

Technical Application Bulletin

TAB Number:	TAB Biol. 003v1
Product:	B-CLEAR [®]
Subject:	B-CLEAR [®] : Cholestatic Hepatotoxicity
Reference:	B-CLEAR [®] Manual / TAB Anal. 001v1 (d ₈ -taurocholate LC/MS/MS)
Date:	31 January 2008
Purpose:	Describe the use of B-CLEAR [®] uptake/efflux studies to evaluate a compound's potential to cause cholestatic hepatotoxicity

Introduction

Drug-induced liver toxicity is the single most common reason for withdrawal of FDA-approved drugs from the market. Recent data suggest that hepatic transport proteins may be an important site of toxic interactions, and inhibition of the hepatic uptake and biliary excretion of bile acids (cholestasis) by drugs is one of the most common clinical liver toxicities. Current screens focus primarily on hepatic uptake, and cannot differentiate between effects on hepatic uptake and biliary excretion.

Because the B-CLEAR[®] (sandwich-cultured hepatocytes) technology maintains the expression and function of key uptake and efflux transporters relative to *in vivo*, it is the optimal system to evaluate and predict the potential of a compound to cause transporter-based liver toxicity. We used taurocholic acid as a bile acid probe substrate to evaluate the potential for Test Compounds (putative inhibitors) to cause cholestasis (alteration of the hepatic uptake and/or efflux of bile acids) in B-CLEAR[®]-RT (rat).

Method Overview

The method described in this application bulletin uses d₈-taurocholate (d₈-TC) as a probe to assess bile acid uptake and efflux in hepatocytes. Hepatocytes were preincubated with Test Compound (5 and 50 μM, except for troglitazone, which was evaluated at 1 and 10 μM) for a fixed period in parallel studies using either Plus (+) (calcium-containing) Buffer or Minus (-) (calcium-free) Buffer. Incubation in Plus (+) Buffer maintains the integrity of the tight junctions, while incubation in Minus (-) Buffer opens the tight junctions. Following the initial incubation, the hepatocytes were washed, and d₈-TC (2.5 μM) and Test Compound were added to the hepatocytes. Following a predetermined co-incubation time, the hepatocytes were washed and lysed, and the lysate was analyzed for d₈-TC. d₈-TC, measured by LC/MS/MS analysis, was used to distinguish between the probe and the endogenous taurocholate produced in sandwich-cultured hepatocytes (radiolabelled taurocholate can also be used in this assay). The amount of d₈-TC excreted

into the bile pockets was determined by subtracting the amount of d₈-TC in the Minus (-) Buffer lysate (hepatocytes) from the amount of d₈-TC in the Plus (+) Buffer incubation lysate (hepatocytes + bile pockets). For a detailed, step-by-step procedure on performing the B-CLEAR[®] assay to evaluate cholestatic hepatotoxicity, see the Application Methodology section. For a detailed procedure on LC/MS/MS analysis of d₈-TC, see Qualyst Technical Application Bulletin, TAB Anal. 001v1.

Results

The effects of Test Compounds on the accumulation, biliary excretion index (BEI), and *in vitro* biliary clearance (Cl_{biliary}) of d₈-taurocholate (d₈-TC) were evaluated in B-CLEAR[®]-RT; the results are presented in Tables 1 and 2.

The accumulation of d₈-TC in hepatocytes and bile pockets [Plus (+) Buffer] was 131 ± 31.4 pmol/mg protein, and the accumulation in hepatocytes [Minus (-) Buffer] was 15.1 ± 2.47 pmol/mg protein in the absence of inhibitor (Table 1). Erythromycin-estolate and troglitazone inhibited the accumulation of d₈-TC into hepatocytes [Minus (-) Buffer] compared to controls (no inhibitor), whereas cyclosporine and glyburide (50 μM concentration) increased the accumulation of d₈-TC in hepatocytes; nefazadone had no effect on d₈-TC accumulation. Salicylate was used as a negative control to demonstrate that compounds that do not display *in vivo* cholestatic potential have no effect on the accumulation of d₈-TC in the B-CLEAR[®] system.

Table 1. The effect of Test Compounds on the uptake of d₈-TC expressed as accumulation and as a percentage of control (no inhibitor).

Test Compound		Accumulation (+) Buffer mean ± standard deviation		Accumulation (-) Buffer mean ± standard deviation	
		pmol/mg protein	% control	pmol/mg protein	% control
No inhibitor		131 ± 31.4	100	15.1 ± 2.47	100
Salicylate	5 μM	152 ± 36.9	118 ± 34.7	17.0 ± 3.58	113 ± 21.8
	50 μM	136 ± 32.3	105 ± 24.2	16.4 ± 3.53	109 ± 22.6
Cyclosporine	5 μM	80.2 ± 19.0	61.2 ± 8.40	38.3 ± 6.96	253 ± 27.6
	50 μM	44.1 ± 9.21	33.7 ± 4.20	30.7 ± 6.21	202 ± 15.2
Erythromycin-estolate	5 μM	45.0 ± 9.48	35.5 ± 11.6	9.67 ± 2.74	62.7 ± 12.1
	50 μM	9.82 ± 2.19	7.75 ± 2.65	4.38 ± 1.32	29.1 ± 9.42
Glyburide	5 μM	108 ± 26.2	83.7 ± 18.3	12.0 ± 3.02	78.7 ± 16.6
	50 μM	50.4 ± 10.6	38.7 ± 2.29	40.7 ± 6.79	271 ± 32.8
Nefazodone	5 μM	103 ± 18.2	79.6 ± 14.7	12.8 ± 1.74	85.1 ± 8.28
	50 μM	56.9 ± 11.0	43.8 ± 6.98	18.3 ± 4.36	121 ± 30.3
Troglitazone	1 μM	105 ± 25.8	80.1 ± 7.11	11.7 ± 2.99	76.3 ± 10.7
	10 μM	15.5 ± 1.84	12.2 ± 2.49	4.99 ± 2.63	32.9 ± 9.31

The effects of Test Compounds on the biliary efflux of d₈-TC were measured and calculated as both the BEI and Cl_{biliary} (Table 2). The BEI for d₈-TC was 88.3 ± 1.32 %, indicating that 88 % of the d₈-TC taken up by the hepatocytes was effluxed into the bile. Of the six test compounds evaluated, glyburide, and, to a lesser extent, cyclosporine, had the greatest inhibitory effect on the BEI. Erythromycin-estolate, nefazodone, and troglitazone demonstrated a dose-dependent decrease in the BEI of d₈-TC; salicylate (negative control) had no effect on the BEI.

The Cl_{biliary} of d₈-TC in the absence of inhibitor was 37.1 ± 9.54 mL/min/kg. All compounds, with the exception of salicylate, showed a substantial decrease in Cl_{biliary} of d₈-TC at the higher concentration of Test Compound. A decrease in Cl_{biliary} is the result of a decrease in the amount (mass) of d₈-TC excreted into the bile, which results from inhibition of either (1) canalicular efflux transporters, or (2) basolateral uptake transporters lowering the intracellular accumulation of d₈-TC, and indirectly affecting the biliary efflux of d₈-TC. The Cl_{biliary} is an indicator of the overall effect of the compound on bile acid excretion. The BEI, in conjunction with the accumulation, can be used to determine the site of action (basolateral/uptake versus canalicular/efflux) of a particular compound on the hepatobiliary disposition of bile acids in the B-CLEAR[®] system.

Table 2. The effect of Test Compounds on the biliary efflux of d₈-taurocholate expressed as BEI or Cl_{biliary} and as a percentage of control (no inhibitor).

Test Compound	BEI mean ± standard deviation		Cl _{biliary} mean ± standard deviation	
	%	% control	mL/min/kg	% control
No inhibitor	88.3 ± 1.32	100	37.1 ± 9.54	100
Salicylate	5 µM	88.7 ± 0.462	101 ± 1.42	43.2 ± 10.9
	50 µM	87.9 ± 1.25	100 ± 0.421	38.4 ± 9.69
Cyclosporine	5 µM	51.6 ± 4.58	58.8 ± 4.07	13.4 ± 4.62
	50 µM	29.9 ± 3.86	34.0 ± 4.69	4.26 ± 1.27
Erythromycin- estolate	5 µM	78.3 ± 6.71	88.6 ± 8.70	11.3 ± 3.01
	50 µM	55.7 ± 4.07	62.9 ± 3.72	1.74 ± 0.361
Glyburide	5 µM	89.0 ± 0.689	101 ± 1.32	30.9 ± 7.20
	50 µM	18.4 ± 12.7	20.8 ± 14.2	3.10 ± 2.53
Nefazodone	5 µM	87.4 ± 0.933	99.0 ± 1.19	28.7 ± 5.36
	50 µM	67.7 ± 6.00	76.7 ± 5.62	12.3 ± 3.08
Troglitazone	1 µM	88.9 ± 0.916	101 ± 2.18	30.0 ± 7.89
	10 µM	67.5 ± 10.7	76.4 ± 13.1	3.36 ± 0.722

Conclusions

Transporter-based drug interactions that affect basolateral uptake or canalicular efflux in the liver may lead to the alteration of the *in vivo* hepatobiliary disposition of bile acids. The inhibition of the hepatobiliary disposition of bile acids is one mechanism by which drugs may cause cholestasis. Unlike other *in vitro* transporter models, the B-CLEAR[®] technology can simultaneously determine the potential for a compound to alter the basolateral uptake and/or the canalicular efflux of bile acids as well as predict the overall effect. The following scenarios illustrate three possibilities:

Scenario 1. Efflux is Primarily Affected (cyclosporine)

- The BEI will decrease: the mass of d₈-TC transported into the bile will decrease, which is consistent with inhibition of canalicular efflux transporters, e.g. Bsep.
- Accumulation in Plus (+) Buffer will decrease (decrease in total mass processed).
- Intracellular concentration [accumulation in Minus (-) Buffer] may increase, especially if no other processes are affected.

Scenario 2. Uptake is Primarily Affected (erythromycin-estolate)

- The BEI should not change so long as sufficient mass was taken up to detect biliary excretion.
- Accumulation in Plus (+) Buffer will decrease (decrease in total mass processed).
- Intracellular concentration may also decrease.

Scenario 3. Mixed Effects (troglitazone, glyburide)

- The BEI will decrease, consistent with inhibition of canalicular efflux transporters.
- Accumulation in Plus (+) Buffer will decrease, which is consistent with inhibition of basolateral uptake transporters (decrease in total mass processed).
- Accumulation in Minus (-) Buffer may increase, which is consistent with inhibition of canalicular efflux to a greater extent than basolateral uptake, i.e. glyburide.
- Accumulation in Minus (-) Buffer could also stay the same or decrease, which is consistent with inhibition of basolateral uptake to a greater extent than canalicular efflux, i.e. troglitazone.
- No change in the accumulation in Minus (-) Buffer may be observed if the inhibition of basolateral uptake and canalicular efflux occurs in parallel.

The clinical definition of cholestasis is any condition in which substances normally excreted into bile are retained. The most common method for the clinical determination

of cholestasis is to measure serum concentrations of bile acids or conjugated bilirubin. Increased serum concentrations of bile acids may not be reflective of increased hepatocyte concentrations of bile acids. It is generally assumed that it is the concentration of bile acids inside the hepatocyte that is the primary determinate of hepatotoxicity. It is important to differentiate between effects on the uptake or efflux of bile acids, since inhibition of uptake will result in decreased hepatocyte concentrations of bile acids, whereas, inhibition of efflux will result in increased hepatocyte concentrations of bile acids. However, inhibition at either site will result in increased serum concentrations. Knowledge of the site of inhibition is important in understanding the relationship between elevated serum concentrations of bile acids and the concentration of bile acids in the hepatocyte.