In Vitro and in Vivo Studies of the Antileukemic Action of \( N,N'\)-bis\[ p-(N'-methylamidino)phenyl\]terephthalamidine, tetrahydrochloride (NSC 57155) on P815 Leukemic Cells

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Summary

The effect of various concentrations of \( N,N'\)-bis\[ p-(N'-methylamidino)phenyl\]terephthalamidine, tetrahydrochloride (NSC 57155) on various incubation periods on P815Y cells in tissue culture and on sensitive P815 and resistant P815/NSC 57155 cells in mice was studied. A 50% growth inhibition in tissue culture after 72 hr was produced by incubation with 300 \( \mu g/ml \) for 15-30 min, 100 \( \mu g/ml \) for 1-2 hr, or 1 \( \mu g/ml \) for 72 hr.

The incorporation of NSC 57155\( ^{14}C \) into cells and its distribution in nuclei and cytoplasm were studied with a 2-hr incubation at a concentration of 100 \( \mu g/ml \). After that period most of the activity was found in the cytoplasm and only very little in the cell nuclei. More than 50% of this activity could be extracted with nonpolar solvents.

Incorporation of NSC 57155\( ^{14}C \) occurred at a slower rate in resistant P815/NSC 57155 cells than in sensitive P815 cells.

When P815 and P815/NSC 57155 cells were bioassayed after in vitro incubation with 100 \( \mu g/ml \) of NSC 57155, the cytostatic effect was found to be time dependent. P815/NSC 57155 cells were somewhat sensitive to these high concentrations of the drug, but a longer incubation period was required than with the sensitive cells.

Introduction

The terephthalamidine derivative, \( N,N'\)-bis\[ p-(N'-methylamidino)phenyl\]terephthalamidine, tetrahydrochloride (NSC 57155) is a member of the group of terephthalic acid derivatives (8) which have shown considerable activity against many experimental leukemias (2, 12, 16, 20, 24). In man, however, several of these compounds have produced undesirable side effects which have been a limiting factor in their clinical trial (13-15, 18).

Although much work has been done concerning the physiologic distribution (4, 11, 21, 23), biologic (13, 14, 16), and biochemical effects (7, 10, 17, 19, 22) of these compounds, no clear-cut picture of the mechanism of action has been found as yet. Most of the information available so far concerns \( 4',4''\)-bis\[ 2-imidazolin-2-yl\]2-chloroterephthalamidine, dihydrochloride (NSC 38280), a typical early member of the terephthalamidine group, whose structure differs from that of NSC 57155 as shown in Chart 1.

Both these compounds show marked antileukemic activity in mice, but there are some differences in the spectrum of leukemias affected and a lack of cross-resistance in some leukemia systems (1, 10), which may indicate a difference in the mechanisms of action of the 2 compounds. This view is supported by the observation that cross-resistance, which exists in some mouse leukemias, between the Vinca rosea alkaloids and NSC 38280 is lacking between the former and NSC 57155 (1).

Effects of NSC 38280 on various biochemical systems have been described (DNA, RNA, and protein metabolism) (7, 10, 17, 19), but the correlation of these with the mechanism of the antileukemic action is not clear. The problem of the mechanisms of action of the whole group becomes complicated, since we probably face a multiple action, where the limit between trivial and significant reactions is hard to define. These changes in different biochemical systems may also be the consequence of one basic action. From this point of view the 1st reaction to occur may be of particular importance. In the study reported below an attempt has been made to establish this by using various incubation periods of P815 leukemic cells with NSC 57155 and by studying its uptake at the 1st critical stage at which activity could be demonstrated. NSC 57155 was chosen for its relatively high solubility, as compared to NSC 38280, and to characterize some of its experimental effects preliminary to clinical trial. Its availability in labeled form was of considerable help.

Materials and Methods

For experiments in tissue culture, P815Y cells (25) (kindly supplied by Dr. Glenn A. Fischer) were grown in Fischer's medium (6) (again, supplied by Dr. Fischer). P815Y cells were derived from P815 leukemic mice and were 1st cultivated in...
Studies on P815 Leukemic Cells

Chart 1. Asterisk indicates the $^{14}$C-labeled C atom.

Studies on P815 Leukemic Cells

When P815 cells freshly harvested from mice are transferred to Fischer's medium, they usually will not grow in vitro, whereas P815Y cells from the mouse will grow in vitro with a high degree of regularity. P815Y cells, retransferred into mice, are less sensitive to NSC 57155 than the original P815 cells grown in vivo by transfer from mouse to mouse. Although P815 and P815Y cells are histologically identical, it has so far not been possible to achieve any in vitro resistance to P815Y cells with any of the terephthalanilide derivatives. A suspension of $5 \times 10^5$ cells/ml of medium was prepared and incubated with NSC 57155 at $37^\circ$C. After incubation the cells were washed 3 times with 5 ml of medium and then distributed into 3-8 screw-cap culture tubes with 5 ml of medium each, so that each tube contained $10^4$ cells/ml. All cell cultures were counted with an electronic cell counter (Coulter counter, model A) for 3 consecutive days, using a constant size threshold in the counter. No other observations, however, were made on cell size. Control cell cultures were treated the same way, using the same amount of distilled water instead of the NSC 57155 solution.

Sensitive P815 (5) and resistant P815/NSC 57155 (3) leukemic cells, inoculated into BDF1 hybrid (C57 x DBA2) mice, were used for the in vivo experiments. Before transplantation the cells were incubated in vitro for a given time with the compound which had been dissolved in Fischer's medium. They were then washed 3 times with 5 ml of medium, and injected i.p., using $10^6$ cells/inoculum. Eight to 10 mice were used in each group. Control cells were similarly treated, but no NSC 57155 was used in the incubation medium. Prolongation of survival time over the control group was evaluated in terms of T/C %.

For uptake studies, NSC 57155-14C with a specific activity of 0.75 mc/mmole was used. Five ml of medium containing $25-30 \times 10^6$ cells/ml were incubated for a given time with 0.0732 /µc of NSC 57155-14C at a concentration of 100 /µg/ml. After incubation, the cells were washed 7 times with 5 ml of medium. No radioactivity could be found in the last 2 washings. The cells were then resuspended in 5 ml of medium and counted. One ml of this suspension was centrifuged, and the sedimented cells were extracted consecutively with 1 ml each of alcohol, ether, and acetone. The washings were collected, and 1 ml was applied to a square filter paper (Schleicher and Schuell, No. 292), dried, and analyzed for radioactivity with a combustion method as described by Kalberer and Rutschmann (9). The remaining cells were hydrolyzed in 2 ml of 0.2 N NaOH at $37^\circ$C for 24 hr; 0.2 ml and 0.4 ml of the hydrolysate were dried on filter paper and combustion analyses made.

A 2nd 1-ml sample of the original cell suspension was centrifuged. All sedimented cells were hydrolyzed in 2 ml of 0.2 N NaOH at $37^\circ$C for 24 hr, and 0.2 ml and 0.4 ml were used for combustions.

The remaining 3 ml of cell suspension were used for isolation of nuclei according to the method of Srinivasan et al. (27). All washing fluids used for the isolation of nuclei were collected, and again 0.2 ml and 0.4 ml were used for combustions. Isolated nuclei were counted and centrifuged. Sedimented nuclei were hydrolyzed in 2 ml of 0.2 N NaOH at $37^\circ$C for 24 hr, and 0.2 ml and 0.4 ml were used for combustions. Radioactivity determinations were performed on a Packard Tri-Carb scintillation counter, model 314 EX-2.

Results

As was reported previously (1), P815Y cells in tissue culture are able to continue to grow for the 1st 24 hr in the presence of high concentrations of other terephthalanilides. We therefore investigated this phenomenon with NSC 57155. Chart 2 indicates the ability of the cells to undergo at least 1 division, even when in continuous contact with 100 /µg/ml of NSC 57155.

Chart 3 shows the growth of P815Y cells in tissue culture when they were in continuous contact with various concentrations of NSC 57155. One /µg/ml produced approximately 50% inhibition after 72 hr. The 2 highest doses used, namely 100 /µg/ml and 300 /µg/ml, were able to completely stop cell growth within 72 hr.

Charts 4 and 5 show the cell growth when 100 /µg/ml and 300

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1 Kindly supplied by Dr. A. Wander AG, Berne, Switzerland, through the Cancer Chemotherapy National Service Center, National Cancer Institute, USPHS.
**TABLE 1**

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>Incubation (hr)</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated/control</td>
<td>Treated/control %</td>
</tr>
<tr>
<td>P815</td>
<td>2</td>
<td>11.3/10.3</td>
</tr>
<tr>
<td>P815/NSC 57155</td>
<td>2</td>
<td>9.8/9.0</td>
</tr>
<tr>
<td>P815</td>
<td>4</td>
<td>17.3/9.0</td>
</tr>
<tr>
<td>P815/NSC 57155</td>
<td>4</td>
<td>9.6/9.1</td>
</tr>
<tr>
<td>P815</td>
<td>7</td>
<td>25.5/10.0</td>
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<tr>
<td>P815/NSC 57155</td>
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<td>10.8/8.9</td>
</tr>
<tr>
<td>P815</td>
<td>24</td>
<td>47.3/10.6</td>
</tr>
<tr>
<td>P815/NSC 57155</td>
<td>24</td>
<td>18.6/10.5</td>
</tr>
</tbody>
</table>

* Experiment calculated at 60 days.

When P815 and P815/NSC 57155 cells from ascitic fluid were incubated in vitro for various periods in 100 µg/ml of NSC 57155 and then injected into mice, a 2-hr incubation was not long enough to produce prolongation of survival time of the mice (Table 1). The 1st significant prolongation could be observed after 4-hr incubation of P815 cells and after 24-hr incubation of P815/NSC 57155 cells with the compound.

Chart 6 shows the uptake of the radioactivity after injection of NSC 57155-¹⁴C in P815 and P815/NSC 57155 cells in vitro following various incubation periods with 100 µg/ml. As can be seen, the uptake in the resistant line was slower and somewhat
lower, even after 24 hr. No attempts were made in these studies to identify the unchanged compound NSC 57155, or any possible complexes or metabolites of the injected compound.

Table 2 gives the uptake data in P815 and P815Y whole cells, the distribution in cytoplasm and nuclei, and the activity extracted with alcohol, ether, and acetone. It is worth pointing out that the amount of NSC 57155 absorbed by P815Y cells after 2-hr incubation with 100 µg/ml is essentially the same as that after 72-hr incubation with 1 µg/ml, and that both values have approximately the same biologic effect on cells in vitro, i.e., approximately 50% growth inhibition.

<table>
<thead>
<tr>
<th>Incubation (hr)</th>
<th>P815 cells</th>
<th>P815Y cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.43</td>
<td>0.82</td>
</tr>
<tr>
<td>2</td>
<td>4.87</td>
<td>1.62</td>
</tr>
<tr>
<td>2</td>
<td>90.0</td>
<td>99.0</td>
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<tr>
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<tr>
<td>2</td>
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<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Discussion

Various incubation times of cells with NSC 57155 showed a distinct dose-time relationship. The higher the dose, the shorter the incubation time required. The same growth inhibition could be achieved when incubating the cells with 300 µg/ml of NSC 57155 for 15–30 min, or with 100 µg/ml for 1–2 hr, or with 1 µg/ml for 72 hr.

The results in Table 1 are of interest in that the resistant P815/NSC 57155 cells also demonstrated some sensitivity to a high concentration of NSC 57155. Longer incubation times than with P815 cells were necessary, however, to make this sensitivity evident.

In Chart 6 the resistant cells are also shown to incorporate a considerable amount of NSC 57155, but again at a slower rate than the sensitive cells.

These data suggest that the resistance seen in the mouse after daily i.p. treatment may be due to the fact that the drug is rapidly excreted in vivo and that therefore the levels of NSC 57155, obtained after daily injections, are too low and last too short a time to be active against the relatively resistant P815/NSC 57155 cells. In normal tissue culture studies with P815Y, however, the cells were exposed to a steady drug concentration over a 72-hr period. This could explain the failure to demonstrate any resistance to the terephthalanilide derivatives in such tissue culture studies (1).

Another possible explanation might be the differences in growth rates of the 2 lines. No information regarding the rates of growth of these 2 lines in vitro during the incubation period is available, but the rate of growth in vivo would seem to be approximately equal, as seen by the controls in Table 2, where the mean survival time with P815 is 9–11 days and that with P815/NSC 57155 is 8.9–10.5 days.

Uptake studies of NSC 57155-14C at 100 µg/ml after 2-hr incubation, and at 1 µg/ml after 72-hr incubation, showed approximately the same level. This finding suggests that in

TABLE 2

<table>
<thead>
<tr>
<th>NSC 57155 (µg/ml)</th>
<th>Incubation (hr)</th>
<th>P815 cells</th>
<th>P815Y cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>2</td>
<td>1.43</td>
<td>0.82</td>
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<tr>
<td>100</td>
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<tr>
<td>1</td>
<td>72</td>
<td>1.75</td>
<td></td>
</tr>
</tbody>
</table>

* The difference in the uptake found in whole cells [2], that separated in cytoplasm and nuclei [7], and the one extracted with alcohol, ether, and acetone [8], lies within the range to be expected from this type of experiment.

* The amount (µg/10⁶ cells) is calculated by dividing the estimated radioactivity by the specific activity of NSC 57155-14C, disregarding the possible metabolic change of the compound. No qualitative analyses were done.
addition to other factors the drug level inside the cell may play an important role. As mentioned above, it is interesting to note that the resistant cells absorbed a considerable amount of the compound as well. The values for NSC 57155 were lower than those found by others (10, 22, 26) for NSC 38280 in L1210 and P388 ascitic cells in vivo.

In distribution studies of the compound within the cell, very low values of radioactivity in the nuclei were found after 2-hr incubation. Considering that approximately 20-25% of microscopically tested, isolated nuclei contain some cytoplasmic contamination, the values would even be lower. However, Kensler (10) has shown by chromatographic techniques that NSC 38280, after 24 hr, is mostly located in the nuclear and mitochondrial fractions. He has also demonstrated the presence of a fluorescent 2-amino-N'-bis(imidazolin) terephthalanilide in the nuclei of kidney and liver cells 24 hr after injection (22). Using a fluorescent derivative, 2-amino-4',4''-bis(4-methylimidazolin) terephthalanilide, in P815 leukemia both in vivo and in vitro (Burchenal, J. H., Lancaster, S. P., and Adams, H. H. Unpublished observations), we have demonstrated that after incubation periods of less than 0.5 hr the fluorescence is mostly in the form of large aggregates in the cytoplasm, whereas after 4 hr it is almost entirely in the nucleus. It is possible that, if our studies had been carried for longer incubation periods, a greater portion of NSC 57155 would have been found in the nuclei. It must also be taken into consideration that a possible weak attachment or binding of the compound to the nuclei could be destroyed during the isolation procedure and the compound washed out. It is of interest that a large percentage of the radioactivity could be extracted from the cells with nonpolar solvents. The formation of drug lipid complexes of NSC 38280 has been described by Yesair et al. (28), and this finding with NSC 57155 also suggests the presence in the cell of a drug lipid complex.

References


25. Schindler, R. S., Day, S. M., and Fischer, G. A. Culture of...


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