CC-1065 (NSC 298223), a Most Potent Antitumor Agent: Kinetics of Inhibition of Growth, DNA Synthesis, and Cell Survival

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ABSTRACT

CC-1065 (NSC 298223) is the most cytotoxic agent tested against cells in culture in our laboratory. The 50% lethal doses for exponentially growing B16 melanoma and Chinese hamster ovary cells were 0.44 and 0.14 ng/ml, respectively, as compared to 35 and 500 ng/ml for Adriamycin. In the human tumor-cloning assay, 1-hr exposure to CC-1065 (0.1 ng/ml) caused ≥50% lethality in a broad spectrum of tumors. The dose-survival curves for B16 and Chinese hamster ovary cells were characterized by an initial shoulder followed by an exponential decline with increasing dose. CC-1065 was more lethal to exponentially growing B16 cells (50% lethal dose = 0.44 ng/ml) than to plateau-phase cells (50% lethal dose = 1.2 ng/ml).

CC-1065 inhibited DNA synthesis much more than did RNA or protein synthesis. After a 2-hr incubation with drug, inhibition of DNA synthesis was low immediately (0 hr) after drug exposure and reached maximum inhibition about 20 hr later. The doses for 50% inhibition of growth (0.18 ng/ml), survival (0.44 ng/ml), and DNA synthesis (0.15 ng/ml) were in the same range, whereas RNA synthesis was inhibited 50% at a much higher dose (5 ng/ml).

INTRODUCTION

CC-1065, a new antibiotic produced by Streptomyces zelenis, Dietz and Li nov. sp., was discovered and characterized at The Upjohn Company, Kalamazoo, Mich. (4). It has significant cytotoxicity in vitro and antitumor activity in vivo (4, 6). It is the most potent agent tested in our laboratories against L1210 cells in culture. It caused 90% inhibition of the growth of L1210 cells at 0.05 ng/ml. This may be compared to the 90% inhibition of the growth by some of the more cytotoxic antitumor agents in this assay system: actinomycin D, 4 ng/ml; and Adriamycin, 20 ng/ml (5). It was also active in vivo against the murine tumors, P388 and L1210 leukemia, B16 melanoma, CD3F, mammary, and Colon 26 at doses ranging from 1 to 50 µg/kg (6, 8). The structure of CC-1065 was reported by Martin et al. (7) and is shown in Chart 1.

Previous studies by Li et al. (5) showed that CC-1065 is much more effective in inhibiting DNA synthesis than in inhibiting RNA or protein synthesis. The inhibition of DNA synthesis is probably mediated through the strong binding of CC-1065 to DNA on the basis of studies of thermal melting temperature of DNA, difference circular dichroism spectra, and Sephadex chromatography (5). The drug binds only to double-stranded DNA and not to heat-denatured DNA (5). The mechanism of interaction of CC-1065 with DNA has been described by Swenson et al. (9).

Inasmuch as CC-1065 was so highly cytotoxic and interacted strongly with DNA, it was assayed against human tumor cells in the cloning assay (3) and against B16 melanoma and CHO cells in culture. This paper compares the lethality of CC-1065 to that of several antitumor drugs and describes the kinetics of growth inhibition, survival, and DNA synthesis in B16 cells. An abstract of this study has appeared elsewhere (1).

MATERIALS AND METHODS

CHO2 Cell Culture. CHO cells were maintained in Ham's F-10 supplemented with 15% fetal calf serum. CHO cells grow exponentially up to a cell density of about 5 x 104/75-sq-cm flask.

B16 Melanoma Cell Culture. The B16 (clone F-10) cell line was obtained from Dr. I. J. Fidler (Frederick Cancer Research Center, Frederick, Md.). The cells were grown as a monolayer in MEM (MEM with Earle's salts) supplemented with 10% fetal calf serum, 1 mm sodium pyruvate, 2 mm L-glutamine, penicillin G (60 µg/ml), and streptomycin (10 µg/ml). This medium was further supplemented with MEM nonessential amino acids (10 ml of 100 x concentration per liter of medium) and essential MEM vitamins (10 ml of 100 x concentration per liter). Medium constituents were purchased from Grand Island Biological Co., Grand Island, N. Y.

Drugs. The drugs used were obtained from the sources indicated. Adriamycin and actinomycin D were obtained from Bristol Laboratories, Syracuse, N. Y. Streptomycin was from Upjohn Company, Kalamazoo, Mich. CC-1065 (NSC 298223) was obtained from the National Cancer Institute, Bethesda, Md.

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1 The abbreviations used are: CHO, Chinese hamster ovary; MEM, minimal essential medium (Eagle's); LD50 and LD90, drug concentration to kill 50% and 90% of the cells, respectively; T-CFU, tumor colony-forming units.
the treated cells was expressed as a percentage of control survival. The coefficient of variation (standard deviation expressed as a percentage of the mean) in determining cell survival was about 15% within each experiment. All experiments were repeated at least once.

For growth inhibition experiments, B16 cell monolayers (about 10^5 cells/75 sq cm) were exposed for different periods to the drug. The cell monolayer was then washed to remove extracellular drug and was incubated for 72 hr. At the end of 72 hr, the cells were harvested and counted with a Coulter Counter. The percentage of growth inhibition was calculated as:

\[
100 \times \frac{\text{Cell no. in treated flask at 72 hr} - \text{cells inoculated}}{\text{Cell No. in control at 72 hr} - \text{cells inoculated}}
\]

**Stability of CC-1065**

CC-1065 was stable when stored as a solution in dimethylformamide in the freezer. The drug (at 0.2 ng/ml) was stable in H_2O in a glass vial for at least 24 hr. It was unstable when stored in a plastic vial under the same condition, probably due to binding to the plastic. In B16 medium containing serum, about 15 to 20% of CC-1065 was lost after 2 hr and 50 to 60% were lost after 24 hr at 37°.

**Inhibition of Growth of B16 Cells by CC-1065**

**Comparison of Glass and Plastic Culture Flasks.** Although CC-1065 binds to plastic, similar growth inhibition was observed when the drug was added to cell monolayers growing in plastic or glass flasks. Therefore, we used plastic flasks in all subsequent experiments. However, glass pipets and glass vials were used to dilute and dispense CC-1065.

**Time Course of Growth Inhibition.** The effect of 72-hr exposure to CC-1065 on the growth of B16 cells is shown in Chart 2. Cells treated with 0.2 ng/ml grew exponentially at a rate slower than the untreated control. Cells exposed to 0.4- ng/ml of drug grew at a much slower rate, and cell growth stopped after 2 cell divisions (i.e., 2 doublings of cell number) subsequent to drug addition. At 0.8 ng/ml, cell growth stopped after 1 cell division.

**RESULTS**

**Determination of Macromolecule Synthesis by B16 Cells.** DNA, RNA, and protein syntheses were monitored by adding 1 ml of 10 μCi [3H]thymidine (2 μg/ml), or 20 μCi [5-3H]uridine (10 μg/ml), or [14C]-leucine (5 μCi/ml) to 9 ml medium covering the cell monolayer of about 10^6 cells/25-sq cm flask. To stop uptake of radioactivity, the radioactive medium was removed, and the cells were washed with 5 ml of cold 0.9% NaCl solution containing the appropriate unlabeled precursor (thymidine, uridine, or leucine) at 100 μg/ml. The cells were then harvested and resuspended in cold medium containing appropriate unlabeled precursor (100 μg/ml).

One aliquot was counted in the Coulter Counter to give the cell number, while 2 other aliquots of cells were filtered through glass microfiber (Whatman GF/C) filters. The filters were washed 4 times with 3 ml cold 10% trichloroacetic acid and once with 3 ml ethanol. The filters were then placed in scintillation vials and heated with 0.5 ml of 0.5 n perchloric acid at 70° for 1 hr. Diethyl (15 ml) was added, and the filters were counted in a scintillation counter.

**Preparation of CC-1065 Solution.** CC-1065 was dissolved in dimethylformamide at 10 to 100 μg/ml and stored in a glass vial in the freezer. CC-1065 is stable under these conditions. Since the drug binds rapidly to plastic, glass pipets and vials were used in preparing dilute solutions. The CC-1065 solution was diluted in medium prior to adding to the cells.

**Human Tumor-cloning Assay.** The human tumor-cloning assay was done according to procedures described in detail previously (3, 12). In brief, tumor cell suspensions were adjusted to a final concentration of 10^5 cells/ml and then incubated at 37° with either CC-1065 (0.1 ng/ml) or the appropriate vehicle for 1 hr. The cells were then washed and resuspended in enriched Connaught Medical Research Laboratories Medium 1066 supplemented with 15% horse serum and 0.3% agar to yield a final concentration of 5 x 10^5 cells/ml. One ml of this mixture was pipetted on top of 1 ml of enriched McCoy’s Medium 5A solidified with 0.5% agar. Conditioned medium was not added. Cultures were incubated at 37° in 7% CO_2 in humidified air. All assays were set up in triplicate.

Colonies (≥50 cells) usually appeared in 10 to 15 days. The number of colonies on control and drug-treated plates was counted on an inverted-stage microscope at 30-fold magnification. A minimum of 30 colonies/plate were required for an experiment to be considered adequate for measurement of drug effect. Colony counts of the 3 plates for a particular drug concentration were averaged to obtain one data point. The standard error of the mean for individual data points averaged 14% of the mean.

The results are presented as a percentage of decrease in T-CFU in the drug-treated samples as compared to the control. A decrease of ≥70% in T-CFU was used because, in a prior retrospective study, it was satisfactory for predicting which patient would respond to a particular chemotherapeutic agent (11).
Effect of Contact Time on Growth Inhibition by CC-1065.
The growth inhibition obtained at different doses, with exposure times ranging from 10 min to 72 hr, is shown in Chart 3. The results are shown below.

CC-1065 acts very rapidly and irreversibly at high doses of the drug. Thus, only 10 min exposure to CC-1065 (5 and 1 ng/ml) resulted in inhibition of growth during subsequent 72 hr incubation. However, the action of the drug could be reversed completely in cells exposed to 0.5 ng/ml for 10 min.

The extent of growth inhibition was dose and exposure time dependent. Growth inhibition reached a plateau after a 2-hr exposure. The dose for 50% growth inhibition (Table 1) ranged from 1.8 ng/ml for 10 min exposure to 0.22 ng/ml for 72 hr exposure. The concentration x time value was similar for exposures ranging from 10 min to 2 hr (see Table 1), which indicates that up to 2-hr growth inhibition was proportional to dose and exposure time. Since there was no further increase in growth inhibition after 2 hr, there was a sharp increase in the concentration x time value for 72 hr as compared to 2 hr.

Complete degradation of CC-1065 by the end of a 2-hr incubation would account for the absence of any further increase in growth inhibition after 2 hr. Only 20% of CC-1065 was lost when the drug was incubated with medium alone for 24 hr at 37°C. However when CC-1065 was incubated with a monolayer culture of B16 cells, 90% of the CC-1065 was removed from the supernatant medium (Table 2). This suggests that CC-1065 was either bound to the cells or was degraded by the cells during the 2-hr incubation. Since there was no CC-1065 in the supernatant medium after 2 hr, there was no further increase in growth inhibition after a 2-hr exposure.

Growth Inhibition in Medium with and without Fetal Calf Serum. Because CC-1065 binds to a variety of substances, its effect on cell growth was examined in serum-free medium. The results (Table 3) are shown below.

CC-1065 was 10 to 20 times more cytotoxic in the absence of serum than in its presence. Thus, after 10 min exposure, 5 ng/ml inhibited growth 85% in the serum-containing medium compared to 91.7% inhibition by 0.5 ng/ml in the serum-free medium.

In the absence of serum, even very low levels (0.25 ng/ml) caused irreversible effects in 10 min.

### Chart 3

Inhibition of growth of B16 cells resulting from varying periods of drug exposure. B16 cell monolayers were exposed to CC-1065 for periods ranging from 10 min to 2 hr. After drug exposure, the cell monolayer was gently washed, fed with fresh medium, and incubated for 70 hr. The growth of the drug-treated samples was expressed as a percentage of the growth in the untreated (washed) control. For the 72-hr time point, cells were continuously exposed to CC-1065 for 72 hr. This experiment was repeated once and gave similar results.

### Table 1

<table>
<thead>
<tr>
<th>Exposure time (hr)</th>
<th>ID₅₀ (ng/ml)</th>
<th>C x t</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.166</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>0.5</td>
<td>0.91</td>
<td>0.46</td>
</tr>
<tr>
<td>1</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>72</td>
<td>0.22</td>
<td>15.8</td>
</tr>
</tbody>
</table>

* ID₅₀: concentration to inhibit growth by 50%. These values were calculated from the data shown in Chart 3. C x t: ID₅₀ x time of exposure.

### Table 2

Growth inhibition by CC-1065 after incubation with medium alone or medium plus B16 cells

<table>
<thead>
<tr>
<th>CC-1065 (ng/ml)</th>
<th>% of control growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>Medium + cells</td>
</tr>
<tr>
<td>0.06</td>
<td>9</td>
</tr>
<tr>
<td>0.125</td>
<td>3</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>0.6</td>
<td>9</td>
</tr>
</tbody>
</table>

* Medium alone or medium plus (1.75 x 10⁶) B16 cells was incubated with different levels of CC-1065 for 2 hr at 37°C. The supernatant medium was then removed, and its ability to inhibit growth of a B16 culture was measured. CC-1065 (0.6 ng/ml) incubated with medium plus cells gave the same growth inhibition as CC-1065 (0.06 ng/ml) in medium alone. Therefore, 90% of the CC-1065 was removed from the supernatant medium during a 2-hr incubation with medium plus cells at 37°C.

### Table 3

Growth inhibition after drug exposure in medium with and without serum

<table>
<thead>
<tr>
<th>CC-1065 (ng/ml)</th>
<th>10 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>No serum</td>
<td>Serum</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
<td>99.3</td>
</tr>
<tr>
<td>0.5</td>
<td>2.2</td>
<td>91.7</td>
</tr>
<tr>
<td>0.25</td>
<td>4.5</td>
<td>74</td>
</tr>
</tbody>
</table>

### Cell Kill by CC-1065

Lethality for Human Tumor Cells. The lethality of CC-1065 (1-hr exposure to 0.1 ng/ml) for a number of human tumors was determined by the human tumor-cloning assay; the results are shown in Table 4. A decrease of ≥70% in the number of T-CFU has been assumed to be necessary for this assay to predict for results in the clinic (11). On this basis (≥70% decrease in T-CFU, CC-1065 was active against tumors from patients with cancer of the lung (1 of 9 patients tested), pancreas (1 of 2 patients tested), stomach (1 of 1 patient tested), small bowel (1 of 1 patient tested), and adenocarcinoma of unknown origin (1 of 2 patients tested). For many more tumors, the percentage of decrease in T-CFU was ≥50%. On the basis of ≥50% decrease in T-CFU, CC-1065 was active against tumors from patients with cancer of the breast (3 of 3 patients), lung (3 of 9 patients), ovary (2 of 8 patients), pancreas (1 of 2 patients), multiple myeloma (2 of 2 patients), stomach (1 of 1 patient), testis (1 of 1 patient), small bowel (1 of 1 patient), and adenocarcinoma of unknown origins (1 of 2 patients). CC-1065 was inactive (<50% decrease in T-CFU) against melanoma (3 patients tested) and breast cancer (1 of 2 patients tested).
samples tested), acute myelogenous leukemia (2 samples tested), esophagus (1 sample tested), non-Hodgkin’s lymphoma (1 sample tested), colon (3 samples tested), and sarcoma (2 samples tested).

**Lethality for B16 and CHO Cells.** The dose-survival curves for B16 and CHO cells are shown in Chart 4. With both cell lines a sigmoid-survival curve was obtained which was characterized by an initial shoulder followed by an exponential (log-linear) decline in survival as the dose increased. These curves were used to determine the \( D_0 \) (1/slope) of the exponential portion of the curve and the LD\(_{50}\) and LD\(_{90}\) values (Table 5). Plateau B16 cells were much less sensitive than were exponentially growing cells.

Table 5 compares the lethality of CC-1065 to several widely used antitumor drugs. The results clearly show that CC-1065 is 50 to \( \geq 1000\) times more cytotoxic than are most other drugs.

B16 cell-kill increased only slightly when exposure to CC-1065 was increased from 2 to 24 hr. These results corroborate the time course of growth inhibition and suggest that CC-1065 action was completed during a 2-hr exposure.

**Inhibition of Macromolecule Synthesis in B16 Cells**

The effect of CC-1065 on DNA, RNA, and protein syntheses was determined immediately after a 2-hr exposure to the drug. In these experiments, labeled precursors were added simultaneously with the drug and the cells were harvested 2 hr later to determine radioactivity incorporation into DNA, RNA, and protein. DNA synthesis was inhibited 19.5 \( \pm \) 5.3% (S.D.), 32.3 \( \pm \) 5.4%, and 65.7 \( \pm \) 4.7% at 0.5, 1, and 5 ng/ml, respectively. RNA and protein synthesis were not inhibited even at 5 ng/ml. Similar results have been reported by Li et al. (5) using L1210 cells.

The dose needed for inhibition of DNA synthesis was much greater than the doses needed for inhibition of cell growth and survival. Thus, the doses required for 50% inhibition of growth,
The results of several experiments to measure DNA inhibitions are shown by uridine, ["C]leucine, and ["H]thymidine were added at 18 hr post-drug exposure. Chart 6) shows that, at 18 hr postexposure to 1 ng/ml, DNA synthesis was inhibited 80% with no inhibition of RNA, or protein synthesis. Significant inhibition (53%) of RNA synthesis was seen only at 5 ng/ml. At 18-hr postdrug exposure, the doses for 50% inhibition of growth (0.25 ng/ml), survival (0.44 ng/ml), and DNA synthesis (0.15 ng/ml) were similar, whereas about a 10-fold higher dose (5 ng/ml) was needed for inhibition of RNA synthesis.

DISCUSSION

Comparative Cytotoxicity of CC-1065 and Adriamycin. Several factors, including lethality to cells in culture and in the human tumor-cloning assay, influence the selection of cancer chemotherapeutic drugs for clinical trial. Adriamycin is being compared to CC-1065, since it is a cytotoxic antitumor agent that interacts with DNA and is active against a broad spectrum of tumors in humans. CC-1065 (LD50 = 0.6 nm) was 100 times more cytotoxic than was Adriamycin (LD50 = 64 nm) to B16 cells. Greater cytotoxicity of CC-1065 as compared to Adriamycin was also seen with CHO and L1210 cells (1, 5).

CC-1065 can also be considered to be at least 100 times more potent than Adriamycin in the human tumor-cloning assay, in which CC-1065 was used at 0.1 ng/ml and Adriamycin was used at 40 to 400 ng/ml. Many of the human tumors (Table 4) were tested simultaneously for their sensitivity to CC-1065 and Adriamycin. For 6 lung tumor samples, the percentages of survival after exposure to CC-1065 and Adriamycin, respectively, were as follows: 33, 90; 18, 56; 46, 73; 61, 100; 97, 77; and 81, 59. These results show that 4 of the 6 tumor samples were more sensitive to CC-1065 than the Adriamycin. CC-1065 (34 and 50% survival) and Adriamycin (40 and 60% survival) were equally lethal to 2 of the breast tumor samples. For 4 ovarian tumor samples, the percentages of survival after exposure to CC-1065 and Adriamycin, respectively, were as follows: 31, 100; 60, 100; 63, 54; and 100, 75. Therefore, CC-1065 was significantly more active than Adriamycin against at least one ovarian tumor. These results also show that tumors that were resistant to Adriamycin were sensitive to CC-1065; i.e., the drugs were not cross-resistant.

Kinetics of CC-1065 Cytotoxicity. Both growth inhibition and cell kill studies showed that maximum effect was obtained after a 2-hr exposure to B16 cells to CC-1065. There was no further increase in growth inhibition or cell kill for drug exposure periods greater than 2 hr. This cessation of CC-1065 effect was not due to degradation of the drug, since only 15 to 20% of the cytotoxicity of CC-1065 was lost after 2 hr of incubation in medium at 37°. The reason for this limitation of CC-1065 activity is probably due to the removal of the drug from the medium due to drug binding to the cells.

These results in vitro correlate well with in vivo experiments in which the percentage of increase of life span of leukemic (P388 or L1210) mice was the same irrespective of whether the mice were given a single dose or several doses of the drug.

Kinetics of Inhibition of DNA Synthesis. The primary site of CC-1065 action is presumed to be its interaction with DNA based on the following evidence. (a) There was marked inhibition of DNA synthesis with minimal inhibition of RNA and no inhibition of protein synthesis (Chart 6; Ref. 5). (b) The dose for 50% inhibition of DNA synthesis, growth, and cell survival were similar and ranged between 0.15 and 0.45 ng/ml. The 50% inhibitory dose for RNA synthesis was about 10-fold higher. Preferential inhibition of DNA synthesis as compared to RNA or protein synthesis was also seen in L1210 cells (5).
Further studies by Swenson et al. (9) have indicated that CC-1065 does not intercalate with DNA but binds in the minor groove of double-stranded DNA at A-T-rich sites. Therefore, the inhibition of DNA synthesis probably occurs as a result of the binding of CC-1065 to the DNA template.

The percentage of inhibition of DNA synthesis in cells exposed to 1 ng/ml was minimum at 4-hr postdrug exposure (Chart 5). Preliminary experiments indicated that this decrease was an artifact of the method of expressing DNA synthesis rate as cpm/cell. Cell progression studies showed that CC-1065 caused cells to accumulate in S (1). Therefore, the percentage of cells in S at 4 hr postdrug was higher than that in the control. To take into account the varying percentages of S-phase cells, the rate of DNA synthesis was recalculated as cpm/S-phase cell. When this correction was made, the decrease in percentage of inhibition of DNA synthesis at 4-hr postdrug exposure was not observed, rather the percentage of inhibition gradually increased to reach a maximum at 19 hr.

Maximum inhibition of DNA synthesis was seen at 18 to 20 hr after termination of drug exposure. Such an effect could be caused by extensive cell death at this time, resulting in generalized loss of activity of enzymes involved in macromolecule synthesis. However, RNA and protein synthesis continued at this time, indicating that a generalized loss of metabolic activity had not occurred. Therefore, maximum inhibition of DNA synthesis at 18 to 20 hr after drug exposure suggests delayed expression of drug toxicity. Delayed expression of drug toxicity was also seen in vivo as delayed deaths of drug-treated animals (8).

REFERENCES

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