bryo, magnetic resonance imaging does not capture data at high enough resolution (Fig. 5), and serial sections cannot be aligned well enough to recreate structures as fine as nerve fibers (*19*). Any colored stain can be imaged in the same way as the in situ analysis shown in Fig. 2, allowing for the 3D visualization of results from many other types of experiments (for example, tissue-specific activity of enhancers and promoters in LacZ reporter constructs, cell lineage studies, and gene-trap experiments). The main disadvantage of OPT microscopy, as compared to μMRI, is that high-resolution reconstructions depend on the specimen being transparent and its tissues possessing a homogenous refractive index.

The increasing demand for comprehensive gene expression data has led to the creation of large-scale bioinformatics databases, some of which aim to store data on the 3D expression patterns of thousands of genes (*1*). We believe OPT microscopy will dramatically increase the rate at which such data is gathered as well as facilitate the detailed examination of mutant phenotypes from large-scale mutagenesis screens.

In addition to its use in developmental biology, OPT microscopy has the potential for use in medical applications for which knowledge of the 3D structure of a specimen may be useful, but not readily accessible from histopathological sections.

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Divergent Regulation of Dihydrofolate Reductase Between Malaria Parasite and Human Host

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For half a century, successful antifolate therapy against *Plasmodium falciparum* malaria has been attributed to host-parasite differences in drug binding to dihydrofolate reductase–thymidylate synthase (DHFR-TS). Selectivity may also arise through previously unappreciated differences in regulation of this drug target. The DHFR-TS of *Plasmodium* binds its cognate messenger RNA (mRNA) and inhibits its own translation. However, unlike translational regulation of DHFR or TS in humans, DHFR-TS mRNA binding is not coupled to enzyme active sites. Thus, antifolate treatment does not relieve translational inhibition and parasites cannot replenish dead enzyme.

Malaria caused by various species of protozoan parasites, *Plasmodium*, results in about 900 million acute cases and 2.7 million deaths every year (*1*). With the emergence of drug resistance, there is a continual need for new antimalarial agents that are potent and selective. Development of new drugs will be greatly facilitated by a complete understanding of the molecular mechanisms underlying previously successful antimalarials. Antifolates, including pyrimethamine, have been used in the treatment of malaria for about 50 years (*2*). These drugs target DHFR-TS, a specific bifunctional protein in the parasite (*3*). DHFR and TS are expressed as separate proteins in mammalian cells, but the functional importance, if any, of the difference seen in the protein configuration has remained elusive.

The selective activity of pyrimethamine has traditionally been attributed to higher affinity of the drug for *Plasmodium* DHFR-TS than for human DHFR (*2, 4–6*). However, other DHFR-TS inhibitors, which lack parasite-specific affinity, also show selective toxicity for malaria (*7–9*). WR99210 is a potent inhibitor of *P. falciparum* proliferation in culture (median inhibitory concentration IC_{50} = 0.1 nM; Fig. 1A) (*9, 10*). In contrast, human fibroblast HT1080 cells tolerate this compound (IC_{50} = 6300 nM; Fig. 1A) (*10*). The large difference in sensitivity to WR99210 is not due to differential affinity for DHFR: Enzyme kinetic assays revealed a mere 10-fold difference in binding of WR99210 between *Plasmodium* DHFR-TS (inhibition constant K_i = 1.1 nM) and human DHFR (K_i = 12 nM) (*10*). Integration of one human DHFR coding sequence in *P. falciparum* is sufficient to shift the IC_{50} of WR99210 from 0.1 nm to 860 nM, as previously reported (*11, 12*). This degree of resistance is similar to that of human cells (Fig. 1A). Thus, WR99210 selectivity does not arise from host-parasite differences in drug uptake, metabolism, or export.

Host-parasite differences in the regulation and expression of DHFR and TS may play a role in drug selectivity. Mammalian DHFR and TS levels increase with specific drug treatment (*13–15*): Methotrexate causes accumulation of DHFR protein (*13*), and 5-fluorouracil and D1694 increase TS levels (*15, 16*). Translational control determines such drug-induced target overproduction in mammalian cells. In the absence of substrates or drugs, mammalian DHFR and TS bind their cognate mRNA within the coding region and block translation (*16–18*). In the presence of substrates [dihydrofolate (DHF) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) for DHFR; deoxyuridine monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (mTHF) for TS] or inhibitors [methotrexate for DHFR; 5-fluoro-dUMP (FdUMP) for TS], the host enzymes disassociate from their cognate mRNA, relieving translational arrest (*16–18*). Thus, drug treatment reverses natural, autologous, translational inhibition and causes overproduction of the target protein. Such innate cellular buffering ensures that mammalian cells are resistant to inhibitors of DHFR and TS. The absence of such cellular buffering may ac-

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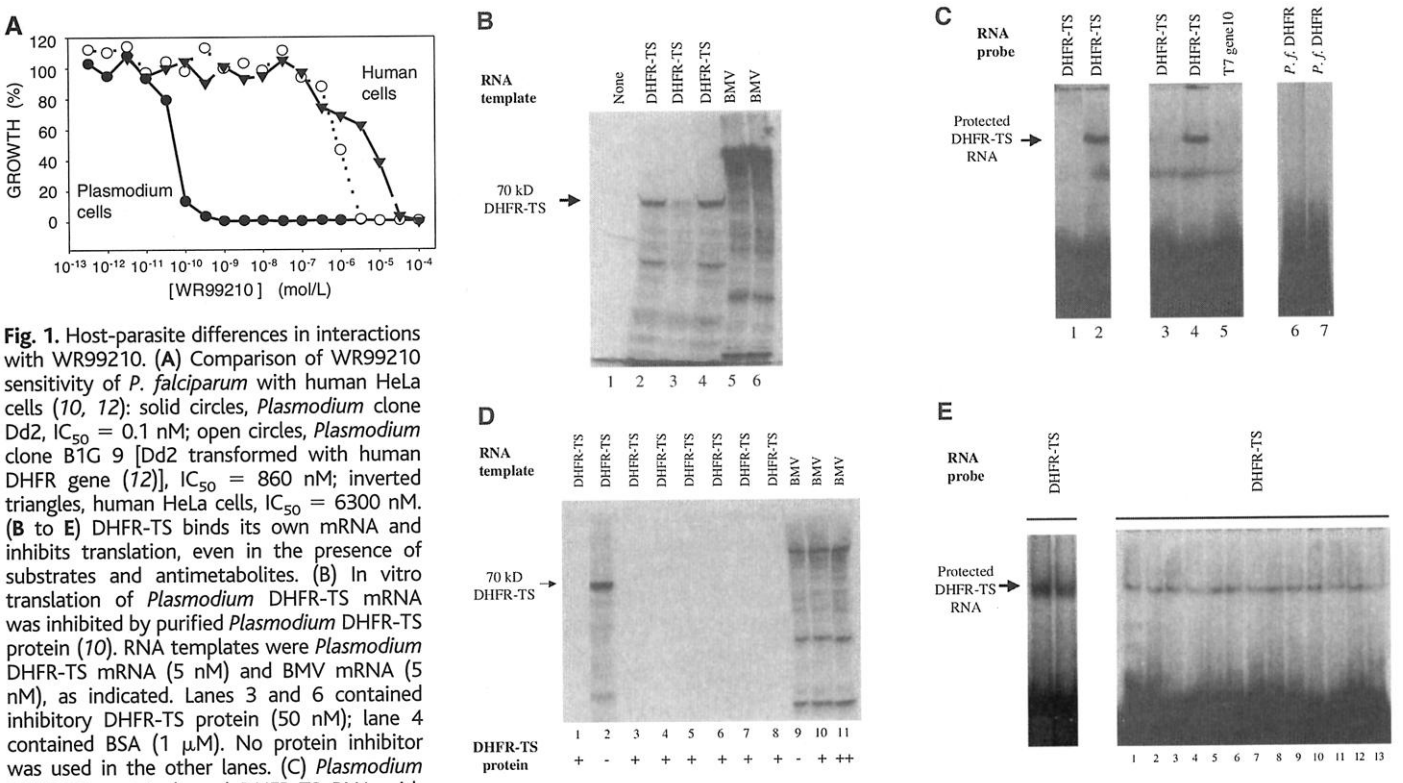

Fig. 1. Host-parasite differences in interactions with WR99210. (**A**) Comparison of WR99210 sensitivity of *P. falciparum* with human HeLa cells (*10, 12*): solid circles, *Plasmodium* clone Dd2, IC_{50} = 0.1 nM; open circles, *Plasmodium* clone B1G 9 [Dd2 transformed with human DHFR gene (*12*)], IC_{50} = 860 nM; inverted triangles, human HeLa cells, IC_{50} = 6300 nM. (**B** to **E**) DHFR-TS binds its own mRNA and inhibits translation, even in the presence of substrates and antimetabolites. (B) In vitro translation of *Plasmodium* DHFR-TS mRNA was inhibited by purified *Plasmodium* DHFR-TS protein (*10*). RNA templates were *Plasmodium* DHFR-TS mRNA (5 nM) and BMV mRNA (5 nM), as indicated. Lanes 3 and 6 contained inhibitory DHFR-TS protein (50 nM); lane 4 contained BSA (1 μM). No protein inhibitor was used in the other lanes. (C) *Plasmodium* DHFR-TS protein bound DHFR-TS RNA with specificity. ^{32}P-labeled mRNAs (0.5 nM) for DHFR-TS (lanes 1 to 4), T7 gene10 (lane 5), or *Plasmodium* DHFR (lanes 6 and 7) were mixed with proteins, and binding was determined by a gel mobility shift assay (*10*). Lanes 2 to 5 contained DHFR-TS binding protein (75 nM); lane 7 contained *Plasmodium* DHFR protein (3 μM). No protein was used in the other lanes. Lane 3 also contained competing nonradioactive DHFR-TS RNA (47 nM), and lane 4 also contained competing nonradioactive T7 gene10 RNA (96 nM). (D and E) DHFR-TS substrates and inhibitors do not reverse binding interactions between protein and cognate mRNA. (D) During in vitro translation (*10*), lanes 1, 3 to 8, and 10 each contained inhibitory DHFR-TS protein (75 nM); lane 11 had more DHFR-TS protein (150 nM). Lanes 3 to 6 contained substrates for DHFR-TS (100 μM each): NADPH (lane 3), DHF (lane 4), mTHF (lane 5), and dUMP (lane 6). Lanes 7 and 8 contained DHFR-TS inhibitors (100 nM each): MTX (lane 7) and 5-FdUMP (lane 8). (E) Binding between DHFR-TS RNA probe (0.5 nM) and DHFR-TS protein (750 nM) was not disrupted by substrates or inhibitors in a gel-shift assay (*10*). Left panel: RNA-protein interaction with (right lane) and without (left lane) 500 nM WR99210. Right panel: RNA-protein interaction. Lane 1 contained no additives. Lanes 2 to 8 contained substrates (100 μM each): NADPH (lane 2), DHF (lane 3), dUMP (lane 4), mTHF (lane 5), NADPH + DHF (lane 6), dUMP + mTHF (lane 7), and all four substrates (lane 8). Lanes 9 to 13 contained inhibitors (100 nM each): MTX (lane 9), pyrimethamine (lane 10), 5-FdUMP (lane 11), 1843U89 (lane 12), and all four inhibitors (lane 13).

count for the sensitivity of *Plasmodium* to DHFR-TS inhibitors.

Like the mammalian enzymes, *Plasmodium* DHFR-TS specifically blocked its own synthesis (Fig. 1B). An in vitro translation system synthesized the expected 70-kD protein (*8*) from *Plasmodium* DHFR-TS mRNA. Purified *Plasmodium* DHFR-TS protein prevented translation of DHFR-TS mRNA but not the translation of mRNA for brome mosaic virus (BMV) (Fig. 1B). Gel-shift assay showed direct binding of full-length *Plasmodium* DHFR-TS to its cognate mRNA but not to the unrelated gene10 mRNA from bacteriophage T7 (Fig. 1C). Binding of labeled DHFR-TS mRNA was reversed with nonlabeled DHFR-TS mRNA but not by nonspecific T7 gene10 mRNA.

Closer examination revealed important differences between the regulation of *Plasmodium* and mammalian DHFRs. In contrast to mammalian DHFR, catalytically active *Plasmodium* DHFR domain failed to bind DHFR mRNA or DHFR-TS mRNA (Fig. 1C, lanes 6 and 7). To determine whether the receptor for *Plasmodium* DHFR-TS mRNA lay outside the DHFR domain (TS-40) (*19*), we used an *E. coli* expression system (Fig. 2A). The difficulties in heterologous expression of full-length *Plasmodium* DHFR-TS in *E. coli* are well known and are usually attributed to poor use of malaria codons (*20, 21*). The following results show that underexpression of full-length *Plasmodium* DHFR-TS is better explained by invoking RNA-protein interactions. Truncated DHFR domain of *P. falciparum* was readily overexpressed (Fig. 2B, lanes 2 and 5), as was a truncated TS-31 fragment (Fig. 2D, lane 4). Truncated DHFR expression with *P. falciparum* codons produced just as much protein as did constructs made with *E. coli* codons (*22*). In sharp contrast, full-length DHFR-TS (70 kD) was poorly expressed even though its mRNA was as abundant as that of DHFR alone in parallel *E. coli* cultures (Fig. 2C). Most important, overexpression of *Plasmodium* DHFR domain was suppressed when TS-40 was produced in trans from a separate plasmid (Fig. 2D, lane 7). The TS-40 fragment itself was poorly expressed (Fig. 2D, lane 5). These expression studies are consistent with the hypothesis that *Plasmodium* DHFR-TS protein binds its own RNA through a site distant from the DHFR catalytic site.

Because the DHFR domain of malaria did not bind mRNA, we hypothesized that inhibitors of DHFR would not reverse interactions between full-length *Plasmodium* DHFR-TS and its cognate mRNA. This proved to be the case. Inhibition of *Plasmodium* DHFR-TS synthesis in an in vitro translation system could not be reversed by substrates or inhibitors of DHFR-TS (Fig. 1D). Similarly, direct binding of *Plasmodium* DHFR-TS to its mRNA could not be reversed with substrates (NADPH and DHF for DHFR; dUMP and mTHF for TS) nor with inhibitors [methotrexate (MTX), pyrimethamine, and WR99210, which inhibit DHFR; F-dUMP and 1843U89, which inhibit TS)] (Fig. 1E). The drug binding studies show directly that, unlike the host enzymes, the bifunctional *Plasmodium* protein binds mRNA at a site distinct from the DHFR as well as the TS catalytic site.

Together, these results predicted that WR99210 treatment of intact cells would induce overexpression of mammalian DHFR

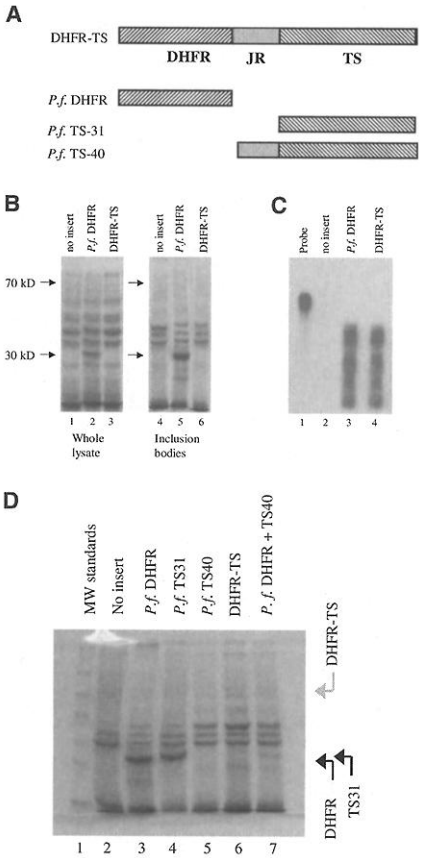

Fig. 2. Translational repression of full-length *Plasmodium* DHFR-TS, but not smaller domains, in a heterologous *E. coli* expression system. **(A)** Schematic representation of DHFR-TS fragments used in the *E. coli* expression system [JR, "joining region"; see (D)]. **(B)** Monofunctional *Plasmodium* DHFR protein, but not full-length DHFR-TS, can be overexpressed in *E. coli*. Host *E. coli* BL21(DE3) cells were transformed with different derivatives of the protein expression vector pET23D: pET only (lanes 1 and 4), pET-DHFR (lanes 2 and 5), or pET-DHFR-TS (lanes 3 and 6). Whole-cell lysate (lanes 1 to 3) and inclusion bodies (lanes 4 to 6) were resolved by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue (*10, 19*). **(C)** mRNA levels for pET-DHFR and pET-DHFR-TS were similar in parallel *E. coli* cultures. Host *E. coli* cells containing pET only (lane 2), pET-DHFR (lane 3), or pET-DHFR-TS (lane 4) were grown to late log phase. mRNA levels were determined by RNA protection assay (*10*). Lane 1 shows the undigested full-length probe. **(D)** A "TS-40" insert, coding for a 40-kD protein that includes the "joining region" and the TS region (*19*), suppressed *Plasmodium* DHFR overexpression in trans (lane 7). The TS-40 protein itself could not be overexpressed (lane 5). In contrast, TS-31 (TS without the "joining region") was readily overexpressed (lane 4). The expected position of full-length DHFR-TS, had it been overexpressed, is shown with a gray arrow.

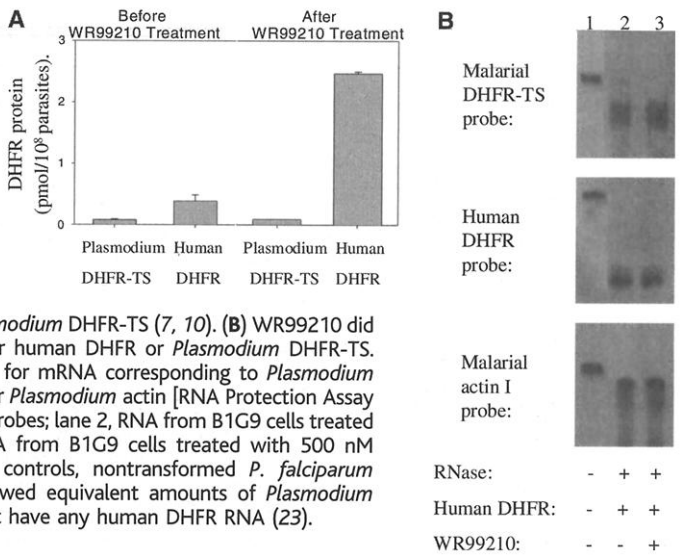

Fig. 3. **(A)** WR99210 treatment induces expression of human DHFR but not *Plasmodium* DHFR-TS protein. *Plasmodium falciparum* clone B1G9 (Dd2 transformed with human DHFR) was treated with 500 nM WR99210 or solvent (control). After 24 hours, parasite lysates were analyzed for human DHFR and *Plasmodium* DHFR-TS (*7, 10*). **(B)** WR99210 did not alter mRNA levels for human DHFR or *Plasmodium* DHFR-TS. Clone B1G9 was assayed for mRNA corresponding to *Plasmodium* DHFR-TS, human DHFR, or *Plasmodium* actin [RNA Protection Assay (*10*)]. Lane 1, undigested probes; lane 2, RNA from B1G9 cells treated with solvent; lane 3, RNA from B1G9 cells treated with 500 nM WR99210. In additional controls, nontransformed *P. falciparum* clones Dd2 and 3D7 showed equivalent amounts of *Plasmodium* DHFR-TS RNA but did not have any human DHFR RNA (*23*).

but not *Plasmodium* DHFR-TS. This prediction was tested in *P. falciparum* cells transformed with a single copy of human DHFR (*12*). Even before drug treatment, the expression level of human DHFR was higher than that of *Plasmodium* DHFR-TS by a factor of ~6 (Fig. 3A). After treatment with 500 nM WR99210, there was another factor of ~6 increase in human DHFR protein but no change in *Plasmodium* DHFR-TS (Fig. 3A). Even though human DHFR protein expression increased, the levels of human DHFR mRNA and *Plasmodium* DHFR-TS mRNA were comparable and unchanged, before and after WR99210 treatment (Fig. 3B). No changes in degradation rates of *Plasmodium* DHFR-TS or human DHFR were observed during WR99210 treatment (*23*).

Our findings point to an important paradigm for antimicrobial drug selectivity. Relatively nonspecific antimetabolites can selectively inhibit pathogen functions if the pathogen uniquely expresses limiting quantities of the drug target and lacks a mechanism to readily replenish the inhibited target. In the *Plasmodium* DHFR-TS system, host-parasite differences in target levels are partially realized through intrinsic RNA-protein interactions that are insensitive to cellular metabolites in the parasite; they are fully realized after drug administration. Another drug target that shows selective sensitivity without a host-parasite difference in affinity for the inhibiting drug is the African trypanosome enzyme ornithine decarboxylase (*24*). During treatment with difluoromethylornithine (DFMO), selectivity is achieved, at least in part, through rapid replenishment of the host ornithine decarboxylase but not the parasite enzyme. Although increases in target expression through gene amplification are known to affect drug sensitivity (*25*), our studies show that host-parasite differences in cellular responses to small ligands can affect target levels and drug sensitivity. Such properties may be of interest in future searches for good drug targets.

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