Antifolate Studies. Activities of 40 Potential Antimalarial Compounds against Sensitive and Chlorguanide Triazine Resistant Strains of Folate-Requiring Bacteria and *Escherichia coli*

Clara S. Genther and Carl C. Smith*

Department of Environmental Health and Christ Hospital Institute of Medical Research, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267. *Received March 15, 1976*

As part of the search for new antimalarial drugs, a screening program was developed using sensitive and chlorguanide triazine (CGT, cycloguanil) resistant strains of the folate-requiring bacteria, *Streptococcus faecium* durans, *Lactobacillus casei*, and *Pediococcus cerevisiae*. The activities of 40 compounds have been studied against these strains and *Escherichia coli*. Observations have been made on the points of 50% growth inhibition, the fold increase of resistance shown to each compound by the resistant strains as compared with the parent sensitive strains, and the reversal of growth inhibition by folic acid with *S. faecium* and *L. casei* by folinic acid with *P. cerevisiae* and by *p*-aminobenzoic acid with *E. coli*. Comparisons have been made of the activities of the test compounds with those of the standard antimalarial antifolates, CGT and pyrimethamine (PM), and the antibacterial results have been compared with the activities of the compounds against *Plasmodium berghei* infections in the mouse and against human malaria infections where data are available. Of the 17 compounds reversed by folates, five had patterns of activity similar to CGT and PM in that they were most active against *S. faecium* and nine compounds exhibited a different pattern, being highly active against all four test bacteria. This suggests that these latter compounds either have different pharmacokinetic properties or have additional modes of action. The three CGT-resistant organisms responded to antifolates in different ways. *S. faecium* (R) and *P. cerevisiae* (R) strains were cross resistant to 4,6-diaminotriazines, 2,4-diaminopyrimidines, 2,4-diaminoquinazolines, and active 2,4-diaminopteridines. *L. casei* (R) was cross resistant to the triazines but was collaterally sensitive to all the other antifolates. Most of the compounds not reversed by folates were much less inhibitory for the test organisms; they were most active against *L. casei*. In general, their growth inhibitory concentrations varied less for the four test organisms and the responses of the sensitive and CGT_R strains were similar. However, there was some cross resistance to five compounds and some collateral sensitivity to five others. Comparison of the bacteriological data with the activities of the compounds against *Plasmodium berghei* in the mouse showed little correlation between the two test systems; each appears to provide independent and useful information.

The advent of chloroquine-resistant strains of malaria in Southeast Asia led to the establishment of the current antimalarial program under the sponsorship of the Walter Reed Army Institute of Research.[1] As part of this study we developed a screening program for antifolate compounds using folate-requiring bacteria. The purposes of the program were threefold: (1) to find more active antifolates; (2) to find antifolates with different patterns of activity from those in present use; and (3) to find compounds which would be active against strains resistant to treatment with standard antimalarials.

To answer these questions we designed a screening program using a spectrum of bacterial strains each of which enters the folic acid cycle at a different point; these included *Escherichia coli*, *Streptococcus faecium*, *Lactobacillus casei*, and *Pediococcus cerevisiae*. *E. coli* makes its own folic acid starting with very simple precursors; it is inhibited by sulfonamides, which compete with *p*-aminobenzoic acid (PABA),[2] but only slightly by active antimalarial antifolates such as chlorguanide triazine (CGT, cycloguanil) and pyrimethamine (PM). *S. faecium* can use pteroic acid which resembles folic acid except that it lacks the glutamic acid portion; it can also use folic and folinic acids. This organism is strongly inhibited by CGT and PM. *L. casei* requires the intact folic acid molecule and can also use 5-methylfolic acid which cannot be used by *S. faecium*.[3] *L. casei* is inhibited to a moderate degree by CGT and PM. *P. cerevisiae* requires not only the complete folic acid moiety but, in addition, the molecule must be reduced to tetrahydrofolate.[4] *P. cerevisiae* is also inhibited to a moderate degree by CGT and PM.

In addition to the parent strains of these bacteria, we isolated substrains which are highly resistant to certain of the more important antifolate antimalarial agents.[5] It was reasoned that these resistant bacteria might prove to be useful models of some of the drug-resistant (i.e., CGT- or PM-resistant) malarial strains uncovered in the field. In the present report we are presenting the results of tests with the chlorguanide triazine resistant (CGT_R) strains of *S. faecium*, *L. casei*, and *P. cerevisiae*.

This paper includes data on 40 compounds; about half are antifolates and the remainder are compounds whose activity was not expected to be reversed by folates. These latter compounds were included in order to detect any possible antifolate action and to see whether they would exhibit any unusual activity against the antifolate-resistant strains.

Results and Discussion

The reproducibility of the results with the various test organisms is illustrated by the data in Table I. Over 200 determinations of activity of PM against the sensitive strains of *S. faecium*, *L. casei*, and *P. cerevisiae* have been made over a period of 10 years since this compound was used as a control with each lot of compounds tested. Less extensive data on CGT, amethopterin, and trimethoprim are also included. The means of the micromolar concentrations required to reduce growth by 50% (IC_{50}) and their standard deviations are listed. The standard deviations as expressed in percent range from 22 to 57%, a range considered quite acceptable for this type of test.

The results obtained with the 40 chosen compounds against both the parent sensitive and CGT_R strains of *S. faecium*, *L. casei*, *P. cerevisiae*, and *E. coli* are given in Table II. The data include the IC_{50}, the reversal or nonreversal by folic acid with *S. faecium* and *L. casei*, by folinic acid with *P. cerevisiae* and by *p*-aminobenzoic acid (PABA) with *E. coli*, and the FIR (fold increase in resistance). The FIR was calculated by dividing the IC_{50} of the CGT_R strain by the IC_{50} of the sensitive strain. The compounds are grouped roughly by structure. The results obtained with the same compounds in the Rane malaria test, which employs mice infected with *Plasmodium berghei*,[6,7] are given in Table III.

Table I. Reproducibility of Results Obtained from 1966 to 1976

	IC_{50}[a]					
	S. faecium		*L. casei*		*P. cerevisiae*	
Compd	No. of trials	Mean	No. of trials	Mean	No. of trials	Mean
PM[b]	286	0.008 ± 0.004	214	5.0 ± 1.49	205	2.75 ± 0.66
CGT	138	0.028 ± 0.011	18	15.8 ± 4.08	12	32 ± 6.9
MTX	9	0.0033 ± 0.001	8	0.00007 ± 0.00004	9	0.27 ± 0.15
TMP	10	0.11 ± 0.028	12	0.18 ± 0.049	10	6.67 ± 1.56

[a] Micromolar concentration of compound required to reduce growth to 50% of the control. [b] PM, pyrimethamine; CGT, chlorguanide triazine (cycloguanil); MTX, amethopterin, and TMP, trimethoprim.

Compounds 1–4 are 4,6-diamino-2,2-dimethyl-*s*-triazines; 1 is CGT, the active antimalarial metabolite of chlorguanide (proguanil)[8] and the compound to which the bacterial strains were made resistant. This compound is highly active against *S. faecium* and moderately active against *L. casei* and *P. cerevisiae*, but is not active against *E. coli* up to the limits of the test (1000 μg/ml). The CGT_R strain of *S. faecium* was 194 000 times more resistant to CGT than the sensitive organism; *L. casei* (R) was 147 times more resistant and *P. cerevisiae* (R), 26 times more resistant. CGT is reversed by folates and is highly active in the mouse malaria test and in human infections.[9]

Replacing Cl with an Et yields **2** which is more active than CGT in the *L. casei* (R) strain and shows measurable activity against *E. coli*. However, this compound has low activity in the mouse test and is not sufficiently different from CGT to merit further study.

Compounds **3** and **4** have the phenyl group linked to the triazine with $-OCH_2-$ and $-O(CH_2)_3O-$ and have additional Cl substitutions. These compounds differ widely from CGT or PM in activities. They are highly active against both sensitive and resistant strains of all four test organisms. The FIR is smaller than that of CGT for the *S. faecium* (R) and *L. casei* (R) strains but the figure is larger in the case of *P. cerevisiae* (R). These two compounds are reversed by folates but not by PABA (*E. coli*). Both are highly active against the mouse malaria and have been tested against simian and human malaria.[10]

Compounds **5**–**7** are 2,4-diaminopyrimidines, **5** being the well-known drug PM. Against our sensitive organisms, PM is more active than CGT but has a similar activity profile. Interestingly, PM has a pattern of activity against the resistant cultures which distinguishes it clearly from CGT. Although *S. faecium* (R) and *P. cerevisiae* (R) are cross resistant to PM, the FIR is only $^1/_{100}$th that of CGT with *S. faecium* (R) and one-ninth that of CGT with *P. cerevisiae* (R). The greatest difference is seen in the case of *L. casei* (R) where the resistant strain is collaterally sensitive to PM.[11] This interesting and unexplained phenomenon is under study. The *L. casei* (R) strain although resistant to triazines is collaterally sensitive to pyrimidines, quinazolines, and pteridines. PM is reversed by folates but not by PABA with *E. coli*. PM is highly active against mouse, monkey, and human malaria.[9,12]

Trimethoprim (TMP, **6**) is both an antimalarial and antibacterial agent.[13] Its pattern of activity against the test bacterial strains is quite different from that of either CGT or PM. It is only slightly more active against *S. faecium* than *L. casei* and is highly active against *E. coli*. Compared to PM it is only one-tenth as active against *S. faecium* and one-third as active against *P. cerevisiae*, but it is approximately 30 times as active against *L. casei* and 190 times as active against *E. coli*. The FIR for *S. faecium* (R) is only 15 and for *P. cerevisiae* (R) the FIR is 6. *L. casei* (R) is collaterally sensitive to TMP. TMP is reversed by folates against *S. faecium*, *L. casei*, and *P. cerevisiae* but not by PABA against *E. coli*. TMP is not active in the mouse malaria test but does show activity in simian and human malaria, particularly in combination with a sulfonamide.[10,14]

If one replaces the trimethoxyphenyl group of TMP with a piperonyl, **7**, one retains activity against *S. faecium*, *L. casei*, and *P. cerevisiae*. However, the compound was much less active against *E. coli* and was inactive against mouse and human malaria.[10]

Compounds **8**–**13** are 2,4-diaminoquinazolines with substitutions at the 6 position: **8**, 3′,5′-$Cl_2C_6H_3O-$; **9**, 4′-ClC_6H_4S-; **10**, 4′-$ClC_6H_4CH_2S-$; **11**, 2′-naphthyl-SO_2-; and **12**, 3′,4′-$Cl_2C_6H_3CH_2NH-$, or a benzo[*f*]quinazoline, **13**. These are remarkably active compounds whose pattern of activity differs widely from that of CGT and PM. They were highly active against all four test organisms and only slightly more active against *S. faecium* than against *L. casei* and *P. cerevisiae*. **12** was moderately active against *E. coli* while the remaining members of the group were highly active. It is difficult to make structure comparisons within this set of compounds since in most cases more than one group is varied. In comparing **10** and **9** it can be seen that substituting a benzyl for the phenyl group lowered activity against all four organisms and against mouse malaria. In general **11** was the most active compound; even against the resistant strains only very small amounts of this drug were required for growth inhibition. **11** was also much more active than PM and CGT in the mouse test. Activities against the resistant strains showed that with this group of compounds the FIR with *S. faecium* (R) was considerably less than that with PM especially with **10** and **11**; with *P. cerevisiae* (R) the FIR of **12** and **13** were in the same range as that of PM and with the remaining compounds the FIR was higher, being about the same as CGT. *L. casei* (R) was collaterally sensitive to all of the group, being most sensitive to **12** and **13**. These compounds were reversed by folates but not by PABA (*E. coli*). **9**, **11**, and **12** were active in the mouse malaria test and have been tested in simian malaria, and **11** has been approved for clinical trial in combination with sulfadiazine.[10]

14–**17** are 2,4-diaminopteridines; **14** has Me_2-C_6H_4- at the 6 position and NH_2- at the 7 position. This compound has the familiar CGT–PM pattern of activity. However, its FIR to *S. faecium* (R) was only one-tenth that of PM, the FIR to *P. cerevisiae* (R) was about the same as PM, and *L. casei* (R) was collaterally sensitive. It was reversed by folates but not by PABA (*E. coli*). It has moderate activity against mouse malaria, but in human malaria infection it effected only temporary suppression of the parasitemia and had some toxic effects.[10]

Introducing Me_2CH- groupings at both the 6 and 7 positions provided a compound, **15**, which had a pattern of activity similar to CGT or PM and was much more active against all of the bacterial strains than was **14**. The cross resistance pattern of this compound was similar to that of **14**. It was reversed by folates but not by PABA (*E. coli*). It was not active against mouse malaria.

16 is amethopterin (MTX, methotrexate), an active

antitumor agent. This compound was very highly active against the three folate-requiring bacteria with a pattern of activity different from that of CGT and PM in that it was most active against *L. casei*.[15] It was approximately 10^5 times as active as PM against *L. casei* and more active than CGT but less active than PM against *E. coli*. The FIR with *S. faecium* is much less than with PM and CGT; the FIR with *P. cerevisiae* is the same as PM. *L. casei* (R) is collaterally sensitive to MTX. It is not reversed by folic acid against *S. faecium* and *L. casei* but is reversed by folinic acid with *P. cerevisiae*.[15] It is not reversed by PABA (*E. coli*). MTX is not active in mouse malaria. Although a report indicating some activity of MTX in human *Plasmodium vivax* malaria has appeared in a relatively uncontrolled study,[16] the results are suspect since extensive testing of MTX and aminopterin in both blood-induced and sporozoite-induced *Plasmodium cynomolgi* showed them to be totally inactive.[17]

17 is sodium tetrahydrohomopteroate. This compound is highly active against *S. faecium*, moderately active against *L. casei*, and inactive against *P. cerevisiae*. The FIR against *S. faecium* was high and *L. casei* (R) was collaterally sensitive. It was reversed by folic acid. It had no activity in the mouse malaria test but did show activity against *Plasmodium cynomolgi*.[18] This compound must be kept in the reduced form which makes it difficult to use as a chemotherapeutic agent.

Blocking or substituting the triazine amino groups, **18** and **19**, greatly reduced activity against *S. faecium* confirming earlier findings.[15,19] However, their activities against *L. casei* and *P. cerevisiae* were equal to that of PM. There was no cross resistance or collateral sensitivity shown to these compounds by any of the three resistant strains. Their activities were not reversed by folic or folinic acids. Both compounds were moderately active in mouse malaria.

20 and **21** are pyrimidines with no free NH_2– groups. **20** had no activity against *S. faecium*, *P. cerevisiae*, or *E. coli*. It had low activity against *L. casei* which was not reversed by folic acid. *L. casei* (R) was collaterally sensitive to this compound. **20** had moderate activity in mouse malaria. **21** had activity against all four organisms, being least active against *S. faecium*. There was no cross resistance or collateral sensitivity to this compound and its activities were not reversed by folates or PABA (*E. coli*). This compound had high activity in the mouse malaria test, being as good as CGT. It was active against human malaria induced by drug-sensitive strains but was not effective against drug-resistant strains.[10]

22, **23**, and **24** are quinazolines without free NH_2– groups. **22** had moderate activity against all four test organisms. There was no cross resistance shown by *S. faecium* (R) or *P. cerevisiae* (R) and the activities of this compound were not reversed. It had moderate activity in the mouse malaria. **23** and **24** were very similar in activities. These compounds are higher in activity than **22**, being almost as active as PM against *L. casei* and *P. cerevisiae*. They are much higher in activity than PM against *E. coli*. There was no cross resistance with *S. faecium* (R) or *P. cerevisiae* (R) and slight collateral sensitivity with *L. casei* (R). Their activities were not reversed by folates or by PABA (*E. coli*) and they were not tested against mouse malaria.

Dapsone **25** was inactive against *S. faecium* and of low to moderate activity against *L. casei*, *P. cerevisiae*, and *E. coli*. There was low-grade cross resistance with *P. cerevisiae* (R) and collateral sensitivity with *L. casei* (R). Its activity was not reversed by folates but was reversed by PABA with *E. coli*. It was highly active in the mouse malaria test. Dapsone is effective against leprosy and is active in human malaria.[9,10]

26–28 are the three sulfonamides—sulfalene, sulfathiazole, and sulfadiazine. All three were inactive against *S. faecium* and *P. cerevisiae*.[20] **26** (sulfalene) had a very low-grade activity against *L. casei*. These sulfonamides had high activity against *E. coli* which was reversed by PABA. They were moderately to highly active against mouse malaria. Sulfonamides have long been known to have some antimalarial activity and are particularly useful in combination with antifolates in human infections.[9,10]

Clopidol (**29**) had a low-grade activity against *S. faecium*, *L. casei*, and *P. cerevisiae* and no activity against *E. coli*. There was no cross resistance or collateral sensitivity with the resistant strains. This compound was not reversed by folates. It was moderately active as a suppressive in the mouse malaria test. This active coccidiostat had negligible activity in human malaria.[10]

The antihelminthic compound **30** had low activity against all four test organisms, being most active against *E. coli*. There was no cross resistance or collateral sensitivity and it was not reversed by folates or by PABA (*E. coli*). It had low-grade activity in the mouse and human malaria.[10]

The only interesting aspect of **31**, terephthalyldihydroxamic acid, was the unexpected finding of some cross resistance of *S. faecium* (R) (threefold) in a nonreversed and relatively nonactive compound. It had low activity against mouse malaria.

Cycloleucine (**32**) is an unnatural amino acid with antitumor activity. It was inactive against all four bacterial strains and mouse malaria. In studies with human malaria it produced only minimal effects.[10]

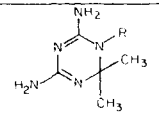
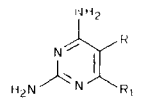
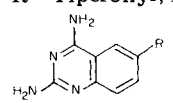
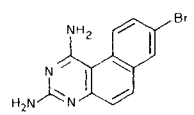
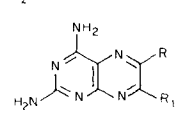
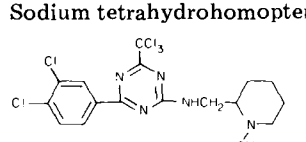
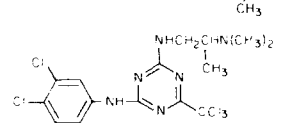
33–35 are guanidine derivatives. **33** (nitroguanil) is an antihelminthic agent. The interesting finding with this compound was the cross resistance exhibited by *L. casei* (R) (12-fold). This compound was not reversed by folates and it had very low activity against mouse malaria. It is active in human malaria but is judged to have no advantage over proguanil.[9] Dypnone guanylhydrazone (**34**) was considerably more active than **33** against all four organisms. There was some cross resistance shown by the *S. faecium* (R) strain, the FIR being 6. This compound was reversed by folic acid in the case of *S. faecium* but not by folates with *L. casei* and *P. cerevisiae* or by PABA (*E. coli*). It had no significant effect against human malaria and produced gastrointestinal side effects.[10] The activities of **35** are in the same range as those of **34**. There was no cross resistance or collateral sensitivity and its activity was not reversed by folates or PABA. This compound was more highly active in mouse malaria than **34**.

Menoctone (**36**), a naphthoquinone, had moderate activity against *L. casei* (R), low activity against *S. faecium* and *P. cerevisiae*, and slight collateral sensitivity with *L. casei* (R). It was not reversed by folates. In the mouse malaria it showed moderate activity but in human malaria it was ineffective.[10]

The 4-quinolinemethanol **37** had low and closely similar activities against all four test organisms. There was no cross resistance or collateral sensitivity nor was it reversed by folates or PABA. It was highly active against mouse and human malaria.[10]

The last three compounds are chloroquine (**38**), primaquine (**39**), and quinine (**40**), all well-known antimalarial quinoline derivatives. Against the test bacteria all three compounds had low activity, primaquine being the most active. With *S. faecium* (R) there was a slight cross re-

Table II. Inhibitory Concentrations, Increase in Resistance, and Reversibility of Compounds against Sensitive (S) and Chlorguanide Triazine Resistant (R) Strains of Three Folate-Requiring Bacteria and *E. coli*

No.	Compound	IC_{50},[a] FIR,[b] and reversibility[c]									
		S. faecium			*L. casei*			*P. cerevisiae*			*E. coli*
		S	R	FIR	S	R	FIR	S	R	FIR	S
											
1	R = 4-ClC_6H_4[d]	0.017 (+)	3300 (+)	194 000	16.3 (+)	2400 (+)	147	35 (+)	900 (+)	26	>3470
2	R = 4-EtC_6H_4	0.036 (+)	>350	>9700	27.3 (+)	291 (+)	11	42.6 (+)	>355	>8	1900 (-)
3	R = 3,4-$Cl_2C_6H_3CH_2O$	0.0009 (+)	3.4	3800	0.057 (+)	3.1	54	0.14 (+)	8.2	59	7 (-)
4	R = 2,4,5-$Cl_3C_6H_2O(CH_2)_3O$	0.0001 (+)	0.019 (+)	190	0.021 (+)	0.4 (+)	19	0.003 (+)	0.8 (+)	267	1.7 (-)
											
5	R = 4-ClC_6H_4; R_1 = Et[e]	0.008 (+)	10.5 (+)	1300	5.1 (+)	0.24 (+)	0.05	4 (+)	12 (+)	3	76 (-)
6	R = 3,4,5-$(CH_3O)_3C_6H_2CH_2$; R_1 = H[f]	0.08 (+)	1.2 (+)	15	0.19 (+)	0.017 (+)	0.09	11.7 (+)	67.5 (+)	6	0.4 (-)
7	R = Piperonyl; R_1 = H	0.25 (+)	24.2 (+)	96.8	0.94 (+)	0.25 (+)	0.27	12.3 (+)	90.1 (+)	7.3	45 (-)
											
8	R = 3,5-$Cl_2C_6H_3O$	0.009 (+)	1.3 (+)	144	0.25 (+)	0.04 (+)	0.16	0.12 (+)	1.4 (+)	11.7	2 (-)
9	R = 4-ClC_6H_4S	0.001 (+)	0.18 (+)	180	0.028 (+)	0.01 (+)	0.36	0.028 (+)	0.4 (+)	14.3	0.4 (-)
10	R = 4-$ClC_6H_4CH_2S$	0.04 (+)	1 (+)	25	0.2 (+)	0.04 (+)	0.2	0.05 (+)	1.4 (+)	28	2.9 (-)
11	R = 2-Naphthyl-SO_2	0.002 (+)	0.08 (+)	40	0.04 (+)	0.009 (+)	0.23	0.002 (+)	0.05 (+)	25	<0.2 (-)
12	R = 3,4-$Cl_2C_6H_3CH_2NH$	0.006 (+)	2 (+)	330	0.4 (+)	0.03 (+)	0.08	0.2 (+)	0.7 (+)	3.5	33 (-)
13		0.006 (+)	0.7 (+)	117	1 (+)	0.06 (+)	0.06	0.04 (+)	0.3	7.5	4.8 (-)
											
14	R = 2-MeC_6H_4; R_1 = NH_2	1.1 (+)	124	113	68 (+)	1.9	0.03	8.5 (+)	44	5	209 (-)
15	R = Me_2CH; R_1 = Me_2CH	0.005 (+)	0.3 (+)	60	3.2 (+)	0.5 (+)	0.16	0.07 (+)	0.5 (+)	7	8.1 (-)
16	Amethopterin	0.002 (-)	0.01 (-)	5	0.00006 (-)	0.00001 (-)	0.17	0.4 (+)	1.5 (+)	4	143 (-)
17	Sodium tetrahydrohomopteroate	0.009 (+)	7.2 (+)	800	6 (+)	0.2 (+)	0.03	>2900	>2900	Indet[g]	
18		132 (-)	128 (-)	0.97	3 (-)	3.2 (-)	1.1	4.9 (-)	3.8 (-)	1	
19		14.2 (-)	14.6 (-)	1	5.7 (-)	4.9 (-)	0.9	5.9 (-)	7.5 (-)	1	

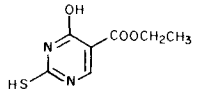
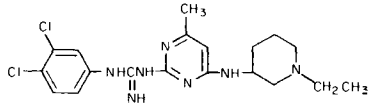
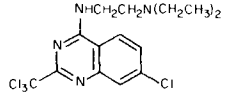
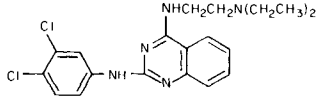
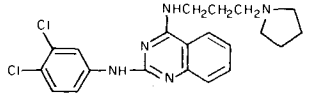
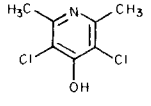
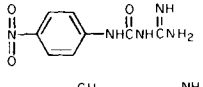
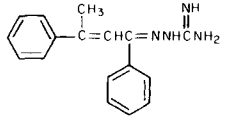
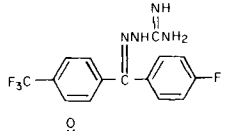
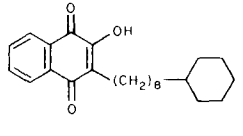
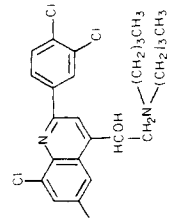
20		>5000	>5000	Indet	3000 (−)	574	0.19	>5000	>5000	Indet	>5000
21		227 (−)	121 (−)	0.53	65.1 (−)	55.7 (−)	0.85	32 (−)	39 (−)	1	85 (−)
22		76 (−)	67	0.9	30 (−)	8	0.27	32 (−)	35	1	50 (−)
23		31 (−)	61	2	12 (−)	5	0.4	6.5 (−)	8.2	1	11 (−)
24		32 (−)	22	0.7	10 (−)	5.7	0.6	3.5 (−)	7.9 (−)	2	10 (−)
25	**4,4′-Diaminodiphenylsulfone (DDS)**	>4000	>4000	Indet	314 (−)	75	0.24	105 (−)	>400	>4	137 (+)
26	**2-Sulfanilamido-3-methoxypyrazine**	>3600	>3600	Indet	8200	8200	1	>3600	>3600	Indet	13.5 (+)
27	**Sulfathiazole**	>3900	>3900	Indet	>3900	>3900	Indet	>3900	>3900	Indet	7.8 (+)
28	**Sulfadiazine**	>4000	>4000	Indet	>4000	>4000	Indet	>4000	>4000	Indet	12 (+)
29		3700 (−)	2200 (−)	0.6	1700 (−)	885	0.52	833 (−)	1000 (−)	1	>5200
30	**1,4-Bis(trichloromethylbenzene)**	2000 (−)	1900 (−)	1	1000 (−)	990 (−)	1	959 (−)	2000 (−)	2	262 (−)
31	**Terephthalyldihydroxamic acid**	1700 (−)	>5000 (−)	3	2600 (−)	3000 (−)	1	>5000	>5000	Indet	>5000
32	**1-Aminocyclopentanecarboxylic acid (cycloleucine)**	>7700	>7700	Indet	>7700	>7700	Indet	>7700	>7700	Indet	>7700
33		1300 (−)	2500 (−)	2	157 (−)	1800 (−)	12	83 (−)	197 (−)	2	>4480
34		11 (+)	60	6	48 (−)	41 (−)	1	25 (−)	13 (−)	0.5	51 (−)
35		64 (−)	35 (−)	0.6	31 (−)	30 (−)	1	22 (−)	21 (−)	1	17 (−)
36		407 (−)	394 (−)	1	61 (−)	19 (−)	0.3	1000 (−)	570 (−)	0.6	>2700

Table II (*Continued*)

No.	R	R_1	IC_{50},[a] FIR,[b] and reversibility[c]									
			S. faecium			*L. casei*			*P. cerevisiae*			*E. coli*
			S	R	FIR	S	R	FIR	S	R	FIR	S
37			327 (-)	572 (-)	2	170 (-)	250 (-)	2	290 (-)	354 (-)	1	182 (-)
38	Chloroquine		6600 (-)	7500	1	6400 (-)	6000	1	2500 (-)	2500	1	
39	Primaquine		1200 (-)	3000	3	630 (-)	1300	2	580 (-)	540	1	
40	Quinine		>2500	>2500	Indet	980 (-)	1200	1	780 (-)	850 (-)	1	1300

[a] IC_{50} = micromolar concentration of compound required to reduce growth to 50% of the control. All data points are at least the average of duplicate determinations. [b] FIR = fold increase in resistance = IC_{50} R/IC_{50} S. [c] Reversed (+) or not reversed (-) by folic acid (*S. faecium* and *L. casei*), folinic acid (*P. cerevisiae*), or *p*-aminobenzoic acid (*E. coli*). [d] Cycloguanil (CGT). [e] Pyrimethamine (PM). [f] Trimethoprim. [g] Indet = indeterminate.

Table III. Antimalarial Activity against *Plasmodium berghei*[a] in the Mouse

No.	Min active dose, mg/kg	Min curative dose, mg/kg
1	20	40
2	320	>640
3	2.5	20
4	20	160
5	10	40
6	>640	>640
7	640	640
8	320	640
9	5	40
10	320	640
11	0.63	1.25
12	20	80
13	640	>640
14	40	320
15	640	640
16	>640	>640
17	>640	>640
18	160	320
19	80	160
20	40	320
21	20	40
22	160	320
23		
24		
25	20	40
26	40	160
27	40	160
28	160	640
29	160	>640
30	640	640
31	320	640
32	>640	>640
33	640	640
34	160	>640
35	40	160
36	40	160
37	5	20
38	20	80
39	40	160
40	640	>640

[a] The activity was determined by the Rane Laboratory, University of Miami. Mice are given a standard inoculum of a blood-induced *P. berghei* infection and are treated with a single subcutaneous dose of compound suspended in peanut oil.[6,7] The lowest dose which effects an increase of 100% in mean survival time is considered the minimum active dose. Survivors for a period of 60 days are considered cured. The lowest dose which effects cure of any of the mice is considered the minimum curative dose.

sistance to primaquine, the FIR being 3. The activities of the three compounds were not reversed by folates. Against mouse malaria, chloroquine was highly active, primaquine was moderately active, and quinine had low activity. These compounds are of established value in the treatment of human malaria.[9,10]

Side by side comparison of the bacteriological data with the activities of the compounds against *P. berghei* in the mouse showed little correlation. However, each test appears to provide independent and useful information.

Experimental Section

The bacteria used in this study were *Streptococcus faecium* durans (*Streptococcus faecalis* ATCC 8043), *Lactobacillus casei* ATCC 7469, *Pediococcus cerevisiae* ATCC 8081, and *Escherichia coli* ATCC 10536.

The assay methods and media employed for the folate-requiring organisms were the same as those described earlier.[5] The concentration of folic acid used was 0.002 μg/ml for *S. faecium* and 0.001 μg/ml for *L. casei*. Folinic acid in a concentration of 0.001 μg/ml was used for *P. cerevisiae*. With *E. coli* the medium used

was that of Sahyun et al.[21] to which 0.6% acid hydrolyzed casein was added. Growth of the test organisms was read as turbidity in the Klett-Summerson photoelectric colorimeter. The results are expressed as the micromolar concentration required to produce 50% growth inhibition (IC_{50}) of the strain under the conditions used. Where sufficient drug was available, concentrations up to 1000 μg/ml were tested; when this was not possible the highest concentration used was 100 μg/ml.

In addition to antibacterial activity, tests were made for the reversal of the drug inhibition by PABA in the case of *E. coli*, folic acid with *S. faecium* and *L. casei*, and folinic acid with *P. cerevisiae*. The reversal was indicated as positive when addition of either 10 μg/ml of PABA or ten times the amounts of folic or folinic acids normally used produced at least a fourfold decrease in activity of the compound.

Strains of *S. faecium*, *L. casei*, and *P. cerevisiae* were made resistant to CGT by serial transfer in increasing amounts of the drug.[5] The most highly resistant strains obtained with each organism were used in the tests reported here. From these data the fold increase in resistance (FIR) of the CGT_R strains compared to the respective sensitive strains for each compound has been calculated (IC_{50} R/IC_{50} S). Thus, for most compounds ten quantitative and seven qualitative (reversal) parameters have been determined.

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References and Notes

(1) K. E. Kinnamon and W. E. Rothe, *Am. J. Trop. Med. Hyg.*, **24**, 174 (1975).
(2) J. Lascelles and D. D. Woods, *Br. J. Exp. Pathol.*, **33**, 288 (1952).
(3) E. L. R. Stokstad and J. Koch, *Physiol. Rev.*, **47**, 83 (1967).
(4) H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, **176**, 165 (1948).
(5) C. C. Smith and C. S. Genther, *Antimicrob. Agents Chemother.*, **2**, 103 (1972).
(6) L. Rane and D. S. Rane, *Abstr., 9th Int. Congr. Trop. Med. Malaria*, 1, 281 (Abstr. No. 406) (1973).
(7) T. S. Osdene, and P. B. Russell, and L. Rane, *J. Med. Chem.*, **10**, 431 (1967).
(8) A. F. Crowther and A. A. Levi, *Br. J. Pharmacol.*, **8**, 93 (1953).
(9) Report of a WHO Scientific Group, *W.H.O., Tech. Rep. Ser.*, **No. 375** (1967).
(10) C. J. Canfield and R. S. Rozman, *Bull. W.H.O.*, **50**, 203 (1974).
(11) C. C. Smith and C. S. Genther, *Ann. N.Y. Acad. Sci.*, **186**, 185 (1971).
(12) L. H. Schmidt and C. S. Genther, *J. Pharmacol. Exp. Ther.*, **107**, 61 (1953).
(13) S. R. M. Bushby and G. H. Hitchings, *Br. J. Pharmacol. Chemother.*, **33**, 72 (1968).
(14) L. H. Schmidt, J. Harrison, R. Ellison, and P. Worcester, *Proc. Soc. Exp. Biol. Med.*, **131**, 294 (1969).
(15) G. H. Hitchings and J. J. Burchall, *Adv. Enzymol.*, **27**, 417 (1965).
(16) T. W. Sheehy and H. Kempsey, *J. Am. Med. Assoc.*, **214**, 109 (1970).
(17) L. H. Schmidt, unpublished results.
(18) R. L. Kisliuk, M. Friedkin, L. H. Schmidt, and R. N. Rossan, *Science*, **156**, 1616 (1967).
(19) G. H. Hitchings, *Trans. R. Soc. Trop. Med. Hyg.*, **46**, 467 (1952).
(20) J. O. Lampen and M. J. Jones, *J. Biol. Chem.*, **166**, 435 (1946).
(21) M. Sahyun, P. Beard, E. W. Schultz, J. Snow, and E. Cross, *J. Infect. Dis.*, **58**, 28 (1936).

Inhibition of Squalene Synthetase by Farnesyl Pyrophosphate Analogues[1]

Paul R. Ortiz de Montellano,* Jeng Shu Wei, Rafael Castillo, Charles K. Hsu, and Amrit Boparai

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.
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The pyrophosphates of the following farnesol analogues have been synthesized: 2-methylfarnesol; 7,11-dimethyl-3-ethyl-2,6,10-dodecatrien-1-ol; 3-demethylfarnesol; 4-methylthiofarnesol; 7,11-dimethyl-3-iodo-2,6,10-dodecatrien-1-ol; 7,11-dimethyl-2-iodo-2,6,10-dodecatrien-1-ol; 7,11-dimethyldodeca-6,10-dien-2-yn-1-ol; phytol; 3,7,11-trimethyl-2-dodecen-1-ol; 3,7,11-trimethyldodecan-1-ol; and geraniol. The double bonds in all the above compounds were in the *E* configuration, except phytol, which was a 7:3 mixture of 2*E* and 2*Z* isomers. Each of the pyrophosphates inhibits the incorporation of labeled farnesyl pyrophosphate into squalene by a yeast enzyme preparation. Free alcohols and monophosphates are inactive. The analogues, listed in order of decreasing inhibitory strength, are, by kinetic analysis, competitive or mixed inhibitors. Irreversible inhibition is not observed. The results suggest that binding to the enzyme is primarily mediated by the pyrophosphate moiety assisted by relatively nonspecific lipophilic interactions. Decreasing the chain length and saturating double bonds severely reduces binding, while substitution at the 2, 3, and 4 positions, and lengthening of the chain, is well tolerated.

The incidence of atherosclerotic disorders, a major cause of death in the United States and other industrial societies, is closely correlated with occurrence of elevated plasma cholesterol levels.[2] Methods for effective reduction of plasma cholesterol levels are therefore of high interest, even though the role of this sterol in the etiology of the disease is not clearly defined.[2,3] In theory, cholesterol concentrations can be reduced by lowering the dietary intake of the sterol, by enhancing its metabolism and elimination, and by decreasing its rate of biosynthesis. Cholesterol synthesis, however, is subject to feedback regulation,[4] so that decreases in cholesterol levels tend to be compensated for by increased biosynthesis. Removal of sterols in rats due to cholestyramine feeding, for example, causes a 200–300% increase in hepatic cholesterol synthesis.[5] The most effective approaches to lowering physiological cho-