

The Role of *In Vitro* ADME Assays in Antimalarial Drug Discovery and Development

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Abstract: The high level of attrition of drugs in clinical development has led pharmaceutical companies to increase the efficiency of their lead identification and development through techniques such as combinatorial chemistry and high-throughput (HTP) screening. Since the major reasons for clinical drug candidate failure other than efficacy are pharmacokinetics and toxicity, attention has been focused on assessing properties such as metabolic stability, drug-drug interactions (DDI), and absorption earlier in the drug discovery process. Animal studies are simply too labor-intensive and expensive to use for evaluating every hit, so it has been necessary to develop and implement higher throughput *in vitro* ADME screens to manage the large number of compounds of interest.

The antimalarial drug development program at the Walter Reed Army Institute of Research, Division of Experimental Therapeutics (WRAIR/ET) has adopted this paradigm in its search for a long-term prophylactic for the prevention of malaria. The overarching goal of this program is to develop new, long half-life, orally bioavailable compounds with potent intrinsic activity against liver- and blood-stage parasites. From the WRAIR HTP antimalarial screen, numerous compounds are regularly identified with potent activity. These hits are now immediately evaluated using a panel of *in vitro* ADME screens to identify and predict compounds that will meet our specific treatment criteria. In this review, the WRAIR ADME screening program for antimalarial drugs is described as well as how we have implemented it to predict the ADME properties of small molecule for the identification of promising drug candidates.

Keywords: ADME, antimalarial drugs, metabolism, mass spectrometry, drug-drug interactions, permeability, modeling.

INTRODUCTION

The cost of drug discovery and development has reached exorbitant levels and is currently estimated to be greater than 800 million dollars per drug entity reaching the market [Discovery.com, FDA.com]. The majority of this cost is incurred during the clinical phase of drug development. Having a compound fail in the later stages of clinical development or, even worse, after release into the market can be devastatingly expensive even to the largest pharmaceutical organizations. In addition to toxicity, one of the primary reasons for the clinical failure of a compound is a poor pharmacokinetic profile [1, 2]. The pharmacokinetic properties of a compound are the summation of its absorption (A), distribution (D), metabolism (M), and excretion (E) characteristics, collectively described as ADME.

Pharmaceutical development of small molecules for the treatment of disease must carefully consider the pharmacokinetic properties of each compound prior to pursuing it as a drug candidate. Antimalarial drug candidates are no exception. Although small molecules that exhibit potent antimalarial properties are regularly discovered in various screening programs, most of these compounds will never reach clinical use, primarily because of poor pharmacokinetic and/or toxicity profiles. Before pharmacokinetic studies are undertaken, several factors

related to the desired indication must be considered. For instance: What is the goal of the antimalarial drug program? Will the drug be a treatment, prophylactic, or both? What is the intended route of administration? What is the potency of the compound? What are the potential toxicities associated with the compound? Once the desired indication is defined, selected candidates must then be evaluated for properties that are appropriate to the indication such as half-life, membrane permeability, and mechanisms of elimination. Traditionally, these properties have been evaluated in labor- and resource-intensive animal studies. With the implementation of rapid synthetic processes and high-throughput (HTP) screening systems, however, it is now implausible for even the most highly-funded pharmaceutical companies to conduct animal studies on the number of active compounds being identified. Although animal studies will continue to be required for pre-clinical development for the foreseeable future, several pharmaceutical organizations have implemented a battery of *in silico* and *in vitro* test systems to screen out and prioritize compounds based on desirable pharmaceutical properties. To be effective, these screening systems are placed early in pre-clinical development in order to limit the number of compounds requiring expensive evaluations in animal models and to provide a rational approach to lead optimization.

Development of antimalarial compounds, particularly for prophylaxis, requires that a number of ADME considerations be met. First and foremost, administration of antimalarial compounds for treatment (with the exception of severe malaria) or prophylaxis must be for the most part through a "user friendly" route. Thus, priority must be given to develop drugs that can be administered through an oral or

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possibly transdermal route. This requirement is primarily due to the lack of medical facilities, compliance issues, and other logistical problems associated with the locations and populations affected by malaria. Secondly, the half-life of antimalarial drugs is of critical concern. For long-term prophylactic treatments, a long half-life is desirable so that treatment regimens can be set to a minimum of once a day up to once a month. On the other hand, a drug intended to be used as a treatment for malaria in endemic areas should have a shorter half-life [3-6]. A shorter half-life allows the drug to be rapidly cleared from the body once the patient is cured of the disease. The characteristic helps to prevent the development of resistant parasites from new infections that are exposed to sub-therapeutic levels of the drug. Another important concern is that patients being treated for malaria as well as those taking long-term prophylactics are likely to be exposed to other drugs during the treatment period. A significant interaction with one of the CYP enzymes could easily prevent final approval of an antimalarial drug, especially with the trend toward combination therapies intended to prevent the development of drug resistance [7]. For this reason, it is important to consider potential drug-drug interactions (DDI) early in the antimalarial drug development process [8].

This review will introduce a number of commonly used *in vitro* ADME screening assays that are particularly relevant to the development of antimalarial drugs. The techniques mentioned here have been extensively published and reviewed elsewhere so detailed methods of each are not presented. In addition, this review will also present the ADME program at Walter Reed Army Institute of Research, Division of Experimental Therapeutics (WRAIR ET) currently employed for the discovery and development of antimalarial drugs.

ABSORPTION

Oral administration is the most common desired pathway for the delivery of therapeutic compounds. Due to several confounding considerations including patient compliance and healthcare related issues found in developing countries, oral bioavailability is especially critical for antimalarial drugs. For drugs delivered via the oral route, the first barrier to systemic exposure is the membrane lining the gut wall. For this reason, the ability of a molecule to be orally absorbed is one of the most important criteria for deciding whether the molecule is a potential lead candidate. The ability of a compound to pass through the gut membrane is due to a number of structurally-related factors including molecular weight, polar surface area, lipophilicity, and others [9]. While individually these are all important parameters, it is difficult to predict the sum of their effects on the overall membrane permeability of a particular compound. Several established *in vitro* assays are described below that provide a reasonably good prediction of oral bioavailability. In order to fully understand the advantages and disadvantages of each model, however, it is helpful to be aware of the normal physiology of and the absorption process in the gut.

There are several possible mechanisms for the absorption of small molecules. Passive paracellular transport is an aqueous extracellular route across the epithelium. The

driving forces for passive diffusion are the electrochemical potential gradients derived from differences in concentration, electrical potential, and hydrostatic pressure between the two sides of the epithelium. Passive transcellular transport involves the movement of solute molecules across the apical membrane, through the cell cytoplasm, and across the basolateral membrane. This mechanism is the main route of permeation for hydrophobic compounds. Carrier-mediated transport is a process involving active transport via membrane associated transporters such as p-glycoproteins (PgPs). The small intestinal mucosa express a large number of absorption transporters, which are responsible for the absorption of nutrients and vitamins. As their names suggest, influx transporters can increase intestinal drug absorption whereas efflux transporters can have the opposite effect. Finally, enterocytes possess vesicular transport processes that can facilitate drug absorption. These processes include fluid-phase endocytosis (FPE), where solute molecules dissolved in the luminal fluid are incorporated by bulk transport into the fluid-phase of endocytic vesicles, and receptor-mediated endocytosis (RME), generally of importance only for the mucosal permeation of macromolecules, but not for small molecules [10].

As discussed above, membrane permeability in the gut occurs via several possible mechanisms. However, passive diffusion is by far the most important for small molecule therapeutics. For this reason, most of the *in vitro* models for predicting oral absorption focus on passive diffusion. Also, active transporters are highly expressed in certain regions of the gut and can play a significant role in preventing the adequate absorption of some molecules. It is therefore important to consider incorporating a permeability model that will provide information regarding a compound's susceptibility to efflux mechanisms [10].

Currently, there are two widely utilized methods for modeling intestinal permeability that do not involve biological preparations, the Parallel Artificial Membrane Permeation Assay (PAMPA) and the Immobilized Artificial Membrane (IAM). First, PAMPA is a method described by Kansy *et al.* [11] that utilizes phospholipid-coated filters to estimate membrane permeability. The assay is typically carried out in a 96-well plate format and measures the ability of compounds to diffuse from a donor to an acceptor compartment. The compartments are separated by a 0.45 μm pore size PVDF membrane filter impregnated with a solution of egg lecithin dissolved in dodecane. Immediately following the addition of the artificial membrane to the PVDF membrane filter, the 96-well filter plate (known as the "Donor" plate) is filled with buffer solutions containing the compounds to be tested. The Donor plate is then placed upon a 96-well Acceptor plate filled with sufficient buffer so that there is liquid contact between the buffer in the Acceptor plate and the PVDF membrane filter. The Donor and Acceptor plates are incubated together for 12–16 hours after which the plates are separated and the concentration of compound in the Acceptor compartment is determined by UV/Vis measurements or LC/MS when necessary [11-13]. The primary advantages of techniques utilizing artificial membranes are their reproducibility and potential for higher throughput. The 96-well format PAMPA assays can usually be read by most modern plate readers in under a minute, rather than the hours it would take to analyze each sample

individually by LC/MS. In addition, unlike biological systems, it is possible to conduct experiments at a wide range of pH levels, which is often an important factor in the absorption of certain compounds. Also, experiments using artificial membranes are relatively easy and much less resource-dependent than cell culture models. The major disadvantages, however, include the lack of enzymes, influx and efflux transporters, and modeling of paracellular pathways. These disadvantages can lead to misleading results and should always be considered when relying solely on artificial membranes to predict oral bioavailability.

The IAM is a solid-phase membrane mimetic system prepared by covalently binding membrane-forming lipids to an amorphous silica substrate. Essentially, an IAM is a reverse-phase liquid chromatography column. Instead of a hydrocarbon based substrate, IAMs utilize lipids suggested to mimic the lipid environment of the cell membrane. More lipophilic compounds will interact with the lipid phase of the IAM resulting in a longer retention in the column (or higher capacity, 'k') and are therefore considered to have better permeability properties. IAMs have been correlated to Caco-2 cell permeability assays [11, 14]. Although IAMs do not require cell culture facilities, LC/MS analysis is still required. Unlike cell culture models, the LC/MS analysis is not suitable to fast gradients or other rapid analytical techniques and can lead to lengthy analytical times. In addition, Hidalgo [10] suggested IAMs were not predictive across classes of compounds and may only be valid for ranking compounds of similar structures.

As mentioned above, artificial membranes provide the potential for higher throughput capability and are less resource dependent but can lead to misleading absorption predictions. For this reason, cell culture permeability models remain an important part of ADME screening programs in the pharmaceutical industry. Cell culture models for drug absorption are based on the assumption that passage of drugs across the intestinal epithelium is the main barrier for drugs to reach the portal circulation [15]. The Caco-2 cell line, derived from a human colorectal carcinoma, has been established as an *in vitro* model for predicting drug absorption across the human intestine [16].

Caco-2 cells cultured on semi-permeable membranes differentiate into a highly functional epithelial barrier with remarkable morphological and biochemical similarities to the small intestinal columnar epithelium. The Caco-2 assay system facilitates the measurement of drug absorption rates and has been used for several years to predict and rank-order compounds throughout the pharmaceutical industry. The apparent permeability coefficients (P_{app}) obtained from Caco-2 transport studies have been shown to correlate well to human intestinal absorption [17-20]. As an added advantage, these cells express a number of P-gP-type transporters. By performing apical-to-basal (A to B) and basal-to-apical (B to A) transport studies, these cells provide valuable information regarding a drug's susceptibility to active transport [10]. Figure 1 displays a representative diagram of the Caco-2 cell assay system. Although widely used in the pharmaceutical industry to predict oral bioavailability, Caco-2 cells have only previously been used on a limited basis for antimalarial drugs. Augustijns *et al.*, 1996 [21], and Augustijns, 1996 [22] utilized Caco-2 cells to evaluate the transport of Artemisinin compounds and chloroquine, respectively. Recently, we have implemented the use of Caco-2 cells at WRAIR to aid in the rank ordering of compounds for oral absorption. The utility and predictability of this assay for antimalarial drugs is currently being evaluated.

The next most common cell culture permeability model is the use of the Main Darby Canine Kidney (MDCK) cell line in a similar fashion to that of the Caco-2 system. MDCK cells are a common model for studying drug transport mechanism in distal renal epithelia. Permeability (P_{app}) measurements with this system have been correlated to those observed for Caco-2 cells [10, 17]. This primary disadvantage of the MDCK cell line is that their expression of various transporters and relevant enzymes has not been well characterized. MDCK cells transfected with human MDR1 cDNA, however, are commercially available and have been used to measure both passive transcellular diffusion and P-glycoprotein mediated efflux [23-27]. As a consequence, MDCK cells are gaining acceptance within the pharmaceutical industry as an alternate model to Caco-2

Caco-2 or MDCK Cell Culture Membrane Permeability

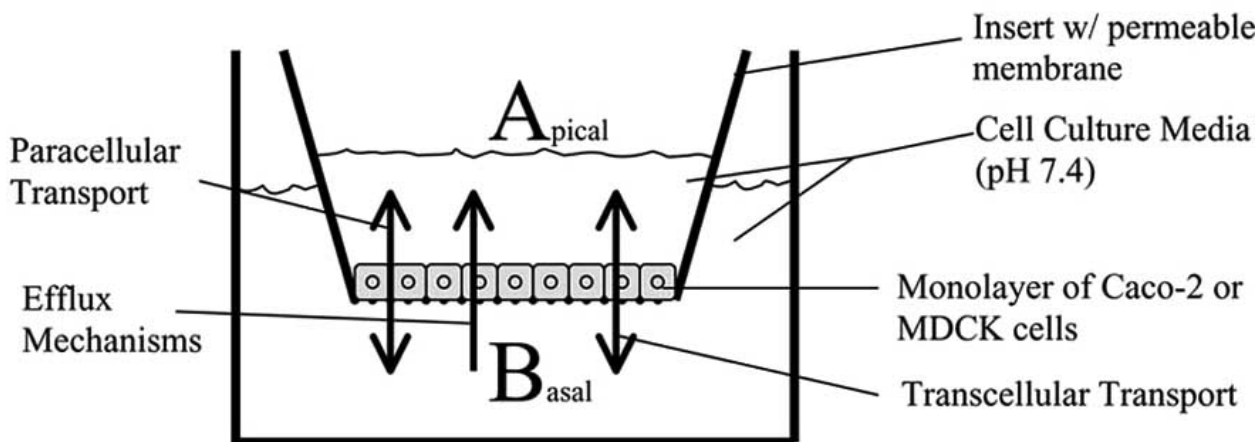


Fig. (1). Diagram representing the major components of the cell culture membrane permeability models.

cells. One of the most significant advantages of this system is that the MDCK cells are much easier to culture and grow more rapidly than Caco-2 cells making the MDCK assay more amenable to HTP operations.

The major advantages of these cell culture models are the functional characteristics, especially with regard to transporter mechanisms. On the other hand, these assays are much more technically difficult, resource- and time-consuming, and require extensive sample analysis by LC/MS. Due to the typical sample load, numbers of different compounds, and low drug concentrations, it is impractical to analyze Caco-2 or MDCK permeability samples in a high-through-put mode by LC/UV or most other analytical techniques. Therefore, LC/MS analytical development and assay time are common bottlenecks for these screens.

METABOLIC STABILITY

Once a compound is absorbed through gut membrane, it enters the circulation and is immediately subjected to clearance mechanisms. As with permeability, the clearance mechanism(s) and rate of a particular drug are directly related to its structural characteristics. Among others, molecular weight, lipophilicity, solubility, metabolic liability, and plasma protein binding play important roles in determining the clearance of drugs [28-31]. Unbound drugs are cleared from the body by a number of mechanisms. First, they can be removed directly by excretion into the urine or the bile (subsequently the feces). Alternatively, they can be cleared after chemical modifications by the enzymatic activities of a number of different metabolizing enzymes found in several different organ systems [32].

The primary purposes of *in vitro* metabolism assays in drug discovery and development are to guide lead prioritization and lead optimization. First, *in vitro* metabolism can be used to rank order a series of candidates by predicting the *in vivo* half-life. Using a variety of methods (discussed in greater detail below), *in vitro* metabolism can predict the *in vivo* hepatic clearance rate [28, 33, 34]. Secondly, by combining metabolic stability with structural analysis and metabolite identification, *in vitro* metabolism can be used to identify structural liabilities within promising compounds that could be modified to improve half-life without sacrificing the compound's potency [35].

In general, xenobiotic metabolism makes lipophilic compounds more water soluble and thus more easily excreted in the urine. Although significant metabolism can occur in the small intestine and other organs, the liver possesses the most and highest concentration of metabolizing enzymes and plays the greatest role in metabolic clearance of most compounds. For this reason, *in vitro* drug metabolism assays have focused on various preparations of liver tissues to predict metabolic stability [31]. Several different methods are available for determining metabolic stability of compounds and are reviewed extensively elsewhere. For this reason, this review will briefly describe the most commonly used systems and the advantages of each.

The most common method for determining metabolic stability is through the use of human and/or animal liver microsomes. Microsomes are easily generated by homogenizing liver tissues following by differential centrifugation steps to isolate the broken endoplasmic reticulum membranes. These membranes form spherical particles that contain several metabolizing enzymes including cytochrome P450 (CYP) enzymes, flavin-containing monooxygenases, glucuronyl transferases, as well as several others. In addition, both pooled human and animal liver microsomes are commercially available providing a convenient and reliable source. The primary advantage of microsomes is their ease of use and the ability to store them for extended periods (years) without losing metabolic activity.

Microsomes are typically used in the drug screening process for determining metabolic stability (a.k.a *in vitro* intrinsic clearance), which can then be correlated to *in vivo* metabolic clearance. *In vitro* intrinsic clearance is most accurately determined by measuring the kinetic parameters (K_m and V_{max}) associated with the formation of known metabolites. In drug discovery, however, the metabolites are often not known and standards are not available for quantification. Therefore, *in vitro* $t_{1/2}$ (the time point at which 50% of the parent compound is remaining) is typically used by measuring the disappearance of the parent compound. Recently, Li *et al.*, 2003 [36], utilized a combination of human and rat liver microsomes to determine the intrinsic clearance of a number of antiparasitic and antimalarial drugs. Using this *in vitro* data, they were able to classify the compounds as high clearance, low clearance, and intermediate clearance drugs. With the exception of artemisinin, the *in vitro* data accurately predicted what is observed for these drugs *in vivo*.

As an alternate to the use of microsomes, the use of S9 liver fractions has become increasingly popular in metabolic stability assays. The S9 fraction is a cellular liver fraction that contains both the microsomal and some of the cytosolic fraction, and as a result, contains both Phase I and Phase II metabolizing enzymes. Although not as well characterized as liver microsomes, the S9 fraction has the potential to provide more information about the *in vivo* metabolism of a candidate, especially if metabolic stability samples are to be used in conjunction with metabolite identification. Metabolic stability assays using S9 fractions are conducted similarly to those conducted with microsomes [37, 38].

Metabolic stability experiments are typically conducted in miniaturized assays between 0.1-2 mL containing 0.1 to 2 mg/mL of microsomal protein, 0.2-0.5 units/mL of glucose-6-phosphate dehydrogenase (G6PD), and a NADPH regenerating system in a phosphate or Tris buffer (pH 7.4) incubated at 37°C. Assays can be conducted using a variety of drug concentrations, however, a range of 1-5 μ M is typically used to avoid saturation or inhibition of specific enzymes systems by the parent compound. Metabolic stability assays can be conducted for as long as the integrity of the enzyme system (microsomes as well as regenerating system) remains intact (up to 2-4 hours), but they are typically completed in 30 to 60 minutes. For general considerations in HTP screens, Masimirembwa *et al.* [39] suggest that microsomal protein concentrations not exceed

0.5 mg/ml, test compound concentrations not exceed 10 μ M, and incubation times not go beyond 60 minutes. There are a variety of means for stopping the reaction, but it generally involves either solvent quenching using acetonitrile or methanol, or addition of a buffer containing TCA. Both means are designed to eliminate the enzymatic activity and assist in the precipitation of microsomal proteins. Assay tubes or plates are then centrifuged or filtered to remove proteins and much of the buffering salts that would interfere with LC/MS analysis. For detailed methods on the use of HTP *in vitro* metabolism assays see Masimirembwa *et al.* [39] or Korfmacher *et al.* [40].

Depending on the throughput required and the resources available, some ADME programs utilize human or rat liver hepatocytes for conducting metabolic stability assays. Several groups have reported success using both fresh and cryopreserved hepatocytes. The use of hepatocytes provides significant advantages to that of microsomes or S9 fractions. For a complete review of the use of hepatocytes see Sinz (1999) [41]. First of all, properly cultured hepatocytes express the Phase I and Phase II metabolizing enzymes and therefore provide a more realistic approximation of liver metabolism. In addition, metabolic stability samples incubated with hepatocytes can easily be used for metabolite ID to identify both Phase I and Phase II metabolites. Perhaps most importantly, unlike microsomes and S9 fractions, hepatocytes have intact cell membranes and express membrane transporters providing a more realistic barrier that the compound must pass through in order to be metabolized. The use of hepatocytes thus helps to screen for the ability of a compound to be transported through the hepatocyte membrane due to poor membrane permeability or active transport processes. This helps to alleviate the inherent problem in cell-free systems of the possible overestimation of clearance. Hepatocytes have been used successfully by several groups to predict *in vivo* clearance.

Liver slices are also used by some groups for conducting metabolic stability assays, although to a much lesser extent. In this technique, drugs are incubated with thinly sliced pieces of freshly isolated livers (usually mouse or rat) in culture media for selected periods of time. Although this technique can provide valuable information towards predicting the *in vivo* metabolic rate and primary metabolic pathways for drugs, it is not readily amenable to high-throughput. The use of liver slices has previously been reviewed by Ekins *et al.* [42].

METABOLITE IDENTIFICATION

Once candidate compounds have been evaluated for their metabolic stability, the *in vitro* metabolic profile of selected drugs is often investigated. The goal of these studies may be to discover species differences in metabolism that can aid in understanding animal efficacy and pharmacokinetic results. In addition, in compounds with a short metabolic half-life, these studies may identify the metabolic pathway responsible and can be used to suggest sites of chemical modification to help optimize the structure-property relationship while maintaining good activity. As an integral part of lead optimization, these new compounds can be synthesized and screened, and the results used to optimize the structure-activity relationship of the class of compounds.

In vitro metabolite ID experiments are usually performed as described above for metabolic stability with incubations of drug and species microsomes. Analysis of samples is typically done using liquid chromatography tandem mass spectrometry (LC-MS/MS), and preliminary structural elucidation can be done. LC-MS/MS has an appropriate balance of speed, selectivity, reliability, and sensitivity that make it suitable for a fast-paced discovery and development program [35]. Mass/charge ratios of metabolites, in addition to the knowledge of the chemical structure of the parent compound, allow for preliminary assignment of the route of metabolism, e.g. hydroxylation, dealkylation, hydrolysis, and so forth. In the case of incubations with hepatocytes or S9, Phase II conjugation reactions such as sulfation, acetylation, and glucuronidation may also be observed. Tandem mass spectrometry (MS/MS) can then be used to attempt to pinpoint the sites of metabolism, although structure determination is not always unambiguous. The limiting factor for this identification is the correct interpretation of MS/MS fragmentation data, and this can be quite time-consuming for the scientist. Use of software that predicts fragmentation, such as Mass Fragmenter (Advanced Chemistry Development) or Mass Frontier (Thermo Electron Corp.) can facilitate this type of analysis, although hands on expertise is still usually needed. Other tools include databases such as the Metabolite Database (MDL Information Systems Inc.) that contains a library of transformations of >8000 parent structures, or Metabolite ID (Thermo Electron Corp) that aids in processing MS/MS data and in making assignments of metabolites by relation to the parent fragmentation.

Other mass spectrometric techniques or tools can be used to help distinguish compound-related signals from endogenous background. At the simplest level, metabolites of compounds containing atoms with more than one abundant isotope, such as chlorine or bromine, are easy to distinguish from background because of their distinctive isotope patterns. Other techniques, such as use of inductively-coupled plasma MS (ICP-MS), allow LC-MS chromatograms to be specifically generated for compounds possessing a limited number of heteroatoms. This can facilitate the identification of metabolites containing these particular atoms; specifically sulfur, bromine, and chlorine [35]. High resolution MS can also help overcome problems with selectivity that result either from the inability to distinguish isobaric ions or because of endogenous interferences. Time-of-flight (TOF) mass spectrometers can be used to generate high-resolution mass data and assign a molecular formula. With this type of instrument, however, tandem mass spectrometry must be performed separately: this has led to the development of hybrid quadrupole-TOF (Q-TOF) instruments, which can collect high-resolution MS data of both parent and product (MS/MS fragment) ions.

For most compounds taken through the screening process, the level of identification achievable by these methods is sufficient. For more detailed investigations, either for *in vitro* or *in vivo* metabolism studies, confirmation of the structure (including regioselectivity of hydroxylation) can be accomplished using nuclear magnetic resonance spectroscopy (NMR) or synthesis of putative metabolites followed by LC-MS/MS [35]. Either of these two types of analysis is much more time consuming and

would only be done on a limited number of compounds. Briefly, for NMR studies there are two main approaches: either generation and isolation of an appropriate amount of metabolite (mg quantity) for direct NMR spectroscopy, or LC-NMR. In the first approach, scaled-up incubations are used to produce larger amounts of metabolites, and the metabolites can then be isolated by preparative or semi-preparative HPLC. The fraction containing the metabolite is concentrated, dissolved in an appropriate deuterated solvent, and placed in a tube for analysis. In LC-NMR, the NMR spectrometer must be equipped with a flow probe and the HPLC utilized must use deuterated solvents for the mobile phase. The metabolism incubation is chromatographically separated, then the peak of interest is either held in the active volume of the probehead ("stopped-flow") or the peak is stored in a small loop and then transferred to the probehead ("loop-storage") [35]. These in-depth structural elucidation studies are not easily adaptable to high-throughput because of the large amount of preparative and analytical time required for each step.

DRUG-DRUG INTERACTIONS

Antimalarial drugs, particularly those being used for prophylaxis, are likely to be administered in the presence of other therapies. In addition, the current trends to combat antimalarial drug resistance through combination therapies make understanding the potential drug-drug interactions of antimalarial drugs extremely important early in the development process. Several antimalarial drugs currently in use today have significant drug-drug interaction potential. Several types of drug-drug interactions can occur but most often they are related to an interaction with one of the CYP isozymes. Quinine and quinidine, for example, are classic CYP2D6 inhibitors. These compounds have also been shown to inhibit the metabolism of another antimalarial drug, halofantrine, by inhibiting CYP3A4, which subsequently leads to cardiotoxicity from prolonged exposure to halofantrine [43]. Inhibition of CYP3A4 by grapefruit juice can have a similar effect on halofantrine metabolism [44]. On the other hand, quinidine and quinine have been implicated in the activation of CYP3A4 metabolism of other 3A4 substrates [45, 46]. In addition, chloroquine, one of the most commonly used treatment drugs for malaria in the world, has been shown to significantly reduce CYP2D6 activity in humans [47]. Bapiro *et al.*, 2001 [8], identified a number of antimalarial drugs in a high-throughput *in vitro* DDI screen that significantly inhibited two CYP isozymes, including artemisinin, dihydroartemisinin, and primaquine (CYP1A2) and proguanil, cycloquanil, amodiaquine, and desethylamodiaquine (CYP2D6). Dapsone, currently being considered as a combination partner for several antimalarial drugs, has been shown to enhance metabolism of CYP2C9 [48-50]. Additionally, activation of the prodrug proguanil to its active metabolite cycloquanil is highly dependent on the activity of CYP2C19. Inhibition of this isozyme by other drugs [51], estrogen, and hormones associated with late pregnancy [52] can significantly reduce plasma levels of cycloquanil. Finally, compounds that induce the activity of CYP3A4 have been shown to decrease plasma levels of the common antimalarial prophylactic mefloquine [53]. These examples confirm the importance of understanding the

potential for drug-drug interactions with antimalarial drugs. Because CYP isozymes are commonly involved, this review will focus on methods for detecting potential interactions with CYP isozymes.

Drug-drug interaction has been defined as an increase or reduction in the enzyme activity in a specific metabolic pathway mediated by a different compound. CYP metabolizing enzymes have taken relevant importance because of their role in the metabolism of most of the drugs on the market. It has been estimated that the CYP isozyme 3A4, for example, is responsible for the metabolism of between 30-60 % of all drugs [54, 55]. Inhibition is the most frequent source for drug-drug interactions, where the compound of interest is either unable to be eliminated through metabolism, reaching toxic levels, or in the case of a prodrug, is not converted to the active metabolite and therefore can not achieve the desired therapeutic level. Such interactions can have a devastating effect on the utility of certain compounds especially when there is the possibility of concomitant therapy. For this reason, enzyme inhibition (especially for CYP enzymes) has been considered a characteristic that should be evaluated early in the development of a new drug.

Induction of a specific metabolic activity typically involves an increase in the synthesis of the particular metabolizing enzyme. Enzyme induction causes drugs metabolized by that particular enzyme to be cleared at a faster rate, thus having a reduced or short-lived therapeutic effect. In the case of a prodrug, increased metabolism may result in a toxic level of the active component. When compared to inhibition, the number of drugs affected by this mechanism is small. While this is an important characteristic to consider, methods for evaluating this phenomenon are not easily employed or amenable to high-throughput screening and will therefore not be addressed in this review [39, 56-58].

In recent years, drug-drug interactions resulting from inhibition of CYP enzymes have led to numerous product withdrawals [55, 59-62]. As a result, the pharmaceutical industry has placed a high priority on screening for these characteristics early in the discovery and development process. In addition, methods for evaluating the inhibition of CYP metabolizing enzymes are readily available, require limited resources, and are highly predictable. The ability of a drug to inhibit CYP enzymes is described through the determination of the K_i or IC_{50} . Apparent K_i or inhibition constant is the affinity of a substance to bind an enzyme. Generally, compounds with K_i values $> 20 \mu\text{M}$ have been defined as weak inhibitors, and those with K_i values of $< 1 \mu\text{M}$ as potent inhibitors. The significance of inhibition by compounds resulting in K_i values between 1 and $20 \mu\text{M}$ is difficult to determine. An IC_{50} value is a measure of the concentration of the test drug that is able to decrease the metabolic capacity of the enzyme in the study by half. It has been also defined that an $IC_{50} < 1 \mu\text{M}$ indicates a potent inhibitor and values $> 50 \mu\text{M}$ are weak inhibitors [56, 63]. Differentiation should be made also between direct inhibition (reversible) and metabolism/mechanism-based (irreversible) [7, 57, 64].

Currently, there are several methods that allow the quantification of K_i or IC_{50} values for new chemical entities

(NCEs). Traditionally, known substrates of specific enzymes are incubated with CYP recombinant isoforms, and the reduction in the concentration of the substrates or the increase in metabolite concentration over time is monitored using LC/UV or radioactivity. Test compounds are added to the reaction in a range of 6-8 concentrations and the ability of these compounds to inhibit either the metabolism of the substrate or the production of specific metabolites is determined. Typically, the metabolic reaction is compared to one in the presence of a known inhibitor for the particular enzyme being studied. These kinetics studies are relatively expensive, time consuming, and allow the evaluation of just one enzyme at a time [56, 65].

Recently, fluorometric assays have been developed and commercialized as kits for easy use and the screening of more than 30 compounds at a time. These kits include substrates that are metabolized to fluorescent products. Substrates are incubated in a 96-well plate with microsomes and cofactors and plate can be read in minutes using a fluorescent plate reader. This technique has proven to be accurate and efficient. The most significant advantage of this approach is the ability to rapidly screen large numbers of compounds without the need to develop analytical methods. The primary disadvantage of the use of fluorescent substrates is the likelihood that test compounds and/or their metabolites may fluoresce, thus confounding the results of the fluorescent assay. Typical known inhibitors previously reported to be used successfully with this technique include furafylline (CYP1A2); sulfaphenazole (CYP2C9); tranlycypromine (CYP2C19); quinine (CYP2D6); and ketoconazole (CYP3A4) [7, 39, 66].

Currently, improvements in analytical methods such as LC-MS/MS are greatly improving the throughput of traditional drug-drug interaction protocols by increasing selectivity, sensitivity and efficiency. Carefully selecting specific substrates for each CYP enzyme also allows the use of human liver microsomes, reducing the cost for the analysis. Several recent studies have proven the reliability and utility of this technique. Reduced run time in the LC method using ultra-fast chromatography systems and/or the incorporation of staggered or parallel LC systems has greatly enhanced the number of samples that can be analyzed without compromising the integrity of the assay [7, 65, 67, 68]. While these systems require significant investments in technology and highly qualified personnel, the ultimate payoff is the prevention of expenditures of large sums of resources in later development.

IN SILICO ADME PREDICTIONS

Currently, it remains a difficult task for most ADME programs to keep pace with combinatorial chemistry and high-throughput *in vitro* efficacy screens. Major bottle necks include poor compound solubility, drug stability, analytical method development, and limited analytical resources. Following the lead of the *in vitro* efficacy screening systems, ADME programs have begun to employ *in silico* techniques to pre-screen compounds prior to conducting *in vitro* ADME testing. Major advances have been made in computer systems and modeling techniques that drug development scientists use as a means for predicting important ADME properties such as solubility,

lipophilicity, and even metabolic lability. The major advantage of these techniques is that they can be employed prior to the synthesis or purchasing of compounds from compound libraries. Considering that synthesis of particular compounds is often a lengthy and expensive process, *in silico* modeling can be a valuable addition to drug discovery and development programs. In addition to screening out compounds that will be too insoluble for ADME *in vitro* screening systems or that will be poorly orally bioavailable based on inherent structural characteristics, *in silico* modeling can also be used for virtual lead optimization where the effect of specific structural modifications can be predicted prior to synthesis.

The main goal of *in silico* techniques in the drug development process is to provide computational models that can optimize the selection of candidate drugs from large pools of available compounds. *In silico* models are generated from large amounts of ADME data for candidate compounds from which structure-activity relationships (SARs) as well as structure-metabolism relationships (SMRs) are developed. SARs are based on descriptors, or attributes of a compound that are responsible for certain physiological properties of ADME. Descriptors encompass a wide range of structural moieties and chemical properties of compounds [69]. Properties such as aqueous solubility, alcohol/water partition coefficient, or lipophilicity can be used to model an ADME property such as absorption, while similarities among compounds known to inhibit P-gp can be used to evaluate efflux pumps of the blood brain barrier [70] and GI tract [70, 71]. Other more complex models employ algorithms involving a variety of quantum principles that are then combined to evaluate the property of interest. The resulting SARs are then subjected to regression analysis, mathematical algorithms, or evaluated by neural networks which 'mimic the pattern-recognition properties of the brain [72]. The ensuing models are trained to make quantitative structure-property relationships (QSPR) followed by a rigorous validation process. The results are computational models capable of robust physicochemical ADME predictions. Guided by these predictions, medicinal chemists can select compounds with the appropriate physicochemical and physiological properties, alter features to optimize desired properties, or have a basis to exclude a compound from development.

Some of the issues encountered by *in silico* modeling concern the simplicity or complexity of the descriptors used as well as the quality of the data employed to generate a particular model, since these features lend to the degree of predictability or the accuracy of the model. The models are centered on quantitative structure-property analysis (QSPR) of individual descriptors subjected to regression analysis or a wide variety of descriptors may be combined in complex multi-linear regression analysis (MLR) frameworks. Other more complex models employ algorithms involving a variety of quantum principles that are then combined to evaluate a property of ADME. In addition, the availability of significant data, the variability of the data used, and filtering of datasets for use for particular descriptors necessary to generate a reliable model have proven to be complicated [73]. A number of detailed surveys of various modeling approaches have revealed discrepancies amongst the source and quality of data used [9, 73-75]. Although

some models have given rise to robust predictions for properties such as solubility for which ample data exists, other properties, especially those for human studies, are severely limited. Complicated models can be difficult to interpret, especially in relating which particular characteristics of a compound should be adjusted to achieve a desired ADME property. Nevertheless, *in silico* techniques are projected to streamline the lead optimization process by drastically increasing the efficiency of pre-clinical evaluations and thus reduce costly drug failures at later stages of development.

WRAIR'S ADME PROGRAM

The Division of Experimental Therapeutics at the Walter Reed Army Institute of Research contains one of the world's largest and best-funded antimalarial drug discovery development programs. Through internal capabilities and external collaborations, this organization is perhaps one of the only in the world capable of taking an antimalarial drug from discovery through clinical development. Although primarily funded through government agencies and non-profit organizations, the structural and functional aspects of the division are being modeled after the pharmaceutical industry paradigm. It is believed that discovery and development of new antimalarial drugs can best be achieved by incorporating the most recent technological and strategic advances made in drug discovery and development in the private pharmaceutical companies. The incorporation of ADME screening early in the discovery and development process has been an example of this policy.

The ADME program at WRAIR/ET is divided into two main components designated Tier 1 and Tier 2, as seen in (Fig. 2). Tier 1 consists of high-throughput ADME

screening. The primary goal of the Tier 1 program is to quickly evaluate potential lead compounds for desired pharmacological properties. The primary mission for the WRAIR/ET malaria program is to develop a long-term, orally bioavailable prophylactic drug to prevent malaria. At a minimum, this drug will be administered no more than once per day, however, once per week or once per month is preferred. Given these considerations, the Tier 1 high-throughput screens have focused on predicting oral bioavailability and metabolic stability. In addition, because soldiers in battlefield situations are often injured or subjected to other therapeutic treatments to include bioterrorism prevention and/or radioactivity protection agents, a high-throughput screen from drug-drug interactions is also employed.

Currently, compounds determined to be "hits" in the *in vitro* antimalarial screen are initially evaluated through *in silico* modeling for solubility, lipophilicity, and other desired characteristics. Unless significant problems such as extremely low solubility (*i.e.* less than 1 µg/ml) are identified, the compounds are evaluated in the DDI and metabolic stability screens. Both the fluorometric and microsomal LC/MS methods are utilized for the DDI screening. The DDI screen evaluates potential interactions with five common CYP isozymes: 1A2, 2C19, 2D6, 2E1, and 3A4. Any compound exhibiting an IC₅₀ of less than 10 µM for any of the isozymes is flagged for further consideration. If that compound proves to be a compound of additional interest (*i.e.* good ADME profile, good efficacy in animal models), additional studies are performed to determine the nature of the interaction.

Metabolic stability assays are initially performed in both human and mouse liver microsomes. Mouse liver microsomes are employed at this stage primarily because the

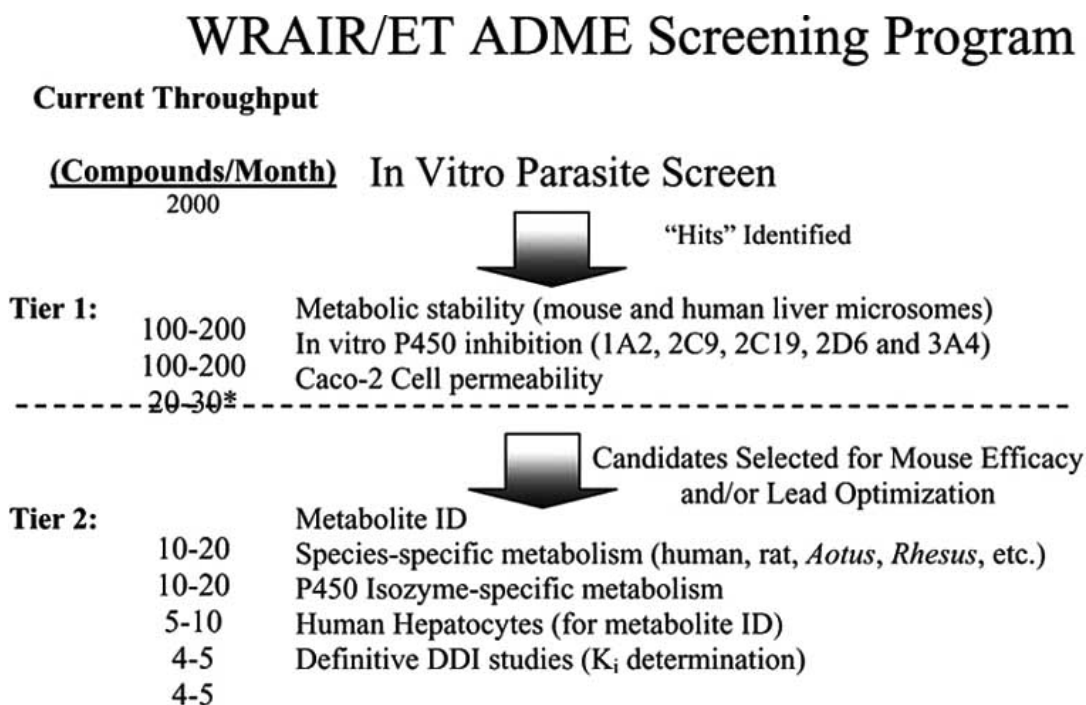


Fig. (2). Organization and current capacity of the WRAIR/ET ADME screening program. *Due to current bottle-necks in the sample analysis, the throughput of the Caco-2 cells is not equivalent to the other Tier 1 screens. Both PAMPA assays and ultra-fast analytical techniques are being evaluated to achieve a higher-throughput permeability screen.

mouse efficacy model (Thompson Test) is the next step in the WRAIR/ET drug development paradigm. Typically, metabolic stability assays are conducting using 1-5 μ M of test compound and 0.5 mg/ml of microsomal protein. Time points included in the assay are 0, 15, 30, and 60 minutes. *In vitro* half-lives of less than 30 minutes are considered metabolically unstable (in need of lead optimization), 30-60 minutes marginally stable (potential lead optimization necessary), or greater than 60 minutes are considered to be metabolically stable.

Following the DDI and metabolic screens, drugs are then screened through the Caco-2 permeability assay. The current throughput of this assay is not yet equivalent to the DDI or the metabolic stability assays and therefore it is reserved for only the metabolically stable or potentially the marginally stable compounds. The Caco-2 cell assay is conducted using 24-well plates with collagen coated inserts (BD BIOCOAT HTS CaCo-2 Assay System, Invitrogen Corp., Cat#354802). Both apical to basal (A to B) and basal to apical (B to A) permeability and permeability + transport, respectively is evaluated. Due to current resource and analytical constraints only approximately 8 drugs/week are evaluated through this system. Currently, a 96-well Caco-2 assay and ultra-fast LC methods are being evaluated to improve the throughput of this extremely valuable and informative assay. In addition, the use of an artificial membrane system (PAMPA) is being evaluated to provide an additional pre-screen to the Caco-2 cell assay.

Once the Tier 1 assays have been completed, the data is reported to medicinal chemistry and the project managers. These groups determine which of the compounds will proceed to mouse efficacy testing or be recycled for lead optimization or termination. At this point, Tier 2 ADME analysis is employed at the request of medicinal chemist or project managers. In general, medicinal chemists may request metabolite identification on particular compounds to aid in lead optimization for metabolic stability. Project managers may request isozyme specific analysis for potential prodrugs, or species specific metabolic stability and/or metabolite ID to aid in toxicological species selection. In addition to *in vitro* ADME studies, Tier 2 also encompasses early pharmacokinetic studies to evaluate oral bioavailability and *in vivo* metabolic properties. Although beyond the scope of this review, these studies are not only critical to the final decision regarding the development of specific compounds but are also correlated back to the high-throughput screens. The goal is to ultimately evaluate the utility of the *in vitro* screens and further optimize their predictability.

CONCLUSIONS

In a similar fashion as the pharmaceutical industry, WRAIR/ET's malaria drug discovery and development program has pushed ADME screening earlier and earlier into the discovery process. It is believed that implementation of these screens will reduce the need for expensive animal testing, increase the number of compounds evaluated through the discovery/development process, and hence increase the probability of finding a successful antimalarial clinical drug candidate. WRAIR/ET has implemented a two-tiered approach encompassing both high-throughput ADME screening (Tier 1) and compound specific ADME studies

directed at lead optimization, selection, and development (Tier 2). Currently, new technologies such as 96-well Caco-2 assays, PAMPA permeability assays, and rapid LC/MS analysis are being considered to increase the throughput of WRAIR/ET's ADME program. In addition, *in silico* modeling technologies for physicochemical properties, toxicity, and ADME are being evaluated for a future role in the screening process. Finally, as this program becomes increasingly efficient and integrated into the malaria program, the data generated for compounds currently in early development will be compared with *in vivo* pharmacokinetic and efficacy data once they reach the later stages. As the discovery and development pipeline continues, the accumulation of both *in vitro* and *in vivo* data should be analyzed to determine the utility of the *in vitro* screens.

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