FLUORESCENCE ASSAYS FOR CYTOCHROME P450 ENZYMES

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ABSTRACT

A large number of pharmacologically active compounds synthesized in the discovery phase of pharmaceutical R&D are rejected because they interact with the metabolism of existing therapeutic drugs or because they have poor bioavailability caused by rapid metabolism. In many cases this is because the compounds are either substrates or inhibitors of one or more cytochrome P450 (CYP450) isozymes. To detect these interactions, assays of CYP450 isozymes generally use either separation methods involving HPLC, or fluorogenic substrates. However, existing fluorogenic substrates either have poor kinetics, or the enzymatic products do not have the desired optical properties to allow reduction of the amount of enzyme needed to levels that would make large scale screening affordable.

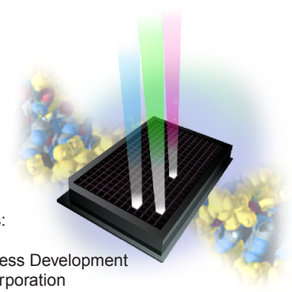
Here we present data from assays based on fluorogenic substrates that are efficiently metabolized by human CYP450 enzymes 3A4, 2D6, 2C9, and 2C19 to yield highly fluorescent products. These assays allow high throughput screening of large numbers of compounds.

The information generated has several uses:

· Allows early detection of compounds with potential metabolic liabilities

· Enables SAR analysis of compound-cytochrome P450 interactions

· Provides guidance to medicinal chemists for drug lead optimizations.



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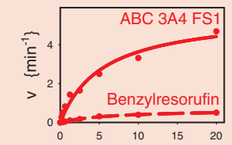
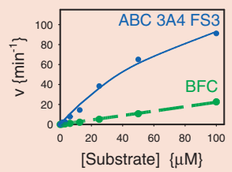
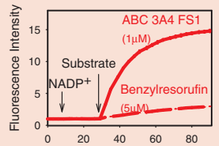
3A4

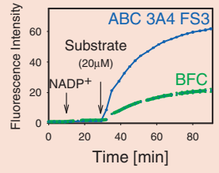
KINETICS OF SUBSTRATES



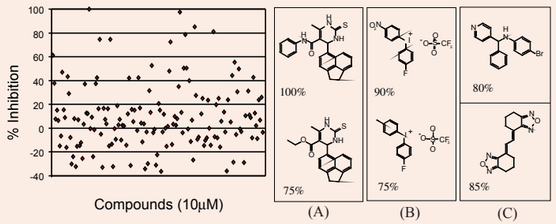


FLUORESCENCE EMISSION OVER INITIAL SIGNAL





SCREENING OF AURORA COMPOUNDS AND STRUCTURES OF HITS



160 Compounds from Aurora's compound collection were assayed using ABC 3A4 FS1. About 5% of the compounds inhibited CYP 3A4 activity by 60% or more. Structures of 6 hits included planar hydrophobic thioureas (A), iodonium compounds (B), and hydrophobics with aniline or heterocyclic moieties (C).

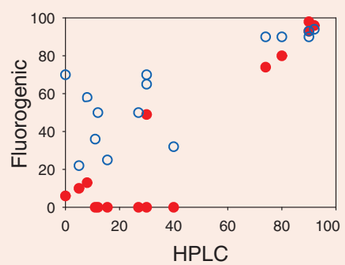
COMPARISON OF Ki VALUES WITH LITERATURE

DRUG	lit. ki (μM)	3A4 FS1	3A4 FS3
Miconazole	0.9-1.3	0.3	0.4
Nifedipine	10-22	0.9	1.6
Troleandomycin	10-51	1.8	15
Ethinylestradiol	34	27.5	1.6
Verapamil	24-82	12.7	8
Diltiazem	50-75	9.0	17
Progesterone	8-45	> 40	23
Erythromycin	16-194	8.4	> 50
Amiodarone	47	> 30	7

Fluorogenic substrates were used to measure apparent Ki values for a series of drugs know to interact with human CYP3A4 and compared to literature values*. Both substrates (FS1 & FS3) detected the more potent inhibitors (top 3 drugs) while the Ki values differed to varying degrees. This is an expected result, as CYP3A4 is known to exhibit substrate-dependent cooperativity and/or allosterism.

* (K.E. Thummel & G.R. Wilkinson, Annu. Rev. Pharmacol. Toxicol. 1998, 38: 389)

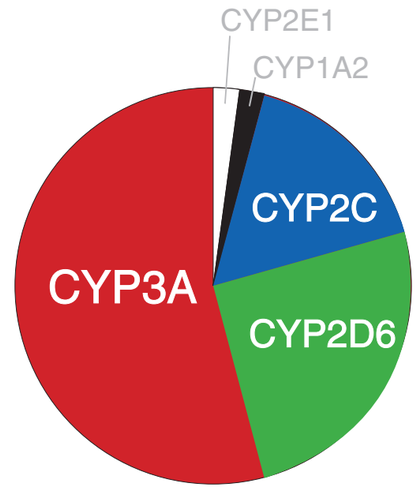
FLUOROGENIC SUBSTRATES VERSUS HPLC METHOD



Values represent % inhibition with 10μM compound from a drug lead series.

Insect cell-expressed CYP 3A4
● ABC 3A4 FS1
○ ABC 3A4 FS3

HPLC data obtained with human liver microsomes and 80μM Testosterone (^{14}C)
(Sylvie Marc, Christine Julien-Larose, I.R.J., Parke-Davis, FR.)



Relevance of human cytochrome P450s to drug metabolism

LEGEND

SUBSTRATE KINETICS AND SIGNAL TRACES
To demonstrate the superior kinetic and/or optical properties of Aurora's substrates, side-by side comparisons with the current best commercially available fluorogenic substrates (from Gentest, Molecular Probes, Sigma, Aldrich) were performed. Solid curves depict results from Aurora substrates, broken curves represent data for commercially available substrates. The color of the traces indicates the color of the emitted fluorescence from the product of substrate metabolism (Grey indicates the necessity of UV excitation of the fluorescent metabolite). The graphs on the left of each panel display kinetic properties of substrates, with turnover of substrate molecules per enzyme and minute graphed against substrate concentrations. The graphs to the right depict the signal from the metabolism of substrate by identical concentrations of enzyme in a microtiter plate well. All signal traces are normalized to the initial signal from plate fluorescence and scatter (set arbitrarily to 1 in graphs depicting signal intensities).

SUMMARY

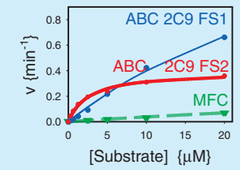
Aurora's cytochrome P450 isozyme assays employ proprietary fluorogenic enzyme substrates that enable fast compound profiling in standard microtiter plate formats as well as in highly miniaturized assay formats.

Assays are designed for use with individually expressed enzymes and are available for all cytochrome P450 enzymes involved in human drug metabolism. The concentration of fluorogenic substrate in every assay is below the substrate's Km for the isozyme, which permits detection of strong and weak inhibitors. Kinetic and optical properties of substrates permit use of enzymes at concentrations of 20nM or less. At these low enzyme concentrations, test compound metabolism during the assay period is minimal. For several isozymes, multiple substrates with non-overlapping spectra are available, which can be used in multiplexed assays.

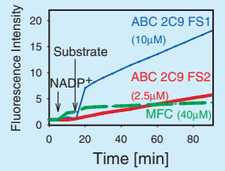
The inhibition data obtained with Aurora's assays enable SAR analyses for cytochrome P450 isozyme inhibition, which is useful in guiding medicinal chemists and making them aware of potential drug lead interactions with cytochrome P450s early in the drug discovery process.

2C9

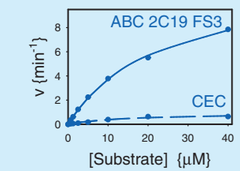
KINETICS OF SUBSTRATES

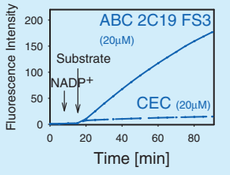


FLUORESCENCE EMISSION OVER INITIAL SIGNAL



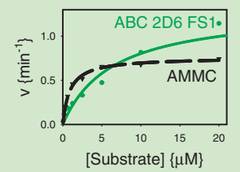
2C19





2D6

KINETICS OF SUBSTRATES



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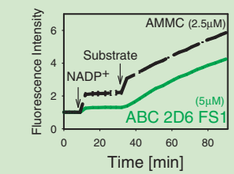

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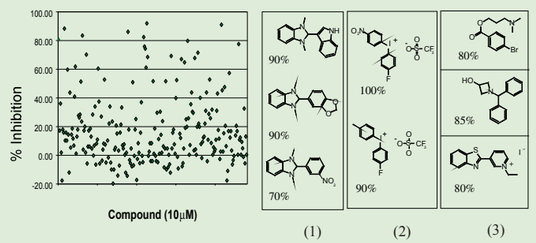

240 Compounds from Aurora's compound collection (includes 160 from the 3A4 assay) were assayed using substrate ABC 2D6 FS1. About 10% of the compounds in this sampling inhibited CYP 2D6 activity by 60% or more. Structures of 7 hits are displayed. Notably, hits in the 2D6 assay differed from the ones found in the 3A4 assay except for the iodonium compounds (2) that are expected to inhibit all CYP450 enzymes. Also, a cluster of ortho-phenylenediamines (1) was inhibitory in the assay. In general, CYP 2D6 preferentially binds compounds that carry a positive charge under physiological conditions and are otherwise hydrophobic. Three hits fitting these criteria are depicted (3).