

## Effects of Some Antitumor Agents on Growth and Glycolytic Enzymes of the Flagellate *Crithidia*

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Some antitumor agents known to specifically inhibit certain tumor cell enzymes were examined for activity against glycolytic enzymes and growth of the insect trypanosomatid, *Crithidia fasciculata*. The cytoplasmic enzymes hexokinase,  $\alpha$ -glycerophosphate dehydrogenase, malic dehydrogenase, and glucose-6-phosphate dehydrogenase were tested. Agaric acid (2-hydroxy-1,2,3-nonadecane tricarboxylic acid) was highly inhibitory (50 to 100%) to malic and  $\alpha$ -glycerophosphate dehydrogenases at  $\sim 3 \times 10^{-5}$  M; 2-(*p*-hydroxyphenyl)-2-phenylpropane ( $2 \times 10^{-4}$  M), and 5,6-dichloro-2-benzoxazolinone ( $5 \times 10^{-4}$  M) were less effective (50% inhibition) against them. The antiprotozoal agents primaquine ( $4 \times 10^{-4}$  M) and Melarsoprol ( $8 \times 10^{-4}$  M) were 30 to 40% inhibitory. Agaric acid, 2-(*p*-hydroxyphenyl)-2-phenylpropane, and 5,6-dichloro-2-benzoxazolinone inhibited growth of *Crithidia* at less than  $10^{-4}$  M. Eight other test compounds from the Cancer Chemotherapy National Service Center (CCNSC) were not toxic to cell growth, although two (4-biphenylcarboxylic acid and 1-[*p*-chlorobenzyl]-2-ethyl-5-methyl-indole-3-acetic acid) inhibited *Crithidia*  $\alpha$ -glycerophosphate dehydrogenase below 1 mM. All of the compounds used specifically inhibited cancer cell  $\alpha$ -glycerophosphate dehydrogenase. The corresponding enzyme in pathogenic African trypanosomes is important in their terminal respiration. *C. fasciculata* may be useful in preliminary evaluation of chemotherapeutic agents as potential trypanocides.

Chemotherapy of African sleeping sickness is unsatisfactory, and pharmacological leads are few. This paper describes some inhibitors of certain soluble enzymes of a nonpathogenic trypanosomatid, *Crithidia fasciculata*, with the hope of uncovering points at which metabolic inhibitors of potential chemotherapeutic interest can be applied.

Bloodstream trypanosomes of the *Trypanosoma brucei* subgroup are both intensely glycolytic and aerobic but have neither functional cytochromes nor a Krebs cycle (15). Pyruvate and glycerol are the main end products of respiration (30). Bloodstream forms have malate (MDH; EC 1.1.1.37) and  $\alpha$ -glycerophosphate ( $\alpha$ -GPDH; EC 1.1.1.8) dehydrogenases (31), on which they depend for reoxidation of the reduced nicotinamide adenine dinucleotide (NADH) produced by the glyceraldehyde phosphate dehydrogenase step of glycolysis. Terminal respiration is mediated by an  $\alpha$ -GP-dihydroxyacetone phosphate (DHAP) cycle involving an NAD-linked  $\alpha$ -GPDH and an  $\alpha$ -GP oxidase system (19, 20).

Arsenicals (e.g., tryparsamide) have for many years dominated treatment of late trypanosomiasis, but they are dangerous to the host (17, 33).

Their mode of action is unclear, and their inhibition of trypanosome glycolytic enzymes is incompletely charted (18, 35). Few practical drugs have been developed for African human trypanosomiasis during the past decade. Present drugs are expensive, and their prolonged administration is difficult and often ineffectual, especially when the central nervous system is invaded (17, 34).

Metabolic pathways of pathogenic trypanosomes and of cancer cells seem similar in certain respects: both metabolize large amounts of glucose through the Embden-Meyerhof pathway, both need dehydrogenases to recycle NADH, and both rely conspicuously on  $\alpha$ -GP metabolism (5, 13, 24). Cancer cells, low in  $\alpha$ -GPDH, use it for phospholipid production (5); trypanosomes seem to use this enzyme to regenerate NAD and synthesize lipids (2). Since inhibition of  $\alpha$ -GPDH and other glycolytic enzymes is being investigated from the standpoint of cancer chemotherapy (11, 12), we determined the effect of some known antitumor agents (specific  $\alpha$ -GPDH inhibitors; 11, 12) and arsenicals on glycolytic enzymes and cells of *C. fasciculata*. This trypanosomatid

flagellate is a nonpathogenic gut parasite of Diptera and is far more easily cultivated in the quantity required for enzyme analysis than is any pathogenic trypanosome (2, 22, 23). *C. fasciculata* has a soluble, NAD-linked  $\alpha$ -GPDH; preliminary drug inhibition studies directed at this enzyme seem promising (1, 2).

#### MATERIALS AND METHODS

**Organism and mass cultures.** *C. fasciculata* (*Anopheles* isolate; ATCC 11745) was cultured in complex medium (2).

**Soluble enzyme fractions.** Soluble preparations, free of mitochondria and kinetoplasts, were obtained by grinding cells with neutral alumina, followed by differential centrifugation (2). Samples (0.5 to 1.0 ml) of these fractions, stored at  $-20^{\circ}\text{C}$ , were thawed at room temperature for use. Activity of any of the assayed enzymes did not decrease during 6-months frozen storage.

**Protein determination.** A biuret method (26) was used with bovine serum albumin, fraction V, from Sigma Chemical Co., St. Louis, Mo., as standard.

**Enzyme analyses.** Reaction rates were determined spectrophotometrically on a recording spectrophotometer with a temperature-controlled chamber. All reactions (at  $25^{\circ}\text{C}$ ) were monitored as oxidation or as formation of reduced pyridine nucleotide cofactors, NADH and nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. Reactions were in 0.2 M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.1. This pH, near the original pH of the soluble enzyme fractions, was chosen to approximate *in vivo* conditions. Drug activity was tested on  $\alpha$ -GPDH, with 0.125 mM NADH, 100  $\mu\text{g}$  of protein, and 3.82 mM DHAP (obtained from Sigma Chemical Co. and prepared according to the manufacturer's instructions) added to start the reaction. The MDH assay had 0.125 mM NADH and 45  $\mu\text{g}$  of protein; 1.65 mM oxalacetate was added to start the reaction. The glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) assay had 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP; in 1%  $\text{NaHCO}_3$ ) and 450  $\mu\text{g}$  of protein; 1.25 mM G6P was added to start the reaction. Hexokinase (HK; EC 2.7.1.1) was tested in 590  $\mu\text{g}$  of protein, 0.5 mM  $\text{Mg}^{++}$  (as  $\text{Cl}^-$ ), 5.0  $\mu\text{g}$  of pure G6PDH (from yeast; obtained from Boehringer-Mannheim Corp., New York, N.Y.), 3.0 mM adenosine triphosphate (ATP), 40 mM glucose, and was incubated for 5 min at  $25^{\circ}\text{C}$ . To start the G6PDH assay reaction, 0.5 mM NADP was added. *Crithidia* G6PDH did not affect the reaction rate because the concentration of the commercial G6PDH in the system was high compared to the *Crithidia* enzyme—much in excess of the concentration needed for the reaction. Each drug assay was done on two different soluble-enzyme preparations.

**Growth inhibition.** Drugs were tested for inhibition of growth in a defined medium (Table 1). This medium is physiologically minimal (K. M. O'Connell, unpublished data), i.e., the constituents are in the lowest concentrations compatible with maximal

TABLE 1. *Crithidia fasciculata* defined medium

Drugs	Amt <sup>a</sup>
$\text{K}_3$ citrate $\cdot$ $\text{H}_2\text{O}$ .....	0.1 g
Citric acid (anhydrous).....	0.045 g
L-Malic acid.....	0.02 g
Succinic acid.....	0.1 g
$\text{MgCO}_3$ .....	0.1 g
Trace elements <sup>b</sup> .....	0.01 g
$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot \text{H}_2\text{O}$ .....	1.0 mg
Ca succinate $\cdot$ $\text{H}_2\text{O}$ .....	3.0 mg
Guanosine $\cdot$ $\text{H}_2\text{O}$ .....	2.5 mg
Adenosine.....	2.5 mg
Uracil.....	0.4 mg
Thymine.....	0.2 mg
Orotic acid.....	0.2 mg
L-Arginine $\cdot$ $\text{HCl}$ .....	0.05 g
L-Histidine $\cdot$ $\text{HCl}$ .....	0.01 g
L-Isoleucine.....	0.02 g
L-Leucine.....	0.03 g
L-Lysine $\cdot$ $\text{HCl}$ .....	0.02 g
L-Methionine.....	0.015 g
L-Phenylalanine.....	0.015 g
L-Threonine.....	0.01 g
L-Tryptophan.....	0.01 g
L-Tyrosine ethyl ester $\cdot$ $\text{HCl}$ .....	0.01 g
L-Valine.....	0.015 g
Nicotinamide.....	0.05 mg
Ca pantothenate.....	0.03 mg
Na riboflavine $\text{PO}_4 \cdot 2\text{H}_2\text{O}$ .....	0.05 mg
Pyridoxamine $\cdot 2\text{HCl}$ .....	0.006 mg
Biotin.....	0.2 $\mu\text{g}$
Thiamine $\text{HCl}$ .....	0.03 mg
Biopterin <sup>c</sup> .....	0.3 $\mu\text{g}$
Folic acid.....	0.03 mg
Sorbitol.....	1.5 g
$\text{Na}_2$ DL-glycerophosphate $\cdot 5\text{H}_2\text{O}$ (25% $\alpha$ , 75% $\beta$ ).....	1.0 g
Hemin <sup>d</sup> (in Quadrol) <sup>e</sup> .....	1.5 mg

<sup>a</sup> Expressed as weight per 100 ml of final medium; pH 7.4 with KOH.

<sup>b</sup> To yield (mg/100 ml): Fe, 0.6; Mn, 0.5; Zn, 0.5; Mo, 0.2; Cu, 0.04; V, 0.02; Co, 0.01; B, 0.01; Ni, 0.01; Cr, 0.01.

<sup>c</sup> Crude ( $\sim 6\%$ ), Sigma Chemical Co.

<sup>d</sup> Type III, equine ( $\sim 98\%$ ), Sigma Chemical Co.

<sup>e</sup> Quadrol is *N,N,N',N'*-tetra-kis(2-hydroxypropyl) ethylenediamine (Wyandotte Chemical Corp., Wyandotte, Mich.).

growth. Excess nutrient metabolites mask anti-metabolite activity. The medium (at 10/9 concentration) was distributed in 4.5-ml portions in 25-ml micro-Fernbach flasks and autoclaved at  $121^{\circ}\text{C}$  for 15 min. Drugs (1% solutions) were dissolved, Seitz-filtered, diluted with distilled water, and aseptically added to the medium in 0.5-ml portions, bringing the solution to final concentration. The flasks were inoculated with one drop of log-phase culture, and incubated for 5 days at  $28^{\circ}\text{C}$ . Growth was scored as absorbance on a Welch Densichron (W. M. Welch

Mfg. Co., Chicago, Ill.) fitted with a special cuvette holder and light source.

**Drugs.** Many drugs poorly soluble in water were dissolved in concentrated KOH; the pH in enzyme work was adjusted by gassing with CO<sub>2</sub>. Drugs for whole-cell work were dissolved in KOH, and uninoculated control flasks were checked for pH change. Since most drugs inhibited at low concentrations, drug-induced changes in pH were usually negligible. The following drugs (all specific inhibitors of mammalian  $\alpha$ -GPDH) obtained from the CCNSC also are being tested on animals: 4-biphenylcarboxylic acid (BCA, CCNSC 23040); (4-biphenyl)-glycolic acid (GCA, 16282); 1,4-dichloroglycouril (GD, 84986); (phenylazo)-thio-2-phenylhydrazide formic acid (FPT, 4275); 2-(*p*-hydroxyphenyl)-2-phenylpropane (HPP, 6237); 5,6-dichloro-2-benzoxazolinone (BAD, 24962); 1-(*p*-chlorobenzyl)-5-methoxy-2-(*p*-methoxyphenyl)-indole-3-acetic acid (IAC, 66575); 1-(*p*-chlorobenzyl)-2-ethyl-5-methylindole-3-acetic acid (ICM, 66574); 2-hydroxy-1,2,3-nonadecane tricarboxylic acid (agaric acid, AA, 65690); (*p*-cyclohexylphenyl)-acetic acid (ACP, 89393); 1-hydrazino-phthalazine hydrochloride (PHM, 89394).

Several are available commercially, e.g. AA (K & K Laboratories, Plainview, N.Y.), and HPP (as 4-cumylphenol from Aldrich Chem. Co., Inc., Milwaukee, Wis.).

Melarsen [*N*-(4,6-diamino-*s*-triazine-2-yl)arsanilic acid] and Melarsoprol [*p*-(4,6-diamino-*s*-triazin-2-yl)amino] dithiobenzenearsonous acid 3-hydroxypropylene ester) were gifts from May & Baker, Ltd., Dagenham, Essex, England. Tryparsamide [*N*-(carbamoylmethyl)arsanilic acid] was bought from K & K Laboratories. Primaquine diphosphate [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline] was a gift from Sterling-Winthrop (Rensselaer, N.Y.).

**Expression of results.** Two concentrations of the effective drugs were used on each enzyme system. Pharmacological dose curves were plotted as straight-line functions of logarithmic drug concentration against linear percentage of inhibition; only concentrations in the 20 to 80% inhibitory range were used. The 50%-inhibition concentrations were derived from these plots. Wide concentration ranges were used for initial whole-cell toxicity estimations; 50% inhibitory levels were determined from narrow concentration curves.

## RESULTS

**Growth studies.** Three of the 11 CCNSC compounds inhibited *C. fasciculata* below 100  $\mu$ M (Table 2); one (GD) was less active. Known anti-protozoal drugs served as controls. The anti-malarial primaquine was effective at low concentrations. The trypanocide Melarsoprol inhibited at higher concentrations, approaching 1 mM. Melarsoprol was dissolved in 100% propylene glycol as administered clinically (33); control experiments showed no interaction between solvent and drug.

Drugs that did not inhibit growth are in Table

TABLE 2. Effect of drugs on *C. fasciculata* growth

Drugs	Concn (10 <sup>-6</sup> M) <sup>a</sup>
<i>Inhibitory</i>	
HPP .....	24
BAD .....	51
AA .....	70
Primaquine .....	150
GD .....	380
Melarsoprol .....	560
<i>Noninhibitory</i>	
GCA .....	450 <sup>b</sup>
PHM .....	310
FPT .....	120 <sup>c</sup>
BCA .....	150 <sup>d</sup>
ACP .....	460
ICM .....	300
IAC .....	140 <sup>c</sup>
Tryparsamide .....	3,300
Melarsen, Na .....	2,700

<sup>a</sup> Values for inhibitory drugs are concentrations causing 50% inhibition.

<sup>b</sup> Values for noninhibitory drugs are for the highest concentration tested.

<sup>c</sup> Precipitates at higher concentrations. The solution was highly alkaline, causing pH toxicity at higher concentrations.

2. Of the latter compounds, only BCA and ICM were inhibitory to  $\alpha$ -GPDH below 10<sup>-3</sup> M. Many CCNSC compounds required strong alkali for solution, thus limiting trial of higher concentrations of several drugs (FPT, BCA, IAC). Melarsen and tryparsamide did not inhibit growth.

**Enzyme inhibition.** HK, G6PDH, MDH, and  $\alpha$ -GPDH figure prominently in the incomplete glycolysis of *brucei*-subgroup trypanosomes. Inhibition experiments with drugs are summarized in Table 3. Agaric acid was highly inhibitory to  $\alpha$ -GPDH and MDH. HPP, the most effective of the whole-cell inhibitors (Table 2), is rather specific for  $\alpha$ -GPDH, but it inhibits the other enzymes at higher concentrations. GD, although inhibiting growth and  $\alpha$ -GPDH activity, was not stable and, hence, was not tested further. Primaquine was generally effective against pyridine nucleotide-mediated reactions, but it was not specific for G6PDH as it is in mammalian systems (16).

**Additional biochemical studies.** A Lineweaver-Burk plot (Fig. 1) of the *Crithidia*  $\alpha$ -GPDH gave a  $K_m$  of  $2.17 \times 10^{-3}$  M for DHAP, a high value compared to that for enzymes from other organisms (4, 6).

During this study, investigations were started on other glycolytic enzymes mainly by methods like those detailed. Enzymes present included aldolase (EC 4.1.2.7), glyceraldehydephosphate

TABLE 3. Toxicity ranges of drugs on enzymes of *Crithidia fasciculata*

Drug	Concn ( $10^{-6}$ M)	Per cent inhibition			
		$\alpha$ -GPDH	MDH	HK	G6PDH
AA <sup>a</sup>	7	50	0		0
	33	~100	50		0
	100	~100	86		15
	160	~100	~100		50
	230	~100	~100		90
HPP	100	~2	~16	~12	0
	240	50	40	31	0
	340	77	50	38	0
	500	~100	62	50	30
	660	~100	68	53	50
	800	~100	74	57	77
GD	400	50			
	500	65			
BAD	550	50	~8	21	0
	1,200	~100	43	40	0
	1,500	~100	50	45	
	1,700	~100	54	50	
Prima- quine <sup>b</sup>	400	42	33		~12
	470	45	50		25
	490	50	54		30
	600	74	75		50
Melarso- prol	820	34	37	~10	50
	1,400	50	49	22	62
	1,670	58	50	25	65
	5,420	~100	~83	50	~89

<sup>a</sup> AA did not inhibit HK below 0.300 mM; above this it inhibited the yeast G6PDH used in the assay system.

<sup>b</sup> Primaquine did not inhibit HK below 0.250 mM; above this it inhibited the yeast G6PDH used in the assay.

dehydrogenase (EC 1.2.1.13), and glycerol kinase (EC 2.7.1.30). Lactic dehydrogenase was not present, confirming Schwartz (32). Alcohol dehydrogenase was not detected, although it has been reported in *Crithidia* (3).

### DISCUSSION

The  $\alpha$ -GP system mediating terminal respiration and the recycling of NADH in *brucei*-subgroup trypanosomes has been proposed as a likely chemotherapeutic target, since it is essential for survival of the parasite but not for the survival of the host (18). Williamson (35) suggested that anticancer agents might find use in trypanosome chemotherapy. The drugs used here specifically inhibit mammalian cancer cell

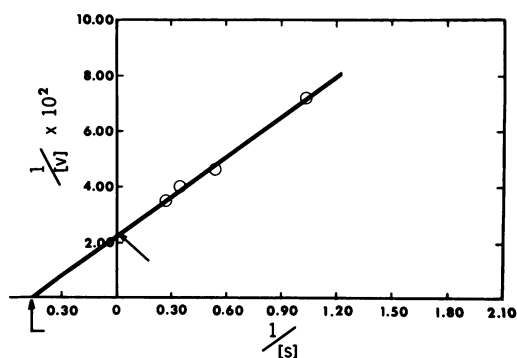


FIG. 1. Lineweaver-Burk plot for *C. fasciculata*  $\alpha$ -glycerophosphate dehydrogenase;  $v$  is the initial change in optical density (340 nm)/100  $\mu$ g of protein per min, and  $S$  is the concentration of dihydroxyacetone phosphate (mM). Arrows indicate  $K_m$  (2.17 mM) and  $V_{max}$  (0.47).

$\alpha$ -GPDH (11, 12). The three antitumor drugs (AA, HPP, and BAD) effective against *Crithidia* and its enzymes in this study have passed stage 1 and are near stage 2 of the CCNSC evaluation system (28).

AA has been used as an anhydrotic agent in the symptomatic treatment of tuberculosis, although it is known to be a toxin in mushroom poisoning (14, 36). The 50% inhibitory concentration of AA to whole cells was of the same order as that for 50% or greater inhibition of  $\alpha$ -GPDH and MDH. It was more toxic to *Crithidia*  $\alpha$ -GPDH (50% inhibition at  $7 \times 10^{-6}$  M) than to the tumor enzyme (40% at  $7 \times 10^{-5}$  M; 13). AA was more inhibitory to enzymes; HPP was more toxic to whole cells. This difference could be due to better permeation by HPP. AA is a potent aconitase inhibitor (9); since *Crithidia* has a functional Krebs cycle (25), AA, if freely penetrating, should be more effective than HPP. BAD, although not highly inhibitory to the *Crithidia* enzymes, was toxic to whole cells at low concentrations; the primary effect of this drug may be at enzyme sites other than those tested, or inhibition of growth may reflect cumulative, but low, toxicity to several enzymes. These results indicate that the three drugs are potent inhibitors of a trypanosomatid and that they are potential inhibitors of enzymes essential to pathogenic trypanosomes.

Effects of trypanocides, especially arsenicals, on trypanosome glycolytic enzymes have hardly been investigated (35). The few previous investigations of their mode of action have not correlated growth inhibition with enzyme inhibition (7, 8, 10, 21, 27).

Melarsoprol did not strongly inhibit growth of *C. fasciculata*. This may denote inability to concentrate the drug. Susceptible trypanosomes may preferentially absorb arsenicals (35). Melarsoprol was more toxic to *T. rhodesiense* kinases than to other enzymes (18); however, it was more inhibitory to *Crithidia*  $\alpha$ -GPDH and MDH. Direct comparison of the Melarsoprol concentrations needed to inhibit *Crithidia* and *T. rhodesiense* enzymes is not immediately possible, since values for the latter have not been published (18).

The pentavalent arsenicals melarsen and tryparsamide inhibited neither *Crithidia* cells nor enzymes, a confirmation of the theory that such compounds must first be activated by reduction to the trivalent state by the host (33, 35). *C. fasciculata* is not refractory to organic arsenicals: preliminary results indicate that mapharsen inhibits growth by 50% at  $2 \times 10^{-4}$  M.

Since the metabolism of bloodstream and culture forms of trypanosomes differs profoundly, use of an insect parasite to predict drug action could be misleading. Newton (29), however, favors use of an easily cultured trypanosomatid for initial drug mode-of-action studies to rapidly eliminate possible drug action hypotheses. The present studies may encourage the use of *C. fasciculata* for exploring application to trypanosomiasis of drugs now under consideration for other purposes.

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