The Impact of Incomplete Dose-Response Curves on EC_{50} and E_{max} Determinations in Enzyme Induction Assessment

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Introduction

Induction of cytochrome P450 (CYP) enzymes is one of the principal mechanisms of drug-drug interactions. It is for this reason that the FDA recommends that new drug candidates be evaluated for their ability to induce CYP enzymes. The FDA recently revised the guidelines for evaluating CYP induction (FDA, 2012). According to this new guidance document, a new chemical entity (NCE) can be classified as a CYP inducer based on the results from calculation of CYP induction parameters with the basic method (R3 value calculation) or the mechanistic static model (FDA, 2012), amongst others. In addition, CYP induction potential of an NCE can be predicted by comparing the relative induction score of an NCE to a predefined threshold against a set of known inducers for a given test system (Fahmi *et al.*, 2008) (*i.e.,* the calibration approach) as well as the mechanistic models. These include mechanistic static modeling, which takes into consideration induction and inhibitory effects of the NCE in the gut and the liver, or a dynamic modeling approach, which takes into consideration *in vitro* drug disposition data (*e.g.*, protein/tissue binding, metabolism, transport and drug-drug interaction) and physicochemical properties, and population-based absorption, distribution and excretion of the NCE (FDA, 2012). These methods for predicting CYP induction by an NCE all require the calculation of the maximal fold induction (E_{max}) and the concentration at which there is a 50% maximal induction effect (EC_{50}). Therefore, it is now common practice to calculate EC_{50} and E_{max} values to aid in the prediction of drug-drug interactions. Typically, EC_{50} and E_{max} values are calculated when an increase of two-fold or higher is observed versus the vehicle control, and these values are used to assess clinical induction potential with the aforementioned approaches. There are multiple statistical models that can be used to calculate E_{max} and EC_{50} values from an experimental data set. In literature, two of the most common approaches to calculate these parameters are 1) the Sigmoid 3-parameter equation and 2) Hill 3-parameter equation. In general, the Sigmoid 3-parameter equation forces the calculated E_{max} towards the maximal experimentally observed value regardless of whether a 'true' E_{max} has been reached. Conversely, the Hill 3-parameter equation extrapolates the data set to calculate E_{max} and EC_{50} values that are closer to those obtained with a comprehensive data set.

In the present study, these two statistical approaches were evaluated for their fidelity to estimate EC_{50} and E_{max} when experimental E_{max} was not reached. We compared the two equations by applying each to various sets of CYP3A4 mRNA data, which demonstrates that these equations can generate disparate E_{max} and EC_{50} values with data sets where maximal induction was not reached.

Materials & Methods

Buffer RLT (Qiagen), DMSO (Sigma), Gene Expression Assay (Applied Biosystems), High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), Supplemented MCM (MCM+; XenoTech), Proteinase K (Qiagen), Rifampin (Sigma), RNase-free water (Fisher), RNase Inhibitor (Applied Biosystems), RNeasy Mini Kit (Qiagen), TaqMan Fast Advanced Master Mix (Applied Biosystems)

Cryopreserved human hepatocytes were seeded and cultured at concentrations ranging from 0.22 x 10^6 to 0.3 x 10^6 cells/well in a collagen-Matrigel sandwich configuration in 48-well tissue culture plates in a humidified culture chamber (37 ± 1°C at 95% relative humidity and 95/5% air/CO_2) for 24 hours. Cultures were then treated once daily for 3 consecutive days with MCM+ medium containing either 0.1% DMSO or one of six concentrations of rifampin (0.01, 0.05, 0.1, 0.5, 5 and 10 µM). Following treatment, cells were harvested with Buffer RLT. Total RNA was isolated from the cell lysates according to the Buffer RLT procedure (Qiagen) and purified using the RNeasy Mini Kit (Qiagen). RNA concentrations were determined by measuring absorbance at 260 and 280 nm on the NanoDrop. Single-stranded cDNA was prepared with the RT Master Mix using the AB 7900HT Fast Real-Time PCR System (Applied Biosystems). For the qRT-PCR assay, to assess the mRNA expression of CYP3A4, each PCR was performed in triplicate. A Primer Mix was prepared for each Gene Expression assay, which contained TaqMan Fast Advanced Master Mix (1X), Gene Expression Assay (1X, 900 nM primers) and RNase-free water. The Reaction Mix was prepared by adding the Primer Mix to cDNA. Reactions were analyzed on an Applied Biosystems Real-Time PCR sequence detection system (AB 7900HT). The relative quantity of the target cDNA compared with that of the endogenous control cDNA (GAPDH) was determined by the ΔΔCt method (Applied Biosystems User Bulletin #2). Relative quantification measures change in mRNA expression in a test sample relative to that in a vehicle control sample (*e.g.*, 0.1% v/v DMSO).

Data processing

Dose-response curves were generated by plotting the fold increase in CYP3A4 mRNA (y-axis) against the concentrations of the compound tested (x-axis). The EC_{50}, E_{max} and standard errors for each parameter for CYP induction response were calculated using SigmaPlot 12.0 based on the following equations:

Sigmoid 3-parameter

$$f = a/(1+\exp(-(x - x_0)/b))$$

where a = E_{max}, b = slope and x_0 = EC_{50}

Sigmoidal Hill 3-parameter

$$f = a*x^b/(c^b + x^b)$$

where a = E_{max}, b = slope and c = EC_{50}

Fold increase = Fold change -1

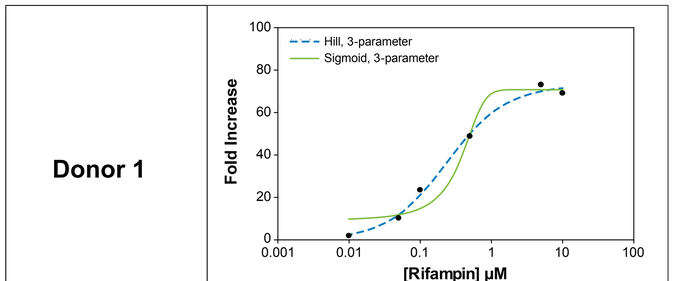
Results

To compare the Sigmoid 3-parameter and Hill 3-parameter equations, CYP3A4 dose-response curves were obtained from three preparations of cryopreserved human hepatocytes treated with rifampin as described above from which EC_{50} and E_{max} values were calculated (**Figure 1**, **Tables 1** and **2**).

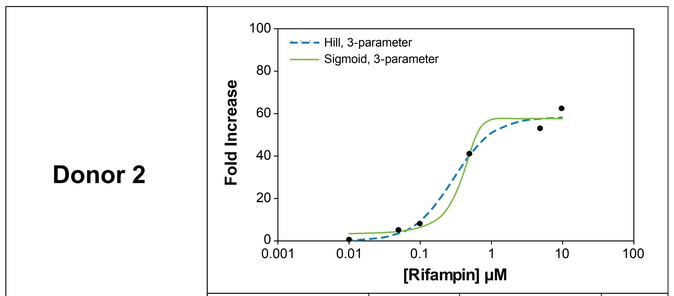
To demonstrate the effect of incomplete dose-response curves caused by toxicity or solubility limitations, CYP3A4 dose-response curves were obtained from three cultures of cryopreserved human hepatocytes treated with rifampin as described above (**Figure 2**, **Tables 1** and **2**). The dose-response curves were generated by systematically removing the highest concentration prior to a subsequent calculation of E_{max} and EC_{50} values. This method mimics data sets obtained when E_{max} cannot be reached experimentally (either due to cytotoxicity or compound insolubility).

The fidelity of each equation was evaluated by comparing EC_{50} and E_{max} results obtained from dose-response curves wherein a compound caused a CYP induction response resulting in a plateau (*i.e.*, E_{max} was reached). These results (**Tables 1** and **2**) demonstrate that when E_{max} is reached, use of the Hill or Sigmoid 3-parameter equation to generate the dose-response curves yield similar EC_{50} and E_{max} values. Furthermore, these results show that failure to reach a plateau causes changes in the resulting efficacy (E_{max}) results as well as an increase in the observed potency of the compound (EC_{50} shift to the left).

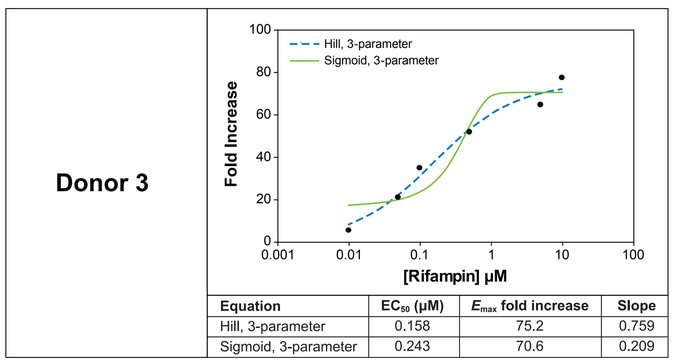
Figure 1. Complete dose-response curves for CYP3A4 mRNA induction by rifampin (0.01 – 10 µM) in three cultures of cryopreserved human hepatocytes



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.237	72.8	1.05
Sigmoid, 3-parameter	0.343	70.7	0.182



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.295	58.7	1.53
Sigmoid, 3-parameter	0.378	58.0	0.134



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.158	75.2	0.759
Sigmoid, 3-parameter	0.243	70.6	0.209

Table 1. Summary of CYP3A4 mRNA induction parameters (E_{max}, EC_{50} and slope values) calculated with the Hill 3-parameter equation

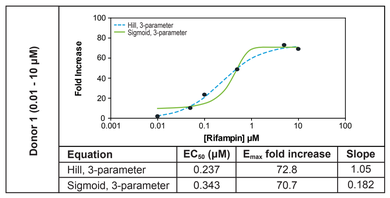
Hill, 3-parameter		Rifampin Concentrations			
		0.01 – 10 µM (6 points)	0.01 – 5 µM (5 points)	0.01 – 0.5 µM (4 points)	0.01 – 0.1 µM (3 points)
Donor 1	E_{max} (fold)	72.8	76.8	52.9	NC
	EC_{50} (µM)	0.237	0.273	0.116	
	Slope	1.05	0.984	1.67	
Donor 2	E_{max} (fold)	58.7	53.7	NC	NC
	EC_{50} (µM)	0.295	0.252		
	Slope	1.53	1.71		
Donor 3	E_{max} (fold)	75.2	65.3	56.7	127
	EC_{50} (µM)	0.158	0.101	0.0718	0.283
	Slope	0.759	0.961	1.24	0.932

NC = Not calculated (calculation failed due to insufficient data)

Table 2. Summary of CYP3A4 mRNA induction parameters (E_{max}, EC_{50} and slope values) calculated with the Sigmoid 3-parameter equation

Sigmoid, 3-parameter		Rifampin Concentrations			
		0.01 – 10 µM (6 points)	0.01 – 5 µM (5 points)	0.01 – 0.5 µM (4 points)	0.01 – 0.1 µM (3 points)
Donor 1	E_{max} (fold)	70.7	72.6	48.6	26.0
	EC_{50} (µM)	0.343	0.355	0.102	0.0586
	Slope	0.182	0.186	0.035	0.019
Donor 2	E_{max} (fold)	58.0	53.1	41.3	8.17
	EC_{50} (µM)	0.378	0.343	0.175	0.0433
	Slope	0.134	0.124	0.0549	0.0133
Donor 3	E_{max} (fold)	70.6	58.2	51.5	36.9
	EC_{50} (µM)	0.243	0.081	0.0701	0.0445
	Slope	0.209	0.0392	0.0351	0.0195

Figure 2. The effect of various data processing methods on CYP3A4 mRNA induction parameters following sequential removal of rifampin concentrations to mimic the potential effect of toxicity and solubility limitations



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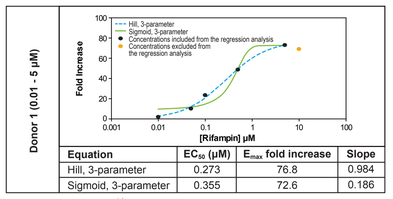

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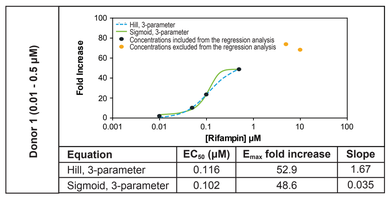
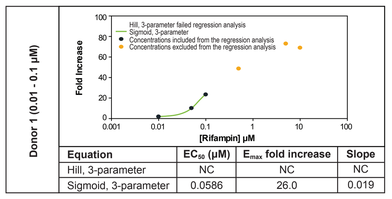
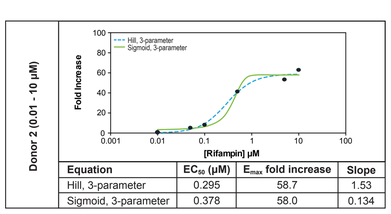


Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.116	52.9	1.67
Sigmoid, 3-parameter	0.102	48.6	0.035



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	NC	NC	NC
Sigmoid, 3-parameter	0.0586	26.0	0.019



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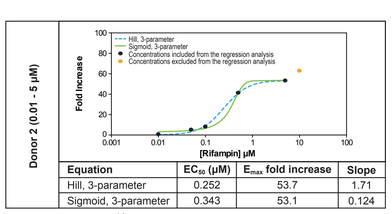
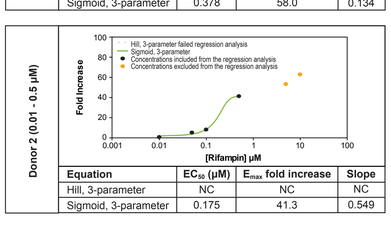
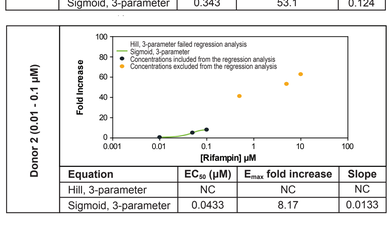


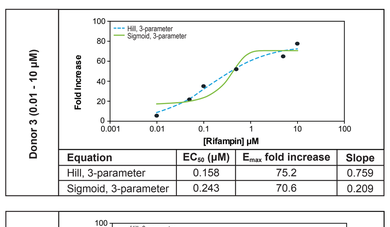
Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.252	53.7	1.71
Sigmoid, 3-parameter	0.343	53.1	0.124



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	NC	NC	NC
Sigmoid, 3-parameter	0.175	41.3	0.549



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	NC	NC	NC
Sigmoid, 3-parameter	0.0433	8.17	0.0133



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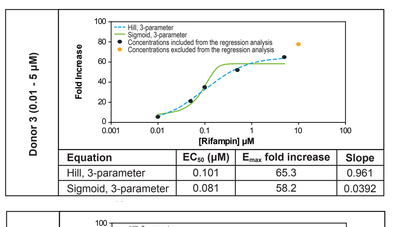
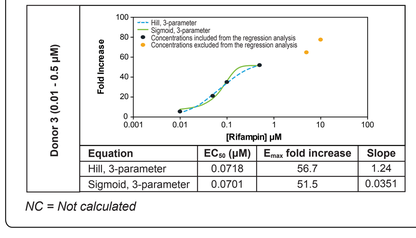
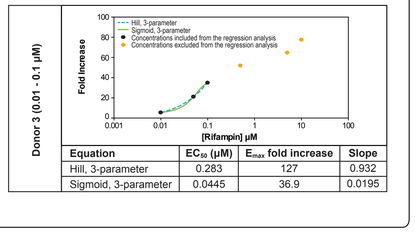


Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.101	65.3	0.961
Sigmoid, 3-parameter	0.081	58.2	0.0392



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.0718	56.7	1.24
Sigmoid, 3-parameter	0.0701	51.5	0.0351



Equation	EC_{50} (µM)	E_{max} fold increase	Slope
Hill, 3-parameter	0.283	127	0.932
Sigmoid, 3-parameter	0.0445	36.9	0.0195

NC = Not calculated

Conclusions

- In cases where maximal induction was reached in the *in vitro* experiments, use of the Hill or Sigmoid 3-parameter equation yielded similar EC_{50} and E_{max} results.
- When the experimental induction curve reaches a plateau, the calculated EC_{50} was consistent across cultures, whereas the E_{max} exhibited intra-culture variability commonly associated with donor variability.
- When E_{max} was not reached experimentally, the Hill equation exhibited a tendency to over-predict E_{max}, while the Sigmoid 3-parameter exhibited a tendency to under-predict E_{max} and to increase the potency (lower EC_{50} values).
- In general, the Hill 3-parameter equation resulted in a better prediction of the 'true' E_{max} and EC_{50} value compared to the predictions of the Sigmoid 3-parameter equation when an incomplete curve was used.
- When the experimental E_{max} was not reached, the resulting E_{max} and EC_{50} values may be subject to variability based on data processing methods.
- In these cases, it is important to understand and consider the pitfalls of these equations when evaluating the potential for clinical enzyme induction from *in vitro* data sets with further prediction modeling.

References

1 Fahmi O.A., Sherri Boldt S., Kish M., Obach S.R., and Tremaine L.M. (2008) Prediction of Drug-Drug Interactions from *In Vitro* Induction Data: Application of the Relative Induction Score Approach Using Cryopreserved Human Hepatocytes. *Drug Metab Dispos.* **36(9):**1971-1974.

2 [FDA] Food and Drug Administration (2012) Draft Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, U.S. Department of Health and Human Services, Rockville, MD. 79 p.