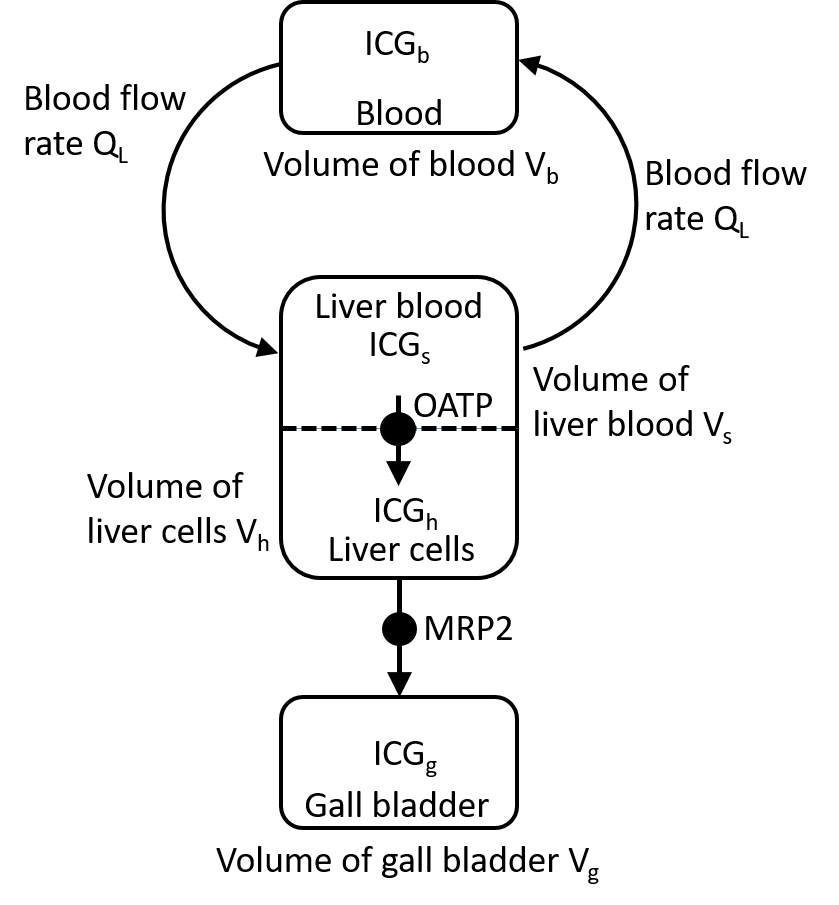
APAP-ILI normally resolves after undergoing a phase of hepatocyte necrosis, inflammatory response and regeneration. When there is necrosis in the centrilobular vein, neutrophils and macrophages are recruited, extravasation of cells is facilitated by vasodilation and blood flow velocity is reduced (Mitchell, 2015). Liver architecture is disrupted due to the accumulation of dead hepatocytes which further impair the blood flow through the liver sinusoid. These factors may contribute to an effective delay in the clearance of ICG from the blood. Furthermore, ICG uptake by the liver parenchyma is carried out by non-ATP dependent transporters and its excretion is carried out by ATP-dependent transporters (Huang & Vore, 2001, de Graaf et al., 2011). Therefore, the clearance of ICG may also be influenced by changes in hepatocyte homeostasis, energy storage and membrane transporter activity. Multidrug Resistance-Associated Protein 4 (Mrp4) is an efflux membrane transporter involved in excreting APAP from hepatocytes, and mice pre-treated with APAP have been shown to overexpress Mrp4, resulting in significantly reduced toxicity following a second (higher) dose (Aleksunes et al., 2008). Therefore, changes in membrane transporter expression due to APAP treatment could also occur for ICG transporters and modify its clearance.

There remain many uncertainties regarding how ICG clearance measured by MSOT can be pre-clinically and clinically applied in APAP-ILI scenarios. There is still a lack of knowledge about which physiological processes are most likely to have the most impact upon ICG elimination from the body during APAP-ILI. *In silico* modelling is a useful tool for enhancing mechanistic understanding of biological systems and toxicological events (Valerio, 2009, Combes, 2012, Raies & Bajic, 2016). Such applications are generally based on the mathematical construction of a cell or organ network that can then be manipulated towards theoretical or normally inaccessible pharmacological scenarios allowing for the analysis and quantification of parameters that would normally not be possible with *in vitro* or *in vivo* experiments (Diaz Ochoa et al., 2012, Remien et al., 2012). Additionally, this approach is a cost-effective way of understanding physiological and pathophysiological events while complying with the principles of the 3Rs in decreasing the number of animals required in a given study (NC3Rs, 2017). In order to further study ICG as a metric of liver function, we have developed a mathematical model of ICG clearance from the blood through the liver parenchyma to better understand the *in vivo* processes that are most likely to influence the rate and characteristics of this clearance during APAP-ILI. We further complement our analysis with statistical modelling to assess ICG parameters in combination with other DILI biomarkers to determine the capacity of ICG for refining predictions of either early injury or hepatocyte necrosis caused by APAP.

# Materials and Methods

## A mechanistic mathematical model of ICG dynamics

A four-compartment pharmacokinetic (PK) model was developed to simulate the *in vivo* dynamics of ICG. The compartments denote: i) blood (with volume Vb = 0.002 L; sources for all parameter values are indicated in Table 1); ii) liver sinusoid/blood (with volume Vs = 1.34×10-4 L); iii) liver cells/hepatocytes (with volume Vh = 4.14×10-4 L); iv) gall bladder (with volume Vg = 1.4×10-5 L). ICG concentration (rather than signal intensity) is modelled in each of these compartments with notation ICGb, ICGs, ICGh, ICGg for concentrations (nM) of ICG in the blood, sinusoid, hepatocytes and gall bladder, respectively. In line with the experiments, an initial (t = 0) level of ICG in the blood (ICGb) of 4 nM and zero initial ICG concentration elsewhere (i.e., ICGs = ICGh = ICGg = 0) is assumed. Transport to/from the blood into the liver sinusoid is governed by first order (linear) kinetics and QL denotes the liver blood flow rate (0.165 L/h). A schematic of the model is shown in Figure 1 with corresponding model parameter values in Table 1.



**Figure 1: Schematic of the ICG PK model.** The model includes four PK compartments for: i) rest of body (i.e., non-liver) blood (with volume Vb); ii) liver sinusoid/blood (with volume Vs); iii) liver cells/hepatocytes (with volume Vh); iv) gall bladder (with volume Vg). Liver blood flow rate is denoted by QL. Concentrations of ICG are represented by ICGb, ICGs, ICGh, ICGg in the blood, sinusoid, hepatocytes and gall bladder, respectively. OATP and MRP2 denote transport from liver blood into the hepatocytes and from hepatocytes into the gall bladder, respectively.

Uptake of ICG from the liver sinusoid into the hepatocytes is assumed to be governed by non-ATP dependent membrane transport proteins (e.g., OATP, NTCP; this transport in the model is denoted via the term ‘OATP’) whereas efflux from the hepatocytes into the bile, and ultimately the gall bladder, is governed by ATP-dependent transporters (e.g. MRP2; denoted in the model via the term ‘MRP2’). ICG does not return to the circulation from the gall bladder and so, for the purposes and timescales of this study, the gall bladder represents an effective sink compartment. The PK model represents a simplification of the processes involved in the distribution and clearance of ICG, intended to provide enough mechanistic information to describe necessary process relevant for effective parameterisation and to maximise the utility of available experimental data. As such, additional physiological processes such as spatial dynamics arising from liver sinusoid zonation or intercellular transport along gap junctions between neighbouring hepatocytes are omitted to avoid additional complexity but are implicit within the more abstract, simplified model structure.

Standard Michaelis-Menten saturating kinetics are used for the transport terms (OATP and MRP2), namely:

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| --- | --- |
|  | (1) |
|  | (2) |

The parameters denote maximum transport rates and parameters reflect the reciprocal of the substrate-transporter affinity, e.g. a large value denotes a low substrate:transporter affinity and vice-versa. Transport parameter rates (see Table 1) are assigned as = 6.24×103 nmol/h, = 120 nmol/h, = 2.92×104 nM, = 1×105 nM. The following system of ordinary differential equations (ODEs) describe how the concentrations of ICG in the four compartments change over time:

|  |  |  |
| --- | --- | --- |
|  |  | (3) |
|  |  | (4) |
|  |  | (5) |
|  |  | (6) |

Model parameters can be found in Table 1.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **Description** | **Value** | **Units** | **Reference** |
|  | Blood volume | 2×10-3 | L | (Utturkar et al., 2013) |
|  | Sinusoid (liver blood) volume | 1.34×10-4 | L | (Kusuhara & Sugiyama, 2010) |
|  | Hepatocyte volume | 4.14×10-4 | L | (Kusuhara & Sugiyama, 2010) |
|  | Gall bladder volume | 1.4×10-5 | L | (Wang et al., 1997) |
|  | Hepatic blood flow rate | 1.67×10-1 | L/h | (Utturkar et al., 2013) |
|  | Maximum (ATP-independent) influx rate of ICG into hepatocytes | 6.24×103 | nmol/h | (Paumgartner, 1970) |
|  | Sinusoid ICG concentration at which ICG hepatic influx is half-maximal | 2.92×104 | nmol/L | (Ott, 1998) |
|  | Maximum (ATP-dependent) efflux rate of ICG into from hepatocytes into bile | 120 | nmol/h | (Huang & Vore, 2001) |
|  | Hepatocyte ICG concentration at which ICG hepatic efflux is half-maximal | 1×105 | nmol/L | Estimated |

**Table 1: Summary of parameters for a mechanistic mathematical model of ICG clearance.**

Note that all of the model parameters can be sourced from the literature except for , which represents the hepatocyte ICG concentration at which ICG hepatic efflux into the bile is half-maximal. We find, however, that varying has negligible effect on the ICG blood concentration (see sensitivity analysis) and therefore we choose an arbitrary value for this parameter to allow for relatively slow clearance of ICG from the liver (e.g., see Figure 2).

As part of the sensitivity analysis, it is of particular interest to examine how sensitive ICG blood concentration dynamics are to changes in the number of viable hepatocytes, liver flow rate, hepatocyte uptake kinetics and ATP-dependent hepatocyte efflux kinetics. The effect of liver flow rate can be simply determined by varying the parameter QL. However, viable hepatocytes, hepatocyte uptake kinetics and hepatocyte ATP-dependent efflux kinetics are more difficult with the model in its current form as both transporter terms implicitly depend on the number of viable hepatocytes. For example, in terms of their physiological interpretation, the and terms are equal to the products of their respective maximum transport rates (units per hour) and the amount of corresponding transport proteins (units nmole), that is in both cases:

This can be rewritten as:

Or equivalently:

In the model, total hepatocyte volume is denoted by . Therefore, denoting the bracketed term by , the maximum transporter rates can be rewritten as , namely: and . Substituting these into our system of ODEs (equations (3)-(6)) gives:

|  |  |  |
| --- | --- | --- |
|  |  | (7) |
|  |  | (8) |
|  |  | (9) |
|  |  | (10) |

where, by using our estimates for and we obtain = 1.51×107 nmol/L/h and = 2.90×105 nmol/L/h. In this new form, the dependency of hepatocyte volume on transporter kinetics is made explicit and therefore viable hepatocytes, hepatocyte uptake kinetics and hepatocyte ATP-dependent efflux kinetics can now be explored directly by varying parameters (for viable hepatocytes); and (for hepatocyte uptake kinetics); and and (for hepatocyte ATP-dependent efflux kinetics).

## Calculation of model parameter sensitivity

Sensitivity analysis can determine which model output (e.g., a dependent variable such as ICG blood concentration) is most sensitive to a specific condition (e.g., change in hepatic flow rate), defined by model input (e.g., parameter ). Time-dependent coefficients are used to quantify these sensitivities. For example, the coefficient denotes the scaled sensitivity of to changes in parameter :

|  |  |
| --- | --- |
|  | (11) |

Here, represents model parameters and , and each coefficient is normalised so that each output is dimensionless and the relative sensitivity for each parameter can be assessed. Using these coefficients, corresponding time-integral sensitivity coefficients () are calculated to give an indication of the total sensitivity over the entire time course of the simulation:

|  |  |
| --- | --- |
|  | (12) |

Details of this method can be found in full elsewhere (Martins, 2000, Martins, 2001, Ingalls & Sauro, 2003).

## Measuring the impact of APAP toxicity upon ICG clearance

The impact of APAP upon liver functionality was assessed via measurements of ICG clearance through the liver by means of MSOT imaging. Briefly, mice were dosed with APAP and then subsequently administered ICG for imaging and measurement of liver function impairment at various time-points (3, 6, 24, 48, 72 and 96 hours post-APAP dose). Vehicle control measures of ICG dynamics were also measured at the same time-points. Full details of the experiments are provided below.

### Experimental methods

Experimental protocol was described and published by Brillant et al. (2017). Briefly, C57BL/6J mice (Charles River, Margate, UK), aged 7 - 8 weeks old were group housed (n = 5) with free access to food and water with a seven-day acclimatisation period. Male mice were fasted for 14 h prior to APAP dosing. For the time course study, groups of randomly selected animals were administered 300 mg/kg of APAP (Sigma Aldrich, UK) in 0.9% saline (Braun, Melsungen, Germany) or 0.9% saline (vehicle control) with a single intraperitoneal (i.p.) injection. For the assessment of liver function impairment, mice were imaged at different time-points (3 h, 6 h, 24 h, 48 h, 72 h and 96 h) post-APAP dosing and euthanised immediately after imaging for blood and liver harvesting. For MSOT imaging purposes, mice were administered 40 nM of ICG (i.v.) for the measurement of ICG clearance from the blood, and 5 nM for the measurement of ICG accumulation and clearance from the liver and gallbladder as described by Brillant, et al. (2017).

### Optimal parameter perturbations to describe effects of APAP toxicity on ICG blood dynamics

Results of the model sensitivity analysis informed which two parameters in the model are likely to best explain variations in ICG dynamics following dosing with APAP. These parameters were varied within the model across a range based on their default value (0 to 1,000% of default at intervals of 1%) to generate 1,001×1,001 combined outputs for 6 scenarios (3, 6, 24, 48, 72 and 96 h post-APAP dose). These outputs were compared with the data to determine which parameter combination provided the best fit between model and experiment. This comparison was quantified with the normalised relative error metric:

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| --- | --- |
|  | (13) |

where represents the relevant experimental data for ICG concentration in the blood obtained via MSOT and denote the model parameters being optimised. The “best fit” is defined as the parameterisation,

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| --- | --- | --- |
|  |  | (14) |

such that

|  |  |  |
| --- | --- | --- |
|  |  | (15) |

A secondary measure of best fit was used to ascertain which optimised parameter sets provided good fits with minimal deviations from the default model parameterisation. “Good fits” were defined as error metric values that were within 5% of the absolute minimum error. That is the set,

|  |  |  |
| --- | --- | --- |
|  |  | (16) |

such that

|  |  |  |
| --- | --- | --- |
|  |  | (17) |

A further fitting measure is defined using the minimum distance in parameter space between the default parameter coordinates (, ) and parameter coordinates that correspond with a “good fit” error metric. This “minimum distance” fit is thus defined,

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| --- | --- | --- |
|  |  | (18) |

such that

|  |  |  |
| --- | --- | --- |
|  |  | (19) |

## Numerical simulation

All numerical simulations of the model were performed using MATLAB R2019a software.

## ICG as diagnostic biomarker for liver injury and necrosis

To better understand the pre-clinical utility, translatability and potential of ICG measurements by means of MSOT imaging as a diagnostic tool to predict and identify drug-induced liver injury (DILI), statistical analysis (details below) was employed to assess and compare the predictivity of ICG data and conventional serum biomarkers with regard to DILI.

### Experimental methods

In order to assess liver toxicity, the measurement of serum biomarkers such as alanine aminotransferase (ALT), total bilirubin (TBIL), micro-RNA122 (miR122) and high mobility group box 1 (HMGB1) was performed as in Brillant et al. (2017) and as previously described (Antoine et al., 2009, Antoine et al., 2010, Starkey Lewis et al., 2011, Antoine et al., 2012, Antoine et al., 2013). Briefly, serum ALT activity was determined by kinetic assay according to the manufacturer's instructions (Thermo Fisher) and assayed on a Varioskan Flash machine (Thermo Fisher). miR122 was extracted and purified using a miRNeasy kit followed by an RNeasy MiniElute Cleanup Kit (Qiagen, Venlo, Netherlands), in accordance with the manufacturer's instructions. Reverse transcription was performed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) and miR-22 primers using a GeneAmp PCR9700 machine and qPCR reactions were run using Taqman PCR primers and Master mix (Applied Biosystems) on a ViiA7 machine (Life Technologies). Total HMGB1 measurement was determined by ELISA according to the manufacturer's instructions (Shino-Test/IBL international) and total bilirubin (TBIL) was measured using a Bilirubin Assay Kit (Sigma-Aldrich) following the manufacturer's instructions (Brillant et al., 2017).

### Multinomial logistic regression

Multinomial logistic regression aims to predict the probability of falling into an outcome category based on predictor variables. The relationship between predictor (biomarkers) and outcome variables (HD or DILI score) can be represented using a generalised linear model,

|  |  |
| --- | --- |
|  | (20) |

The objective of the regression is to optimise the parameters , , such that the model provides the best fit to the observed data. For various values of , , and , the probability of falling into a particular class can be estimated by applying a logistic transformation to equation (20) according to the logistic cumulative distribution function, resulting in the following:

|  |  |
| --- | --- |
|  | (21) |

The following key assumptions were assessed prior to analysis:

* **The dependent variable is measured on a nominal level.** This assumption holds for both scenarios since the HD scores are categories ranging from 0-4 and the DILI scores are categories ranging from 0-2.5.
* **One or more of the independent variables are continuous, ordinal or nominal.** The predictor variables in our model are represented by biomarker concentrations (ALT, miR122, HMGB1, TBIL) and ICG, all of which are measured on a continuous scale.
* **Observations must be independent, with the dependent variable having exclusive/exhaustive categories.** In the data used for this analysis, each mouse is independent of one another, and each mouse must fall into category [0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4] for HD score or [0, 0.5, 1, 1.5, 2, 2.5] for DILI score. For both models, each mouse can only be assigned one score.
* **Multi-collinearity should not be present between any predictor variables**. This assumption was tested using the Variance Inflation Factor (VIF) test, all predictor variables remained within the recommended reasonable range for the VIF (1 – 10).
* **Outliers/highly influential points should not be present in the observed data.** No outliers were detected.
* **Adequate sample size.** A recommended sample size is 10 x the number of predictor variables. For the model predicting HD, there are 12 observations and therefore only one predictor variable may be used at each time. For the model predicting DILI score, there are 29 observations, therefore a maximum of 3 predictor variables may be used at one time.

Following assumption validity assessment, ordinary-least-squares (OLS) was used for parameter optimisation. For tests with multiple panels, a forward stepwise regression analysis was conducted. For the model predicting HD, each biomarker was individually trialled for predictive capability and results of each are reported. For the model predicting DILI score, each potential combination of up to three biomarkers was tested for predictive capability, with only successful models reported.

# Results

## Compartmental ICG dynamics and the effects of APAP-ILI

MSOT imaging data of ICG concentration in different compartments of the body reveal characteristic clearance of the dye in control conditions when administered with an initial concentration of 4 nM (blood) and 0.5 nM (liver and gall bladder) (Figure 2). Simulating these experimental conditions in the parameterised mathematical model illustrates the good agreement between mathematical model and experimental data dynamics, validating the choice of PK model and parameter values. Model simulations indicate rapid uptake of ICG from the blood into the liver and subsequently into the hepatocytes. The hepatic ICG concentration then decreases over time due to efflux into the bile and gallbladder via the ATP-dependent transporters. Note that sinusoid and hepatocyte compartments in the model are combined for comparison with MSOT data for the liver.

Following an APAP overdose of 300 mg/kg, MSOT imaging records how clearance of ICG is reduced as blood concentrations remain higher for an extended duration (Figure 2). This effect is most notable in the transient blood dynamics. It should also be noted that while the mathematical model is fully quantitative, simulating molar concentrations in dimensional units of time, the MSOT data is only semi-quantitative (time units only) and is measured in arbitrary (pixel intensity) units. Therefore it is appropriate to focus on the temporal, transient dynamics and in particular, the blood compartment since this is where the initial dose (with known quantity) is supplied.

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| **Figure 2: Compartmental ICG dynamics with and without liver injury.** ICG concentrations were measured in the blood, liver and gall bladder via MSOT (control data, red). Clearance of ICG from the blood is used as an indicator of liver functionality. Following an overdose of APAP (300 mg/kg), MSOT imaging reveals a reduced rate of ICG clearance from the blood (APAP data, yellow). Corresponding simulations using the parameterised mathematical model (blue) demonstrate good agreement between model dynamics and the control data. |

## Model parameters affecting ICG concentration in the blood

In order to investigate how the mechanisms of ICG clearance are affected by liver injury and toxicity brought about by APAP overdose, it is necessary to determine which model parameters affect ICG dynamics in the blood when they are perturbed. Parameter perturbations therefore represent a potential disruption to the mechanism of ICG clearance and parameter sensitivity analysis allows for the discovery of the relevant parameters as well as the degree of sensitivity. Time-integral sensitivity coefficients are calculated to effectively provide a number for each parameter indicating how sensitive ICG blood concentration is to perturbations of this parameter. A relatively large coefficient value indicates a higher sensitivity and, therefore, small changes in this model parameter would result in relatively large changes in blood concentrations of ICG. The results of the sensitivity analysis show that ICG blood concentration is insensitive to the rate of ATP-dependent hepatic efflux into the bile (Figure 3). The remaining parameters (, , , ), however, do demonstrate sensitivity with a slightly greater effect observed for perturbations to the rate of hepatic blood flow ().

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| **Figure 3: Sensitivity analysis of the mathematical model of ICG clearance.** Time-integral sensitivity coefficients () give an indication of the total sensitivity of the model parameters , , , , and on ICG blood concentration over the entire time course of the simulation (20 minutes). |

## Mechanistic interpretation of APAP-ILI affecting ICG clearance

The investigation into the impact of APAP-ILI on liver functionality through the use of MSOT imaging was carried out based on imaging of ICG in the blood at 3, 6, 24, 48, 72 and 96 h post-challenge with a toxic dose of APAP. Mechanistic processes affected by this toxicity were proposed based on model parameters identified in the sensitivity analysis. ICG in the blood is sensitive to hepatic flow, liver volume and hepatic uptake kinetics. This corresponds to model parameters , , and . Parameter (representing affinity between ICG and non-ATP dependent membrane transport proteins) would not be expected to vary significantly due to APAP-ILI. Furthermore, changes in either or represent the exact same mathematical process in the model in terms of affecting ICG dynamics in the blood compartment. Varying has much greater implications for the dynamics in other model compartments for which there is no data. Consequently, two mechanisms were considered for explaining the impact of APAP overdose in ICG dynamics: changes in hepatic flow rate () and hepatic uptake rate ().

Discrepancies between MSOT data measured post-APAP dose and APAP-free control data were accounted for in the model via optimisation of parameters and . These parameters were varied as specified in Section 2.3.2 in order to identify various minimal values of the error term in equation (13) to denote the “best fit” () and “minimum distance” () fitting metrics. The results of this analysis are visualised in Figure 4A by plotting the error metric (in terms of percentage of the minimum error) across the explored parameter space for each time-point (post-APAP dose). Note that the extent of plot colouration is reduced to show only good fits (within 5% of the minimum error value). The percentage changes required in both parameters to adequately describe the different ICG dynamics for each time-point post-APAP dose are summarised in Figure 4B. The data showing dynamics of ICG concentration in the blood at each time-point are compared with both the best fit and minimum distance fitting metrics (Figure 5). This illustrates that relaxing the fitting criteria from absolute minimum error (best fit) to the minimum distance metric does not significantly reduce the quality of fit.

The results of Figure 4B suggest that a reduction in hepatic flow rate alone is sufficient to describe the ICG dynamics at 3, 6 and 24 h. This parameter change replicates the reduction in ICG clearance observed at these time-points in Figure 5. The ICG blood dynamics are not sufficiently different at 48 h and 72 h to justify any mechanistic changes from the default parameterisation (APAP-free control). At 96 h, the analysis suggests that both hepatic flow and uptake rates must be increased to sufficiently describe the ICG dynamics of the data. In Figure 5 it is observed that this corresponds with a somewhat unexpected increase in the clearance of ICG, perhaps due to some adaptive response. It should be noted that not all best fits across the 6 time-points are equally good at describing the data and the 96h-time-point data represents the least good fit. R2 values of 0.85, 0.97, 0.98, 0.91, 0.93 and 0.72 were obtained for the best fits to data representing 3, 6, 24, 48, 72 and 96 h time-points post-challenge with APAP overdose.

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| **A** |
|  |
| **B** |
|  |
| **Figure 4: Error analysis of parameter perturbations to describe changes in ICG clearance following APAP-ILI.** Model parameters representing hepatic flow () and hepatic uptake () rates were varied in order to identify minimum parameter deviations such that the resulting fit is still within 5% of the best fit when compared with ICG blood data. (A): Small errors (within 5% of minimum) are plotted across the 2D parameter space for each time-point post-APAP overdose. The position of the “best fit” (red dot) and “minimum distance” (blue dot) fitting metrics in parameter space are also indicated. (B): The percentage change in hepatic flow (red) and hepatic uptake (blue) parameters required to adequately describe the observed ICG blood dynamics is summarised illustrating how these mechanisms might change and adapt over time. |

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| **Figure 5: Comparing ICG blood concentration data to mathematical model dynamics with optimised changes in hepatic flow and hepatic uptake.** The mathematical model was independently fit to the data using both “best fit” (red, solid) and “minimum distance” (green, dashed) fitting metrics for each time-point post APAP overdose. |

## Predicted impact of APAP-ILI on ICG dynamics in the liver

The impact of changes made to the mechanism of ICG clearance from the blood may have downstream consequences for ICG dynamics in both the liver and gall bladder compartments. When the availability of sufficient experimental data is limited, predictive mathematical model simulations can be particularly useful (Figure 6). The results of Figure 6 suggest that only subtle changes are made to ICG dynamics in the liver and gall bladder following APAP overdose. This corresponds well with what has been observed previously for the limited data available for these compartments (e.g., see Figure 2).

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| **Figure 6: Model predictions of ICG dynamics for downstream compartments.** Model simulations were carried out based on optimised parametrisations for each time-point post-APAP scenario (“minimum distance” fitting metric). The control (APAP-free) scenario is also plotted for comparison. The impact of APAP on ICG clearance in the blood is reflected in the rate of accumulation in the liver compartment. |

However, it should be noted that the effects of APAP overdose are only implemented in the model in terms of the impact upon ICG blood concentrations and therefore any other changes that may occur in downstream compartments (such as ATP-dependent efflux transport for example) are not explicitly accounted for and remain speculative and uncertain. To emphasise this point, and further justify the prior decision to vary hepatic uptake () rather than liver volume (), it is interesting to consider the implications of using changes in liver volume that suitably describe ICG blood data but have severe and unrealistic consequences for ICG liver data. This is illustrated by simulating the model with optimised changes in hepatic flow and volume (rather than uptake) and, to further stress the importance of being mindful of potential implications for downstream compartments, the “best fit” metric is used rather than the “minimum distance” (Figure 7). Using the best fit metric results in a slightly more distinct reduction in clearance of ICG from the blood (Figure 7B). This is because the minimum distance metric marginally overestimates clearance for shorter post-APAP times (see Figure 5). However, the rapidly changing volumes of the liver, that are suitable to recapitulate ICG blood dynamics (Figure 7A), result in significant and unrealistic fluctuations in ICG concentration in the liver (Figure 7C).

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| **A**  **B**  **C**  **D** |
| **Figure 7: Model predictions made with liver volume fluctuations optimised to fit ICG blood dynamics.** (A): Parameter changes required in hepatic flow and liver volume (rather than hepatic uptake) to fit ICG dynamics in the blood following APAP overdose (“best fit” fitting metric). Note that required fold-changes in parameter value are equivalent for hepatic uptake () and liver volume () when considering the effect on blood dynamics only. Model simulations with the scenario parameterisations of (A) are plotted for blood (B), liver (C), and gall bladder (D) compartments. |

## Using ICG and DILI biomarkers to predict hydropic degeneration and hepatocyte necrosis

To investigate the potential utility of ICG half-life, multinomial logistic regression was applied, comparing ICG with gold-standard DILI biomarkers with regards to their predictivity of two hepatic function endpoints, hydropic degeneration and DILI (represented by level of necrosis). Note that, although measurements of necrosis are used here to derive a DILI score for APAP, in a more general context, DILI and necrosis are not synonymous. Markers were investigated individually for their capability to predict HD score (Table 2), and as a panel for their capability in predicting DILI score (Table 3), to compare with the clinical protocol.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Goodness of fit** | | **Likelihood ratio tests** | |
| **Predictor** | **Chi-square** | **Sig (p-value)** | **Chi-square** | **Sig (p-value)** |
| **ALT** | 40.984 | 0.814 | 7.205 | 0.206 |
| **TBIL** | 39.402 | 0.707 | 8.447 | 0.133 |
| **miR-122** | 46.010 | 0.634 | 4.624 | 0.623 |
| **HMGB1** | 55.201 | 0.285 | 4.273 | 0.511 |
| **ICG** | 34.702 | 0.951 | 12.173 | 0.032 |

**Table 2: Suitability of biomarkers/ICG half-life in predicting HD.** Degrees of freedom; 50 (goodness of fit); 5 (likelihood ratio tests). The significance (p-values) are calculated by using the chi-square distribution and the given degrees of freedom. For the goodness of fit tests, significant results (p < 0.05) indicate a poor fit to the data. We therefore require the p-value to be > 0.05 in order for the model to fit the data well. For the likelihood ratio tests however, significant results (p < 0.05) indicate that the model including predictor variable is an improvement on the intercept only model. We therefore require the p-value to be < 0.05 in order for the model to be useful.

All of the logistic regression models aiming to predict HD score (ALT, TBIL, miR-122, HMGB1 and ICG as predictors) fit the data well, as indicated by all p-values > 0.05 (Table 2). Interestingly though, ICG is the only predictor resulting in a statistically significant improvement in predictions when compared to an intercept only model. This is indicated by a significant likelihood ratio test (p = 0.032).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Goodness of fit** | | **Likelihood ratio tests** | | **R2** |
| **Predictor** | **Chi-square** | **Sig (p-value)** | **Chi-square** | **Sig (p-value)** |
| **ICG** | 168.780 | 0.026 | 11.448 | 0.043 | - |
| **ALT+ICG** | 55.529 | 1.000 | 50.578 | 0.000 | 0.568 |
| **TBIL+ICG** | 161.418 | 0.032 | 17.386 | 0.066 | - |
| **ALT+TBIL+ICG** | 8.067 | 1.000 | 79.369 | 0.000 | 0.892 |
| **ICG+HMGB1+miR-122** | 14.298 | 1.000 | 73.809 | 0.000 | 0.829 |

**Table 3: Suitability of biomarkers/ICG in predicting DILI score**. The significance (p-values) are calculated by using the chi-square distribution and the given degrees of freedom. For the goodness of fit tests, significant results (p < 0.05) indicate a poor fit to the data. For the likelihood ratio tests however, significant results (p < 0.05) indicate that the model including predictor variable is an improvement on the intercept only model. For any models which are an improvement on the intercept only model, R2 values are provided to describe how well the model can represent the variation in the data.

Most of the logistic regression models aiming to predict DILI score (Table 3) provide reasonable fits to the data, as indicated by goodness of fit p-values > 0.05. Of these models, whilst all provide an improvement over the intercept only model (likelihood ratio p-value <0.05), some better represent the variability of DILI score observed. If the APAP component of the current clinical panel (APAP + ALT + TBIL) were to be replaced with ICG, a non-invasive marker, the novel panel (ALT+TBIL+ICG) represents variability in DILI score well, with an R2 of 0.892. A panel including solely novel biomarkers (ICG + miR-122 + HMGB1) also accurately reflects variability in DILI score (R2 0.829).

# Discussion

The clearance of Indocyanine green (ICG) from blood plasma is correlated to the functionality of the liver (Faybik et al., 2004, Zoller et al., 2014). ICG is primarily taken up by hepatocytes and excreted into the liver canaliculi and gall bladder without any metabolic change and without enterohepatic circulation. Therefore the clearance of this dye mainly depends on blood flow, hepatocyte integrity and biliary excretion. In order to investigate the nature of these clearance processes from a mechanistic viewpoint and establish the utility of ICG as an effective biomarker for liver function impairment, a suite of experimental, mathematical modelling and statistical analysis techniques were combined in a systems toxicology approach. The aims of this approach were to quantitatively assess the mechanisms in the compartmental pharmacokinetic system that can affect and delay ICG clearance in APAP-ILI and investigate the optimal panel of biomarkers in combination with ICG half-life to predict APAP-ILI. Furthermore, this systems toxicology approach is an important example of how the utility of existing animal data can be maximised via integration with new computational modelling studies in line with the principles of the 3Rs (NC3Rs, 2017).

A fully parameterised mathematical model of ICG clearance compares well with default (healthy liver) data to describe the control kinetics in multiple compartments (blood, liver and gall bladder). The impact of APAP overdose on these clearance kinetics was measured experimentally and accounted for in the model by suitable parameter modifications, informed by sensitivity analysis. This analysis allowed us to discount inhibition of ATP-dependent efflux as a mechanism of APAP-ILI affecting ICG blood clearance and is an important finding of the *in silico* model. The hyperbolic shape of the error analysis plots (Figure 4), indicating where good fits can be located in parameter space, imply that variations in ICG clearance following APAP overdose can be simulated in the model by suitable changes in either hepatic uptake rate (*)* or hepatic flow rate (*)* or a combination of both. A minimum distance fitting metric was defined in order to identify parameter adjustments that were sufficient in order to fit the data with minimal deviation from the default parameter set (i.e., from APAP-free control conditions). The model fits the data relatively well for all data except perhaps the 96h time-point post-APAP dose (R2~0.72). This data differs from the rest in that clearance of ICG is significantly increased beyond that of normal control conditions. This is in contrast with earlier time-points whereby ICG clearance is reduced due to suspected effects of liver injury and a reduction in liver functionality. The minimal changes in model parameters that provide a good fit to the data include reduced hepatic flow () at 3, 6, 24h post-APAP; no changes from control/default at 48 and 72h; and increases in both hepatic flow and ICG uptake into the liver ()*.* The model prediction that the dynamics of ICG in the blood are significantly, and perhaps primarily, affected by hepatic flow rate as a consequence of DILI are consistent with studies that report an increase in hepatic congestion and the accumulation of red blood cells affecting flow following APAP-induced hepatotoxicity (Hinson et al., 2010).

The model could be improved upon by taking into account apparent adaptation 96 h after dosing with APAP, potentially as a result of a response to liver injury. Alternatively, the model could be further expanded by introducing an extra distribution compartment to account for biphasic pharmacokinetics seen in some data, although this would require more extensive and abstract parameterisation. A critical component of the modelling approach was to be cautious about fitting metrics and extrapolation of data-driven modelling results. It is vitally important to be aware of the limitations of data used for modelling in a systems toxicology approach and to understand what evidence it provides for modelling hypotheses. Using the “best fit” is not always the most informative metric to understand how mechanisms within the system influence key biological outcomes. Indeed there are merits to using a “minimum distance” fitting metric whereby minimal parameter changes from the default scenario that still provide minimal error terms are identified. For example, the results of Figure 4 suggest that the ICG dynamics at 72h post-APAP are not significantly different to control conditions, but the “best fit” metric is found for a reduction in hepatic uptake by -50% and increase in hepatic flow by +855% with only a marginal reduction in the error term (R2~0.932 vs. R2~0.916 and Figure 5). Furthermore, one must be cautious when modifying parameters to explain observed data to also account for any downstream consequences for other parts of the model. For example, adjusting hepatic uptake rate or liver volume are equivalent in terms of their impact upon ICG blood dynamics and either can be modified to fit the data. However, altered liver volumes have much more significant effects on other compartmental dynamics such as the concentration of ICG in the hepatocytes (Figure 6 and Figure 7).

Statistical modelling identified the combination of ALT, TBIL and ICG as the optimal panel for predicting hepatocyte necrosis (DILI score) in our APAP-related study. This pre-clinical finding warrants further investigation, since clinically, introduction of a non-invasive marker of hepatic injury would improve patient experience whilst obtaining similar, and potentially greater scientific understanding. ICG alone was also able to better explain variability in hydropic degeneration when compared with the conventional biomarker panel. With early markers of injury still in high demand in the DILI community, our study highlights the potential utility of ICG as a marker for hepatic dysfunction.

DILI is a major human health concern and one of the principal reasons is that currently used biomarkers lack sensitivity and specificity (Kaplowitz, 2001, Lee & Senior, 2005). Hy’s law still remains the gold standard panel used for the diagnosis of severe liver injury, which includes ALT, TBIL and other parameters such as APAP concentration in the case of APAP overdose or protein adducts (Davern II et al., 2006, Temple, 2006). However, these biomarkers provide little insight into the underlying mechanisms of DILI and APAP concentration or protein adducts for instance are only applicable in APAP-ILI cases. For these reasons, developing and validating as well as evaluating the translatability of novel tools is important. Whilst ICG was investigated here in the context of necrosis as a form of DILI, the approach taken has potential utility for assessment of the relationship between ICG and other mechanisms of DILI, e.g., inflammation, apoptosis, cholestasis, etc. Using a systems toxicology approach, the results of this study support and enhance the value and utility of ICG kinetics measured by means of non-invasive MSOT imaging in DILI scenarios.

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Author Contributions

JAL, CLM & NB wrote the manuscript; NB performed the experiments; JAL & SDW performed the mathematical modelling; CLM performed the statistical analysis; NB, JWD & SDW designed the research. All authors read and approved the final manuscript.

Declaration of Interests

Chantelle L. Mason is an employee of AstraZeneca and has interests in the company.

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