Identification of Drugs Causing Severe Drug-induced Liver Injury (DILI) Using *in Vitro* Approach with Primary Human Hepatocytes

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Abstract

DILI is a major cause of early termination of drug development and of FDA regulatory action against marketed drugs, of which severe DILI is of great concern. In this study, the utility of primary human hepatocytes in assessing drugs causing severe DILI was evaluated. Based on the FDA Drug Labeling on DILI coupled with acute liver failure (ALF) reports, 80 drugs were divided as severe DILI or not. Severe DILI drugs are those withdrawn from the markets or labeled with a Black Box Warning due primarily to hepatotoxicity as well as those in the Warnings and Precautions or Adverse Reactions with associated ALF confirmed in USA alone or in two other countries. Human hepatocytes were treated at seven concentrations and luminescence and fluorescence assays performed with four endpoints (ATP content, GSH depletion, Caspase 3/7 activity and ROS) at 24 hours. The data was normalized and AUC calculated. Assay performance was evaluated with Receive Operating Characteristic (ROC) and the results quantitatively reported as sensitivity and specificity with 95% confidence intervals. We found that ROS normalized with ATP content predicted severe DILI very well with 90% and 88% of sensitivity and specificity respectively, indicating that oxidative stress is one of the critical factors related to severe DILI and combined toxic endpoints (ROS/ATP) performed better than individual measures. The proposed *in vitro* system can be useful in preclinical phase for early detection of the drugs with potential to cause severe DILI and for prioritization of the drugs based on the type of DILI the drugs may elicit .

Introduction

The unique aspects of this study are:

- A large number of drugs surveyed to predict severe DILI, not overall DILI
- Multiple doses used such that a cumulative measure of drug effect can be quantified by area under the curve (AUC)
- Individual mechanistic endpoints evaluated to associate mechanisms underlying severe DILI
- Closest *in vitro* model to human liver due to the presence of full complement of xenobiotic metabolizing enzymes.

Materials and Methods

Human Hepatocytes and Drug Treatment: Cryopreserved human hepatocytes were used in the study. The cells had high (>85%) viability and plating efficiency. Human hepatocytes pooled from 10 individual donors were used (thawed and pooled immediately before the study). A volume of 10 µl of the cell suspension (5000 cells) was added to each well of 384 well plate and the cells were allowed to attach for 4h followed by addition of 10 µl of 2X dosing solution starting at 250 µM for 24 hours.

Assays: Cellular ATP contents were measured using ATPlite kit (Perkin Elmer). Caspase-GLO® 3/7and GSH-Glo™ Glutathione Assay kit (Promega, WI) were used to measure caspase levels and total cellular glutathione, respectively following manufacturer's instructions. ROS were measured using the indicator H_2DCFDA (Molecular Probes, Invitrogen, CA)

Definition of severe DILI (Ref. 1,2)

Severe DILI (SD) are drugs
- withdrawn or having Black Box Warning **due to DILI**
- having Warnings & Precautions or Adverse Reactions for DILI **with ALF** in at least two countries

Non-Severe DILI (NSD) are drugs
- withdrawn or with Black Box Warning **not** due to DILI
- having Warnings & Precautions or Adverse Reaction for DILI, but ether no **ALF** or only one country reported
- with no DILI indication mentioned in the drug labels

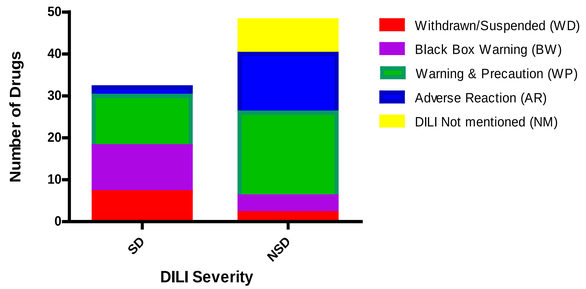

Figure 1. The distributions of marketed drugs with drug labeling in severe DILI (SD) and non-severe DILI (NSD) groups.

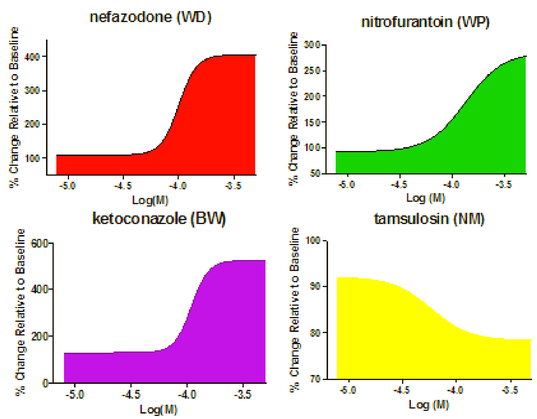

Figure 2. Representatives of dose-response curves fitted with A four-parameter logistic and AUC calculated with Trapezoidal method. The curves enables the usage of AUC as a systematic And cumulative quantification of drug's toxicity

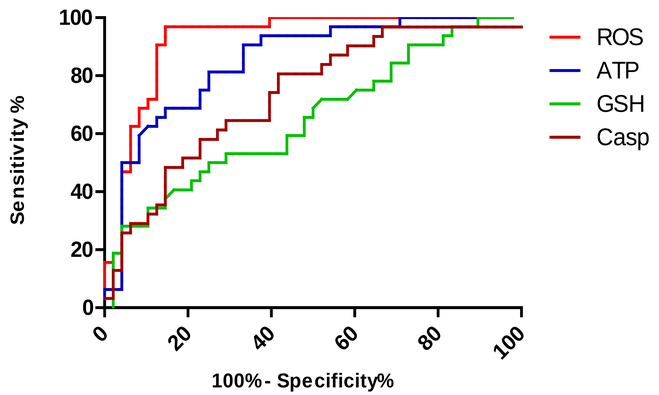

Figure 3. The assay performance evaluated by Receive Operating Characteristic (ROC) curves.

Table 1. Assay Performance

	Cut-off	Sensitivity%	95% CI	Specificity%	95% CI	LR
ROS/ATP	> 111.2	90.6	75% to 98%	87.5	75% to 95%	7.25
GSH	< 169.0	65.6	47% to 81%	52.1	37% to 66%	1.37
ATP	< 163.3	81.3	64% to 93%	75.0	60% to 86%	3.25
Casp3/7	> 118.2	74.2	55% to 88%	60.4	45% to 74%	1.87

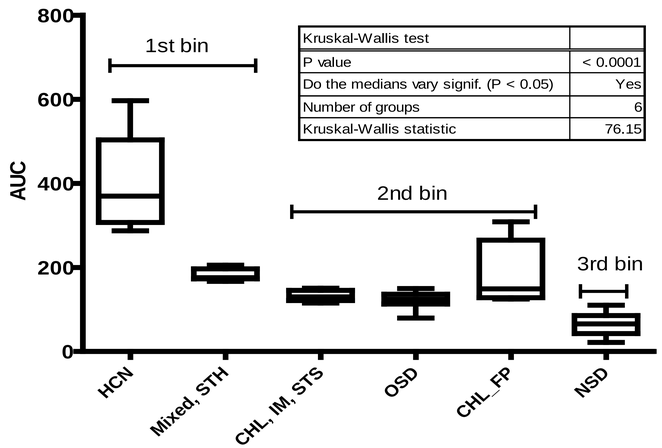

Figure 4.The respose distribution of drugs with different DILI types in ROS/ATP. HCN: hepatocellular necrosis, CHL: cholastasis, Mixed: nectosis/cholastasis, STH: steatohepatitis, IM: immune mediated, STS:steatosis, OSD: other severe DILI, CHL_FP: cholastasis fals positively identified, NSD: non-severe DILI

Table 2. Proposed reference drugs for different bins

	Drug Name	ROS	GSH	Casp	ATP	DILI Type	Label
1st bin	cyclofenil	1	1	1	1	HCN	WD
	nefazodone	1	1	1	1	Mixed	WD
	ketoconazole	1	1	1	1	HCN	BW
	tamoxifen	1	1	1	1	HCN,STH	WP
2nd bin	troglitazone	1	1	1	1	CHL	WD
	valproic acid	1	0	1	1	STS	BW
	propylthiouracil	1	0	0	0	IM	BW
	chlorpromazine	1	1	1	1	CHL_FP	AR
	toremifene	1	1	1	1	CHL_FP	WP
3rd bin	ifosfamide	0	0	0	0	NSD	AR
	primidone	0	0	0	0	NSD	NM
	cimetidine	0	0	0	0	NSD	NM

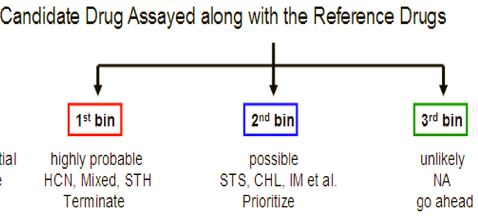

Figure 5. Proposed scheme of early screening of severe DILI potential drugs

Conclusion

- Using a quantitative measure of dose response relationship and the assay endpoints with clear underlying mode of action, a robust *in vitro* screening system was developed to distinguish severe DILI drugs from others with high accuracy.
- Very few false positives and false negatives: several drugs of cholestatic nature were false positively identified as severe DILI dugs while only one drug (carbamazepine) was false negative.
- Based on the intensity of severe DILI drugs' response in ROS assay, the drugs were grouped into three bins and each bin is associated with certain specific DILI types. A scheme of early screening is proposed to first distinguish the severe DILI drugs from others and second to prioritize the drug candidates based on the DILI types.
- Further work is under way to develop the assay endpoints to distinguish the false positives (i.e., cholestasis DILI drugs) from the true severe DILI drugs

Reference

1. Suzuki et al., Drug Safety 33(6): 503-22 (2010)
2. Mindikoglu et al., Liver Transplantation 15:719-729 (2009)

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