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Communication

Application of Covalent Binding Body Burden in the HμREL Human Hepatocyte Coculture Model for Reactivity Risk Assessment of Metabolically Low Turnover Drugs

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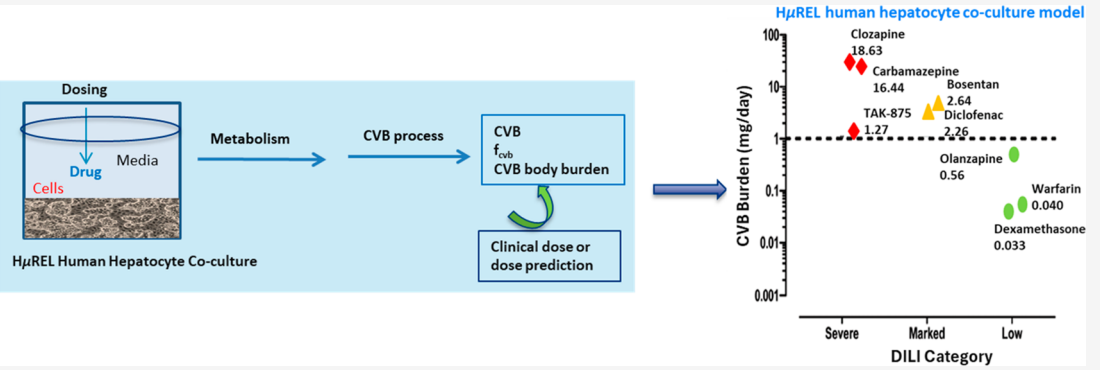

ABSTRACT: The human hepatocyte suspension model has been a valuable tool to study covalent binding (CVB) for compounds that form reactive metabolites. However, accurately measuring CVB values with the suspension model becomes challenging for metabolically low turnover compounds. In this study, we evaluated the HμREL human hepatocyte coculture model relative to existing literature using human hepatocyte suspension for drugs of known drug-induced liver injury category. Our results indicate that this coculture model provides ample metabolic turnover to reproducibly measure CVB. It is sufficiently robust to apply a predefined 1 mg/day CVB body burden threshold for risk assessment to guide our discovery programs, allowing for expanded coverage to include metabolically low turnover compounds.

Drug-induced liver injury (DILI) is a major concern in drug development, often leading to the attrition of potential therapeutic candidates.[1] Multiple factors or mechanisms may contribute to the occurrence of DILI.[2] The covalent binding (CVB) of reactive metabolites to proteins is recognized as one of several mechanisms underlying DILI.[3] The level of covalent binding is conventionally measured in the hepatocyte suspension model with radiolabeled compounds. Covalent binding (CVB) body burden, which quantifies the fraction of covalent binding relative to the metabolic turnover and clinical daily dose, can be used in the risk assessment of compounds generating reactive species. Richard Thompson et al. developed a methodology to determine CVB body burden for 36 commercial drugs using a hepatocyte suspension model and established a CVB body burden threshold of 1 mg/day to differentiate drugs subject to bioactivation as either bearing low or high risk potential to cause idiosyncratic adverse drug reactions (IADRs).[4] The conventional hepatocyte suspension model is often limited to a 4-h maximum incubation due to loss of cell viability and enzymatic activity. As such, it has limited utility for slowly metabolized compounds due to insufficient metabolic turnover, impeding the conduct of CVB studies for their risk assessment. In light of the successful advancements in drug discovery to optimize and identify very metabolically stable lead molecules, a growing need exists to establish a hepatocyte model that can assess the CVB body burden for low turnover compounds that generate reactive metabolites.

Over the past decade, approaches such as hepatocyte relay, monolayer plated hepatocyte culture, hepatocyte coculture, and 3D-spheroid culture have been developed to support long-term hepatocyte incubations to increase metabolic turnover.[5−7] While these approaches are widely used for accurately predicting in vivo clearance as well as producing in vivo

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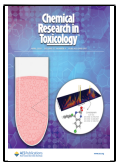

Table 1. CVB for the Drug Test Set and JNJ-Compounds (10 μM) in HμREL Human Hepatocyte Coculture

Compounds	DILI Category	Incubation Time (h)	Turnover (%)	CVB_{net} (N = 3) (pmol/mg protein)	f_{cvb} (N = 3)	Daily Dose (mg/day)	Mean CVB daily burden[a] (mg/day)
CVB assessment for commercial drug set							
^{14}C-Carbamazepine	Severe	168	38.1	101.4 ± 21.2	0.0137 ± 0.0003	1200	16.44 ± 0.33
^{14}C-Clozapine	Severe	48	35.7	172.5 ± 24.3	0.0207 ± 0.0007	900	18.63 ± 0.62
^{3}H-Tak-875	Severe	12	70.4	549.9 ± 47.9	0.0254 ± 0.0010	50	1.27 ± 0.049
^{3}H-Bosentan	Marked	24	48.7	181.41 ± 82.1	0.0106 ± 0.0025	125 bid	2.64 ± 0.63
^{14}C-Diclofenac	Marked	12	100	274.5 ± 9.7	0.0113 ± 0.0027	200	2.26 ± 0.31
^{3}H-Dexamethasone	Low	48	49.2	37.0 ± 7.6	0.0033 ± 0.0005	10	0.033 ± 0.005
^{14}C-Olanzapine	Low	72	44.4	419.0 ± 41.2	0.00281 ± 0.0022	20	0.56 ± 0.04
^{14}C-Warfarin	Low	168	76.9	122.8 ± 10.1	0.0040 ± 0.0008	10	0.040 ± 0.008
CVB assessment for JNJ-compounds							
^{14}C-JNJ-A		48	67.5	31.3 ± 10.9	0.0013 ± 0.0004	832[b]	1.00
^{3}H-JNJ-B		48	42.5	86.6 ± 8.9	0.0059 ± 0.0004	170[b]	1.00
^{3}H-JNJ-C		24	21.4	6.8 ± 0.7	0.0018 ± 0.0002	568[b]	1.00

[a]Intra-assay variation of CVB burden for commercial drug set ranged from 2.0 to 23.8%. [b]Estimated clinical dose to reach 1 mg/day CVB burden assuming f_a and f_m were equal to 1 for the most conservative assessment.

relevant metabolites for low turnover compounds, their utility for CVB assessment has not been widely reported outside of an application in plated hepatocytes.[8] Furthermore, some of these models are poorly suited to conduct CVB studies. For example, the hepatocyte relay method involves the successive transfer of supernatant from 4-h hepatocyte incubations to freshly thawed hepatocytes to extend incubation times up to 24 h. However, the requirement to introduce multiple rounds of freshly thawed hepatocytes in the relay method prohibits the cumulative measurement of CVB in a common hepatocyte source. There are also drawbacks in monolayer cultures of plated cryopreserved hepatocytes, which tend to dedifferentiate after 24 or 48 h in culture, resulting in a loss of drug metabolizing enzymatic activity over time. Hepatocyte coculture systems like HepatoPac and HμREL, as well as 3D-spheroid cultures, may be more suitable to conduct CVB studies given their ability to maintain enzymatic activity for up to 7 days or longer using a single hepatocyte preparation.

For our CVB investigations, we selected the HμREL coculture of human hepatocytes and nonparenchymal stromal cells in 24-well format (Visikol Inc., Hampton, NJ) as our preferred platform, given its greater hepatocyte density over other platforms to maximize our ability to collect and measure covalent binding resulting from reactive metabolites formation.[6] Eight commercially available drugs known to cause severe or marked DILI (carbamazepine, clozapine, TAK-875, bosentan, and diclofenac) or bearing low DILI concern (dexamethasone, olanzapine, and warfarin) were selected to validate this model.[4,9,10] With the exception of diclofenac, these drugs demonstrate relatively low turnover in suspension hepatocytes, and all bear an established CVB value, thereby serving as a bridge between suspension to coculture hepatocyte systems.[4,9,10] Diclofenac, a known high turnover drug in hepatocyte suspension, was included as part of this test set due to its close proximity to the 1 mg/day CVB burden threshold in suspension hepatocytes[4] and, thus, provides an important calibration point for the HμREL system. Overall, our goal was to test the feasibility and robustness of this coculture model to utilize for risk assessment of metabolically low turnover compounds, thereby expanding the scope of compound coverage for the hepatocyte suspension model first described by Thompson et al.[4]

Experimentally, the radiolabeled test compounds were incubated at 10 μM in 24-well microtiter plates containing HμREL cocultured human hepatocytes at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. To ensure adequate time to achieve measurable CVB levels, incubation times were selected based on the metabolic turnover for each compound. Each well initially contained 0.188 million viable hepatocytes. Control incubations were carried out to evaluate nonspecific binding to proteins. After incubation, both the cells and medium were collected, and proteins were precipitated. The resulting supernatants were dried, reconstituted, and filtered prior to their injection onto an LC-Radio-detector to measure metabolic turnover. The remaining protein pellets were subjected to extensive wash cycles to remove unbound radioactivity, then solubilized and, finally, measured for both radioactivity and protein concentration. Covalent protein binding of the incubated test compounds was determined by normalizing radioactivity to protein content and subtracting nonspecific binding for accurate assessment, as shown in the following equation.[4]

$$\text{CVB} = \frac{\text{Amount of radioactivity in the sample(pmol equiv)}}{\text{Amount of protein in the sample(mg)}}$$

The fraction of metabolism leading to covalent binding (f_{cvb}) was calculated with the following formula.

$$f_{cvb} = [\text{CVB(pmol eq/mg)} \times \text{Amount of protein(mg) in the incubation}] / [\text{Turnover} \times \text{Amount of drug dosed(pmol)}]$$

Following the Thompson et al. approach, the daily CVB body burden was calculated using the following formula

$$\text{CVB Burden} = D \times f_a \times f_m \times f_{cvb}$$

where D is the maximum prescribed daily dose, f_a is the fraction of the dose absorbed, and f_m is the fraction of the dose eliminated via metabolism. This model is intended to serve as an in vitro tool for reactivity risk assessment of molecules progressing to regular preclinical development, where obtaining precise values for f_a and f_m during this stage is often unfeasible for most molecules. Therefore, given the recognized limitation, the conservative assumption of setting f_a and f_m to 1 is adopted to ensure the model's applicability to molecules at the preclinical stage, aligning with the assumption outlined in

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the Thompson publication for the most conservative assessment.[4]

Table 1 displays the results of CVB assessments for the drug test set in HμREL coculture hepatocytes. Notably, all compounds exhibited robust turnover rates exceeding 35% in this system. For instance, warfarin, typically known for its exceptionally low turnover in suspension hepatocytes (<10% turnover after 4-h incubations generally in our lab) demonstrated remarkably high turnover of 76.9% in HμREL coculture hepatocytes following 168-h incubations.

Each test set drug exhibited detectable CVB values ranging from 37.0 to 549.9 pmol/mg protein, well-above the lower limit of detection at 3.6 pmol/mg protein. Similar to the conclusions made by Thompson et al., distinguishing drugs between low- or high-concern categories was not possible using either the CVB value or the fraction of CVB (f_{cvb}) alone and required the daily dose to derive the CVB burden.

Drugs classified in the low-risk category (dexamethasone, olanzapine, and warfarin) displayed CVB burdens of less than 1 mg/day, while those categorized as high or marked risk concern categories (carbamazepine, clozapine, TAK-875, bosentan, and diclofenac) exhibited CVB burdens exceeding 1 mg/day in the HμREL coculture model. Thus, the application of the 1 mg/day CVB body burden threshold first proposed by Thompson et al. remains applicable in the HμREL coculture model.

To assess the robustness of the CVB study in HμREL coculture hepatocytes, we conducted n = 3 replicates in a single run to evaluate intra-assay variation (Table 1). Additionally, we tested carbamazepine and warfarin in four separate studies run on different days to assess interassay variation (Table S1). The coefficient of variation (CV) for intra-assay deviation ranged from 2.0% to 23.8% across the commercial drug test set, while the CV for interassay deviation was 35.3% for carbamazepine and 32.7% for warfarin. These values fall within the interassay CV range of 29% to 49% observed during in-house CVB assays using suspension hepatocytes.

To evaluate the fidelity of the f_{cvb} value over the course of prolonged HμREL incubations, we measured the metabolic turnover, CVB, and f_{cvb} for warfarin and bosentan at various time points (Figure 1). Figure 1 illustrates that warfarin and bosentan both demonstrate a notable trend for increased turnover and CVB as the incubation time is extended, consistent with sustained metabolic capacity of the HμREL

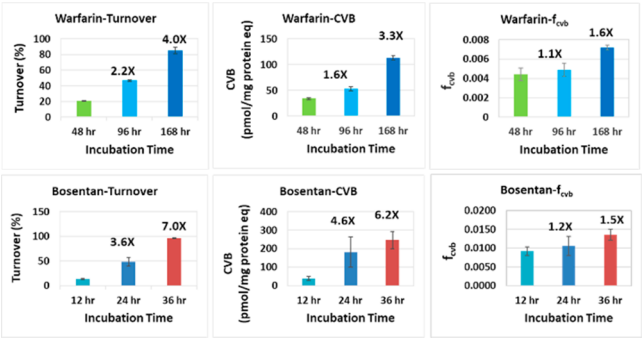

Figure 1. Impact of incubation times on metabolic turnover, CVB, and f_{cvb} of warfarin and bosentan (10 μM) using HμREL human hepatocyte coculture model. Fold increases of values compared to those at the first time point are labeled on the graph.

incubations. Meanwhile, f_{cvb}, the fraction of metabolism leading to covalent binding, normalized by metabolic turnover, remains fairly consistent across the extended incubation time points. Similar trends were observed for warfarin when examining additional time points across the 7-day incubations for metabolic turnover, CVB, and f_{cvb}, as depicted in Supporting Information Figure S1. For both warfarin and bosentan, only a marginal increase of 1.5−1.6 fold was observed in the f_{cvb} at the last time point relative to the initial time point. Such increase is slightly beyond the intra-assay variability and could be attributable to the generation of reactive metabolites through continued sequential metabolic processes; nonetheless, the f_{cvb} remained within 2-fold irrespective of the time point. For cautionary purposes, it is suggested to target 50% turnover or less to minimize extensive sequential metabolism, which may bear less in vivo relevance, from contributing to the f_{cvb} determination.

Figure 2 presents a comparison of the CVB burden of test drug set obtained using the HμREL model vs the hepatocyte

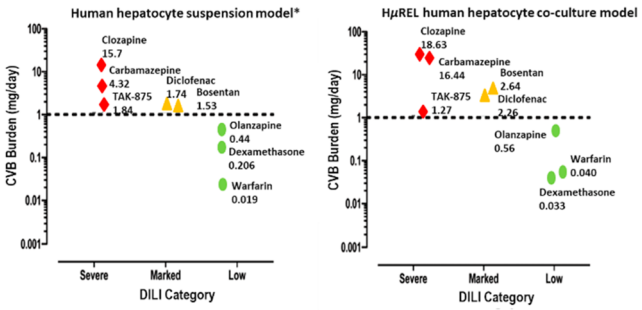

Figure 2. Comparison of CVB daily burden of test compounds from human hepatocyte suspension models and HμREL human hepatocyte coculture model. *Data obtained from refs 4, 9, and 10.

suspension model.[4,9,10] Most compounds exhibited similar CVB burden values in both models, differing by no more than 2-fold, with the notable exceptions of carbamazepine and dexamethasone. Carbamazepine, a drug associated with severe liver injury, demonstrated a significantly higher CVB burden in the HμREL model compared to the suspension model. Conversely, dexamethasone, a drug with no reported liver toxicity, exhibited a markedly lower CVB burden in the HμREL model than in the suspension model. Thus, relative to suspension hepatocytes, the HμREL model does not exhibit any clear bias in the derived CVB burden values.

Upon validating the HμREL coculture model, we applied this approach to our discovery programs as an issue-driven strategy for metabolically low turnover compounds, enabling risk assessment based on program needs, with consideration given to program indication, reactive metabolite formation, and the anticipated clinical dose and dosage regimen. JNJ-A, JNJ-B, and JNJ-C are internal lead compounds in the discovery phase that exhibit low metabolic turnover in human hepatocytes (CLint <4 μL/min/million cells) and form glutathione or postcursor adducts in human hepatocytes. In the HμREL model, in addition to an increase in metabolic turnover, these compounds produced similar metabolite profile and thiol-related adducts. Consequently, we employed the HμREL model to assess their reactivity risk potential using their radiolabeled analogues.

Table 1 provides a comprehensive overview of the CVB assessments of JNJ-A, B, and C in HμREL human hepatocyte

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coculture. All three JNJ compounds exhibited a turnover rate exceeding 20%, with net covalent binding ranging from 6.8 to 86.6 pmol eq/mg protein, surpassing the lower limit of detection. The resulting f_{cvb} values for JNJ-A, B, and C were determined to be 0.0013, 0.0059, and 0.0018, respectively. Given that clinical dose predictions remain preliminary at this stage, we calculated the maximum clinical dose required to achieve a 1 mg/day body burden. This resulted in estimates of 832, 170, and 568 mg/day for JNJ-A, B, and C, respectively. Overall, the HμREL model allowed us to assess their reactivity risk, which would have been challenging using the conventional suspension hepatocyte approach.

In conclusion, our study has successfully validated the HμREL hepatocyte coculture model using a commercial set of drugs and established a reference threshold to qualify reactivity risk potential for low-turnover drugs. Through enhanced metabolic turnover, the HμREL model produced robust and reproducible f_{cvb} values to apply to low-turnover drugs by employing the previously defined 1 mg/day CVB body burden threshold originally established for hepatocyte suspension models. Furthermore, the model and approach induced confidence to apply to our discovery projects, expanding our ability to conduct risk assessment for the growing precedence of low turnover drugs. Of note, as many different mechanisms may contribute to the onset of DILI in humans, CVB burden is not used in isolation but rather as part of an integrated approach, incorporating CVB body burden, dose, indication, other DILI-relevant mechanistic assays, and preclinical safety findings to make informed decisions on risk assessment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.4c00046.

Materials and methods. Table S1: assessment of interassay variation for carbamazepine and warfarin in HμREL human hepatocyte coculture. Figure S1: impact of incubation times on metabolic turnover, CVB and f_{cvb} of warfarin using HμREL human hepatocyte coculture model (PDF)

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Author Contributions

‖J.S. and K.J.C. contributed equally. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DILI, drug-induced liver injury; IADR, idiosyncratic adverse drug reaction; CVB, covalent binding.

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