Journal of Nematology 25(4):573-577. 1993.
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Fluoroacetic Acid Is a Potent and Specific Inhibitor of Reproduction in the Nematode *Caenorhabditis elegans*

PAUL J. MIDDENDORF[1] AND DAVID B. DUSENBERY[2]

Abstract: Fluoroacetic acid is known to lead to inhibition of aconitase and block both the Krebs and glyoxylate cycles. In this study, we discovered it to be a potent and specific inhibitor of reproduction in a bioassay using the nematode *Caenorhabditis elegans.* Fluoroacetic acid added to the growth medium reduced reproduction in the second generation by 50% at concentrations 3,000 times lower than the concentrations that reduced 24-hour survival by 50%. Four concentrations (2, 4, 8, and 17 mM) of fluoroacetic acid were tested thoroughly. At the two lower concentrations, the survival rates were unaffected, and first-generation reproduction was greatly reduced but not completely eliminated. Survival was reduced at the higher concentrations. Malonate, which inhibits the Krebs cycle, and itaconate, which inhibits the glyoxylate cycle, were tested individually and in combination. The combination did not specifically inhibit reproduction, suggesting another mode of action for fluoroacetic acid. Fluoroacetic acid shows promise as a tool in studies requiring age synchrony.
Key words: aconitase, aging, *Caenorhabditis elegans,* fluoroacetic acid, fluorocitrate, lethality, nematode, reproduction.

In the course of developing and testing toxicity assays using lethality and reproduction as endpoints and *Caenorhabditis elegans* as the test organism (12-14), experiments were performed with fluoroacetic acid. Fluoroacetate is a metabolic precursor of fluorocitrate, a compound that inhibits the action of aconitase, an enzyme central to both the Krebs and glyoxylate cycles (6). Because fluoroacetic acid strongly inhibited reproduction at much lower concentrations than it inhibited survival, its use could supplement other methods of inhibiting reproduction and enable processes and phenomena (such as aging) that require synchronous populations for experimentation to be studied.

To determine whether fluoroacetic acid inhibited reproduction because it interfered with both the Krebs cycle and the glyoxylate cycle, metabolic poisons that interfere with only one of the cycles were tested individually and in combination against *C. elegans.* Malonate is a specific competitive inhibitor of succinate dehydrogenase, a Krebs cycle enzyme. Itaconate noncompetitively inhibits isocitrate lyase, an enzyme of the glyoxylate cycle. Isocitrate lyase is found in *C. elegans* at high levels during embryogenesis and in first-stage juveniles, but is not detectable again until, as adults, eggs are produced (9).

MATERIALS AND METHODS

Caenorhabditis elegans var. Bristol (strain N2) were grown on 5-cm-d petri plates containing 10 ml of K agar (KCl, NaCl, peptone, cholesterol, $CaCl_2$, and $MgSO_4$) (12). A thin lawn of a uracil-requiring mutant strain (OP50) of *Escherichia coli* was added to the surface of the medium as a food source and allowed to grow for 24 hours. All experiments were performed at 20 C. Plates were prepared by spreading 133 µl of aqueous solution of test compound (for treatment plates) or deionized water (for control plates) with a glass rod on plates containing the medium and bacteria. Plates were stored in a desiccator over a saturated solution of $CaCl_2$ for 24 hours to remove excess moisture and to allow the test compound to diffuse throughout the medium.

Dauer larvae were prepared by seeding egg white plates (1) with adult nematodes and incubating at 20 C for at least 1 week. Dauers were rinsed from the plates using a buffer (19.5 g $Na_2HPO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, and 1.0 g NH_4Cl in 1 liter distilled water) and centrifuged at

Received for publication 5 April 1993.
[1] Environmental Science and Technology Laboratory, Georgia Tech Research Institute, Georgia Institute of Technology, Atlanta, GA 30332. [2] School of Biology, Georgia Institute of Technology, Atlanta, GA 30332.

650*g*. The supernatant was aspirated and the dauers were resuspended in 10 ml of 1% sodium dodecyl sulfate for 30 minutes to kill nematodes not in the dauer stage. The dauers were again centrifuged, and the supernatant was aspirated. The pelleted worms were layered in centrifuge tubes on top of a 15% ficoll solution. The solutions were centrifuged again, the supernatant was aspirated, and the pelleted dauers were resuspended in the buffer solution. The final rinse with the buffer and centrifugation was repeated three times to clean the dauers. The dauers were then stored in the buffer at 20 C until needed.

In the survival assay, seven adult (24 hours after dauer) nematodes were placed on each plate 24 hours after the test solution was added; 24 hours later the number of living nematodes on each plate was counted under a dissecting microscope. Nematodes were scored as alive if they were motile or if they responded to a light stroke with a platinum wire. Nematodes not found on the plate were considered to have died and decomposed. Ten replicates at each concentration were performed.

In the reproduction assay, one adult (24 hours after dauer) nematode was placed on each treatment plate. Three days later, one of the adult offspring (when available) was transferred to a new, identical treatment plate. Four days later, the number of offspring at all stages beyond the egg on each of the second set of plates was counted under a dissecting microscope. Ten replicates at each concentration were performed.

Fluoroacetic acid, malonic acid, itaconic acid, sodium azide (Sigma Chemical Co., St. Louis, MO), and sodium cyanide (Aldrich Chemical Co., Milwaukee, WI), were tested individually at various concentrations in both the survival and reproduction assays. Three combinations of malonic acid and itaconic acid were tested. In one experiment, a stock solution containing the concentration of itaconic acid that killed 50% of the exposed worms (LC_{50}) and the LC_{50} of malonic acid was made and then the following dilutions were made with distilled water: 1/8, 1/4, 3/8, 1/2, 5/8, 3/4, 7/8, and 1/1. In a second experiment, the stock solution contained the LC_{16} of malonic acid and the LC_{84} of itaconic acid, and the same dilutions were used on treatment plates. In a third experiment, the stock solution contained the LC_{84} of malonic acid and the LC_{16} of itaconic acid, and the same dilutions were used on treatment plates. The three experiments were repeated using the EC_{50}, EC_{16}, and EC_{84} for reproduction. The effects of combinations of chemicals were determined to be synergistic, additive, or antagonistic using the method of Marking and Dawson (7).

Concentration–response curves for the survival assay and the reproduction assay were developed for each test compound and combination of compounds. The probit method was used to calculate the LC_{50} and EC_{50} for reproduction using a computer program (10) based on the method of Finney (2).

For mixtures, calculations were made using the dilution of the mixture as the concentration. To determine the contribution of each compound to the LC_{50} of the mixture, the dilution that gave the LC_{50} was multiplied by the actual concentration of each of the compounds in the mixture. The ratio of the LC_{50} to EC_{50} was calculated for each of the compounds added in combination by dividing its contribution to the LC_{50} by its contribution to the EC_{50}. An example for the experiment using the LC_{50} for malonic acid and the LC_{50} for itaconic acid is shown in the following equation

$$Ratio = \frac{\left(\frac{D_L \times LC_{50:mal}}{D_R \times EC_{50:mal}} + \frac{D_L \times LC_{50:itcn}}{D_R \times EC_{50:itcn}}\right)}{2}$$

where D_L = dilution of combination that resulted in 50% lethality, and D_R = dilution of combination that reduced reproduction by 50%.

To determine the optimal concentrations of fluoroacetic acid at which reproduction was strongly inhibited and survival

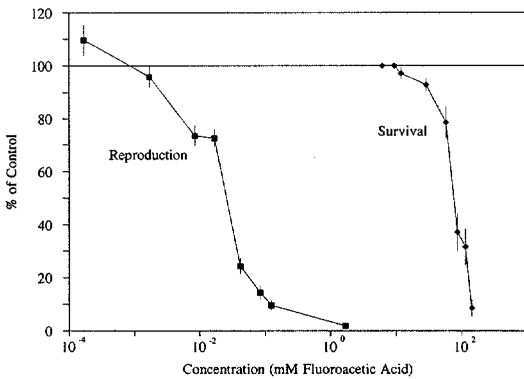

FIG. 1. Concentration–response curves of fluoroacetate for survival and second-generation reproduction of *Caenorhabditis elegans*. The calculated LC_{50} is 76 mM, and the calculated EC_{50} for reproduction is 23 μM. Error bars represent the standard error of the mean.

was not affected, four concentrations (1.7, 4.2, 8.5, and 17 mM in the final media) were tested. In the survival assay, the nematodes on each plate were scored at 24-hour intervals (±1 hour) for 6 days. The reproduction assay was performed as in the other experiments. Fifteen control and 10 treatment plates at each concentration were used throughout the experiment. For statistical analysis of the survival study, each plate was considered a replicate. The binomial distribution of the ratio of living to dead nematodes on the treatment plates was compared to the control distribution by goodness of fit analysis.

To determine whether the living bacteria modified fluoroacetic acid, autoclaved *E. coli* OP50 were added to 5 control plates and 10 plates each containing fluoroacetic acid at the EC_{84}, EC_{50}, and EC_{16} for reproduction. The reproduction assay was performed as described previously.

RESULTS

The concentration–response curves for fluoroacetic acid effects on 24-hour survival and second-generation reproduction are presented in Figure 1. The LC_{50} was calculated as 76 mM, whereas the EC_{50} for reproduction was calculated as 23 μM, over 3,000-fold lower. The LC_{50}/EC_{50} ratio was far greater for fluoroacetic acid than for all but one of 18 other chemicals tested (unpubl. results), including five other known metabolic poisons. The exception was fluorodeoxyuridine, with a ratio greater than 12,000.

The absence of bacteria did not appear to change the effect of fluoroacetic acid on reproduction. With autoclaved bacteria added in place of live bacteria, reproduction at the EC_{16} was reduced by 28% (±11%), 45% (±12%) at the EC_{50}, and 73% (±10%) at the EC_{84}.

Additional tests were performed at four final concentrations in the medium (1.7, 4.2, 8.5, and 17 mM) in the range where fluoroacetic acid did not affect survival, and where second generation reproduction was essentially zero (Table 1). At the two higher concentrations, the nematode survival rate was lower ($P < 0.01$). Reproduction at each concentration was not completely suppressed on all plates. Numerous first-generation (G_1) offspring were observed in all 1.7 and 4.2 mM fluoroacetic acid treatment plates beginning

TABLE 1. Percentage survival of *Caenorhabditis elegans* exposed to fluoroacetic acid.

Concentration (nM)	Day 1 %	Day 2 %	Day 3 %	Day 4 %	Day 5 %	Day 6 %	G†
Control	100	100	100	100	99	97	
1.7	100	99	99	99	99	99	7.2
4.2	100	100	100	99	98	98	0.1
8.5	100	100	99	98	96	89	39‡
17	100	100	98	96	89	77	68‡

Nematodes were cultured on *Escherichia coli* on plates containing K medium and fluoroacetic acid for 6 days, and the number of living *C. elegans* was counted at 24-hour intervals.
† G = Calculated goodness of fit to controls on day 6 of the binomial distribution of number of dead animals per plate.
‡ Different ($P < 0.01$) from controls on day 6 using goodness of fit test with 7 degrees of freedom (critical value = 18.5).

TABLE 2. Ratios of LC_{50} to reproductive EC_{50} against *Caenorhabditis elegans* from some metabolic poisons.

Compound	LC_{50} (mM)	EC_{50} (mM)	LC_{50}/EC_{50}	Nominal site of inhibition
Sodium cyanide	2.0	1.4	1.4	Cytochrome aa_3 of electron transport
Sodium azide	1.3	0.22	6.0	Cytochrome aa_3 of electron transport
Fluoroacetic acid	76	0.023	3474	Aconitase of citric acid and glyoxylate cycles
Malonic acid	9.9	3.3	3.0	Succinate dehydrogenase of citric acid cycle
Itaconic acid	21	4.3	5.0	Isocitrate lyase of glyoxylate cycle

on day 4, and the number of offspring observed generally increased from day 4 through day 6. The G_1 offspring were similar in size to normal J1 through J4 juvenile stages. The G_1 offspring transferred to parent the second generation remained small, did not advance to the adult stage, and produced no offspring. At 8.5 mM, only some of the plates had G_1 offspring, and the numbers were small. These offspring did not appear to advance beyond the J1 stage. No offspring were observed on 17 mM fluoroacetic acid treatment plates.

The LC_{50}/EC_{50} ratios for some other metabolic poisons are presented in Table 2. In all cases the ratios were between one and six. The ratios for the combinations of itaconic acid and malonic acid (Table 3) were similar to the ratios for the individual metabolic poisons tested.

DISCUSSION

If the potent reproduction effect of fluoroacetic acid in *C. elegans* relative to the lethality effect is caused by inhibiting both the Krebs and glyoxylate cycles, then the combination of malonic acid and itaconic acid would be expected to produce results qualitatively similar to those obtained with fluoroacetic acid. This combination would indicate synergy between the chemicals, and the ratio of the LC_{50} to EC_{50} for reproduction would be very large. However, this was not observed.

TABLE 3. Ratios of LC_{50} to EC_{50} of combination of itaconic and malonic acids against *Caenorhabditis elegans.*

Combination	D_L†	D_R†	Ratio
Itaconic Acid:Malonic Acid‡			
50:50	0.71	0.97	2.9
16:84	0.64	0.44	5.8
84:16	0.87	0.92	3.9

† Dilutions as defined in text.
‡ Dilutions of the respective concentrations added to plates based on LC_{50} or EC_{50}; e.g., 16:84 indicates that the undiluted mixture used in lethality tests was composed of itaconic acid at its LC_{16}, and malonic acid at its LC_{84}, whereas the mixture used for reproduction testing had itaconic acid at its EC_{16} and malonic acid at its EC_{84}.

The specific effect of fluoroacetic acid in inhibiting reproduction in *C. elegans* while a Krebs cycle inhibitor and a glyoxylate cycle inhibitor individually and in combination did not specifically inhibit reproduction suggests that fluoroacetic acid acts with great potency on an unidentified pathway specific to reproduction. Because fluoroacetic acid acts through fluorocitrate to inhibit aconitase, aconitase may be essential in some stage or some metabolic cycle of reproduction.

Although *C. elegans* can be age-synchronized by several methods, including sodium dodecyl sulfate treatment of cultures to kill all but dauer larvae, the synchrony is lost once the populations start reproducing. Therefore, several methods have been investigated to exclude progeny from a population. Inhibitors of DNA synthesis such as fluorodeoxyuridine and hydroxyurea (3,5) have been used, as well as filtration (3,11). Temperature-sensitive mutants are available that do not reproduce at 25 C (4). However, each of these methods has drawbacks making them either untenable for handling large numbers of nematodes or suspect in terms of altering normal structures or biochemical processes (3,8,11).

Although aging reportedly progresses normally at 400 μM fluorodeoxyuridine

(8), nematode biochemistry may be altered by its presence. Fluoroacetic acid, however, affects different biochemical processes than does fluorodeoxyuridine. Experiments requiring age synchrony and performed independently in the presence of fluorodeoxyuridine or fluoroacetic acid may allow researchers to observe changes and compare the results from the two treatments as a means of identifying unintended side effects.

The small number of first-generation offspring that are still produced in the presence of nonlethal concentrations of fluoroacetic acid may minimize the usefulness of this technique. However, where absolute inhibition of reproduction in the first generation is not necessary, the technique may be useful. The technique may facilitate producing large numbers of age-synchronous nematodes.

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