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Studies on the biochemical mechanism of the novel antitumor agent, CI-920

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Summary. *Biochemical studies on a new antitumor antibiotic, CI-920, have been directed toward understanding its mode of action. The most striking effect brought on by CI-920 was a marked inhibition of macromolecular synthesis. L1210 leukemia cells exposed to 10 μM CI-920 exhibited a decreased rate of DNA, RNA, and protein synthesis within 45 min, and maximal inhibition occurred within 60 min. The reduction in nucleic acid synthesis was not due to precursor depletion, since ribonucleoside and deoxyribonucleoside triphosphate levels in cells exposed to 10 μM CI-920 for 2 h either remained unchanged relative to control cells or were elevated, suggesting a block more directly at the level of nucleotide incorporation. Nevertheless, CI-920 (50 μM) had no effect on DNA or RNA polymerase activity as assessed in permeabilized L1210 cells. However, if viable cells were exposed to 20 μM CI-920 for 1 h prior to permeabilization and then the polymerases assayed in the absence of drug, there was a 60% depression in enzyme activity. The inhibition of RNA polymerase appears to result from an effect on the enzyme rather than the template, since inhibition of RNA polymerase activity in cell-free systems from drug-treated cells could not be restored by addition of excess DNA template. DNA polymerase, however, was at least partially restored by addition of template and therefore was inconclusive in this respect. The data, then, suggest that CI-920 inhibits nucleic acid synthesis directly at the level of nucleotide incorporation, either by direct inhibition of DNA or RNA polymerase or by inactivation of an essential component of these enzyme systems. Since the drug in its parent form did not inhibit nucleic acid synthesis in cell-free systems the effects may possibly be mediated through conversion of this agent to another chemical form within viable cells.*

Introduction

A new antitumor antibiotic, CI-920, has been isolated [10] and structurally characterized [3] from a species of Streptomyces [11]. This agent effectively inhibits the growth of a wide spectrum of tumor models in vitro and exhibits strong antitumor activity versus L1210 and P388 in vivo [5,6]. The novel structure of this agent (Fig. 1) has generated considerable interest in its mode of action, mainly in anticipation of identifying a unique target site [2]. In these studies we have discerned several key biochemical effects of CI-920 and have established that this agent appears to act specifically at the level of macromolecular synthesis.

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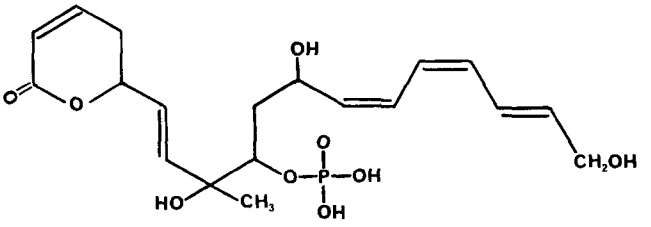

CI-920

Fig. 1. Chemical structure of CI-920, 2*H*-pyran-2-one, 5-6-dihydro-6-[3,6,13-trihydroxy-3-methyl-4-(phosphonooxy)-1,7,9,11-tridecatetraenyl]-, sodium salt

Materials and methods

Chemicals. Purine and pyrimidine nucleotides, native calf thymus DNA, and poly-(deoxyadenylate-deoxythymidylate) were purchased from Sigma Chemical Company, St. Louis, Mo. DNA polymerase (*Micrococcus luteus*) was obtained from Miles Laboratories, Elkhart, Ind. *E. coli* DNA and RNA polymerases and activated DNA (calf thymus) were purchased from PL Biochemicals, Incorporated, Milwaukee, Wis. [Methyl-^{3}H]thymidine, [5-^{3}H]uridine, L-[4,5-^{3}H]leucine, deoxy-[8-^{3}H]adenosine 5′-triphosphate, [methyl-^{3}H]thymidine 5′-triphosphate, and [5-^{3}H]uridine 5′-triphosphate were purchased from Amersham, Arlington Heights, Ill.

Cell culture. All experiments were performed in L1210 mouse leukemia cells grown as a suspension culture in RPMI 1640 supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin.

Incorporation of radioactive precursors into macromolecules. Incorporation of radiolabeled precursors into DNA, RNA, and protein was monitored by exposing logarithmically growing cells to [methyl-^{3}H]thymidine, [5-^{3}H]uridine, or L-[4,5-^{3}H]leucine, respectively, at a concentration of 1 μm and a specific activity of 1 μCi per nmol. At regular intervals the cells from a 1-ml aliquot were injected into two volumes of ice-cold TCA and the precipitate collected on glassfiber filters. The filters were washed five times with 2-ml aliquots of ice-cold 15% TCA, dried, and placed in scintillation vials along with 10 ml Ready-Solv (Beckman, Irvine, Calif.). Radioactivity was determined in a Beckman LS 6800 scintillation counter.

Deoxyribonucleoside triphoshate assay. Approximately 5×10^7 cells were extracted with 60% aqueous methanol as described previously [12]. dCTP and dGTP were measured enzymatically, DNA polymerase being used along with calf thymus DNA as a template [9]. dATP and TTP were determined in an identical manner, exept that poly(deoxyadenylate-deoxythymidylate) was used as a template [7].

Ribonucleoside triphosphate analysis. Approximately 10^7 cells were extracted with 0.5 ml 0.7 *M* perchloric acid. Extracts were centrifuged to remove precipitated protein, neutralized with solid potassium bicarbonate, and centrifuged once more to remove potassium perchlorate. Then 50 µl of the supernatant was analyzed by anion-exchange chromatography in a Perkin-Elmer series 4 liquid chromatograph equipped with a Whatman Pellicular anion exchange precolumn (0.4 × 6 cm) and a Whatman Partisil PSX 10/25 SAX column (0.46 × 25 cm). Nucleosides were resolved with an ammonium phosphate gradient starting with 5 m*M* pH 2.8 and ending at 0.5 *M* pH 4.8. Precise details of the elution procedure are given elsewhere [8]. Peaks were detected using a Kratos spectroflow 773 variable wavelength detector and electronically integrated using a Perkin-Elmer Sigma 15 integrator calibrated against known standards.

DNA and RNA polymerase assays. DNA and RNA polymerase activities were determined in permeabilized L1210 cells by a modification of the method of Berger [1]. Cells were suspended for 30 min at a concentration of 2×10^6 per ml in ice-cold permeabilization buffer containing 10 m*M* Tris, pH 7.8, 10 m*M* EDTA, 4 m*M* $MgCl_2$, and 30 m*M* 2-mercaptoethanol. The cells were collected by centrifugation and resuspended in permeabilization buffer at 10^7 cells per ml. Substrates for the assay were dissolved in reaction buffer containing 100 m*M* Hepes, pH 7.8, 20 m*M* $MgCl_2$, and 150 m*M* NaCl at the following concentrations: DNA polymerase – 15 m*M* ATP, 0.3 m*M* dATP, 0.3 m*M* dCTP, 0.3 m*M* dGTP, 3 µ*M* [methyl^3]TTP, 5 µCi/nmol; RNA polymerase – 15 m*M* ATP, 0.3 m*M* CTP, 0.3 m*M* GTP, and 3 u*M*[5-^{3}H]uridine 5′-triphosphate, 5 µCi/nmol. The reaction was started by adding two parts of cell suspension to one part of substrate mixture and incubating at 37°C. At regular intervals 1-ml aliquots were removed and injected into 2 ml ice-cold TCA. The precipitate was collected and processed as described in the incorporation studies.

Results

The effect of CI-920 on the incorporation of precursors into DNA, RNA, and Protein

Logarithmically growing L1210 cells were exposed to tritiated thymidine, uridine, or leucine with or without 10 µ*M* CI-920 added at time zero, and the incorporation of these precursors into the TCA-insoluble fraction was determined as a measure of DNA, RNA, and protein synthesis. Figure 2 shows that 10 µ*M* CI-920 caused a time-dependent inhibition of the incorporation of all three precursors. The rate of macromolecular synthesis was decreased by 45 min and maximum inhibition occured by 60 min. Figure 3 shows the relative rates of DNA, RNA, and protein synthesis in cells exposed for 2 h to varying concentrations of CI-920. The concentrations of drug necessary to inhibit DNA, RNA, and protein synthesis by 50% were 2.5, 2.8, and 4.5 µ*M*, respectively.

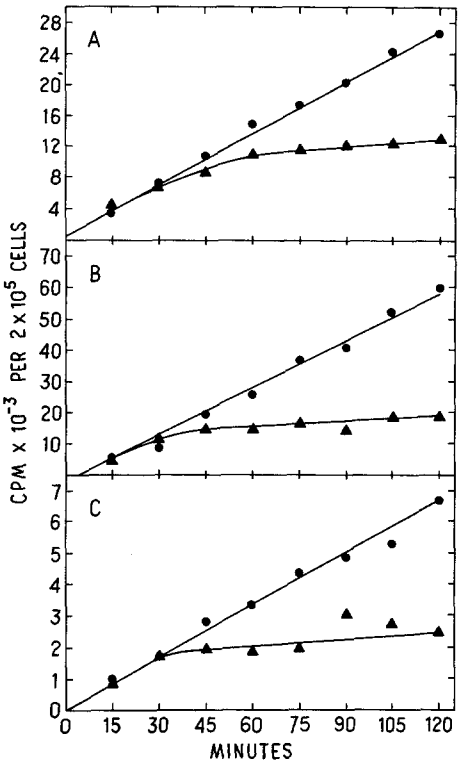

Fig. 2A–C. Effect of CI-920 on **A** thymidine incorporation into DNA, **B** uridine incorporation into RNA, and **C** leucine incorporation into protein in L1210 leukemia. ● control; ▲, cells exposed to 10 µ*M* CI-920 at time 0

Table 1. Effects of CI-920 on the intracellular ribonucleoside triphosphate pools in L1210 leukemia in vitro

Exposure time (min)	ATP (1,620 ± 54)	GTP (390 ± 30)	CTP (183 ± 22)	UTP (434 ± 24)
15	116	115	125	120
30	108	113	114	109
60	113	127	139	138
120	107	137	181	153

Cells were exposed to 10 µ*M* CI-920 for the indicated times. Data in parentheses represent control values expressed as nmoles/10^9 cells and represent the mean of four experiments ± standard error
Treated cell data represent percent of controls

Effect of CI-920 on the intracellular levels of ribonucleoside and deoxyribonucleoside triphosphates

It was clear that CI-920 was a rapid and potent inhibitor of nucleic acid synthesis, an effect that could result from one of several mechanisms, including precursor depletion. Therefore, the intracellular levels of nucleic acid precursors were measured in drug-treated cells and compared with untreated controls. Table 1 shows the intracellular levels of ribonucleoside triphosphates (expressed as percent of control) in L1210 cells exposed to 10 µ*M* CI-920 for varying lengths of time. It is apparent that at no time did any of these precursors fall below control levels, and in fact at the longer exposure times some of the nucleotides had increased, especially the pyrimidine pools.

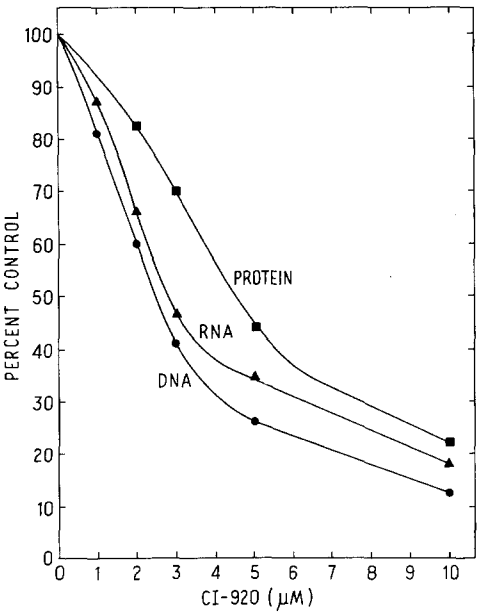

Fig. 3. Dose response for the suppression of macromolecular synthesis by CI-920 in L1210 leukemia. Cells were exposed for 2 h to the indicated concentrations of CI-920, after which the rate of incorporation of radiolabeled precursors was monitored over a 20-min interval. ● thymidine; ▲, uridine; ■, leucine

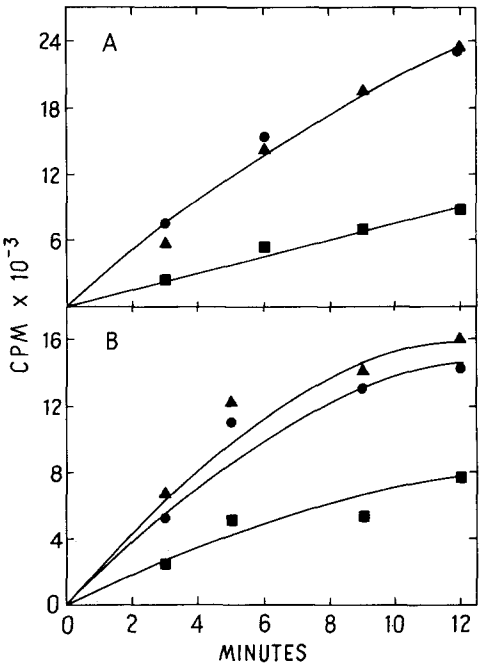

Fig. 4A, B. Effect of CI-920 on **A** DNA and **B** RNA polymerase as measured in permeabilized L1210 leukemia cells. ● control; ▲, cells exposed to 50 μ*M* CI-920 after permeabilization and during the assay; ■ cells exposed to 20 μ*M* CI-920 for 1 h before permeabilization but absent during the assay

Table 2. Effect of CI-920 on the intracellular levels of deoxyribonucleoside triphosphates in L1210 cells

	dATP	dGTP	dCTP	TTP
Control	13.9 ± 3	6.0 ± 3	30.8 ± 7	21.1 ± 6
CL 1565A	13.6 ± 3 (98)	4.9 ± 1 (82)	24.8 ± 9 (81)	31.7 ± 10 (150)

Cells were exposed to 10 μ*M* CI-920 for 2 h. Data are expressed as nanomoles/10^9 cells and represent the mean of four experiments ± standard error
Numbers in parentheses are percentages of control values

Similarly, deoxyribonucleoside triphosphate levels were measured in cells exposed to 10 μ*M* CI-920 for 2 h (Table 2). Again, there was no decrease in any of the precursors significant enough to account for the observed inhibition of DNA synthesis, and in fact there was a 50% increase in TTP.

Effect of CI-920 on DNA and RNA polymerase

Clearly the inhibition of DNA and RNA synthesis by CI-920 was not due to precursor depletion, which suggests that this drug interacts either with DNA and RNA polymerase or with DNA template function. The effects of CI-920 on DNA or RNA polymerase in permeabilized L1210 cells is shown in Fig. 4. The validity of these assays was verified by the fact that Adriamycin and actinomycin D completely abolished DNA and RNA polymerase activity, respectively. CI-920 (50 μ*M*) added directly to the permeabilized system had no effect on either polymerase. The results were the same whether the permeabilized cells were exposed to the drug simultaneously with the substrates or preincubated with drug 5–30 min prior to the addition of the substrates. However, if intact cells were exposed to 20 μ*M* CI-920 for 1 h prior to permeabilization and then the polymerases assayed in the absence of drug there was a 60% depression in enzyme activity.

Discrimination between the inhibition of either polymerase activity or template function by CI-920

Whereas CI-920 did not inhibit DNA or RNA polymerase activity when added to these assays directly, this drug did cause an apparent irreversible inhibition of these activities in viable cells. The following experiments were designed to test whether the drug was affecting the polymerases directly or inhibiting the template.

The procedure was identical with that used for the previously described DNA and RNA polymerase assays, except that after the cells had been exposed to the permeabilization buffer they were gently homogenized with a Dounce homogenizer in such a way that the polymerases could be exposed to additional macromolecules. Table 3 summarizes the effect of introducing excess DNA template to the L1210 DNA and RNA polymerase assay. Addition of supplemental template caused an increased activity in both control and drug-treated cells; however, in reference to RNA synthesis, the drug-treated cell fraction maintained the same degree of

Table 3. Effect of supplemental DNA template on the activity of DNA and RNA polymerase inhibited by CI-920 in L1210 leukemia

Assay	Addition	CPM		% Control
		Control	CI-920	
DNA Polymerase	–	13,832	5,558	(40%)
DNA Polymerase	Activated DNA	29,824	25,115	(84%)
RNA Polymerase	–	7,446	3,561	(48%)
RNA Polymerase	Activated DNA	11,425	5,339	(47%)

DNA and RNA polymerase assays were performed as described in *Methods,* except that the cells were gently homogenized with a Dounce homogenizer to disrupt the cell membrane. Treated cells were exposed to 20 μ*M* CI-920 for 1 h and then disrupted for the assay. The quantity of supplemental DNA was 40 μg and polymerase activity was measured over 15 min. The data in parentheses represent percentages of control values and are the average of two separate experiments in each case

inhibition with respect to the control as without added DNA, indicating that added template could not restore the lost activity. In contrast, the inhibition of DNA synthesis was at least partially relieved by added template, possibly implicating an interaction with DNA.

To further investigate an effect on the template, DNA damage in the form of single-stranded breaks was investigated by the alkaline elution method [4]. Treatment of cells for 1 h with concentrations of CI-920 as high as 50 μ*M*, however, caused no DNA strand breakage, an effect that might be expected if this drug bound or interacted with DNA in a destructive manner (data not shown).

Discussion

CI-920 clearly has a marked inhibitory effect on macromolecular synthesis, which appears to be similar both in magnitude and in time of onset for DNA, RNA, and protein synthesis. Whereas the similarity makes it difficult to distinguish between primary and secondary effects, it does suggest that this drug functions by mechanism which is rather nonspecific for any single macromolecular process.

There are several possible explanations for the decrease in nucleic acid synthesis. These include direct inhibition of DNA or RNA polymerase, inactivation of the DNA template, or depletion of an essential precursor. The last possibility seems unlikely, since CI-920 caused no significant decrease in ribonucleoside triphosphates or deoxyribonucleoside triphosphates. Indeed, some of these substrates were elevated in the presence of drug, indicating a more direct block at the level of nucleic acid assemblage. Assessment of this possibility has proven complex, since CI-920, when added directly to DNA or RNA polymerase assays in permeabilized cells, had no effect on these activities. This seemingly rules out a direct effect not only on the polymerases but also on the DNA template, since DNA binders and damaging agents such as Adriamycin and actinomycin D abolish these activities. However, if intact cells were preincubated with CI-920 just long enough to impair macromolecular synthesis (1 h), DNA and RNA polymerase activity remained severely inhibited when the cells were then permeabilized and assayed even in the absence of drug. Thus the above data do not rule out the possibility that an active metabolite which inhibits these enzymes may be formed within viable cells. This possibility has been investigated further through experiments in which a heavy suspension of cells (10^8/ml) was incubated at 37° C with 200 μ*M* CI-920 for 4 h. After the cells had been disrupted by sonication and centrifuged at 100,000 *g* for 1 h, as much as 0.5 ml of the supernatant was added to the polymerase assays. No inhibition was observed, however, indicating that if an active metabolite was formed it was unstable to isolation (D.W. Fry, unpublished results).

A distinction between effects of CI-920 on either DNA or RNA polymerase or on the DNA template in pretreated cells has been difficult to demonstrate. The inhibition of RNA polymerase in drug-treated cells was not abolished by addition of excess DNA, implying a more direct effect on the enzyme. In contrast, the addition of DNA restored DNA polymerase activity, indicating that this enzyme was intact and that the limiting factor was the quantity of functional primer DNA. Although the latter observation suggests that CI-920 affects the DNA template in some manner, this drug produced no strand breakage as assessed by alkaline elution.

Based on the above data our tentative conclusions are that (1) CI-920 inhibits nucleic acid synthesis directly at the level of nucleotide incorporation rather than by precursor depletion; and (2) CI-920 does not itself inhibit DNA or RNA polymerase nor cause apparent damage to the DNA template.

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