AUTOMATED CYTOCHROME P450 INHIBITION SCREENING

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ABSTRACT

Modern drug discovery laboratories have extensively used automated liquid handling systems (LHS) to develop rapid and robust assays to screen increasing numbers of discovery compounds for various important parameters such as metabolic stability, P450 inhibition and permeability. We present a method, developed at BioDynamics for rapid throughput cytochrome P450 inhibition screening, using fluorescent substrates and cDNA expressed enzymes in conjunction with an 8-tip Multiprobe 2 instrument (Packard BioSciences). The LHS performs a serial dilution of up to 10 different compounds to give a range of different concentrations (to generate IC50 values) in a 96 well plate (test compound plate). The test compound plate is then kept for use with all of the different enzyme assays. The LHS also prepares the incubation plate so that each well contains buffer, cDNA expressed microsomal protein and fluorescent substrate. The diluted test compounds are then transferred from the test compound plate to the heated incubation plate by the LHS. After a pre-incubation period, metabolic reactions are started by the LHS which adds pre-warmed cofactor to each well of the incubation plate. The incubation plate is then monitored continuously for fluorescence production over 10 minutes in a microplate fluorescence plate reader. This assay enables the rapid inhibition screening of up to 10 compounds in each of the major human drug metabolising enzymes.

INTRODUCTION

Undesirable DMPK properties can be a major cause of new candidate drug failures in the drug development process [1]. Acceptable pharmacokinetics in man is an essential requirement to the development of any new chemical entity. Therefore, highly permeable, low clearance compounds are generally desired for development and which do not interact with major drug metabolising enzymes in an inhibitory manner.

To help select such compounds, *in vitro* drug metabolism techniques have been developed which can allow the screening of a relatively large number of compounds using automated methods. One assay which readily lends itself to automation, is cytochrome P450 inhibition screening using cDNA expressed human CYP450-enzymes.

Inhibition of cytochrome P450 by xenobiotics has been implicated in clinically relevant, and sometimes fatal, drug-drug interactions in vivo [2]. Therefore, it is desirable to screen out potent inhibitors of cytochrome P450 during the lead optimisation process. This presentation outlines the method used at BioDynamics to assess the potential of new test compounds towards the inhibition of CYP450-enzymes using cDNA expressed microsomes and fluorescent substrates. In particular, the method has been developed to support drug discovery programmes which often require the rapid screening of a relatively large number of compounds to hasten the lead optimisation process.

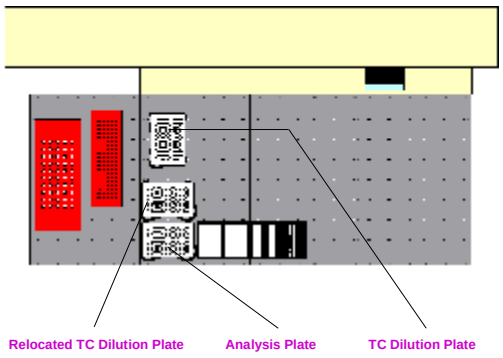
OUTLINE

Using an automated liquid handling system (8-tip, Multiprobe 2, Packard BioScience, Pangbourne, Berkshire, UK) test compounds at various concentrations are incubated at 37˚C with cDNA expressed human CYP450 enzymes, buffer, NADPH and commercially available substrates which undergo CYP-mediated metabolism to a highly fluorescent metabolite. The incubations are monitored continuously during 10 minutes (i.e. kinetic) for the production of fluorescence. The rate of fluorescence production is then compared in the presence and absence of the test compounds. The process is controlled by use of dedicated excel spreadsheets and well maps. Positive control incubations with known cytochrome P450 inhibitors are included. Our current method allows for 7-point (concentration) IC50 values to be generated for up to 10 test compounds with each of the major human drug metabolising CYP-enzymes per assay.

METHODOLOGY

A Test Compound Plate and Incubation Plate are prepared by the Multiprobe 2 (MP2). Figure 1 presents the deck of the MP2 and shows the location of these plates.

Figure 1 The deck of the Multiprobe 2

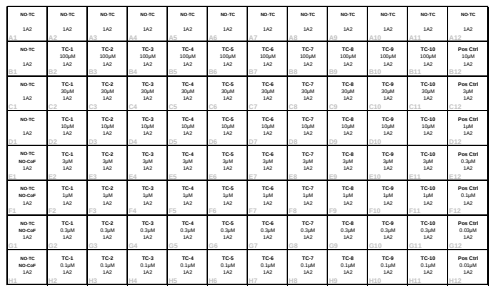


An Excel spreadsheet contains all the relevant study information and to enable safe sample tracking, various Plate Maps are then generated showing the location of each test compound at each stage of the process.

TEST COMPOUND PLATE

Test compounds and the positive control inhibitor are serially diluted by the MP2 in columns to give 7 concentrations of each test compound and inhibitor based upon a log scale. Test compound dose vehicle is present at the top of each column and row of the plate, so that control enzyme rates are reflective over the entire plate (to minimise pate-edge effects). This plate is then covered by film and kept for use with all assays. An example Test Compound Plate is shown below:

Figure 2 Test Compound Plate



TC = test compound, CoF = cofactor (NADPH)
Pos Ctrl = Positive Control Inhibitor

INCUBATION PLATE

The MP2 aspirates the required volumes of cDNA expressed microsomes, buffer and fluorescent substrate from the Reagent Rack and combines these components into a central reservoir. This mixture is then dispensed into the appropriate wells of a 96 well polystyrene plate and an aliquot from each well of the Test Compound Plate is transferred by the MP2 into the appropriate wells of the Incubation Plate.

Following a pre-incubation period of 5 minutes, pre-warmed enzyme cofactor solution is added by the MP2 to start metabolic reactions. The Incubation Plate is immediately placed into a heated microplate fluorescence reader (BioTek FL600) and the plate scanned at the appropriate excitation and emission wavelengths (dependant upon fluorescent substrate) every minute for up to 10 minutes.

The exact amounts of each component and the nature of the fluorescent substrate will depend upon which enzyme activity is being assayed for. However, each assay has been designed so that the following common incubation conditions apply:

Table 1 Common Incubation conditions

Parameter	Values
Final concentrations of test compounds	0, 0.1, 0.3, 1, 3, 10, 30 and 100 µM
Final volume of organic solvent	1% (v/v)
Final incubation volume	250 µL
Incubation time	10 minutes

The rate of fluorescence production is then calculated in each well containing test compound and displayed graphically to check for linearity. Example data for a test compound (TC-1) recently screened at BioDynamics for inhibition activity against CYP3A4 is presented below:

Figure 3 Example data showing inhibitory effects of a recently screened test compound

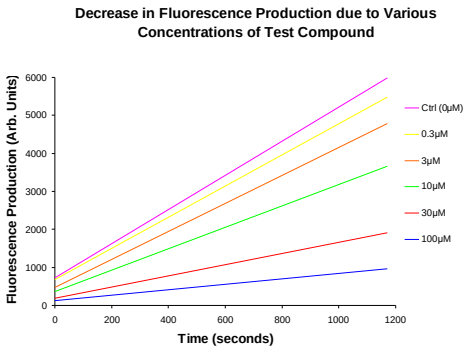


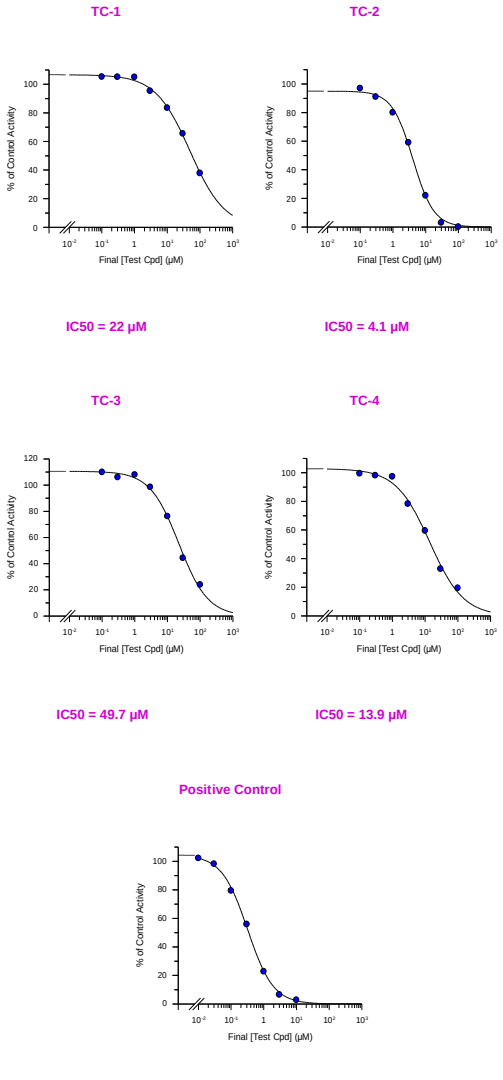
Figure 3 shows that the rate of fluorescence (i.e. metabolism) decreases as the concentration of test compound increases in a dose-related manner. It is presumed therefore that TC-1 in this example is acting as an inhibitor of CYP3A4-mediated metabolism.

The relative potency of this inhibition can be quantified by calculating the IC50 for the reaction under study. The rate of fluorescence production in the presence of each concentration of test compound is expressed as a percentage of total control activity (ie all wells containing dose vehicle only). These data are then plotted against each concentration of test compound on a log scale to generate the IC50 value.

Example data for a batch of compounds (TC-1 to TC-4) recently screened at BioDynamics for inhibition against CYP3A4 is given in Figure 4.

From these results TC-2 would appear to be a relatively potent inhibitor of CYP3A4.

Figure 4 Example Data showing the effects of Test Compounds TC-1 to TC-4 on fluorescence production in comparison to a positive control.



DISCUSSION

The use of fluorescence-based cytochrome P450 inhibition screening assays offers marked advantages over more traditional HPLC-based methods where, typically, radioactive substrates are incubated with human liver microsomes. The major advantage is the increased throughput and shortened analysis times. To generate 7-point IC50 data for 10 test compounds in each of the major human drug metabolising CYP450 enzymes would be a considerable undertaking and HPLC analysis times of up to 1 hour per sample are not unusual. These data could be generated very rapidly using the method presented in this poster. Furthermore, the assays do not involve the use of radioactivity.

However, as with all screening methods there are some limitations. Typically, the substrates used in these kinds of assays are not clinically relevant and therefore for compounds in development, the HPLC-based assays would be more appropriate where the data may be used to support a regulatory submission. Furthermore, some test compounds themselves exhibit fluorescent properties which can interfere with the assays and activation of enzyme activity, especially with CYP3A4, is sometimes observed.

At BioDynamics we routinely screen for CYP1A2, 2C9, 2C19, 2D6 and 3A4 activity.

Figure 5 The 8-Tip Multiprobe 2

REFERENCES

1. Caldwell, G.W., Compound optimisation in early- and late-phase drug discovery: Acceptable pharmacokinetic properties utilising combined physicochemical, In vitro and In Vivo screens, *Curr. Opin. Drug Discovery Dev*. (2000), (3) pp 30-41.

2. Lin, J.H and Lu A.Y Inhibition and induction of cytochrome P450 and the clinical implications, *Clin. Pharmacokinet.* (1998), (35) pp 361-390.

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