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Efficacies of Lipophilic Inhibitors of Dihydrofolate Reductase against Parasitic Protozoa

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Competitive inhibitors of dihydrofolate reductase (DHFR) are used in chemotherapy or prophylaxis of many microbial pathogens, including the eukaryotic parasites *Plasmodium falciparum* and *Toxoplasma gondii*. Unfortunately, point mutations in the DHFR gene can confer resistance to inhibitors specific to these pathogens. We have developed a rapid system for testing inhibitors of DHFRs from a variety of parasites. We replaced the DHFR gene from the budding yeast *Saccharomyces cerevisiae* with the DHFR-coding region from humans, *P. falciparum*, *T. gondii*, *Pneumocystis carinii*, and bovine or human-derived *Cryptosporidium parvum*. We studied 84 dicyclic and tricyclic 2,4-diaminopyrimidine derivatives in this heterologous system and identified those most effective against the DHFR enzymes from each of the pathogens. Among these compounds, six tetrahydroquinazolines were effective inhibitors of every strain tested, but they also inhibited the human DHFR and were not selective for the parasites. However, two quinazolines and four tetrahydroquinazolines were both potent and selective inhibitors of the *P. falciparum* DHFR. These compounds show promise for development as antimalarial drugs.

The treatment of diseases caused by eukaryotic pathogens is particularly difficult because of the similarity between their cell biology and that of their human host. The selection for pathogens resistant to currently effective drugs and the increase in immunocompromised individuals have added urgency to the search for new therapies directed specifically against these pathogens. One fruitful avenue for identification of chemotherapeutic drugs is to screen compounds that have already been synthesized in order to identify those that might be active against these increasingly important pathogens. We have adopted this strategy and screened a large library of compounds that are directed against the enzyme dihydrofolate reductase (DHFR) (EC 1.5.1.3). DHFR is a central enzyme in nucleic acid and amino acid synthesis in all cells, but the active sites of enzymes from different organisms show subtle differences that allow the identification of inhibitors specific for a particular species (3, 16–18, 24). For example, pyrimethamine is a selective inhibitor that is effective in the nanomolar range against the DHFRs from *Plasmodium falciparum* and *Toxoplasma gondii*, but the human enzyme is relatively insensitive to the drug (8, 14, 24). Thus, pyrimethamine has been used in malaria and toxoplasmosis therapy for many years (9, 49).

We have designed an easy and inexpensive system to test in budding yeast (*Saccharomyces cerevisiae*) potential DHFR inhibitors against the enzymes from a variety of parasites. Function of the endogenous *dfr1* gene was eliminated from the yeast (15), and the defect was complemented by expression of a heterologous DHFR gene from *P. falciparum*, *T. gondii*, *Pneumocystis carinii*, *Cryptosporidium parvum*, or humans (4). DHFR inhibitors function principally as competitive inhibitors of the enzyme. We have shown that the sensitivity of our engineered yeast strains to DHFR inhibitors depends on the interaction of the drug and the heterologous DHFR enzyme (4, 44, 48). For example, point mutations within the coding region of the *P. falciparum* DHFR gene can render the enzyme resistant to pyrimethamine. As one would expect, yeast that depends on a pyrimethamine-sensitive (Pyr^s) allele of the *P. falciparum* DHFR gene are killed by treatment with nanomolar concentrations of pyrimethamine, but the same yeast strain dependent upon a mutant pyrimethamine-resistant (Pyr^r) allele of DHFR is resistant to the drug. We have expanded this approach to design a rapid screen to identify DHFR inhibitors that are effective against yeast strains that depend upon a series of Pyr^r alleles of *P. falciparum* and against DHFR enzymes from other parasites.

In this paper, we report the analysis of 84 compounds to determine their efficacy against the *P. falciparum*, *T. gondii*, *C. parvum*, *P. carinii*, and human DHFR enzymes. We have identified six compounds that are potent inhibitors of all of the enzymes. Several of the compounds show selective inhibition of one or several of the parasite enzymes compared with the human DHFR. Among these selective inhibitors, a large number were effective against pyrimethamine-resistant alleles of *P. falciparum*. These data will allow further refinement of the structure-resistance profiles of these parasite enzymes and the design of more effective, selective inhibitors.

MATERIALS AND METHODS

Yeast strains. The *S. cerevisiae* strain used as a recipient of all of the plasmids was the *dfr1* mutant TH5 (*MAT***a** *leu2-3,112 trp1 ura3-52 dfr1::URA3 tup1*), generously provided by Tun Huang (15). Yeast was cultured for all experiments at 30°C on minimal, dropout, or rich (yeast extract-peptone-dextrose medium, using standard yeast genetics techniques (1, 13). Growth of the *dfr1* mutant was

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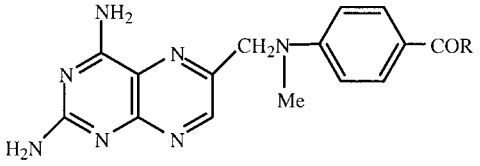
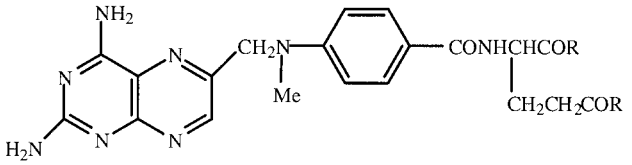


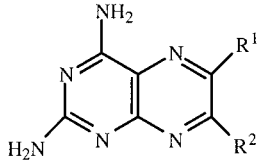
Entries 1-6 (refs. 6 and 7)

1 : R = 2-pentylamino
2 : R = 2-(1,5-dihydroxypentyl)amino
3 : R =1-adamantylamino
4 : R = morpholino
5 : R = cyclohexylamino
6 : R = 4-carbethoxy-1-piperazinyl



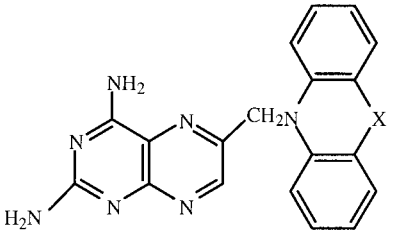
Entries 7-15 (ref.29)

7 : R = n-propylamino
8 : R = n-butylamino
9 : R = n-hexylamino
10 : R = 1-pyrrolidino
11 : R = 3,4-dimethoxybenzylamino
12 : R = 3-hydroxypropylamino
13 : R = benzylamino
14 : R = 2-phenylethylamino
15 : R = 3-(N,N-dimethylamino)propylamino



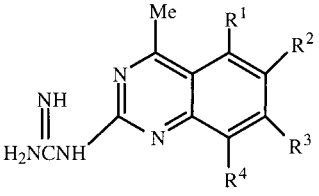
Entries 16-24 (refs. 27 and 41)

16 : R^1 = Me, R^2 = H
17 : R^1 = H, R^2 = Me
18 : R^1 = R^2 = 4-chlorobenzyl
19 : R^1 = R^2 = 3,4-dichlorobenzyl
20 : R^1 = R^2 = 2-naphthylmethyl
21 : R^1 = R^2 = 3-fluoromethylbenzyl
22 : R^1 = R^2 = 4-n-butylbenzyl
23 : R^1 = R^2 = 2-phenylethyl
24 : R^1 = R^2 = 3-phenylpropyl



Entries 25-30 (ref. 28)

25 : X = CH_2CH_2
26 : X = CH=CH
27 : X = no bond
28 : X = CH_2
29 : X = O
30 : X = S



Entries 31-35 (Refs. 31 and 36)

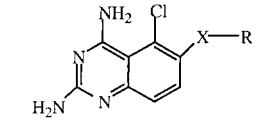
31 : R^1 = R^2 = R^4 = H, R^3 = OMe
32 : R^1 = R^4 = OMe; R^2 = R^3 = H
33 : R^1 = R^4 = H; R^2 = R^3 = OMe
34 : R^1 = R^3 = H; R^2 = R^4 = OMe
35 : R^1 = R^3 = OMe; R^2 = R^4 = H

FIG. 1. Structures of test compounds (entries 1 to 35).

supported by supplementation of the medium with 100 μg of dTMP (Sigma, St. Louis, Mo.) per ml.

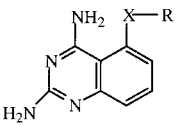
The TH5 yeast strain was transfected with a set of vectors that each expressed a heterologous DHFR enzyme. The parent expression vector, pEH2, is derived from pRS314 (45, 48). The DHFR-coding region from *P. carinii* (Pc-yeast) (11), *S. cerevisiae* (Sc-yeast) (12), or humans (Hu-yeast) (26) was cloned into the vector flanked at its 5′ end by a portion of the yeast DHFR promoter region and at its 3′ end by a portion of the yeast DHFR terminator, as described in detail by Brophy et al. (4). In Apicomplexan parasites, the DHFR enzyme is one domain of a bifunctional protein that also contains the thymidylate synthase (TS) activity (5, 18, 46). The same plasmid that contains the DHFR and TS domains from *T. gondii* was a gift from David Roos and Mary Reynolds (27). The DHFR domains from two strains of *C. parvum* were used, one derived from an infected human (hCp-yeast) and the other from a bovine infection (bCp-yeast) (46). Although the two differ at nine positions, none of these differences occur in amino acids that would be expected to cause changes in drug sensitivity; we have detected no differences in this study or in a previous one (4, 46). A set of yeast strains that expressed the DHFR domain from *P. falciparum* was also constructed (48). Each strain expressed an allele of *P. falciparum* DHFR whose sensitivity to the DHFR inhibitor pyrimethamine was known (18). The *P. falciparum*-derived strains are designated by their amino acid differences compared with the pyrimethamine-sensitive allele (S108). The S108N, N51I + S108N, C59R + S108N, N51I + C59R + S108N, and N51I + C59R + S108N + I164L alleles exhibit progressively higher levels of resistance to pyrimethamine (18). Two novel DHFR alleles (Y57H and I164M) were also tested; both show somewhat higher pyrimethamine resistance than the wild type (22). All of these heterologous DHFR enzymes complemented the *dfr1* mutation in the TH5 yeast strain.

Synthesis of the test compounds. The 84 compounds tested in this work are listed by structure in Fig. 1 and 2. These were archival samples with a purity of ≥90% as determined by thin-layer chromatography. The compounds in entries 1 to 6 were made from 4-[*N*-(2,4-diaminopteridin-6-yl)methyl-*N*-methyl]aminobenzoic acid and amines by the mixed anhydride method using isobutyl chloroformate and triethylamine (6, 7), whereas those in entries 7 to 15 were made by reaction of amines with diethyl *N*-[4-(2,4-diaminopteridin-6-yl)methyl-*N*-methyl]aminobenzoyl-L-glutamate (methotrexate diethyl ester) (30). 2,4-Diamino-6-methylpteridine (entry 16) and its 7-isomer (entry 17) were synthesized from 2,4,5,6-tetraaminopyrimidine and 1,3-dihydroxyacetone as described previously (43). The 6,7-disubstituted pteridines (entries 18 to 24) were made from 2,4,5,6-tetraaminopyrimidine and 1,2-diketones (28). The pteridines with a diarylamine side chain (entries 25 to 30) were obtained from 2,4-diamino-6-bromomethylpteridine and the appropriate diarylamine (29). The guanidinoquinazolines (entries 31 to 35) were prepared from arylamines, cyanoguanidine, and acetone (32, 38). The 2,4-diamino-5-chloroquinazolines were obtained by reductive coupling of 2,4-diamino-5-chloroquinazoline-6-carbonitrile with an arylamine (entries 36 and 37), by reductive coupling of 2,4,6-triamino-5-chloroquinazoline with an aromatic aldehyde (entries 38, 41, and 42), or by N methylation of a preformed 6-arylmethylaminoquinazoline with formaldehyde and sodium cyanoborohydride (entry 40) (37). The 2,4-diaminoquinazolines with an aromatic substituent at the position 5 were obtained from 2,4-diamino-5-iodoquinazoline and an arylalkene or arylalkyne via a palladium-catalyzed coupling reaction followed by catalytic hydrogenation (entries 43 to 45), by reductive coupling of 2,4-diaminoquinazoline-5-carbonitrile with an arylamine (entries 46 and 47), or by N methylation of a preformed 5-anilinomethylquinazoline (entries 48 and 49) (35). The 5- and 6-substituted 2,4-diaminothieno[2,3-*d*]pyrimidines (entries 50 to 54) were made from the corresponding 2-aminothiophene-3-carbonitriles and chloroformamidine hydrochoride (33, 36), whereas the 2,4-diamino-6-anilinomethylthieno[2,3-*d*]pyrimidines (entries 55 to 58) were made via a four-step sequence from 2,4-diamino-5-methylthieno[2,3-*d*]pyrimidine (41). One member of the latter group (entry 58) was obtained by reductive dehalogenation of the corresponding 6-bromo compound (41). The pyrido[4,3-*d*]pyrimidines (entries 59 to 61) were made by alkylation of the unsubstituted amine (33, 41), whereas the compounds in entries 62 to 65 were made from 2,4-diamino-6-bromomethylpyrido[3,2-



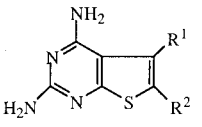
Entries 36-42 (refs. 32 and 35)

36 : X = CH_2NH, R = 2,5-dimethoxyphenyl
37 : X = CH_2NH, R = 2-bromo-3,4,5-trimethoxyphenyl
38 : X = $NHCH_2$, R = 2,5-dimethoxyphenyl
39 : X = $NHCH_2$, R = 3,4,5-trimethoxyphenyl
40 : X = $N(Me)CH_2$, R = 3,4,5-trimethoxyphenyl
41 : X = $NHCH_2CH_2$, R = 2,5-dimethoxyphenyl
42 : X = $NHCH_2CH_2$, R = 3,4,5-trimethoxyphenyl



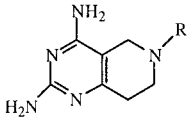
Entries 43-49 (ref. 33)

43 : X = CH=CH, R = phenyl
44 : X = CH_2CH_2, R = phenyl
45 : X = CH_2CH_2, R = 3,4,5-trimethoxyphenyl
46 : X = CH_2NH, R = 2,5-dimethoxyphenyl
47 : X = CH_2NH, R = 3,4,5-trimethoxyphenyl
48 : X = $CH_2N(Me)$, R = 2,5-dimethoxyphenyl
49 : X = $CH_2N(Me)$, R = 3,4,5-trimethoxyphenyl



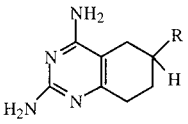
Entries 50-58 (refs. 32,33, and 39)

50 : R^1 = Me, R^2 = 2,5-dimethoxybenzyl
51 : R^1 = Me, R^2 = 2-(2,5-dimethoxyphenyl)ethyl
52 : R^1 = Me, R^2 = 2-bromo-3,4,5-trimethoxybenzyl
53 : R^1 = 2-(2,5-dimethoxyphenyl)ethyl, R^2 = H
54 : R^1 = 2-(2-bromo-3,4,5-trimethoxyphenyl)ethyl, R^2 = H
55 : R^1 = 3,4,5-trimethoxyanilinomethyl, R^2 = Br
56 : R^1 = 3,4,5-trimethoxy-N-methylanilinomethyl, R^2 = Br
57 : R^1 = 3,5-dichloro-4-pyrroloanilinomethyl, R^2 = Br
58 : R^1 = 3,5-dichloro-4-pyrroloanilinomethyl, R^2 = H



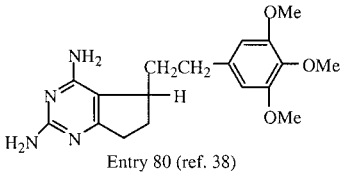
Entries 59-61 (refs. 32 and 33)

59 : R = 2-bromo-3,4,5-trimethoxybenzyl
60 : R = 2-(3,4,5-trimethoxyphenyl)ethyl
61 : R = 3-(3,4,5-trimethoxyphenyl)propyl

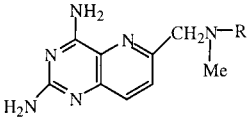


Entries 66-79 (ref. 37)

66 : R = 3-trifluoromethylbenzyl
67 : R = 2,5-dimethoxybenzyl
68 : R = 3-trifluoromethoxybenzyl
69 : R = phenyl
70 : R = t-butyl
71 : R = 3-methoxybenzyl
72 : R = 3-methylbenzyl
73 : R = 3-thenylmethyl
74 : R = 3,4-dichlorobenzyl
75 : R = 4-methoxybenzyl
76 : R = 4-methoxybenzylidene
77 : R = 2-methoxybenzyl
78 : R = 4-trifluoromethoxybenzyl
79 : R = 2-methylbenzyl

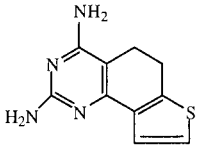


Entry 80 (ref. 38)



Entries 62-65 (ref. 30)

62 : R = 3,4,5-trimethoxyphenyl
63 : R = 4-chlorophenyl
64 : R = 3-chlorophenyl
65 : R = 3,4-dichlorophenyl



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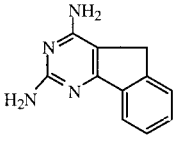

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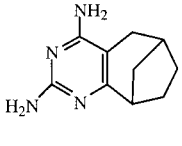
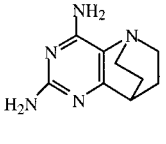


Entry 83 (ref. 40)



Entry 84 (ref. 18)

FIG. 2. Structures of test compounds (entries 36 to 84).

d]pyrimidine and anilines or *N*-methylanilines (31). The tetrahydroquinazolines (entries 66 to 79) and other several other di- and tricyclic pyrimidines (entries 80 to 84) were made from cyclic ketones and cyanoguanidine (39, 40, 42).

Drug sensitivity assays. The radial assay used here has been described in detail by Sibley et al. (44) and Brophy et al. (4). Briefly, drugs were dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and stored at −70°C until use. Drug dilutions in DMSO were made just before use and added to the growth medium or plate at a maximum final concentration of 1% DMSO in any solution. Addition of sulfanilamide increased the sensitivity of the yeast strains to the test drugs on plates. Therefore, for drug sensitivity experiments on solid medium, sulfanilamide was spread on the surface of the plate at a final concentration of 1 mM for tests of the complete set of heterologous strains. For some experiments testing only the *P. falciparum* set, 0.4 mM sulfanilamide was used on the plates. Drug sensitivity tests were made using a double replica plating procedure because this improved the discrimination of growth. A 10-μl volume of the test drug was added directly to the center of the plate. After 3 days of growth, each strain was scored for sensitivity by comparison with growth on the control plate without drug. Each drug was tested in triplicate.

The quantitative drug sensitivity assays were also conducted as previously described (44). Log-phase yeast cells were diluted uniformly into wells of a 96-well plate to generate the final concentrations required. Control wells lacked drug but contained a concentration of DMSO equal to that used in drug treatment; these were scored as 100% growth. The DMSO concentration was always <1%. The optical densities at 650 nm of the various drug dilutions were divided by this control value to determine percentage growth at each drug concentration. The 50% inhibitory concentration (IC_{50}) was calculated using the two values that flanked the 50% mark and the formula $y = mx + b$, where m and b were the slope and y intercept, respectively, calculated using the two flanking drug concentrations. The solution for x at $y = 50\%$ yielded the IC_{50}.

RESULTS AND DISCUSSION

The overall goal of this study was to rapidly screen a set of lipophilic compounds that were designed as inhibitors of DHFR in order to identify those effective against several human pathogens. We were especially interested in identifying inhibitors that are effective against the alleles of DHFR from Pyr^r *P. falciparum*, but the DHFR enzymes from *C. parvum*, *P. carinii*, and *T. gondii* were included as well. The gene that encoded the enzyme from each pathogen was expressed in the same strain of DHFR-deficient *S. cerevisiae*; the growth of each yeast strain was dependent upon the activity of the heterologous enzyme (4). Two additional specificity controls were included: the same yeast strain dependent on the human DHFR or the *S. cerevisiae* enzyme expressed from the same single-copy plasmid as the heterologous DHFR genes.

The compounds are listed in Fig. 1 and 2. Each compound was tested in a simple radial assay; the results from a typical experiment are shown in Fig. 3 to illustrate the strategy for screening. The yeast strains were streaked on master plates in a radial pattern as depicted at the bottom of Fig. 3. Eight compounds were tested in a set with two kinds of controls. Trimetrexate is a potent inhibitor of all DHFR enzymes (20) and was included in each series as a positive control. In order to increase sensitivity to the DHFR inhibitor, all plates con-

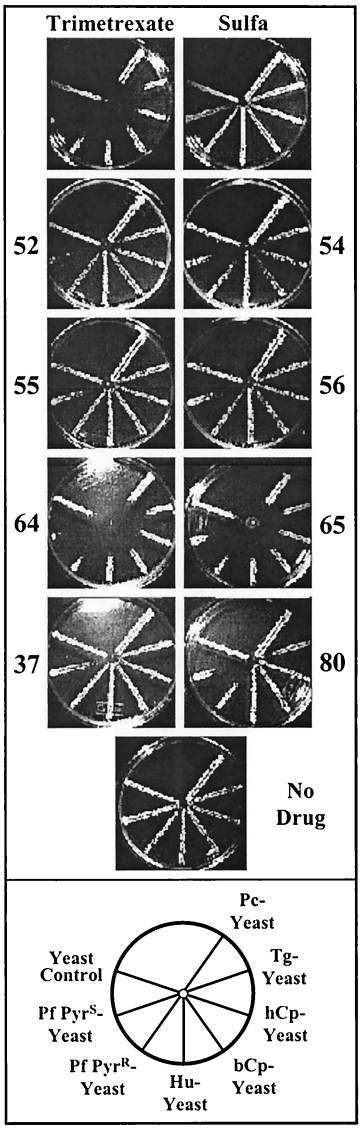


FIG. 3. Outline of basic screening protocol. Plates shown at the top are those normally included in each set. All plates except the no-drug control contain 1 mM sulfanilamide. The sulfa control shows growth of each yeast strain with no additional drug, and each plate with a designated compound was spotted with 10 μl of a 10-mg/ml solution of the compound dissolved in DMSO as described in Materials and Methods. Each set also contained a test plate with trimetrexate, a drug known to efficiently inhibit all test strains. At the bottom, a map of the location of each strain on the plates is shown. Pc-yeast, *dfr1* yeast dependent upon the DHFR enzyme from *P. carinii*; Tg-yeast, DHFR-TS from *T. gondii*; hCp-yeast, DHFR-TS from human-derived *C. parvum*; bCp-yeast, DHFR-TS from bovine-derived *C. parvum*; Hu-yeast, DHFR from human; Pf Pyr^R-yeast, DHFR domain from the pyrimethamine-resistant (N51I + S108N) allele of *P. falciparum*; Pf Pyr^S-yeast, DHFR domain from the pyrimethamine-sensitive (S108) allele of *P. falciparum*; yeast control, DHFR gene from *S. cerevisiae*.

tained 1 mM sulfanilamide. As a result, the yeasts were also replica plated from a master plate to a plate with sulfanilamide alone to ensure that growth was not inhibited by that addition. Each plate was then made from the master plate and spotted with 10 μl of a 10 mM solution of a test drug dissolved in DMSO. To ensure that the transfer was complete, a final plate with no drug was also included, as shown at the bottom of Fig. 3. In each case, the relative growth of the yeast strain reflected the relative inhibition of the DHFR enzyme expressed by the test strain.

Figure 3 also illustrates that different drugs showed different patterns of inhibition against the set of yeast strains. For example, the two pyrido[3,2-*d*]pyrimidines, compounds 64 and 65, were potent inhibitors of all of the strains, similar to the pattern displayed by trimetrexate. In contrast, the quinazoline, compound 37, and the thieno[2,3-*d*]pyrimidines, compounds 55 and 56, inhibited only the pyrimethamine-sensitive enzyme from *P. falciparum*, while 2,4-diamino-5-[3-(3,4,5-trimethoxyphenyl)ethyl]-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidine, compound 80, was effective against both the Pyr^s and Pyr^r alleles of the *P. falciparum* enzyme. Differences of this sort in the patterns of inhibition reflect variations in drug potency (4) and were the basis for our classification of the 84 compounds into five categories. These are summarized in Fig. 4. A table with data for all of the compounds is located on our website (http://depts.washington.edu/genetics/spokeassay.htm).

There are presently no effective drugs for treatment of *C. parvum* infections, so we specifically searched for compounds that would inhibit yeast expressing the *C. parvum* DHFR. Figure 4A shows the typical pattern for the six tetrahydroquinazolines that were extremely potent inhibitors of all of the yeast strains, including those dependent upon *C. parvum*. The nine compounds shown in Fig. 4B were effective against the human enzyme and at least some others but showed no inhibition of the *C. parvum*-dependent strains. This group contained three quinazolines, two tetrahydroquinazolines, and four pyrimidine derivatives. The nine compounds listed in Fig. 4C, comprised of pyrimidine derivatives and quinazolines, inhibited *P. falciparum* and the related pathogen *T. gondii*. Figure 4D lists a large number of compounds that were specific inhibitors of the *P. falciparum* enzyme alone. Most of the pteridines tested fell into this category, and a large number of the quinazolines did so as well.

It was of particular interest that a number of compounds were effective inhibitors of the yeast strain that expressed a pyrimethamine-resistant enzyme from *P. falciparum*; these are displayed in Fig. 4E. Some compounds in this category are also listed in Fig. 4B because they were ineffective against the *C. parvum*-dependent strains, but these represent an important subset of lead compounds to be studied as potential antimalarial drugs. Some of the pyrido[3,2-*d*]pyrimidines and most of the tetrahydroquinazolines fell into this category. Some of these compounds had been tested in vitro against the purified DHFR enzymes of *P. carinii*, *T. gondii*, or rat liver (39). We assumed that the inhibition of the rat and human enzymes would be similar, and this allowed us to compare the radial assay with the in vitro assay. The correspondence was excellent. For example, the tetrahydroquinazolines were generally potent inhibitors of all of the yeast strains tested and were scored as positive in the radial assay. We noted that neither compound 69 [(6*R*, 6*S*)-2,4-diamino-6-phenyl-5,6,7,8-tetrahydroquinazoline] nor compound 74 [(6*R*, 6*S*)-2,4-diamino-6-(3,4-dichlorobenzyl)-5,6,7,8-tetrahydroquinazoline] fell into this group. Both of these compounds had shown little or no inhibition in vitro of the purified DHFR enzymes of *P. carinii*, *T. gondii*, or rat liver (39), demonstrating the congruence of this

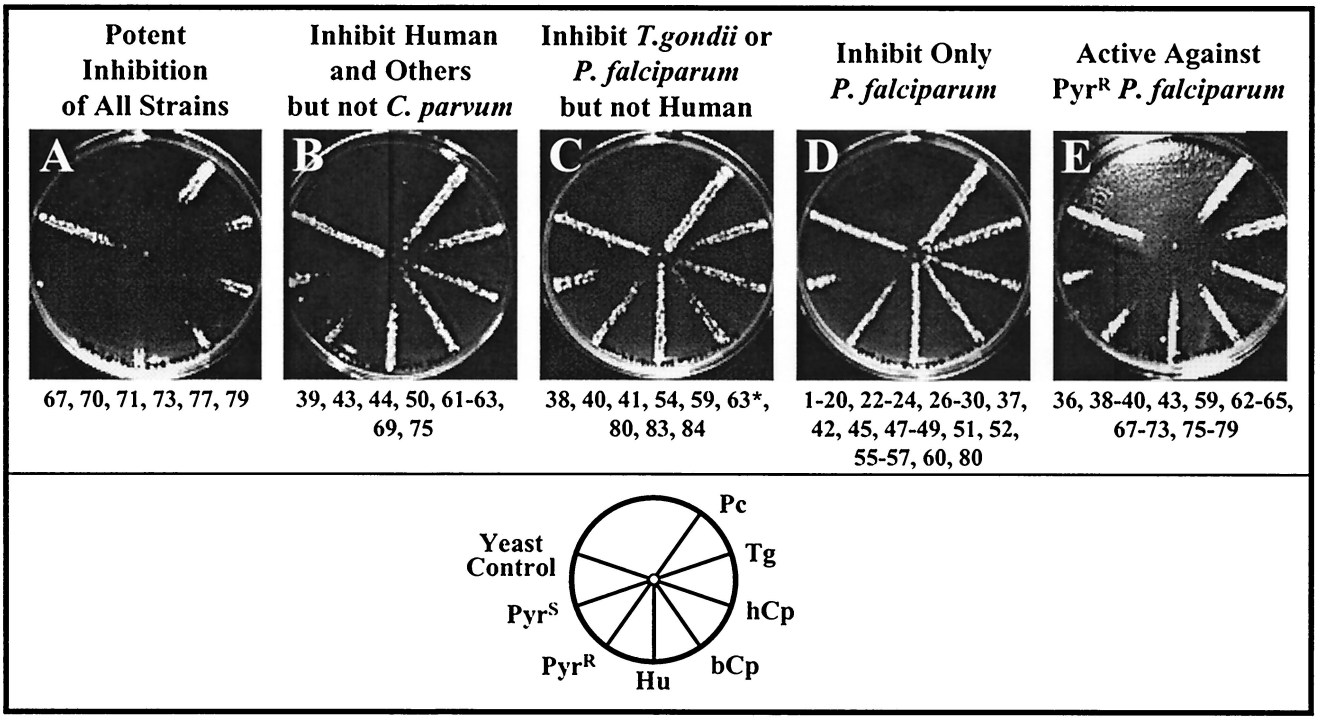


FIG. 4. Summary of patterns of drug activity observed on radial assay. The diagram at the bottom shows a typical pattern used to define each category. All drugs were tested three times, and if minor differences were observed, the category was determined by two of three patterns. The protocol was as described in Fig. 3 and in Materials and Methods. *, the Hu-yeast was more sensitive to compound 63 than the two Cp-yeast strains but was more resistant than the Pf- or Tg-yeasts, and thus the drug appears in categories panels B, C, and E. A complete table of the data for all of the compounds is available on our website (http://depts.washington.edu/genetics/spokeassay.htm).

yeast-based screening system with the results observed from direct assay of the purified enzyme.

Due to their highly lipophilic nature, some of the compounds were only sparingly soluble in the aqueous agar and precipitated as they were spotted on the plate. For this reason, six compounds (compounds 20, 47, 49, 53, and 55) were not amenable to testing by this assay method, which requires a very high initial concentration of the drug to be spotted in the center of the plate. However, any of these drugs might be effective if tested in vitro, where a lower initial concentration could be used.

These experiments also illustrate an interesting advantage of testing the compounds against living cells. Each assay included the same yeast strain dependent upon expression of the *S. cerevisiae* DHFR from the same plasmid used to express the heterologous enzymes. Even trimetrexate showed an extremely modest inhibition of this strain (Fig. 3B). If no inhibition of the yeast control was observed, this demonstrated that nonspecific toxicity of that compound was not a problem, at least for yeast cells. Compounds 36, 63 to 68, 70 to 76, 77, and 79 showed this kind of pattern (see Fig. 4B and D for examples). Whenever a compound did inhibit the yeast control, that compound was retested to determine whether addition of dTMP and the drug would restore the yeast growth. This reversal of inhibition was the case for these compounds, and we concluded that the growth deficit resulted from specific inhibition of the folate pathway by the drug. Most of the drugs in this category were in the tetrahydroquinazoline group. The tetrahydroquinazoline compound 78 showed incomplete reversal of growth inhibition in the presence of dTMP; this likely reflects at least some nonspecific inhibition of the yeast growth. Although the inhibition of the yeast enzyme was generally modest, compounds in this group may show promise as leads for development of antifungal agents.

The radial assay is only semiquantitative; to more precisely test the potency and specificity of promising compounds, we grew the yeast strains in a range of drug concentrations in liquid and measured their growth relative to that of the same strain without drug. We first tested the relative effectiveness of the compounds against *C. parvum* and human DHFR. An example of these data is shown in Fig. 5. Compound 36 (Fig. 5A) had been shown to be a reasonable inhibitor of the *P. carinii*, *T. gondii*, and rat liver enzymes in vitro (39), and it was a potent inhibitor of all three strains tested in this assay, showing IC_{50}s in the 10^{-7} M range. Both compounds 63 and 69 were in the category shown in Fig. 4B and more effectively inhibited the Hu-yeast than the two *C. parvum* strains. In this liquid growth assay, compound 69 inhibited the Hu-yeast in the micromolar range but was ineffective against the two Cp-yeasts (Fig. 5B). Compound 63 inhibited all three strains, but the IC_{50} was about 10-fold lower against the Hu-yeast (Fig. 5C). We next focused on correlating the qualitative categories defined by the radial assay with the IC_{50}s for the same compounds. We concentrated on comparing the human- and *C. parvum*-dependent yeast strains, and the data for 14 compounds are summarized in Table 1. The three compounds (compounds 36, 63, and 69) displayed in Fig. 5 are in boldface in Table 1. In addition, Table 1 lists the published IC_{50}s for the same com-

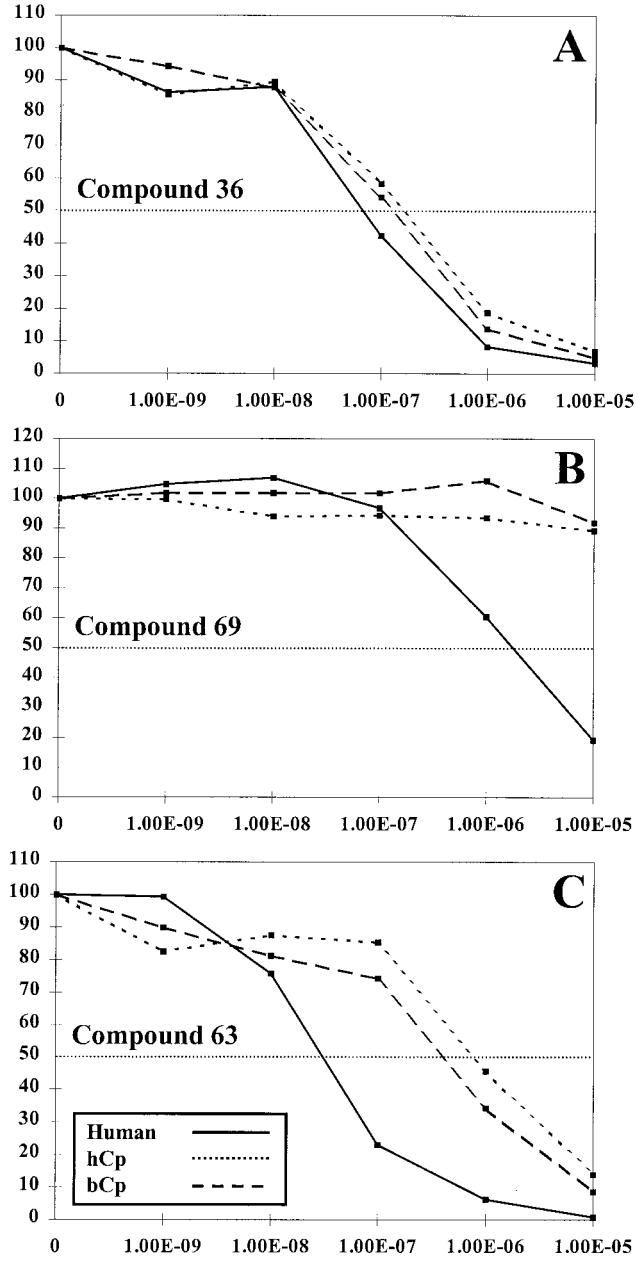


FIG. 5. Quantitative determination of efficacy of drugs against Hu-yeast, hCp-yeast, and bCp-yeast. Each strain was grown to log phase and then resuspended in growth medium containing 1 mM sulfanilamide and one of the indicated drugs at a concentration range of 0 to 10^{-5} M. The growth at each concentration relative to that of the no-drug control was calculated and graphed. Each determination was done in triplicate, and the mean value was used to calculate the growth. The IC_{50} was calculated as described in Materials and Methods.

pounds tested in vitro against the purified enzyme from rat liver. Several conclusions can be drawn. First, compounds that were ineffective in vitro were equally ineffective in the yeast assay (compounds 43, 50, 52, 54, 69, and 81). However, there was one compound, compound 28, that apparently did not penetrate the yeast, since it was without activity against any of the yeast strains, but showed good potency in vitro. Second, the relative effectiveness of the compounds against the rat liver enzyme and the Hu-yeast was similar, as one would expect for two mammalian enzymes. The most effective against rat liver were compounds 36 and 39, and these were also the most potent against the Hu-yeast. This direct comparison allows assessment of the relative sensitivity of the human enzyme and the DHFR from bovine-derived and human-derived *C. parvum*. Compounds 36 and 39 were also the most effective in this group against the two Cp-yeast strains, but the efficiency against the parasite DHFR was three- to fourfold lower than that against the Hu-yeast. No differences in sensitivity of the two forms of the *C. parvum* enzyme were observed. The correlation between the qualitative radial assay and the IC_{50} determinations, along with their correspondence with earlier assays against the purified enzyme, support the utility of the rapid screen as a first step in identification of drugs that are effective against these pathogens.

The most promising lead that emerged from the qualitative screen was the effectiveness of several categories of compounds against the Pyr^r allele of *P. falciparum*. To examine this lead in more detail, we tested the compounds that were effective against the Pyr^r allele further against a set of yeast strains that carry eight different alleles of *P. falciparum* DHFR with known sensitivity to pyrimethamine. These were arrayed in a radial pattern with the pyrimethamine-sensitive allele (S108) at the upper right and five progressively more pyrimethamine-resistant forms in a clockwise pattern, as shown in Fig. 6D. In addition, two novel alleles with a fivefold elevation in pyrimethamine resistance were included (Met164 and His57) (22). This set of *P. falciparum*-dependent strains was classified in comparison to their sensitivity to pyrimethamine and to the potent experimental DHFR inhibitor 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[3′-(2,4,5-trichlorophenoxy)-propyloxy]-1,3,5-triazine hydrochloride (WR99210) (21), as shown in Fig. 6B and C. The yeast that carried the human DHFR allele was not included in this part of the screen because the human enzyme is much more resistant to the test compounds, and we could not have assayed all of the *P. falciparum* alleles on the same

TABLE 1. Comparison of IC_{50}s of 14 compounds[a]

Compound	IC_{50} (μM) for:				Radial assay (Fig. 4)[b]
	In vitro rat enzyme	Hu-yeast	hCp-yeast	bCp-yeast	
4	ND[c]	10.0	>10	>10	D
28	0.003	>10	>10	>10	D
36	**0.004**	**0.08**	**0.29**	**0.2**	**E**
39	0.006	0.07	4.61	3.75	B, E
40	0.038	0.5	0.81	0.78	C, E
43	0.51	3.0	>10	>10	B, E
50	0.4	3.8	>10	>10	B
52	0.93	10.0	10.2	10.0	D
54	2.8	>10	>10	>10	C
63	**0.022**	**0.05**	**0.91**	**0.65**	**B, C, E**
66	0.19	0.7	5.75	3.84	—[d]
69	**1.9**	**3.0**	**>10**	**>10**	**B, E**
75	0.077	0.09	0.53	0.36	B, E
81	30.0	>10	>10	>10	—

[a] The values for compounds tested in vitro against the rat liver enzyme are from published work (33–37, 39, 42). Boldface indicates data shown in Fig. 5.
[b] The letters indicate the panels in Fig. 4.
[c] ND, not determined.
[d] —, not displayed.

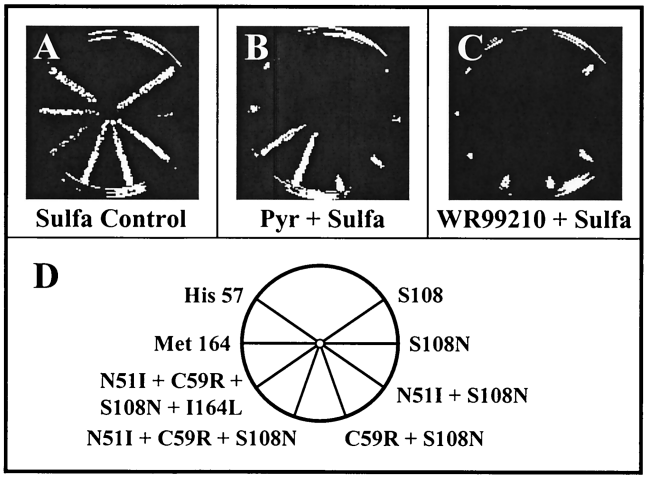


FIG. 6. Protocol for testing DHFR alleles from *P. falciparum*. Yeast strains dependent upon the DHFRs from eight different *P. falciparum* alleles were radially arrayed as shown in panel D. The master plates were grown for 2 to 3 days at 30°C and replica plated to plates that contained 0.4 mM sulfanilamide. Each test plate was spotted with 10 μl of the drug to be tested, and the results were tabulated after an additional 3 days of growth. The control plate with sulfanilamide alone is shown for each set of test plates, since growth of two of the strains was slowed somewhat by the sulfanilamide. Each strain is designated by the genotype of the allele, with the amino acid change from the wild type indicated. The DHFR domains from the first six strains are derived from standard reference strains of *P. falciparum* whose sensitivity to pyrimethamine has been established. The Met 164 and His 57 alleles are novel mutations that confer a low level of pyrimethamine resistance (21).

plate had it been included. For these experiments, a lower level of sulfanilamide, 0.4 mM, was present in the plates for the first 20 of these compounds, and no sulfanilamide was used for the remaining 13 compounds. When sulfanilamide was added, a sulfa-only control was always included, and this is depicted in Fig. 6A. In each case, the effectiveness of the test compound was measured against WR99210 or pyrimethamine under the same conditions.

Figure 7A summarizes these data for the 33 compounds that showed activity against the Pyr^r *P. falciparum*. We were interested both in the potency of the compounds and in whether the strains with more mutations showed higher levels of resistance to these drugs, as they do against pyrimethamine (18). Because of the chemical similarity of the compounds, we assumed that diffusion would be similar enough for all compounds to allow us to categorize the drugs qualitatively. Strains were placed in two categories: less or more potent than pyrimethamine (Fig. 7A and B), but with a pattern of resistance similar to that observed for pyrimethamine. The third group (Fig. 7C) included compounds that had a novel pattern of resistance. For example, on the plate shown, the most resistant strain carries the N51I + S108N allele, whereas for most compounds tested, the resistance was highest in strains that carried three or four mutations. Compounds 43, 72, and 80 were then tested in liquid culture to determine the IC_{50}s against four of these DHFR alleles: the most pyrimethamine-sensitive (S108), two double mutant alleles that confer intermediate pyrimethamine resistance (N51I + S108N and C59R + S108N) and the highly resistant allele (N51I + C59R + S108N + I164L). Compounds 26, 70, and 77 were tested against only the highly resistant allele. Table 2 summarizes these data in comparison with both pyrimethamine and WR99210. As expected, pyrimethamine is extremely effective against the S108 allele but shows about a 10-fold higher IC_{50} against both double mutants and no effect on the quadruple mutant. In contrast, WR99210 was effective against all of the alleles tested, even the highly pyrimethamine-resistant mutant form (21). Among the test compounds, compound 72 showed excellent potency, in the same range as pyrimethamine and WR99210, against all of the alleles except the quadruple mutant. This compound, (6*R*, 6*S*)-2,4-diamino-

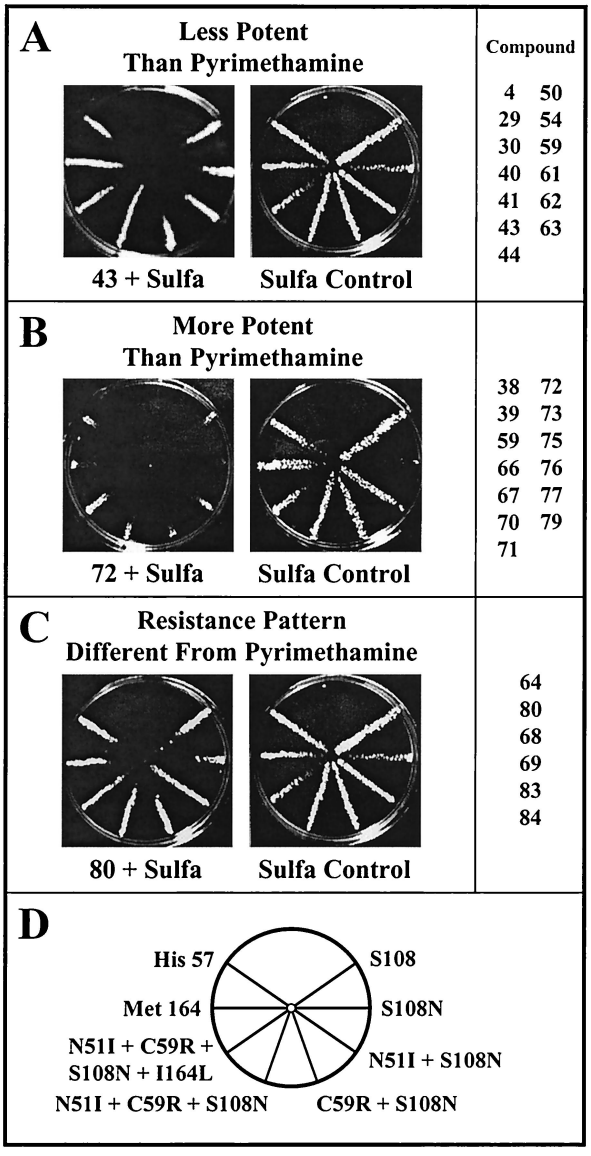


FIG. 7. Summary of efficacy of drugs tested against eight different *P. falciparum* alleles of DHFR. The drugs tested were categorized in comparison to the potency of pyrimethamine and the experimental DHFR inhibitor WR99210. (A and B) Compounds in which the effectiveness of the drug against the set of reference alleles was similar to the pattern for pyrimethamine. (C) Compounds in which the relative effectiveness of the drug against the reference alleles was different from the pattern for pyrimethamine.

TABLE 2. Efficacy of selected compounds against four reference strains of *P. falciparum* DHFR

DHFR allele	IC_{50} (μM) of compound:							
	Pyrimethamine	WR99210	26	43	70	72	77	80
S108	0.007	0.01	ND[a]	0.5	ND	0.017	ND	4.4
N51I + S108N	0.07	0.06	ND	3.0	ND	0.05	ND	7.2
C59R + S108N	0.07	0.06	ND	4.4	ND	0.08	ND	9.5
N51I + C59R + S108N + I164L	>10	0.07	>10	>10	10.0	2.9	3.7	>10

[a] ND, not determined.

6-(3-methylbenzyl)-5,6,7,8-tetrahydroquinazoline, showed about fourfold selectivity when the purified DHFR of *T. gondii* was compared in vitro with the human DHFR as well (39). The quinazolines, compounds 38 and 39, and the related tetrahydroquinazolines, compounds 67, 70, 71, and 75, all showed a similar excellent potency and reasonable selectivity, even against the triple (N51I + C59R + S108N) mutant *P. falciparum* DHFR allele.

The pattern of resistance of *P. falciparum* DHFR to pyrimethamine is well studied. In that case, the loss of potency is gradual as one examines DHFR alleles with increasing numbers of mutations. The pattern for the tetrahydroquinazolines is different; the drugs efficiently inhibit all of the alleles with one to three mutations. The change from isoleucine to leucine at amino acid 164 has a profound effect, abrogating the effectiveness of all drugs in this class. Only WR99210 was effective against the quadruple mutant allele (N51I + C59R + S108N + I164L). This mutant has not yet been observed in African populations of *P. falciparum* (2, 19, 23, 25, 47), and thus a compound effective against the common double and triple mutant alleles is an extremely interesting lead for further development.

While DHFR inhibitors are extremely effective drugs, point mutations have been rapidly selected in *P. falciparum* populations whenever they have been used. The mutations selected by pyrimethamine have been clearly identified, and their pattern of sensitivity seems to be similar in the set of compounds studied here (10, 18). However, identification of inhibitors still effective against the most pyrimethamine-resistant alleles holds the potential for reversing this pattern. WR99210 is one example of such a situation; some mutations that confer resistance to WR99210 increase the sensitivity to pyrimethamine (48). If other compounds also show this "opposing selection," it may be possible to slow the selection of parasites resistant to these newer DHFR inhibitors.

The large libraries of already-synthesized inhibitors of DHFR are a potentially fruitful source of lead compounds. Several of the tetrahydroquinazolines surveyed here show real promise both for more detailed analysis of the active site of *P. falciparum* and for further development as antimalarials or drugs effective against *T. gondii*. The ease with which this yeast-based assay is performed and the opportunity to screen compounds against DHFR enzymes from a variety of pathogens make it a reasonable first step in narrowing the search for drugs that are effective against these infectious diseases of humans or domestic animals.

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