

## THE PREDICTIVE NATURE OF HIGH-THROUGHPUT TOXICITY SCREENING USING A HUMAN HEPATOCYTE CELL LINE

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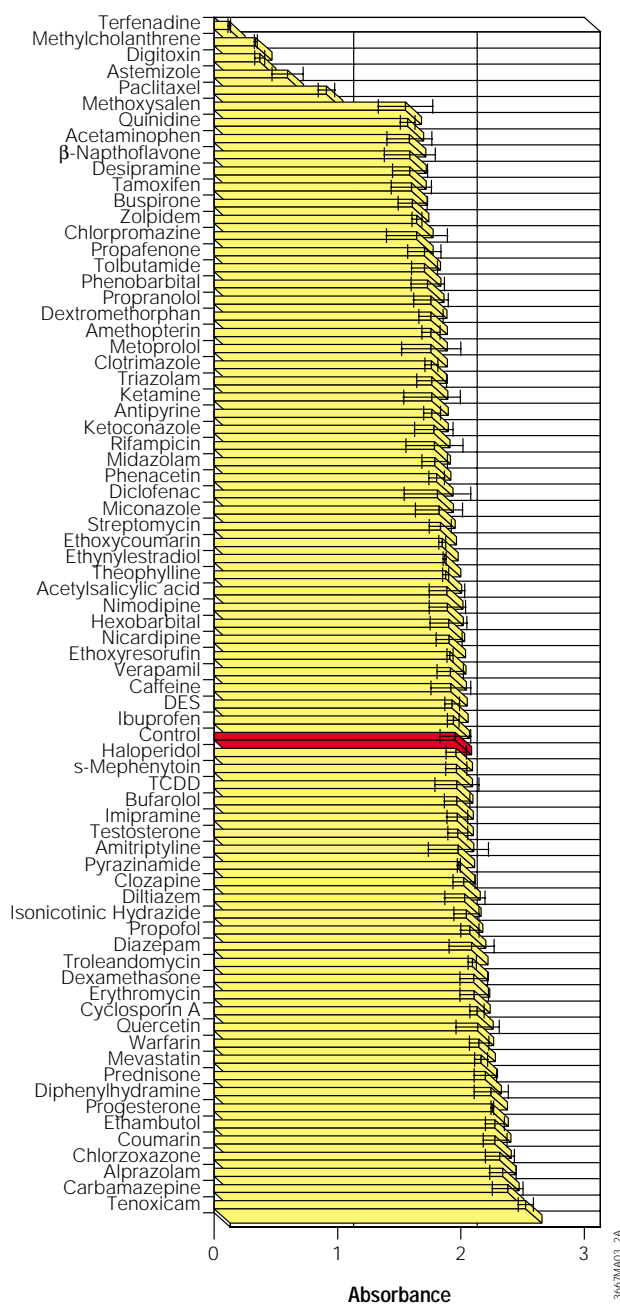
### Abstract

*Drug discovery has become an industrialized process in which vast libraries of compounds are screened for activity against a chosen target. The wealth of active compounds that emerge from these primary screens has created a bottleneck in drug development. First-round hits do not often meet the safety and efficacy criteria required for human therapeutics, so sequential rounds of optimization are required before a product can be administered to humans. Optimization requires assays that test Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADME/Tox). In this paper we demonstrate the utility of high-throughput testing in a human liver cell line. If the technologies and reagents presented in this article had been available earlier, ADME/Tox screening could have prevented deaths and costly drug recalls by two major pharmaceutical companies.*

### Introduction

Historically, antihistamines have been a very safe class of drugs (1). However, two drugs, terfenadine (Seldane<sup>®</sup>) and astemizole (Hismanal<sup>®</sup>) were withdrawn from the market because of serious side effects. Both drugs were found to cause life-threatening cardiac arrhythmias when given in high doses or co-administered with certain antibiotics like erythromycin and ketoconazole (2). This surprising toxicity provides an excellent case study in light of the number of safe antihistamines currently on the market and the growing interest among pharmaceutical scientists in early ADME/Tox screening.

The presumption that chemical libraries contain compounds with a spectrum of positive and negative effects forms the foundation of ADME/Tox screening. Beneficial features of a drug candidate include high specificity, low toxicity, good oral absorption and half-life, among others. The goal of early high-throughput ADME/Tox screening is to distinguish between good and bad compounds early in the discovery process. The identification of problems early in drug screening



**Figure 1. Cell proliferation assay.** The ability of 73 randomly chosen compounds to inhibit cell proliferation was tested in the ACTIVTox<sup>®</sup> cell line. Plating density was 15,000 cells per well in a 96-well plate, and all compounds were tested in quadruplicate at 10 $\mu$ M except for TCDD, which was tested at 1nM. The CellTiter 96<sup>®</sup> AQ<sub>UEOUS</sub> One Solution MTS reduction assay was performed 72 hours after adding the compounds. With this assay, the metabolic reduction of MTS, which is expressed as an absorbance, is proportional to cell number. Five compounds, shown at the top of the graph, significantly inhibited cell proliferation.

represents the single largest cost-saving opportunity in the pharmaceutical industry at the present time.

Once identified in a screening campaign, a favorable compound can provide the building blocks for directed libraries, hundreds to thousands of variants that are synthesized around the core structure of the compound of interest. An iterative process of screening and resynthesis eventually identifies the compounds with the best mix of therapeutic activity and ADME/Tox properties.

**Tools to Achieve High-Throughput ADME/Tox Screening**

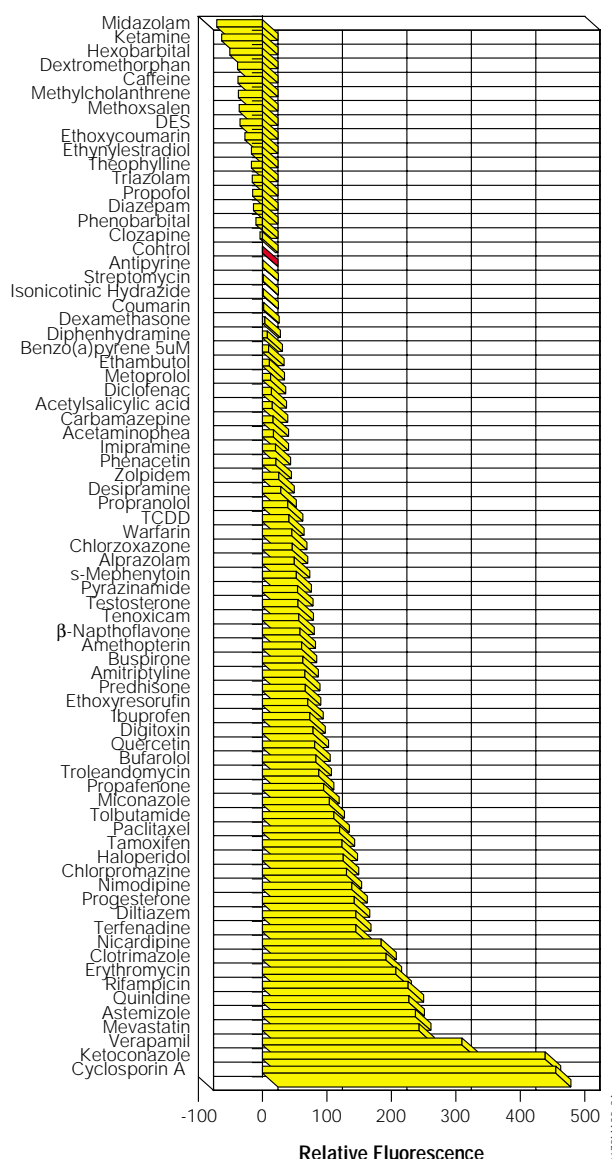
ADME/Tox screening demands high-throughput. Assays should be fast and reflect normal human responses. The industry has responded by developing robotics capable of over 100,000 assays per day and by designing simple, easily automated assays. Scientists have identified Caco-2, a human intestinal cell line, as a standard cellular target for predicting drug absorption. However, the major determinant of drug metabolism and toxicity is the liver, a target that has been difficult to standardize.

**We demonstrate the utility of high-throughput testing in a human liver cell line.**

Much of the screening for metabolism and toxicity is done in primary human hepatocytes (cells isolated from normal liver) or human liver surrogates including animal hepatocytes and whole animals. None of these models are completely satisfactory. Toxicology studies performed in animals are occasionally misleading but, more importantly, are too slow to be used for real-time feedback in a drug discovery campaign. Optimal lead development requires that the pharmacological properties of the compound be maximized simultaneously with the therapeutic properties. Early hits need to be ranked and examined quickly so that the information can be used to guide new synthesis.

Primary hepatocytes, whether animal or human, are problematic, since they do not divide significantly in culture and require constant fresh isolation. Human liver is very scarce, and each donor is unique in terms of genetics and environmental factors. Donors are typically trauma victims who would have been organ donors but were rejected for reasons such as liver disease, sepsis or prolonged shock. These patients have typically received a cocktail of drugs including high doses of steroids, antibiotics, diuretics, and pressors. Experiments done on primary hepatocytes from human donors are essentially anecdotal.

Amphioxus Cell Technologies has developed a proprietary human liver cell line that reflects normal human liver



**Figure 2. P-glycoprotein inhibition.** The same 73 compounds used in Figure 1 were tested for their ability to inhibit P-glycoprotein. In this study, control is subtracted to allow ranking by degree of inhibition.

metabolism (3). This cell line is grown according to defined procedures and is subject to strict quality control and release criteria. The cell line is as reliable and consistent as the existing machinery and assays available to drug discovery scientists. In this paper we show how this cell line might be used with high-throughput assays to avoid dangerous and costly mistakes in drug discovery.

Methods

Compounds

We tested a group of 73 compounds based on their chemical diversity and their availability from Sigma Chemicals (St. Louis, MO). No effort was made to preselect compounds, but known cytochrome P450 inducers were recognized among them. The compounds are indicated in Figures 1 and 2.

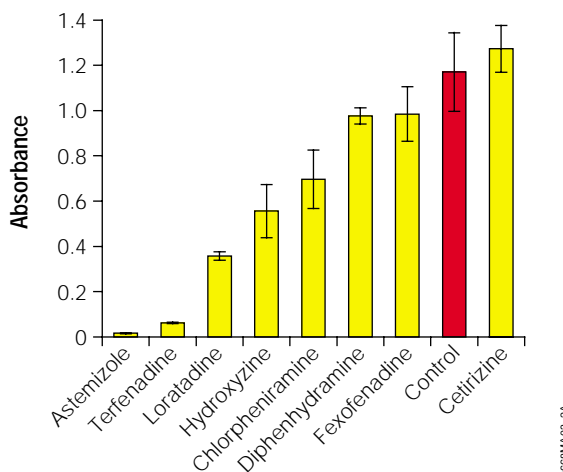
Cells

All studies were performed using Amphioxus Cell Technologies' ACTIVTox<sup>®</sup> human liver cell line (C3A; ATCC# CRL-10741; info@amphioxus.com; 281-679-7900). Cells were fed on a three-times-weekly schedule using proprietary medium. Assays were performed in 96-well plates at a cell density of 15,000/well for anti-proliferative effects or at confluence (150,000/well) for P-glycoprotein competition studies.

Assays

**Cell Proliferation:** Inhibition of cell proliferation was measured using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay<sup>(a)</sup> (Cat.# C3580). In this homogeneous, colorimetric assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) is reduced to a soluble formazan in the presence of an electron-coupling reagent (phenazine ethosulfate; PES) as a result of dehydrogenase activity found in metabolically active cells. Absorbance by formazan at 490nm is directly proportional to the number of viable cells. Assays were performed after a 72-hour incubation with the test compounds. All readings were performed in quadruplicate (mean of four wells) and measured in a Model 550 microplate reader (Bio-Rad<sup>®</sup> Laboratories, Hercules, CA).

**P-glycoprotein Inhibition:** P-glycoprotein (P-gp), the product of the multidrug resistance (MDR) gene, is an ATP-dependent pump that extrudes certain drugs from the cell. P-gp inhibition was measured with calcein-AM (Molecular Probes, Eugene, OR). Compounds that are P-gp substrates compete with calcein-AM for P-gp binding, effectively inhibiting the ability of P-gp to extrude calcein-AM from the cell. Consequently, the calcein-AM accumulates in the interior of the cell where the AM group is cleaved by cellular esterases, converting it to the fluorescent compound, calcein. Fluorescence increases proportionally to the inhibition of calcein-AM binding to P-gp by the test compound. Fluorescence was measured in a FL600 microplate fluorescence plate reader (Bio-Tek Instruments, Winooski, VT).



**Figure 3. Survey of H1-Antagonists.** A set of first and second generation H1 antagonists was tested for the ability to inhibit proliferation in the MTS assay. All drugs were tested in quadruplicate at 80 μM, and all assays were performed at 72 hours. The rank order is shown with maximal inhibition on the left (astemizole) and minimal inhibition on the right (cetirizine).

Results

We screened a random set of 73 compounds for toxicity (inhibition of cellular proliferation) by the MTS assay described above. As shown in Figure 1, five compounds inhibited cell growth, including terfenadine and astemizole. Diphenhydramine, a structurally related antihistamine, was not toxic.

Figure 2 shows the results of P-gp screening on the same set of 73 compounds. Note that many of the known P-gp substrates are identified at the bottom of the graph (e.g., cyclosporin A, ketoconazole) and that astemizole and terfenadine are once again identified. Erythromycin is also identified as a P-gp-dependent compound, but diphenhydramine is a fairly low-level competitor of calcein-AM.

A successful screening campaign eventually leads to a collection of closely related analogues that must be ranked for optimal activity and ADME/Tox properties. To mimic this situation, we tested a variety of first-generation (chlorpheniramine, diphenhydramine, and hydroxyzine) and second-generation (astemizole, terfenadine, loratadine, fexofenadine, and cetirizine) H-1 antagonists. Figure 3 demonstrates the toxicity profile of the panel. Astemizole and terfenadine were clearly the most toxic of the class; loratadine was somewhat less toxic, and cetirizine and fexofenadine the least toxic.

Discussion

This study demonstrates the utility of a metabolically active cell line in high-throughput ADME/Tox screening. Terfenadine and astemizole were not chosen specifically, but they were identified in a mock screening campaign. These two antihistamines and erythromycin, an antibiotic that is frequently co-administered with antihistamines, were shown to block P-gp drug export. Finally terfenadine and astemizole were shown to be more toxic than closely related compounds. Knowledge of these results would have prevented their release; alternative choices were available at the time, and both drugs have now been replaced by safer alternatives.

**The point of ADME/Tox screening is to identify toxic compounds at the lowest possible cost.**

An interesting point in this screening is the fact that terfenadine and astemizole are cardiac toxins; both agents produced a dangerous arrhythmia known as *torsade de pointes*. This is now recognized as a common form of toxicity shared by other classes of drugs including antidepressants. The cause is blockade of a potassium rectifier channel encoded by the human Ether-a-Gogo Related Gene (hERG) (4) and identified in the whole organism by prolongation of the QTc interval on electrocardiography. Detection of toxicity due to known cardiotoxins by a liver cell line is an important observation for two reasons. First, it suggests that multiple types of toxicity might be predicted in a small number of cellular models, thus reducing costs and time for testing; and second, it shows the importance of biological systems in testing toxicity. A general toxicity model is more likely to predict a wide range of toxicities, including novel mechanisms. The point of ADME/Tox screening is to identify toxic compounds at the lowest possible cost.

Terfenadine and astemizole toxicity was reported in people taking erythromycin. The reason was assumed to be competition for the metabolizing cytochrome P450 (CYP 3A4). This is probably an oversimplification, because astemizole is only partially metabolized by CYP 3A4 (5). Another likely explanation, competition for P-gp, was demonstrated in this study. P-gp has specificity for amphiphilic molecules, the same characteristics frequently found in CYP 3A4 substrates (6). Modern drug screening is very sensitive to the issue of CYP 3A4 specificity. The data presented here encourage the addition of P-gp to ADME/Tox screening.

Safety ranking of the remainder of the antihistamine class is demonstrated in Figure 3. Loratadine has a better safety profile than terfenadine and astemizole, but it is significantly more toxic than cetirizine, fexofenadine, or diphenylhydramine. Consistent with the results shown in Figure 3, loratadine has been shown to cause QTc prolongation when co-administered with nefazodone, a calcium channel blocker (7).

Ideally ADME/Tox screening involves a suite of assays that include CYP inductions, P-gp inhibition, and multiple toxicity assays. The most toxic compounds are identified in all assays, but some mechanism-based toxins require specific testing. A broad-based approach will more likely identify all toxins. Further study of this hypothesis is under-way.

In summary, a metabolically active cell line coupled with a simple set of high-throughput tests can distinguish between closely related compounds. In the case of the antihistamines described in this paper, lives would have been saved, and expensive drug recalls would have been avoided. Simple, meaningful tests of this caliber should be routine in every drug development program.

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Ordering Information

Product	Size	Cat.#
CellTiter 96® AO <sub>ueous</sub> One Solution Cell Proliferation Assay <sup>(a)</sup>	1,000 assays	G3580
	5,000 assays	G3581
	200 assays	G3582

For Laboratory Use.

<sup>(a)</sup>The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.

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