Potentiated Cytotoxicity of Glycolytic Inhibitors by Phenethylbiguanide in Cell Culture.* (27599)

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We have shown that phenethylbiguanide at critical concentrations enhances lactic acid production and glucose utilization of cell cultures without appreciably inhibiting growth (1). This property was held in common with respiratory inhibitors or uncouplers of phosphorylation. Since the primary sites of action of phenethylbiguanide have been shown to be in these areas(2,3,4,5), it appeared likely that cultures growing in media containing phenethylbiguanide were characterized by impaired energy transfer related to respiration. It was postulated that such cultures with increased dependence of glycolytic processes for energy would be especially sensitive to inhibitors of glycolysis. A suitable inhibitor of glycolysis in the study of this question appeared to be oxamate, an inhibitor of lactic acid dehydrogenase, which was shown by Papaconstantinou and Colowick(6) to inhibit HeLa cell cultures. The present report concerns the potentiated cytotoxicity of oxamate and other glycolytic inhibitors by phenethylbiguanide.

Materials and methods. Two procedures were used for appraisal of response to test compounds. In the first procedure, one ml of whole medium containing 10,000 cells was pipetted into 16 × 125 mm tubes placed in a slanted position in racks. The cells were established (plated) by incubation for 24 hours at 37°C in a humidified atmosphere of 8% carbon dioxide - 92% air. The medium was removed and the tubes rinsed 2 times with Earle's solution. The cells then were overlaid in duplicate tubes with one ml of growth medium containing graded amounts of test compounds. Incubation was continued for 4-5 days until maximal growth was attained in untreated tubes. Growth was measured by the method of Oyama and Eagle(7). The media were collected and glucose content determined by the procedure of Park and Johnson(8). Lactic acid was determined according to the procedure of Barker and Summerson(9). In the second test procedure, 10,000-15,000 cells were inoculated directly in one-ml growth medium containing the test compounds at graded concentrations. The remainder of the test was conducted as described above. The 2 procedures yielded equivalent results. Composition of media and other details of cell culture were described in a preceding communication(1).

Oxamic acid was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Phenethylbiguanide · HCl (DBI) was a product of U.S. Vitamin & Pharmaceutical Corp., N. Y. Other compounds were obtained from miscellaneous sources.

Results. In preliminary experiments, the previously observed growth inhibition of HeLa cells by oxamate(6) was also observed for KB cells. This compound inhibited growth of both KB and HeLa cells 50% at concentrations of 20-40 mM (2.2-4.5 mg sodium oxamate/ml). The concentration of oxamate required to inhibit growth and to kill the cells was decreased by further addition of microgram quantities of phenethylbiguanide as shown in Fig. 1. The effectiveness of phenethylbiguanide increased over the range of 1-5 μg/ml so that progressively smaller concentrations of oxamate were required for cytotoxic effects. Glucose utilization and acid production were proportional to growth obtained. In Fig. 2 is shown the activity of pyruvate in antagonizing the cytotoxicity of phenethylbiguanide - oxamate combination. The pyruvate appeared to compete with oxamate and progressively overcame the cytotoxicity of oxamate in media containing phenethylbiguanide. The effects of other metabolites are shown in Table I. Alpha-ketobutyrate and oxalacetate also overcame the inhibi-

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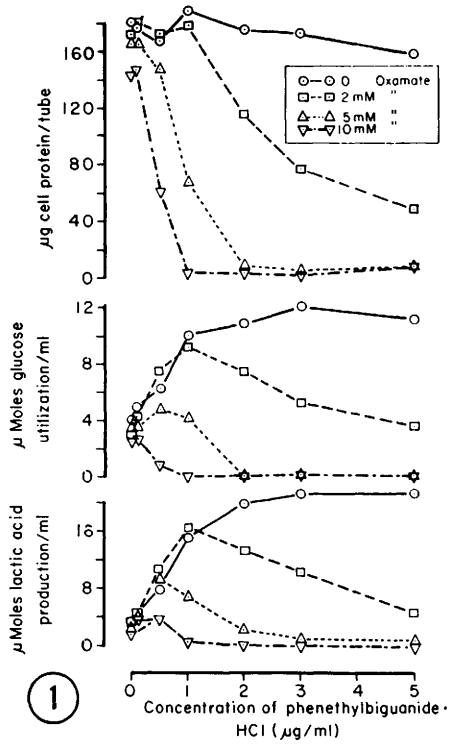

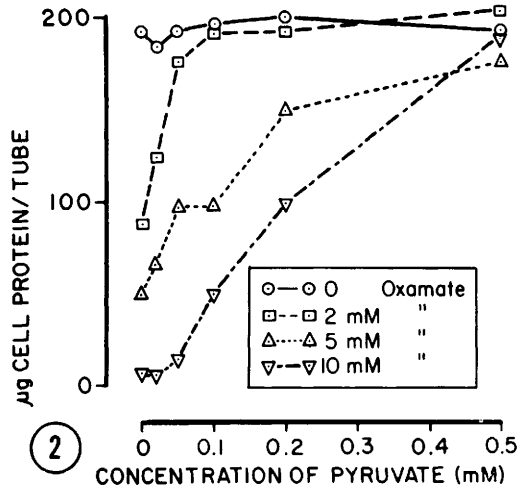

FIG. 1. Effect of oxamate on growth, glucose utilization, and lactic acid production of KB cell cultures in growth medium containing varying concentrations of phenethylbiguanide. Cell protein of 24-hr plated cells at time of treatment was 12 μg/tube. Growth was continued for 5 days.

FIG. 2. Suppression of oxamate-phenethylbiguanide cytotoxicity by pyruvate. Cell protein of 24-hr plated cells at time of treatment was 14 μg/tube. Growth medium contained 2 μg phenethylbiguanide · HCl/ml. Incubation was continued for 5 days.

TABLE I. Effect of Metabolites in Overcoming Cytotoxicity of Oxamate plus Phenethylbiguanide.

Conc. of metabolite (mM)	α-keto-butyrate	Oxal-acetate	α-keto-glutarate	α-hydroxy-butyrate	Lactic acid
	(μg cell protein/tube)				
0	4	4	4	4	4
.1	8	32	2	8	6
.5	104	178	4	4	4
1	164	194	20	4	4
2	170	192	10	4	4
5	180	194	32	4	2
10	162	180	36	4	0
20	92	160	14	4	0

Medium contained 10 mM concentration of oxamate and 3 μg phenethylbiguanide · HCl. Incubation was 5 days at 37°C. Initial cell protein was 12 μg/ml. Growth in media containing phenethylbiguanide or oxamate alone was 190 μg cell protein/tube.

tion of the combination. Higher concentrations of these compounds, themselves, were slightly inhibitory for growth. Alpha-ketoglutarate was relatively inactive. Lactic acid and α-hydroxyglutarate were inactive. The reversal of cytotoxicity was in all instances accompanied by a corresponding increase in glycolysis. Pyruvate and α-ketobutyrate also overcame the inhibition of high concentrations of oxamate, alone, as reported by Papaconstantinou and Colowick(6). None of these compounds influenced the effects of phenethylbiguanide alone.

In Table II are recorded other compounds which demonstrated significantly increased cytotoxicity in presence of phenethylbiguanide. The routine test cells were inoculated directly into tubes containing growth medium and graded quantities of test compounds. Period of growth was 4-5 days. The ratio of the concentrations of the compounds which inhibited growth 50% (ID_{50}) in the absence of phenethylbiguanide to the ID_{50} in the presence of 2 μg phenethylbiguanide · HCl was designated the potentiation index. Values of unity indicated that cytotoxicity of a test compound was not increased by phenethylbiguanide. Values between 1 and 2 were considered of minor significance. Oxamic acid and soluble esters of oxamic acid yielded potentiation indices of 5.5 to 11.7. The esters were observed to hydrolyze readily. Analogs

TABLE II. Potentiation of Cytotoxicity of Compounds by Phenethylbiguanide in Cell Culture.

Cytotoxic compound	1 ID_{50} (with 0 phen*) (mM)	2 ID_{50} (with 2 μg phen*) (mM)	Potentiation index Column 1 / Column 2
Oxamic acid	20	3	6.7
Oxamic acid, ethyl ester	17.5	1.5	11.7
Oxamic acid, tertiary butyl ester	10	1.8	5.5
Azapyruvate, sodium salt	7.3	.73	10.0
Azapyruvate, ethyl ester	2.8	.7	4.0
Fluoropyruvate, sodium salt	2.0	.5	4.0
2-deoxy-D-glucose	3.0	1.0	3.0
2-deoxy-D-galactose	3.0	1.0	3.0
Sodium bisulfite	.6	.1	6.0

Period of growth was 5 days at 37°C.
* Phenethylbiguanide.

of pyruvate, *i.e.*, azapyruvate and fluoropyruvate, yielded a potentiation index of 4 to 10. Sodium bisulfite, which has been shown to be an inhibitor of lactic acid dehydrogenase(10, 11), was potentiated. Cytotoxicity of 2-deoxy-D-glucose which inhibits elsewhere in the glycolytic pathway, *i.e.*, at hexosephosphoisomerase(12) (as well as at glucose-6-phosphate dehydrogenase) and of 2-deoxy-D-galactose was potentiated.

The majority of compounds tested yielded potentiation indices of approximate unity and were considered to be not significantly potentiated by phenethylbiguanide. These included compounds structurally related to oxamic acid or to pyruvate:

N-methyloxamic acid; N-benzyloxamic acid; N-octyloxamic acid; N,N-dicyclohexyloxamic acid; N,N′-dimethyloxamide; oxamide; oxalic acid; oxalic acid, dimethyl ester; oxaldihydrazide; N,N′-dicarbethoxyhydrazide; dithiooxamide; N,N′-didodecyldithiooxamide; N,N-biscarboxydithiooxamide; dithioloxalate; glycollic acid; glyoxylic acid; 3,4-dihydroxyphenylglyoxylic acid; 2-p-biphenylglyoxylic acid; mandelic acid; 2,3-butane dione, 3-oxime; tartaric acid; dioxosuccinate osazone; mucochloric acid; dihydroxyacetone; glyceraldehyde; pyruvic aldehyde; hydroxymalonic acid; α-hydroxybutyric acid; α-hydroxy-α-methylbutyric acid; propionamide; methylene glycinonitrile; cyanoacetic acid; butyric acid; betaine.

A variety of enzyme inhibitors and metabolic analogs also yielded potentiation indices of unity:

Potassium fluoride; potassium iodoacetate; sodium fluoroacetate; amethopterin; 2,6-diaminopurine; puromycin; tetraethylthiuramdisulfide; chalcone; chloromycetin; azaserine.

A number of carbohydrate derivatives, in contrast to 2-deoxy-D-glucose and 2-deoxy-D-galactose, were inactive:

D-ribose; D-glucosamine; N-acetyl-D-glucosamine; D-galacturonic acid; D-glucuronic acid; galacturonolactone; glucuronolactone; mucic acid; glucosephenylhydrazone.

Cytotoxicity of the following compounds was not potentiated by phenethylbiguanide:

Pyridoxal; 3-pyridine sulfonic acid; isonicotinic hydrazide; pyridine-3-aldoxime; 3,4-pyridinedicarboxylic acid; urethane; ethyl ethylcarbamate; 1-phenylsemicarbazide; 1,2-naphthoquinone-4-sulfonic acid; spermidine; isatin; hydroxyurea; chloral hydrate.

The cytotoxicity of mercuhydrin, an inhibitor of α-glycerophosphate dehydrogenase but not of lactic dehydrogenase(13), was not potentiated by phenethylbiguanide.

Sodium azide and antimycin A, which are electron transport system inhibitors(14), potentiated the cytotoxic effects of oxamate and 2-deoxy-D-glucose when substituted for phenethylbiguanide. The cytotoxicity of oxamate was not enhanced by 2,4-dinitrophenol, which uncouples phosphorylation as well as stimulating respiration(14). In contrast, the cytotoxicity of 2-deoxy-D-glucose was potentiated by 2,4-dinitrophenol.

Discussion. The concentration of oxamate required to inhibit growth 50% (2.2-2.5 mg/ml) was high compared with that of the majority of compounds considered cytotoxic in cell culture (less than 1 to 100 μg/ml)(15, 16). However, oxamate is of considerable interest because of the specificity of its action upon lactic acid dehydrogenase. The concurrent use of phenethylbiguanide potentiated the cytotoxic effects of oxamate. The potentiation most likely consisted of simultaneous

inhibition of glycolysis in cells already subjected to a respiratory inhibitor. Thus, 2 major pathways of energy supply of the cells were blocked. Inhibition by high concentrations of oxamate or by phenethylbiguanide, alone, may be due to more complete suppression of glycolysis or of respiration or to additional effects. The specificity of oxamate for lactic acid dehydrogenase in the cell cultures in media containing phenethylbiguanide was indicated by the capacity of pyruvate, α-ketobutyrate and oxalacetate to prevent the cytotoxicity and inhibition of glycolysis. Papaconstantinou and Colowick drew similar conclusions in inhibition studies of HeLa cells by oxamate alone. Soluble esters of oxamate, which also were readily hydrolyzed, were potentiated. Analogs of pyruvate, *i.e.*, azapyruvate and fluoropyruvate, also were potentiated. Further, sodium bisulfite, a lactic acid dehydrogenase inhibitor, and 2-deoxy-D-glucose, which inhibits glycolysis at a different level (hexosephosphoisomerase), were potentiated by phenethylbiguanide. The cytotoxicity of other compounds tested was not significantly increased. Conversely, other respiratory inhibitors, sodium azide and antimycin A, potentiated the cytotoxicity of oxamate as well as 2-deoxy-D-glucose. It was interesting to note that oxamate and 2-deoxy-D-glucose acting at different sites in the glycolytic pathway could be distinguished by their contrasting effects in the presence of 2,4-dinitrophenol. The combined action of 2-deoxy-D-glucose acting effectively on hexosephosphoisomerase and respiratory inhibitors or phosphorylation uncouplers may have sequentially blocked glucose utilization. The inhibitory effect of 2-deoxy-D-glucose on glucose-6-phosphate-dehydrogenase also may be important in this relation. The mechanisms responsible for the ineffectiveness of 2,4-dinitrophenol to potentiate cytotoxicity of oxamate remain to be determined. The data obtained demonstrate that KB cell cultures in the presence of respiratory inhibitors or uncouplers of phosphorylation displayed an increased susceptibility to the cytotoxic effects of certain inhibitors of glycolysis. The cell culture system containing phenethylbiguanide may be suitable for detection and study of similar glycolytic inhibitors.

Summary. Cultured cells exposed to phenethylbiguanide and other respiratory inhibitors or uncouplers of phosphorylation exhibited an increased susceptibility to cytotoxicity of certain inhibitors of glycolysis.

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