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A New Standardized Electrochemical Array for Drug Metabolic Profiling with Human Cytochromes P450

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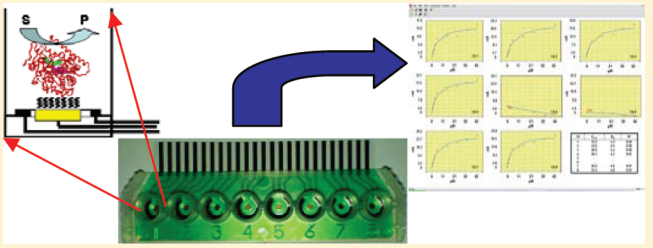
ABSTRACT: Over the past two decades, a wealth of information on the human cytochrome P450 enzymes and their role in drug metabolism both in vitro and in vivo has been gathered. Our understanding of this area has progressed greatly, but our confidence in the development of quantitative projections of drug interactions, made from in vitro data, is somehow still shaky. There are therefore no doubts in the necessity for reliable and fast methodologies for P450 drug metabolism analysis, capable of providing accurate and precise in vitro data. This paper reports on the first integration of a P450-electrode into a microtiter plate format for the rapid determination of the affinity parameters (K_M) for a set of known drugs. The most relevant human drug metabolizing cytochromes P450, isoforms 3A4, 2D6, and 2C9, have been covalently bound to a gold electrode via a 10-carboxydecanethiol and 8-hydroxyoctanethiol (1:1) self-assembled monolayer at the bottom of an eight-well microtiter plate. The electrochemical response of the P450-electrode and the performance of the platform have been validated using a set of 30 known drugs with K_M values spanning from less than 1 to more than 100 μM. The K_M values obtained using this platform show an excellent error, and their ranking is within the range of those present in the literature determined from conventional incubation experiments with cytochrome P450s 3A4, 2D6, and 2C9.



Human cytochromes P450 are responsible for the metabolism of the large majority of known drugs.[1] Characterization of the specific metabolic pathways and of the P450 isoenzymes involved constitutes an essential part of the drug development process. There are 11 major drug-metabolizing P450s that are expressed in the human liver, and among these the isoforms, 3A4, 2D6, and 2C9 are responsible for the metabolism of nearly 90% of all drugs.[2] Metabolic stability of a drug is a major factor that will ultimately determine the concentration of the drug found in the systemic circulation. Since the practice of simultaneous prescription of more than one drug to treat one or more conditions in a single patient has become a common practice, drug−drug and drug−P450 interactions have been cited as one of the major reasons for hospitalization and even death.[3] Many drug−drug interactions are metabolism based and result from two or more drugs competing for the same enzyme that in the majority of the cases involves P450s.[4] Therefore, it has become increasingly important to develop methods that enable screening for metabolic stability and P450 inhibition in a simple, rapid, high-throughput format. At present, high-throughput screening of drug−P450 interaction is achieved using a competitive fluorescence assay with fluorogenic substrates.[5,6] This approach, based on a competitive reaction, can only yield indirect values for the kinetic parameters and is unable to distinguish between substrates and inhibitors. An approach that does not possess these limitations and that, thanks to the full metabolic characterization, is more information rich, but also more costly and slow, is the use of mass spectrometry.[4] One of the major challenges encountered when screening drugs for metabolic studies is that a great variety of chemicals with distinct molecular structures must be analyzed using a generic method. For a method to achieve high-throughput rates, the measurements of the output signal should be preferably compound-independent and the data acquisition must be rapid. This has proved to be challenging for the mass spectrometry-based methodologies, though progress is constantly being made, and sparked the need for investigating alternative methodologies more likely to be high throughput.[4]

Recombinant enzymes, microsomes, hepatocytes, and liver slices have all been used as in vitro model systems for testing the metabolic stability of new drug candidates leading to a certain controversy when attempting to compare data obtained using enzymes from different sources.[4] Human liver microsomes have in the past provided the most convenient way to study drug metabolism. They consist of a subcellular fraction of tissue obtained by differential high-speed centrifugation and can be obtained commercially, with or without prior phenotyping, for most

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important drug metabolizing enzymes. All of the P450 enzymes are collected together in the microsomal fraction. Microsomes from different donors are used, either individually or pooled together, to avoid reliance on microsomes that are deficient in one or more metabolic pathways. With the use of selective chemical inhibitors for each major pathway, the metabolic pathways for a new drug can be demonstrated or ruled out. With the cloning of most of the common P450s,[1] the recombinant human enzymes have been expressed in a variety of cells allowing a more controlled system and becoming the new standard for drug metabolism and toxicological studies. Incubations with these enzymes still requires a lengthy, multistep procedure followed by either fluorescence or mass spectrometry analysis.

The reaction catalyzed by cytochromes P450 involves the use of two reducing equivalents donated by NADPH via a reductase. Electrodes can be used as an alternative source of electrons in place of NADPH: here an electric current is measured with an intensity that is proportional to the rate of substrate turnover by the P450 enzymes. Reports can be found in the most recent literature on the electrochemical characterization and potential exploitation of P450 for the characterization of specific substrates.[7−20] Our laboratory has previously reported the successful covalent immobilization of cytochromes P450 on gold electrodes by using self-assembled monolayers that exploited different methods to tether the protein to the surface, and their activity was demonstrated in terms of product formation.[7,18−21] The bioelectrochemistry of cytochromes P450 has been adopted in some cases for the determination of unknown quantities of specific analytes in a classical biosensor setup. In these approaches the sensors were designed to work with a linear response to increasing concentration of substrate with the specific aim of determining the unknown concentration of such analyte in biological samples.[22−27] The novelty of the approach presented in this work stands on the design of a system that responds hyperbolically to increasing concentrations of substrate, following the enzymatic Michaleis−Menten model that allows the determination of the substrate-specific parameters such as K_M that is important in guiding the determination of the dosage for each drug.

In this paper we present the construction and characterization of an array that draws together the requirements for a high-throughput methodology with output that does not require substrate-dependent analytical methods. It uses a controlled and reproducible source of recombinant human cytochrome P450 isoforms, enabling the investigation of the specific contribution of different enzymes in a microtiter plate format where no reagents, reductases, or NADPH are needed, but simply requires increasing concentrations of pure drug for titration in the well.

The platform presented in this paper is not a biosensor intended to detect an analyte in a complex mixture, but rather a device for the identification of specific metabolic parameters (K_M) of pure samples of new potential drugs in controlled experimental conditions. For this reason, a set of drugs, used as pure samples, with K_M values for the three P450s ranging over 1 order of magnitude, represents the ideal set for the validation of the array.

MATERIALS AND METHODS

Materials. Cytochrome P450 3A4, 2D6, and 2C9 were expressed and purified as previously described.[28] The proteins were stored at −20 °C in 50 mM phosphate buffer, 500 mM potassium chloride, and 50% glycerol until use. 10-carboxydecanethiol (CDT) and 8-hydroxyoctanethiol (HOT) were purchased from NBS Biological Ltd. (UK). All other chemicals were obtained from Sigma and were of highest purity available and used as received. All solutions were prepared using Milli-Q water purifying system (Millipore, UK)

Array Preparation. The surface of the gold working electrode was cleaned before modification with the self-assembled monolayer (SAM) using oxygen plasma. The array was placed in a K1050X Plasma Treatment Unit (Emitech, UK), and oxygen plasma with 90 W RF was applied for 10 min. Subsequent to the plasma treatment, ethanol was added to each well and incubated for 20 min. A 200 μL amount of ethanolic solution containing 1 mM CDT and 1 mM HOT was added to each well and incubated for 48 h. The excess solution was then removed, and the electrodes washed three times with ethanol to remove the unbound alkanethiols. The ethanol was removed, and 250 μL of water was added to each well to wash out the ethanol. This washing step was repeated twice. A 200 μL amount of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer containing 1 M NaCl (pH 6) was added to each well. A 200 μL amount of 25 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 25 mM *N*-hydroxysulfosuccinimide (S-NHS) dissolved in 50 mM MES buffer was added to each well to activate the SAM's carboxylic groups. After 20 min, the EDC solution was removed and washed twice with water. Protein solution of either P450 3A4, 2D6, or 2C9 was diluted with 50 mM phosphate buffer containing 500 mM potassium chloride (pH 7.4) to the concentration of 5 μM. Ten microliters of protein solution was added to each well, thereby making sure the whole working (gold) electrode was covered by the protein solution. The array was incubated with the protein for 1 h at 4 °C in a high-humidity chamber to limit evaporation of the droplet. After the protein immobilization, the electrodes were washed three times with phosphate buffer and stored in the same buffer until use.

Electrochemical Measurements. No commercially available electrochemical arrays with the desired characteristics of gold working, carbon counter, and silver/silver chloride reference electrodes in each well are available. Two electrode systems are commercially available, but none have reference electrodes in each well. Therefore, it was chosen to design and construct an array with the desired characteristics as described in Results and Discussion. The choice of materials and design was guided by integration with the existing technology, electrodes design, disposability, and materials compatibility with the final goal of constructing a disposable array with a cost predicted to be no more than 10 US$ per unit. The array was manufactured according to the specifications by Conductive Technologies Inc. (York, PA).

Furthermore, only few multichannel potentiostats are equipped with more than 8 independent channels and in general no more than 16. The commercially available potentiostats with a higher number of working electrodes normally have a common reference for all the working electrodes. This configuration is not ideal for our application; therefore, all the electrochemical measurements were performed using an eight-channel multipotentiostat PG580-RM commercially available from Uniscan (Uniscan, UK) interfaced to a PC. This instrument is capable of controlling eight independent working, counter, and reference electrodes, making it ideal for our application. The array's connector was custom designed and constructed in collaboration with Uniscan, allowing easy connection of the array to the potentiostat (Figure 1). All potentials are reported versus the 0.5 M Ag/AgCl reference electrode. Data fittings were carried

out using a custom-made macro that integrates with the acquisition software of the Uniscan multichannel potentiostat. The macro was written in collaboration with Uniscan and is based on a Michaelis−Menten model.

Cyclic Voltammetry. Insulation properties of SAM-modified electrodes were tested by cyclic voltammetry from −0.2 V to +0.65 V using a solution containing 10 mM potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) with 1 M KCl as supporting electrolyte.

Cyclic voltammograms of P450 modified arrays were performed under anaerobic conditions in a Belle technology glovebox. Buffer solutions containing 50 mM phosphate and 500 mM potassium chloride (pH 7.4) were used. The scan range was from 0 V to −0.65 V versus Ag/AgCl.

Titration of P450 with Specific Drug Substrates. Electrochemical measurements were carried out at 25 °C in 50 mM potassium phosphate pH 7.4 containing 500 mM potassium chloride (working buffer). Drugs were purchased at the purest grade from Sigma Aldrich and were dissolved in the appropriate solvent: either methanol, ethanol, or DMSO. The choice of the appropriate solvent was made according to the solubility of the specific drug and the potential interference of the solvent with the P450 metabolism. Stock solutions of 1, 10, and 50 mM of the drugs were used to generate the desired dilutions into the aforementioned buffer.

The prepared arrays with the immobilized P450 containing 200 μL of 50 mM phosphate and 500 mM potassium chloride (pH 7.4) buffer were inserted in the specifically designed connector fitted on the platform of a microtiter plate shaker. The wells were left uncovered for full oxygenation of the buffer during the titration.

An initial cyclic voltammetry scan was recorded before any addition to determine the catalytic current in resting state. The substrate was then added with a multichannel pipet in 1 μL aliquots to minimize the addition of large quantities of organic solvents. After each addition, the array was stirred with the plate shaker and rested for 1 min to allow for substrate binding to the enzyme. Cyclic voltammetry was then performed after each addition. All CVs were recorded between 0 and −0.65 V vs Ag/AgCl. Control experiments were carried out by titrating equivalent volumes of buffer containing the solvents used to dissolve the drugs. In a second set of control experiments, arrays modified with only the SAM but no protein were used and titrated with the drugs.

RESULTS AND DISCUSSION

Design and Construction of the Array. The design of the array was driven by the following considerations: (1) need of integration with the existing automated fluidic technologies existing in the pharma industry; (2) the constraints in the three-electrode design; (3) the disposability of the array; (4) the compatibility between the materials used. Most of the P450 assays used in the pharma industry are run in microtiter plates. It was therefore decided to construct an eight-well array with the standard dimensions of a row of a 96-well microtiter plate. The housing forming the eight wells was molded in polypropylene (Figure 1). Other materials, such as polycarbonate and polystyrene, were also tested, but polypropylene was chosen because of its excellent properties of chemical resistance and biocompatibility.

In all previously published electrochemical applications of cytochromes P450, a three-electrode configuration, working, counter, and reference, has been adopted as the set up of choice[7,18,29]

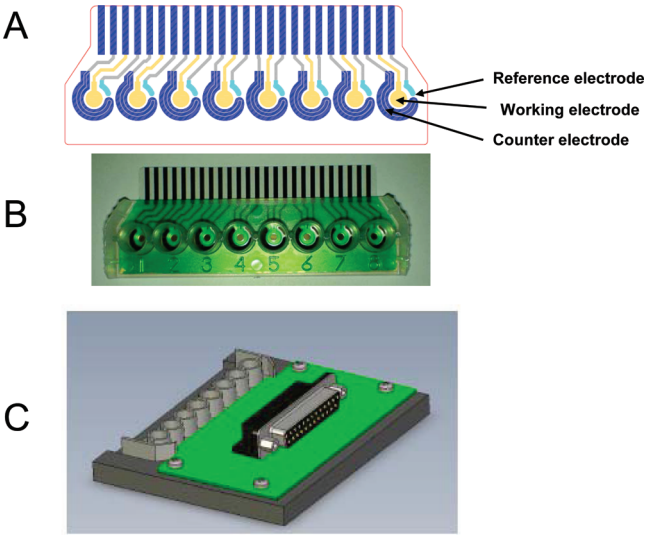

Figure 1. (A) Scheme of the design of the electrode array on the PET layer. The materials used for the construction of the array are color coded: yellow for the gold of the working electrode and the track; blue for the carbon ink of the counter and of the contacts pads; cyan for the Ag/AgCl ink of the reference electrode; gray for the silver ink used for the connecting tracks. (B) Photograph of the array from the top. It is possible to observe the polypropylene housing and the green layer of insulation ink sandwiched between the housing and the polyethylene terephthalate (PET)/contacts layer. The comb structure of the contacts is also noticeable. (C) Side view of the array housed in the holder for measurement.

because precise control is needed over the applied potential in order to limit the adverse effect of nonspecific reactions, mainly associated with oxygen, at the electrode surface. Therefore, it was decided to keep the three-electrode configuration in each well with every electrode independently wired to a multichannel potentiostat (Figure 1).

The electrode materials are crucial for the performance of the amperometric sensor and were therefore chosen with care. The working electrode was constructed by laser ablation of the desired features, working area, and contacts, from a sheet of gold (50 nm thick) sputtered on polyethylene terephthalate (PET). The choice of gold stems from the successful immobilization of several P450s onto gold surfaces modified with a self-assembled monolayer (SAM),[7,18,20,29] while the low cost of the sputtered material makes it ideal for a disposable consumable. The reference and counter electrodes were instead built by screen printing methodologies on the PET support. Inks of carbon and silver/silver chloride were used for the counter and reference electrodes, respectively, while silver was used to draw the contacts (Figure 1). An initial design making use of a gold counter electrode fabricated by laser ablation together with the working electrode was adopted to minimize the number of fabrication steps but was abandoned because of the instability of such electrodes in buffers containing a high concentration of chlorides. A layer of insulating ink was printed in the area outside the wells in order to improve the adhesion of the PET support to the polypropylene housing.

The geometric layout of the electrodes on the bottom of the well was chosen in order to maintain the working electrode disk at the center of the well, the reference electrode on the left side, and the counter electrode extending for about two-thirds of the

circumference. Positioning the working electrode at the center of the well facilitates the deposition of the protein during the preparation of the array.

The stability of the screen printed reference electrode was tested. Its potential stability was measured in the working buffer containing 0.5 M KCl using a saturated calomel electrode as standard. A medium value of the potential of 7 ± 0.2 mV with a drift of 0.25 mV h^{-1} was recorded for 24 h. An elapse time of 20 min was measured due to electrode hydration. The long-term stability was checked by measuring the potential value in a 0.5 M KCl solution, and it was found to be stable for more than two weeks. Furthermore, the mechanical stability of the printed electrode was investigated before and after plasma treatment and incubation in the ethanolic solution. The DuPont ink 5870 was found to possess the best combination of mechanical and electrochemical stability.

Electrochemical Characterization of the Array. As the P450 catalytic reaction is driven by the application of a negative potential bias, some of the drugs that have electroactive properties could be directly reduced at the gold surface. Therefore, we identify this as the only potential source of interference because the array platform is intended to be titrated with increasing concentrations of the pure drug. Nevertheless, in order to minimize this possible interference, the SAM was deemed to function not only as the anchoring point for the protein but also as a barrier limiting the diffusion of molecules to the gold surface. For this reason, a long chain alkenthiol was used to improve the insulation properties of the SAM, as the strong van der Waals interactions between the long aliphatic chains will make a compact and uniform layer (Figure 2).

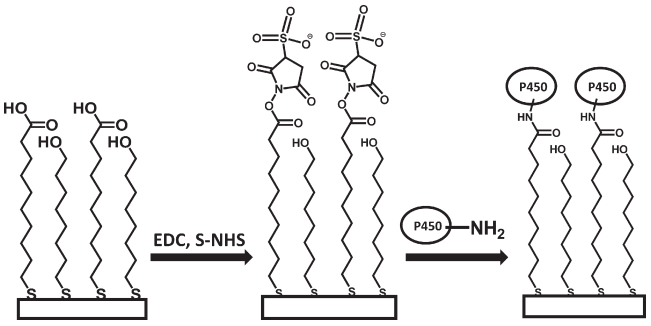

Figure 2. Scheme of the electrode modification. Deposition of 10-carboxydecanethiol and 8-hydroxyoctanethiol on the gold electrode to form a compact self-assembled monolayer, followed by the activation of the carboxylic groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (S-NHS). The P450 is then covalently linked via its surface amino groups.

Figure 3A shows the cyclic voltammograms (CVs) obtained at 50 mV/s, in a solution of 10 mM $K_3[Fe(CN)_6]$ in 1 M KCl, on a bare gold electrode and on an electrode on which the SAM was immobilized. The dramatic disappearance of the peaks in the second sample modified with the 10-carboxydecanethiol and 8-hydroxyoctanethiol (SAM, C_8/C_{10}) is a confirmation of the presence of an insulating layer on the gold surface. The self-assembled monolayer is creating a barrier that limits the diffusion of the redox molecules from the solution to the electrode but allows electron transfer to and from the protein by covalently binding the P450 enzyme to the electrode surface at a distance that is compatible with electron tunneling to the heme. Diffusion-controlled electron transfer is therefore limited in favor of the tunneling from the covalently bound protein.

Following the immobilization of the P450 onto the (C_8/C_{10}) SAM-modified gold surface, the electrochemical activity of the bound protein was investigated by CV in anaerobic conditions. Figure 3B shows the CV recorded in the absence of oxygen for P450 3A4 immobilized onto the C_8/C_{10} modified gold electrode in one of the wells of the array. The background signal of the solely SAM modified electrode is represented for comparison (dotted line). The CV of the bound enzyme shows two peaks at −480 mV and −290 mV with a resulting midpoint potential of −395 mV versus 0.5 M Ag/AgCl. These values are in the range of those reported in the literature for P450 on different electrode and different immobilization approaches.[10]

Testing of the Array. The pharmaceutical industry has shown a high interest in the ability to determine the K_M in vitro and in a high throughput way because the drug's specific K_M is an important parameter that contributes to the determination of the dosage in vivo. At present, K_M values are obtained from lengthy and costly LC-MS measurements carried out after incubation of the drug with a P450 reconstituted system, i.e., P450 plus a reductase and NADPH.

In our previous work we have shown that all the P450 chosen for this work, 3A4, 2D6, and 2C9, respond in the presence of

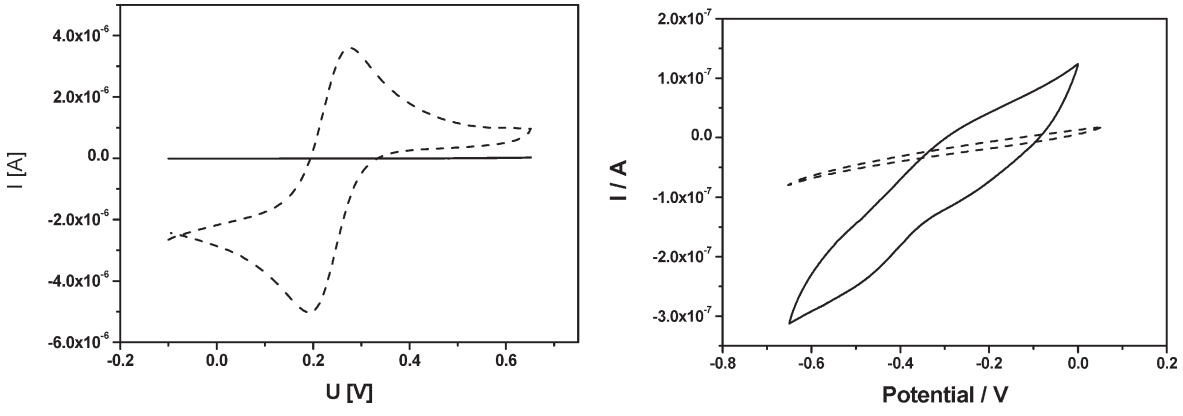

Figure 3. (A) Cyclic voltammetry of a solution of 10 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl on bare gold (dotted) and on 10-carboxydecanethiol and 8-hydroxyoctanethiol modified gold (solid line). Scan rate was 50 mV/s. (B) Cyclic voltammetry recorded from one well in the absence of oxygen using the 10-carboxydecanethiol and 8-hydroxyoctanethiol modified electrode with (solid line) and without (dotted line) bound P450 3A4. The CVs were recorded at 50 mV/s in 50 mM phosphate and 500 mM potassium chloride (pH 7.4) buffer.

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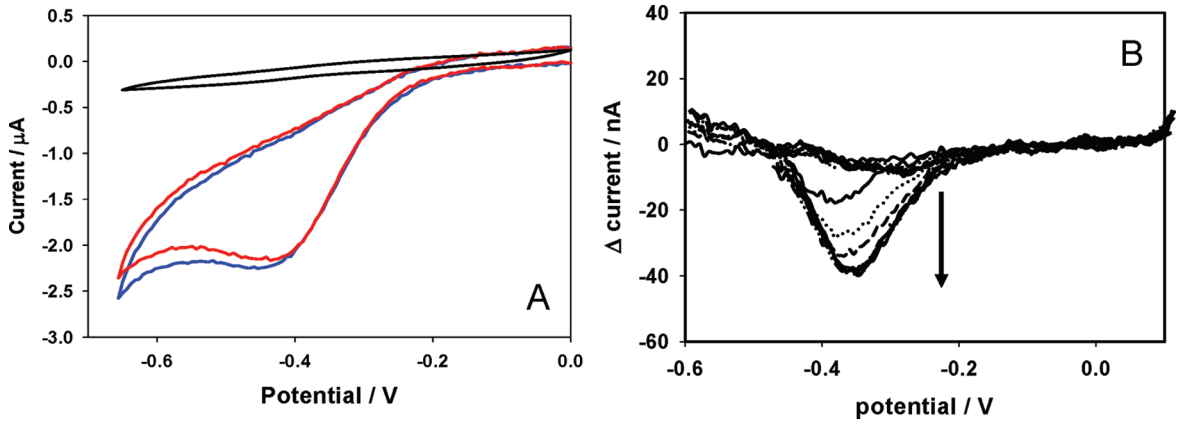

Figure 4. (A) Cyclic voltammetry recorded from one well of the electrode modified with 10-carboxydecanethiol and 8-hydroxyoctanethiol with bound P450 3A4 in the absence (black thin line) and in the presence (red line) of oxygen. The blue line represents the catalytic current recorded in the presence of oxygen and saturating concentrations (50 μM) of quindine. The CVs were recorded at 50 mV/s in 50 mM phosphate and 500 mM potassium chloride (pH 7.4) buffer. (B) Δ current as a function of the applied potential obtained by subtracting the CV recorded prior to any addition of substrate from the CVs recorded upon addition of substrate. The arrow indicates the direction of the catalytic current during the titration.

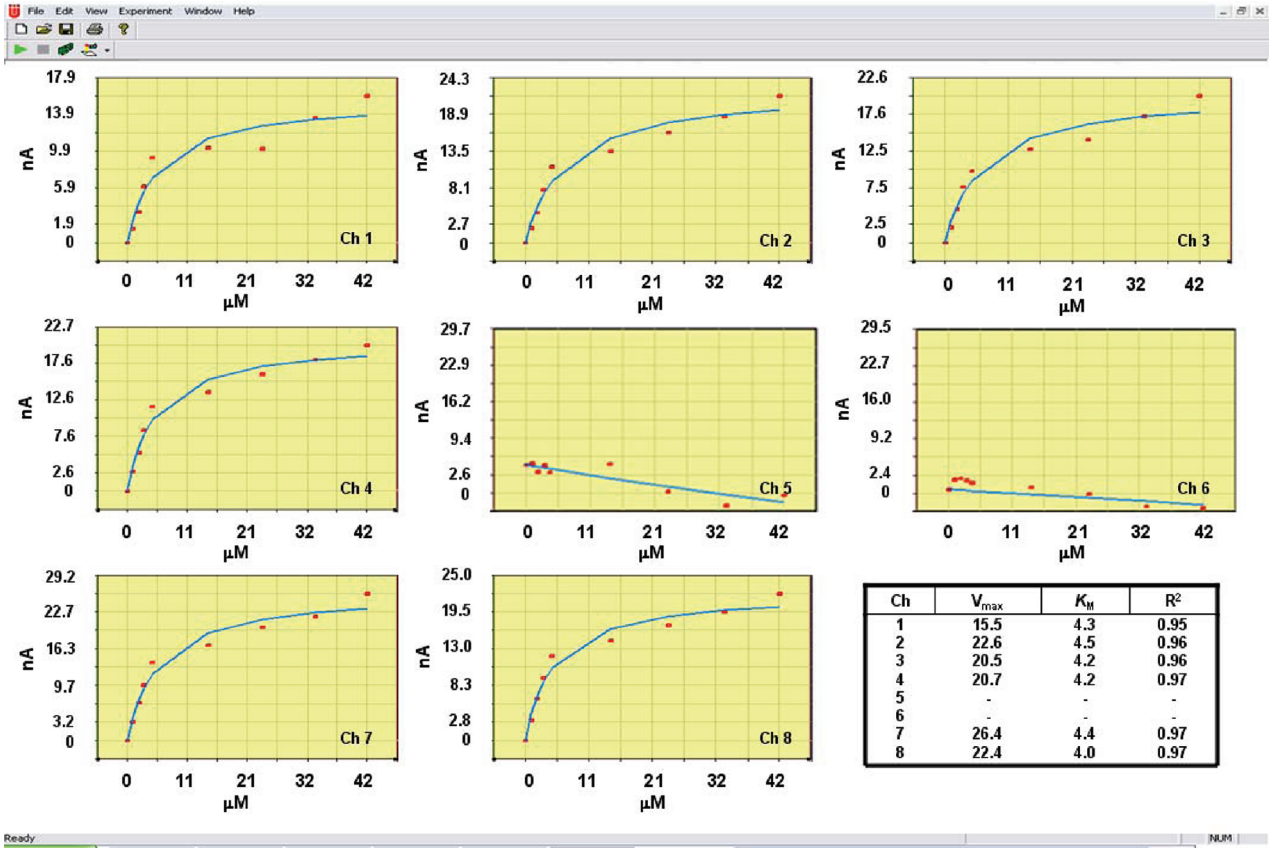

Figure 5. Screenshot example of the data analysis carried out by the custom-made macro in the Uniscan software. The eight panels represent the catalytic currents recorded in each individual well and plotted as a function of the added substrate. The data points are fitted with the Michaelis−Menten model, and the best fit is represented by the blue line. The fitting parameters are listed in the bottom right panel. All the wells contain the immobilized P450 2C9. Well number 5 and 6 are the control wells where equivalent volumes of buffer and solvent but no substrate are added. In all the other wells, fluoxetine was added and the concentrations (in μM) are indicated on the x-axis. The fitting parameters are given in the table on the bottom right. The V_{max} and K_M values are expressed in nA and μM respectively.

specific substrates with catalytic currents that increase as a function of the concentration of substrate, with a hyperbolic behavior in a Michaelis−Menten fashion.[18,21] Figure 4A shows the CVs recorded in the presence of oxygen and with saturating concentrations of substrate for P450 3A4 immobilized onto the C_8/C_{10} modified gold electrode in one of the wells of the array. The CV recorded in the absence of oxygen is also shown for comparison. The measured cathodic current increases significantly as expected

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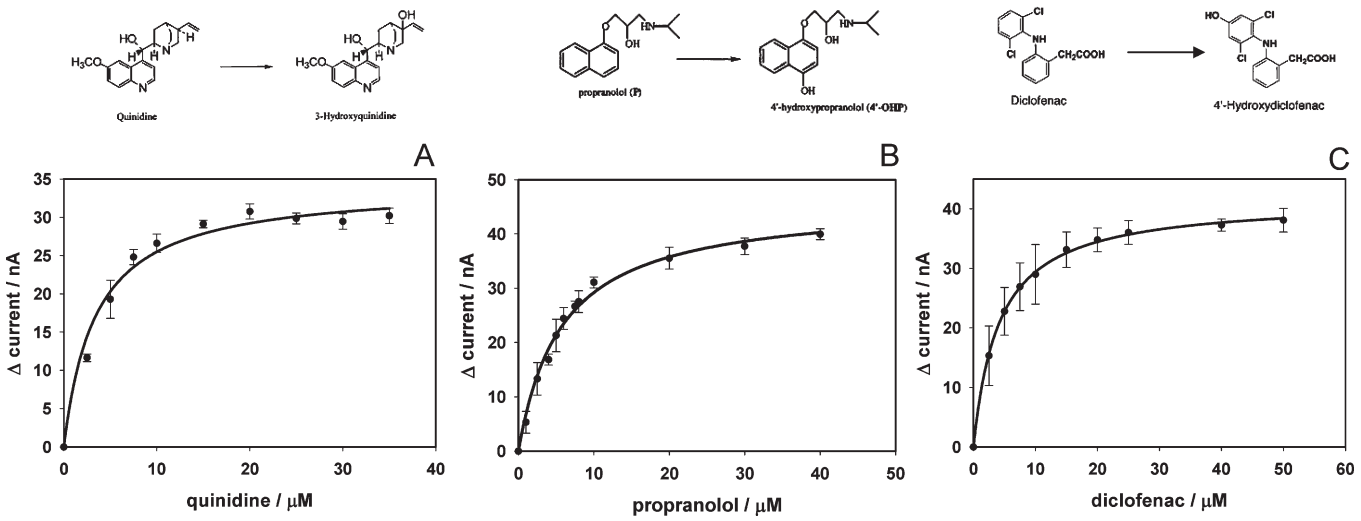

Figure 6. Michaelis−Menten plot based on the current measured at −390 mV upon addition of aliquots with increasing concentrations of substrate. (A) P450 3A4 titrated with quinidine, (B) P450 2D6 titrated with propranolol, and (C) P450 2C9 titrated with diclofenac. Structures of the substrates and products formed in the respective P450-catalyzed reactions are also shown.

from the consumption of electrons in the P450 catalytic cycle as represented by the overall reaction mechanism in eq 1.

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \qquad (1)$$

Figure 4B shows the current/voltage profiles as a function of increasing concentrations of substrate recorded in one well and represented as the current difference by subtracting at each CV the one recorded before any addition of substrate. The maximum difference in catalytic current occurs at values below −390 mV.

Plotting the values of catalytic current at −390 mV as a function of the concentration of added substrate shows a hyperbolic behavior that can be fitted with a Michaelis−Menten model. Figure 5 shows an example, as a screenshot, of the output recorded and analyzed from the eight wells. Using a custom-made macro written in collaboration with the manufacturer of the multichannel potentiostat, Uniscan Ltd., the series of CVs recorded from each channel in each well are analyzed in parallel. The catalytic currents are plotted as a function of the concentration of the added substrate, and the values are fitted with the Michaelis−Menten model. The fitting parameters are displayed together with the R^2 of the fitting.

The data obtained from the titrations with the same substrate in different wells were collated and refitted to improve the statistical significance of the determined K_M. Three examples of collated data obtained from the titration of three different substrates to their respective P450 are shown in Figure 6. The values of current are expressed as the current difference, and the structure of the products produced during the catalytic reaction are represented.

The data points were fitted to a Michaelis−Meneten model, and the calculated K_M values are presented in Table 1. The measured K_M values for each substrate (Table 1) are compared to the values reported in the literature.

These drugs are all known substrates of either P450 3A4, 2C9, or 2D6 for which K_M have been determined in vitro. They have been chosen to span a range of affinity from high (from less than 1 to 10 μM) to low (from 10 to more than 100 μM) for each respective P450 enzyme. The values reported from the literature are often indicated as a range because very often incubations with cytochromes P450 carried out on the same substrate but by different groups yield different results. One of the sources of variability has been associated with the source of the P450 enzymes used to determine the kinetic data, for example purified P450, microsomes, or hepatocytes. For this reason, the source of the enzyme used in the cited reference has been added to Table 1. Another source of variability are the conditions of the assay, for example the ratio between the P450 and its reductase. The range of values found in the literature, often quite large, is the motivation in the use of the standardized electrochemical array proposed here but at the same time makes it difficult to directly compare the results. For these reasons here it is proposed to look at these data in terms of ranking of the various substrates and not as absolute values.

From this point of view, the K_M values measured with the array rank the substrates in accordance to the ranking obtained from the literature values. This indicates the ability of the array to correctly determine the affinity of drugs for the specific P450, opening the possibility for its use in the early stage drug discovery process.

Array validation in terms of intra- and interarray variability was carried out for all the substrates. Precision is more difficult to evaluate, as there is no accepted gold standard in the literature where often imprecise values of K_M are accepted. If a more reliable method, such as the one proposed in this work, will be adopted, it will be possible to attain a set of data on which precision evaluation can be carried out. We therefore ranked and clustered the compounds according to defined ranges, and we used the standard deviation as indication of the performance of the assay. For each substrate, the standard deviation calculated across the wells of an array compare well with the standard deviation between wells of different arrays. The standard deviations indicated in Table 1 for each substrate were determined from the titrations in 24 individual wells (4 arrays) and are an indicator of both intra- and interarray variability. Titrations were performed as described in Materials and Methods , and in each array two wells were dedicated to the control experiments. In the control experiments, aliquots of buffer containing the same solvent used to dissolve the drugs were added to the well containing the

Table 1. Michaelis−Menten Constants (K_M) for P450 3A4, 2D6, and 2C9 with Their Respective Specific Substrates Determined Using the Microtiter Plate Electrochemical Platform[a]

		literature		
	substrate	system used	range of K_M (μM) [reference]	electrochemistry K_M (μM)
		P450 3A4		
high affinity	cyclosporine A	insect microsomes	1.2−2.2[30]	1.8 ± 0.8
	terfenadine	3A4/CPR fusion	7.32−14.1[31]	3.8 ± 2.2
	quinidine	Iinsect microsomes, HLM	1.5[32]	4.1 ± 0.6
	domperidone	insect microsomes HLM	3−15.7[33]	7.1 ± 1.9
low affinity	omeprazole	insect and human liver microsomes	60[34]	14.6 ± 4.8
	chlorzoxazone	HLM	160[35]	15.3 ± 6.5
	verapamil	microsomes	5.4−127[36]	39.0 ± 6.7
	clozapine	Insect microsomes	100−230[37]	41.1 ± 1.3
	propafenone	yeast microsomes, HLM	81−128[38]	43.7 ± 14.2
	theophylline	HLM microsomes	1400−2500[39]	206.4 ± 29.4
		P450 2D6		
high affinity	perhexiline	HLM	3.3[40]	2.4 ± 0.6
	fluoxetine	HLM	1.4−3.8[41]	4.4 ± 1.1
	propranolol	HLM	5.9−31.9[42]	4.6 ± 0.6
	propafenone	HLM	0.5[43]	7.1 ± 2.1
low affinity	clozapine	baculosomes reconstituted system	25[37]	9.3 ± 1.2
	sparteine	human insect microsomes	54.8−64[44]	9.7 ± 1.0
	metoprolol	yeast microsomes	19−46[45]	16.5 ± 2.8
	dextromethorphan	mice baculosomes	2.8−22[46]	33.0 ± 5.4
	debrisoquine	HLM baculosomes	12.1[47] 130[46]	62.7 ± 8.2
	codeine	HLM	1500[48] 150[46]	204.2 ± 27.8
		P450 2C9		
high affinity	warfarin	HLM	2.5[49] 18[50]	1.3 ± 0.6
	diclofenac	HLM	1.8[50] 15[51]	4.3 ± 2.4
	fluoxetine	HLM	9.7[41]	4.3 ± 1.5
low affinity	flurbiprofen	baculosomes	19.4[52]	13.6 ± 5.1
	ibuprofen	HLM	27.9[53]	17.5 ± 6.8
	piroxicam	baculosomes	30.5[52]	36.1 ± 7.1
	tolbutamide	yeast and *E.coli* microsomes	120−192[54] 60−580[46]	84.7 ± 6.7
	dextromethorphan	HLM	222−343[55]	109.1 ± 10.6
	naproxen	insect microsomes	116−307[56] 92−160[46]	117.1 ± 31.3
	melatonin	HLM	114−385[57]	203.7 ± 23.3

[a] The K_M values are compared to the literature data obtained from conventional incubation methodologies. The source of P450 used in the literature is also indicated.

immoblized P450. The position of the two control wells in the array was changed in different experiments to avoid the bias toward specific positions in the array. No catalytic current was detected in these control experiments, confirming that the measured catalytic current in the presence of the drug is generated by the P450 turnover.

A second type of control was also performed using unmodified arrays where only the SAM was deposited and no protein was added. Using these arrays, the substrates were titrated in order to assess any potential interference of the drugs on the electrochemical signal. For all tested drugs, no signal was detected in these experiments, as expected thanks to the insulating properties of the SAM.

Similarly to what was previously observed in an electrochemical microfluidic platform, the uncoupled nature of the human cytochrome P450s prevented the calculation of accurate k_{cat} values.[29] The intensity of current measured during catalysis is proportional to the turnover rate. Nevertheless, since in the P450 catalytic cycle the reducing equivalents can also be used for uncoupling reactions where oxygen is converted into superoxide radicals, hydrogen peroxide, or water, the proportionality factor that would allow the determination of the k_{cat} from the catalytic current remains unknown.

The time necessary to obtain a K_M value for the compound under investigation is 15 min. This is a very short time when compared with typical fluorescent measurements that are normally limited to end point measurements. Furthermore, assays making use of fluorescent substrates are based on competitive inhibition experiments with the drug yielding only indirect measurements of the affinity for a specific P450. Conversely, the platform presented in this work allows for a direct, reagentless, and fast determination of the K_M.

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CONCLUSIONS

The lack of a gold standard for the high throughput in vitro determination of the kinetic parameters for P450 drug metabolism results in the inability to predict with confidence the toxicological and dosage parameters for new potential drugs. The platform presented in this paper addresses some of these issues by developing a validated assay based on recombinant purified P450s, on highly pure reagents, and by having a disposable and standard format. The results of the titrations with the specific substrates clearly show how the electrochemical determination of the K_M with the array accurately ranks the affinity for the different drugs with high precision. Furthermore, the microtiter plate format allows for easy integration in the systems currently employed in the early drug discovery process with ease of handling and incorporation.

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REFERENCES

(1) Ortiz de Montellano, P. *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed.; Kluwer Academics/Plenum Publishers: New York, 2005.

(2) Rendic, S.; Di Carlo, F. J. *Drug Metab. Rev.* **1997**, *29*, 413–580.

(3) Walsky, R. L.; Obach, R. S. *Drug Metab. Dispos.* **2004**, *32*, 647–660.

(4) Ansede, J. H.; Thakker, D. R. *J. Pharm. Sci.* **2004**, *93*, 239–255.

(5) Crespi, C. L.; Miller, V. P.; Stresser, D. M. *Methods Enzymol.* **2002**, *357*, 276–284.

(6) Cohen, L. H.; Remley, M. J.; Raunig, D.; Vaz, A. D. N. *Drug Metab. Dispos.* **2003**, *31*, 1005–1015.

(7) Fantuzzi, A.; Fairhead, M.; Gilardi, G. *J. Am. Chem. Soc.* **2004**, *126*, 5040–5041.

(8) Bistolas, N.; Wollenberger, U.; Jung, C.; Scheller, F. W. *Biosens. Bioelectron.* **2005**, *20*, 24082423.

(9) Fleming, B. D.; Johnson, D. L.; Bond, A. M.; Martin, L. L. *Expert Opin. Drug Metab. Toxicol.* **2006**, *2*, 581–589.

(10) Sadeghi, S. J.; Fantuzzi, A.; Gilardi, G. *Biochim. Biophys. Acta* **2011**, *1814*, 237–248.

(11) Joseph, S.; Rusling, J. F.; Lvov, Y. M.; Friedberg, T.; Fuhr, U. *Biochem. Pharmacol.* **2003**, *65*, 1817–1826.

(12) Shumyantseva, V. V.; Ivanov, Y. D.; Bistolas, N.; Scheller, F. W.; Archakov, A. I.; Wollenberger, U. *Anal. Chem.* **2004**, *76*, 6046–6052.

(13) Johnson, D. L.; Lewis, B. C.; Elliot, D. J.; Miners, J. O.; Martin, L. L. *Biochem. Pharmacol.* **2005**, *69*, 1533–1541.

(14) Peng, L.; Yang, X.; Zhang, Q.; Liu, S. *Electroanal.* **2008**, *20*, 803–807.

(15) Liu, S.; Peng, L.; Yang, X.; Wu, Y.; He, L. *Anal. Biochem.* **2008**, *375*, 209–216.

(16) Yang, M. L.; Kabulski, J. L.; Wollenberg, L.; Chen, X. Q.; Subramanian, M.; Tracy, T. S.; Lederman, D.; Gannett, P. M.; Wu, N. Q. *Drug Metab. Dispos.* **2009**, *37*, 892–899.

(17) Mie, Y.; Suzuki, M.; Komatsu, Y. *J. Am. Chem. Soc.* **2009**, *131*, 6646–6647.

(18) Dodhia, V. R.; Sassone, C.; Fantuzzi, A.; Di Nardo, G.; Sadeghi, S. J.; Gilardi, G. *Electrochem. Commun.* **2008**, *10*, 1744–1747.

(19) Ferrero, V. E. V.; Andolfi, L.; Di Nardo, G.; Sadeghi, S. J.; Fantuzzi, A.; Cannistraro, S.; Gilardi, G. *Anal. Chem.* **2008**, *80*, 8438–8446.

(20) Mak, L. H.; Sadeghi, S. J.; Fantuzzi, A.; Gilardi, G. *Anal. Chem.* **2010**, *82*, 5357–5362.

(21) Panicco, P.; Dodhia, V. R.; Fantuzzi, A.; Gilardi, G. *Anal. Chem.* **2011**, *83*, 2179–2186.

(22) Alonso-Lomillo, M.; Gonzalo-Ruiz, J.; Domínguez-Renedo, O.; Muñoz, F. J.; Arcos-Martínez, M. J. *Biosens. Bioelectron.* **2006**, *23*, 1733–1737.

(23) Hendricks, N. R.; Waryo, T. T.; Arotiba, O.; Jahed, N.; Baker, G. L.; Iwuoha, E. I. *Electrochim. Acta* **2009**, *54*, 1925–1931.

(24) Ignaszak, A.; Hendricks, N.; Waryo, T. T.; Songa, E.; Jahed, N.; Ngece, R.; Al-Ahmed, A.; Kgarebe, B.; Baker, P.; Iwuoha, E. I. *J. Pharm. Biomed. Anal.* **2009**, *49*, 498–501.

(25) Shumyantseva, V. V.; Bulko, T. V.; Kuznetsova, G. P.; Samenkova, N. F.; Archakov, A. I. *Biochemistry (Moscow, Russ. Fed.)* **2009**, *74*, 438–444.

(26) Shumyantseva, V. V.; Supurun, E. V.; Bulko, T. V.; Dobrinina, O. V.; Archakov, A. I. *Biochemistry (Moscow, Russ. Fed.) Suppl.* **2010**, *4*, 25–36.

(27) Shumyantseva, V. V.; Bulko, T. V.; Suprun, E. V.; Chalenko, Y. M.; Vagin, M. Y.; Rudakov, Y. O.; Shatskaya, M. A.; Archakov, A. I. *Biochim. Biophys. Acta* **2011**, *1814*, 94–101.

(28) Dodhia, V. R.; Fantuzzi, A.; Gilardi, G. *J. Biol. Inorg. Chem.* **2006**, *11*, 903–916.

(29) Fantuzzi, A.; Capria, E.; Mak, L. H.; Dodhia, V. R.; Sadeghi, S. J.; Collins, S.; Somers, G.; Huq, E.; Gilardi, G. *Anal. Chem.* **2010**, *82*, 10222–10227.

(30) Dai, Y.; Iwanaga, K.; Lin, Y. S.; Hebert, M. F.; Davis, C. L.; Huang, W.; Kharasch, E. D.; Thummel, K. E. *Biochem. Pharmacol.* **2004**, *68*, 1889–1902.

(31) Rodrigues, A. D.; Mulford, D. J.; Lee, R. D.; Surber, B. W.; Kukulka, M. J.; Ferrero, J. L.; Thomas, S. B.; Shet, M. S.; Estabrook, R. W. *Drug Metab. Dispos.* **1995**, *23*, 765–775.

(32) Ngui, J. S.; Tang, W.; Stearnis, R. A.; Shou, M.; Miller, R. R.; Zhang, Y.; Lin, J. H.; Baillie, T. A. *Drug Metab. Dispos.* **2000**, *28*, 1043–1050.

(33) Ward, B. A.; Morocho, A.; Kandil, A.; Galinsky, R. E.; Flockhart, D. A.; Desta, Z. *Br. J. Clin. Pharmacol.* **2004**, *58*, 277–287.

(34) Yamazaki, H.; Inoue, K.; Shawn, P. M.; Checovich, W. J.; Guengerich, P. F.; Shimada, T. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 434–442.

(35) Shimada, T.; Tsumura, F.; Yamazaki, H. *Drug Metab. Dispos.* **1999**, *27*, 1274–1280.

(36) Tracy, T. S.; Korzekwa, K. R.; Gonzalez, F. J.; Wainer, I. W. *Br. J. Clin. Pharmacol.* **1999**, *47*, 545–552.

(37) Linnet, K.; Olsen, O. V. *Drug Metab. Dispos.* **1997**, *25*, 1379–1382.

(38) Botsch, S.; Gautier, J.-C.; Beaune, P.; Eichelbaum, M.; Kroemer, H. K. *Mol. Pharmacol.* **1992**, *43*, 120–126.

(39) Zhang, Z-Y; Kaminsky, L. S. *Biochem. Pharmacol.* **1995**, *50*, 205–211.

(40) Sorensen, L. B.; Sorensen, R. N.; Miners, J. O.; Somogyi, A. A.; Grgurinovich, N.; Birkett, D. J. *Br. J. Clin. Pharmacol.* **2003**, *55*, 635–638.

(41) Ring, B. J.; Eckstein, J. A.; Gillespie, J. S.; Binkley, S. N.; Vandenbranden, M.; Wrighton, S. A. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 1044–1050.

(42) Johnson, J. A.; Herring, V. L.; Wolfe, M. S.; Relling, M. V. *J. Pharmacol. Exp. Therap.* **2000**, *294*, 1099–1105.

(43) Kroemer, H. K.; Botsch, S.; Heinkele, G.; Schick, M. *Methods Enzymol.* **1996**, *272*, 99–105.

(44) Evert, B.; Eichelbaum, M.; Haubruck, H.; Zanger, U. M. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1997**, *355*, 309–318.

(45) Ellis, S. W.; Rowland, K.; Ackland, M. J.; Rekka, E.; Simula, A. P.; Lennard, M. S.; Wolf, C. R.; Tucker, G. T. *Biochem. J.* **1996**, *316*, 647–654.

(46) Pelkonen, O.; Turpeinen, M.; Hakkola, J.; Honkakoski, P.; Hukkanen, J.; Raunio, H. *Arch. Toxicol.* **2008**, *82*, 667–715.

(47) Granvil, C. P.; Krausz, K. W.; Gelboin, H. V.; Idle, J. R.; Gonzalez, F. J. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 1025–1032.
(48) Yue, Q. Y.; Sawe, J. *Eur. J. Clin. Pharmacol.* **1997**, *52*, 41–47.
(49) Tatsumi, A.; Ikegami, Y.; Morii, R.; Sugiyama, M.; Kadobayashi, M.; Iwakawa, S. *Biol. Pharm. Bull.* **2009**, *32*, 517–519.
(50) Yamazaki, H.; Inoue, K.; Chiba, K.; Ozawa, N.; Kaway, T.; Suzuki, Y.; Goldstein, J. A.; Guengerich, F. P.; Shimada, T. *Biochem. Pharmacol.* **1998**, *56*, 243–251.
(51) Mancy, A.; Antignac, M.; Minoletti, C.; Dijols, S.; Mouries, V.; Duong, N-T. H.; Battioni, P.; Dansette, P. M.; Mansuy, D. *Biochemistry* **1999**, *38*, 14264–14270.
(52) Tracy, T. S.; Hutzler, J. M.; Haining, R. L.; Rettie, A. E.; Hummel, M. A.; Dickmann, L. J. *Drug Metab. Dispos.* **2002**, *30*, 385–390.
(53) Andersson, T. B.; Bredberg, E.; Ericsson, H.; Sjoberg, H. *Drug Metab. Dispos.* **2004**, *32*, 715–721.
(54) Iwata, H.; Fujita, K.; Kushida, H.; Suzuki, A.; Konno, Y.; Nakamura, K.; Fujino, A.; Kamataki, T. *Biochem. Pharmacol.* **1998**, *55*, 1315–1325.
(55) Yu, A.; Haining, R. L. *Drug Metab. Dispos.* **2001**, *29*, 1514–1520.
(56) McGinnity, D. F.; Griffin, S. J.; Moody, G. C.; Voice, M.; Hanlon, S.; Friedberg, T.; Riley, R. J. *Drug Metab. Dispos.* **1999**, *27*, 1023.
(57) Facciola', G.; Hidestrand, M.; von Bahr, C.; Tybring, G. *Eur. J. Clin. Pharmacol.* **2001**, *56*, 881–888.