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Development of a cell viability assay to assess drug metabolite structure–toxicity relationships

Payal Rana [a], Yvonne Will [a], Sashi Nadanaciva [b,†], Lyn H. Jones [c,*]

[a] Drug Safety Research & Development, Pfizer, Eastern Point Road, Groton, CT 06340, USA
[b] Compound Safety Prediction, Pfizer, Eastern Point Road, Groton, CT 06340, USA
[c] Medicine Design, Pfizer, 610 Main St., Cambridge, MA 02139, USA

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ABSTRACT

Many adverse drug reactions are caused by the cytochrome P450 (CYP)-dependent activation of drugs into reactive metabolites. In order to reduce attrition due to metabolism-induced toxicity and to improve the safety of drug candidates, we developed a simple cell viability assay by combining a bioactivation system (human CYP3A4, CYP2D6 and CYP2C9) with Hep3B cells. We screened a series of drugs to explore structural motifs that may be responsible for CYP450-dependent activation caused by reactive metabolite formation, which highlighted specific liabilities regarding certain phenols and anilines.

The high rate of clinical candidate attrition and the immense cost of development have urged the pharmaceutical industry to embed drug safety research and screening as early as possible in the discovery process. At Pfizer, we have introduced in silico predictive tools and cell-based screens (including general cytotoxicity, apoptosis and mitochondrial toxicity assays), to ensure potential safety liabilities are understood before the progression of compounds into animal exploratory toxicity studies.[1–3]

Drug-induced hepatotoxicity is a serious concern during drug development[4] and one of the mechanisms suggested for this liability is related to reactive metabolites produced by drug-metabolizing enzymes such as cytochrome P450.[5] As a result of drug metabolites reacting with intracellular proteins, cellular toxicity pathways are triggered, causing cell swelling, a decline in ATP levels, actin disruption, and apoptosis.[6,7] There are several reported examples of drug reactive metabolites leading to adverse drug reactions (ADRs). Some of these ADRs were so serious that drug treatment had to be limited and in some cases the drug was withdrawn from the market.[8,9] As a result, pharmaceutical companies are making significant efforts to predict ADRs and several tools and strategies are considered to address the formation of reactive metabolites and the potential consequences for the overall safety profile of candidate drugs.[10,11]

In the present study, we describe the modification and development of a cell-based assay system based on the work of Vignati et al.[12] that evaluated the activation of drugs resulting in the formation of toxic metabolites using CYP3A4 in HepG2 cells through transient transfection, or through the addition of microsomes containing the enzyme. CYP3A4, CYP2C9 and CYP2D6 are the major CYP450 enzymes involved in drug metabolism;[13] therefore, we decided to explore the effects of all three CYPs. In our method, Hep3B cells were selected as target cells for evaluating toxicity because they have minimal levels of CYP450 enzymes,[14] thus allowing structure–toxicity relationships to be explored more readily for individual CYPs. To minimize the effect of detoxification through the role of reduced glutathione (GSH), Hep3B cells were treated with L-buthionine-*S*,*R*-sulfoximine (BSO) in order to deplete GSH levels.[12] We tested a series of drugs classified by the FDA according to their drug-induced liver injury (DILI) potential,[15] to explore structural motifs that may be responsible for CYP450-dependent reactive metabolite formation resulting in cytotoxicity in our assay.

The assay involved the addition of test compounds at various concentrations to generate a dose–response in Hep3B cells that had been treated with the CYP enzyme and BSO, and cell viability was assessed through the measurement of ATP levels. Compounds that increased cytotoxicity >2-fold in the presence of the CYP were classified as undergoing toxic reactive metabolite formation. To validate the assay, we screened troglitazone, a well-studied drug that was withdrawn due to DILI caused by CYP-mediated reactive

* Corresponding author. Tel.: +1 617 460 1254.
E-mail address: lyn.jones@pfizer.com (L.H. Jones).
† Present address: Pfizer Oncology, Eastern Point Road, Groton, CT 06340, USA.

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metabolite formation (via quinone methide and thiazolidinedione oxidation, and subsequent ring scission adducts).[16] Troglitazone had also demonstrated a robust response in the Vignati study.[12] We included propranolol in the assay validation study since 2D6 is known to be involved in the formation of reactive metabolites for this drug.[17] As a negative control we chose metaproterenol, a structurally-related amino alcohol, but with no evidence of reactive metabolite formation and no embedded structural alert.[18] As expected, troglitazone (3A4), and propranolol (2D6) exhibited CYP-mediated increases in toxicity, whilst metaproterenol had no effect, providing confidence in the relevance of the assay set-up. The Supplementary material contains full details of the cytotoxicity data for compounds in the DILI list which provides a useful resource for drug metabolism research. Table 1 summarizes the fold-increases in cytotoxicity for selected drugs when incubated with the CYP enzymes, and a brief discussion of the observed effects is provided below.

The structure–toxicity relationships within the adrenergic/-beta-blocker class appear subtle—as mentioned above, propranolol and metaproterenol possess very different effects. Interestingly, fenoterol, which is very similar in structure to metaproterenol, but with an embedded tyramine substituent, possesses 2D6-mediated cytotoxicity. Similarly, labetalol, dobutamine and isoproterenol (an isomer of metaproterenol) also contain a tyramine moiety and undergo CYP-induced cytotoxicity in the assay, in line with reactive metabolite formation for this class via quinone/quinone methide formation.[14,18] Salbutamol however also contains a tyramine unit, but is clean, consistent with there being no evidence of the drug forming a reactive metabolite and its intrinsic stability towards oxidative metabolism.[19] Additionally, it is also worth highlighting that the beta-blockers metoprolol and esmolol do not possess CYP-mediated toxicities, presumably due to their inability to form reactive *para*-quinones (due to the presence of alkyl groups at the 4-position relative to the ether oxygen atom), unlike propranolol (Fig. 1).[17]

Sequential oxidation of the benzofuran motifs in benzarone, benzbromarone and benziodarone can lead to the formation of catechols and then reactive quinone intermediates,[20] and all three compounds are positive in the assay. From our data, benzarone looks particularly vulnerable to 2D6-mediated cytotoxicity. Fenoprofen is metabolized to 4′-hydroxyfenoprofen,[21] and its structural similarity to the probe 2C9 substrate flurbiprofen, and the 2C9-mediated oxidation of several anionic non-steroidal anti-inflammatory drugs,[22] is in line with the observed 2C9-induced cytotoxicity for this compound. Ibuprofen, which lacks the oxidized benzene ring present in fenoprofen, is clean in this assay.

Nimesulide, trazodone and nefazodone contain the aniline structural alert and form quinone imine reactive metabolites following metabolic activation, and our results corroborate these observations since all three compounds undergo 3A4 and 2D6-mediated increases in cytotoxicity.[23–26] Pazopanib also contains an aniline and possesses CYP-induced cytotoxicity, although there are no previous reports of reactive metabolite formation for this compound.[18] However, compounds containing the aniline/anilide structural alert, e.g., bicalutamide and leflunomide, do not always show an effect in this assay (potentially due to electron withdrawing groups ameliorating deleterious oxidation chemistry, or resilience of the anilide towards hydrolysis).[18,27]

Beyond phenol and aniline structural alerts, a number of other drug effects are worth highlighting. Iproniazid is an antidepressant that was withdrawn due to hepatotoxicity. The compound is known to form highly reactive acylating and alkylating species following metabolism of the hydrazine motif,[28] and our results highlight cytotoxicity liabilities through 3A4 and 2D6 metabolism. The nitrofuran motif in nitrofurantoin is known to form reactive radical metabolites in vivo,[29] and we show this compound also undergoes CYP-induced cytotoxicity. Tasosartan was withdrawn from clinical trials as it showed elevated levels of transaminase enzymes.[30] Interestingly, there are no reports of this compound forming reactive metabolites, but we show here significant CYP-mediated cytotoxicity, particularly via 2D6. Similarly, although there are no reports of the withdrawn drug zimelidine possessing reactive metabolites, it undergoes CYP-mediated cytotoxicity in our assay. Metabolic N-demethylation and subsequent oxidation of zimelidine leads to an acrylic acid metabolite,[31] which may undergo adduct formation with biomolecules leading to toxicity.

Table 1
CYP3A4, 2D6 and 2C9-induced cytotoxicity data for selected drugs

Drug	3A4-induced cytotoxicity (fold change)[a]	2D6-induced cytotoxicity (fold change)[a]	2C9-induced cytotoxicity (fold change)[a]
Troglitazone	5.5	3.1	1.2
Propranolol	4.7	121	0.6
Metaproterenol	1.0	1.0	1.0
Fenoterol	1.0	140	1.0
Labetalol	46	1.0	1.0
Dobutamine	1024	89	108
Isoproterenol	136	100	11.9
Salbutamol	1.0	1.0	1.0
Metoprolol	1.0	1.0	1.0
Esmolol	1.0	1.0	1.0
Benzarone	0.8	7.5	0.9
Benzbromarone	1.9	2.0	1.2
Benziodarone	2.2	2.3	2.1
Fenoprofen	1.0	1.0	4.2
Ibuprofen	1.0	1.0	1.0
Nimesulide	9.5	13.2	1.0
Trazodone	34	23	1.0
Nefazodone	4.1	3.1	0.6
Pazopanib	2.5	1.6	8.0
Bicalutamide	1.7	0.7	0.1
Leflunomide	1.0	1.0	1.0
Iproniazid	2.2	2.6	1.0
Nitrofurantoin	44	37	15
Tasosartan	14	52	1.0
Zimelidine	2.8	1.8	1.1

[a] Fold-change in the concentration of drug required to reduce Hep3B cell viability by 50% when incubated with the specified CYP P450 enzyme and BSO as compared to cells with BSO only (drug cytotoxicity concentrations, and data for the broader DILI set of 154 compounds, are reported in the Supplementary material).

In conclusion, we have developed CYP-induced cytotoxicity assays that report on the potential for small molecule drugs to undergo deleterious bioactivation by 3A4, 2D6 and 2C9. Further development of the assay could involve the exploration of other CYP enzymes involved in xenobiotic metabolism (1A2, 2B6, 2C8, 2C19, 2E1, and 3A5)[32] and identification of the metabolites being produced that cause cytotoxicity. Toxicity in the assay may result from specific pharmacology of the metabolite, rather than non-specific biomolecule reactivity or redox chemistry. Additionally, the assay is an artificial system due to the use of BSO to dis-enable an intrinsic detoxifying mechanism. There are also examples of hepatoxic drugs known to form reactive metabolites, such as bromfenac[33] and ketoconazole,[34] which are negative/weakly positive in the assay, and further information would need to be generated to understand the reasons behind these findings (e.g., cytotoxicity profiling of isolated metabolites). Equally, many marketed drugs are known to form reactive metabolites, yet do not possess idiosyncratic toxicities.[11] However, the simplicity of the assays described here, and their relevance to the bioactivation of small molecule drugs should be of broad interest to those working in drug discovery.

Cell culture for Hep3B cells: Hep3B cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in a culture medium containing RPMI-1640 medium