Use of the Trypanosomid Flagellate, *Crithidia fasciculata*, for Evaluating Antimalarials.*† (20803)

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Since the Trypanosomid flagellate, *Crithidia fasciculata* (*Anopheles* strain), a parasite of mosquitoes, requires an exogenous source of folic acid (or closely related compounds) for growth(2) to which it responds quantitatively, it is useful for studying antimetabolites directed at the folic acid group. This strain is not known to have a vertebrate host, but as a member of the Trypanosomidae, it is related to several important intra- and extracellular pathogens.

Earlier investigations in our laboratory with folic acid analogs such as Aminopterin§ and 10-formyl-folic acid§ were inconclusive as these compounds had folic acid rather than antimetabolite activity. Several substituted pyrimidines, (structurally more dissimilar to folic acid) among them potent antimalarials (3,4) which also display anti-leukemic activity(5), were next studied. Excellent correlation had been found between the *in vivo* inhibition of *Plasmodium bergei* (in mice) and of *P. gallinaceum* (in chicks)(6) and, in our laboratory, of *in vitro* inhibition of crithidia, by the pyrimidine antimalarials. This contrasts with dissimilar inhibition patterns observed with bacteria and suggests that crithidia, as described here, may be a better guide for preliminary evaluation of antimalarials.

Method. The completely defined medium devised earlier(2) for this strain was modified for these studies (Table I). All cultures were allowed to grow past the logarithmic growth phase, which required between 10-18 days. Growth was measured as optical density on a Welch Densichron. The antimetabolites‖ used

TABLE I. Basal Medium for *Crithidia fasciculata*. Grams (unless otherwise specified) in 100 ml of final medium.

K_2PO_4	.02
Ethylenediamine-tetra-acetic acid (EDTA)	.06
$MgSO_4 \cdot 7H_2O$	.04
Fe (as SO_4)	.1 mg
Ca (as Cl)	.2 mg
Mo (as Na)	.4 mg
Metals mix*	2.5 ml
L-arginine HCl	.025
L-histidine HCl	.02
DL-isoleucine	.005
DL-lysine	.02
DL-leucine	.005
DL-methionine	.008
DL-phenylalanine	.004
DL-tryptophan	.002
L-tyrosine	.005
DL-valine	.005
Triethanolamine	.5
Hemin	2.5 mg
Vit. mix†	1.0 ml
Adenosine‡	3.0 mg
Glucose (aseptically)§	1.0

Folic acid or folinic acid omitted from the base.‖ pH 8.1; 2 ml distributed in 10 ml flasks.¶

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* 1 ml contains: Zn (as $ZnSO_4$) 1 mg, Mn (as $MnSO_4$) 1 mg, Co (as $CoSO_4 \cdot 7H_2O$) 0.02 mg, Cu (as $CuSO_4 \cdot 5H_2O$) 0.01 mg, B (as H_3BO_3) 0.004 mg, I (as NaI) 0.001 mg, EDTA 1 mg. Concentrations refer to metal ions alone.

† 1 ml contains: Thiamine · HCl 0.6 mg, riboflavin 0.1 mg, Ca pantothenate 0.3 mg, nicotinic acid 0.3 mg, biotin 1.0 μg, pyridoxamine · 2HCl 0.1 mg.

‡ The commercial adenosine used had a 10% impurity of guanosine as determined chromatographically and spectrophotometrically. This impurity did not interfere with this particular study as *Crithidia* can utilize adenosine or guanosine interchangeably(2).

§ One drop of sterile 50% glucose/flask. Preparation: a 50% (w/v) solution of Mallinckrodt C. P. glucose (devoid of traces of yellow impurities found in other C.P. samples from other manufacturers) was brought to pH 3.0-3.6 with H_2SO_4, distributed in 125 × 20 mm screw-capped tubes and autoclaved for 20 min. at 118-120°C.

‖ Supplied as "Leucovorin" (Lederle), the Ca · $5H_2O$ salt.

¶ Special "micro-Fernbach" flasks, Kimble catalog No. 26020-S-43.

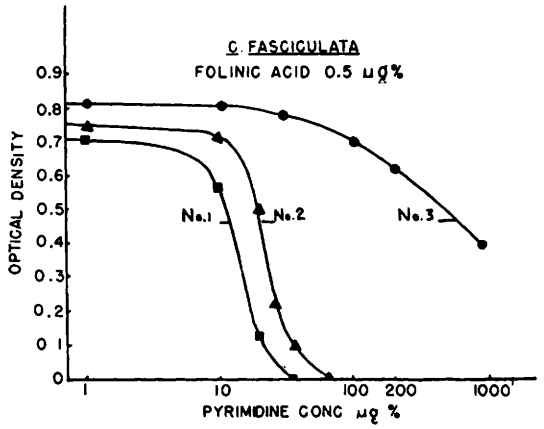

FIG. 1. Graph showing the competitive nature of folinic acid antagonism by 2,4-diaminopyrimidines.

No. 1 5-*p*-chlorophenyl-6-ethyl-2,4 diaminopyrimidine
2 5-(3′4′-dichlorophenyl)-6-methyl-2,4 diaminopyrimidine and
3 5-*p*-chlorophenoxy-6-methyl-2,4 diaminopyrimidine.

were: (1) 5-*p*-chlorophenyl-6-ethyl-, (2) 5-(3′4′-dichlorophenyl)-6-methyl-, and (3) 5-*p*-chlorophenoxy-6-methyl-2,4-diaminopyrimidine. Growth of the organism in the presence of various levels of drug was determined as a function of folic or folinic acid.

Results. Folic and folinic acids are competitively inhibited by these substituted pyrimidines (Table II, Fig. 1). This inhibition of *C. fasciculata* shows patterns closer to the *in vivo* results with plasmodia obtained by the other workers mentioned, and diverges from the results with bacterial test systems. The bacterial systems had failed to distinguish between pyrimidine No. 1 ("Daraprim"), an extremely potent antimalarial, and pyrimidine No. 2 which has only moderate clinical value (Table III).

The failure of quinine or atabrine to antagonize either folic or folinic acid under our experimental conditions indicates, as had been reported(8,9), that these drugs interfere with a system other than the folic acid.

Discussion. The site of action of antifolics is more accessible to analysis in organisms requiring folic acid exogenously. In our unpublished experiments, growth of crithidia in the presence of limiting folic acid was sensitively influenced by other metabolites whose metabolism was linked to that of folic acid, *e.g.*, purine ribosides, thymine, and amino acids such as threonine, methionine, and serine. Details of these interactions will be reported elsewhere.

Since plasmodia requires PABA(10), and all members of the Trypanosomidae thus far studied require folic acid or closely related compounds(11), these requirements may point to vulnerable sites for attack by chemotherapeutic agents. Pathogenic trypanosomes differ widely in sensitivity to drugs. The biochemical differences underlying these species-to-species and strain-to-strain variations in susceptibility to chemotherapeutic agents may have parallels in various non-pathogenic members of this group (such as the strain used in this work) which differ obviously from each other in morphology and nutrition.

The effectiveness of antimalarials is claimed to correlate with their ability to inhibit adenosine-induced auriculoventricular block in rabbits(12). Our preliminary experiments indi-

TABLE II. Antimalarials as Antagonists to Folic Acid in *Crithidia*. Antagonism measured as reduction in final growth (growth expressed as optical density).

Drug in µg %		Folic acid 0.05 µg % Optical density	Folic acid 0.5 µg % Optical density
No addition		.90	1.
Quinine (calculated as free base)	.1	.94	1.
	1.	1.18	1.
	1000.	1.18	1.
Atabrine (Quinacrine HCl)	.1	.92	1.22
	1.	1.30	1.30
	10.	1.20	1.28
	100.	1.22	1.26
	1000.	1.14	1.32
Pyrimidine No. 1*	1.	.60	.92
	3.	.52	1.
	10.	0	.96
	30.	0	.72
	100.	0	0
" No. 2	1.	.92	.82
	3.	.65	.78
	10.	.33	.76
	30.	0	.74
	100.	0	.74
	300.	0	0
" No. 3	3.	.66	.89
	10.	.62	1.02
	30.	.26	.72
	100.	0	.88
	300.	0	.24
	1000.	0	0

* Commercially known as "Daraprim."

TABLE III. Inhibitions of Various Organisms by Substituted Pyrimidines. Results for each organism are relative with pyrimidine No. 3 arbitrarily set at 1; the other values represent multiples of this standard. See references in Table for experimental detail.

Pyrimidine No.	*Plasmodium bergei*(6)	*Plasmodium gallinaceum*(6)	*Crithidia fasciculata*	*Streptococcus faecalis*(7)	*Lactobacillus casei*(7)	*Leuconostoc citrovorum**(6)
1	285.7	150.	33.3	12.5	0.89	2.7
2	185.7	35.	10.	12.5	1.2	2.7
3	1.0	1.0	1.0	1.0	1.0	1.0

* Felton and Niven(13) showed that "*L. citrovorum*" is actually a typical strain of *Pediococcus cerevisiae*.

cate that effective drugs interfere with the utilization of adenosine by crithidia, for which, under certain conditions, adenosine or guanosine are the only compounds which satisfy the nucleic acid component requirement(2). Whether this unusual specificity of ribosides is linked with the special role assigned to adenosine as an antagonist of antimalarials remains to be seen.

Summary. *Crithidia fasciculata* is competitively inhibited by several 2,4-diaminopyrimidine antagonists of folic and folinic acids. These *in vitro* inhibition patterns obtained in a chemically defined medium parallel the order of relative drug activities obtained in other laboratories on animal infections by any of several species of plasmodia. Poor correspondence exists, however, between bacterial inhibitions *in vitro* and inhibitions of plasmodia *in vivo* by these pyrimidines. The results suggest that this flagellate may be preferable to bacteria hitherto used for preliminary *in vitro* evaluation of antimalarials.

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