Cancer Chemother Pharmacol (1989) 24: 251–255

Cancer Chemotherapy and Pharmacology

A study of the mechanisms of cytotoxicity of Ara-C on three human leukemic cell lines

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Summary. The main biochemical determinants involved in cytosine arabinoside (Ara-C) metabolism were studied in one lymphoblastic (Reh) and two myeloid (HL60 and K562) human leukemic cell lines exhibiting various sensitivities to Ara-C, Reh being the most and HL60 the least sensitive. The level of intracellular Ara-C accumulation and Ara-CTP formation was far more important in Reh cells than in myeloid cell lines but was not closely related to deoxycytidine kinase activity or to deoxycytidine triphosphate pool size. The level of Ara-C incorporated into DNA was similar in the three cell lines. Ara-CTP formation correlated better with the cytotoxicity to clonogenic cells than did Ara-C incorporation into DNA. DNA polymerase α was moderately inhibited to various degrees, depending on the cell line; this moderate inhibition does not seem sufficient to explain the inhibition of DNA synthesis. The activity of DNA ligase, the enzyme joining the Okazaki fragments, which was not detected in Reh cells, was strongly inhibited by Ara-C in HL60 and to a lesser degree, in K562 cells. The inhibition of DNA ligase probably also contributes to the inhibition of DNA synthesis and, thus, to the cytotoxic effect of Ara-C and may explain the smaller size of DNA fragments observed following Ara-C treatment. The variations in each critical determinant observed in these three cell lines increase the complexity and plurality of the mechanisms of Ara-C action.

Introduction

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine; Ara-C) is an analog of deoxycytidine (dCyd) currently used for the treatment of acute leukemia. Cellular uptake of the drug is mediated through a nucleoside-specific transport mechanism [29] and is a prerequisite for the metabolic conversion into its mono-, di-, and triphosphate forms (Ara-CMP, Ara-CDP, and Ara-CTP, respectively). The enzyme responsible for the monophosphate synthesis is the cytoplasmic dCyd-kinase, which is feedback-inhibited by the natural nucleotide, deoxycytidine triphosphate (dCTP). Ara-CTP competes with dCTP and inhibits DNA polymerase [8, 9, 19, 20]; small amounts of Ara-CTP are also incorporated into DNA and result in further inhibition of DNA synthesis [11, 17]. However, the precise mechanisms by which Ara-C induces a cytotoxic effect is still a matter of debate. A study of the metabolic changes induced by the drug in cells exhibiting various sensitivities toward Ara-C could contribute to the elucidation of the main mechanism of action of this drug.

The aim of the present work was to determine whether cytotoxicity induced by Ara-C in three human leukemic cell lines exhibiting different sensitivities to the drug was more selectively related to one of the critical determinants involved in Ara-C metabolism. Therefore, we analyzed the following main biochemical determinants in one lymphoblastic and two myeloid cell lines exposed to Ara-C: intracellular drug accumulation and Ara-CTP formation, incorporation into DNA, dCTP pool sizes, and dCyd kinase and DNA polymerase activities. Moreover, as Ara-C treatment of cells may induce fragments of DNA smaller than those in controls [30], we also studied the effect of this analog on DNA ligase, the enzyme joining the Okazaki fragments, which has not yet been studied in such a situation.

Materials and methods

Chemical compounds. Ara-C and its phosphorylated forms were obtained from Sigma. Deoxyribonucleoside triphosphates (dNTP), "large fragment" DNA polymerase from *Escherichia coli*, and poly d(I-C) were obtained from Boehringer. Radiolabelled compounds [^{3}H]-Ara-C, [^{3}H]-dCyd (sp. act. for each, 27 Ci/mmol), [^{3}H]-dGTP, and [^{3}H]-dTTP (sp. act. 17 and 50 Ci/mmol, respectively) were supplied by Amersham, and sheep antiserum anti-Ara-C, by the University of Surrey.

Cell cultures. Three human leukemic cell lines were used in these experiments: HL60, derived from a promyelocytic leukemia [3], K562, from a chronic myelogenous leukemia in blast crisis [15], and Reh [24], derived from an acute lymphoblastic leukemia (CALLA+, TdT+). Cells were grown at 37° C in a 5% CO_2 atmosphere in RPMI-1640 medium containing 10% heat-inactivated dialyzed fetal calf serum and antibiotics (100 IU penicillin/ml and 100 μg streptomycin/ml). All experiments were carried out with the cells in the logarithmic growth phase. Log-growing

* Supported by a Grant de l'Université Paris Val-de-Marne sur un programme priviligié (J. Z.)

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cells were exposed in their culture medium to 10^{-5} *M* Ara-C for 3 h. Control cells were incubated simultaneously without drug.

In preliminary experiments, cell were exposed for 1, 3, 6, and 24 h to different concentrations of Ara-C, ranging from 10^{-8} to 10^{-4} *M*. As the metabolic changes reached a maximum after 3 h exposure with 10^{-5} *M* Ara-C and then reached a plateau, this time and concentration was selected for all incubations. After incubation of the cells, the drug-containing medium was removed and the cell pellet, washed. An aliquot was resuspended in 1 ml 60% ice-cold methanol and stored for 16 h at −20° C to extract Ara-C derivatives and dCTP pools. The methanol-insoluble material was removed by centrifugation at 0° C; the resulting supernatant was then evaporated in a vacuum spin centrifuge until the volume was between 50 and 100 µl. The samples were then stored at −20° C until analysis.

Intracellular total Ara-C and Ara-CTP quantitation. Ara-C and its phosphorylated forms were measured on the methanol extract by a radioimmunoassay (RIA) as previously described by Piall et al. [21]. The sheep antiserum reacts with free Ara-C and its phosphorylated forms. For separation of Ara-C nucleotides, cells were incubated under the same conditions as above, but [^{3}H]-Ara-C (2 µCi/ml) was also added to cold Ara-C. After centrifugation and washing in saline, nucleotides were extracted in ice on a cell pellet with 6% PCA; after 15 min storage in an ice bath, the acid-soluble material was recovered by centrifugation and the supernatant was neutralized to pH 7 with 2 *N* KOH. After centrifugation, the supernatant was stored for chromatography. The fractionation was done on cellulose F plates (20 × 20 cm; Merck). The solvent system was composed of 66% isobutyric acid, 23% distilled water, 10% acetonitrile, and 1% ammoniac (v/v). Each sample was mixed with the standard mixture, comprising 10^{-2} *M* Ara-C, Ara-CMP, and Ara-CTP. After migration for 4 h and drying, the spots were identified in UV light and the radioactivity of each spot was quantified.

Ara-C incorporation into the acid-insoluble fraction. As above, [^{3}H]-Ara-C (2 µCi/ml) was mixed with cold Ara-C and introduced into the culture medium at a concentration of 10^{-5} *M*. After an incubation of 3 h with [^{3}H]-Ara-C, the cells were centrifuged and washed three times with cold phosphate-buffered saline (PBS). Cells were transferred to 3-mm filter discs, then dried; macromolecules were precipated by 10% trichloracetic acid for 20 min and washed twice with methanol for 10 min and then with acetone. Dried discs were counted in 10 ml scintillation fluid. In preliminary experiments, pure DNA was obtained after digestion with proteinase K followed by extraction wih phenol and treatment with ribonuclease [23]. We determined that the bulk of the radioactivity (>95%) was found in DNA.

Measurement of dCTP pools. The assay of dCTP pools was also carried out on the methanol extract according to the enzymatic technique previously described by Skoog [26]. After incubation at 37° C for 30 min in a water bath, the mixture reaction was chilled and poured onto DE 81 paper discs (Whatman); the discs were dried, then treated by the method previously described by Lindell et al. [16]. Using poly d(I-C) as a template, we found standard curves to be linear from 1 to 10 pmol triphosphate. The results were expressed in pmol/10^6 cells.

Enzymes assays. For the enzyme assays, cell pellets containing between 20 and 50×10^6 cells were suspended in 0.5 ml extraction buffer containing 20 m*M* Tris HCl (pH 7.4), 0.5 *M* KCl, 2 m*M* dithiothreitol (dTT), and 0.2% Nonidet NP40 in 0.8 m*M* phenylmethylsulfonylfluoride. The cell suspensions were subjected to three cycles of rapid freezing and thawing, then sonicated before being centrifuged at 10,000 *g* for 5 min. The supernatants were used for the enzyme assays.

Deoxycytidine (dCyd) kinase activity was assayed using a previously described method [22]. Volumes of supernatant containing between 5 and 25 µg protein were tested in the mixture reaction. The activity of dCyd kinase was expressed as the amount (in pmol) of [^{3}H]-dCMP generated per mg protein/min. DNA polymerase activities were measured as previously reported [13]. The activity of this enzyme was measured using volumes of supernatant corresponding to a range of between 0.1 to 1.10^6 cells. In some experiments, 5 m*M* NEM or 1.25 µg/ml aphidicolin, specific inhibitors of DNA polymerase α, were added to the mixture reaction. In one experiment, DNA polymerases were purified by chromatography on phosphocellulose [4] to evaluate the relative activity of DNA polymerases α and β. DNA polymerase activity was expressed as the amount (in nmol) of [^{3}H]-dTMP incorporated into DNA per 10^8 cells/h.

DNA ligase activity was tested according to the method previously described by Modrich and Lehman [18], with slight modification [4]. Prior to the assay, an aliquot of supernatant corresponding to 0.8–4 mg protein was partially purified by absorption onto a column of phosphocellulose (P11; Whatman) and eluted with a linear gradient of 0.15–0.7 *M* KCl in a buffer containing 20% glycerol, 1 m*M* 2-mercaptoethanol, 0.1 m*M* ethylenediaminetetraacetate (EDTA), and 50 m*M* KCl [4]. The bulk of the enzyme eluted as a large peak with 0.4 *M* KCl. Fractions of 1 ml were collected and assayed for DNA ligase activity (1 unit DNA ligase was defined as the amount converting 1 nmol d(A-T)n/mg protein to the exonuclease III resistant form in 30 min.

Clonogenic survival of leukemic cells. After drug exposure, the cells were washed twice with PBS and resuspended in RPMI-1640 medium containing 20% fetal calf serum at a concentration of 2×10^3–2×10^4 cells/ml. The cells were then mixed with a solution of 1.4% methylcellulose in the same medium (v/v), then plated. Viability was determined after 7 days by scoring colonies as well as clusters

Table 1. Deoxycytidine (dCyd) kinase activity and dCTP pool size in three human leukemic cell lines[a]

Cell line	dCyd kinase activity	dCTP pools
HL60	48 ± 12	3.1 ± 0.2
K562	275 ± 11	24.5 ± 2
Reh	157 ± 24	12.5 ± 0.7

[a] dCyd kinase activity is defined as the amount (in pmol) of dCMP generated per min/mg protein. dCTP values are expressed in pmol/10^6 cells. Values represent the mean ± SD from 4 different experiments

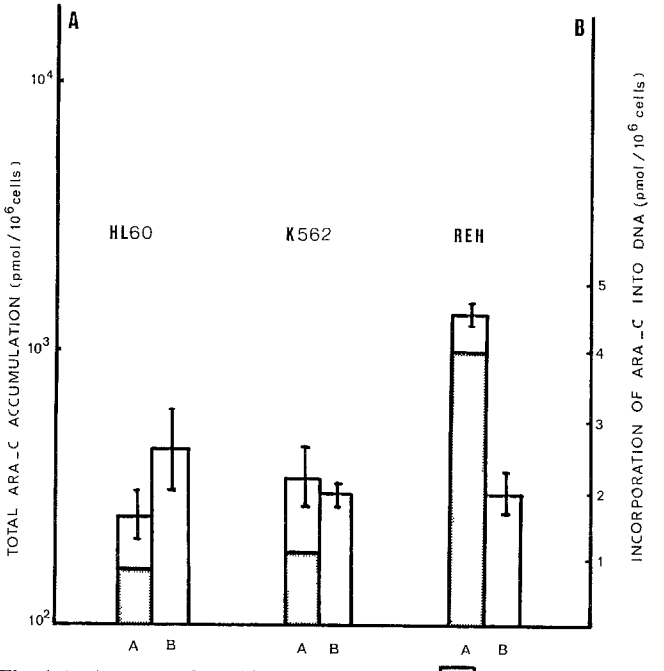

Fig. 1 A: Amount of total intracellular Ara-C and Ara-CTP (in pmol/10^6 cells) in three human leukemic cell lines (*HL60, K562* and *Reh*) after exposure to 10^{-5} *M* Ara-C for 3 h. **B:** Amount of Ara-C incorporated into DNA (in pmol/10^6 cells) after exposure to 10^{-5} *M* Ara-C for 3 h. Values represent the mean ± SD of 3 independent experiments

(< 10 cells). The cloning efficiency of untreated cells in this system was 100% for K562, 50% for Reh, and 20% for HL60.

Results

Figure 1A shows the Ara-C accumulated into the cells after exposure to 10^{-5} *M* drug for 3 h. The intracellular accumulation and retention of Ara-C did not vary significantly in the time course from 1 to 6 h (data not shown). The intracellular Ara-C content, mostly comprising Ara-CTP, especially in Reh cells, was far more important in this line (1,400 pmol/10^6 cells) than in K562 (350 pmol/10^6 cells) or HL60 (250 pmol/10^6 cells). Less than 5% free Ara-C was found in the three cell lines, the remainder being Ara-CMP and Ara-CDP. The Ara-CTP formation seemed to be independent of both dCTP pool size and dCyd kinase activity: in fact, the Reh cell line, which exhibits the highest intracellular level of Ara-CTP, has a dCTP pool size and dCyd kinase activity intermediate between those of the two myeloid cell lines (Table 1).

A very small amount of intracellular Ara-C was incorporated into the acid-insoluble fraction, reflecting drug incorporation into DNA (Fig. 1B). After 3 h incubation with Ara-C, the amount of drug incorporated into DNA was very similar in the three cell lines (1.9 pmol/10^6 cells in K562 and Reh, and 2.6 pmol/10^6 cells in HL60), despite wide variations in the levels of Ara-CTP uptake.

DNA polymerase activity decreased after exposure to Ara-C (Table 2). The decrease was due to the inhibition exerted by intracellular Ara-CTP and not to a diminished de novo synthesis of the enzyme; in fact, after dialysis the extracts recovered normal enzymatic activity. However, DNA polymerase inhibition was not directly related to the

Table 2. Effect of Ara-C on DNA polymerase and DNA ligase activities in human leukemic cell lines[a]

Cell line	DNA polymerase activity		DNA ligase activity	
	A[b]	B[c]	A[b]	B[c]
HL60	17.4 ± 1.4	10.8 ± 2.3	0.011 ± 0.002	0.0017 ± 0.0008
K562	31.3 ± 1.5	7.4 ± 0.8	0.015 ± 0.003	0.008 ± 0.002
Reh	15.4 ± 0.4	6.6 ± 0.75	No activity	No activity

[a] 1 unit DNA polymerase is defined as 1 nmol dTMP incorporated into DNA/h per 10^8 cells; 1 unit DNA ligase is defined as the amount converting 1 nmol/mg protein d(A-T)n to the exonuclease III resistant form in 30 min. Values represent the mean ± SD from 4 different experiments
[b] Untreated cells
[c] Cells treated with 10^{-5} *M* Ara-C for 3 h

Table 3. Cytotoxic action of Ara-C in human leukemic cell lines, measured by the clonogenic survival of cell lines[a]

Treatment	HL60	K562	Reh
None	210 ± 46	115 ± 30	45 ± 14
10^{-5} *M* Ara-C for 3 h	122 ± 20[b]	12 ± 4	0

[a] Viability was determined after 7 days by scoring colonies of clonogenic cells. Values represent the mean ± SD from 3 different experiments
[b] Clusters of < 10 cells were counted

level of Ara-CTP formation; the decrease in DNA polymerase activity was less important in Reh cells than in the K562 cell line (near 50% and 25% of the basal activity, respectively), in spite of a higher intracellular formation of Ara-CTP in the Reh line. The enzyme activity evaluated was mainly related to DNA polymerase α, since this activity was decreased by 85% when potent inhibitors of this enzyme (aphidicolin or NEM) were added to the reaction mixture. Moreover, in one experiment in which DNA polymerases α and β were fractionated by chromatography, the activity assayed on the α peak was similar to that inhibited by aphidicolin on the unfractionated extract.

DNA ligase activity is shown in Table 2. No activity was detected in the Reh cell line, even before drug exposure. The incubation of HL60 and K562 cells with Ara-C induced a marked decrease in DNA ligase activity. The activity found in Ara-C-treated cells was about 15% of that found in untreated HL60 cells and about half of that in untreated K562 cells.

Exposure of cells to 10^{-5} *M* Ara-C for 3 h induced a net decrease in the number of clonogenic cells (Table 3). The Reh cell line was very sensitive to Ara-C, since no clonogenic cells survived after 3 h. The number of clonogenic cells was decreased to 90% in K562 cells; HL60 cells were the most resistant, although the colonies persisting after Ara-C exposure consisted of clusters of < 10 cells.

Discussion

Ara-C is known to produce a marked inhibition of DNA synthesis through its active metabolite, Ara-CTP, but the precise mechanism of the cytotoxicity of this drug is still unclear. Previous works have demonstrated that Ara-CTP acts as a competitive inhibitor of DNA polymerase relative to the normal substrate, deoxycytidine triphosphate [9];

other works have concluded that the inhibition of DNA synthesis is mainly related to the proportion of Ara-C residues incorporated into DNA that were considered to be poor primer termini for further chain elongation [19, 20, 27, 28]. One could suggest that these different hypotheses are not mutually exclusive and do not exclude other cellular mechanisms of action; as Ara-C treatment was shown to induce a reduction in the size of DNA fragments [7], this drug may also affect other enzymes of the replication complex such as DNA ligase, the enzyme capable of joining newly synthesized DNA fragments. To determine whether cytotoxicity induced by Ara-C was related more specifically to one of the cellular determinants involved in the mechanism of action of this drug, we analyzed some of these critical determinants in one lymphoid (Reh) and two myeloid (HL60 and K562) leukemic cell lines.

Ara-C accumulation into cells and Ara-CTP formation varied from one cell line to the other. This intracellular content of Ara-CTP was found to be more important in the Reh than in the myeloid cell lines. This high Ara-CTP formation is in agreement with the extreme sensitivity of this cell line to Ara-C shown by the absence of clonogenic cells after 3 h exposure to the drug. The cytotoxicity exerted by Ara-C on K562 cells was lower, since 10% of the clonogenic cells persisted after drug exposure; the HL60 line, which presented the lowest level of intracellular Ara-C, was more resistant to the drug, although the persisting clonogenic cells consisted of small clusters of cells. In any case, the level of Ara-CTP in the cell lines was not closely related to dCyd kinase activity or to the size of dCTP pools, since in Reh cells these last two parameters were intermediate between those found in the two myeloid cell lines. Other metabolic determinants probably account for the level of Ara-CTP pools, such as Ara-C/Ara-CMP deamination and Ara-CTP catabolism.

Despite the differences in Ara-CTP formation observed in the three cell lines, the amounts of Ara-C incorporated into DNA after Ara-C exposure were very similar. Therefore, in contrast to the results of other studies [12] as shown by clonogenic cell survival, Ara-CTP formation and retention seem to correlate better with cytotoxicity, than Ara-C incorporation into DNA.

The moderate effect of Ara-C on DNA polymerase activity does not seem sufficient to explain fully the inhibition of DNA synthesis in the cell lines analyzed in this study. Inhibition of DNA polymerase is one of the most widely accepted hypotheses to explain the cellular effects of Ara-C. However, as shown previously [20], Ara-CTP is not a potently competitive inhibitor of this enzyme, since the binding activity of Ara-CTP and dCTP for the catalytic site of DNA polymerase is about the same; moreover, high concentrations of deoxycytidine may induce a reversal of Ara-C-mediated cytotoxicity [1]. In our experiments, at concentrations that affect DNA synthesis, Ara-C decreased DNA polymerase activity only between 40% and 75%; moreover, this inhibition was not directly related to Ara-CTP formation, as shown by a lower decrease of activity in Reh than in K562 cells despite a higher Ara-CTP formation in the former.

The interactions of Ara-C with other enzymes of the replication complex, such as DNA ligase [14], may also account for the cytotoxic effects of this drug. DNA ligase functions in both the replication and repair of DNA in mammalian cells [14, 25]. In fact, no DNA ligase activity was detected in Reh cells, a finding in agreement with results reported for blasts originating from patients with common or acute T-lymphoblastic leukemia [4]. This absence of DNA ligase could account for the extreme sensitivity of this cell line to Ara-C and may also explain the great thymidine sensitivity of cultured leukemic lymphoid cells previously reported [6]. Furthermore, its absence in immature cells of the lymphoid lineage may constitute an important factor in the therapeutic effect of Ara-C used in combination with other drugs for the treatment of acute lymphoblastic leukemia and high-grade malignant lymphoma.

In the two myeloid cell lines, especially in HL60 cells, DNA ligase activity was found to be greatly decreased by Ara-C. In separate experiments, we have observed that Ara-C inhibits the formation of the intermediate complex ligase-adenylate, which is essential to the gap-filling step (data not shown). Therefore, the inhibition of DNA ligase may constitute an important factor in the inhibition of DNA replication induced by Ara-C; it may also explain previous data related to the reduced size of DNA fragments after Ara-C treatment [2]. Observing a smaller size of primary pieces from Ara-CTP-treated nuclei compared with those seen in controls, Wist et al. [30] concluded that the rate of ligation was reduced and suggested that the reduced rate of elongation was an important component of the inhibitory effect of Ara-CTP; however, no direct evidence has thus far been presented as to the mechanism of this reduced rate of elongation. More recently, Fram and Kufe [7] have detected DNA strand breaks in L1210 cells after Ara-C exposure. Indeed, as DNA ligase is involved in joining Okazaki fragments at replication forks [5], the inhibition of this enzyme by Ara-C prevents the gap-filling step but may also contribute to the inhibition of DNA repair observed after exposure to this drug [10].

In conclusion, our study shows that Ara-CTP formation – and probably retention – more likely accounts for the cytotoxicity of Ara-C than does Ara-C incorporation into DNA; the highest level of Ara-CTP was found in the cell line most sensitive to the drug, whereas no difference was found in the amounts of Ara-C incorporated into DNA in the three cell lines. In fact, the Ara-CTP pool exerts its cytotoxic effect through the inhibition of DNA polymerase activity and, thus, of DNA synthesis. However, DNA polymerase activity was only partially inhibited, even in the cell line showing the highest formation of Ara-CTP. The activity of DNA ligase, another enzyme of the replication complex, is also inhibited by Ara-C. The absence of detectable DNA ligase activity in the lymphoblastic cell line could explain the extreme sensitivity of this type of cell to Ara-C. The cytotoxicity of Ara-C appears to be the result of conjugate factors that could act synergistically, with one or the other being predominant according to the cellular type.

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Received 21 June 1988/Accepted 2 February 1989