5. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., "Methods in Immunology," Benjamin, New York, 1963.

6. Sober, H. A., Gutter, F. J., Wycoff, M. M., and Peterson, E. A., J. Am. Chem. Soc. 78, 756-763 (1956).

Received Sept. 5, 1967. P.S.E.B.M., 1968, Vol. 127.

Inhibition of Friend Leukemia Virus Splenomegaly by an Acetylenic Carbamate (32819)

D. C. DELONG, L. A. BAKER, N. R. EASTON, AND R. D. DILLARD
(Introduced by I. S. Johnson)
The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206

Previous reports from our laboratories have described the synthesis (1–3), the oncolytic activity (4), and the anti-influenza activity (5) of a series of 1,1-diaryl-2-propynyl carbamates commonly called as a group the acetylenic carbamates. The antitumor studies indicated that the acetylenic carbamates were active against 16 of 21 transplantable tumors tested. The compounds were inactive against three types of ascites tumors indicating that the apparent activity was not due to contact cytotoxicity. Studies employing virus infecions in mice indicated that the acetylenic carbamates also possessed anti-influenza activity but were ineffective against infections in mice produced by polio, Semliki Forest, herpes, or pseudorabies viruses. Since anti-influenza studies had ranked 1-(4-fluorophenyl)-1-phenyl-2-propynyl *N*-cyclohexylcarbamate (FPPC) as the most interesting compound, FPPC was used in studies with Friend leukemia virus infections in DBA/2mice.

Materials and Methods. Plan of experiments. Mice were treated with FPPC either intraperitoneally by injection or orally by gavage. A standard dose schedule of drug administration at 24- and 4-hour preinfection and at 24- and 48-hour postinfection was used except when otherwise noted. Day of infection was considered day 0 and animals were autopsied for spleen removal and bled either 21 or 42 days after infection except where noted. Weight changes between start of drug treatment and time of autopsy were measured. Friend virus used in these studies came from a single lot prepared as the supernatant of a 10% spleen homogenate with a titer of about 10^5 infectious doses/ml. For each experiment a frozen ampul of virus was thawed, and diluted 1–100 in sterile media 199. A volume of 0.25 ml of diluted virus was inoculated intraperitoneally into DBA/2 mice (obtained from Jackson Laboratories, Bar Harbor, Maine) weighing between 14 and 16 gm.

Virus titrations. Spleens from treated and control mice 21 days after infection were homogenized with a Potter-Elvehjem type tissue homogenizer in media 199. The 10% spleen homogenate supernatants or harvested serums were diluted in 10-fold steps to 10^{-6} and then each dilution was injected into groups of 8 DBA/2 mice. After 21 days, recipient mice spleens were removed and weighed. Dilution of tissue was plotted against the log of spleen size and virus reduction was calculated as described by Chirigos (6).

Inhibition of splenomegaly. Since initial experiments indicated a decreased virus multiplication due to drug treatment, inhibition of splenomegaly was used in later studies to measure drug effect. The degree of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Infected control spleen size—Treated spleen size}}{\text{Infected control spleen size—Normal spleen size}} \times 100.$$

Drug studied. Investigations (1–3) of the intramolecular reactions of acetylenic compounds led to the synthesis of a series of 1,1-diaryl-2-propynyl carbamates. The series of compounds was originally extended to include a study of structure–activity relationships based on the oncolytic activity. The combination of anti-influenza activity and

TABLE I. Effect of 1-(4-Fluorophenyl)-1-phenyl-2-propynyl *N*-cyclohexylcarbamate (FPPC) on Friend Leukemia Virus Splenomegaly in DBA/2 Mice.

Expt. no.	Treatment	Dosage (mg/kg × 4)[a]	Wt. gain at autopsy (gm)	Mean spleen wt. (mg)
I	FPPC + virus	30	ND	100
	Virus control	0[b]	ND	2145
	Uninfected control	0[b]	ND	70
II	FPPC + virus	30	+2.0	142
	Virus control	0[b]	+3.5	1485
III	FPPC + virus	20	+1.4	87
	Virus control	0[b]	+2.8	1730

[a] Treatment given 24 and 4 hours preinfection and 24 and 48 hours postinfection.
[b] Same treatment schedule with suspending agent only.

oncolytic activity of FPPC made it an obvious choice for study with an oncogenic virus. The acute LD_{50} of FPPC by the intraperitoneal and oral routes was approximately 300 mg/kg and 2000 mg/kg, respectively.

Deaths at toxic doses occurred on days 3 through 7 and were preceded by depression, lethargy, and reduced activity in mice. Animal behavior studies indicated central nervous system depressant activity near the toxic dose level. In contrast to the report for 1,1-dialkyl-2-propynyl carbamates (7,8) no hypnotic properties were observed. Since FPPC is insoluble in solvents suitable for injection into animals, a suspension in 2.5% Methocel (viscosity: 400 cP, Dow Chemical Co., Midland, Michigan) was used. In all instances, control animals were treated with suspending agent at times corresponding to drug treatment.

Results and Discussion. Table I summarizes initial experiments to determine the effect of FPPC on the spleen enlargement induced by Friend leukemia virus in DBA/2 mice 21 days after infection. In all three experiments FPPC-treated animals had spleens only slightly larger than the normal control animals. The inhibition due to treatment was extremely uniform. These data represent groups of 14–18 animals in each treated group and at least 28–36 animals in each control group. Sidwell *et al.* (9) have indicated that toxic drugs which depress weight gain can also reduce spleen enlargement without having a true effect on virus multiplication. The differences between weight gains of the treated and untreated animals in this study can be attributed to increased spleen size adding to the total weight of the untreated animal and to general biological variation and not to toxicity.

To determine the effect on virus multiplication, the spleens from the treated infected group and from the infected control group of Exp. I were titrated as pools in additional DBA/2 mice. Plots of mean spleen weight versus dilution of virus, as described by Chirigos (6), were used to compare the two preparations. The graph plot indicated a three-log reduction of virus per unit of wet tissue due to drug treatment. The virus control animal spleens were about 21 times as large as the treated animal spleens, therefore, on a virus per spleen or virus per animal basis the reduction is even greater. The 1–10 dilution of spleen homogenate supernatant from treated animals did cause a definite enlargement of spleens in recipient mice indicating that virus was decreased but still present.

Serum was obtained from the two groups of mice of Exp. III at autopsy 21 days after infection and titrated in groups of 8 DBA/2 mice. Spleens were removed from recipient mice and weighed 21 days after infection. A graph plot of these data indicated a 1.7 log reduction of virus due to treatment. Spleen enlargement was obtained with the 1–10 dilution of serum from treated animals. The lower degree of virus reduction of the serum compared to that seen with spleen supernates was influenced by the low virus titer of serum of the control animals, since the 10^{-3} dilution

TABLE II. Oral FPPC Treatment of DBA/2 Mice Infected with Friend Leukemia Virus.

Dosage (mg/kg × 4)[a]	No. of animals	Wt. gain at autopsy (gm)	Mean spleen wt. (mg)
0	32	+3.2	1703
20	8	+4.7	106
40	8	+4.7	106
60	8	+4.8	78
80	8	+4.6	81
100	8	+2.0	75
Uninfected controls[b]		+4.3	62

[a] Treatment given 24 and 4 hours preinfection and 24 and 48 hours postinfection.

[b] Same treatment schedule with suspending agent only.

was the highest dilution to cause spleen enlargement. The virus reduction of the serum was influenced by a lower drug level than used in the experiment in which the spleens were assayed for virus content.

Table II summarizes an experiment when FPPC was administered orally by gavage to infected mice. The FPPC was selectively inhibitory over the complete dose range with weight gains equal to that of uninfected controls. The degree of activity by both ip and oral routes was compared in the same experiment. These data are presented in Table III. The amount of inhibition of splenomegaly was dose-dependent and independent of route of drug administration. The FPPC has almost the same specific activity by the oral route as the ip route. It should be pointed out that the oral LD_{50} was about six times as high as the ip LD_{50}. The therapeutic ratio by the oral route thus is about six times higher than the therapeutic ratio by the ip route. Higher oral therapeutic ratios were also observed in the antitumor and anti-influenza studies but the increase was of the order of two to three times higher.

The results of an experiment employing single ip doses of 30 mg/kg of FPPC are presented in Table IV. Single doses both pre- and postinfection reduced spleen enlargement but not to the degree obtained with the four-dose regime as seen at the bottom of the table. Activity from a single dose 24-hours preinfection is probably due to slow excretion of the drug. The doses postinfection are at least as effective as doses preinfection indicating a therapeutic effect of this drug. The mortality in the infected control group was probably due to ruptured spleens which is quite common when spleens exceed 2 gm in weight. In general, mortality is not a good criterion of infection in this virus system.

A study was made to determine the effect of FPPC on multiple infections with Friend leukemia virus. The results are tabulated in Table V when 20 mg/kg ip doses were used. Groups 1 and 2 are a repeat of the normal assay for activity in this system. Group 3 indicates that by 42 days after infection and 40 days after the last drug treatment, there is a spleen enlargement. This agrees with the spleen and serum titrations which indicated that virus was still present. Group 4 shows the spleen enlargement by day 42 after two infections at day 0 and day 21. Animals that

TABLE III. Comparison of Oral and ip Dose Response in the Same Experiment.

	Oral			ip		
Dose of FPPC (mg/kg)	Wt. gain (gm)	Spleen wt. (mg)	Inhibition (%)	Wt. gain (gm)	Spleen wt. (mg)	Inhibition (%)
0.0[a]	+3.8	1820		+3.8	1820	
1.25	+5.5	980	49.1	+5.5	1130	40.3
2.5	+4.2	880	54.9	+4.5	237	92.5
5.0	+3.8	530	75.4	+4.5	450	80.0
10.0	+5.0	283	89.8	+3.1	142	98.0
15.0	+4.9	200	94.6	+2.8	121	99.2
20.0	+4.0	158	97.1	+3.0	92	100.9
Uninfected controls[a]	+4.9	108		+4.9	108	

[a] Suspending agent given ip.

TABLE IV. Activity of Single Doses of FPPC on Friend Leukemia Splenomegaly in DBA/2 Mice.

Time of dose (30 mg/kg)	S/N[a]	Wt. gain at autopsy (gm)	Mean spleen wt. (mg)	Inhibition (%)
24-hours preinfection	10/10	+2.6	370	85
4-hours preinfection	10/10	+2.4	550	77
24-hours postinfection	10/10	+5.1	230	93
48-hours postinfection	10/10	+2.3	380	84
Control	7/10	+2.6	2057	—
24 + 4 preinfection, 24 + 48 postinfection	10/10	+2.5	130	98

[a] Number of survivors/number of animals on test.

TABLE V. Treatment of Multiple Infections of Friend Leukemia Virus with 20 mg/kg Doses of FPPC.

Group no.	Day of infection	Time of drug treatment[a]	Day of autopsy	Wt. gain at autopsy (gm)	Mean spleen wt. (mg)
1	0	none	21	+2.8	1730
2	0	0	21	+1.4	87
3	0	0	42	+6.9	1264
4	0 + 21	none	42	+5.2	2705
5	0 + 21	0	42	+7.4	1069
6	0 + 21	0 + 21	42	+6.4	105
7	21	none	42	+8.4	2144
8	none	none	42	+9.9	77

[a] Treatment given 24 and 4 hours before and 24 and 48 hours after day indicated.

were infected twice and treated only at the time of the initial infection had spleens that were slightly smaller or equal to that of the group that was infected and treated only once. It would appear that the second virus infection was completely rejected while some multiplication of the initial infecting virus was taking place. Rejection of the second infection was probably due to a host defense mechanism possibly an antigen:antibody reaction. Group 6 indicates that FPPC is effective against two infections of Friend virus when two drug treatments are given and that the drug is effective against spleen enlargement during the relapse from the initial infection. Group 7 provides data to show that animals of the same age were susceptible to virus infection at day 21 and spleens increased to 2144 mg by day 42. This is in contrast to group 5 where spleen size was 1069 mg and spleen enlargement was probably due to the initial infection.

In the same type of experiment using 30 mg/kg doses given ip, the same trends were magnified. The ability to overcome the effect of the initial drug treatment by day 42 was much less with the higher drug level. The 21-day assay could detect little difference between 20 mg/kg and 30 mg/kg but ability to overcome drug treatment by day 42 appeared to be dose dependent. The group that was infected twice and treated once had slightly larger spleens than the group that was infected once and treated once but the observation of rejection of the second infection is again demonstrated in this experiment.

Figure 1 presents a study where we attempted to follow the course of splenomegaly by autopsying and measuring spleen size from groups of five treated and five control animals every 5 days. The graph shows that splenomegaly is inhibited for the first 21 days by drug treatment but then spleen enlargement occurs.

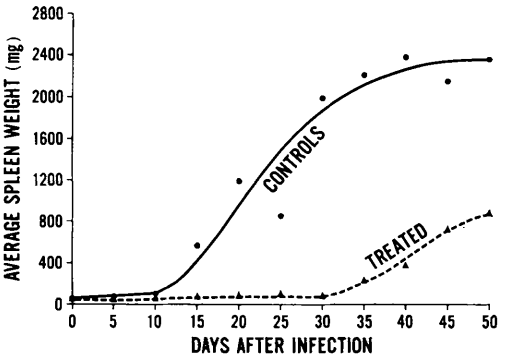

FIG. 1. Effect of FPPC treatment on the course of spleen enlargement.

Numerous workers (10–12) have suggested the use of oncogenic viruses for the detection and study of antiviral compounds. These studies would seem to have even greater value in the light of increasing amounts of evidence indicating a possible relationship between viruses and neoplastic growth in humans. In general, however, previous studies (13) have indicated that some antitumor compounds are active against oncogenic viruses but antiviral compounds have little activity. The present study employed a compound that possessed both antitumor and antiviral activity. This compound had the most effective selective action of any compound we have tested in this system. We hope to be able to determine through structure–activity relationship studies whether the oncogenic virus inhibitory action of the acetylenic carbamates is due to the antitumor effect or the antiviral effect or a combination of the two.

Summary. The compound, 1-(4-fluorophenyl)-1-phenyl-2-propynyl *N*-cyclohexylcarbamate (FPPC), was shown to be a specific inhibitor of Friend leukemia virus splenomegaly *in vivo*. The compound was almost equally effective when administered orally or by injection. Although FPPC suppressed virus multiplication, complete eradication was not observed under the dose schedules used. Virus multiplication during relapse could be inhibited by further FPPC treatment.

1. Easton, N. R., Cassady, D. R., and Dillard, R. D., J. Org. Chem. **27**, 2927 (1962).

2. Easton, N. R., Cassady, D. R., and Dillard, R. D., J. Org. Chem. **29**, 1851 (1964).

3. Easton, N. R. and Dillard, R. D., J. Org. Chem. **28**, 2465 (1963).

4. Dillard, R. D., Poore, G. A., Cassady, D. R., and Easton, N. R., J. Med. Chem. **10**, 40 (1967).

5. DeLong, D. C., Dillard, R. D., Easton, N. R., Streightoff, F., Boniece, W. S., and Redman, C. E., Antimicrobial Agents Chemotherapy **1966**, 509.

6. Chirigos, M. A., Luber, E., March, R., and Pettigrew, H., Cancer Chemotherapy Rept. **45**, 29 (1965).

7. Mehta, M. D. and Catlin, E. R., U.S. Patent 3,062,870, 1962.

8. Keil, W., Muschaweck, R., Rademacher, E., Arzneimittel-Forsch. **4**, 477 (1954).

9. Sidwell, R. W., Dixon, G. J., Sellers, S. M., Maxwell, C. F., and Schabel, F. M., Jr., Proc. Soc. Exptl. Biol. Med. **119**, 1141 (1965).

10. Chirigos, M. A., Ann. N. Y. Acad. Sci. **130**, 56 (1965).

11. Mirand, E. A., Back, N., Prentice, T. C., Ambrus, J. L., and Grace, J. T., Jr., Proc. Soc. Exptl. Biol. Med. **108**, 360 (1961).

12. Sugiura, K., Gann **50**, 251 (1959).

13. Sidwell, R. W., Dixon, G. J., Sellers, S. M., and Schabel, F. M., Jr., Cancer Chemotherapy Rept. **50**, 299 (1966).

Received Sept. 5, 1967. P.S.E.B.M., 1968, Vol. 127.