A modified *in vitro* sulfadoxine susceptibility assay for *Plasmodium falciparum* suitable for investigating Fansidar resistance

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SUMMARY

The combination of pyrimethamine and sulfadoxine (PSD or Fansidar) represents one of the most important chemotherapeutic agents currently used to treat falciparum malaria. To investigate the molecular basis of resistance to PSD, reliable *in vitro* drug assays are required to permit correlation of resistance levels with different genotypes. We describe here protocols that permit accurate evaluation of IC_{50} values for sulfadoxine (SDX) inhibition of *Plasmodium falciparum*. Historically, tests for this drug have suffered from poor reproducibility and extreme variability in reported values. We have examined a series of variables, including serum-containing versus serum-free media, erythrocyte source, pre-test growth conditions, test components and post-test processing. We define conditions which better control the levels of the drug antagonists folate and *p*-aminobenzoate, yielding reproducible differences between lines of *P. falciparum* with differing alleles of the dihydropteroate synthetase gene, which encodes the target enzyme of SDX. We also use this assay to demonstrate a marked difference in the response of different parasite lines to antagonism of SDX inhibition by exogenous folate. The ability to measure reliable IC_{50} values for SDX inhibition should greatly facilitate further experiments to explore the molecular basis of Fansidar resistance.

Key words: *Plasmodium falciparum*, malaria, sulfadoxine, Fansidar resistance, dihydropteroate synthetase, folate.

INTRODUCTION

The combination of pyrimethamine (PYR) and sulfadoxine (SDX) is currently being used in an increasing number of African countries as first-line treatment for *Plasmodium falciparum* malaria, as resistance to the major therapeutic agent, chloroquine, continues to spread (Bloland *et al*. 1993). However, resistance to the antifolate combination (PSD; Fansidar) is already appearing with alarming frequency in these regions. Recently, we and others have investigated the basis for parasite resistance to the SDX component of PSD. This has entailed characterization of the target of the drug, dihydropteroate synthetase (DHPS), and the description of several mutations in conserved regions of this molecule associated with elevated levels of SDX resistance (Brooks *et al*. 1994; Triglia & Cowman, 1994). In subsequent work, we have presented strong evidence for a causal relationship between mutations in DHPS and drug resistance, and demonstrated that at least 1 other gene product, associated with exogenous folate utilization, can also play an important role in determining the level of SDX resistance in a given parasite line (Wang *et al*. 1997).

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As part of the above studies, it was necessary to develop an assay for SDX resistance in cultured parasites more accurate and robust than hitherto described. It has been known for many years that *in vitro* tests for SDX resistance require culture medium depleted of folate and *p*-aminobenzoic acid (PABA), which antagonize the action of SDX, and which can have a marked effect on measured IC_{50} values (Rollo, 1955; Chulay, Watkins & Sixsmith, 1984; Spencer *et al*. 1984; Watkins *et al*. 1985; Milhous *et al*. 1985; Nguyen Dinh *et al*. 1985; Schapira *et al*. 1986; Tan-Ariya, Brockelman & Menabandhu, 1987). However, even with the use of such medium, reported IC_{50} values show enormous variation (*ca*. 5 orders of magnitude), ranging over *ca*. 10 nM (Milhous *et al*. 1985), 150 nM (Watkins *et al*. 1987), 1 μM (Chulay *et al*. 1984; Tan-Ariya *et al*. 1987) up to *ca*. 3 mM (Schapira *et al*. 1986) for strains classified as SDX sensitive. The poor reproducibility reflected by these disparate values also seriously affects the ability to accurately quantitate differences in susceptibility between sensitive and apparently resistant parasites. Given the extreme variability in PABA (Schapira *et al*. 1986) and, to a lesser extent, folate levels (Chanarin, 1980; Milhous *et al*. 1985; Tan-Ariya *et al*. 1987) found in different samples of human plasma, some workers have employed exhaustive dialysis (e.g. for 72 h, with 5 changes of

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dialysis buffer) to completely deplete the plasma or serum used in the culture medium of these molecules. However, such material, combined with RPMI 1640 medium also devoid of PABA and folate, does not support the growth of all strains of *P. falciparum* (Milhous *et al*. 1985; Tan-Ariya *et al*. 1987). To avoid this problem, supplements of 0·5 ng/ml PABA and 10 ng/ml folic acid (Tan-Ariya *et al*. 1987; Nguyan-Dinh *et al*. 1985), 7 ng/ml folic acid alone (Spencer *et al*. 1984) or 10 ng/ml folic acid alone (Chulay *et al*. 1984) have been added back to medium to ensure reasonable growth while keeping levels very low, but published IC_{50} values using this modification were still highly variable between groups.

These earlier studies were also compromised by the fact that they pre-dated the discovery of structural variation in the DHPS enzyme. It is now known from extensive DNA sequencing studies that there are at least 5 residues of DHPS in which mutations can be found (positions 436, 437, 540, 581 and 613). To date, at least 10 different allelic variants of the wild-type molecule have been identified (Brooks *et al*. 1994; Triglia & Cowman, 1994; Reeder *et al*. 1996; Wang *et al*., manuscript submitted), with the great majority of mutant parasite lines having from 1 to 3 amino acid changes per molecule. As we began attempts to measure the effect of the various DHPS mutations on SDX resistance, it became clear that a much higher degree of resolution and reproducibility was required than attainable by published variants of the *in vitro* SDX test. In particular, it was necessary to control folate levels more rigorously, as minute concentrations of exogenous folate, in particular, could have profound effects on the SDX resistance levels of certain parasite lines (Milhous *et al*. 1985; Chulay *et al*. 1984). Here, we report in detail the modified SDX assay that has permitted us to demonstrate the individual contributions of mutations in DHPS, both in unrelated parasite lines, and in the parents and progeny of the genetic cross between SDX-sensitive HB3 and SDX-resistant Dd2 (Wang *et al*. 1997), as well as to quantitate the additional influence of exogenous folate on SDX resistance. By a close examination of variables affecting the SDX assay, we have improved it to the point of giving consistently reproducible data, where differences of 3 orders of magnitude are measurable between the most sensitive and most resistant parasite lines.

MATERIALS AND METHODS

Parasite culture

All parasite lines used in this work have been described previously (Brooks *et al*. 1994; Wang *et al*. 1997, and references therein). For routine culture after retrieval from storage in liquid N_2, parasites were grown at 37 °C in standard RPMI 1640 medium (Gibco-BRL), 10 % human plasma and supplements as described (Read & Hyde, 1993). In preparation for testing their SDX susceptibility, they were transferred into medium containing 0·5 % Albumax I (Gibco-BRL; added from 10 % stock in sterile water; filter sterilized) instead of plasma or serum, custom RPMI 1640 liquid medium (containing glutamine and HEPES) supplied free of folic acid and PABA (Gibco-BRL), 0·2 % glucose, 1 μg/ml hypoxanthine (Sigma; 1 mg/ml stock in sterile water), 50 μg/ml gentamycin, with 100 ng/ml of folic acid added back as a supplement (100 μg/ml stock in 1 M $NaHCO_3$). The haematocrit in this culture was 2·5 %. After they were sufficiently adapted to this medium (as judged by a healthy morphological appearance and a normal growth rate that enabled parasitaemias of $\geqslant 5$ % to be easily achieved), the parasites were transferred to the same medium without folic acid, at a parasitaemia of 0·5–1·0 %, 2 days before entering the SDX test described below.

In vitro *sulfadoxine assay*

Parasites were set up in the above folate-free medium at an initial 0·5 % parasitaemia, 2·5 % haematocrit, 225 μl volume, in 96-well microtitre plates (Costar). For each parasite line, a column of 8 wells was reserved. In the first row of each column, before adding parasites, 1 μl of dimethylsulfoxide (Me_2SO) without drug was added (control samples). In the next 7 rows, SDX (stock solution 12–25 mg/ml in tissue-culture grade Me_2SO) was added to samples in ascending order of $2\times$ concentration, with the volume of added drug solution kept at 1 μl in all cases. For parasite lines established to be SDX sensitive in preliminary experiments, the concentration range used to establish accurate IC_{50} values was 0–0·2 μg/ml (0–0·64 μM); for resistant lines, this was 0–12 μg/ml (0–38·7 μM). The latter range was extended when the effect of exogenous folate on IC_{50} values was investigated (see Results section). Wells were also set up identically but without inoculation of parasites (i.e. using uninfected erythrocytes in medium only) as controls for background incorporation of radioactive label. The microtitre plate was placed in a box, gassed with 5 % CO_2, 5 % O_2, 90 % N_2, and sealed. After 48 h incubation with the drug at 37 °C, 0·5 μCi [^{3}H]hypoxanthine (Amersham; 10–30 Ci/mmol) in 25 μl of test medium was added to each well to monitor growth (Chulay, Haynes & Diggs, 1983) and samples processed after a further 16 h. This entailed harvesting of the well contents into 15 ml of distilled water to lyse the cells and parasites, and suction onto 24 mm Whatman GF/C filters. Excess label was removed by washing the filters with a further 15 ml of distilled water. After a final wash in 100 % ethanol to remove residual water,

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the filters were counted in 4 ml of Ecoscint scintillant (National Diagnostics) and data were analysed using locally developed curve-fitting software. IC_{50} values were defined as the concentration of SDX at which parasite growth was reduced to half that of the untreated controls, as measured by tritium incorporation.

RESULTS

The main modifications found to be necessary in the improved assay procedure involved substitution of quality-controlled lipid-rich albumin for culture plasma or serum, regularizing the supply of erythrocyte host cells from a single known donor, and culturing the parasites through several growth cycles in 2 modified media before entry into the drug test. The first of these is a medium devoid of PABA, but with a low level of folate, followed by a final growth cycle of 48 h in a medium devoid of both these reagents immediately before entry into the drug test. However, we found that a number of other factors could also compromise the quality and credibility of the resulting inhibition curves. The variables we investigated are described below. The criteria we used to assess the importance of these variables were the reproducibility of the IC_{50} values for a given parasite line, together with the magnitude of the difference between parasites carrying wild type DHPS (typically HB3) and those with one of the triply mutated forms, represented by Dd2 (S436F, A437G, A613S) (Wang *et al*. 1997).

Red blood cells

Normally, red blood cells for routine parasite culture are obtained on a 3–4 weekly basis from a blood transfusion service. A degree of consistency in the SDX test amongst donated samples could be obtained by washing and dialysis of the cells against PBS overnight as described below. However, we received blood samples where this procedure was insufficient to allow reproducible measurement of IC_{50} values. As shown in Fig. 1, IC_{50} values measured for Dd2 as an example varied by more than 6-fold in 4 different batches of blood, with a 5th batch (batch 3) rendering measurement impossible over the range of SDX concentrations used. We concluded from these data that, while the wash and dialysis steps should, in principle, reduce residual PABA and unbound folate in the erythrocytes to a very low level before entry into the drug test, there are factors sufficiently variable between individual donors such that this is not achieved. Alternatively, other, unknown blood constituents could be contributing irreproducibly to antagonism of the SDX inhibition. We therefore found it necessary to utilize a single known source of erythrocytes. Ten ml of blood were removed from the same donor every 3–4 weeks, immediately dispersed into 1 ml of CPD anticoagulant (2·3 mM citric acid, 13·2 mM Na citrate, 2 mM NaH_2PO_4, 19 mM glucose, pH 5·1) and stored at 4 °C for a maximum of 4 weeks. Each time red cells were required, a 1 ml aliquot was washed briefly with 15 ml of PBS, the cells spun down, then resuspended in 50 ml of PBS at 4 °C overnight. After spinning down the next day, cells were resuspended to 50% haematocrit in test culture medium. Fifty μl of these cells were used per ml of test culture (2·5% haematocrit). By confining measurements to cultures utilizing erythrocytes from a single donor, we were able to collect much more reproducible data, where inhibition curves were consistently superimposable and corresponded to the lowest curve (batch 2) in Fig. 1. This we interpret as representing the minimum degree of antagonism to SDX inhibition from blood-associated factors.

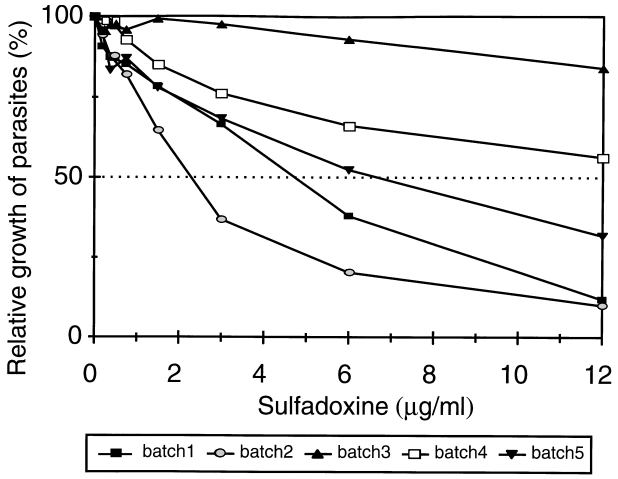

Fig. 1. Measurement of IC_{50} values for SDX inhibition of the Dd2 line in 5 batches of dialysed blood from different (unknown) donors. No IC_{50} value could be derived from the uppermost curve (batch 3) and only an approximate estimate from batch 4.

Substitution of plasma/serum with lipid-rich albumin

Different samples of human serum or plasma contain highly variable levels of folate of *ca*. 2–25 ng/ml (Chanarin, 1980; Milhous *et al*. 1985; Tan-Ariya *et al*. 1987) and PABA (< 10 ng/ml to > 800 ng/ml; Schapira *et al*. 1986). Consistent with this, and similar to the situation with the erythrocytes described above, we found that IC_{50} values measured in different batches of pooled sera were poorly reproducible. Exhaustive dialysis is able to reduce the concentrations of these small molecules to near-undetectable levels (Milhous *et al*. 1985; Tan-Ariya *et al*. 1987), but these regimes required 54–72 h of dialysis with a number of buffer changes. Moreover, the observation of Milhous *et al*. (1985), that dialysis can alter the measured IC_{50} value by up to 4 orders of magnitude, strongly suggests that to achieve

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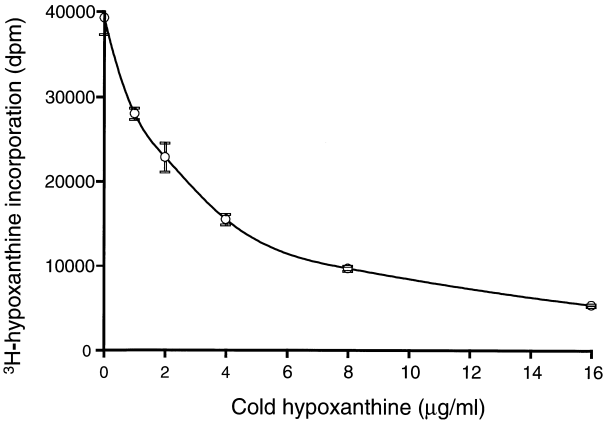

Fig. 2. The effect of titrating cold hypoxanthine into a mock SDX test (no drug added) on the eventual recovery of ^{3}H-counts from labelled hypoxanthine used to monitor parasite growth. Parasites grew sufficiently well for a short period to permit measurement of the zero hypoxanthine point, but required $\geqslant 1$ μg/ml for sustained growth (see text). Data points are averaged from 4 determinations.

reproducibility, a successful dialysis regime would need to be strictly adhered to in every detail. We concluded that greater reproducibility and convenience could be achieved by dispensing with donated serum and plasma from uncontrolled sources, and substituting Albumax I, a commercially available, chromatographically purified, lipid-rich preparation of bovine serum albumin which acts as a carrier for essential fatty acids, lipids, trace elements and other components required for cell growth. Furthermore, Albumax dissolves readily and is easier to filter-sterilize than plasma or serum. Although the exact levels of any residual PABA and folate in Albumax were not determined, and are not measured by the manufacturers, our experiments where minute levels of these compounds added back to cultures caused marked alteration of IC_{50} values (see below) indicated that they must be negligible. We have used several different batches of this preparation, and found to date no indication of batch-to-batch variation.

Parasite growth rate and hypoxanthine incorporation

In its radioactive form, the purine base hypoxanthine is widely used as a monitor of parasite growth rates in the presence or absence of drug (Chulay *et al*. 1983). However, when using Albumax as a serum substitute as described above, under the conditions of the pre-test and test cultures, we found this compound also to be a necessary growth additive. This is consistent with a recent report in which *P. falciparum* was grown in a different serum-free medium (Asahi *et al*. 1996). The higher the degree of incorporation of the radioactive hypoxanthine into parasite macromolecules, the more reliable are the inhibition curves measured in the presence of SDX. Therefore, to minimize dilution of the specific activity of the label, cold hypoxanthine was titrated into cultures to determine the effect on recovered counts, as well as to establish the minimum amount that would reliably support healthy growth (over several weeks) in different strains, as monitored by microscopical examination of the parasites. This was found to be 1 μg/ml, although 2 μg/ml could also be used without reducing the incorporated counts to an undesirable level (Fig. 2).

We found that an increase of parasitaemia of at least 4-fold over the 2-day period prior to transfer into zero folate medium was necessary to achieve sufficient incorporation of radioactivity in the actual drug test. Such growth rates generally resulted in *ca*. 20000 dpm in untreated controls, up to a maximum of about 100000 (using 1 μg/ml cold hypoxanthine). If less than 5000 dpm were seen in these controls, curves were not calculated, as the counts were then insufficient to yield smooth plots of inhibition, and hence reliable IC_{50} measurements.

Use of pre-test cultures in depleted media

Earlier workers had adopted the principle of growing the parasites in medium depleted of folate and PABA before putting them into the SDX susceptibility test (Chulay *et al*. 1984; Schapira *et al*. 1986). We found that this procedure represents a precarious balance, where too great a reduction in folate and PABA levels led to the death or very slow growth of some parasite lines, making it impossible to carry out the drug test, but where too generous a back-supplementation with PABA and/or folate to ensure healthy growth led to (sometimes severe) upward distortion of IC_{50} values, as the antagonists are carried over to the drug test at too high a concentration. Importantly, moreover, different parasite types responded in the test to highly varying degrees to such prior supplementation. By systematically testing cultures in a range of PABA (0 and 5 ng/ml) and folate concentrations (0, 2, 5, 10, 25 and 100 ng/ml) in different combinations, we found that growth in depleted medium with 100 ng/ml folate, zero PABA, could be sustained for a minimum of several months by all parasite strains that we tested (a total of 24, including 16 progeny of the HB3-Dd2 cross). The period of adaptation to low folate (which took from a few days to a few weeks, depending upon the particular line) was necessary to ensure negligible interference with the eventual IC_{50} measurements, while permitting good growth over the 48 h period before the drug test (and during the test itself) when the parasites are transferred to the pre-test/test media that are completely devoid of PABA and folate. This in turn resulted in a sufficiently high incorporation of radioactive label with the lowest possible (and hence most credible) IC_{50} values when the parasites were finally inoculated into the 18 h

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drug test. We also found that the dilution factor used when transferring inocula from the low (100 ng/ml) folate medium into the pre-test (zero folate) medium was important. This was kept to a maximum of 5 μl of inoculum into 200 μl of fresh culture, otherwise the carry-over of folate was again too high, leading ultimately to artificially high IC_{50} values.

Solvents used for label and drug

Depending upon the supplier, [^{3}H]hypoxanthine is obtained either as a dried powder or as a 50% ethanolic solution. Although only 0·5 μl (0·5 μCi) of the latter was added to each microtitre well in the test plate, we found a significant perturbation of the assay by the presence of the resulting ethanol (0·1%), resulting again in skewing of IC_{50} values to higher levels compared to cultures where label was added in an entirely aqueous solution. Therefore, 20 μl (20 μCi) of an ethanolic solution was removed from stock as required, dried down, and redissolved in 1 ml of culture medium before use. This was sufficient for testing 4 parasite lines, each at 8 different concentrations of drug (i.e. a complete dilution series necessary to obtain a reliable curve for IC_{50} determination, plus controls with uninfected cells). Label supplied as dry powder was dissolved in sterile water to 1 mCi/ml and stored at 4 °C.

Given the above results, we avoided ethanol as a solvent for SDX (Spencer *et al*. 1984), using tissue-culture grade Me_2SO instead, in which the drug is freely soluble at high concentration. This also gave more reproducible results than the alternative method of suspending the drug in water (in which it is poorly soluble) and adding concentrated NaOH until the compound is completely dissolved. The Me_2SO caused no detectable elevation of IC_{50} levels, and in controls at the same concentration but with no dissolved drug, had no noticeable effect on parasite growth rate or morphology.

Processing of post-labelled samples

After 48 h of label incorporation, the parasite samples in each microtitre well were processed for scintillation counting. This involved diluting the contents of each well into a large volume of water to effect lysis and pouring through a glass-fibre filter attached to a vacuum suction device, followed by a second wash step. We found that the background counts of unincorporated hypoxanthine could be unacceptably high unless quite stringent washing of the filters was employed. Thus, on increasing wash volumes up to 15 ml, background counts progressively diminished in control experiments. However, above this volume, the background remained constant. We therefore routinely fixed on an aqueous wash volume of 2 × 15 ml as standard, followed by an ethanol wash to quickly dry the filters.

Reproducibility of IC_{50} measurements

Implementing all of the modifications described above, we were able to achieve a high degree of reproducibility, not only within a series of tests conducted simultaneously or in close succession, but also over long time-periods, when parasites were taken out of liquid N_2 storage on different occasions. Fig. 3 shows examples of the marked improvement achieved relative to measurements made using an unmodified protocol, where serum, blood from an unknown donor and extensive washing of blood cells (rather than pre-test culturing in low folate) were employed. The difference between the IC_{50} values for the 3D7 and K1 lines illustrated here was reproducible using the modified protocol, but both absolute values and even the relative order of resistance were highly variable using the conventional procedure. Representative IC_{50} curves for different parasite lines using the modified assay are shown in Fig. 4, where a broad correlation is seen between IC_{50} values and the degree of mutation (0, 1, 2 or 3 altered residues) in the DHPS sequences of the respective lines. In particular, we were able to consistently measure differences of between 4 and 10-fold (depending upon exactly which lines were being compared) between parasites differing by only 1 in their total number of DHPS mutations. For the most SDX-resistant line, Dd2, 6 determinations from 6 different cultures yielded a mean IC_{50} value of 4·0 μg/ml with a standard deviation of 1·6 μg/ml, i.e. 40% of the mean value. For the most SDX-sensitive line, HB3, 6 determinations yielded a mean IC_{50} of 4·3 ng/ml, with a standard deviation of 1·9 ng/ml (44% of the mean). Reproducibility was therefore consistently achieved over a range of SDX concentrations covering a 3 orders of magnitude difference in susceptibility.

The sensitivity of IC_{50} values to exogenous folate

The above IC_{50} values were the lowest we were able to attain for a given parasite line, and are likely to reflect predominantly the alterations in drug binding brought about by mutation of the DHPS enzyme. The dramatic effect of adding very low concentrations of exogenous folic acid to these cultures during the drug test is shown in Fig. 5A and 5B. In Fig. 5A, where folate was titred into the assay, a concentration as low as 12·5 ng/ml had a profound effect on the inhibition curve. Moreover, the curves in Fig. 5B show clearly that different parasite lines were affected to markedly differing degrees by this addition. Thus, for example, 3D7 and K1 show a pronounced effect of folate, while HB3 shows almost none. Even when the SDX concentration was raised to 100 μg/ml (323 μM), it was not possible to measure an IC_{50} for GC-03, 3D7, K1 and other parasites lines that display this 'folate effect'. Although we do not

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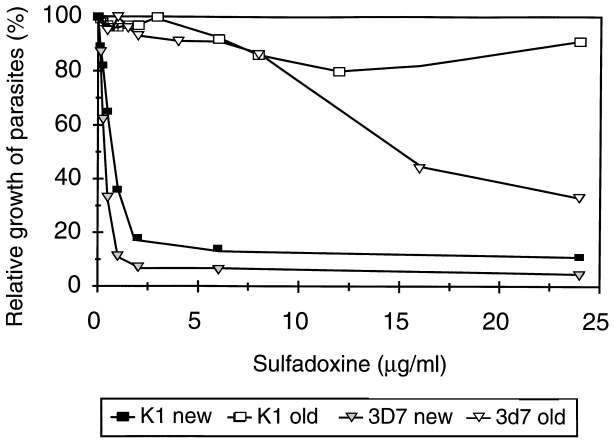

Fig. 3. Comparison of representative data obtained using an older SDX assay method (see text) with those obtained adopting all of the modifications described in the text for 2 parasite lines (3D7 and K1) of different drug susceptibilities.

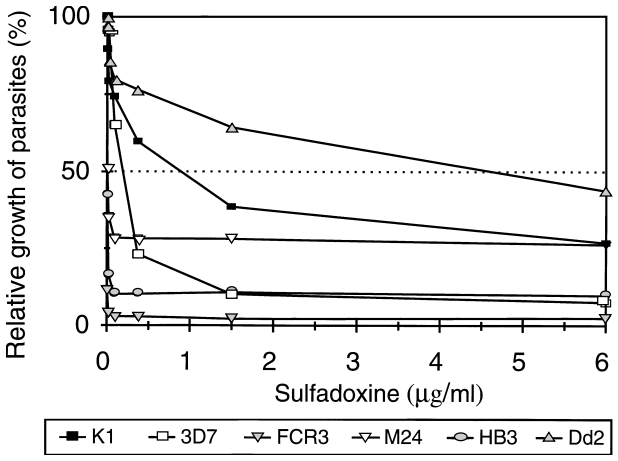

Fig. 4. Representative IC_{50} plots for parasite lines carrying wild-type DHPS (M24, FCR3 and HB3), 1 mutation (3D7; A437G), 2 mutations (K1; A437G and A581G), and 3 mutations (Dd2; S436F, A437G and A613S). For accurate calculation of the IC_{50} values quoted in the Results section, similar plots were carried out, but the range of drug concentrations used was narrowed to the region of interest, to give greater resolution (see Materials and Methods section). A computer program was then used to optimize curve fitting to the data points.

yet understand the precise molecular basis of this phenomenon, the extreme sensitivity of IC_{50} values observed here emphasizes the crucial importance of rigorously controlling folate levels in the assays.

PCR verification of samples

In tests such as that described here, where several parasite lines are being assayed simultaneously in the wells of a microtitre tray, the possibility of cross-contamination must always be borne in mind. One

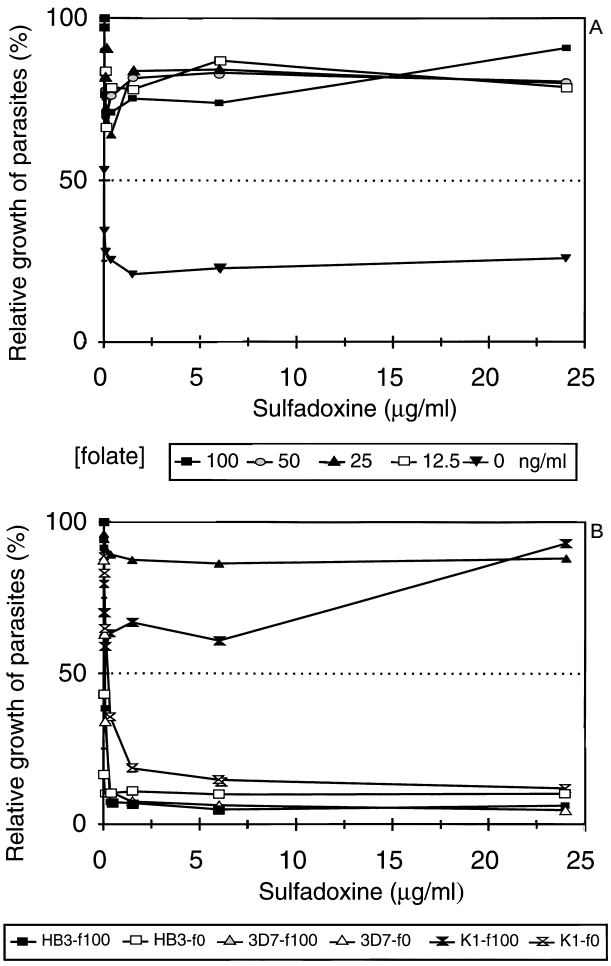

Fig. 5. The effect of (A) increasing concentrations of folate on IC_{50} curves of the GC-03 parasite line, and of (B) 100 ng/ml folate on IC_{50} curves of K1, 3D7 and HB3 parasite lines.

procedure to verify the identity of cultures is to use polymerase chain reaction (PCR) amplification on a diagnostic DNA sequence to type each sample after it has been through the drug test, and to check that the pattern is of the predicted type. We found that this could be conveniently achieved by sampling the culture medium above the settled parasitized erythrocytes. The optimal volume of this medium to achieve satisfactory amplification in a standard 100 μl PCR reaction was observed to be 5–20 μl, using primers that can detect the various mutation patterns in the parasite *dhps* gene (Wang *et al.* 1995) (data not shown). This technique has the advantage of leaving all of the parasitized erythrocytes intact to go into the counting of incorporated label, as well as avoiding the problems of inhibition of the PCR sometimes observed when whole blood samples are used.

DISCUSSION

Problems with achieving reproducible data for SDX inhibition values have for many years rendered it difficult to investigate the molecular basis of re-

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sistance to this drug, and to assess the relative importance of SDX resistance in the clinical failure of PSD. With the recent identification of a series of mutations in the *dhps* gene (Brooks *et al*. 1994; Triglia & Cowman, 1994), whose protein product is the target of SDX and other sulfa drugs, it became imperative to refine the SDX *in vitro* test to a degree that would allow a clear differentiation between parasites with differing degrees of susceptibility, and sufficient reproducibility to permit a proper evaluation of the relative roles of individual mutations.

The procedures we describe here represent a significant improvement over the protocol used in our previous work (Brooks *et al*. 1994), and in the earlier studies of SDX resistance referred to in the Introduction section. They represent conditions where folate and PABA concentrations are reproducibly reduced to the lowest possible levels compatible with sufficient parasite growth for entry into the SDX test. They consistently yield the lowest attainable IC_{50} values, and have allowed us to monitor accurately differences in SDX responses among different lines, and between parental levels and those of the individual progeny of the HB3-Dd2 cross (Wang *et al*. 1997). The modified assay should also prove useful where different sulfa drugs are being compared for their inhibitory activity.

We have also used this test to examine the effect of adding back folate to different cultures. In this way, we have extended older work that demonstrated the importance of this compound in the context of SDX inhibition, and shown that parasites of different origins vary greatly in their ability to make use of exogenous folate, thus losing their susceptibility to SDX. It is important to be able to explore this phenomenon in more quantitative detail, as the malaria parasite *in vivo* is clearly not in a folate/PABA-free environment. Moreover, it has been shown recently that folate supplementation in the diet, given to help relieve anaemia brought on by falciparum malaria, had a significantly adverse effect on the efficacy of Fansidar treatment, whereas intake of an iron supplement or placebo did not (van Hensbroek *et al*. 1995). The next stage in which the SDX assay described here will play an important role will be in measuring the relative contribution of combined mutations in DHFR and DHPS to resistance to Fansidar itself, as well as in assessing how the effect of folate, clearly a major factor in the SDX resistance of certain parasites, is modulated by the presence of both SDX and PYR together, as in the Fansidar combination. The molecular basis of the synergistic relationship between these two drugs (Chulay *et al*. 1984; Watkins *et al*. 1985) is still poorly understood. An ability to confidently measure the susceptibility of parasites to sulfa-drugs both singly and in combinations should permit further progress to be made in exploring these important phenomena.

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