Jpn. J. Cancer Res. 81, 1314–1319, December 1990

# Metabolism and Toxicity of Electroporated 1-β-D-Arabinofuranosylcytosine Triphosphate in a Human Leukemia Cell Line

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**The metabolism and toxicity of 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP) directly injected into cells by electroporation was studied in human leukemia cell lines. The intracellular accumulation of ara-CTP (ara-CTP-Ep) was dependent on the cell type, extracellular ara-CTP concentration and pulse voltage on electroporation. In a promyelocytic leukemia cell line, HL-60, ara-CTP-Ep revealed a cytotoxic effect in a dose-dependent manner, although electroporation alone did not have any significant toxicity. Furthermore, simultaneous injection of dCTP, or continuous exposure to deoxycytidine, but not to other deoxyribonucleosides, immediately after electroporation rescued the cells from the toxicity of ara-CTP-Ep. The degradation of ara-CTP-Ep consisted of an early rapid phase followed by a slower phase with a half life of 1.5 h. The addition of dipyridamole (10 μM), an inhibitor of nucleoside transport, retarded this degradation process. These data indicate that transfer of ara-CTP by electroporation is a useful method for the study of ara-CTP metabolism.**

Key words: Electroporation — 1-β-D-Arabinofuranosylcytosine triphosphate — Cytotoxicity

Several synthetic nucleosides are currently in use as anti-tumor or anti-viral agents. To exhibit biological activity, these compounds must be transported into cells and phosphorylated, usually to their triphosphates, by the activity of several cytoplasmic enzymes.[1, 2] Thus, the metabolic study of a nucleoside analog is hampered when cells are deficient in one of these activation steps. For example, leukemic blasts isolated from patients were shown to have much lower 1-β-D-arabinofuranosylcytosine (ara-C) transport ability than exponentially growing tumor cell lines.[3] Takimoto *et al.* reported that ara-C transport was cell cycle-dependent, being 30–50% higher in the S phase than in the $G_1$ phase.[4] Moreover, B lymphoblastoid cell lines can accumulate less deoxyribonucleotides than T lymphoblastoid cell lines following incubation with corresponding deoxyribonucleosides, presumably because of lower phosphorylating activity or a higher level of catabolic enzyme, 5′-deoxynucleotidase.[5, 6] Therefore, the development of a method to inject directly into cells a nucleotide, which is normally incapable of passing the plasma membrane, is potentially important.

Various techniques of introducing macromolecules, especially DNA, into viable cells have contributed to the progress of molecular biology. Among them is electroporation, which employs a high-voltage electrical pulse as the injection tool.[7, 8] The simplicity of the method and the minimum effect on cell viability are considered to be advantageous. Recently, electroporation has also been used to transfer proteins, such as monoclonal antibodies[9] or phosphorylated nucleotides.[10] Our present study was carried out to determine whether electroporation of nucleotides into cells would be a useful technique for the study of the toxicity and metabolism of nucleosides by using ara-CTP as a model compound.

## MATERIALS AND METHODS

**Cell cultures** A human promyelocytic leukemia cell line, HL-60, and a T lymphoblastoid cell line, Molt 4, were kindly provided by the Japanese Cancer Cell Type Collection. These cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 m*M*), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cultures were kept at 37°C in a humidified atmosphere of 95% air/5% $CO_2$ and checked regularly for mycoplasma.[11]

**Electroporation** Cells in their late logarithmic phase were suspended in 0.25 ml of electroporation buffer (137 m*M* NaCl, 5.4 m*M* KCl, 1.1 m*M* $Na_2HPO_4 \cdot 12\ H_2O$, 1.47 m*M* $KH_2PO_4$, 0.5 m*M* $MgCl_2 \cdot 6H_2O$, 9 m*M* $CaCl_2$, 6.1 m*M* glucose, pH 7.14).[8] Then various concentrations of ara-CTP or dCTP were added, and an exponentially decaying electrical pulse was applied once to the cells on ice.[12]

**Cytotoxicity study and nucleotide extraction** The electroporated cells were left on ice for another 15 min followed by three washings with ice-cold phosphate-buffered saline (pH 7.4) (PBS). A portion of the cells was resuspended in RPMI medium at a density of $2 \times 10^5$/ml and seeded in a microtiter well. After three days, viable cells were enumerated by the trypan blue dye exclusion test. Cell growth was expressed as a percentage of that of control unelectroporated cells. The remaining cells were

extracted with 0.4*N* perchloric acid and neutralized with $KOH/KH_2PO_4$.

**Measurement of intracellular nucleotides** Following the extraction described above, ara-CTP was separated and quantified by high-performance liquid chromatography (HPLC) on a Partisil 10-SAX column eluted with 0.5 *M* KCl/0.25 *M* $KH_2PO_4$, pH 3.2 at 1.5 ml/min.[13] For the determination of dCTP, ribonucleotides in the cell extracts were destroyed by sodium periodate according to Garrett and Santi.[14] Such periodated samples were subjected separately to HPLC under the same eluting conditions.

## RESULTS

**Effect of pulse voltage on ara-CTP accumulation in HL-60 and Molt 4 cells** First, we determined the accumulation of ara-CTP (ara-CTP-Ep)in HL-60 or Molt 4 cells by changing the pulse voltage in 5 m*M* extracellular ara-CTP concentration. As shown in Fig. 1, HL-60 cells accumulated ara-CTP in proportion to the pulse voltage up to 700 V (1.75 kV/cm). Further increase of the voltage resulted in the decrease of ara-CTP-Ep. On the other hand, ara-CTP-Ep was less efficient in Molt 4 cells; the maximum introduction was about one-third of that in HL-60 cells. Taking these results into consideration, we chose 550 V and HL-60 cells for further experiments.

**Effect of extracellular ara-CTP concentration on ara-CTP accumulation and cytotoxicity** Ara-CTP-Ep was apparently a function of extracellular ara-CTP concentration. Fig. 2 shows that ara-CTP-Ep increased linearly from 0.625 to 2.5 m*M*, and thereafter a prominent increase was observed at 5 or 10 m*M*. Concomitantly, a reciprocal decrease of cell growth was detected. For comparison, we examined the toxicity of ara-CTP accumulated during incubation with ara-C. In these experiments, cells were incubated with various concentrations of ara-C for 1 h and resuspended in the PRMI-medium after three washings with PBS. As shown in Fig. 3, cell growth was inhibited as ara-C concentration increased.

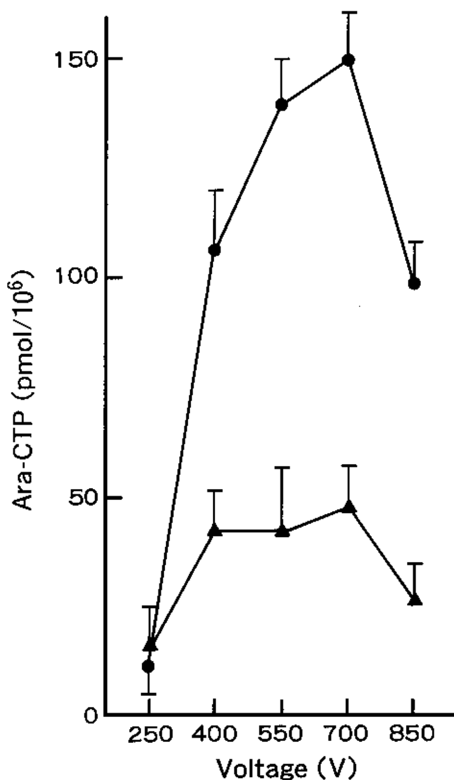


Fig. 1. Intracellular ara-CTP levels following electroporation at different voltages in HL-60 and Molt4 cells. HL-60 (●) or Molt4 (▲) cells were electroporated at different pulse voltages in 5 m*M* extracellular ara-CTP. Then, ara-CTP levels were determined as described in "Materials and Methods." Points indicate the mean values of five experiments: bars, SD

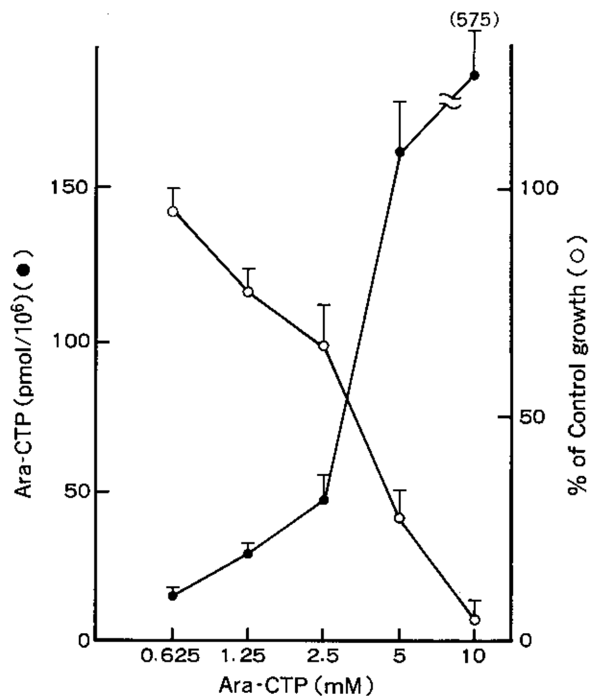


Fig. 2. Effect of extracellular ara-CTP concentration on ara-CTP accumulation and cytotoxicity in HL-60 cells. Cells were electroporated at 550 V with various concentrations of ara-CTP. Subsequently, ara-CTP was determined by HPLC (●) and cell growth after three days was expressed as a percentage of that in control unelectroporated cells (○). The data represent the mean±SD of four experiments.

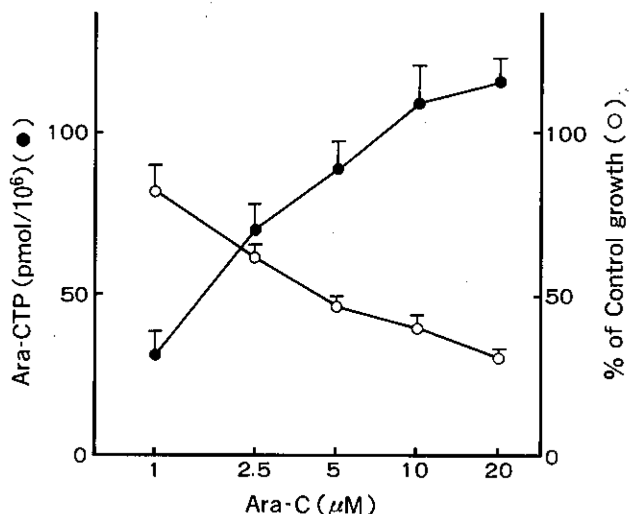


Fig. 3. Ara-CTP levels and cytotoxicity following incubation with various concentrations of ara-C. HL-60 cells were incubated with various concentrations of ara-C for 1 h followed by three washings with cold PBS. Then, ara-CTP (●) and cell growth (○) were evaluated as in Fig. 2. The mean±SD of four experiments are shown.

However, ara-CTP accumulation reached a plateau at 10 $\mu M$.

**Rescue from ara-CTP-Ep toxicity by deoxycytidine or dCTP** A naturally occurring nucleoside, deoxycytidine (CdR), has been reported to protect cells from ara-C cytotoxicity.[15] To examine whether this is also the case for the toxicity of ara-CTP-Ep, we incubated the cells with different concentrations of various nucleosides after electroporation of 10 m$M$ ara-CTP. Fig. 4 reveals that CdR counteracted the toxicity of ara-CTP-Ep in a dose-dependent fashion. However, other deoxyribonucleosides, such as deoxyadenosine with 10 $\mu M$ deoxycoformycin, an inhibitor of adenosine deaminase, deoxyguanosine with 100 $\mu M$ 8-aminoguanosine, an inhibitor of purine nucleoside phosphorylase, and thymidine did not show any protective effect at the highest concentrations used in this study. Cytidine, however, had some protective activity.

The ratio of dCTP to ara-CTP is considered to be a crucial factor in ara-C toxicity.[16] Therefore, we evaluated the changes of ara-CTP-Ep toxicity when dCTP was injected simultaneously. In these experiments,

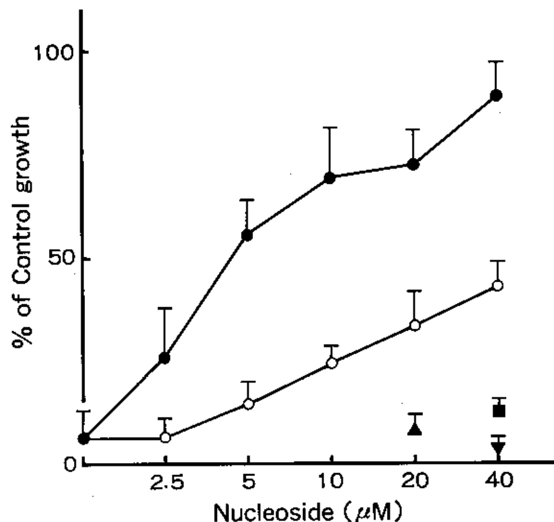


Fig. 4. Rescue from toxicity of electroporated ara-CTP by several nucleosides. HL-60 cells were electroporated at 550 V in 10 m$M$ ara-CTP. After washings with cold PBS three times, the cells were resuspended in fresh RPMI-medium with or without different nucleosides. Cell growth was determined on day 3. Nucleosides tested; deoxycytidine (●), cytidine (○), deoxyadenosine+deoxycoformycin (▼), deoxyguanosine+8-aminoguanosine (▲), thymidine (■). Points indicate the mean values of four experiments; bars, SD.

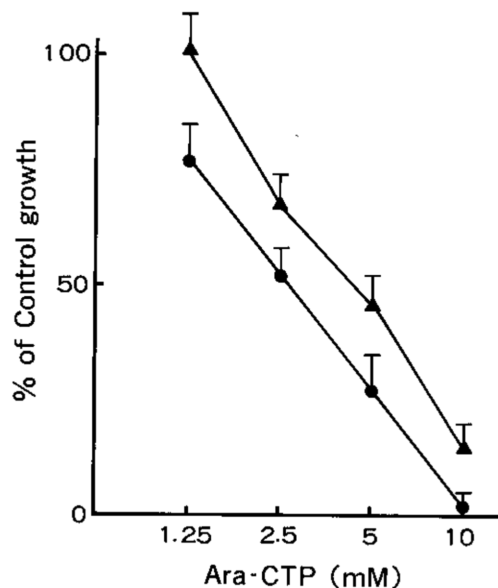


Fig. 5. Modulation of the toxicity of electroporated ara-CTP by simultaneous injection of dCTP. HL-60 cells were electroporated at 550 V under various concentrations of ara-CTP with or without 2.5 m$M$ dCTP. Ara-CTP levels (mean±SD) at different extracellular ara-CTP concentrations with vs without dCTP were as follows; 498±56 vs. 490±31 (10 m$M$), 178±9 vs. 147±23 (5 m$M$), 91±18 vs. 83±15 (2.5 m$M$) and 28±5 vs. 33±3 (1.25 m$M$). Then, cell growth was compared after three days. (●) ara-CTP only, (▲) ara-CTP+dCTP. The data represent the mean±SD of four experiments.

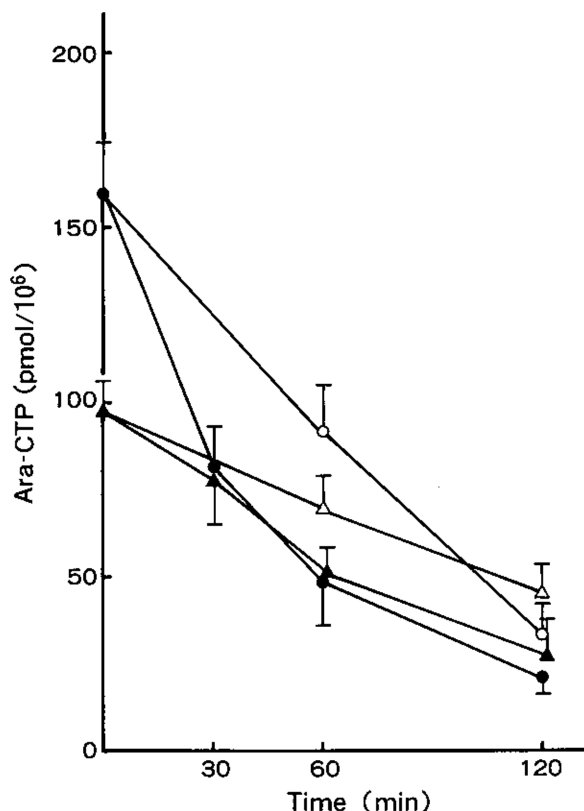


Fig. 6. Degradation of ara-CTP. HL-60 cells were either electroporated at 550 V in 5 m*M* ara-CTP or incubated with 10 $\mu M$ ara-C for 1 h. The cells were washed three times with PBS and transferred to fresh RPMI-medium with or without dipyridamole (10 $\mu M$). Then, aliquots of cells were taken and intracellular ara-CTP levels were estimated. (●) electroporated ara-CTP, (○) electroporated ara-CTP + dipyridamole, (▲) ara-CTP accumulated by ara-C, (△) ara-CTP accumulated by ara-C + dipyridamole. The data show the mean ± SD of four experiments.

2.5 m*M* dCTP and various concentrations of ara-CTP were added to the medium on electroporation. This treatment successfully introduced dCTP of 34.4 ± 4.4 pmol/$10^6$ cells without any significant effect on ara-CTP accumulation. In contrast, dCTP levels were 7.6 ± 2.4 pmol/$10^6$ cells in unelectroporated control cells.[13] As shown in Fig. 5, this had a significant rescue effect, albeit to a lesser degree.

**Ara-CTP degradation** The intracellular retention of ara-CTP has been reported to be an important determinant of ara-C toxicity.[17, 18] Therefore, we measured the degradation of ara-CTP by taking samples at 0, 30, 60 and 120 min after resuspension in fresh medium. The degradation of ara-CTP-Ep consisted of an early rapid phase and a slower second phase. The half life of the latter was approximately 1.5 h, similar to that of ara-CTP accumulation following incubation with ara-C (Fig. 6). In addition, the inclusion of 10 $\mu M$ dipyridamole at time zero significantly retarded the degradation of both ara-CTP-Ep and ara-CTP produced by ara-C.

## DISCUSSION

The injection of nucleotide into viable cells is considered to be a useful tool for the study of naturally occurring nucleosides or their synthetic analogs, since it can bypass several biochemical events necessary for the activation of the nucleoside. In fact, microinjection of nucleotide has been accomplished by Wawra.[19] The difficulty of the technique and the limited applicability to adherent cells are the problems of this method. Recently, Sokoloski *et al.* demonstrated the transfer of deoxyribonucleoside triphosphates into suspension cultures by electroporation.[10] Furthermore, the introduced nucleotide, ara-CTP, was shown to act as an inhibitor of DNA synthesis.[10] In the present investigation, we extended the previous study and examined the toxicity and metabolism of an electroporated nucleotide, ara-CTP.

First, we tried to find the optimal conditions for electroporation of ara-CTP into human leukemia cell lines. The efficiency of ara-CTP introduction depended, as expected, on the cell type, electrical pulse voltage and extracellular ara-CTP concentration on electroporation. When the extracellular ara-CTP concentration was constant (5 m*M*), ara-CTP-Ep was a function of pulse voltage, with a maximum at 700 V. Notably, electroporated cells at 550 V without addition of ara-CTP exhibited approximately 80–90% of the cell growth of unelectroporated cells after three days, suggesting that recovery from metabolic disturbance induced by electroporation, if any, had been achieved easily after the transfer of the cells into the RPMI-medium at 37°C. The generation of ara-CTP following incubation with ara-C was a saturable process with a plateau at 10 $\mu M$.[13, 20] However, electroporation made it possible to accumulate at least five times more ara-CTP, which is obviously an advantage for further metabolic studies.

The injected ara-CTP exhibited biological activity, i.e. cytotoxicity, in a dose-dependent manner, which has been demonstrated here for the first time. Although a direct comparison is difficult, ara-CTP-Ep and ara-CTP accumulated by incubation with ara-C had similar inhibitory effects on cell growth, as depicted in Figs. 2 and 3. This represents further evidence that electroporation of ara-CTP is a good model for the study of ara-C metabolism. Interestingly, CdR, but not three other deoxyribonucleosides, rescued the cells from the cytotoxicity of ara-CTP-Ep. The biochemical mechanism of this protection needs further study, since CdR is consid-

ered to work as a modulator of ara-C toxicity through competition at the level of CdR kinase.[21]

The importance of retention of intracellular ara-CTP has been shown in both *in vitro*[17, 18] and clinical situations.[22] These findings led us to investigate the rate of ara-CTP degradation in electroporated cells. The half-life of ara-CTP accumulated following incubation with 10 $\mu M$ ara-C was about 1.5 h, which is in the range of previous reports.[17, 18] In contrast, ara-CTP-Ep was degraded rapidly during the first 30 min followed by a slower step with a half-life of 1.5 h, which was independent of the initial ara-CTP-Ep concentration. Finally, dipyridamole, a potent inhibitor of ara-C transport,[23] has been reported to retard the degradation of ara-CTP through inhibition of drug efflux.[24] This is also the case for the degradation of ara-CTP-Ep, which may indicate that one of the main catabolic pathways of ara-CTP-Ep is ara-CTP→ara-CMP →ara-C in HL-60 cells.

In conclusion, the electroporation of nucleotides is a promising method for the study of their metabolism for the following reasons: (i) this method is simple and reproducible, (ii) it can be applied to cells in suspension, (iii) the electroporated nucleotide has biological activity, and (iv) the metabolic behavior is similar to that of the accumulated nucleotide following incubation of cells with the corresponding nucleoside.

ACKNOWLEDGMENTS

We thank Dr. Alice S. Cary (Kyoto Baptist Hospital) for her pertinent advice on the manuscript. This work was supported in part by a grant from the Fujiwara Memorial Foundation.

*(Received July 25, 1990/Accepted September 27, 1990)*

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