CI-973, a New Platinum Derivative with Potential Antileukemic Activity

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ABSTRACT

We examined the effects of CI-973 (supplied by Parke-Davis) on several human leukemia cell lines and a Chinese hamster ovary (CHO) line and their drug-resistant counterparts. The cell lines used were HL-60, HL-60/mAMSA, HL-60/DOX, KBM3, KBM3/mAMSA 6, KBM3/mAMSA 6(85), CHO, and CHO/AC-7. DOX, mAMSA, and AC-7 indicate resistance to doxorubicin, amsacrine, or 1-β-D-arabinofuranosycytosine, respectively. Cells were incubated with CI-973, and the effect was evaluated by two methods: growth inhibition assay and inhibition of colony formation.

All cell lines examined were inhibited by CI-973; two of three amsacrine-resistant lines and the one cytarabine-resistant line demonstrated collateral sensitivity. At equivalent dosages, a 4-day exposure provided much greater cell kill than a 1-h exposure. Clonogenic assay showed exponential killing over 3 log units. Maximum CI-973 levels required to kill 50% of cells were 10-fold lower than the peak plasma levels achieved in a phase I solid tumor study. A continuous infusion phase I study in acute leukemia has been initiated.

INTRODUCTION

In adult acute leukemia, the development of resistance in leukemic cells is the ultimate reason for treatment failure in the majority of patients. Exposure of leukemic cells to one drug often results in the development of resistance to that drug but also leads to cross-resistance to other structurally unrelated drugs. With a limited number of active drugs, the search for new ones, particularly drugs lacking cross-resistance to those presently used in the treatment of leukemia, is of primary importance. Platinum and platinum analogues are a group of antitumor agents with substantial activity in solid tumors (1, 2). Investigations using these drugs as antileukemic agents have been limited (3, 4).

CI-973 (Parke-Davis) is a platinum diamine complex that exhibits antitumor activity in vitro. CI-973 has been examined in 37 model tumor systems, including both cisplatin-sensitive and -resistant cell lines. Overall, CI-973 was superior or equivalent to cisplatin in 24 of the 37 systems (5). In each of the seven cisplatin-resistant murine lines tested, CI-973 demonstrated activity superior to that of cisplatin or carboplatin (5–7). In animal studies, dose-limiting toxicity involves mainly the gastrointestinal tract, although in repeated-dose studies with lower doses hematopoietic effects were dose limiting. Unlike cisplatin, and to a lesser extent carboplatin, CI-973 was not associated with nephrotoxicity or ototoxicity.

In two recent phase I studies of CI-973 given as a short infusion on day 1 or daily for 5 days every 3–4 weeks in patients with solid tumors, the dose–limiting toxicity was granulocytopenia, with minimal or no associated thrombocytopenia (8, 9). This suggested the potential usefulness of CI-973 as an antileukemic agent. Prior to initiating a phase I trial of CI-973 in acute leukemia, we were interested in the activity of this drug in human leukemia-derived cell lines, particularly in those displaying resistant phenotypes to drugs used in the first-line treatment of acute leukemia, including those with an MDR phenotype (10) and with resistance associated with a defect in topoisomerase II function. The results of a study with these cell lines and a CHO cell line resistant to ara-C are the subject of this report.

MATERIALS AND METHODS

Cell Lines

Two human myeloid leukemia cell lines, HL-60 and KBM3, and a CHO cell line, and their resistant counterparts, were used in the study. The HL-60 cell line was originally developed from acute myelogenous leukemia, and the cell phenotype is reminiscent of the promyelocytic variant, in spite of the lack of t(15;17) (11). KBM3 is a cell line developed in our laboratory from a patient with acute monocytic leukemia; the blast cells continue to maintain a monocytic phenotype (12).

The leukemic cell lines were maintained in Iscove's modification of Dulbecco's medium supplemented with 10% FCS (Hazelton, Lanexa, KS) and L-glutamine. They were passaged twice weekly, at the initial seeding density of 3 × 10⁶ cells/ml. The cells were continuously monitored for Mycoplasma contamination and were Mycoplasma free when used.

Induction of Resistance

mAMSA resistance was induced by a repeated 60-min exposure to increasing concentrations of the drug, starting with the dose eliciting 50% cell kill (IC₅₀). According to the tolerance of cells, the dose was kept the same, decreased, or increased, as described for HL-60 (13). The KBM3 cells were rendered resistant to mAMSA, using an identical protocol, 1 year after development of the HL-60/mAMSA cell line. The phenotype and drug resistance pattern of HL-60/mAMSA cells are described in detail elsewhere (14). The mAMSA resistance index was between 70 and 100. In the present experiments, we used two sublines of KBM3 mAMSA, differing from each other by the number of passages after development of resistance but showing the same degree of mAMSA resistance.

The characteristic feature of these cell lines was a complete absence of MDR phenotype. There was normal uptake, retention, and outward transport of mAMSA, doxorubicin, and VP-16 and abnormal topo-I-mediated response to mAMSA, indicative of a resistant form of the enzyme (14–17). All mAMSA-resistant lines were characterized by a stable resistant phenotype when growing in the absence of drug for >200 doubling times.

Resistance to doxorubicin was induced in HL-60 cells in suspension cultures by continuous exposure to increasing concentrations of the drug over a period of 1 year. Cell samples were frozen in liquid nitrogen at defined levels of resistance and were thawed shortly before use. The cells were then grown in the absence of doxorubicin and showed a stable multidrug-resistant phenotype characterized by overexpression of the
The mdr-1 gene without gene amplification, as determined by Northern and Southern blots, respectively. Using a modified technique of suspension hybridization/RNase protection assay with an RNA antisense probe (18), it was calculated that the cells expressed approximately 55 copies of the mdr-1 message. The complete description and characterization of the HL60/DOX cell lines will be reported elsewhere.

The CHO cells resistant to ara-C (CHO/AC7), as well as their sensitive parent cells, were kindly provided by Dr. P Saunders (M. D. Anderson, Houston, TX). The resistant cells were deficient in deoxythymidine kinase (19). They had an ara-C resistance index of 100 and displayed cross-resistance to 2-chlorodeoxyadenosine (10-fold) and fludarabine (30-fold). They were grown as adherent cells in McCoy’s medium supplemented with 15% FCS.

**Drug Supply**

CI-973 was provided by Parke-Davis. The drug was dissolved to a stock concentration of 10^6 μM and stored until use at 4°C. CI-973 was tested at concentrations of 0.1 to 100 μM.

**Cytotoxicity Assays**

To evaluate the effect of CI-973 we used two assays: (a) growth inhibition assay and (b) inhibition of colony formation.

**Growth Inhibition Assay with Leukemic Cells.** Exponentially growing cells were resuspended in fresh medium at 3 x 10^5/ml and were distributed to each well of a 24-well culture plate. CI-973 (10 μl) was added to triplicate wells to obtain the desired concentration. No drug was added to control wells. After incubation for 4 days at 37°C in a humidified atmosphere of 5% CO2 in air, cells were carefully pipetted to obtain a single-cell suspension and were counted twice for each well. Cell counts, obtained using a Coulter counter, were compared with those of control cultures.

In two experiments with HL-60, the cells were exposed to CI-973 for 1 h, washed, reseeded in drug-free medium, and cultured for 3 days before counting.

**Colony Inhibition Assay.** In the colony inhibition assay, the effect of CI-973 on clonogenic leukemic cells was studied in semisolid agar. Cells were suspended in Iscove’s modification of Dulbecco’s medium supplemented with 15% FCS, CI-973, and 0.35% agar (Bacto-agar; Difco), in a 35-mm Petri dish. Cells were plated at 1 x 10^4/ml for CI-973 concentrations of 0-4 μM and at 1 x 10^4/ml for concentrations of 5-100 μM. Triplicate dishes were prepared for each drug concentration. After incubation for 7 days in a fully humidified atmosphere of 5% CO2 in air at 37°C, clones of >10 cells were scored with an inverted microscope.

In assays with CHO cells, nearly confluent cells were trypsinized, washed, and resuspended to a final concentration of 250 (CHO/AC7) or 500 (CHO) cells/ml, in McCoy's medium supplemented with 15% FCS. Drug was again added at various concentrations, and a 1-ml cell suspension was added to 35-mm Petri dishes. Triplicate cultures were prepared for each drug concentration. After 4 days of incubation, the medium was decanted and plates were stained with Wright stain. Adherent clones of >10 cells were scored with an inverted microscope.

**Statistical Evaluation**

The surviving fraction of cells or clones was determined by dividing the cell or colony numbers in drug-exposed cultures by values obtained in control cultures. Survival curves were constructed and the IC50 was computed from the curves.

The RI was then calculated as RI = mean IC50 of resistant cell line/mean IC50 of parent (sensitive) cell line. To stabilize the variance, a logarithmic transformation of the data was employed. A two-way analysis of variance was used to compare sensitivity curves.

**RESULTS**

**Exposure Time to CI-973.** Using the same concentrations of CI-973, 1 h of exposure of CI-973 was less inhibitory to HL-60 cells than the 4-day exposure. A representative experiment is shown in Fig. 1. Less than 10% (Fig. 1) killing was seen after 1 h with concentrations ranging from 0.1 to 50 μM. After 1 h at the highest concentration (100 μM), 70% of HL-60 cells were still alive. In contrast, >90% inhibition was observed after a 4-day exposure of cells to concentrations of 5 μM or higher. Based on these results, continuous exposure was used to study CI-973 effects.

**Growth Inhibition Assay.** A continuous 4-day exposure to CI-973 in suspension culture (Fig. 2) resulted in a dose-dependent inhibition of cell growth in the dose range between 0.1 and 10 μM (Fig. 2). The HL-60 cells resistant to doxorubicin (HL-60/DOX) showed a dose-response curve similar to that of parental cells, indicating an absence of cross-resistance. Similarly, HL-60/mAMSA cells lacked cross-resistance and appeared to be more sensitive to CI-973 than the parental cells, with a lower IC50 (Fig. 2A; Table 1). These results were confirmed for mAMSA-resistant cells with the independently derived series of KBM3 cell lines (Table 1). As shown in Fig. 2B, the dose-response curves of sensitive parent cells and mAMSA-resistant derivatives were of the same order of magnitude. Statistical evaluations of repeated experiments revealed that one resistant subline [KB3/mAMSA 6(85)] was significantly more sensitive to CI-973, while the other (earlier passage) showed a slightly, but significantly, lower sensitivity (P < 0.001) (Fig. 2B; Table 1).

**Clonogenic Assay.** Since the malignant population is perpetuated by clonogenic cells, we investigated the sensitivity of these cells in standard clonogenic assay, using agar-medium for leukemic cells and medium alone for adherent CHO cells. For leukemic cells, the results of these studies confirmed those obtained with the growth inhibition assay. The killing of clonogenic leukemic cells with the MDR (HL 60/DOX), as well as the mAMSA-resistant, phenotype is an exponential function of the dose of CI-973, over a wide range of drug concentrations (0.1-100 μM) (Fig. 3). The exponential nature of the cell kill over 4 log units (Fig. 3) excludes the possibility of a small drug-resistant population of clonogenic cells, not detected by the less sensitive growth inhibition assay. As shown in Fig. 3, a >3-log reduction was obtained at 50 μM drug, in both cell lines.

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4. M. Beran, unpublished observations.

**Fig. 1.** Survival of HL-60 cells after exposure to CI-973 for 1 h (---) or 4 days (—).
ANTILEUKEMIC ACTIVITY OF CI-973

Table 1  Sensitivity of leukemic cell lines grown in the presence of CI-973

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of experiments</th>
<th>IC_{50} range (µM)</th>
<th>RI</th>
<th>RI confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>6</td>
<td>0.95–2.60</td>
<td>1.0</td>
<td>0.65–1.54</td>
</tr>
<tr>
<td>HL-60/mAMSA</td>
<td>3</td>
<td>0.50–0.80</td>
<td>0.43</td>
<td>0.29–0.72</td>
</tr>
<tr>
<td>HL-60/DOX</td>
<td>4</td>
<td>1.4–1.9</td>
<td>1.07</td>
<td>0.70–1.60</td>
</tr>
<tr>
<td>KBM3</td>
<td>2</td>
<td>0.65–0.70</td>
<td>1.0</td>
<td>0.80–1.25</td>
</tr>
<tr>
<td>KBM3/mAMSA 6</td>
<td>3</td>
<td>0.85–1.20</td>
<td>1.47</td>
<td>1.06–2.09</td>
</tr>
<tr>
<td>KBM3/mAMSA 6(85)</td>
<td>3</td>
<td>0.20–0.24</td>
<td>0.32</td>
<td>0.26–0.40</td>
</tr>
<tr>
<td>CHO</td>
<td>2</td>
<td>5.2–5.8</td>
<td>1.0</td>
<td>0.69–1.45</td>
</tr>
<tr>
<td>CHO/AC-7</td>
<td>2</td>
<td>1.4–1.6</td>
<td>0.27</td>
<td>0.18–0.41</td>
</tr>
</tbody>
</table>

The sensitivity of Chinese hamster ovary cells sensitive (CHO) and resistant (CHO/AC-7) to a primary antileukemic drug, ara-C, was also investigated using the clonogenic system. The results shown in Fig. 4 and Table 1 demonstrate differences in the CI-973 sensitivity of these lines, with CHO/AC-7 being significantly more sensitive than parental cells (P < 0.001).

To further compare the sensitivity of different cell lines, concentrations of CI-973 that produce 50% growth inhibition or clonogenic cell killing (IC_{50}) were calculated, and comparisons were made between sensitive cell lines and their derivatives with primary resistance to mAMSA, doxorubicin, or ara-C (Table 1). Two of the three mAMSA-resistant cell lines had an average RI below 0.5, an observation compatible with collateral sensitivity, while one subline had an average RI of 1.5. Collateral sensitivity to CI-973 was also seen in the ara-C-resistant CHO.
cells, while leukemic cells with the MDR phenotype had an RI magnitude, ranging from 0.20 to 2.60 μM.

Overall, the cure rates of 15-25% in patients with AML (20). Intensified therapy using dose modulation (high dose ara-C) or maintenance modifications (autologous bone marrow transplants) has not increased the proportion of cured patients (21). One explanation for the lack of progress in AML therapy is that all active agents have belonged to two classes: (a) nucleoside analogues (ara-C) or (b) topoisomerase II-interactive agents. Discovering new anti-AML agents with different mechanisms of action and lack of cross-resistance may enhance the results of therapy.

The introduction of cisplatin to combination therapy for testicular cancer has markedly increased the cure rate in this disease (2). Use of cisplatin in AML therapy is limited by the ototoxicity and renal toxicity seen with higher doses of this compound. Carboplatin, an analogue of cisplatin that produces less renal toxicity and ototoxicity, has shown some success when used in AML (3, 4). However, thrombocytopenia is a major problem with this agent.

CI-973 has recently been explored in phase I studies in patients with solid tumors. Granulocytopenia was the dose-limiting toxicity and, importantly, it was not associated with significant thrombocytopenia. This led to our interest in exploring this drug in acute leukemia studies. The availability in our laboratory of several leukemic cell lines and their resistant counterparts provided an opportunity to investigate the in vitro antileukemic activity of CI-973, as well as potential cross-resistance. We also wished to evaluate its efficacy in cells resistant to ara-C, since ara-C is the most active and most commonly used drug in AML therapy. Because of the lack of leukemic cell lines resistant to ara-C, we used CHO cells.

The first observation was that CI-973 was inhibitory to all cell lines and the effect was a function of the drug concentration and time of exposure. Importantly, two of the three mAMSA-resistant cell lines and the ara-C-resistant CHO cell line demonstrated collateral sensitivity to CI-973; the HL-60/DOX cell line with the MDR phenotype (and extensive and high levels of cross-resistance to numerous drugs) did not differ in sensitivity from the parent line. Thus, resistance to commonly used agents in AML therapy did not confer resistance to CI-973, suggesting the possibility of viable non-cross-resistant AML therapy.

Collateral sensitivity is a known phenomenon that occurs in the form of a moderately increased sensitivity to one drug in cells rendered resistant to unrelated drug(s). Our mAMSA-resistant cell lines show collateral sensitivity to ara-C, which cannot be explained by a change in the intracellular processing of the active metabolite, ara-C triphosphate; ara-C triphosphate is accumulated to the same level in both mAMSA-sensitive and -resistant cells. Similarly, the reason for the increased sensitivity to CI-973 in cells deficient in deoxycytidine kinase is not known; it could be due to differences in cellular uptake, distribution, or metabolism of CI-973, rather than to changes in the interaction of CI-973 with DNA. Recently, it was found that in mAMSA-resistant cells the resistant phenotype is characterized by a change in the expression of a Mr 76,000 cellular protein, which is related to the family of stress/heat shock proteins. Decreased expression of such proteins may have an impact on numerous aspects of cellular metabolism, including the cellular response to DNA-targeting drugs such as CI-973.

The regimens explored in the phase I solid tumor study were short term infusions delivered every 4 weeks. Our in vitro studies comparing a 1-h with a 4-7-day exposure of CI-973 in HL-60 cells suggest that, at equivalent dosages, a prolonged exposure to CI-973 results in greater leukemic cell kill. Finally, our observations that the IC50 of CI-973 ranged from 0.20 to 5.8 μM in different cell lines and that no resistant subpopulation was seen, with exponential killing over a 4-log unit range, may be relevant for clinical studies.

In the phase I study at the tolerable higher doses, peak plasma levels of CI-973 of up to 60 μM have been obtained.6 This suggests that continuous infusion of CI-973 at the higher doses (which would be proposed in the leukemia studies) would be able to produce plasma levels equivalent to those required for in vitro killing of leukemic cells. Based on these encouraging findings with CI-973 in vitro, a phase I study of continuous infusion of CI-973 in acute leukemia has been initiated.

REFERENCES


5. Lee and M. Beran, unpublished observations.
6. R. Boyd, personal communication.


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