Effects of 4,4′-Diaetyl-diphenyl-urea-bis(guanylhydrazone) on Leukemia L1210

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SUMMARY
A new aromatic bisguanylhydrazone derivative, 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone), markedly prolonged the survival of DBA/2Ha and DBA/2J mice inoculated i.p. or s.c. with leukemia L1210. Drug-induced 50-day cures of leukemic mice occurred with a higher incidence in DBA/2Ha than in DBA/2J mice, and in female than in male mice. The drug was active by parenteral but not by oral route of administration. Single or repeated doses were most effective when given early after tumor inoculation. The effectiveness of 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone) was reduced when treatment was started 1-2 days prior to the day of death of untreated leukemic mice or when L1210 was inoculated directly into the brain. The selectivity of the antileukemic action of 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone) was evidenced by the high therapeutic index of the drug, and by data indicating that the compound acted in conjunction with host defenses directed against the leukemia.

By the i.p. route of injection, the potency and the therapeutic index of the new drug were much greater than those of methylglyoxal-bis(guanylhydrazone). No cross-resistance occurred between these 2 compounds, or between 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone) and arabinosyl cytosine. In contrast, the new bisguanylhydrazone was not active against a terephthalanilide-resistant subline of leukemia L1210. Marked synergistic antileukemic effects were observed when 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone) was given in combination with arabinosyl cytosine because of the unusual potency of the new agent alone or in combination with arabinosyl cytosine, the clinical trial of this bisguanylhydrazone has been initiated.

INTRODUCTION
The antitumor and toxicologic effects of CH3-G2 in laboratory animals (3, 11, 19, 21), and in patients with acute myelocytic leukemia (4, 8, 22) and malignant lymphoma (22) are well known and have been reviewed recently (12). The marked toxicity of the drug in man prevented the routine use of the compound. Because of the clinical antileukemic activity of CH3-G, more than 200 compounds chemically related to this agent were tested by the CCNSC and in our laboratory in efforts to select derivatives with a better therapeutic index than that of the parent compound. None of these bisguanylhydrazones was more effective than CH3-G. Only a few aromatic derivatives showed borderline activity when given i.p. to DBA/2Ha mice inoculated with L1210 i.p. (11).

Following the observation that the antileukemic effects of CH3-G are greatly potentiated by OHSB in mice (2, 12, 18), some bisguanylhydrazones with borderline activity were tested against L1210 in combination with OHSB (15, 16, 18). The marked synergistic effects noted in these studies indicated that certain aromatic bisguanylhydrazone and diamidine derivatives have potential antileukemic action when given in combination. The data also inferred that new compounds of this type may be found to have significant antileukemic effects when given alone. This possibility was substantiated by the finding that DDUG, a new aromatic bisguanylhydrazone, has marked antileukemic activity in mice (13). The effects of this new drug against L1210 and L1210 sublines resistant to bisguanylhydrazones, terephthalanilides, and ara-C, and the synergistic antileukemic action of combinations of DDUG with ara-C, are described in this report.

MATERIALS AND METHODS
The chemical structure of 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone) is shown in Chart 1. Two salts of the compound were used, namely, the dihydrochloride dihydrate (HCl), also known as CIBA 32248A-Ba, which was obtained first and was used in most of the experiments reported in this study, and chloro-4',4''-di-2-imidazolin-2-yl-terephthalanilide dihydrochloride (DDUG, 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone); ara-C, arabinosyl cytosine; NSC 38280, 2-

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1 This investigation was supported in part by a research grant (CA-04130) from the National Cancer Institute, USPHS. 2 The abbreviations used are: CH3-G, methylglyoxal-bis(guanylhydrazone) dihydrochloride; DDUG, 4,4′-diaetyl-diphenyl-urea-bis(guanylhydrazone); ara-C, arabinosyl cytosine; NSC 38280, 2-
Antileukemic Effects of 4,4'-Diacetyl-diphenyl-urea-bis(guanylylhydrazone)

Chart 2. Survival of female DBA/2Ha-DD mice inoculated i.p. with L1210 after i.p. treatment with 4,4'-diacetyl-diphenyl-urea-bis(guanylylhydrazone) dihydrochloride (DDUG HCl) at doses of 3.125, 6.25, 12.5, 25 or 50 mg/kg/day or with methylglyoxal-bis(guanylylhydrazone) (CH₃-G). Control mice were injected with saline. mkd, mg/kg/day.

and the dimethanesulfonate (MS), also known as CIBA 32248B-Ba, which became available in purified form only more recently and was compared to DDUG HCl in several experiments. DDUG MS was ultimately chosen for further preclinical and clinical investigations because its solubility permits i.v. administration. Both of these salts were synthesized by Prof. A. Marxer at the CIBA Research Laboratories, Basel, Switzerland (10). The compounds were prepared daily shortly before use: DDUG HCl was finely suspended in distilled water, and DDUG MS was dissolved in distilled water or, in a few comparative experiments, finely suspended in saline. CH₃-G was obtained from the CCNSC and ara-C from Dr. C. G. Smith, the Upjohn Co. Research Laboratories.

The L1210 used in this study was obtained in 1957 from Dr. A. Goldin, National Cancer Institute, and thereafter was transferred every 6-7 days in female DBA/2Ha-DD mice by the i.p. inoculation of 1 X 10⁶ ascites cells. L1210/CH₃-G and L1210/DDUG were developed in this laboratory (15, 16). L1210/CH₃-G is maintained under treatment with CH₃-G 50 mg/kg/day. L1210/DDUG was developed using the HCl salt as the selecting compound (15) and, since March 1967, is maintained under treatment with the MS salt at the dose of 25mg/kg/day. L1210/NSC 38280 and L1210/ara-C were obtained in 1967 from Dr. J. H. Burehenal, the Sloan-Kettering Institute. All these L1210 sublines are transferred in female DBA/2Ha-DD mice following the procedures described for L1210.

The DBA/2Ha-DD and DBA/2Ha-dd mice were obtained from this institute’s breeding colony. The DBA/2Ha-DD subline of DBA/2 strain was derived from intense brown coat color mutants of regular dilute brown DBA/2Ha-dd mice (commonly called DBA/2Ha) and has been maintained by rigorous inbreeding since 1952. DBA/2Ha-DD and DBA/2Ha-dd mice share the same histocompatibility characteristics, as indicated by the acceptance of reciprocal skin graft (T. S. Hauschka, personal communication). The DBA/2Ha mice are DBA/2 mice originally obtained by Dr. T. S. Hauschka in 1943 at Lankenau Hospital Research Institute, where the strain had been obtained directly from Dr. C. C. Little in 1929 (cross No. 212 of DBA mice). The strain has been kept on a sibmated basis since then (T. S. Hauschka, personal communication). The DBA/2J mice used in this study were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Unless otherwise specified, in each experiment 1 X 10⁶ leukemic cells were inoculated i.p. or s.c., and drug treatment was started 24 hours later and given once daily for 6 consecutive days; 5 mice per group were used. Average survival was always calculated excluding mice surviving 50 days.

RESULTS

Effects of DDUG against L1210 and Resistant Sublines in DBA/2 Ha Mice. The prolongation of survival of DBA/2 Ha-DD mice bearing L1210 after treatment with the 2 salts of DDUG is shown in Charts 2 and 3. The effects of DDUG HCl were compared with those of CH₃-G in the same experiments. For both salts of DDUG, the prolongation of survival was proportional to the dose administered. A high percentage of mice survived 50 days, and most of them were resistant to reinoculation of 10⁵ to 10⁶ L1210 cells given 5 to 100 days.
at doses of 15-30 mg/kg/day. In males, however, no 50-day age survival time comparable to that seen in female animals.

HC1 salt in female and male DBA/2Ha mice (7-9 animals per group) were similar to those seen in DBA/2Ha-DD mice. Cures were seen (Table 1). In 1 experiment, the effects of the E. Mihich and A. I. Mulhern bis(guanylhydrazone) dimethanesulfonate, mkd, mg/kg/day.

With either salts at each of the doses which also resulted in a incidence of early mortality caused by 15 mg/kg/day of DDUG MS was greater than that caused by 25 or 50 mg/kg/day of DDUG HC1. For example, at the dose of 30 mg/kg/day of DDUG MS is narrower than that of DDUG HC1. Nevertheless, relatively reduced effects against L1210 implanted s.c. were evident at the lower doses used (15) (see also analogous data on Chart 4, groups treated from Day 1). In 1 experiment, the average survival of mice inoculated i.p. with L1210 and treated i.p. with 25-100 mg/kg/day of DDUG HC1 was prolonged by only 13%, and only 1 of the 5 animals receiving the highest dose survived 50 days. In contrast, average survival of mice inoculated s.c. with L1210 and treated i.p. with 25-100 mg/kg/day of DDUG HC1 was prolonged by 60-211% and 4 of 15 animals receiving the highest dose survived 50 days. Nevertheless, relatively reduced effects against DDUG implanted s.c. were evident at the lower doses used (15) (see also analogous data on Chart 4, groups treated from Day 1). Similarly, i.p. treatment with 15-30 mg/kg/day of DDUG MS was less effective in mice inoculated with L1210 s.c. than in mice inoculated with L1210 i.p. (see data in lower part of Chart 8). Thus, although both salts of DDUG were most active when given i.p. to mice inoculated with L1210 i.p., by this route they also were effective in mice inoculated with L1210 s.c. In contrast, the results of 2 experiments showed that i.p. treatment with DDUG MS, at the dose of 30 mg/kg/day, does not prolong survival caused by 50 mg/kg/day of CH3-G was intermediate between that caused by 3.125 and 6.25 mg/kg/day of DDUG HC1. The antileukemic effects of the 2 salts tested were comparable. In fact, doses of 25 mg/kg/day of the HC1 salt and of 30 mg/kg/day of the MS salt are approximately equimolar. Thus, a close comparison of the data shown in Charts 2 and 3 indicate that the more soluble DDUG MS was slightly more effective than DDUG HC1. For example, at the dose of 30 mg/kg/day of DDUG MS and 25 mg/kg/day of DDUG HC1, median survival was 37.5 and 30 days, respectively, and the incidence of 50-day survivors was 40 and 28%, respectively. At the dose of 60 mg/kg/day of DDUG MS, 5 of 10 mice survived 50 days but 4 of 10 died within the 2nd day of treatment (data not shown in Chart 3). Thus, at this level, the MS salt was much more toxic than the HCl salt at the corresponding dose of 50 mg/kg/day (see Chart 2). The greater toxicity of DDUG MS with respect to that of DDUG HC1 was also evident in terms of the early deaths caused by the 2 salts. In fact, the incidence of early mortality caused by 15 mg/kg/day of DDUG MS (Chart 3) was greater than that caused by 25 or 50 mg/kg/day of DDUG HC1 (Chart 2). It should be noted, however, that a few early deaths occurred among mice treated with either salts at each of the doses which also resulted in a significant number of 50-day survivors. These early deaths were preceded by acute collapse and respiratory paralysis.

Data obtained in 2 experiments indicated that in male DBA/2Ha-DD mice DDUG MS caused prolongation of average survival time comparable to that seen in female animals at doses of 15-30 mg/kg/day. In males, however, no 50-day cures were seen (Table 1). In 1 experiment, the effects of the HCl salt in female and male DBA/2Ha mice (7-9 animals per group) were similar to those seen in DBA/2Ha-DD mice. The LD50's of the two salts of DDUG in DBA/2Ha-DD mice are shown in Table 2. After 5 i.p. injections, a 4-fold difference was evident between the LD50 of the 2 salts, DDUG MS being the more toxic compound. After a single dose, the difference was more than 10 fold. The comparison of these data with those shown in Charts 2 and 3 indicates that, by the i.p. route, the therapeutic range of safety of the more readily soluble DDUG MS is narrower than that of DDUG HC1. After repeated treatments with either salt of DDUG at doses in the LD50 range death occurred almost in each case within 15 days from the last injection. Only 1 of 34 mice surviving longer than 2 weeks died 45 days after treatment with 50 mg/kg/day of DDUG MS. Thus the observed death of leukemic mice 35 days after treatment with DDUG or later (see Charts 2 and 3) was not likely to be due to delayed drug toxicity.

In contrast to the marked antileukemic action of i.p. treatments with DDUG, no therapeutic effect was noted after oral administration of either salt mixed in the diet at levels as high as 1%. In the same experiments CH3-G was effective by this route, in confirmation of previous data (12). In 1 experiment, the average survival of mice inoculated i.p. with L1210 and treated s.c. with 50-100 mg/kg/day of DDUG HC1 was prolonged by only 13%, and only 1 of the 5 animals receiving the highest dose survived 50 days. In contrast, average survival of mice inoculated s.c. with L1210 and treated i.p. with 25-100 mg/kg/day of DDUG HC1 was prolonged by 60-211% and 4 of 15 animals receiving the highest dose survived 50 days.
Antileukemic Effects of 4,4'-Diacetyl-diphenyl-urea-bis(guanylhydrazone)

Table 2

<table>
<thead>
<tr>
<th>No. of successive daily i.p. injections</th>
<th>LD_{90} (mg/kg/day)</th>
<th>19/30 confidence limits (mg/kg/day)</th>
<th>Slope</th>
<th>LD_{90} (mg/kg/day)</th>
<th>19/30 confidence limits (mg/kg/day)</th>
<th>Slope</th>
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<td></td>
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<td>1.15</td>
<td></td>
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<tr>
<td>5</td>
<td>202</td>
<td></td>
<td></td>
<td>42-53</td>
<td>1.11</td>
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</tr>
</tbody>
</table>

Median lethal doses of two salts of 4,4'-diacetyl-diphenyl-urea-bis(guanylhydrazone) (DDUG) in female DBA/2Ha-DD mice. Calculated according to Litchfield and Wilcoxon (9). DDUG HC1, dihydrochloride salt of DDUG; DDUG MS, dimethanesulfonate salt of DDUG.

25% prolongation of average survival was seen in mice inoculated i.p. with L1210 and treated with 15 mg/kg/day of DDUG MS from Day 5.

As shown in Chart 5, a single i.p. injection of 200 mg/kg of DDUG HCl caused marked prolongation of average survival time when given as late as 4 days after an i.p. inoculation of 10^6 L1210 cells. A similar effect was seen in mice inoculated with 10^6 L1210 cells only when the drug was given on Day 1 or 2. Comparable results were obtained in 1 experiment in which DDUG MS was tested at the dose of 60 mg/kg.

The lack of cross-resistance between DDUG HCl and CH_{3}-G was observed in the 3 experiments summarized in Table 3. The effects of the combination of OHSB and NSC 67322, which has synergistic effects against L1210 (16), are also shown for comparison. It is apparent that this combination treatment was effective against L1210/CH_{3}-G but not against L1210/DDUG. In 1 experiment DDUG MS was also found to be as effective against L1210/CH_{3}-G as against L1210 at doses of 15 and 30 mg/kg/day. In 2 other experiments, at the same doses, DDUG MS had only borderline effects against L1210/NSC 38280 but was as active against L1210/ara-C as against L1210 (15 mice per group).

Effects of DDUG in Combination with ara-C against L1210 in DBA/2 Ha Mice. The effects of the 2 drugs are shown in Chart 6. Both the prolongation of survival and the incidence of 50-day survivors were greater in mice treated with 25 mg/kg/day of DDUG HCl in combination with 5 mg/kg/day of ara-C than in mice treated with either drug alone at the same dose (lower part of chart) or at twice that dose (upper part of chart). When both compounds were used at the higher dose, the prolongation of survival and the incidence of 50-day cures were even greater. In 1 experiment, results quite similar to those shown in Chart 6 were obtained using DDUG MS at doses of 15 and 30 mg/kg/day in combination with ara-C at a dose of 5 mg/kg/day. As shown in Table 4, a significant number of male mice survived 50 days after treatment with combinations of DDUG HCl and ara-C, in contrast to the lack of 50-day cures among male mice treated with DDUG MS alone (Table 1). The incidence of cures determined by the combined treatment was somewhat higher in female than in male mice, however.

Delayed administration of the 2 compounds also was effective (Chart 7). In mice treated with either drug alone from Day 3, average survival was prolonged but no 50-day cures were seen. Among 30 mice given combined treatments, 19 survived 50 days. Average survival of mice treated with the combination
Lack of cross-resistance between 4,4'-diacetyl-diphenyl-urea-bis(guanylhydrazone) dihydrochloride (DDUG HCl) and methylglyoxal-bis(guanylhydrazone) dihydrochloride (CH₃-G) in L1210. NSC 67322, pentanedial-bis(guanylhydrazone); OHSB, hydroxystilbamidine.

Given for 6 consecutive days starting the day after i.p. tumor inoculation.

Chart 6. Survival of DBA/2Ha-DD mice inoculated i.p. with L1210 after treatment with 4,4'-diacetyl-diphenyl-urea-bis(guanylhydrazone) dihydrochloride (DDUG) and arabinosyl cytosine (ara-C). mkd, mg/kg/day.

Table 4

<table>
<thead>
<tr>
<th>Treatments i.p.</th>
<th>Male mice (No./Total)</th>
<th>Female mice (No./Total)</th>
</tr>
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<td>(mg/kg/day X 6)</td>
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<td></td>
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<tr>
<td>DDUG ara-C</td>
<td>50/60</td>
<td>55/60</td>
</tr>
<tr>
<td>None</td>
<td>0/10</td>
<td>0/10</td>
</tr>
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Antileukemic Effects of DDUG in Female DBA/2J Mice. Data obtained in the study of the antileukemic effects of the 2 salts of DDUG in DBA/2Ha mice suggested that host defenses participate directly in the therapeutic effects observed. Mice surviving 50 days as a result of drug treatment were resistant to reinoculation of L1210. Moreover, data to be presented in detail separately showed that this state of resistance could be transferred adoptively by spleen-cell suspensions, and that marked prolongation of survival, but no 50-day cure, was caused by either salt of DDUG in combination with ara-C in preirradiated DBA/2Ha-DD mice (14). Therefore, in order to see whether the host response to L1210 was particular for the mouse used, the antileukemic effects of DDUG MS were with combinations of 10 mg/kg/day of ara-C with DDUG at the doses of 25-50 mg/kg/day was 27-28 days, and a total of 4 of 10 mice survived 50 days, whereas none of the mice treated with either drug alone were cured. Survival of controls was 8.4 days.

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**Antileukemic Effects of 4,4'-Diacetyl-diphenyl-urea-bis(guanylhydrazone)**

![Chart 8. Survival of DBA/2 mice inoculated with L1210 i.p. or s.c. after treatment with 4,4'-diacetyl-diphenyl-urea-bis(guanylhydrazone)dimethanesulfonate. mkd, mg/kg/day.](image)

<table>
<thead>
<tr>
<th>Treatments i.p.</th>
<th>Average survival (days)</th>
<th>50-day survivors</th>
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<tr>
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<td>30, 10</td>
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<td>3/10</td>
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Effects of 4,4'-diacetyl-diphenyl-urea-bis(guanylhydrazone)dimethanesulfonate (DDUG MS) in combination with arabinosylcytosine (ara-C) against L1210 in female DBA/2J mice.

- Given once daily starting the day after tumor inoculation.
- Calculated excluding mice surviving 50 days.

In 1 experiment, combined treatment with DDUG MS and ara-C had greater effects than either drug alone (Table 5). The 3 of 10 mice which survived 50 days were resistant to successive inoculation of $10^4$ to $10^6$ L1210 cells. In 2 other experiments, the average survival of mice treated with 30 mg/kg/day of DDUG MS in combination with 10 mg/kg/day of ara-C was 20.4 days; 2 of 30 mice survived 50 days. In contrast, in mice receiving 300 R total body X-irradiation the day before L1210 inoculation, average survival was only 13.8 days and no animal survived. Average survival of controls was 7.3 days regardless of irradiation.

**DISCUSSION**

The results of this investigation indicate that both salts of DDUG have marked and comparable therapeutic effects against L1210 in DBA/2 mice. The more soluble dimethanesulfonate salt of DDUG was chosen for further preclinical (20) and clinical (J. F. Holland, personal communication) study because, in contrast to the HCl salt, it is sufficiently soluble in aqueous media to be administered i.v. Since the comparative experiments carried out in DBA/2Ha mice with the 2 salts of DDUG yielded qualitatively similar data, the results obtained with the HCl or MS salt will be discussed separately only when required to emphasize quantitative differences.

In DBA/2Ha mice implanted i.p. or s.c. with L1210, the marked prolongation of survival seen as a result of early treat-
ment with DDUG was much greater than that induced by CH$_3$-G alone. In mice inoculated i.p. with L1210, however, the increase of survival was comparable to that caused by CH$_3$-G or certain related bisguanylhydrazones in combination with OHSB (12, 13, 15, 16). In contrast, the high incidence of 50-day cures induced by DDUG was unique. Thus, the new drug appeared to have much greater and more selective antileukemic activity than CH$_3$-G.

It would appear that DDUG may be less readily absorbed than CH$_3$-G. For instance, DDUG had no antileukemic effects p.o. whereas CH$_3$-G was active by this route (12). The possibility should be considered, however, that DDUG is deacetylated in the stomach into monoguanylhydrazone derivative and di-cetyl-diphenyl-urea. Hydrolysis of DDUG into these compounds was found to occur at acid pH in vitro (J. Gelzer personal communication). Nevertheless, other evidence cannot be explained readily on this basis. Thus, against L1210 inoculated i.p., the drug was much less active after s.c. than after i.p. injection. Also, when given i.p., it was less effective against L1210 inoculated s.c. than against L1210 inoculated i.p. In contrast, i.p. administration of CH$_3$-G was equally or relatively more effective against L1210 inoculated s.c. (12, 15).

In comparing the effects of i.p. treatments started on Day 5 to those started on Day 1, it appears that DDUG retains relatively little of its effectiveness against L1210 inoculated i.p. when it is given 1–2 days prior to the day of death of controls. Yet, in combination with ara-C, DDUG had therapeutic effects against L1210 even when given from Day 5; these effects were particularly marked in mice inoculated s.c. with the tumor. It should be noted in this respect that on the 5th day mice inoculated s.c. with L1210 are not as near death as mice inoculated i.p. with the same number of tumor cells. Since transplantability tests indicated that in DBA/2Ha mice inoculated i.p. with L1210, i.p. treatment with CH$_3$-G reduced the number of leukemic cells to a relatively greater extent in brain than in spleen and liver (12), the possibility was considered that DDUG is not as effective as CH$_3$-G against intracerebral L1210. Indeed, in 2 experiments, DDUG did not prolong significantly the survival of mice inoculated with the leukemia by this route.

At curative doses, DDUG caused early death in a small number of cases. This early mortality seemed to be related to individual variation in rate of drug absorption or in sensitivity to the acute effects of the drug. In fact it occurred at doses of the HCl salt as low as $1/8$ of and $1/4$ of the LD$_{50}$ and LD$_{10}$ respectively, and, in the case of the more soluble MS salt, at doses of $1/6$ and $1/3$ of the LD$_{50}$ and LD$_{10}$ respectively. These early deaths were preceded by general collapse and respiratory paralysis similar to those noted after i.v. injection of DDUG to DBA/2Ha or Swiss HaICR mice, and reminiscent of the paralytic syndrome seen after i.v. doses in rabbits, dogs, and monkeys (20). It is indicative, however, that increasing therapeutic effects for a portion of the population are accompanied by increasing toxic effects for another portion of the population. In this frame of reference, one may attempt to answer the question which has been posed (1), whether toxicity is necessary to achieve the curative effects, by indicating that, under these experimental conditions, toxicity was really necessary. The possibility of avoiding these early deaths by modifying the schedule of drug treatment is currently being explored (17).

The therapeutic index of DDUG MS was less favorable than that of DDUG HCl. In fact, comparable antileukemic effects were observed with the HCl salt at parenteral doses $1/2$–$1/2$ the LD$_{50}$ and with the MS salt at doses $1/2$–$1/2$ the LD$_{50}$ (see Charts 2, 3; Table 2). The differences in water solubility between these 2 salts may lead to differences in rate of absorption of DDUG after i.p. injection, and these may in turn be at the basis of the differences in therapeutic index noted. Obviously this interpretation requires further validation by experimental data. In spite of these differences, the therapeutic index of either salt was much higher than that of CH$_3$-G. Indeed, parenteral doses of CH$_3$-G effective against L1210 were $1/4$–$1/4$ the LD$_{50}$ (12).

No cross-resistance was apparent between DDUG and CH$_3$-G. Moreover, the 2 drugs appeared to be synergistic against L1210 (20). It was of interest that the combination of OHSB and pentanedial-bis(guanylhydrazone), which has synergistic effects against L1210 (16), was active against L1210/CH$_3$-G but not against L1210/DDUG. Since in this combination OHSB seemed to be primarily responsible for the antileukemic effects seen (16), the question is raised whether DDUG is similar to OHSB and different from CH$_3$-G in terms of intracellular mode of action and/or kinetics of cellular uptake. The fact that DDUG had no activity against L1210/NCS 38280 also poses questions about possible relationships between the action of the new drug and that of the terephthalanilide NCS 38280.

At appropriate doses, synergism was noted between the new drug and ara-C, as indicated by the fact that the therapeutic effects obtained after treatment with the combination were greater than those caused by either drug alone at doses twice those used in combination. Increased antileukemic action was also evident after delayed treatments with the combination and was then more marked in mice inoculated with L1210 s.c. than in those given the tumor i.p. The fact that a somewhat greater number of mice survived 50 days when treatment was started on Day 3 than when it was started on Day 1 (see Chart 7), is reminiscent of the finding that, in CDBA mice, the therapeutic action of dichloroamethopterin against L1210 was mostly evident when the onset of chemotherapy was delayed, namely when synergism between antifolic action and defenses of the host directed against the tumor was most effective (5).

Data other than that just mentioned, which will be reported in detail separately, also suggested that an immunologic response of the host against L1210 participated directly in the therapeutic effects of DDUG alone or in combination with ara-C. In fact, the 50-day cured mice were resistant to re inoculation of L1210, the combination of DDUG with ara-C caused marked prolongation of survival but no cures in DBA/2Ha mice irradiated prior to L1210 inoculation, and the state of resistance to L1210 of 50-day survivors could be transferred adoptively by spleen cell suspensions to DBA/2 mice not previously exposed to L1210 (14). The findings that the incidence of 50-day cures induced by DDUG in combination with ara-C was lower in male than in female DBA/2Ha mice, and that no cures were seen in male mice treated with DDUG alone, are also consistent with the data just mentioned in view of the
generally more vigorous immunological response of female mice, which is well established (6).

Since unequivocal evidence for immunity against L1210 had been obtained previously in CDBA but not in DBA/2 mice (5), the effects of DDUG and its combination with ara-C were also tested in DBA/2J mice, namely in a subline of DBA/2 mouse strain other than the DBA/2Ha subline. In fact, since the DBA/2Ha subline was already being kept by Hausekka in 1948, at the time L1210 originated (7), and since to date it has been propagated only by strict sib-mating (see Materials and Methods), the possibility was considered that DBA/2Ha mice respond to L1210 differently from other DBA/2 mice. Indeed, the data obtained showed that DDUG was less effective against L1210 in female DBA/2J than in female DBA/2Ha mice. This suggests the possibility that antigenic differences were smaller between L1210 and DBA/2J mice than between L1210 and DBA/2Ha mice. That antigenic differences also exist between L1210 and DBA/2J mice was indicated by the fact that, in preliminary experiments, 20-30% of DBA/2J mice survived 30 days after treatment with DDUG in combination with ara-C, and were resistant to subsequent reinoculation of L1210. Moreover, the finding that the prolongation of survival caused by DDUG in combination with ara-C was greatly reduced in female DBA/2J mice which had been irradiated prior to L1210 inoculation supports the conclusion that also in this mouse subline weak host defenses directed against the leukemia take part in the therapeutic effects observed.

Delayed death of some leukemic DBA/2Ha-DD mice occurred 35 days after treatment with DDUG or later. Since delayed death was not seen among nonleukemic mice injected with the drug at doses within the LD50 range it is unlikely that drug toxicity contributed significantly to the delayed death of leukemic mice. Such delayed death did not occur among DBA/2Ha-DD mice irradiated prior to L1210 inoculation or among DBA/2J mice, namely, among animals in which the immunity response to the tumor is absent or very slight. It seems possible, therefore, that delayed death occurred among mice in which the immunity response is capable of destroying leukemic cells beyond the period of drug action, yet is not strong enough to cause complete cures.

The observation that, at therapeutic doses, DDUG alone or in combination with ara-C acted in conjunction with host defenses directed against L1210, and that this synergism also occurred in DBA/2J mice, indicates that these chemotherapeutic treatments are selective enough not to impair the host response to weak antigens present on the leukemic cells. The question whether or not the immunogens involved are L1210 specific cannot be answered on the basis of the data available.

A number of features of the action of DDUG given alone or in combination with ara-C indicate that the new guanylhydrazone may be a useful antileukemic agent. These include the high therapeutic index and the wide range of therapeutic doses of DDUG, the selectivity of antileukemic action of the drug, the greater effectiveness of parenteral administration of DDUG with respect to CH2-G, and the marked synergistic and curative effects of both early and delayed administration of DDUG in combination with ara-C. Possible limitations in the usefulness of DDUG alone may be related to the occurrence of occasional early deaths of leukemic mice at curative doses, to the relatively reduced therapeutic effectiveness of the drug in mice with widely disseminated leukemia, and/or to the paralytic syndrome and hepatotoxicity caused by the compound at toxic doses in rats, dogs, and monkeys (20). The question whether or not these possible limitations are of practical significance must await the results of current clinical trials.

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REFERENCES

Effects of 4,4′-Diacetyl-diphenyl-urea-bis(guanylhydrazone) on Leukemia L1210

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