Interpretation of *in Vitro* Metabolic Stability Studies for Racemic Mixtures

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ABSTRACT: In early drug discovery, where chiral syntheses may not yet have been elucidated or enantiomeric separation is not feasible, screening of racemates in metabolic stability assays may offer a pragmatic approach. To assess the risk of incorrectly deprioritizing enantiomers due to misclassification of apparent *in vitro* intrinsic clearance (CL_{int,app}), we evaluated (1) theoretical simulations; (2) literature on enantiomeric CL_{int,app} differences; (3) historic MSD data; and (4) new data on enantiomers with high eudysmic ratios and low predicted three-dimensional similarity. Overall, the results suggested minimal risk of not progressing an enantiomer due to an appreciably different (>3-fold) racemate CL_{int,app}.

KEYWORDS: Intrinsic clearance, enantiomers, drug discovery, hepatocytes

During drug discovery, a key early measured parameter is often metabolic stability, typically using hepatocytes (heps) or hepatic microsomes (mics) supplemented with NADPH. Metabolic stability (or apparent intrinsic clearance, CL_{int,app}) is used, along with binding estimates, in the prediction of *in vivo* metabolic intrinsic clearance.¹ This, in turn, when contextualized with other key parameters, is used in predictions of oral bioavailability, exposure, half-life, and hence efficacious dose. In this Letter, CL_{int,app} refers to the apparent (measured) CL_{int} without consideration of incubation binding.

Compound specifications for initial screening assays are often based on both chemical and enantiomeric purity (typically >90%), particularly for *in vitro* and *in vivo* drug metabolism and pharmacokinetic (DMPK) studies. This is based on concerns over the nature of contaminants (e.g., possible CYP inhibitors) as well as the known potential for enantiomer-specific metabolism.²

Occasionally, due to synthetic limitations for chiral chemistry and/or analytical challenges with chiral separations, drug discovery project teams may look to reduce the cycle time for *in vitro* potency, selectivity, and DMPK assays by generating early data on racemic mixtures (composed of enantiomers E1 and E2). Such data may be used to inform on the value of progressing any given compound, therefore warranting subsequent chiral separation or chiral resynthesis. In addition, racemate CL_{int,app} could help assess the potential for metabolic vulnerability of the required pharmacophore. Assuming a 50:50 enantiomeric mix (rather than enantiomeric excess >0 or contamination by intermediates or byproducts), the risk of missing active enantiomers by testing racemates in potency assays is low; if only a single enantiomer is active, that activity will be diluted only 2-fold by the inactive enantiomer.

In this investigation, we attempted to understand whether similar assumptions can be made when measuring CL_{int,app}. Throughout the simulations, we have assumed a 50:50 mix of enantiomers in the racemic mixtures, as would be formed by chemical syntheses without the use of chiral solvents or chiral catalysts.

Assays to measure CL_{int,app} almost invariably have LC–MS/MS end points that do not distinguish enantiomers. Diastereomers were not considered in this analysis because they are generally separable under standard purification conditions and submitted to *in vitro* metabolic stability assays as distinct entities. However, when assays are performed with racemic mixtures, data will reflect a combination of the two different enantiomers (Figure 1). When the enantiomer CL_{int,app} values are different, the resulting racemate plot will be biphasic. The initial slope (k_e) is used to determine the CL_{int,app} (where CL_{int,app} = $-k_e$ /protein or cell concentration).

The MSD metabolic stability database was interrogated to find compounds (irrespective of chirality) that had previously been tested more than once. This data (Table 1) was used as a baseline to define assay variability. Over 400 compounds had been repeat tested, with 88–95% showing CL_{int,app} values within 3-fold. This level of reproducibility (2–3-fold) is consistent with both in-house (data not shown) and literature³ values for positive control compounds that are run in each assay.

To understand the risk of misclassification of CL_{int,app}, [i.e., racemate CL_{int,app} (R) > 3-fold higher than the lower CL_{int,app}

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Figure 1. Simulated $\ln(\% \text{ remaining})$ vs time plots for enantiomer 1 (E1, squares), enantiomer 2 (E2, triangles) ($\text{CLint}_{\text{app}}$ of 100 and 1000 $\mu\text{L}/\text{min}/\text{mg}$ microsomal protein, respectively), and corresponding racemate (diamonds) in a 0.25 mg/mL microsomal metabolic stability assay.

Table 1. Replicate $\text{CLint}_{\text{app}}$ Data for Identical Compounds Tested at Least Twice in MSD Metabolic Stability Assays

assay	<i>n</i>	WH rat			<i>n</i>	human		
		>3-fold (%)	>10-fold (%)	fold error		>3-fold (%)	>10-fold (%)	fold error
Heps	471	7	1.5	2.23	594	5	0.2	1.65
Mics	427	12	0.9	1.96	449	10	0.7	1.93

enantiomer (LCE)], we performed simulations of the ratio of the racemate $\text{CLint}_{\text{app}}$ (R) to that of each enantiomer across a wide range of E1/E2 $\text{CLint}_{\text{app}}$ values and ratios. These simulations (not shown) revealed that the $\text{CLint}_{\text{app}}$ value for the more stable enantiomer tended to have greater differences from the racemate values, compared to the enantiomer with higher $\text{CLint}_{\text{app}}$ (i.e., R/LCE [lower $\text{CLint}_{\text{app}}$ enantiomer] > R/HCE [higher $\text{CLint}_{\text{app}}$ enantiomer]). Subsequent simulations focused on the R/LCE $\text{CLint}_{\text{app}}$ ratio for different E1/E2 $\text{CLint}_{\text{app}}$ scenarios.

Figure 2 shows that when the E1/E2 $\text{CLint}_{\text{app}}$ ratio is high (e.g., 30- or 100-fold), the measured racemate $\text{CLint}_{\text{app}}$ would overestimate the lower enantiomer $\text{CLint}_{\text{app}}$ significantly (R/LCE $\text{CLint}_{\text{app}}$ ratio = 12–15 and 42–45 respectively). The plot also shows that when E1/E2 $\text{CLint}_{\text{app}}$ = 10, the R/LCE ratio

Figure 2. Simulated plots: ratio of the racemate CLint to that of the more stable enantiomer (R/LCE) versus the ratio of CLint values for the enantiomers (E1/E2).

can vary widely. To understand this further, we performed additional simulations for E1/E2 $\text{CLint}_{\text{app}}$ ratios of 3 and 10. For example, for E1/E2 $\text{CLint}_{\text{app}}$ ratio = 10, we simulated racemate data for enantiomer pairs having $\text{CLint}_{\text{app}}$ values ranging from 1 and 10 to 1000 and 10000 $\mu\text{L}/\text{min}/\text{mg}$. The results (Figure 3) show that for E1/E2 $\text{CLint}_{\text{app}}$ ratios of 3, the

Figure 3. Simulated R/LCE plotted against racemate $\text{CLint}_{\text{app}}$ for E1/E2 ratios of 3 (squares) and 10 (circles).

R/LCE would be 1.5–2.5. However, when the E1/E2 ratio was 10, at low racemate $\text{CLint}_{\text{app}}$, the R/LCE $\text{CLint}_{\text{app}}$ ratio would be >5, but this decreased to 1 as measured racemate $\text{CLint}_{\text{app}}$ increased.

These simulations were performed assuming MSD in-house microsomal stability screening conditions with initial time-points of 0 and 5 min. To test the hypothesis that these time-points were impacting the data interpretation, we also simulated 0 and 1 min initial time-points for enantiomers with $\text{CLint}_{\text{app}}$ of 300 and 3000 $\mu\text{L}/\text{min}/\text{mg}$ (E1/E2 $\text{CLint}_{\text{app}}$ ratio = 10). The simulated racemate $\text{CLint}_{\text{app}}$ was 577 $\mu\text{L}/\text{min}/\text{mg}$ with 0 and 5 min compared to 1232 $\mu\text{L}/\text{min}/\text{mg}$ with 0 and 1 min, resulting in R/LCE $\text{CLint}_{\text{app}}$ ratios of 2 and 4, respectively. Simulating with earlier time-points allows a more accurate determination of the initial slope resulting in a higher $\text{CLint}_{\text{app}}$. Under standard experimental conditions (e.g., 0 and 5 min time-point), overestimating lower enantiomer $\text{CLint}_{\text{app}}$ using racemates may therefore be less likely.

Chiral inversion and racemization are not usually considered during metabolic stability screening since the analytical methods usually employed do not resolve enantiomers. Chiral inversion, the process whereby one enantiomer converts to the other, through either enzymatic (e.g., *R*-ibuprofen^{4,5}) or chemical (e.g., clopidogrel⁶) means, provides additional complexity when interpreting racemate data. If one enantiomer inverts, then the other enantiomer will have both formation and clearance rates. Under this scenario, initial slopes in theoretical models may be misleading, as shown in Table 2. In this example, racemate $\text{CLint}_{\text{app}}$ provides a good approximation of actual $\text{CLint}_{\text{app}}$ for both the enantiomers until there is a 10-fold difference in the sum of the CLint pathways, as also seen when chiral inversion does not occur.

The simulations demonstrated that if E1/E2 $\text{CLint}_{\text{app}}$ ratios are >10, then racemate $\text{CLint}_{\text{app}}$ data may significantly overestimate the lower $\text{CLint}_{\text{app}}$. However, for E1/E2 $\text{CLint}_{\text{app}}$ ratios of ≤ 10 , using our standard experimental time-points, racemate $\text{CLint}_{\text{app}}$ data will be an acceptable surrogate for the individual enantiomers. Exceptions would occur where E1/E2 $\text{CLint}_{\text{app}}$ ratio = 10 with a low racemate $\text{CLint}_{\text{app}}$ (e.g., < 100

Table 2. Simulation of the Effect of Chiral Inversion on Observed CLInt_{app}^a

sum of E1 CLInt _{app}	E2 CLInt _{app} (initial slope)	racemate CLInt _{app}	R/LCE (initial slope)	R/LCE (actual; E2 CLInt _{app} = 100)
101	10.9	53.5	4.9	0.5
110	11.8	58	4.9	0.6
200	20.1	100	5.0	1.0
1100	65	313	4.8	3.1

^aCLInt_{app} values were fixed to 100 for both E1 to E2 (chiral inversion) and E2 metabolism. Noninversion metabolism CLInt_{app} for E1 was varied from 1 to 1000.

μL/min/mg). However, these compounds are unlikely to be deprioritized from progression to further assays if low CLInt_{app} is the goal. Based on this, we subsequently explored whether E1/E2 CLInt_{app} values >10 are indeed likely.

To explore actual enantiomeric differences in CLInt_{app}, literature data were evaluated for compounds with known stereoselective metabolism. The overall E1/E2 CLInt_{app} ratios, based on either substrate depletion or the sum of all metabolite formation CLInt_{app} values, were found to be <3 for each compound (warfarin⁷ = 1.3, verapamil⁸ < 2, metoprolol⁹ < 1.2, and omeprazole¹⁰ = 2.9), despite some marked differences in the CLInt_{app} for formation of individual metabolites.

A retrospective analysis of MSD screening data was also performed for matched pairs of enantiomers (*n* = 750; randomly assigned as E1 or E2) with CLInt_{app} values determined in rat and human microsomes and/or hepatocytes. Briefly, experimental conditions were as follows: 0.3 μM substrate was incubated with 0.25 mg/mL pooled human liver microsomes (initiated with the addition of 1 mM NADPH) or 1 million cells/mL cryopreserved pooled human hepatocytes. Loss of parent compound was evaluated by LC–MS/MS after quenching with acetonitrile containing internal standard, protein precipitation, and centrifugation of time-point samples. Each compound had a single replicate incubation for these screening assays. Strict criteria were used to select enantiomer pairs requiring each isomer structure to be fully stereochemically defined and annotated as a pure single isomer by the chemist. Compounds came from many programs and represented broad small molecule chemical space. As shown in Figure 4, the CLInt_{app} values for the enantiomeric pairs generally fell within 3-fold of each other with 10-fold differences being rare (<1% of CLInt_{app} values were >10-fold different). The enantiomeric pair CLInt_{app} data is remarkably similar to the assay reproducibility data for identical compounds summarized in Table 1 (>10-fold CLInt_{app} differences of 0.3–0.9% for enantiomers and 0.2–1.5% for the same compound in replicate assays). This suggests that enantiomeric differences do not generally appear to be larger than would be expected from overall assay variability.

Interestingly, compounds that had >10-fold enantiomeric CLInt_{app} differences in one assay type did not consistently show this effect across species or matrices. It was also noted that similar overall data were obtained in both NADPH-dependent microsomal and hepatocyte stability assays, suggesting concordance for both cytochrome P450 (CYP)- and non-CYP-mediated metabolism pathways (although this was not specifically evaluated in this study).

Further analysis of the same MSD database showed that, of these 750 enantiomer pairs, approximately 100 had also been tested in metabolic stability assays as racemates. Figure 5

Figure 4. Retrospective analysis of MSD database comparing CLInt_{app} values for matched pairs of enantiomers in Wistar Han rat and human liver microsomal (a and c, μL/min/mg) and hepatocyte stability assays (b and d, μL/min/10⁶ cells). Lines are unity (solid) and 3-fold (dashed) and 10-fold from unity (dotted).

illustrates that, for these compounds, the CLInt_{app} ratio of the racemate to LCE was generally within 2-fold, which was in agreement with the simulation in Figure 3 (i.e., E1/E2 CLInt_{app} ratio ≤ 3 leads to racemate/LCE CLInt_{app} ratio ≤ 2).

Submission of compounds for metabolic stability assays is often dependent on primary potency criteria. Consequently, if both enantiomers of a compound were tested for metabolic

Figure 5. Histogram showing frequency of measured $CL_{int,app}$ ratios for racemic mixtures compared to the lower $CL_{int,app}$ enantiomer (LCE) from MSD database.

stability, it is likely that they were both active and potentially adopt a similar three-dimensional shape in their target as well as metabolic enzymes. To ensure against bias in the retrospective analysis, pairs of enantiomers with largely different potency and predicted three-dimensional shapes were prospectively selected for measurement of $CL_{int,app}$. Criteria for compound selection were as follows: eudysmic ratio > 100 in any potency assay and low 3D overlay score (<1.5 maximal TanimotoCombo score using ROCS¹¹ after generation of up to 200 conformers of each enantiomer with OMEGA¹²). The resulting selection was further refined based on a range of predicted $CL_{int,app}$ (in-house QSAR model) and structural diversity. $CL_{int,app}$ data was generated in rat and human liver microsomal stability assays for the 10 resulting enantiomer pairs. For this “extreme” compound set, enantiomeric differences in $CL_{int,app}$ were >3-fold for only one pair of enantiomers in human liver microsomes and three pairs in rat liver microsomes. In both rat and human liver microsomes, a single pair showed enantiomeric $CL_{int,app}$ differences >10-fold (data not shown).

In conclusion, using a variety of approaches, the risk of misinforming project teams through generation of metabolic stability data on racemic mixtures is low. Through theoretical considerations, enantiomeric $CL_{int,app}$ differences of >10-fold may cause concern. However, from literature evaluation, retrospective analysis and prospective studies with isomers of high eudysmic ratio and dissimilar shape, such large differences in $CL_{int,app}$ for enantiomers, seems to be a relatively rare event. With the improving throughput of chiral separation technologies¹³ as well as advances in chiral chemistry, program teams may only need to consider testing racemic mixtures for nonroutine workflows. Additionally, teams would need to assess the overall impact of timelines by adding an additional step for measuring racemate $CL_{int,app}$ as well as the subsequent testing of the separate enantiomers. However, understanding the risks should enable pragmatic screening study design decisions when warranted. These results also highlight that physicochemical properties are an important contributor to *in vitro* metabolic stability,¹⁴ as demonstrated by similar stabilities for enantiomers prospectively selected to be as biologically differentiated as possible.

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Notes

The authors declare the following competing financial interest(s): All authors are all employees of Merck Co., Inc.

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ABBREVIATIONS

$CL_{int,app}$, apparent intrinsic clearance; CYP, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; E1, E2, enantiomers 1 and 2; Heps, hepatocytes; LC–MS/MS, liquid chromatography with triple quadrupole mass spectrometry; Mics, microsomes; MSD, Merck Sharp & Dohme, a subsidiary of Merck & Co., Inc.; NADPH, nicotinamide adenine dinucleotide phosphate; R/LCE, racemate $CL_{int,app}$ divided by $CL_{int,app}$ of the most stable enantiomer (“lower $CL_{int,app}$ enantiomer”); R/HCE, racemate $CL_{int,app}$ divided by $CL_{int,app}$ of the least stable enantiomer (“higher $CL_{int,app}$ enantiomer”); QSAR, quantitative structure–activity relationship; WH rat, Wistar Han rat; 3D, three-dimensional

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