PREDICTION OF IN VITRO INTRINSIC CLEARANCE FROM HEPATOCYTES:
COMPARISON OF SUSPENSIONS AND MONOLAYER CULTURES

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ABSTRACT:

Due to the time-dependent loss of cytochrome P450 (P450)-mediated metabolism in freshly isolated hepatocytes, several types of culture systems have been developed to extend their lifespan. The aim of this study was to evaluate the ability of monolayer cultures of rat hepatocytes to determine the in vitro CL\textsubscript{int} compared with suspensions of freshly isolated hepatocytes. Seven compounds were incubated in rat hepatocyte suspensions and monolayer cultures, and in vitro CL\textsubscript{int} was obtained via metabolite formation (12 pathways) or substrate depletion approaches. Only two compounds (tolbutamide and 7-ethoxycoumarin) gave comparable (within 2-fold) in vitro CL\textsubscript{int} in both suspensions and monolayer cultures. Although the overall rank order of compounds was the same in both models (covering a range of 3-4 orders of magnitude), the prediction of in vitro CL\textsubscript{int} for high-turnover compounds (seven pathways) was lower for monolayer cultures compared with suspensions, probably due to an uptake rate limitation leading to increases in K\textsubscript{m}. In general, there was an average 50% loss of the P450 activity in monolayers based on a decrease in V\textsubscript{max} relative to suspensions. However, monolayer cultures gave a higher estimation of in vitro CL\textsubscript{int} for the low-turnover compound S-warfarin compared with fresh cell suspensions due to a decrease in the K\textsubscript{m} of the four individual metabolites. The use of hepatocyte monolayer cultures may offer the potential advantage of extending the lower end of the usable clearance range (below 0.1 µl/min/10\textsuperscript{6} cells) for predicting in vivo CL\textsubscript{int}.

The use of freshly isolated hepatocytes as an in vitro system for the prediction of in vivo clearance is widely accepted because it represents a more realistic physiological model than some other liver preparations, e.g., hepatic microsomes (Houston and Carlile, 1997; Ito and Houston, 2004). Hepatocytes possess the full complement of drug-metabolizing enzymes, do not rely on artificially high concentrations of cofactors, and express cell membrane receptors. An additional advantage of hepatocytes in suspension is the rapid dispersion of compound throughout the incubation, which aids rapid distribution and depletion methods seems to be drug-dependent (Griffin and Houston, 2004).

Hepatocyte models with a lifespan of days rather than hours would be advantageous, and to this end, several approaches to long-term hepatocyte culture have been investigated in recent years. Placing cells in culture allows them to recover from the trauma of isolation and prolongs the maintenance of liver-specific function in an environment that more closely resembles the in vivo situation. However, a major factor in the limited success of cultures as a model for prediction studies is the relatively rapid loss of drug-metabolizing enzymes. Hepatocytes can lose up to 80% of their drug-metabolizing (P450) capacity within the first 24 h of culture (Bissell, 1976), and it is thought that this is due to a degradation of the enzymes by nitric oxide (López-García, 1998) and a failure to synthesize new enzymes (Paine, 1990) by a loss of the transcription of liver-specific genes. Some phase II metabolism is also decreased, but there is a general consensus that, compared with fresh suspensions, it is well preserved in monolayer cultures (Rogiers and Verret, 1993). The use of monolayer cultures on collagen, sandwich cultures, and immobilization cultures (Dunn et al., 1989; Koebel et al., 1994; Lavé et al., 1997) can maintain liver-specific function to differing extents. However, none of these systems can completely restore liver-specific function to levels seen in vivo but simply slow the rate of decline.

Before the utility of long-term cultured hepatocytes for the estimation of kinetic parameters and hence the prediction of in vitro CL\textsubscript{int} can be established, it is important to assess the impact of using a

ABBREVIATIONS: P450, cytochrome P450; TOL, tolbutamide; WAR, 8-hydroxywarfarin; PHE, phenytoin; 7-EC, 7-ethoxycoumarin; DZ, diazepam; DEX, dextromethorphan; PROP, propranolol; HTOL, hydroxytolbutamide; MEM, methoxymorphan; DOR, dextrophan; FCS, fetal calf serum; HMM, hepatocyte maintenance media; NDZ, nordiazepam.
monolayer of cells rather than a suspension. Because the monolayer configuration results in a relatively static system, the kinetics of metabolism may not reflect those seen within a thoroughly mixed suspension of hepatocytes. A study by Lavé et al. (1997) demonstrated the use of monolayer cultures of differing species to predict human CLint, but did not directly compare the data with fresh suspensions. Lau et al. (2002) compared fresh suspensions and sandwich cultures of hepatocytes from various species using a high-throughput approach to calculate clearance based on drug disappearance from a single 2-h time point. Using this high-throughput screen to estimate in vitro CLint resulted in a significant correlation (0.71) between fresh and sandwich cultured rat hepatocytes; however, there was no analysis of the kinetics of metabolites formed. To allow a comprehensive evaluation of these two modes (static and dynamic) for hepatocyte incubation, the kinetics of metabolite formation of a range of prototypic drug substrates is required to provide comprehensive coverage of the full range of enzymes and pathways of metabolism.

The aim of the current study was to compare the ability of rat hepatocytes in monolayer culture and suspensions to provide kinetic parameters for CLint determination. A simple monolayer culture was selected as the mode of cellular incubation, and standard methods for CLint determination. Incubations of the full range of enzymes and pathways of metabolism.

### Materials and Methods

**Chemicals.** DZ and 4'-OH DZ were gifts from Roche Products Ltd. (Welwyn Garden City, Herts, UK). WAR, TZ, and HTOL were gifts from Aventis (Cambridge, UK), Wyeth Laboratories (Maidenhead, Berks, UK), and Celltech (Cambridge, UK). DEX, MEM, and DOR were purchased from Sigma Chemical (Poole, Dorset, UK) or BDH (Lutterworth, Leicester, UK) and were of the highest grade available.

**Isolation of Hepatocytes.** Rat hepatocytes were prepared by the collagenase perfusion method (Berry and Friend, 1969) as described in Griffin and Houston (2004). Only cells with viability greater than 85% as assessed by trypan blue exclusion were used, and dilutions to appropriate cell densities were based on viable cells only. Hepatocytes from the same preparation were used to prepare suspensions and monolayer cultures to allow direct comparisons to be made.

**Incubation of Hepatocytes in Suspensions.** After isolation, for suspension incubations, the cell suspension was diluted to the relevant cell density in Williams’ E media containing 0.03% bovine serum albumin and incubated as described in Griffin and Houston (2004). Incubations were performed in Eppendorf tubes in a Thermomixer (Eppendorf AG, Hamburg, Germany) set at 37°C and 900 rpm. Incubations (volume, 1 ml) were initiated by the addition of 5 µl of substrate in dimethyl formamide [final solvent concentration, 0.5% (v/v)] after 5 min of preincubation of the cell suspension and terminated by snap freezing in liquid nitrogen. The details of optimized incubation time, cell density, and substrate concentration are given in Table 1. The kinetic parameters for hepatocyte suspensions have been previously reported (Griffin and Houston, 2004).

**Incubation of Hepatocytes in Monolayer Cultures.** For monolayer cultures, the final washing and resuspension step of hepatic cell isolation was performed with HMM containing 5% FCS, 0.01 µM dexamethasone, 0.01 µM insulin, 10 units/ml penicillin, and 10 µg/ml streptomycin. Cells were diluted to 0.3 × 10^6 cells/ml, and 0.25 ml was dispensed into each well of a 48-well collagen-coated plate (Becton Dickinson UK Ltd., Oxford, UK), giving a cell density of 7.5 × 10^4 cells/well. Cells were allowed to attach for 2 h at 37°C and 5% CO2 in an incubator (SANYO Gallenkamp PLC, Leicester, UK), after which the extent of attachment was observed under a microscope. The level of attachment was high, and the unattached cells were removed by replacing the media with 200 µl of fresh prewarmed HMM (with supplements as described above). The plates were returned to the incubator for 10 min before starting incubations. Incubations were started by the addition of 200 µl of prewarmed HMM containing 2 µl of substrate in dimethyl formamide [final solvent concentration, 0.5% (v/v)] to give final concentrations in incubations as detailed in Table 1. Monolayer cultures were not shaken or stirred during the incubation to reduce any detachment of hepatocytes from collagen. Linearity studies were performed with respect to incubation time and optimal conditions for metabolite formation selected, as also detailed in Table 1. Incubations were terminated by the removal of 350 µl of media from the well. All incubations were performed in duplicate, and samples were stored at −80°C until analysis.

During optimization experiments, hepatocytes were removed from collagen by scraping with a spatula after the addition of 100 µl of methanol/water (50:50 v/v), which also lysed the hepatocytes. The level of metabolites and parent in hepatocytes was assessed by high-performance liquid chromatography.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>Substrate Concentration µM</th>
<th>Suspensions</th>
<th>Monolayer Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incubation Time</td>
<td>Cell Density</td>
</tr>
<tr>
<td>TOl</td>
<td>HTOL</td>
<td>100–1500</td>
<td>min</td>
<td>10^6 cells/ml</td>
</tr>
<tr>
<td>7-EC</td>
<td>7-HC</td>
<td>0.05–200</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>PHE</td>
<td>4-OH PHE</td>
<td>0.1–100</td>
<td>15</td>
<td>0.5</td>
</tr>
<tr>
<td>DZ</td>
<td>4'-OH DZ</td>
<td>2.5–250</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>DEX</td>
<td>DOR</td>
<td>1–500</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>MEM</td>
<td>TZ</td>
<td></td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>WAR</td>
<td>4-OH WAR</td>
<td>25–1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-OH WAR</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>8-OH WAR</td>
<td></td>
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<tr>
<td></td>
<td>10-OH WAR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROP</td>
<td>—</td>
<td></td>
<td>5</td>
<td>Up to 60</td>
</tr>
</tbody>
</table>

Note: PROP CLint determined by the substrate depletion method.
Sample Hydrolysis, Extraction, and Analysis. Samples for 7-EC, PHE, NDZ, DZ, DEX, and WAR were hydrolyzed with β-glucuronidase as described previously (Griffin and Houston, 2004) to release primary metabolites quantified according to the degree of metabolism found to be insignificant (<1% of level in media).

Data Analysis. All data were analyzed using GraFit 4.0 software (Erithacus Software Ltd., Horley, Surrey, UK). The kinetic model selected for data fitting was based on visual inspection of the data on Eadie-Hofstee or Hanes plots. For TOL, DZ, and WAR metabolites and DOR formation in monolayer cultures, the Michaelis-Menten equation was used, and \( \text{CL}_{\text{int}} \) was estimated from the kinetic parameters \( V_{\text{max}} \) and \( K_{\text{M}} \) or \( S_{50} \) for the high-affinity site only (Ashforth et al., 1995; Carlile et al., 1998; Carlile, 1994; Witherow and Houston, 1999). Where more than one metabolite was measured, the individual \( \text{CL}_{\text{int}} \) values were summed to give total \( \text{CL}_{\text{int}} \). Analysis of PROP was performed assuming monoexponential depletions, where \( \text{CL}_{\text{int}} \) was calculated from the dose to area under the curve ratio.

Results

In vitro \( \text{CL}_{\text{int}} \) was estimated for seven commonly used probe substrates (13 pathways of metabolism) in freshly isolated suspensions, and monolayer cultures of rat hepatocytes are summarized in Table 2. For all compounds with the exception of PROP, in vitro \( \text{CL}_{\text{int}} \) was estimated from the kinetic parameters \( V_{\text{max}} \) and \( K_{\text{M}} \) or \( S_{50} \) for the major metabolites. Due to the number of metabolites formed, the depletions approach was used for PROP, where the disappearance of substrate over time was monitored (10 time points) and the in vitro \( \text{CL}_{\text{int}} \) estimated from the area under the depletion curve. The in vitro \( \text{CL}_{\text{int}} \) values estimated from both hepatocyte models cover a wide range of 3 to 4 orders of magnitude from 0.1 \( \mu\text{M}/10^6 \text{ cells} \).

As demonstrated in a previous report using hepatocyte suspensions (Griffin and Houston, 2004), metabolite formation for these substrates exhibited a range of kinetic profiles, including typical Michaelis-Menten kinetics (HTOL, DZ, DEX, WAR), two-site kinetics (7-EC and 4-OH PHE), and sigmoidal kinetics (DEX metabolites DOR and MEM). In most cases, the same type of kinetics was observed in monolayer culture. The only exception to this was the absence of sigmoidicity for DOR formation in monolayers. This change in kinetics was also evident in cryopreserved hepatocytes (Griffin and Houston, 2004). Some changes in the contribution of each metabolite to total metabolism were also observed. For example, in fresh suspensions both metabolites of DEX metabolism, MEM and DOR, had equal contributions, whereas in monolayer cultures DOR became the dominant pathway. Interestingly, the latter observation was similar to that reported in hepatic microsomes, where DOR contributes to approximately 95% of total metabolism (Witherow and Houston, 1999). However, in monolayer cultures, the level of metabolism may not be sufficiently high for this difference to be significant.

Figure 1 compares the in vitro \( \text{CL}_{\text{int}} \) values determined for the individual metabolites of the compounds in suspensions and monolayer cultures. The metabolites are coded according to the degree of metabolism was found to be insignificant (<1% of level in media).
Fig. 1. In vitro CL_{int} values for individual metabolites in suspensions and monolayer cultures of rat hepatocytes. The metabolites represented are HTOL (1), 7-HC (2), 4-OH PHE (3), 4'-OH DZ (4a), NDZ (4b), TZ (4c), DOR (5a), MEM (5b), 4-OH WAR (6a), 6-OH WAR (6b), 7-OH WAR (6c), 8-OH WAR (6d), and PROP (7). In the case of MEM in both models and DOR in suspensions, the values are CL_{max}. Metabolites for compounds giving a good estimation of in vitro CL_{int} in monolayer cultures are shown by open circles, those showing an underprediction are shown by closed squares, and those showing an overprediction are shown by closed triangles. The solid line is a line of unity, and the dashed line represents the average one-third decrease by monolayers relative to suspensions for data above 50 μM, and the dotted line represents the average one-third decrease by monolayers relative to suspensions for data above 50 μM. Data represent mean ± S.D. of at least three determinations. Parameter values for suspensions of hepatocytes are taken from Griffin and Houston (2004).

Fig. 2. V_{max} estimates for individual metabolites in suspensions and monolayer cultures of rat hepatocytes. The metabolites represented are HTOL (1), 7-HC (2), 4-OH PHE (3), 4'-OH DZ (4a), NDZ (4b), TZ (4c), DOR (5a), MEM (5b), 4-OH WAR (6a), 6-OH WAR (6b), 7-OH WAR (6c), 8-OH WAR (6d), and 10-OH WAR (6e). The symbols used are the same as in Fig. 1. The solid line is a line of unity, and the dashed line represents the average decrease of 50% shown by monolayers relative to suspensions. Data are represented as mean ± S.D. of at least three determinations. Parameter values for suspensions of hepatocytes are taken from Griffin and Houston (2004).

Fig. 3. K_{M} estimates for individual metabolites in suspensions and monolayer cultures of rat hepatocytes. The metabolites represented are HTOL (1), 7-HC (2), 4-OH PHE (3), 4'-OH DZ (4a), NDZ (4b), TZ (4c), DOR (5a), MEM (5b), 4-OH WAR (6a), 6-OH WAR (6b), 7-OH WAR (6c), and 8-OH WAR (6d). In the case of MEM in both models and DOR in suspensions, the values are S_{50}. Symbols used are the same as in Fig. 1. The solid line is a line of unity, the dashed line represents the average increase of 5-fold shown by monolayers relative to suspensions for data below 50 μM, and the dotted line represents the average one-third decrease by monolayers relative to suspensions for data above 50 μM. Data represent mean ± S.D. of at least three determinations. Parameter values for suspensions of hepatocytes are taken from Griffin and Houston (2004).

Discussion

Although freshly isolated hepatocytes are presently regarded as the most superior in vitro model for prediction studies, they lose viability and drug-metabolizing capacity within a few hours of isolation (Skett, 1994). Several types of culture systems have been developed to extend the lifespan of isolated hepatocytes and potentially allow a more accurate assessment of low-clearance drugs. These include monolayer cultures on collagen, sandwich cultures, and immobilization cultures (Dunn et al., 1989; Koebe et al., 1994; Laveé et al., 1997), all of which can maintain liver-specific function to differing extents. However, none of these systems can completely restore liver-specific function to the levels seen in vivo but simply slow the rate of decline. These culture systems are widely used for induction studies, but there is not much literature on the use of these models for estimation of kinetic
parameters and hence prediction of $CL_{int}$. The use of hepatocytes in suspension results in a rapid dispersion of compound throughout the incubation, which aids rapid distribution of the drug to metabolizing enzymes and facilitates sampling. In contrast, the use of the static monolayer configuration of hepatocytes may restrict dispersion and influence kinetic parameters. Therefore, the aim of the current study was to evaluate the ability of monolayer cultures of rat hepatocytes to predict in vitro $CL_{int}$ compared with suspensions of freshly isolated hepatocytes.

In the present study, the in vitro $CL_{int}$ of seven commonly used probe substrates—TOL, WAR, PHE, 7-EC, DZ, DEX, and PROP—was determined in suspensions and monolayer cultures of rat hepatocytes using either metabolite formation (12 pathways) or substrate depletion (PROP) methods. The in vitro $CL_{int}$ values determined for the compounds in freshly isolated rat hepatocytes were reported previously (Griffin and Houston, 2004) and are generally in agreement with others in the literature (Table 2). The exceptions to this were the in vitro $CL_{int}$ values determined for PHE and TOL, which in the present study are lower than those of Ashforth et al. (1995). This was due to increased sensitivity of the analytical methods allowing lower substrate concentrations to be studied and/or inclusion of a larger number of substrate concentrations, which improves the accuracy of the prediction upon modeling of the data. The lower $CL_{int}$ value for PROP can be attributed to the high substrate concentration used to improve analytical sensitivity. Hepatocyte suspensions have been shown to be good predictors of in vivo $CL_{int}$ (Houston and Carlile, 1997); therefore, it can be assumed that these in vitro $CL_{int}$ values will give good estimates of in vivo $CL_{int}$ and hepatic clearance. The results show that monolayer cultures gave comparable (within 2-fold) in vitro $CL_{int}$ for oxidation of 7-EC and TOL compared with suspensions of freshly isolated rat hepatocytes. For the other 11 pathways of metabolism studied, $CL_{int}$ is either lower (for high $CL_{int}$ drugs) or higher (for low $CL_{int}$ drugs) in monolayers than in suspensions, but overall, the same rank order is observed.

On average, there is a 50% decrease in $V_{max}$ values obtained in monolayers relative to suspensions. Vernia et al. (2001a) and Donato et al. (2001) have demonstrated that there is a P450-dependent decrease in the overall P450-mediated metabolism within a culture system. However, the present studies show no definite correlation between the changes in in vitro $CL_{int}$ in monolayer cultures and the specific P450 enzymes, with the exception of CYP3A-mediated metabolism (10-OH WAR, TZ, DOR, and MEM), which seems to show a consistent decrease in monolayer cultures. However, the fact that the in vitro $CL_{int}$ of some compounds can be estimated accurately excludes the possibility that the culture conditions employed in this study do not support liver-specific function. Conditions were optimized with respect to incubation time and cell density to allow enough time for detectable metabolism while limiting any loss of enzyme activity. The consistent decrease in $V_{max}$ values indicates an initial loss of 50% in enzyme activity (relative to suspensions), probably due to the attachment time (2 h) necessary with the monolayer system prior to the kinetic experiment.

7-EC and TOL were the only compounds to give similar in vitro $CL_{int}$ in both suspensions and monolayers. Only in the former case are both $V_{max}$ and $K_M$ similar for both systems (both parameters decrease for TOL). Monolayer cultures gave a higher estimation of in vitro $CL_{int}$ compared with suspensions for WAR, largely as a result of decreased $K_M$ values. Lower in vitro $CL_{int}$ values were observed for PHE, DZ, PROP, and DEX in monolayer cultures either as a result of a decreased $V_{max}$ and/or an increased $K_M$ ($S_{0.5}$). It is of interest to speculate that a “maximum” monolayer in vitro $CL_{int}$ operates, a phenomenon that has been observed in liver slices (Worboys et al., 1997) and ascribed to drug uptake limiting enzyme accessibility. The in vitro $CL_{int}$ values in monolayer cultures for high-turnover compounds (DZ, DEX, PROP, and PHE) may also be rate-limited by drug accessibility. Hepatocytes in a monolayer culture are in a static environment and have a relatively small surface area for diffusion in contrast to suspensions, where the entire surface area is exposed to drug. For high-turnover compounds, an uptake rate limitation may apply, and a concentration gradient between the cells and surrounding media may be generated, which in turn may affect the $K_M$.

Other explanations for the discrepancies in clearance values from suspensions and monolayer cultures maybe related to transporters and active uptake/efflux from hepatocytes. Initial studies demonstrated that there was no accumulation of compound or metabolites in the hepatocytes after culture, suggesting that there is no active uptake. However, there is a possibility that these compounds make be substrates for efflux transporters, which remove the compound from the cells before metabolism can occur and hence decrease clearance. In suspensions, efflux transporters are not retained due to the loss of cell polarity and redistribution of canalicular membranes (Groothuis and Meijer, 1996); therefore, the compound remains in the cell, leading to higher metabolism.

Lavé et al. (1997) performed a study with 10 compounds in monolayer cultures of hepatocytes from different species, including rats. The monolayer culture conditions were very similar to those employed in this study, and although the purpose of this study was to predict clearance in humans by allometric scaling, some of the data generated could be used to confirm the observations. The in vitro data represented a range of in vitro $CL_{int}$ values giving a consistent underprediction of in vivo $CL_{int}$, and the magnitude of the effect depended on in vitro $CL_{int}$.

Although a drug uptake/enzyme accessibility limitation (including the possibility of transporter involvement) may explain the lower estimation of in vitro $CL_{int}$ for high-turnover compounds in monolayer cultures, it does not explain the increased metabolism of WAR, a low-turnover compound. One possible explanation for this latter effect may be linked to the need for longer incubation times for low-turnover compounds to obtain a reasonable level of metabolism. After the initial rapid decline, as culture time increases the liver-specific functions may become stabilized (Vernia et al., 2001b) and hence produce a higher level of metabolism in culture than in suspension. An imbalance between phase I and II metabolism may also contribute, as highlighted by Richert et al. (2002), who demonstrated a higher level of preservation of phase II activity compared with phase I in cultures. Other studies have shown variable results in terms of phase II enzyme preservation, but there is a general consensus that they are better maintained than phase I enzymes (Rogiers and Vercauyse, 1993). It is possible that phase II enzymes may be preserved better in monolayer cultures compared with suspensions; thus, phase I metabolites do not accumulate (and cause a feedback inhibition effect) due to their rapid conversion to glucuronides and other phase II metabolites.

In conclusion, monolayer cultures give a good estimate of in vitro $CL_{int}$ for 7-EC and TOL. The in vitro $CL_{int}$ for high-turnover compounds is lower in monolayer cultures compared with suspensions. This may be explained by an uptake rate limitation (within a static system with a relatively small surface area for diffusion) leading to increases in $K_M$ values and a reduced ability of the P450 enzymes to metabolize, resulting in a decrease in $V_{max}$. In contrast, monolayer cultures give a higher in vitro $CL_{int}$ for the low-turnover compound WAR compared with fresh suspensions. This is due to a decrease in the $K_M$ of the individual metabolites and may be as a result of the increased stability of enzymes in monolayer cultures compared with
suspensions. The use of the in vitro data generated from monolayer cultures for low-turnover compounds (<0.1 μl/min/10⁶ cells) may provide a more accurate prediction of in vivo CLint than suspensions and hence extend the usable range of in vitro CLint values. However, further work is required with a range of low-clearance compounds to confirm the broader applicability of this observation.

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References

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