

ABSTRACT

Based on assessment of liver effects (changes in size/shape, yolk retention, loss of bile or liver degeneration) for 20 reference drugs, Jones et al. (2009) reported an overall predictive value of 84% in identifying mammalian hepatotoxicants using zebrafish (Zf) prior to evaluating the significance of bioanalysis. For this study another reference drug set was sent blinded to Evotec for screening. Using a 6-point concentration response curve and continuous exposure to compounds between 72-120 hours post fertilization (hpf), Tuebingen Zf were exposed to 18 nonproprietary/8 proprietary compounds. Zf were assessed for lethality, lowest observed effect concentration, and gross morphology. Compound uptake (average ng/Zf) was quantified by mass spectrometry. Compounds were classified as non-hepatotoxic (N), flag hepatotoxic (F-low incidence of yolk retention), or hepatotoxic (H-hepatomegaly, liver degeneration and/or prominent yolk retention). The relevance of body burden and acute toxicity was also considered in the final classification. As a general screen to flag compounds with any potential to cause hepatic injury in humans and/or animals the overall predictive value was 89% (sensitivity/specificity of 74/33%, N=26). Mixed results were obtained when trying to differentiate compound pairs based on hepatotoxicity risk. For example, nefazadone(H)/buspirone(F) & ranitidine(F)/famotidine(N) discriminated from each other based on severity of liver changes, while ibuprofen(H)/benoxaprofen(H) & trovafloxacin(N)/levofloxacin(H) did not. The results of this blinded analysis are encouraging for flagging compounds with any potential to cause hepatic injury in mammals (hazard assessment in early compound screening). Other factors in addition to acute toxicity/body burden will need to be considered when trying to differentiate between compounds with different levels of hepatotoxicity risk in man (risk assessment). Therefore, a combination of different assays may need to be utilized in order to reduce the likelihood of attrition due to hepatotoxicity late in the drug discovery and development process.

METHODS

Embryos

- Fertilized eggs obtained from breeding pairs of adult Tuebingen Zf
- Two different clutches arrayed per plate in alternate wells
- 7 fertilized eggs per well at 2-cell stage into 24-well culture plates containing 0.3X Danieau's solution
- 1X Danieau's stock: 58 mM NaCl, 0.7 mM KCl, 0.4mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5mM HEPES, pH 7.1
- Incubated at 28.5°C in humidity controlled environment
- All procedures performed in accordance with the UK Home Office Animals Scientific Procedures Act (1986)

Test Compounds

- Stock solutions were produced by serially diluting test compounds in the appropriate vehicle (DMSO; final concentration exposed to larvae, 0.5%)

Hepatotoxicity Assessment

- Concentration response study using standardized concentrations up to 1mM alongside Evotec's internal and vehicle controls. Larvae were exposed to compounds at 72 hpf with visual assessment of lethality, hepatic degeneration, hepatomegaly and yolk retention at 120 hpf using a dissecting stereomicroscope. A larva was classified as dead if all four of the following endpoints were present: 1) lack of heartbeat; 2) lack of circulating blood; 3) visual evidence of necrotic tissue; and 4) lack of motility (touch response). If lethality was observed across all concentrations in range, the screen was repeated at a lower concentration. Compounds were classified as non-hepatotoxic (N), flag hepatotoxic (F-low incidence of yolk retention), or hepatotoxic (H-hepatomegaly, liver degeneration and/or prominent yolk retention). The level of compound uptake, phenotypes observed at the LOEC and potential secondary/acute toxicity (gross phenotypes consisting of multiple non-liver-specific endpoints in addition to liver abnormalities) were also considered in the final classification.

Bioanalysis

- At non-lethal concentrations of compound, up to fourteen larvae per concentration were collected and subjected to bioanalysis utilizing Evotec's proprietary methodology. In order to quantify the amount of compound in the larvae, a routine mass spectrometry optimization and calibration curve using compound was run prior to testing the samples. Bioanalysis provided the average weight of compound per larva [ng/fish].

RESULTS

DRUG PAIR COMPARISONS (NSAIDs)

(IBuprofen and BENoxaprofen)						(DIClofenac and IBUfenac)						(MELoxicam and SUdoxicam)					
EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %
3	ND	0	0	0	0	10	ND	0	0	0	0	3	ND	0	0	0	0
10	3.1	0	14	0	0	30	ND	0	36	0	0	30	3.1	0	0	0	14
30	7.1	0	29	7	29	40	ND	71	0	7	21	30	6.7	0	0	0	29
100	18.1	0	93	0	86	50	ND	100	0	0	0	100	25.9	0	7	0	21
300	100	100	100	100	100	100	ND	100	100	100	100	300	100	100	100	100	100

IBU = MARKETED CBU = WITHDRAWN

Similar liver toxicity phenotypes were observed at similar parent body burden values. IBUEN caused lethality at lower concentration compared to IBU. CBUEN were classified as hepatotoxic in blinded assessment.

IBU = MARKETED CBU = WITHDRAWN

Similar liver toxicity phenotypes were observed at similar parent body burden values. IBUEN caused lethality at lower concentration compared to IBU. CBUEN were classified as hepatotoxic in blinded assessment.

MELO = PRE-CATION CBU = WITHDRAWN

Low liver toxicity phenotypes were observed at higher IBU concentration compared to DIC. CBU caused lethality at a higher concentration compared to DIC. CBUEN were classified as hepatotoxic in blinded assessment.

DRUG PAIR COMPARISONS (Antibacterials)

(LEVofloxacin and TROfloxacin)						(ERYthromycin-BASE and ERYthromycin-ESTolate)						(ROXithromycin and TELithromycin)					
EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %
30	LEV	0	0	0	0	100	BASE	0	0	0	0	100	ROX	8.5	0	0	0
100	3.8	0	0	0	0	300	7.6	0	0	0	0	300	3.9	0	0	0	
300	13.3	0	0	0	0	1000	20.3	0	64	0	0	1000	16.9	0	0	0	
7000	40.1	0	50	0	0	2000	0	0	0	0	0	2000	0	0	0	0	
2000	0	0	0	0	0	10	0	0	0	0	0	10	6.5	0	0	0	
30	TR	0	0	0	0	30	ND	0	0	0	0	30	25.4	0	0	0	
100	12.1	0	0	0	0	40	ND	50	21	0	0	1000	59.5	0	0	0	
300	14.2	0	0	0	0	50	100	100	100	100	100	1000	59.5	0	0	0	
1000	85.1	0	0	0	0	100	100	100	100	100	100	1000	59.5	0	0	0	

LEV = SERIOUS SIDE EFFECT CBU = WITHDRAWN

LEV liver toxicity phenotypes were not observed, except for single concentration of LEV. CBUEN were not observed with other drug. LEV was classified as hepatotoxic in blinded assessment.

IBASE = ADVERSE REACTIONS CBU = WITHDRAWN

IBASE liver toxicity phenotypes were observed at lower concentration of EST. CBUEN were observed at lower concentration of EST. CBUEN were classified as flag hepatotoxic (hepat) in blinded assessment.

ROX = MARKETED, not in US CBU = WITHDRAWN

Low liver toxicity phenotypes were not observed with other drug. CBUEN were not observed with other drug. ROX and TEL were both classified as non-hepatotoxic in blinded assessment.

DRUG PAIR COMPARISONS (Miscellaneous)

(BUSpirone and NEFazadone)						(ENTacapon and TOLcapone)						(FAMotidine and RANitidine)					
EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %	EXP [µM] LOEC	Uptake [ng/fish]	Lethal %	Yolk Ret %	Hepato megaly %	Liver Degrad %
10	23.7	0	0	0	0	100	ENT	1.3	0	0	0	100	FAM	0	0	0	0
30	48.8	0	0	0	0	300	8.7	0	21-29	0	0-7	100	TEL	1.6	0	0	0
100	114.2	0	29	0	0	500	7	57	0	0	0	1000	6.8	0	0	0	
300	96	14	0	14	0	1000	100	100	100	100	100	1000	6.8	0	0	0	
1000	100	100	100	100	100	1000	100	100	100	100	100	1000	6.8	0	0	0	
3000	34.8	0	0	0	0	3	TOL	1.2	0	0	0	3	RAN	0	0	0	
10	61.1	0	57	0	57	10	7	0	7-14	0	29	10	FAM	0.1	0	50	0
30	100	100	100	100	100	15	0	0	0	29	30	100	0.2	0	50	0	
100	100	100	100	100	100	30	100	100	100	100	100	1000	1.1	0	50	0	
300	100	100	100	100	100	100	100	100	100	100	100	1000	0	0	0	0	

IBUS = MARKETED CBU = WITHDRAWN

Low liver toxicity phenotypes were observed with both compounds. CBUEN was observed at lower concentration for NEF. CBUEN was classified as flag hepatotoxic (acute toxic) while NEF was classified as hepatotoxic in blinded assessment.

IBENT = MARKETED CBU = WITHDRAWN

Low liver toxicity phenotypes were observed at similar parent body burden values. CBUEN caused lethality at a lower concentration compared to ENT. CBUEN was classified as flag hepatotoxic (acute toxic) while ENT was classified as hepatotoxic in blinded assessment.

IBFAM = MARKETED CBU = WITHDRAWN

Low liver toxicity phenotypes were not observed with other drug. CBUEN was not observed with other drug. CBUEN was classified as non-hepatotoxic while RAN was classified as flag hepatotoxic (acute) in blinded assessment.

SECTION AND TABLE LEGEND

EXP = Exposure of drug in media
 PPT = Precipitation
 LOEC = Lowest Observed Effect Concentration
 Yolk Ret = Yolk Retention
 Liver Degrad = Liver Degradation
 N.D. = parent drug not detected due to standardized analytical protocol
 H = Hepatotoxic in blinded Zf assessment
 F = Flag Hepatotoxic in blinded Zf assessment
 N = Non-Hepatotoxic in blinded Zf assessment

TRUTH TABLES

PPV = Positive predictive value
 TP = True Positive
 FP = False Positive
 TN = True Negative
 FN = False Negative
 Current USFDA label was used for the idealized classification system where available

AS HAZARD ASSESSMENT

TP = marketed (with cautions), black box warning OR withdrawn and F/H
 FP = marketed and F/H
 TN = marketed and N
 FN = marketed (with cautions), black box warning OR withdrawn and N

AS RISK ASSESSMENT

TP = black box warning or withdrawn and H OR marketed (with cautions) and F
 FP = marketed (with cautions) and H OR marketed and F
 TN = marketed and N
 FN = black box warning and F/N

Table 1 Compound sets are presented with notations regarding hepatotoxicity risk

DRUG NAME	STATUS	FDA	EVENT CLASSIFICATION	HAZARD POTENTIAL	HAZARD POTENTIAL (relative to reference)	RELATIVE RISK BETWEEN DRUG PAIRS	SEVERITY OF RISK POTENTIAL (based on hazard)	
IBUPROFEN	MARKETED	PRECIPITATION	HEPATOTOXIC	TP	TP	NO DISCRIMINATION BETWEEN DRUG PAIRS	FP	
BENOXAPROFEN	WITHDRAWN		HEPATOTOXIC	TP	TP	REN CAUSES LETHALITY AT LOWER CONCENTRATION. BUT HEPATOTOXIC PHENOTYPES WERE OBSERVED AT SIMILAR BODY BURDEN	TP	
DICLOFENAC	BLACK BOX WARNING		HEPATOTOXIC	TP	TP	APPROPRIATE NONDISCRIMINATION BETWEEN DRUG PAIRS	TP	
IBUFENAC	WITHDRAWN		HEPATOTOXIC	TP	TP	REN CAUSES LETHALITY AND HEPATOTOXIC PHENOTYPES AT 5-FOLD HIGHER CONCENTRATION, BUT WITHOUT BIODATA TO SUPPORT HEPATOTOXIC EFFECTS. RELATIVE TOXICITY OF HEPATOTOXIC EFFECTS CANNOT BE DETERMINED	TP	
MELOXICAM	MARKETED	PRECIPITATION	HEPATOTOXIC	TP	TP	SID BIOSCREEN TESTS DUE TO FREQUENCY OF HEPATOTOXIC PHENOTYPE	FN	
SUDOXICAM	WITHDRAWN		NONHEPATOTOXIC (RETEST)	FN	FN	SID CAUSES LETHALITY AT SIMILAR CONCENTRATIONS	FN	
LEVOFLOXACIN	MARKETED	SERIOUS SIDE EFFECT	HEPATOTOXIC (RETEST)	FP	FP	DISCRIMINATION BETWEEN DRUG PAIRS IS REVERSED	FP	
TROVAFLOXACIN	WITHDRAWN		NONHEPATOTOXIC	FN	FN	NO LETHAL CONCENTRATION ACHIEVED	FN	
ERYTHROMYCIN BASE	MARKETED	PRECIPITATION	FLAG HEPATOTOXIC (RETEST)	TP	TP	NO DISCRIMINATION BETWEEN DRUG PAIRS	TP	
ERYTHROMYCIN ESTOLATE	WITHDRAWN		FLAG HEPATOTOXIC	TP	TP	EST CAUSES LETHALITY AT ~80 FOLD LESS CONCENTRATION, BUT WITHOUT BIODATA TO SUPPORT HEPATOTOXIC EFFECTS. RELATIVE TOXICITY OF HEPATOTOXIC EFFECTS CANNOT BE DETERMINED	FN	
ROXITHROMYCIN	MARKETED		NONHEPATOTOXIC	TN	TN	NO DISCRIMINATION BETWEEN DRUG PAIRS	TN	
TELITHROMYCIN	BLACK BOX WARNING		NONHEPATOTOXIC	FN	FN	NO LETHALITY ACHIEVED AND NO EVIDENCE OF HEPATOTOXIC PHENOTYPES DESPITE EVIDENCE OF BODY BURDEN OF BOTH DRUGS	FN	
BUSPIRONE	MARKETED		FLAG HEPATOTOXIC (ACUTE TOXIC)	FP	FP	NON-TEST DUE TO ACUTE TOXICITY	DISCRIMINATION BETWEEN DRUG PAIRS IS ACHIEVED	FP
NEFAZADONE	WITHDRAWN		HEPATOTOXIC	TP	TP	NEF CAUSES LETHALITY AT 10-FOLD LOWER CONCENTRATION, HOWEVER, PROMINENT HEPATOTOXIC PHENOTYPES OBSERVED AT ~2.5-FOLD LOWER BODY BURDEN	TP	
ENTACAPONE	MARKETED		FLAG HEPATOTOXIC (ACUTE TOXIC)	FP	FP	NON-TEST DUE TO ACUTE TOXICITY	DISCRIMINATION BETWEEN DRUG PAIRS IS ACHIEVED	FP
TOLCAPONE	BLACK BOX WARNING		HEPATOTOXIC	TP	TP	TOL CAUSES LETHALITY AT 10-FOLD LOWER CONCENTRATION, HEPATOTOXIC PHENOTYPES OBSERVED AT SIMILAR BODY BURDEN	TP	
FAMOTIDINE	MARKETED		NONHEPATOTOXIC	TN	TN	DISCRIMINATION BETWEEN DRUG PAIRS IS ACHIEVED	TN	
RANITIDINE	MARKETED	ADVERSE REACTIONS	FLAG HEPATOTOXIC (RETEST)	TP	TP	LETHALITY IS NOT ACHIEVED, YOLK RETENTION IS OBSERVED WITH VERY LOW BODY BURDEN	TN	
PPV (FP + FN)				85%	90%		56%	
Sensitivity (TP + FN)				79%	29%		64%	
Specificity (TN + TP)				50%	80%		25%	

HAZARD ASSESSMENT

As a general screen to highlight compounds with the potential to cause hepatotoxicity in humans the overall PPV was 85% (sensitivity = 79%, specificity = 50%).

DRUG PAIR COMPARISONS

- The ability to ideally differentiate compounds with differences in severity of human hepatotoxicity risk, was modest using the present classification system (PPV=58%, sensitivity (64%), specificity (29%).

In terms of relative risk or severity of hepatotoxicity between each drug pair 4 pairs segregated correctly (diclofenac & ibufenac, buspirone & nefazadone, entacapon & tolcapone and famotidine & ranitidine), 3 pairs were classified incorrectly in terms of relative risk between each other (ibuprofen & benoxaprofen, levofloxacin & trovafloxacin, and roxithromycin & telithromycin) and 2 pairs would require retesting (erythromycin base & erythromycin estolate, meloxicam & sudoxicam).

- Of the 5 drugs withdrawn from the marketplace due to hepatotoxicity, 3 were identified as hepatotoxic (benoxaprofen, ibufenac, nefazadone) while 2 were identified as non-hepatotoxic (trovafloxacin and sudoxicam) during a blinded assessment. Sudoxicam was recommended as a retest.
- Of the 4 marketed drugs with no (minimal) labeling information regarding liver injury, 2 (famotidine and roxithromycin) were identified correctly as non-hepatotoxic while 2 were classified as 'flag hepatotoxic' (buspirone and entacapon) due to acute toxicity in the Zf.

OVERALL CONCLUSIONS

- In a blinded assessment that used a different drug data set and classification system, our results:
 - compare favorably to Jones et al. (2009) wherein the PPVs for flagging potential hepatotoxic compounds were similar (84 vs 85%).
 - showed the ability to discriminate compounds based on severity of human hepatotoxicity risk, was 58%.
 - showed that the PPV for hazard and risk assessment increases to 100 and 70%, respectively, if 'flag hepatotoxicity-acutely toxic' classifications were not considered liver specific.
- These outcomes may be improved by considering additional factors such as non-specific secondary/acute toxicity, limitations in compound uptake, retesting certain compounds (suggested by the testing company).
- Bioanalysis is necessary to confirm and quantitate compound uptake in Zf. Since the maximum body burden detection for some compounds was low or incomplete, it is unknown whether hepatotoxicity would be induced at higher levels if achieved.

REFERENCES

1) Jones M, Ball JS, Dodd A, et al. Toxicol. 2009;262(1):13-4.