Effects of Elevated Temperatures and Drugs on the Viability of L1210 Leukemia Cells

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SUMMARY

The lethal action of elevated temperatures on neoplastic cells has been determined quantitatively by means of an in vitro-in vivo system of L1210 leukemia cells. Temperatures from 37° to 40° have little effect on these cells. Prolonged exposure at 41° impairs their viability. Between 41° and 42° the lethal effect increases markedly; in Fischer medium, a 4-log kill is achieved at 42° in 3 hr. The exposure of L1210 leukemia cells to a temperature of 42° for a short time sensitizes them to the action of lower temperatures. A 3-hr exposure at 40° has a highly lethal effect when applied after an initial treatment at 42°. If the sequence of heating is reversed, no such effect is observed. Many drugs have been tested for combined therapy. L-erythro-α,β-dihydroxybutyraldehyde has been found to act synergistically with heat. DL-Glyceraldehyde, L-phenylalanine mustard, actinomycin D, and sodium oxamate are also active in combination with heat. However, if toxicity to humans is considered, only DL-erythro-α,β-dihydroxybutyraldehyde, DL-glyceraldehyde, and phenylalanine mustard can be considered as suitable for clinical trial in combination with heat.

INTRODUCTION

A selective lethal effect of elevated temperatures on cancer cells has been demonstrated both experimentally (6, 9, 15, 37, 38) and clinically (7, 8, 12, 24, 25, 41, 42). For a more extensive bibliography see Ref. 7. We have tried to determine this lethal effect quantitatively and to explore the possibility of enhancing it by exposing neoplastic cells to heat and chemicals simultaneously.

As an experimental model, we have chosen an in vitro-in vivo system. L1210 leukemia cells, suspended in a complete culture medium, are treated in vitro and then tested for viability by reinoculation into groups of BDF1 female mice. The L1210 leukemia has been selected because of the precise inverse correlation between the number of cells injected and survival of the animals tested (33, 40). Furthermore, the response of L1210 leukemia to tumor-inhibitory drugs is considered to be similar to that of many human tumors (18). We have treated the cells in vitro because it was necessary to use temperatures higher than mice can tolerate. The viability of the cells after treatment was assayed by reinoculation into mice, rather than by the dye exclusion test (36) used by von Ardenne and others (2-6) in similar experiments. We have found that the viability determined by the dye exclusion method does not agree with the bioassay, which is a more precise and direct method.

Experimental studies have shown that some metabolic processes are preferentially affected by heat: respiration (7, 8, 29, 30, 43), DNA synthesis (16, 17, 28, 29), RNA synthesis (28, 29), and protein synthesis (28, 29). Stehlin (41) has demonstrated that the chemotherapeutic effect of phenylalanine mustard, administered by closed circuit perfusion to human melanomas of the limbs, is considerably increased by raising the temperature of the perfusing fluid above 42°.

These findings have prompted us to investigate more extensively the possibility of using chemicals in combination with heat. We have tested metabolic inhibitors of DNA synthesis [FUDR® (20, 21)], RNA synthesis [actinomycin D (23)], protein synthesis [DHBA (19)], glycolysis [DL-glyceraldehyde (26, 27), 2-deoxy-D-glucose (10, 46)], sodium oxamate (35), mitotic poisons [vinblastine (34)], and an alkylating agent [PAM (11, 32)].

MATERIALS AND METHODS

Cells and Media. L1210 leukemia, obtained from the Wisconsin Alumni Foundation, Madison, Wis., was maintained in the ascites form by weekly transplants in female BDF1 mice purchased from A. R. Schmidt Co., Madison, Wis. For maintenance, 0.1 ml of 6-day-old ascites diluted 1:10 with 0.9% NaCl solution was injected i.p. Cells, 5 to 10 days after passage, were suspended in Fischer medium (14) plus 10% horse serum and antibiotics, or in MEM Spinner medium plus 10% calf serum and antibiotics. The cells were then counted in a hemocytometer and the

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suspension adjusted by dilution to the required concentration. The combination of these media plus serum and antibiotics will henceforth be referred to as complete media. It has been reported that Ll210 leukemia cells can be grown in culture in shaker flasks with various media (13, 31) and in soft agar with Fischer medium (22). We have grown them successfully in shaker culture with Fischer medium + 10% horse serum for 5, 5, 10, 12, and 20 passages. In the Spinner medium, L1210 leukemia cells do not reproduce, but can be maintained at 37° without loss of viability for 24 hr or more. For avoidance of agglutination, in some cases, 0.04% Pluronic F 68 was added after it had been shown that such an addition did not change the sensitivity to heat of the leukemic cells. All the media and sera were purchased from the Grand Island Biological Co., Grand Island, N.Y.

Drugs. The drugs used in this work were: FUDR (Hoffmann La Roche, Inc., Nutley, N. J.), actinomycin D (K and K Laboratories, Inc., Plainview, N. Y.), DHBA (donated by Farmitalia, Milano, Italy), DL-glyceraldehyde and 2-deoxy-D-glucose (Aldrich Chemical Co., Milwaukee, Wis.), sodium oxamate (prepared from oxamic acid; Sigma Chemical Co., St. Louis, Mo.), vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind.), and PAM (donated by Burroughs Wellcome Co., Tuckahoe, N. Y.).

Heat Treatment. L1210 leukemia cells were suspended in complete Fischer or Spinner media at concentrations of 10 to 10^5 cells/ml. From 20 to 50 ml of these suspensions were agitated in 50- or 100-ml Erlenmeyer flasks, closed with ground-glass stoppers, in a thermostated gyroatory water bath shaker (Model G76, New Brunswick Scientific Co., New Brunswick, N. J.) for various lengths of time at the desired temperature. The flasks were agitated at 90 rpm. At the end of the treatment, 0.1 ml of the cell suspension was injected i.p. into groups of 10 to 20 female BDF1 mice. The injected animals were inspected daily and the deaths were recorded.

For the dye exclusion test (36), the following technique has been used. To a drop on a microscope slide of a suspension containing 1 X 10^6 cells/ml, a small amount of dry Eosin Y was added and the suspension was agitated until the dye dissolved. A coverslip was superimposed, and the percentage of stained cells was determined by observation at 400X of 400 cells.

We have used the following technique for culturing the leukemic cells: a suspension of L1210 leukemia in complete Fischer medium plus 0.04% Pluronic F 68 was saturated with a mixture of 95% O_2 and 5% CO_2 and was maintained at 37° in Gyrotory Model G25 shaker incubator (New Brunswick Scientific Co., New Brunswick, N. J.) set at 140 rpm. From 1 to 5 X 10^5 cells/ml were added to start the culture. After a lag period of 2 to 3 days, the culture was established and was maintained by a 1:5 dilution with fresh medium when it had reached a level of 5 X 10^6 cells/ml.

RESULTS

In several reports (2—5), the viability of cells after treatment with heat and chemicals has been assayed by dye exclusion methods. We have also applied this technique, which has the advantages of simplicity and rapidity. As shown in Chart 1, a good correlation between exposure to elevated temperatures and number of stained cells was observed. Nevertheless, we decided to test the validity of these results by reinoculation of the heated cells into mice. This will henceforth be referred to as the bioassay. The L1210 leukemia in ascites form is particularly suitable for quantitative experiments of this type because there is a direct inverse correlation between the number of cells injected into mice and their survival, as elegantly demonstrated by Skipper et al. (39, 40) and Dixon et al. (13). Wilkoff et al. (44) have used a similar bioassay to determine the response of cultured L1210 cells to antileukemic drugs.

We have checked this relationship in our strain of L1210 leukemia and found, in a series of experiments involving 1350 mice (Chart 2), that there is such a correlation, but the fluctuation among different experiments was quite large.

Chart 1. Percentage of L1210 leukemic cells unstained by eosin Y after incubation at the temperatures indicated as a function of time. The cells were suspended in MEM Spinner complete medium. Each point has been determined by counting 400 cells.

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Table 1
The number of female BDF1 mice surviving more than 40 days after injection of L1210 leukemia cells

<table>
<thead>
<tr>
<th>No. of cells injected</th>
<th>No. of animals injected</th>
<th>Survivors at more than 40 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^4</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>1 × 10^3</td>
<td>450</td>
<td>6</td>
</tr>
<tr>
<td>1 × 10^2</td>
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<td>35</td>
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<tr>
<td>1</td>
<td>38</td>
<td>27</td>
</tr>
</tbody>
</table>

Chart 2. Fifty % survival of mice as a function of the number of L1210 leukemic cells injected i.p. The numbers of animals used are in parentheses. Central points, arithmetical averages; solid bars, deviations found in various groups tested in each experiment. For each point of every experiment, groups of 10 to 20 mice were used. The total number of mice was 1350.

was also clear that the injection of a single cell gave a 20 to 40% mortality (Table 1). Accordingly, we can state that a 4-log kill has taken place when 20 to 40% of the mice died after inoculation of 1 × 10^4 treated cells/mouse. Charts 3, 4, and 5 show the survival of mice inoculated with 1 × 10^4 L1210 cells that had been exposed to heat for various lengths of time in MEM Spinner. At 37°C (Chart 3), there was no effect on the viability of the leukemic cells; no difference was found in the survival of the control group and the one given injections of cells incubated for 13 hr. At 41°C (Chart 4), some of the effects of the heat become apparent. In the 13-hr group, there was a significant increase in survival time, which according to Chart 2, corresponds approximately to a 2-log kill. The lethal effect becomes dramatically evident at 42°C (Chart 5), at which exposure of the cells for 4 hr gave a

survival curve identical with that obtained when only a single cell was injected. This is equivalent to a 4-log kill.

There was a major discrepancy between the results of the bioassay and those obtained by dye exclusion. By the latter method, the critical temperature at which the major killing effect appears is between 40°C and 41°C (Chart 1); the 41 and 42°C curves were almost equivalent. By the bioassay, the temperature at which a massive lethal effect appears is between 41°C and 42°C (Charts 4 and 5). The quantitative difference is also quite striking. Using the dye exclusion method, after 2 hr at 43°C, 15% of the cells were still unstained, which is equivalent to less than a 2-log kill (Chart 1), whereas with the bioassay we found that, under the same conditions and the injection of 1 × 10^6 treated cells into mice, there were no deaths (Table 2). This is equal to more than a 4-log kill. After finding this 100-fold discrepancy between these 2 methods, we decided to adopt the bioassay because it is more direct and precise, although considerably more tedious. Having observed the lethal effect of heat in a
Table 2

The conditions required to achieve a 3- to 4-log kill of L1210 leukemic cells by hyperthermia alone

Ten thousand cells were injected i.p. into groups of 10 to 20 female BDF1 mice after treatment in vitro. The survival was determined 40 days after inoculation. For this table, 540 mice were used.

<table>
<thead>
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<th>Temperature</th>
<th>Time (hr)</th>
<th>Survivors 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complete MEM Spinner</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42°</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>42°</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>42°</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>42°</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>43°</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>43°</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>43°</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td><strong>Complete Fischer medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42°</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>42°</td>
<td>1</td>
<td>5</td>
</tr>
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<td>100</td>
</tr>
<tr>
<td>43°</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

1. Survivors = less than 1 viable cell present = more than 4-log kill; 60 to 80% survivors = 1 viable cell present = 4-log kill; 20 to 40% survivors = 10 viable cells present = 3-log kill.

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medium (MEM Spinner) in which the L1210 cells survive but do not reproduce, we chose to investigate their heat sensitivity in Fischer medium, in which these cells can be cultured in shaker flasks.

L1210 leukemia cells exposed to elevated temperatures in complete Fischer medium have a greater sensitivity to heat then those in complete MEM Spinner medium. Charts 5 and 6 show 2 identical experiments, except that in 1 the suspending medium was Fischer (Chart 6) and in the other it was MEM Spinner (Chart 5). A striking difference between the 2 complete media was found. As shown in Chart 5, inoculation of mice with cells that had been subjected to 2 hr of exposure to 42° led to a survival of 20%, but in Chart 6 (Fischer) the survival was 80% at 42°. Whereas Charts 5 and 6 illustrate the results of individual experiments, Table 2 lists the average percentages of surviving mice, according to the length of exposure to 42° and 43° of the cells with which they were inoculated. It is evident from this table that at both temperatures there was a significant difference in the sensitivity to heat, depending on the medium in which the leukemic cells were suspended. In MEM Spinner 2 hr at 43° was as lethal as 0.5 hr in Fischer medium, and 4 hr at 42° in MEM Spinner give fewer surviving mice than did 3 hr in Fischer. In Table 2, the killing effect of heat was estimated from the number of mice surviving for more than 40 days. From the data in Table 1, it is evident that the injection of 1 live cell into groups of mice gave approximately 70% survivors, and the injection of 10 cells gave about 30%.

Taking into account some variability from experiment to experiment, we can safely assume that if, in a group of mice given injections of L1210 leukemia cells, 90 to 100% of the animals survive, less than 1 live cell per animal was present in the inoculum. If an average of 60 to 80% survive, 1 live cell has been injected. If 20 to 40% survive, the live cells injected were 10 per mouse. If the inoculum contains 1 X
10^4 cells, these percentages correspond, respectively, to more than a 4-log kill (90 to 100% survivors), to a 4-log kill (60 to 80% survivors), and to a 3-log kill (20 to 40% survivors).

Chart 7 summarizes the results obtained when L1210 cells suspended in Fischer medium were heated at various temperatures. The amount of cell killing was calculated from Chart 2 at the 50% survival time of the mice inoculated with the treated cells. These results are in good agreement with those in Table 2 and 5, where the leukemic cell kill was calculated from the number of survivors at 40 days. In our opinion, the latter is more accurate, but also requires many more animals. The only significant discrepancy between these 2 methods is found in the 43° curve, at which temperature an exposure of 70 min produced a 4-log kill (Chart 7). In Table 2, 30 min at 43° was sufficient to achieve the same effect. This discrepancy can be explained by the fact that, when the period of incubation becomes too short, exact quantitative analysis becomes more difficult. As shown in Chart 7, temperatures of 41° or less are largely ineffective. Nevertheless, we have observed that the lethal effect of such temperatures became considerable if the leukemic cells were treated sequentially, first with a short period of incubation at 42° and then followed by incubation at 40° (Table 3). This result was not observed when the sequence of treatments was reversed.

Table 4 summarizes the results of the combination of hyperthermia and some compounds. At the concentrations and under the experimental conditions used, DHBA, DL-glyceraldehyde, actinomycin D, PAM, and sodium oxamate exhibited some lethality in combination with heat. Vinblastine, FUDR, and 2-deoxy-D-glucose did not kill these cells, whether applied alone or in association with elevated temperatures. With the exception of PAM and vinblastine, which were tested only at the 2 levels listed in Table 4, all the other chemicals were tested at various concentrations for different lengths of time. This was done in order to determine the minimum concentration capable of giving 100% survival after incubation at 37°. Once this concentration had been established, it was tested for shorter periods of incubation at 42°. We also tested lower concentrations of the same drug for various times at 42°.

Having discovered that DHBA has considerable lethal activity when combined with heat, we wished to determine whether there is a synergistic relationship between these agents. We then titrated the lethal effects of different dosages of DHBA and heat applied separately and concomitantly (Table 5). This was done essentially by the same method used in Table 2, with the difference that in the experiments summarized in Table 5 variable inocula were used instead of a fixed one (1 X 10^4 cells), as in Table 2. In this way it was possible to detect and determine quantitatively the lethal effects of less than 3 logs on L1210 leukemia cells, because as shown in Table 2, it was possible to measure a minimum kill of 3 logs when 1 X 10^4 cells were injected. Similarly, a 2-log kill could be measured by injection of 1 X 10^3 cells, and so on. We found (Table 5) that 30 min at 42° in combination with 0.1 M DHBA resulted in more than a 4-log kill, whereas the sum of the lethal effects of both treatments administered separately was less than 2 logs. From these results, we have established that a synergism of action exists between DHBA and heat, which increases the killing effect on L1210 leukemia cells by a factor of about 100.

**DISCUSSION**

The results reported here clearly demonstrate that the lethal effects of heat on L1210 leukemia in ascites form can be effectively determined quantitatively by measuring the number of cells viable after heat treatment by inoculation.
DrugConcentration (M)TemperatureTime of exposure (hr)Survivors (%)

Actinomycin D

2 X 10^{-6} 37 1 100
8 X 10^{-7} 37 2 100
3.2 X 10^{-7} 37 1 0
3.2 X 10^{-7} 42 1 100

PAM

3.3 X 10^{-6} 37 1 100
3.3 X 10^{-6} 37 1 0
3.3 X 10^{-6} 42 1 100

DL-Glyceraldehyde

2.2 X 10^{-3} 37 8 100
2.2 X 10^{-3} 37 1 0
2.2 X 10^{-3} 42 1 100

DHBA

6 X 10^{-3} 37 1 100
1 X 10^{-3} 37 5 100
1 X 10^{-3} 37 1 0
1 X 10^{-3} 41 2 100
1 X 10^{-3} 42 0.5 100

Table 4

The results obtained by combined treatment of L1210 leukemia cells suspended in complete Fischer medium with hyperthermia and drugs.

Groups of 10 to 20 female BDF1 mice were used for each determination. Ten thousand cells were injected i.p. after treatment. The number of survivors was determined at 40 days after inoculation.

into mice and observing their survival. This can be done by utilizing the 50% survival curve (Chart 2), or, more exactly, by calculating the percentages of long-term survivors after inoculation of different numbers of treated cells (Table 5). We have seen that the relationship between temperature increase and lethality is not a linear one. With the periods of heating used, the killing effect becomes evident between 41°C and 42°C. At lower temperatures, this effect is minimal or nonexistent. The medium in which the cells are suspended has a significant effect on their heat sensitivity. Hyperthermia has a greater lethal effect on cells suspended in complete Fischer medium than those in MEM Spinner. In the Fischer medium, L1210 leukemic cells can be cultured, whereas in MEM Spinner, they are unable to multiply. This difference suggests that some of the synthetic processes related to cell division are particularly sensitive to heat injury. Such a conclusion is in good agreement with our biochemical studies (16, 17) and with those of Mondovi et al. (28, 29), which have shown that DNA synthesis is selectively inhibited by heat.

We have found that the effectiveness of hyperthermia of a lesser degree is increased when applied after a short burst of higher temperature, but the reverse is not true. We do not have an explanation for this phenomenon, but its implications relative to clinical uses of heat for cancer therapy deserve further consideration.

It has been shown that several chemicals have an additive lethal effect with heat on L1210 leukemia cells. It is necessary, however, to consider their toxicity in evaluating these results in view of potential clinical applications. Accordingly, the drugs tested can be placed in the following order: DHBA, DL-glyceraldehyde, PAM, actinomycin D, and sodium oxamate. If the concentrations used in our experiments are extrapolated to clinically effective doses (weight/ml incubation medium to weight/kg body weight) only the first 3 drugs could be considered effective in combination with heat (Table 6). Stehlin (41) has demonstrated enhanced effectiveness of PAM against malignant melanomas of the limbs when the drug is used clinically in heated regional perfusions, at doses and temperatures of the same order of magnitude as in these experiments. Sodium oxamate is also quite effective in combination with heat at doses that inhibit 90% of the glycolytic activity of neoplastic cells. This is not surprising in view of the fact that heat strongly inhibits respiration in tumor cells (7, 29, 30). Thus, the simultaneous application of heat and sodium oxamate deprives the leukemic cells of both their sources of energy. Unfortunately, the concentration of sodium oxamate required to produce this effect is so high that this observation has no practical value. However, these results indicate that it might be worthwhile to search for more efficient glycolytic inhibitors to use in combination with hyperthermia.

Of the chemicals that we have tested, only DHBA produces a synergistic lethality in combination with heat. This drug is also the least toxic for man, as is shown in Table 6. DL-Glyceraldehyde would be as effective as DHBA if the dosages reported by von Ardenne (1) have really been used in humans. Unfortunately, it is impossible to tell from his paper whether those doses are purely theoretical or have been tested successfully in man.

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The treatment of L1210 leukemia cells with simultaneous high temperature and DHBA is 100-fold more effective than each applied separately. We are presently investigating the mechanism of this synergism, which is potentially very important in future clinical applications. The use of drugs with low toxicity that exhibit synergism in combination with sequential heating (first at high temperatures for a short
Table 5

The synergistic effect of DHBA and heat on L1210 leukemia cells incubated in complete Fischer medium

The percentages of survivors are shown as a function of the number of cells inoculated. The number of survivors was determined at 40 days after inoculation. For this table, 800 mice were used.

In Section A, L1210 leukemia cells were treated with 6 different concentrations of DHBA and incubated at 37°C for 1 hr. In Section B, L1210 leukemia cells were incubated at 42°C for different lengths of time without DHBA. In Section C, L1210 leukemia cells were heated at 42°C in combination with 0.1 M DHBA.

### A

<table>
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<th>Concentration (M)</th>
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<th>1 X 10³ cells</th>
<th>1 X 10² cells</th>
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<tr>
<td>0.1</td>
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<td>0</td>
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<td>0.2</td>
<td>0</td>
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<td>55</td>
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<td>0.4</td>
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### B

<table>
<thead>
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<th>Time of heating (hr)</th>
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<th>1 X 10³ cells</th>
<th>1 X 10² cells</th>
<th>1 X 10¹ cells</th>
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<td>10</td>
<td>60</td>
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<td>1</td>
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### C

<table>
<thead>
<tr>
<th>Time of heating (hr)</th>
<th>Concentration (M)</th>
<th>No. of cells</th>
<th>% survivors</th>
</tr>
</thead>
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<td>0.1</td>
<td>1 X 10⁴</td>
<td>100</td>
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<tr>
<td>1</td>
<td>0.1</td>
<td>1 X 10⁴</td>
<td>100</td>
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</table>

Table 6

A comparison between maximum clinical doses and the experimental dose required to kill 1 X 10⁴ L1210 leukemia cells in combination with 1 hr of exposure to heating at 42°C.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Experimental dose</th>
<th>Clinical dose</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>DHBA</td>
<td>8 g</td>
<td>20–30 g</td>
<td>(E. Ciaranfi, personal communication)</td>
</tr>
<tr>
<td>DL-Glyceraldehyde</td>
<td>16 g</td>
<td>48–72 g</td>
<td>? (1)</td>
</tr>
<tr>
<td>PAM</td>
<td>80 mg</td>
<td>25 mg</td>
<td>(45)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>32 mg</td>
<td>8 mg</td>
<td>(33)</td>
</tr>
<tr>
<td>Sodium oxamate</td>
<td>880 g</td>
<td>Not determined</td>
<td></td>
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</table>

*aExperimental dose = quantity by weight of the drug required to kill more than 99.99% of L1210 leukemia cells present at a concentration of 1 X 10⁵/ml in 80 liters Fischer medium in combination with 1 hr of heating at 42°C.

*bClinical dose = maximum daily dose tolerated by an 80-kg man (i.v. injection or infusion). Data are taken from the literature.
REFERENCES


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