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Synergistic Cytotoxicity from Drugs and Cytokines in Vitro as an Approach to Classify Drugs According to their Potential to Cause Idiosyncratic Hepatotoxicity: A Proof of Concept Study.

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Running title: drug/cytokine cytotoxic synergy in vitro

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Abbreviations: IDILI, idiosyncratic drug-induced liver injury; TNF, tumor necrosis factor-alpha; IFN, interferon-gamma; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, Phosphate-buffered saline; DMEM, Dulbecco's Modified Eagles Medium; FBS, fetal bovine serum; ABAM, Antibiotic-Antimycotic; Cmax, maximal plasma concentration; VEH, vehicle; LDH, lactate dehydrogenase; ANOVA, analysis of variance; ROC, receiver operating characteristic; AUC, area under the curve.

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Abstract

Idiosyncratic, drug-induced liver injury (IDILI) typically occurs in a small fraction of patients and has resulted in removal of otherwise efficacious drugs from the market. Current preclinical testing methods are ineffective in predicting which drug candidates have IDILI liability. Recent results suggest that immune mediators such as tumor necrosis factor-alpha (TNF) and interferon-gamma (IFN) interact with drugs that cause IDILI to kill hepatocytes. This proof-of-concept study was designed to test the hypothesis that drugs can be classified according to their ability to cause IDILI in humans using logistic regression modeling with covariates derived from concentration-response relationships that describe cytotoxic interaction with cytokines. Human hepatoma (HepG2) cells were treated with drugs associated with IDILI or with drugs lacking IDILI liability and cotreated with TNF and/or IFN. Detailed concentration-response relationships were determined for calculation of parameters such as the maximal cytotoxic effect, slope and EC50 for use as covariates for classification modeling using logistic regression. These parameters were incorporated into multiple classification models to identify combinations of covariates that most accurately classified the drugs according to their association with human IDILI. Out of 14 drugs associated with IDILI, almost all synergized with TNF to kill HepG2 cells and were successfully classified by statistical modeling. IFN enhanced the toxicity mediated by some IDILI-associated drugs in the presence of TNF. In contrast, of 10 drugs with little/no IDILI liability, none synergized with inflammatory cytokines to kill HepG2 cells and were classified accordingly. The resulting optimal model classified the drugs with extraordinary selectivity and specificity.

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Introduction

Idiosyncratic, drug-induced liver injury (IDILI) is a typically rare reaction that occurs at drug doses that are safe in the majority of patients. Cases of IDILI can be severe, leading to liver transplantation or death (Ostapowicz et al., 2002). In addition to public health concerns, IDILI is a common cause of removal of drugs from the pharmaceutical market due to the occurrence and severity of these reactions and to the poor ability of standard toxicity tests to identify drug candidates with IDILI liability before they reach the market (Watkins, 2005; Aithal et al., 2011). The causes of IDILI are unknown, but it is thought that genetic and/or environmental factors predispose patients to toxicity from an otherwise safe dose of a drug (Roth and Ganey, 2011). Because these reactions are usually rare, drugs with IDILI potential are often not identified during clinical trials that employ limited numbers of human subjects. More effective preclinical strategies to identify drug candidates with IDILI potential could inform decisions about whether to allow a drug candidate to proceed through the development process. An approach in vitro that uses cells that are readily available and easily grown in culture, requires little compound, employs a single, relevant endpoint and is amenable to high-throughput format would be highly desirable.

Development of such an approach has been challenging due to the limited knowledge about mechanisms underlying IDILI. Several attempts have been made based on direct effects of drugs on hepatocellular biochemistry and/or on physical-chemical characteristics of drugs (Aleo et al., 2014; Chen et al., 2013 and 2014; Choi et al., 2015; Khetani et al., 2013; Low et al., 2011; Morgan et al., 2013; Sakatis et al., 2012; Schadt et al., 2015; Susukida et al., 2015; Thompson et al., 2012; Usui et al., 2009; Ware et al., 2015; Zhang et al., 2016 a and b; Zhu et al., 2014). For the most part,

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the performance of these (selectivity and/or specificity) has not been ideal, and, although it is commonly believed that activation of the immune system underlies IDILI, the results of such activation are typically not considered in the design. Such consideration could lead to an improved approach.

Activation of immune cells culminates in the release of immune mediators such as cytokines. Some recently developed animal models as well as human genetic association studies suggest that adaptive immunity plays a role in the precipitation of IDILI responses to some drugs (Chakraborty et al., 2015; Lucena et al., 2011). Mice that have impaired immune tolerance developed liver injury after several administrations of IDILI-associated drugs such as halothane and amodiaquine (Chakraborty et al., 2015; Pardoll et al., 2012). Although these models involving activation of the adaptive immune system resulted in only mild liver injury, they could represent an advance in understanding IDILI pathogenesis. So far, very few animal models of IDILI have been developed that recapitulate the severity of hepatocellular injury observed in humans. Most of these are based on the interaction of drugs with an activated innate immune system (Roth and Ganey, 2011). Among the models based on the interaction between drugs and the innate immune system, the inflammatory mediators tumor necrosis factor-alpha (TNF) and/or interferon-gamma (IFN) were critical to the pathogenesis of liver injury (Dugan et al., 2011; Hassan et al., 2008; Lu et al., 2012; Shaw et al., 2009a; Shaw et al., 2009b; Zou et al., 2009).

Both innate and adaptive immune responses culminate in the release of these potentially cytotoxic, pro-inflammatory cytokines. Findings from the animal studies raised the possibility that IDILI-associated drugs sensitize hepatocytes to cell death

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signaling from cytokines such as TNF and IFN (Roth and Ganey, 2011). Indeed, using a series of drugs Cosgrove et al. (2009) found a correlation between IDILI liability and ability of drugs to synergize with cytokines to kill primary human hepatocytes in vitro. Using a smaller subset of drugs, they also found that their results in primary human hepatocytes could be reproduced using HepG2 cells, suggesting that the latter cells hold promise in classifying drugs according to IDILI liability. These and other studies suggest that IDILI-associated drugs act in part by causing stress to hepatocytes, such that they become susceptible to killing mediated by cytokines (Beggs et al., 2014; Beggs et al., 2015; Cosgrove et al., 2009; Fredriksson et al., 2011; Fredriksson et al., 2014; Maiuri et al., 2015; Zou et al., 2009; for review, see Roth et al., 2017).

Using HepG2 cells, we recently studied the cytotoxic interaction of TNF/IFN with a series of nonsteroidal anti-inflammatory drugs (NSAIDs) with various IDILI liabilities and also with an antibiotic, trovafloxacin (Beggs et al., 2014; Beggs et al., 2015; Maiuri et al., 2015). In studies presented here, we expand on those findings with a larger set of drugs. Importantly, elucidation of detailed concentration-response relationships permitted calculation of various parameters (e.g. EC50, maximal response, slope, etc.) that we then incorporated into statistical models to evaluate the ability of this approach to classify drugs according to their association with IDILI. The results suggest a highly promising, in vitro approach to predict IDILI liability.

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Materials and Methods

Materials

All drugs were purchased from Sigma-Aldrich (St. Louis, MO) or Santa Cruz Biotechnology (Dallas, TX) unless otherwise noted. Recombinant human TNF and IFN were purchased from R & D Systems (Minneapolis, MN) or Millipore (Billerica, MA). Phosphate-buffered saline (PBS), Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), Antibiotic-Antimycotic (ABAM) and 0.25% Trypsin-EDTA were purchased from Life Technologies (Carlsbad, CA).

Cell culture

Human hepatoma HepG2 cells (American Type Culture Collection, Manassas, VA) were grown in 25-cm^2 tissue culture flasks, maintained in DMEM supplemented with 10% FBS and 1% ABAM in a humidified incubator at 37°C under 95% air and 5% CO_2. Cells were passed or used for experiments when they reached approximately 80% confluence. Cells were used at passage 6-16; at higher passage number, the responses became less consistent and, in some cases, less robust.

IDILI classification

The set of 24 drugs evaluated in this study were classified as being associated with (IDILI+) or not associated with IDILI (IDILI−). Classification was based on a set of criteria established by Xu et al. (2008). Table 1 lists the drugs evaluated in this study, their maximal plasma concentration (Cmax) after pharmacologic dosing in human patients and their IDILI classification. The drugs that were used span several

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therapeutic classes (i.e., antibiotics, anti-cancer, anti-epilepsy, anti-diabetes, anxiolytic/neuroleptic/antidepressant/antipsychotic, and nonsteroidal anti-inflammatory drugs). Most of the IDILI+ drugs we chose have caused hepatocellular injury in people, although some (flucloxacillin, chlorpromazine, flutamide, clavulanate) have been associated with mixed or cholestatic liver damage. Some of the IDILI+ drugs (chlorpromazine, diclofenac and trovafloxacin) produced liver injury in experimental animals when coupled with an inflammatory stimulus (Buchweitz et al., 2002; Deng et al., 2006; Shaw et al. 2009b).

Cytotoxicity assessment

HepG2 cells were plated at a density of 4 X 10^4 cells per well in black-walled, 96-well, tissue culture plates and were allowed to attach overnight before being treated with compounds. Drugs were reconstituted in vehicles consisting of sterile water or DMSO (concentration less than 0.5%). Cells were treated with various concentrations of the drug or its vehicle (control) alone or in combination with the cytokines TNF (10 ng/ml) and/or IFN (10 ng/ml) or their PBS vehicle (VEH). Cytotoxicity was evaluated as lactate dehydrogenase (LDH) activity released from the cells into culture medium using the Homogeneous Membrane Integrity Assay kit from Promega (Madison, WI). A spectrophotometric method was used to measure percent LDH release in cases in which the drug interfered with the fluorescence-based assay, (Vanderlinde, 1985).

Concentration-response curves were generated for 24 drugs, 14 of which are associated with human IDILI and 10 of which are not. Cells were treated with drug concentrations generally ranging from 0 to 100 times the Cmax observed in human

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patients. This range of concentrations is based on scaling factors described in Xu et al., (2008) and accounts for variability in Cmax as well as exposure of the liver to greater concentrations. The cytokine concentrations used in this study are within 10-fold of the concentrations found in serum of human patients undergoing an inflammatory response (Pinsky et al., 1993; Taudorf et al., 2007). If a cytotoxic response was observed but did not reach a plateau by the 100 X Cmax concentration, further testing was performed with larger concentrations of drug to generate a complete (sigmoidal) concentration-response curve. Typically, the range of drug concentrations included at least two that were without effect, two defining the maximal effect and two surrounding the EC50. This was necessary because four-parameter logistic modeling used in the statistical analysis requires well defined, sigmoidal concentration-response curves. Cells were exposed to drug/cytokine combinations for 24 hours. This time was chosen based on previous results demonstrating that cytotoxicity from drug/cytokine exposure begins within 24 hours (Beggs et al., 2014; Maiuri et al., 2015). For analysis of drug concentration-response data, concentrations of each drug were expressed as a fraction of its Cmax.

Statistical analysis

The statistical approach used in this study can be divided into three phases: 1) drug concentration-response determination and covariate development using four-parameter logistic models, 2) classification model development using logistic regression models, and 3) analysis of classification accuracy with receiver operating characteristic (ROC) curves.

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Defining covariates: four-parameter logistic concentration-response model. In the first phase, variables (potential covariates) for use in the classification analysis were defined from the analysis of drug concentration-response data. Initially, a one-way analysis of variance (ANOVA) was used as an omnibus test to determine if a particular treatment (e.g. drug alone or in combination with TNF and/or IFN) caused a significant change in LDH release relative to baseline (i.e., LDH release in the absence of drug, hereafter designated "min"). The criterion for significance for the ANOVA was set at α=0.01. A 1% level of significance was used to rule out more vigorously marginal relationships between concentration and response. For treatments that did not result in a significant change in LDH above min ($p > 0.01$), the following was assumed for the purpose of concentration-response modeling: the minimum LDH response (min) = the maximum LDH response (max). For drug/cytokine treatment combinations that did result in a statistically significant LDH response, the concentration-response data were modeled using a four parameter logistic function:

$$\mathrm{LDH}(x) = \min + \frac{\max - \min}{1 + \left({}^{x}/_{\mathrm{EC50}}\right)^{\mathrm{slope}}}$$

where *LDH(x)* is the percentage of LDH released at a given concentration x; x = [drug]/Cmax; min = the % LDH release at 0 drug concentration (i.e., baseline); and max = the maximal LDH response (i.e., maximum % LDH release). From this equation, the drug concentration associated with 50% maximal response (EC50) and the slope of the concentration-response curve were calculated. The four-parameter logistic models were

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generated using R statistical software (R package “drc”) (R Core Team, 2015; Ritz and Streibig, 2005).

In addition to slope and EC50, several other “base covariates” were calculated from the concentration-response curves for use in further analyses. These were calculated for each of the 96 drug/cytokine treatment combinations evaluated in this study (24 drugs X 4 cytokine combinations) (Supplemental tables 1-9). Delta was defined as max minus min. In addition to the covariates determined from concentration-response curves, Cmax was considered as another base covariate.

Similar to EC50, the covariate EC10 represents the [drug]/Cmax value associated with a 10% increase above min relative to max and was determined by the equation:

$$\mathrm{EC10} = \mathrm{D10} \cdot \mathrm{EC50} \cdot 9^{1/\mathrm{slope}}$$

where D10 is a categorical variable related to reaching a threshold LDH response above which a drug is classified as positively associated with IDILI. D10 is defined as 0 if Delta < 10 % LDH release and as 1 if Delta $\geq$ 10 % LDH release.

The base covariate R10 represents the [drug]/Cmax value associated with an increase in 10 LDH percentage points above min for a particular treatment condition and was determined by the equation:

$$\mathrm{R10} = \mathrm{EC50} \cdot \left[\frac{\mathrm{Delta}}{10} - 1\right]^{1/\mathrm{slope}}$$

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R10 was considered to be 0 when the Delta < 10 % LDH (i.e. when D10 = 0).

From the base covariates defined above, several other covariates were derived. These included EC50 quotient, EC10 quotient, R10 quotient, Deltadiff and TNF change. Each of these "derived" covariates is explained in more detail below.

EC50 quotient, EC10 quotient and R10 quotient represent the ratio between the EC50, EC10 or R10 of the drug/cytokine concentration-response curve and the respective value for the drug/VEH concentration-response curve. In some instances, the value derived from this calculation is indeterminate (i.e. when the denominator = 0). In order to incorporate the quotient values into the classification models described below, the categorical variable "Q" was used to eliminate the possibility of the quotient being indeterminate. Q is defined as 0 if Delta VEH and/or Delta cytokine is < 10% LDH and 1 if both Delta VEH and Delta cytokine are ≥ 10% LDH. For the purpose of calculating EC50 quotient the following condition was applied: if Q = 0, then EC50 quotient = 0 and if Q = 1, then EC50 quotient = EC50 VEH/EC50 cytokine. The same condition was applied for calculation of EC10 quotient and R10 quotient.

Deltadiff represents the difference between the Delta of the drug/cytokine concentration-response curve and the Delta of the drug/VEH curve. In other words, Deltadiff = (Delta cytokine) – (Delta VEH). Figure 1 graphically illustrates several of the covariates defined above. Supplemental Tables 1-9 list the values of all of the covariates computed in this study. In addition to the covariates derived from the concentration-response curves, the maximal therapeutic plasma drug concentration (Cmax) in human patients was used in some models (Table 1).

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TNF change is a categorical variable related to the alteration in the drug-induced cytotoxic response in the presence and absence of TNF, determined as:

$$\text{TNF change} = \text{D10TNF} - \text{D10VEH}$$

Recall that D10 is defined as 0 if Delta ≤ 10 % change in LDH release, and D10 is defined as 1 if Delta > 10 % change in LDH release. Accordingly, TNF change = 1 if the TNF curve has a Delta > 10% LDH release and the VEH curve has a Delta ≤ 10 % LDH release; TNF change = 0 in all other situations.

Classification modeling using defined covariates. In the second phase of analysis, classification models were developed using logistic regression with covariates as independent variables in the analysis and known IDILI classification as the dependent variable. Ability to classify drugs accurately was evaluated using the known IDILI classifications shown in Table 1. A model selection process was used to determine if a covariate or set of covariates is associated with IDILI liability. Covariates were first evaluated individually to determine how well a particular covariate classified drugs according to IDILI liability, and then covariates were evaluated in combination. Combinations of covariates were selected to maximize the ability of the model to distinguish between drugs associated or not with IDILI. Specifically, covariates that describe changes in efficacy (Delta, Deltadiff, etc.) were paired with covariates that describe changes in potency (EC50, EC10, R10, etc.) to find covariate combinations that led to models that most accurately discriminated between drugs that are and are not associated with IDILI. The best-fit logistic regression models were used as classification models to compute a probability that a given drug is associated with IDILI. The logistic regression models follow the equation:

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$$\hat{y}_i = \text{prob}(\text{IDILI} = 1|\mathbf{x}_i) = \frac{e^{\beta_0 + \Sigma\beta_i\mathbf{x}_i}}{1 + e^{\beta_0 + \Sigma\beta_i\mathbf{x}_i}}$$

where $\hat{y}_i$ is the calculated (predicted) probability that drug *i* with a vector of covariates $\mathbf{x_i}$ is associated with IDILI. The β coefficients (β_0, the regression intercept; and β_i, the regression slopes for model covariates, $\mathbf{x_i}$) were derived from the logistic regression models using 1) combinations of the covariates ($\mathbf{x_i}$) generated by concentration-response modeling for each of the 24 drugs evaluated in this study, and 2) the true IDILI classification for the dependent variable y_i (i.e., 1 for IDILI+ drugs and 0 for IDILI- drugs). For treatments that did not result in a significant change in LDH above min ($p > 0.01$), a value of 0 was assigned for covariates derived from EC50 for purposes of calculating β_i. The regression coefficients (β_i) were calculated using Firth's method, which eliminates bias when estimating the value β_i (Firth 1993). Firth's method was necessary since many of the covariates used in this study exhibited quasi-complete separation. This occurs when a covariate almost perfectly separates observations into the appropriate categories. In this study, several covariates almost completely separated drugs according to their IDILI liability. When separation or quasi-complete separation occurs, use of the standard method (i.e., maximum likelihood estimation) provides biased, unreliable estimates of β_i. Firth's method uses a penalized likelihood regression to rectify this and is an appropriate method to use for estimating β_i when quasi-complete separation of data occurs (Firth, 1993). All logistic regression models were computed using R statistical software (R package "logistf") (R Core Team, 2015; Heinze et al., 2013).

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Receiver operating characteristic (ROC) analysis. In the third phase of the statistical approach, the classification models, generated as logistic regression models using single covariates or combinations of covariates, were evaluated by ROC analysis to determine which model and corresponding sets of covariates led to the most accurate classification of drugs according to their potential to cause IDILI. An ROC curve was created for each model by graphing the true positive rate (sensitivity; i.e., proportion of drugs correctly classified as associated with IDILI) against the false positive rate (1-specificity; i.e., proportion of drugs incorrectly classified as associated with IDILI) at various probability cutoff thresholds (k). ROC curves were generated using the R package, pROC (R Core Team, 2015; Robin et al., 2011). An area under the curve (AUC) and confidence interval was computed for each ROC curve (where each logistic regression model has one ROC curve). Plots depicting the AUCs and 95% confidence intervals of the ROC curves were generated for the purpose of comparing multiple logistic regression models using the R package, Metafor (Viechtbauer et al., 2010). Corresponding to each ROC curve is an optimal threshold value (k*), the threshold yielding the highest point of accuracy on the curve (i.e., the point nearest the point (1,1) on the curve). Thus, each ROC curve has a corresponding AUC and an optimal cutoff (k*) that corresponds to the highest point of accuracy on that ROC curve.

Combinations of covariates were strategically selected for evaluation based on what was deemed to lead to the most accurate classification of drugs. ROC curves and corresponding whisker plots were generated to illustrate graphically the ability of each classification model to classify drugs accurately. This allowed for selection of optimal set(s) of covariates for accurate drug classification according to IDILI liability. Our goal

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was to achieve a classification model and a corresponding set of covariates with an AUC as close to 1 as possible with the narrowest 95% confidence interval. A model that is able to classify drugs perfectly according to their potential to cause IDILI would have an ROC curve with an AUC = 1. DeLong's method was used to determine if there were statistically significant differences among ROC curves (DeLong et al., 1988).

A separate classification analysis was also performed to evaluate the ability of Cmax to classify a larger set of drugs with known IDILI potential. Cmax values were obtained for 272 drugs from a study conducted by Xu et al. (2008) and evaluated using ROC analysis.

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Results

Drug/cytokine cytotoxicity: concentration-response in vitro

HepG2 cells were treated with various concentrations of a drug alone or in combination with TNF and/or IFN, and cytotoxicity was assessed 24 hours later as increased LDH activity in the culture medium. Detailed cytotoxicity concentration-response curves were generated for 24 drugs (Table 1): 14 drugs that are associated with IDILI and 10 that are not (negative comparators) (Figure 2, Supplemental Figure 4). Of the 14 drugs associated with IDILI, almost all synergized with cytokines in causing cell death (Figure 2). Four of these (diclofenac, bromfenac, nimesulide and clavulanate) caused no cytotoxicity on their own but synergized with TNF to cause cytotoxicity. Nine IDILI-associated drugs led to a statistically significant increase in LDH release (relative to no drug) in the absence of cytokines (valproic acid, doxorubicin, telithromycin, ibuprofen, naproxen, chlorpromazine, flutamide, trovafloxacin and isoniazid). Interestingly TNF significantly enhanced the cytotoxicity mediated by eight of these drugs (valproic acid, doxorubicin, telithromycin, ibuprofen, naproxen, chlorpromazine, trovafloxacin and isoniazid). In contrast to TNF, IFN did not interact with any drug to cause cytotoxicity. However, coexposure to IFN enhanced the cytotoxic interaction between TNF and several of the drugs (diclofenac, bromfenac, trovafloxacin, valproic acid, chlorpromazine, telithromycin and isoniazid). Two of the 14 IDILI-associated drugs (flutamide and flucloxacillin) did not synergize with cytokines to kill HepG2 cells. Of the 10 negative comparators, pioglitazone was the only drug that caused cytotoxicity on its own; however, this effect was not enhanced by the addition of cytokines. With the

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exception of azithromycin, which was modestly cytotoxic in the presence of TNF/IFN, none of the remaining negative comparator drugs synergized with cytokines to kill HepG2 cells (Figure 2).

Classification models and ROC analysis

Data used in the classification models consisted of covariate data generated as described above (i.e., parameters of the concentration-response curves for each of the 24 drugs and additional variables derived from these parameters) in addition to daily dose and Cmax for each drug. Daily dose and Cmax values are shown in Table 1. Supplemental Tables 1 – 9 summarize parameters of the concentration-response curves for each drug and all derived covariates. Numerous logistic regression models were constructed beginning with models employing each covariate alone and then with models populated with combinations of covariates (Supplemental Table 10). All classification models were evaluated for their ability to classify the 24 drugs using ROC analysis. Findings are described below.

Cmax is moderately associated with IDILI potential

IDILI reactions were once thought not to be dose-related; however, the observation that most drugs that have been withdrawn from the market or have received a black box warning due to IDILI were prescribed at doses greater than 50 mg/day suggested that daily dose plays some role in the propensity of a drug to cause IDILI (Uetrecht, 1999). Based on this observation, we evaluated how accurately the daily dose or the Cmax of a drug classifies drugs listed in Table 1 according to their potential

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to cause IDILI. Logistic regression modeling and ROC analysis as described in Methods were used. The AUC of the ROC curve generated for the model incorporating daily dose was 0.64 with a 95% confidence interval of [0.37, 0.9] (Figure 3). Since the 95% confidence interval for the ROC curve from the daily dose model contained the value 0.5, representing no better than random classification, it cannot be concluded that the magnitude of the daily dose is predictive of IDILI for this drug set. A larger set of drugs may be needed to determine if daily dose can predict IDILI.

The AUC of the ROC curve generated for the model employing Cmax for our set of 24 drugs was 0.80, with a 95% confidence interval of [0.61, 0.98] (Figure 3). Similar results were obtained by Shah et al. (2015) for a set of 125 drugs. These results suggest that Cmax is associated with IDILI.

To determine if our set of 24 drugs is representative of a larger set of drugs and to evaluate further the ability of Cmax to predict IDILI liability, Cmax values were obtained for 272 drugs from a study conducted by Xu et al. (2008) and converted to μM units. Cmax is significantly associated with IDILI for this larger dataset (β_{Cmax}= 0.044, $p<0.001$). The AUC of the ROC curve generated from this larger set of drugs is 0.70 with a confidence interval of [0.64, 0.76]. The ROC curves derived from the set of 24 drugs and from the set of 272 drugs are depicted along with their 95% confidence intervals in Supplemental Figure 2. The confidence interval corresponding to the ROC curve derived from the set of 272 drugs (shaded red) is contained within the confidence interval for the ROC curve derived from the set of 24 drugs (shaded grey). This suggests that the smaller set of drugs adequately represents the relationship between Cmax and IDILI potential seen in a much larger set of drugs.

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ROC analysis of models incorporating the base covariates

Almost all of the 14 IDILI-associated drugs synergized with TNF to cause death of HepG2 cells, and some of them were cytotoxic by themselves (Figure 2). These results suggested that cytotoxic synergy with TNF might be associated with IDILI liability. Accordingly, classification models were constructed using base covariates from the concentration-response curves to determine whether the presence of TNF improved a model's ability to classify drugs according to IDILI liability. The base covariates were modeled for each drug individually; base covariates that were at least moderately associated with IDILI liability included Delta VEH, Delta TNF, EC50 VEH, EC50 TNF, EC10 VEH, EC10 TNF, R10 VEH and R10 TNF. AUCs and 95% confidence intervals are shown in Figure 4A for each of these covariates. It can be seen that the confidence interval for each of these covariates does not contain the value 0.5, indicating a significantly better than random ability to classify the 24 drugs according to IDILI potential. The model incorporating Delta TNF produced the ROC curve with the greatest AUC (0.93) and narrowest 95% confidence interval (0.83, 1.00) suggesting that, of these models, it provided the most accurate classification of the drugs (Figure 4A). Furthermore, the base covariates that described the response to drug/TNF (labeled 'TNF') led to models that produced ROC curves that had improved AUCs with narrower confidence intervals than those that described the response to drug alone (i.e., labeled 'VEH') (Figure 4A, B).

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ROC analysis of models incorporating derived covariates

Probability models were also generated using the individual covariates that were derived from the base covariates: EC50 quotient, EC10 quotient, R10 quotient, and Deltadiff. In Figure 5A and B, the EC50, EC10 and R10 quotient covariates represent the ratio of the drug/TNF concentration-response curve to the drug/VEH curve, and Deltadiff represents the difference between the Deltas from the drug/TNF concentration-response curve and the drug/VEH curve. Each of these covariates (except Deltadiff) was moderately associated with IDILI liability (Figure 5A, B); however, the ROC curves generated based on these models did not have greater AUCs or narrower confidence intervals than the models produced by incorporating the base covariates (compare Figures 4 and 5).

Addition of IFN data did not improve the classification of drugs

None of the drugs synergized with IFN in the absence of TNF to cause cytotoxicity, but several IDILI-associated drugs synergized with IFN in the presence of TNF (Figure 2). Accordingly, we examined whether incorporation of the TNF/IFN responses would improve the performance of models that employed only TNF responses. The drug/TNF/IFN models tended to have smaller AUCs and larger confidence intervals than the drug/TNF models (Supplemental Figure 3), indicating that the addition of data describing the IFN response did not enhance the ability of models to classify drugs.

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ROC analysis of models incorporating combinations of the base and derived covariates

Although it was illustrative to evaluate the base and derived covariates individually, we hypothesized that incorporation of several covariates into a model would lead to more accurate classification of drugs than incorporation of a single covariate. Accordingly, various combinations of the base and derived covariates were evaluated to identify a set of covariates that led to the most accurate drug classification. Combining base and derived covariates led to several models with greater AUCs and narrower confidence intervals than the models incorporating only a single covariate. A representative set including the best performing models is presented in Figure 6. Furthermore, when Cmax was added as a covariate, it improved the performance (AUC and confidence interval) of some models but not others (Figure 6). Supplemental Table 10 shows the coefficients (beta values) and their p-values for the models shown in Figure 6.

Some of the combination models were associated with remarkably high AUCs, and some of these were associated with small confidence intervals. There were no statistically significant differences among the models with an AUC > 0.95 as determined by DeLong's method for comparing ROC curves ($p > 0.05$). The ROC curves that met this criterion (AUC > 0.95) are shown in Figure 7.

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Discussion

The purpose of this proof-of-concept study was to develop and evaluate an in vitro approach combined with statistical modeling to classify drugs according to their potential to cause IDILI. The overall hypothesis tested was that the ability of a drug to synergize with the cytokines TNF and/or IFN to kill HepG2 cells is associated with the drug's propensity to cause IDILI in humans. Detailed concentration-response curves were generated, and this proved to be critical for development of a statistical classification model with the capacity to classify drugs correctly.

Since it has been suggested that the daily dose of a drug might be associated with its potential to cause IDILI and since dose is often related to Cmax, we evaluated how well daily dose or Cmax classifies drugs according to their IDILI liability. Daily dose was not effective at classifying the set of 24 drugs according to their potential to cause IDILI (Figure 3). Plasma drug concentration is used as a surrogate for tissue or intracellular concentration, which is difficult to obtain. The relationship between Cmax and the concentration of free drug in liver cells can differ among drugs. The intrahepatocellular concentration of drugs can be affected not only by physicochemical properties of the drug, but also by uptake and efflux mechanisms and metabolism as well as binding to cellular or acellular components (Chu et al., 2013). Accordingly, the intrahepatocellular drug concentration can for some drugs be many times the plasma concentration. Despite this potential disconnect Cmax was somewhat effective at classifying the set of 24 drugs, suggesting that the magnitude of plasma drug concentration is a contributor to IDILI. However, it is clear that the use of Cmax as the

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sole covariate did not lead to a model with great classification ability (Supplemental Figure 2).

We then determined whether cytotoxicity induced by treatment with drugs in the absence of cytokines could produce a high performing model. Models employing only individual base covariates describing cytotoxicity in the absence of TNF performed no better than Cmax (compare Figures 3 and 4). In contrast, the models incorporating TNF performed significantly better in classifying the drugs.

The derived covariates, when evaluated individually, did not produce more desirable ROC curves than the base covariates (compare Figure 5 with Figure 4). However, when covariates that account for TNF-induced changes in potency and/or efficacy were combined with those derived from drug alone, much better models resulted (Figure 6, Figure 7). Furthermore, incorporating Cmax into these models led to the ROC curve with the greatest AUC (0.99) and narrowest confidence interval [0.97, 1] (Figure 6, Figure 7). The coefficients and test statistic values for this best performing classification model, which incorporated Deltadiff, EC50VEH, EC50TNF, DeltaVEH and Cmax as covariates, are listed in Table 2. A cutoff value is an estimated probability above which a drug would be classified as associated with IDILI (1 = associated with IDILI) and below which a drug would be classified as not associated with IDILI (0 = not associated with IDILI). The optimal cutoff threshold is the probability cutoff that permits the most accurate classification of drugs according to IDILI liability for a given model, i.e., the point on the ROC curve closest to the coordinate (1,1). Table 3 shows the sensitivity and specificity of the best performing model when the optimal cutoff threshold was applied. Based on this model, the estimated probability that a specific drug from the

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set of 24 drugs is associated with IDILI is shown in Table 4. As can be seen in the Table, this classification model led to almost complete separation between IDILI-associated drugs and drugs that are not associated with IDILI. If this model were to be used in a preclinical safety evaluation setting to predict IDILI potential of a set of drugs, the user could either select the optimal cutoff threshold or choose a cutoff threshold that is either more or less sensitive depending on what false positive rate is deemed acceptable.

IFN contributed to hepatotoxicity in several animal models of IDILI and was therefore of interest to include in our examination (Shaw et al., 2009; Hassan et al., 2008; Dugan et al., 2011; Roth et al., 2017). Interestingly, IFN did not synergize with any of the drugs in the present study to cause cell death (Figure 2); however, as we showed previously for diclofenac (Maiuri et al., 2015), IFN enhanced the cytotoxic interaction between several IDILI-associated drugs and TNF. What underlies this pattern of response to IFN is not known; however, IFN can increase expression of TNF receptors (Wang et al., 2006) and conversely, TNF can increase IFN signaling (Han et al., 1999; Robinson et al., 2003). These actions could explain why IFN had an effect only in the presence of TNF.

We evaluated whether a change in the concentration-response curves due to exposure to IFN could improve the classification of drugs. The classification model developed from the covariates that described the response to drug/TNF/IFN produced ROC curves that were not improved from those incorporating covariates that describe the response to drug/TNF (Supplemental Figure 3). These results indicate that cytotoxic synergy between IDILI-associated drugs and TNF is sufficient to produce a

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statistical model that accurately classifies drugs irrespective of the presence of IFN. They also suggest that the cell killing activity of IFN depends on the presence of TNF.

It is worth considering the possibility that other cytokines play a role in the pathogenesis of IDILI, and it would be interesting to examine whether other cytokines could interact with drugs to cause cytotoxicity in vitro and/or synergize with TNF to enhance cytotoxicity. Cosgrove et al. (2009) performed a study examining drug/cytokine interactions in vitro and found that IL-1β in combination with TNF and IFN interacted with some drugs to cause cytotoxicity. It is unclear to what extent IL-1β contributed to this interaction. However, Shaw et al. (2009) demonstrated that IL-1β levels are elevated in mice cotreated with trovafloxacin and LPS, raising the possibility that IL-1β plays a role in the hepatotoxicity observed in these mice. Whether or not the presence of IL-1β or other immune mediators would improve the ability of the models presented herein to classify drugs is unknown but worth considering in future studies.

We reported recently that IFN-mediated enhancement of NSAID/TNF-induced cytotoxicity occurs with some IDILI-associated NSAIDs but not others, and this effect was related to chemical structure and to the magnitude of clinical concern about IDILI for specific NSAIDs (Maiuri et al. 2015). Specifically, several acetic acid derivatives that are associated with IDILI of greatest clinical concern synergized with TNF to cause HepG2 cell death, and IFN enhanced this effect. In contrast, two propionic acid derivatives, which are associated with IDILI that is of less clinical concern, also synergized with TNF, but IFN was without effect. In the analysis presented here, we were seeking a binary answer – IDILI potential yes or no – and inclusion of IFN did not affect the outcome. It would be interesting if the ability of drugs to sensitize cells to the

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harmful effects of IFN could distinguish drugs of greater concern clinically for IDILI from those of less concern. Clearly, a larger number of drugs would need to be analyzed to evaluate this.

A potential challenge that might be faced when employing this assay during preclinical safety evaluation is the inability to generate complete concentration-response relationships due to solubility limitations of the drug or other factors. Computing covariates using the four-parameter logistic model requires complete concentration-response curves; however, we defined several covariates that can be computed without the need to generate a complete concentration-response curve. One of these is R10, or the drug concentration at which there is an increase of 10% LDH activity above min. Another covariate that could be computed without the need to generate a complete concentration-response curve we defined as “TNF change.” TNF change identifies those drugs only cytotoxic in the presence of TNF without the need for complete concentration-response curves. Interestingly, combining the covariates R10quotient (i.e., R10TNF/R10VEH) and TNF change resulted in an ROC curve with an AUC = 0.88 and a 95% confidence interval of [0.75, 1] (Supplemental Figure 1). This suggests that a model that leads to good classification of drugs according to their potential to cause IDILI can be generated without the need to delineate complete concentration-response relationships. This model might be useful for predicting IDILI-potential of drug candidates when availability of compound is limited or when solubility limitation prevents generation of a complete concentration-response curve.

Although HepG2 cells are human-derived, their use for drug toxicity evaluation has been criticized because they have limited capacity to bioactivate drugs to toxic

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metabolites via cytochrome P450-mediated pathways. Despite this potential limitation, Cosgrove et al. (2009) found that HepG2 cells behave similarly to primary human hepatocytes in their cytotoxic responses to drug-cytokine combinations. We have also observed comparable responses in primary murine hepatocytes (Zou et al., 2009; Beggs et al., 2014; Maiuri et al., 2015). These findings suggest either that (1) metabolic activation of drugs by HepG2 cells, although limited, is sufficient to stress cells so that they respond to cytokine exposure by dying or (2) metabolism is not generally needed for the cytotoxic interaction of drugs with cytokines.

Visual inspection of Figure 2 revealed three IDILI-positive drugs for which there was modest (flutamide, clavulanate) or no (flucloxacillin) response. Despite this, statistical modeling classified flutamide and clavulanate as IDILI positive. In the case of flucloxacillin, analysis of variance of the concentration-response data determined that there was no statistically significant increase in LDH alone or in the presence of TNF. As a result, covariates were set to zero with the exception of Cmax, and the calculated probability for flucloxacillin was low (0.154; see formula in Methods). In contrast, analysis of variance applied to the concentration-response data for flutamide alone and in combination with TNF detected very small but statistically significant increases in LDH release relative to baseline (Figure 2, Supplemental Figure 1). Consequently, nonzero covariates were generated from the concentration-response data. Similarly for clavulanate, there was an interaction with TNF that was small but statistically significant, resulting in nonzero covariates. These nonzero covariates led to calculated probabilities that, at the optimal cutoff, identified flutamide and clavulanate correctly as IDILI-positive. The pronounced reproducibility of even small changes using HepG2 cells and the

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apparent sensitivity of the model in these two cases may be a strength of this approach to classification.

Flutamide is metabolized in vivo to 2-hydroxyflutamide, which is more potent pharmacologically as an anti-androgen (Brogden et al., 1989) and is thought to contribute to IDILI responses (Ball et al., 2016). To strengthen this proof-of-concept study and to evaluate further the modest cytotoxic effect of flutamide, we investigated the performance of 2-hydroxyflutamide in the assay. Concentration-response curves were generated (Supplemental Figure 5), and covariates derived from the curves were used in the best performing classification model. A high probability for association with IDILI was calculated (0.999) for 2-hydroxyflutamide. Further, we replaced the flutamide covariate data in the best-performing model with the 2-hydroxyflutamide covariates. Recalculation of the best-performing classification model yielded coefficients similar to those presented in Table 2 (Supplemental Table 11), the same AUC for the ROC curve, and no change in classification of drugs as IDILI positive or negative. These results strengthen the conclusions based on the modest cytotoxic response to flutamide and support the approach to classifying IDILI-associated drugs.

The observation that flucloxacillin was incorrectly classified as not associated with IDILI suggests that the best-performing model, although seemingly promising, has limitations. It is worth noting that flucloxacillin typically produces liver injury in humans that is classified as cholestatic rather than hepatocellular (Moseley et al., 2013; Enat et al., 1980; Williams et al., 1981; Bengtsson et al., 1985). Clavulanate is also associated predominately with a cholestatic pattern of injury in human patients (Sánchez-Ruiz-Granados et al., 2012; Beraldo et al, 2013) and interacted only weakly with TNF.

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Accordingly, it is possible that the approach described herein is more robust in classifying drugs that cause hepatocellular rather than cholestatic patterns of injury.

In summary, the results add to evidence that drug-induced stress can sensitize hepatocytes to the killing actions of cytokines such as TNF and IFN (reviewed in Roth et al., 2017). Moreover, this could be requisite for the pathogenesis of IDILI, since numerous IDILI-associated drugs show cytotoxic synergy with cytokines in vitro at drug concentrations near those that occur in patients. Currently, effective assays to screen preclinically for IDILI potential are lacking. A method that accurately identifies drug candidates with the potential to cause IDILI could revolutionize preclinical testing strategies. Our results suggest an in vitro assay that could do just that, i.e., by delineating drug concentration-response curves in the absence and presence of TNF and employing resulting covariates in an appropriate statistical model for classification. One of the strengths of this approach is that the user would have discretion to choose a level of risk tolerance guided by the results of ROC analysis. That choice could depend on a variety of factors, including risk tolerance in the context of the therapeutic use, other drug candidates that are in contention for going forward into development, etc. For example, if several potentially effective compounds with no apparent toxicity were identified in early preclinical screens but some returned an “IDILI+” result in a drug-cytokine assay, this might prompt a decision to pursue other candidates for development. The magnitude of interaction with TNF assessed by direct inspection of concentration-response curves (Figure 2) might also be useful to inform such decisions. Overall, this classification approach is attractive because it (1) uses a cell type that is easily obtained and maintained in culture and yields consistent results, (2) requires

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minimal amounts of test compound, (3) employs a single, easily and inexpensively measured phenotypic endpoint that is directly relevant to IDILI (hepatocellular death), (4) is based on interaction between drug and a product of immune system activation likely to be relevant to IDILI pathogenesis and (5) is adaptable to high throughput technology. Validation of this approach as a screening tool will require the evaluation of additional drugs, but the results presented herein are quite promising.

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Authorship Contributions

Participated in research design: Maiuri, Wassink, Hession, Ganey, and Roth.

Conducted experiments: Maiuri, Turkus, Breier, Lansdell, and Kaur.

Contributed new reagents or analytic tools:

Performed data analysis: Maiuri, Wassink, Hession, Ganey, and Roth.

Wrote or contributed to the writing of the manuscript: Maiuri, Hession, Ganey, and Roth.

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Footnotes

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Figure Legends

Figure 1. Depiction of base covariates considered for evaluation. Blue curve represents hypothetical response to drug alone; red curve represents response to drug in the presence of cytokine. Min = LDH release in the absence of drug. Max = maximal LDH release. Delta = max(LDH) – min(LDH) for each curve. Although not depicted in the figure, Deltadiff = Delta cytokine - DeltaVEH. EC50 is the [drug]/Cmax value at 50% Delta. R10 is not depicted but it represents the [drug]/Cmax value associated with a 10% increase in LDH release above min(LDH).

Figure 2. Drug/cytokine-induced cytotoxicity: concentration-response. HepG2 cells were treated with 14 drugs associated with IDILI and 10 drugs not associated with IDILI, alone (VEH) and in combination with TNF and/or IFN. Cytotoxicity (% LDH release) was evaluated 24 hours after treatment. The numbers listed on the x-axis represent the concentration of drug relative to Cmax (fold Cmax). Refer to Table 1 for the Cmax information and IDILI classification for each drug and to the methods section for the rationale concerning the range of drug concentrations evaluated. Each data point represents the mean ± standard error of the mean (S.E.M.) of at least 3 separate experiments. The dotted curves indicate that the treatment condition (i.e. VEH, TNF, IFN or TNF/IFN) resulted in a statistically significant change in LDH from baseline (no drug) at one or more drug concentrations (ANOVA $p<0.01$). Solid lines indicate that the treatment condition did not result in a statistically significant change in LDH relative to baseline at any drug concentration (ANOVA $p \geq 0.01$). The dotted curves were modeled using a four-parameter logistic function as described in Methods in order to

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compute parameters describing curve characteristics (minimum cytotoxic effect, maximum cytotoxic effect, EC50, etc.).

Figure 3. Comparison of the model incorporating daily dose to that incorporating Cmax. A) AUCs and 95% confidence intervals are depicted for the ROC curves derived from the models incorporating either daily dose or Cmax. The covariates are listed on the left, and the AUC for each one is shown on the right next to the 95% confidence interval, which is shown in brackets. B) The ROC curves for the model incorporating daily dose and Cmax are indicated by red line and blue lines, respectively. The 95% confidence intervals for the model incorporating daily dose and Cmax are shaded red or blue, respectively. Overlap between the confidence intervals for the two ROC curves appears violet.

Figure 4. Evaluation of models incorporating the base covariates.
A) AUCs and 95% confidence intervals are illustrated for the ROC curves derived from the models incorporating the base covariates Delta VEH, Delta TNF, EC50 VEH, EC50 TNF, EC10 VEH, EC10 TNF, R10 VEH or R10 TNF. The covariates are listed on the left, and the AUC for each is shown on the right next to the 95% confidence interval, which is in brackets. *denotes a statistically significant difference as determined by DeLong's test ($p<0.05$). B) ROC curves were generated and indicate for each model the 95% confidence interval shaded in grey. The covariates incorporated in the model are listed on the bottom right corner of each ROC curve.

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Figure 5. Evaluation of models incorporating the derived covariates.

A) AUCs and 95% confidence intervals for the ROC curves are depicted for the models incorporating the derived covariates individually. The covariates are listed on the left, and the AUC for each is shown on the right next to the 95% confidence interval, which is in brackets. B) ROC curves were generated and indicate for each model the 95% confidence interval shaded in grey. The covariate incorporated in the model is listed on the bottom right corner of each ROC curve.

Figure 6. Evaluation of models incorporating combinations of the base and derived covariates with and without Cmax.

AUCs and 95% confidence intervals for the ROC curves are depicted for the models incorporating various combinations of base and derived covariates in the absence (upper half) and presence (lower half) of Cmax. The covariates are listed on the left, and the AUC for each is shown on the right next to the 95% confidence interval, which is in brackets.

Figure 7. ROC curves with an AUC $\geq$ 0.95.

ROC curves for which AUC $\geq$ 0.95 are depicted. The 95% confidence interval is shaded grey. The covariates incorporated into the model are listed at the bottom right corner of each ROC curve. The ROC curves shown were not significantly different from each other as determined by DeLong's test ($p > 0.05$).

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TABLES

Drug	IDILI liability	Cmax (μM)	Daily dose (mg)	Cmax Reference
Aspirin	IDILI–	47	1300	Brandon et al. 1986
Azithromycin	IDILI–	0.5	500	Xu et al. 2008
Buspirone	IDILI–	0.005	15	Xu et al. 2008
Idarubicin	IDILI–	0.02	1	Xu et al. 2008
Levofloxacin	IDILI–	15.7	500	Xu et al. 2008
Moxifloxacin	IDILI–	6.2	400	Stass et al. 1998
Pioglitazone	IDILI–	2.67	15	Xu et al. 2008
Promethazine	IDILI–	0.06	25	Xu et al. 2008
Rofecoxib	IDILI–	1	12.5	Gottesdeiner et al. 2003
Sertraline	IDILI–	0.06	50	Xu et al. 2008
Bromfenac	IDILI+	13.5	50	Gumbhir-Shah et al. 1997
Chlorpromazine	IDILI+	0.84	200	Xu et al. 2008
Clavulanate	IDILI+	12	125	Hu et al. 2002
Diclofenac	IDILI+	7.44	100	Xu et al. 2008
Doxorubicin	IDILI+	1	1	Barpe et al. 2010
Flucloxacillin	IDILI+	72.6	250	Roder et al. 1995
Flutamide	IDILI+	0.36	750	Xu et al. 2008
Ibuprofen	IDILI+	164	800	Bramlage et al. 2008
Isoniazid	IDILI+	77	300	Xu et al. 2008
Naproxen	IDILI+	300	500	Setiawati et al. 2009
Nimesulide	IDILI+	21.08	200	Xu et al. 2008
Telithromycin	IDILI+	2.77	800	Xu et al. 2008
Trovafloxacin	IDILI+	5	300	Xu et al. 2008
Valproic Acid	IDILI+	175	60	Rha et al. 1993

Table 1. IDILI classification, daily dose, Cmax and references from which the Cmax values were taken. IDILI classification was determined by a set of criteria described in Xu et al. (2008). IDILI(–) indicates that the drug is not associated with IDILI, whereas IDILI(+) indicates that the drug is associated with IDILI.

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Covariates	Beta	Chi square	p-value
Intercept	-1.924	7.091	0.008
Deltadiff	0.108	2.471	0.116
EC50VEH	-0.066	6.215	0.013
EC50TNF	0.050	4.480	0.034
DeltaVEH	0.081	11.038	0.001
Cmax	0.0031	0.129	0.720

Table 2. Logistic regression coefficients and p-values for the optimal classification model incorporating the covariates Deltadiff, EC50 VEH, EC50 TNF, Delta VEH and Cmax. The coefficients (beta values) were computed using Firth's approach as described in Methods. A p-value <0.05 indicates that the covariate contributes significantly to the prediction of outcome (IDILI liability).

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		95% confidence interval
Optimal cutoff threshold (k*)	0.46	
True negative rate (specificity) using threshold k*	1	(0.7, 1)
True positive rate (sensitivity) using threshold k*	0.93	(0.79, 1)
AUC	0.99	(0.97, 1)

Table 3. Sensitivity and specificity for the optimal classification model incorporating the covariates Deltadiff, EC50 VEH, EC50 TNF, Delta VEH and Cmax. The optimal cutoff threshold (k*) is shown as is the specificity and sensitivity of the model at k*. Also indicated are the area under the ROC curve (AUC) and the 95% confidence intervals for the specificity, sensitivity and AUC.

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Drug	Estimated probability	True classification
Buspirone	0.1275	IDILI −
Idarubicin	0.1275	IDILI −
Promethazine	0.1275	IDILI −
Sertraline	0.1275	IDILI −
Azithromycin	0.1276	IDILI −
Rofecoxib	0.1278	IDILI −
Moxifloxacin	0.1296	IDILI −
Levofloxacin	0.1329	IDILI −
Aspirin	0.1445	IDILI −
Flucloxacillin	0.1545	IDILI +
Pioglitazone	0.2935	IDILI −
Telithromycin	0.6685	IDILI +
Flutamide	0.7036	IDILI +
Trovafloxacin	0.7448	IDILI +
Isoniazid	0.7562	IDILI +
Diclofenac	0.8578	IDILI +
Naproxen	0.8589	IDILI +
Doxorubicin	0.8839	IDILI +
Bromfenac	0.9397	IDILI +
Clavulanate	0.9509	IDILI +
Chlorpromazine	0.961	IDILI +
Ibuprofen	0.9733	IDILI +
Valproic Acid	0.9909	IDILI +
Nimesulide	1	IDILI +

Table 4. Estimated probabilities that a drug is associated with IDILI computed from the best performing logistic regression model employing Deltadiff, EC50 VEH, EC50 TNF, Delta VEH and Cmax as covariates. With regard to the true IDILI classification of drugs, IDILI(−) indicates that the drug is not associated with IDILI and IDILI (+) indicates that the drug is associated with IDILI in human patients.

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Figure 1

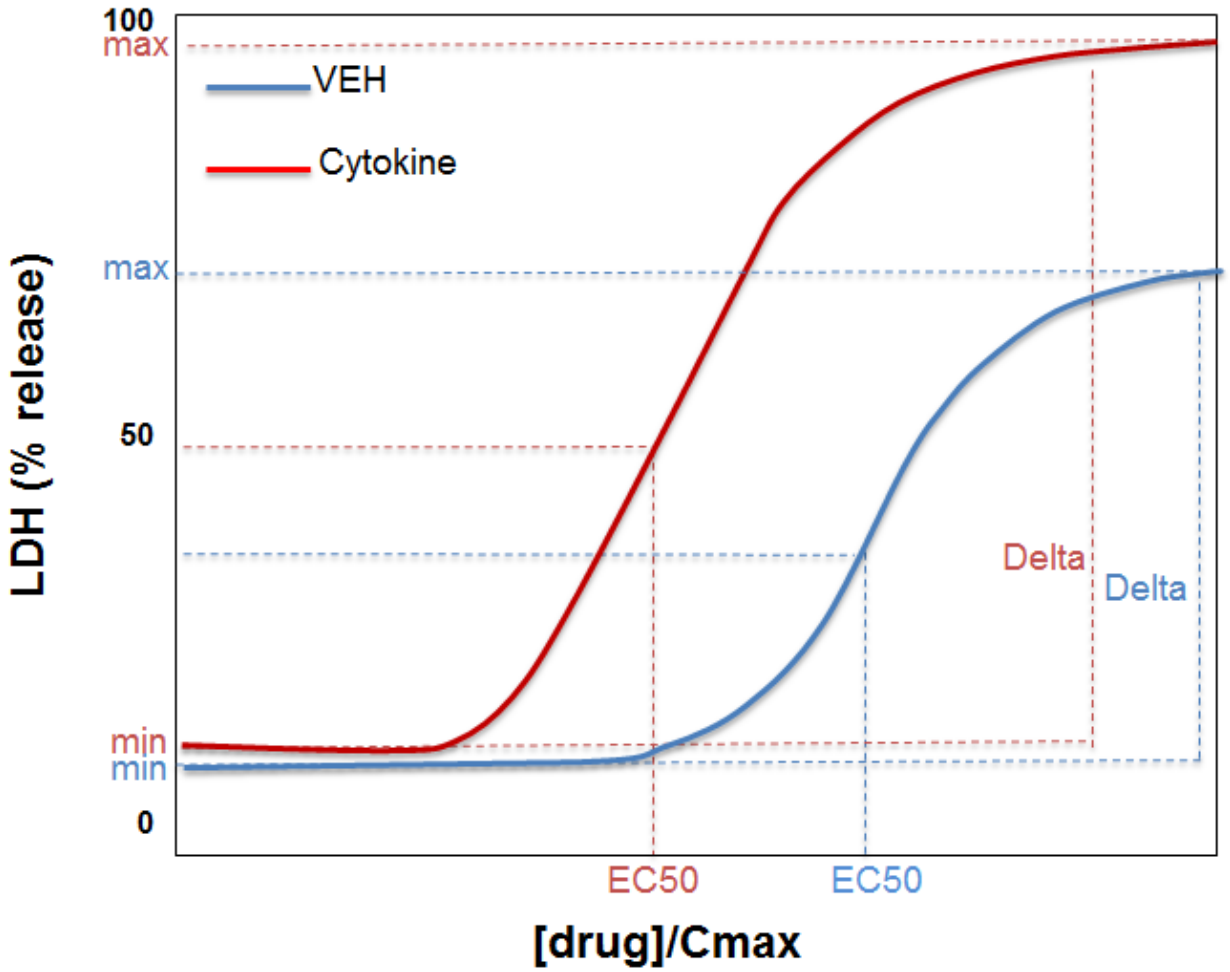

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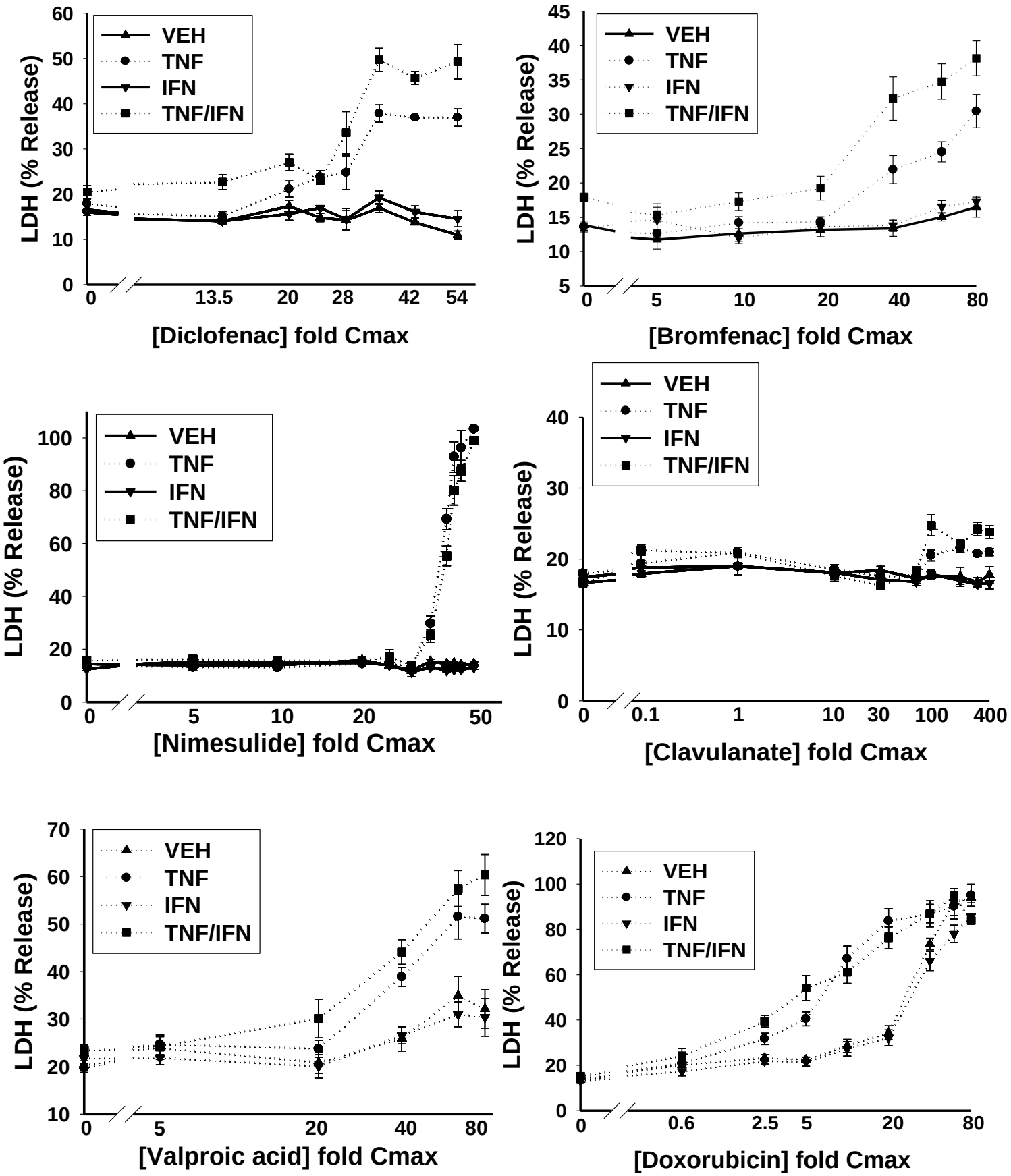
Figure 2



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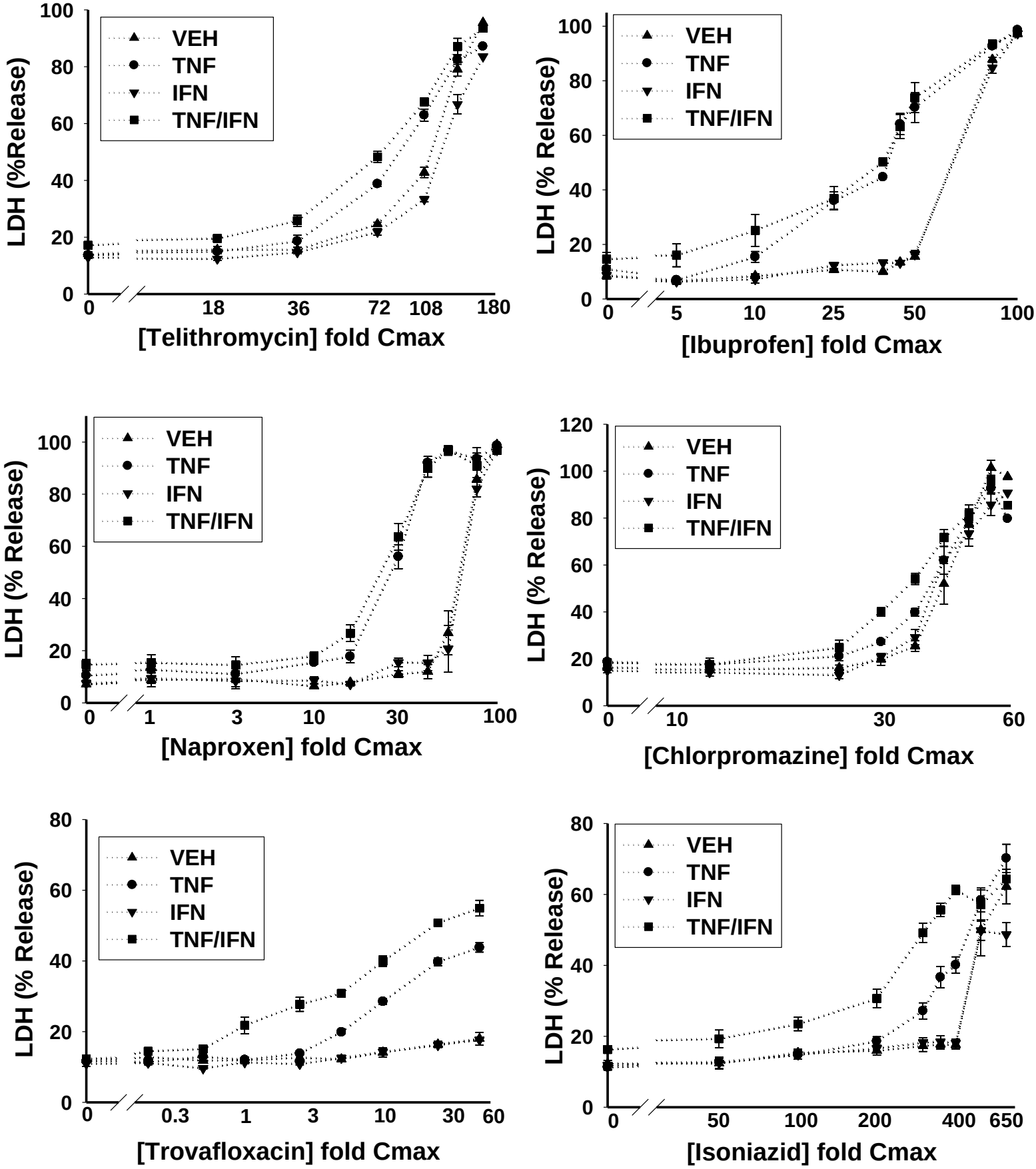

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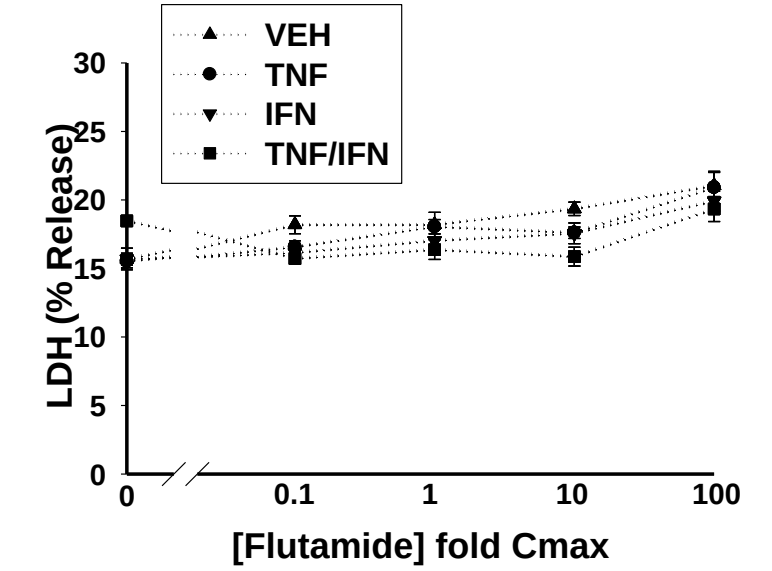

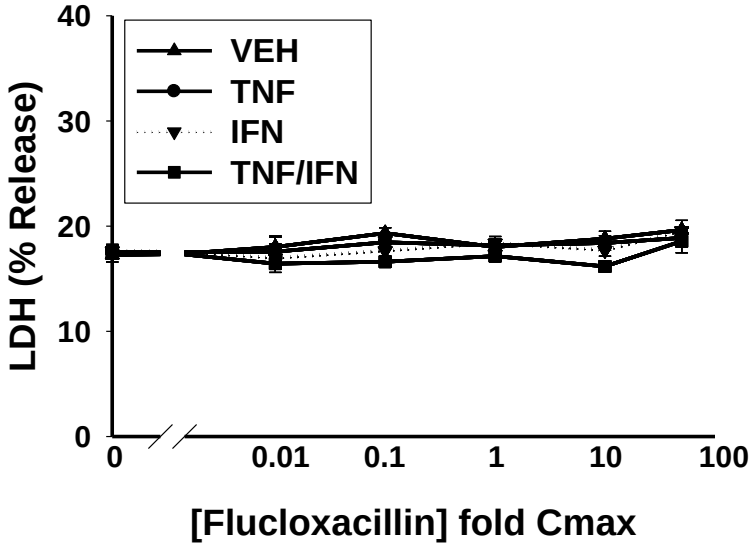

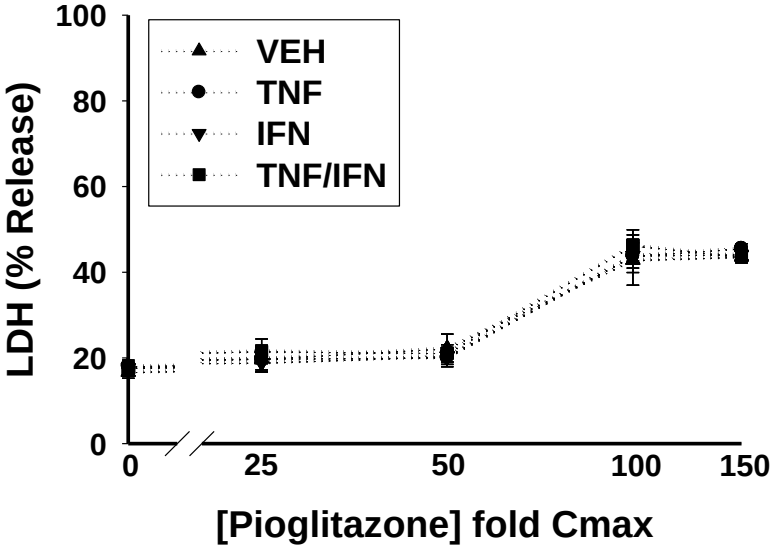

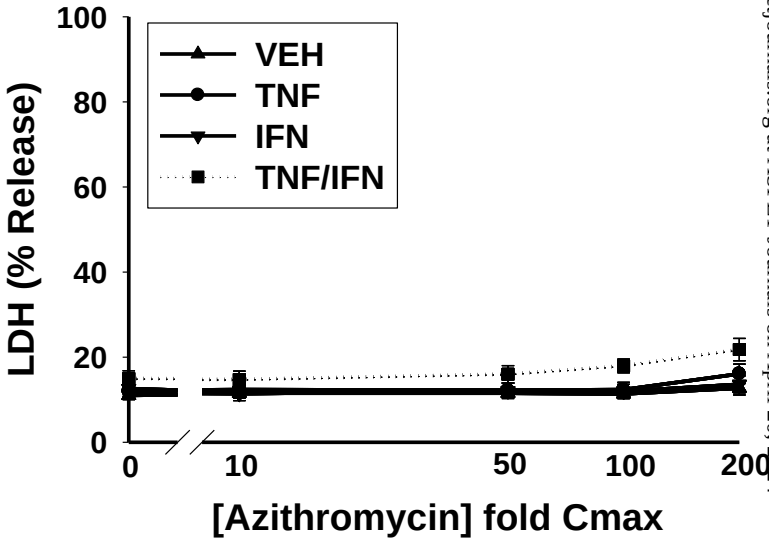

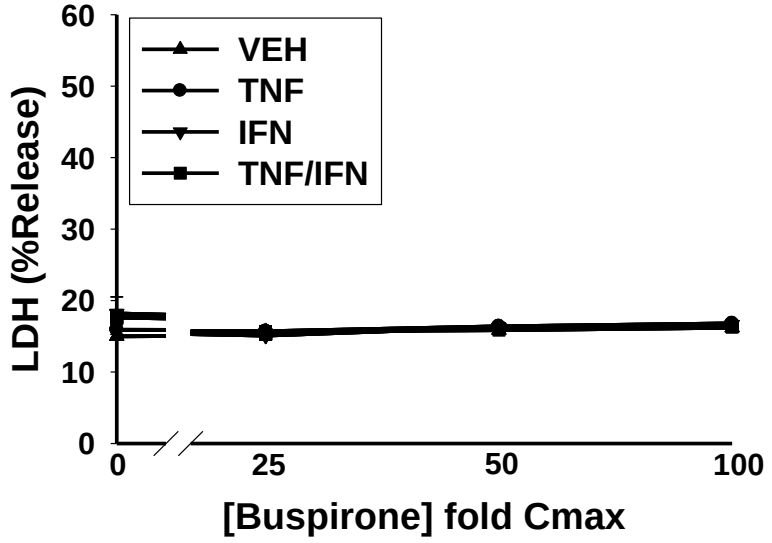

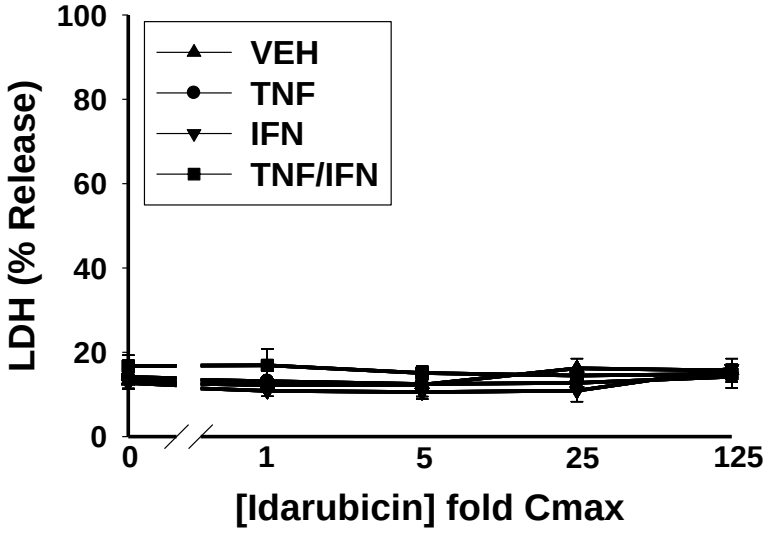

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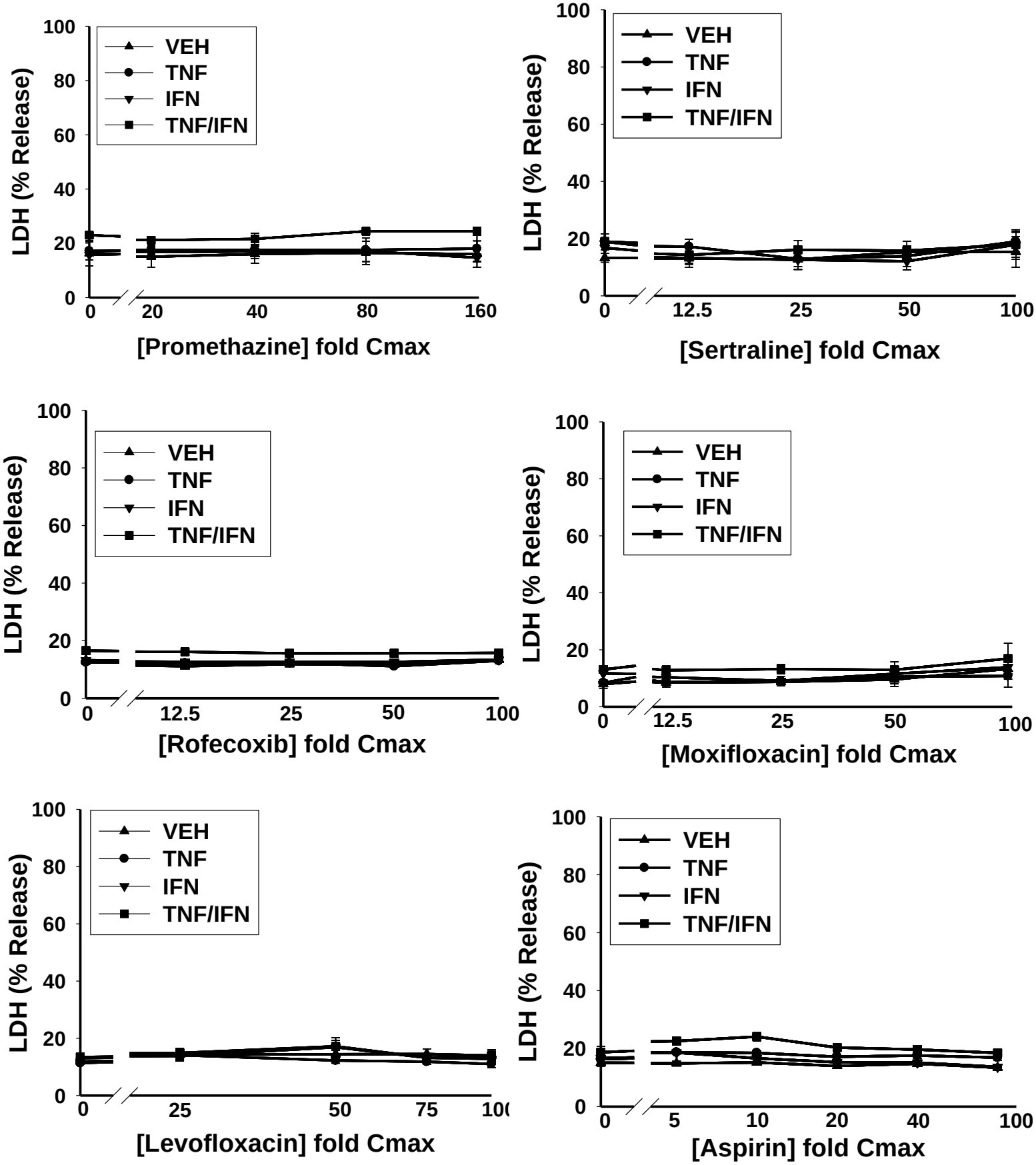

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Figure 3

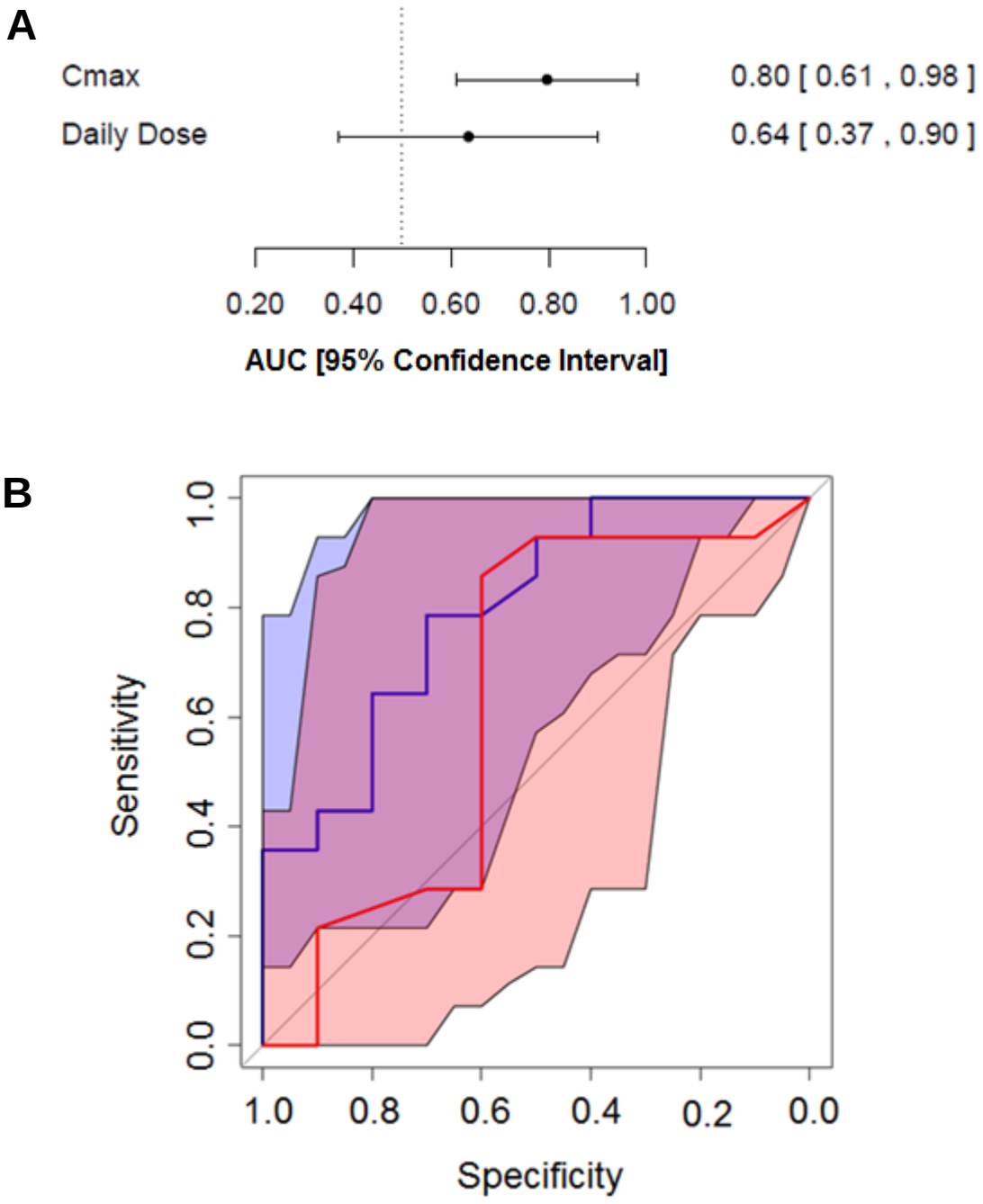

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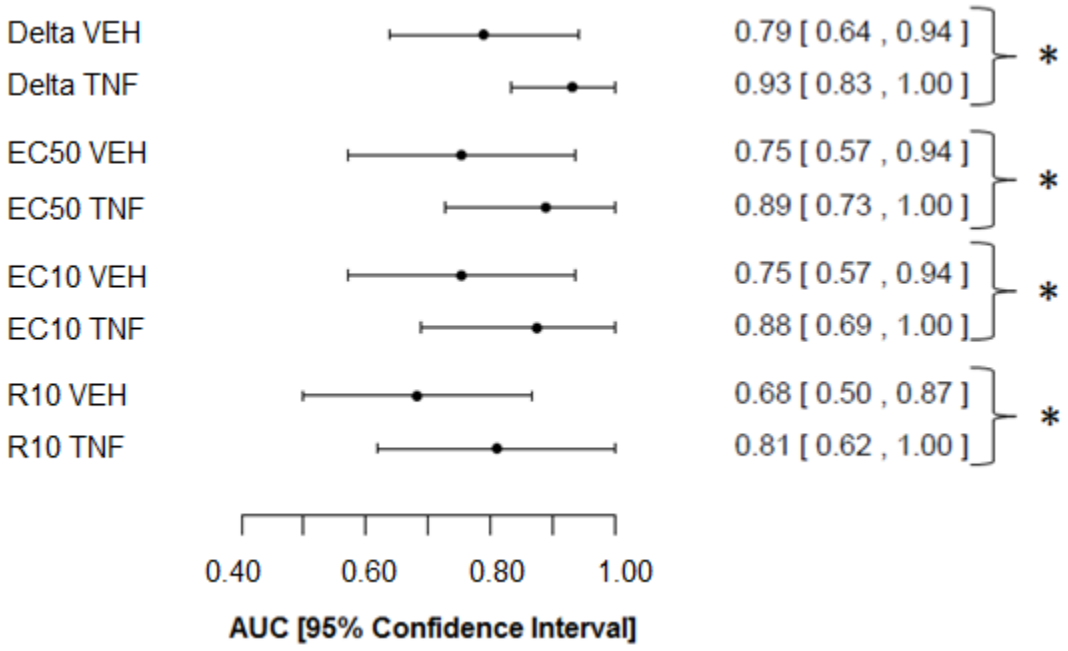
Figure 4

A



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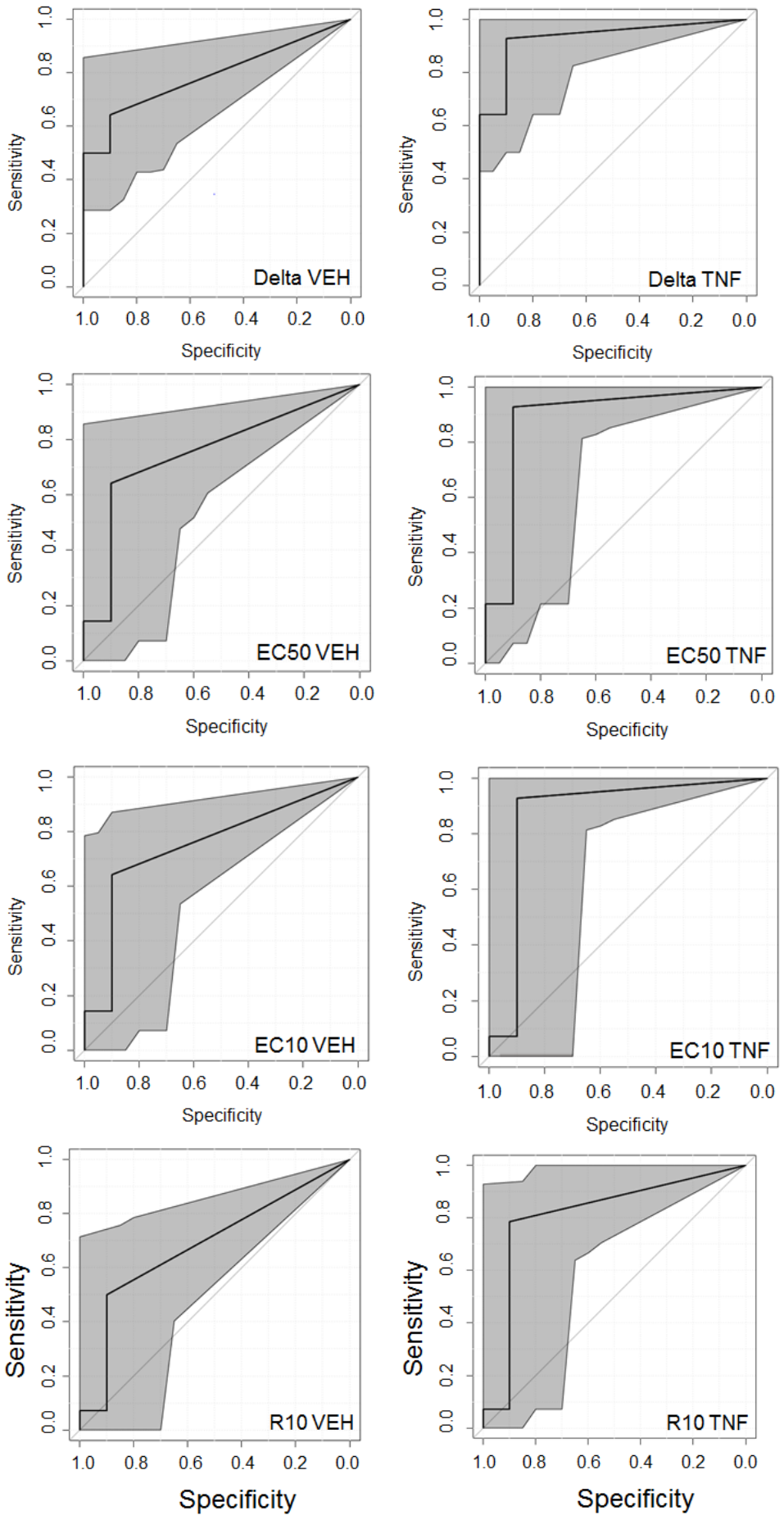

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Figure 5

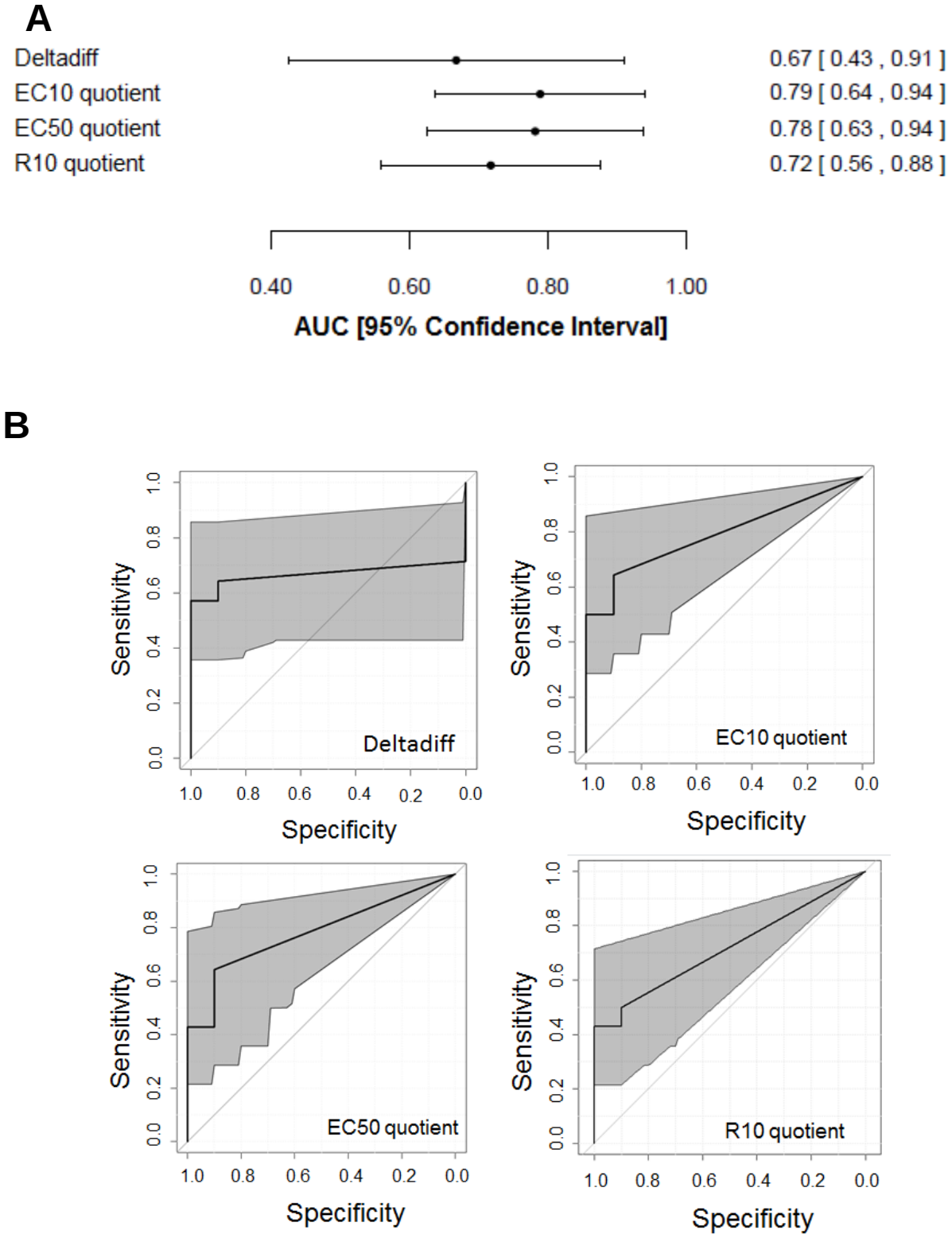

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Figure 6

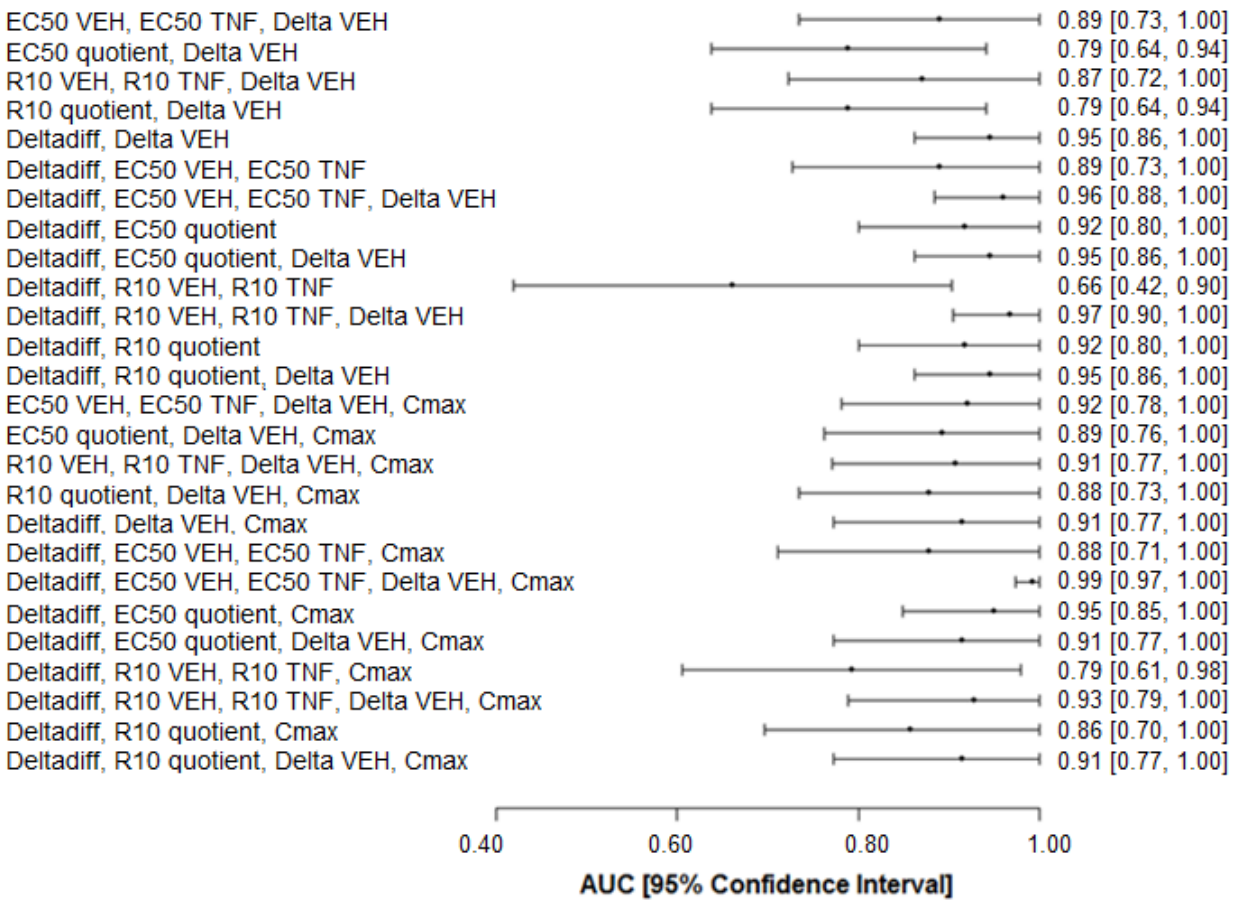

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Figure 7