IN VITRO ASSAY OF ANTIMALARIALS: TECHNOLOGIES, APPLICATIONS, AND PROSPECTS

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In vitro assays of antimalarials have represented some the most valuable techniques for the study of the emergence and changing pattern of drug resistance in many regions of the world and have contributed essential information concerning a rationale treatment program based on the drug sensitivity patterns of the parasites. Although several of these techniques have been standardized, others require specialized procedures which have been developed only during the past few years. Scientists working in Southeast Asia have contributed prominently to the technologies and applications of the *in vitro* assays of antimalarials especially for those drugs to which resistance is emerging in this region.

The first *in vitro* assays of antimalarials involved the evaluation of the effects of drugs on isolates of *Plasmodium falciparum* during short *in vitro* incubation (24 to 48 hours) using schizont maturation as a quantitative end-point. Rieckmann *et al.*, (1968) were the first to apply this short term assay ("macrotechnique") as an *in vitro* field test. A laboratory screening procedure with *P. knowlesi* was also developed based on short term incubation using the incorporation of radioisotopes as a measure of drug effect (Canfield *et al.*, 1970; McCormick, *et al.*, 1971).

One of the most significant contributions to the development of recent technologies and applications of *in vitro* testing of antimalarials was the development of techniques for the *in vitro* continuous cultivation of *P. falciparum* by Trager *et al.*, (1976) and Haynes *et al.*, (1976) based on lowered oxygen tension during incubation. Although these cultivation techniques have since been adapted and employed for research in malaria immunology and biochemistry, *in vitro* assays of antimalarials in both the laboratory and field environments were among the earliest of the applications.

The most serious problem facing the future of successful chemotherapy of malaria, particularly in Southeast Asia, has been the emergence and spread of resistance in strains of *P. falciparum* to standard antimalarials including chloroquine, sulfadoxine/pyrimethamine, quinine, and amodiaquine (Peters, 1985; Spencer, 1985; Pinichpongse *et al.*, 1982). Although mefloquine has been used in only controlled clinical and field trials (Peters, 1985), there have been several documented cases of treatment failures associated with mefloquine-resistant parasites originating in Thailand (Boudreau *et al.*, 1982) and Tanzania (Bygberg *et al.*, 1983). The resistance to antimalarials has emphasized the need to identify and develop promising new antimalarials, especially those with different mechanisms of action to reduce the possibility of cross-resistance with drugs currently in the field.

Technologies for the *in vitro* assay of antimalarials based on continuous cultivation have been used extensively since 1978 and have been widely adapted to support a wide range of studies related to many phases of antimalarial drug research and development. Applications have included screening of

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compounds for activity against continuous lines of *P. falciparum*, supporting Phase II (efficacy) and Phase III (field trials) clinical trials of promising new compounds, monitoring drug sensitivity and cross resistance, evaluating the extent of drug resistance, and new techniques such as cloning of field isolates and the development of drug resistance and its spread within populations. In addition, there have been extensive efforts to develop standardized tests for classes of compounds such as antibiotics or antifolate antimalarials which may require specialized cultivation procedures.

Drug screening for antimalarial activity

Screening of compounds for antimalarial activity using *in vitro* techniques requires the capability to routinely assay large numbers of drugs against one to three strains of *P. falciparum* with well-defined and diverse patterns of drug sensitivity. Such *in vitro* tests provide data on the sensitivity or cross-resistance of parasite lines to compounds without *in vitro* effects; however, results of these *in vitro* screening systems should be complemented with results of other screening systems using standardized animal models such as *P. berghei* in the mouse (Osdene *et al.*, 1967) to fully evaluate other factors such as drug toxicity and metabolism.

Semiautomated systems based on the use of radioisotope and microtiter technology were developed by Desjardins *et al.*, (1979) to support large scale antimalarial drug screening programs. Viability of the continuously cultured parasites following incubation with serial dilution of compounds was determined by the inhibition of uptake of radiolabelled hypoxanthine, a precursor of DNA and RNA metabolism in the parasite (Webster *et al.*, 1981; Chulay *et al.*, 1983), following incubation in defined gas mixtures (5% CO_2, 5% O_2, 90%N_2) in gas-tight chambers (Desjardins *et al.*, 1979). Microtiter technology has allowed antimalarial activity to be accurately determined using only milligram quantities of drug.

The *in vitro* screening system based on the radioisotope technology has been used for evaluating large numbers of compounds for antimalarial activity representing a wide range of structural classes (Desjardins *et al.*, 1979). In particular, studies have been reported using the *in vitro* screening system to evaluate structure-activity relationships among analogues of 2-acetylpyridinethiosemicarbazones (Lambros *et al.*, 1982) and N-benzyloxydihydrotriazines (Childs *et al.*, 1986), to identify the role of substituents involved in drug absorption of 9-phenanthrene-carbinols (Childs *et al.*, 1984), and to support identification of active antimalarial compounds derived from medicinal plants (Pavanand *et al.*, 1986).

Support of phase II and III clinical trials

One of the more important recent applications of *in vitro* drug sensitivity testing has been support of Phase II and Phase III clinical trials of new antimalarials. During these clinical phases drugs are tested for the first time against infections (induced or naturally acquired) of *P. falciparum* in human subjects. It is essential to determine whether treatment failures in these early clinical trials could be related to innate parasite resistance or to pharmacokinetic factors; e.g., drug metabolism or bioavailability.

Development of drug resistance in the Vietnam Smith strain was identified as a factor in a treatment failure of enpiroline during Phase II studies (Cosgriff *et al.*, 1985). A recrudescent isolate of the strain which was initially sensitive to aminoalcohols was isolated from a patient following administration of enpiroline. Analysis of its *in vitro* drug sensitivity pattern revealed that it had became resistant to not only enpiroline but

also showed cross-resistance to mefloquine and halofantrine (Cosgriff *et al.*, 1985).

During early Phase III trials of halofantrine in Thailand there were a significant number of treatment failures (7 failures of 20 patients). Comparison of the drug sensitivities of the isolates following treatment failures as compared to the pretreatment isolates from treatment successes showed that there were no significant differences in their sensitivities to halofantrine. These observations suggested that the failures were probably related to the pharmacokinetics of the drug (i.e., inadequate absorption from a large initial single dose of drug) rather than reduced parasite sensitivity (Webster *et al.*, 1985).

Monitoring drug resistance in the field

The technologies involved in assaying the *in vitro* antimalarial activities of drugs in the field may be contrasted to those developed for screening techniques. Field studies generally require evaluating the sensitivity of large numbers of isolates against a few standard drugs (e.g., chloroquine, quinine, mefloquine). Equipment is usually minimal and portable (small incubators, candle jars, microscopes). Because isolates of *P. falciparum* are synchronized upon blood collection, parasite viability is uaually determined by the inhibition in the development of ring stages to schizonts in predosed plates.

Most field studies have objectives of evaluating the extent or changing patterns of drug resistance within a geographical area or population, examining factors associated with variations in the drug response (WHO, 1984), or determining patterns of cross-resistance between standard and new compounds under development.

One of the earliest field assays techniques was the "macrotechnique" developed by Rieckmann *et al.*, (1968). This test was among the earliest to provide a rapid and reliable method of estimating *in vitro* sensitivity to chloroquine under field conditions (Colwell *et al.*, 1972). Although there were some limitations of this test, primarily the volumes of blood and parasitemia required, the "macrotechnique" was eventually incorporated into the World Health Organization's Global Monitoring Programme(Wernsdorfer and Kouznetov, 1980).

The technology used for the continuous cultivation of *P. falciparum* was adapted in 1978 to develop the "microtechnique"(Rieckmann *et al.*, 1978). This test had a number of significant advantages for field testing over the "macrotechnique" including lower volumes of blood and the use of predosed microtiter test plates. This test has since been widely used in many regions of the world by the World Health Organization to assay *in vitro* sensitivity to chloroquine, quinine, mefloquine, and amodiaquine.

Cloning

One aspect of *in vitro* sensitivity assays of field isolates of *P. falciparum* which is not generally considered is that these results may in fact reflect a composite response of different populations of clones each with possibly its own pattern of drug sensitivities (Rosario, 1981; Thaithong *et al.*, 1984; Webster *et al.*, 1985). Based on differences among a series of defined characteristics including drug sensitivity to chloroquine and pyrimethamine, antigen typing, presence or absence of knobs, and electrophoresis of seven different proteins, Thaithong *et al.*, (1984) demonstrated that a single isolate of *P. falciparum* from northwest Thailand actually consisted of populations of seven distinct clones. In addition to these characteristics, variations were also noted among the growth rates of different clones (Thaithong *et al.*, 1984) supporting observations that prolonged *in vitro* cultivation may result in a significant changes in the drug sensitivity patterns

(Le Bras *et al.*, 1983). Although the isolate and most of the clones were resistant to pyrimethamine, one highly sensitive clone was isolated (Thaithong *et al.*, 1972). Conversely, it has been suggested that isolates which appear sensitive by *in vitro* testing to a particular drug, such as mefloquine may contain a low proportion of drug resistant clones which could be subsequently selected by drug treatment (Webster *et al.*, 1985).

Inducement of drug-resistant lines

An important factor which must be evaluated during the initial stages of drug development is the potential for strains of *P. falciparum* to develop resistance to candidate compounds. Inducing drug resistance *in vitro* not only indicates that the parasite has the genetic capability to develop resistance in the field but may also provide a line of resistant malaria for evaluating patterns of cross-resistance to other promising compounds.

The ability to induce resistance *in vitro* to chloroquine in a series of strains of *P. falciparum* collected from Africa using continuous and increasing *in vitro* exposure to the drug suggested that chloroquine-resistance may have occurred as a result of drug pressure within African strains as well as being introduced from other regions (Nguyen-Dinh and Trager, 1978).

Inducement of mefloquine-resistance in cultured lines of *P. falciparum* from Thailand provided the first *in vitro* evidence that resistance to mefloquine could also be developed in this species (Brockelman *et al.*, 1981) and predicted the eventual appearance of mefloquine-resistance in isolates from human treatment failures (Boudreau *et al.*, 1981). Mefloquine-resistant lines were also developed from chloroquine-sensitive (Malayan Camp) and chloroquine-resistant (Vietnam Smith) lines to support drug screening studies of new antimalarials (Lambros and Notsch, 1984).

Results of studies on the inducement of mefloquine resistance in rodent models suggested that the emergence of resistance to mefloquine could be effectively reduced by using a judiciously selected combination of antimalarials (Peters *et al.*, 1984). The triple combination of mefloquine plus sulfadoxine plus pyrimethamine (MSP) was eventually selected for clinical trials (Peters, 1985) based in part on the ability of the sulfadoxine/pyrimethamine to prevent the development of mefloquine resistance in lines of *P. falciparum* cultured *in vitro* (Tanariya and Brockelman, 1983).

Assay of antibiotics and antifolate antimalarials

Development of *in vitro* assays for the antimalarial activities of antibiotics and antifolate antimalarials requires significant modifications of the current procedures due to differences in these drug's mechanisms of action, use in combination therapy, and special requirements for *in vitro* cultivation.

In spite of evidence for increasing resistance to quinine in many region of Southeast Asia (Chongsuphajaisiddhi *et al.*, 1981), the combination therapy of quinine and tetracycline (Reacher *et al.*, 1981) still remains highly effective for the treatment of multidrug-resistant falciparum malaria. Recent studies on the *in vitro* activity of a series of antibiotics suggested that the mechanisms of action of most of these drugs were relatively slow. At *in vitro* concentrations of tetracyline comparable to serum levels significant antimalarial activity was noted only after 48 and 96 hours of exposure (Geary and Jensen, 1983).

Development of an *in vitro* test suitable for assaying sensitivity of field isolates to the combination of quinine and tetracycline may

present several major problems related to both the technology of the test and interpretation of the results. Technical aspects which have to be evaluated include extended incubation for up to 96 hours. Problems of interpretation of results involve evaluating the effects of quinine and tetracycline (singly and/or in combination) and correlation of the *in vitro* effects with *in vivo* responses.

During the past several years there has been considerable research on the development of a standardized system to assay the sensitivity of isolates of *P. falciparum* to sulfadoxine/pyrimethamine (Fansidar). Fansidar has been one of the most important antimalarials used in areas where chloroquine resistance has recently emerged; however, resistant strains have been reported from many areas of Southeast Asia, Africa, and South America (Peters, 1985).

Development of a standardized *in vitro* field test for Fansidar has presented several difficulties. Fansidar is a combination drug composed of pyrimethamine and sulfadoxine. Although each component has a different mechanism of action the combination has been shown to be potentiating both *in vivo* (Hurly, 1959) and *in vitro* (McCormick and (Canfield, 1972; Eastham and Rieckmann, 1983). Resistance has apparently developed to each of the components (Childs *et al.*, 1986). There was also a difference between the ratio of the pyrimethamine to sulfadoxine which resulted in maximum potentiation *in vitro* (1:80) (Milhous *et al.*, 1985) compared to the ratio of the two drugs in the human serum following oral administration (1:200) (Weidekamm *et al.*, 1982).

Although the mode of action of sulfadoxine involves competition with para-aminobenzoic acid (PABA) for formation of dihydropteroate thus blocking synthesis of tetrahydrofolate (Ferone, 1977), the *in vitro* response of both sulfadoxine and pyrimethamine was inhibited by the excess levels of both PABA and folic acid in the RPMI-1640 medium (Chulay *et al.*, 1984). Several alternative medium formulations have been evaluated including Waymouth's Medium with lacked PABA (Brockelman and Tan-ariya, 1982) and RPMI-1640 with reduced or depleted levels of PABA or folic acid (Childs *et al.*, 1986; Milhous *et al.*, 1985; Spencer *et al.*, 1984).

In contrast to drugs such as chloroquine or mefloquine the *in vitro* activity of sulfadoxine/pyrimethamine is highly stage-specific affecting primarily the late trophozoites or early schizonts (Eastham and Rieckmann, 1983; Gutteridge and Trigg, 1971). This may also contribute to the requirement for longer incubation periods, up to 48 hours. Reinvasion at 48 hours rather than schizont maturation at 24 to 30 hours as an end-point for pyrimethamine sensitivity *in vitro* has been evaluated (Nguyen-Dinh and Payne, 1980).

Results of several field studies have consistently demonstrated that the *in vivo* response to Fansidar correlates more closely with the *in vitro* response to pyrimethamine than to either sulfadoxine or the combination of sulfadoxine and pyrimethamine (Chulay *et al.*, 1984; Lamont and Darlow, 1982; Sabchareon *et al.*, 1985).

In vitro cultivation of *P. vivax*

Although drug resistance to *P. vivax* has not emerged and thus does not yet represent a serious health hazard, it is possible that such may not be true in the future. *In vitro* studies on the drug sensitivity patterns of isolates of *P. vivax* have been seriously limited by the inability to adapt isolates to continuous culture. One factor appears to be the specific requirement for the merozoites to invade immature erythrocytes (Kitchen, 1939). *In vitro* cultivation procedures adapted from cultivation techniques of *P. falciparum* have been generally inconsistent. However, a

recent study (Brockelman *et al.*, 1985) has demonstrated that 99% of all isolates of *P. vivax* could successfully and consistently be maintained through schizogony using an improved medium with elevated glucose levels, higher salt concentrations (particularly $MgCl_2$ and $CaCl_2$), and additional hypoxanthine. These improved procedures should provide a basis for assaying the *in vitro* drug sensitivity patterns of isolates of *P. vivax* at least through one or two cycles.

Prospects for *in vitro* assay of antimalarials

Among the most immediate problems for the *in vitro* assay of antimalarials is the need to develop effective *in vitro* assays for Fansidar and quinine/tetracycline. Tests for the *in vitro* sensitivity of Fansidar are still undergoing development and field evaluation. But it is expected that assays to antifolate antimalarials will be standardized for field use in the near future. Reports of research on *in vitro* tests for tetracycline sensitivity have been generally limited to basic laboratory studies using continuous lines (Geary and Jensen, 1985).

Eventually assays will have to be developed and standardized to support testing of new antimalarials as they are introduced into the field. These would include such drugs as halofantrine and enpiroline. Adaptation of these drugs into a "microtechnique" will require standardization of the test system to preclude possible problems such as binding to the test plates as originally noted in early field trials with mefloquine (Kouznetsov *et al.*, 1980). The use of plates prepared in the field rather than predosed plates was shown to be very effective for the assay of halofantine (Webster *et al.*, 1985).

A serious problem would be the widespread development of resistance of strains of *P. vivax* to standard and new antimalarials. Research is being conducted to develop new technologies to successfully cultivate continuous lines of this parasite in the laboratory; however, it appears that technologies used for the cultivation of *P. falciparum* represent only a starting point and that novel approaches may eventually be required.

New technologies may also be a significant factor in designing field tests which are easier and faster to interpret and generally more sensitive. Assays based on the inhibition of radiolabelled hypoxanthine were especially well suited to evaluating the sensitivities of large numbers of field isolates against six to twelve standard and new antimalarials (Webster *et al.*, 1985). A novel approach for a field test involved pigment production as an endpoint (Rieckmann, 1982); however, the procedure has proven inconsistent under field conditions (WHO, 1984).

The ideal of a simple, reliable, inexpensive, and easy to interpret *in vitro* assay remains a challenge. Nevertheless, it is a challenge that must be met if we are to deal effectively with monitoring and evaluating the continued emergence of multidrug-resistant *P. falciparum*. This requires that we continue to build our basic knowledge of *Plasmodium* biochemistry and to take advantage of unique biochemical features for the development of new and improved *in vitro* assays.

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