Differential incorporation of 1-β-D-arabinofuranosylcytosine and 9-β-D-arabinofuranosylguanine into nuclear and mitochondrial DNA

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Abstract The anti-leukemic nucleoside analogs 1-β-D-arabinofuranosylcytosine (araC) and 9-β-D-arabinofuranosylguanine (araG) are dependent on intracellular phosphorylation for pharmacological activity. AraC is efficiently phosphorylated by deoxycytidine kinase (dCK). Although araG is phosphorylated by dCK in vitro, it is a preferred substrate of mitochondrial deoxyguanosine kinase. We have used autoradiography to show that araC was incorporated into nuclear DNA in Molt-4 and CEM T-lymphoblastoid cells as well as in Chinese hamster ovary cells. In contrast, araG was predominantly incorporated into mitochondrial DNA in the investigated cell lines, without detectable incorporation into nuclear DNA. These data suggest that the molecular targets of araG and araC may differ.

Key words: Nucleoside analog; Nucleoside kinase; 1-β-D-Arabinofuranosylcytosine; 9-β-D-Arabinofuranosylguanine; Mitochondrial DNA synthesis

1. Introduction

1-β-D-Arabinofuranosylcytosine (araC, Cytosar) is commonly used in combination chemotherapy of acute myeloid and lymphoid leukemias. Similar to other nucleoside analogs, araC is dependent on intracellular phosphorylation for pharmacological activity. The initial and rate-limiting phosphorylation step is catalyzed by deoxycytidine kinase (dCK). AraC-MP is further phosphorylated by UMP-CMP kinase and nucleoside diphosphate kinases to araC-TP, which is incorporated into DNA and subsequently induce cell death [1]. AraC's guanine congenitor, 9-β-D-arabinofuranosylguanine (araG) is, similarly to araC, cytotoxic to lymphoid cells. However, araG is selectively cytotoxic towards T-lymphoblasts compared to B-lymphoblasts whereas araC exhibits equal toxicity toward T- and B-cells [2–4]. The selective T-lymphocyte toxicity of araG suggests that the compound may be useful in therapy of T-cell malignancies. However, the poor water solubility and complicated chemical synthesis of araG have long prevented it from being evaluated in clinical trials. Recently, a water soluble prodrug of araG, 2-amino-6-methoxypurine arabinoside (GW506U78), was developed [5], and initial clinical trials showed promising results in treatment of several lymphoid malignancies of T-cell lineage [6,7]. AraC and araG are substrates of dCK in vitro, and it is proposed that both nucleoside analogs are phosphorylated by dCK in vivo [2,8]. However, araG is a very poor substrate of dCK, exhibiting a K_m 100-fold higher than the K_m of araC [8,9]. In contrast, araG is an efficient substrate of deoxyguanosine kinase (dGK) [9]. A major difference between dCK and dGK is their subcellular location: dCK is located in the cytosol or nucleus whereas dGK is located in the mitochondria [10–12]. Accordingly, araC and araG may be phosphorylated in different subcellular compartments depending on which nucleoside kinase catalyzes the phosphorylation. There is evidence that the mitochondrial deoxyribonucleotide pool is separated from the cytosolic/nuclear pool [13,14]. Thymidine and the pyrimidine nucleoside analog 5-bromo-2′-deoxyuridine are incorporated into mitochondrial, but not nuclear, DNA in cells deficient in expression of cytosolic thymidine kinase 1 but with retained expression of mitochondrial thymidine kinase 2 [14,15]. These studies suggest that nucleosides and nucleoside analogs phosphorylated in the mitochondria may become trapped in this subcellular compartment. Because of the different subcellular location of dCK and dGK, we decided to study the incorporation of araC and araG into nuclear and mitochondrial DNA. In summary, we showed that araG is predominantly incorporated into mitochondrial DNA whereas araC was incorporated into nuclear DNA.

2. Materials and methods

2.1. Cell culture

The dCK-deficient Chinese hamster ovary (CHO) cell line was a gift from Dr. W. Plunkett. Molt-4 and CEM T-lymphoblast cell lines were gifts from Dr. J. Balzarini. The CHO cells were cultured in McCoy 5 A modified medium, the CEM cells and the Molt-4 cells were cultured in RPMI 1640. All medium was supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The stably transfected CHO cell line expressing dCK fused to the green fluorescent protein was generated as described [10].

2.2. Autoradiography

The cells were cultured on poly-L-lysine-coated chamber slides (Nunc, Inc.) for 24 h. Cells were labeled with 0.25 μM [^{3}H]thymidine (DuPont), 0.3 μM [^{3}H]araC or 0.7 μM [^{3}H]araG (Moravek Biochem) for 6–20 h. The slides were rinsed with phosphate-buffered saline, fixated 10 min in methanol:acetic acid (3:1), and washed three times with ice-cold 10% TCA, once with water and once with methanol. The slides were coated with Hypercoat photoemultion (Amersham) and exposed 1–3 weeks at 4°C. The autoradiographs were developed using D-11 developer (Kodak).

2.3. Cell proliferation assays

The stably transfected cells were plated in 96-well microtiter plates at a density of $\approx 2\times10^3$ cells/well. After 24 h, araC (Sigma) or araG (a gift from Prof. J. Balzarini, Leuven, Belgium) were added at in-

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Abbreviations: dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; araC, 1-β-D-arabinofuranosylcytosine; araG, 9-β-D-arabinofuranosylguanine; CHO, Chinese hamster ovary

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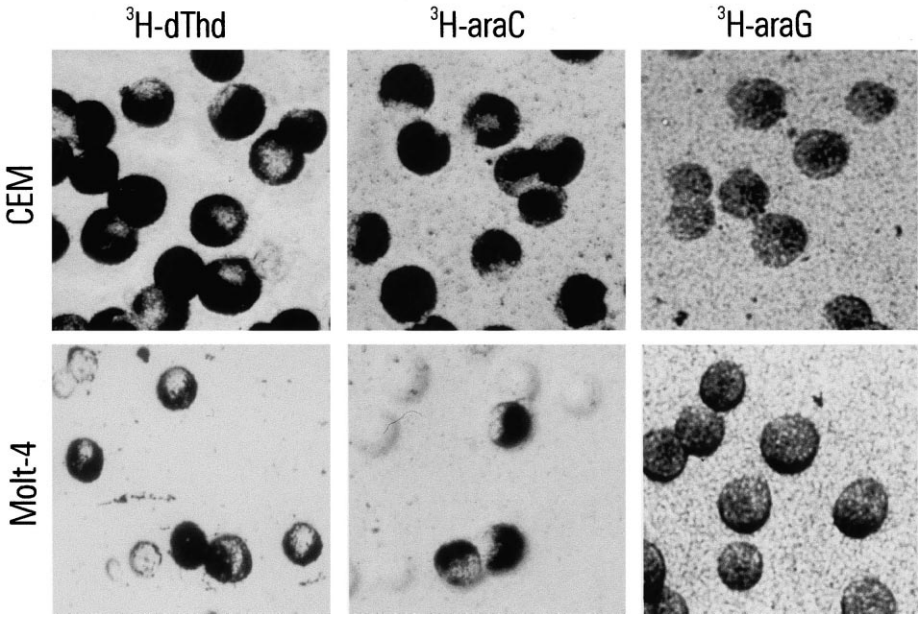

Fig. 1. Autoradiography of CEM and Molt-4 T-lymphoblast cell lines incubated with [^{3}H]dThd, [^{3}H]araC or [^{3}H]araG.

dicated concentrations. Cell survival was determined by the metrotetrazolium assay (Cell Proliferation kit I, Boehringer Mannheim) after 4 days of drug exposure.

3. Results

We incubated the T-lymphoblast cell lines CEM and Molt-4 with radiolabeled dThd, araC and araG. The nucleosides incorporated into DNA were detected in situ by autoradiography (Fig. 1). CEM and Molt-4 cells incubated with [^{3}H]dThd exhibited, as expected, efficient incorporation of the nucleoside analogs into nuclear DNA, as indicated by a dark staining in the part of the cells corresponding to the nuclear region. Cells incubated with araC showed similar autoradiography patterns as the cells incubated with dThd, indicating incorporation of araC into nuclear DNA. In contrast, cells incubated with araG showed a dotted autoradiography pattern distributed throughout the cells indicating incorporation of the nucleoside analog into mitochondrial DNA. In a few cells, a darker border was observed close to the plasma membrane. However, by examining a large number of cells incubated with araG, we were unable to find cells that showed autoradiography patterns similar to those observed for dThd or araC. These data suggested that araG was predominantly incorporated into mitochondrial DNA whereas araC was mainly incorporated into nuclear DNA of the lymphoblast cell lines.

We have previously transfected a mutant dCK-deficient CHO cell line with a plasmid expressing the cDNA of human

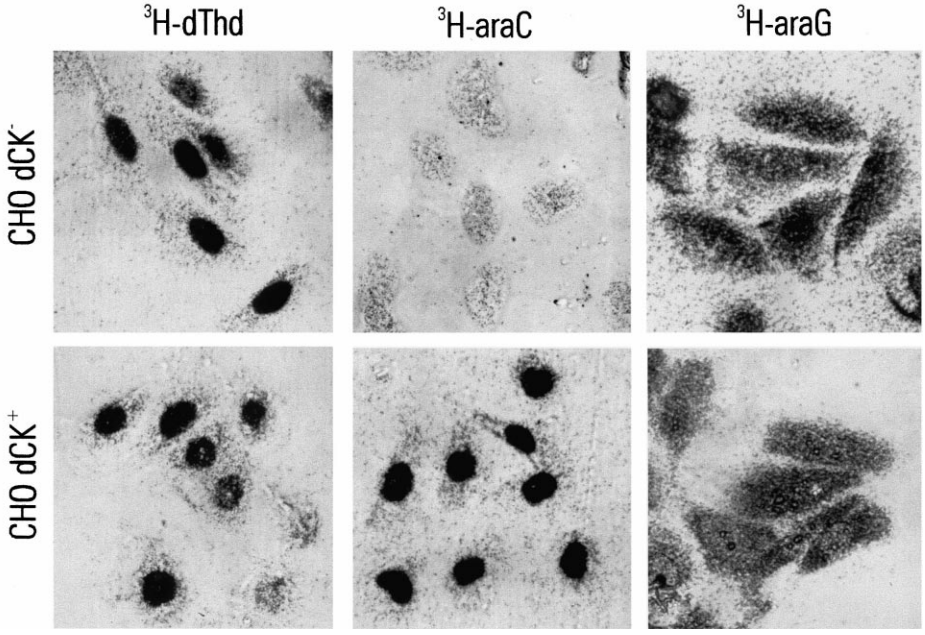

Fig. 2. Autoradiography of dCK-deficient (dCK$^-$) and dCK-expressing (dCK$^+$) CHO cells incubated with [^{3}H]dThd, [^{3}H]araC and [^{3}H]araG.

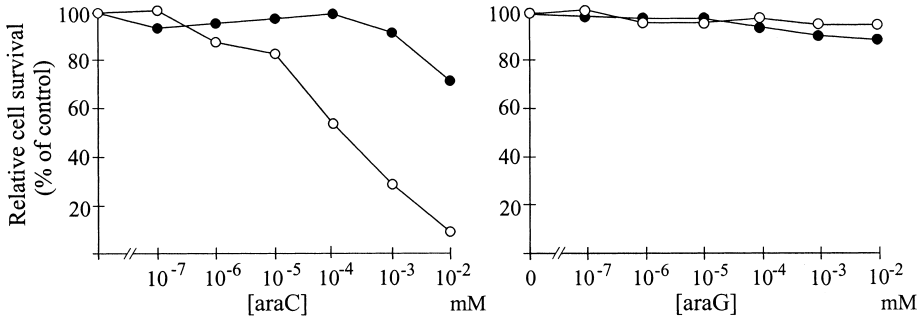

Fig. 3. Sensitivity of dCK-deficient (●) and dCK-expressing (○) CHO cells to the nucleoside analogs araC and araG.

dCK [10]. We used the transfected and untransfected CHO cells to further study the differential incorporation of araC and araG into nuclear and mitochondrial DNA. Autoradiography of CHO cells incubated with [^{3}H]dThd showed as expected dThd incorporated into nuclear DNA independently of dCK expression (Fig. 2). A faint dotted autoradiography pattern was detected in the cytoplasm of the cells incubated with [^{3}H]dThd, representing dThd phosphorylation by mitochondrial thymidine kinase 2, and its subsequent incorporation into mitochondrial DNA [13,14]. No incorporation of araC into either nuclear or mitochondrial DNA was detected in the dCK-deficient cells, whereas the cells reconstituted with dCK expression efficiently incorporated [^{3}H]araC into nuclear DNA. CHO cells incubated with [^{3}H]araG showed a dotted irregular autoradiography pattern distributed throughout the cells, indicating that the nucleoside analog was incorporated into mitochondrial DNA. We were unable to detect any cells that incorporated araG into nuclear DNA. The araG autoradiography pattern was similar in both untransfected dCK-deficient cells and the cells reconstituted with dCK expression.

The exclusive incorporation of araG into mitochondrial DNA in CHO cells, independently of dCK expression, suggests that araG is predominantly phosphorylated by mitochondrial dGK in these cells. We further determined the araC and araG sensitivity of the CHO cell lines with and without dCK expression (Fig. 3). The dCK-deficient cells were resistant to both araC and araG within the tested drug concentration range. Expression of dCK restored sensitivity to araC but no increase in araG sensitivity was observed.

4. Discussion

It is generally believed that cytotoxic nucleoside analogs exert their pharmacological effects by interfering with nuclear DNA replication and repair. We have in the present study shown that the anti-leukemic nucleoside analog araG was incorporated into mitochondrial DNA without detectable incorporation into nuclear DNA in the investigated cancer cell lines. In contrast, the cytosine analog araC was only incorporated into nuclear DNA. The different incorporation of araC and araG into nuclear and mitochondrial DNA can be explained by the intramitochondrial phosphorylation of araG catalyzed by mitochondrial dGK. Once phosphorylated, the nucleoside analog becomes trapped in the mitochondria and cannot be exported to the nuclear/cytosolic deoxyribonucleotide pool. This hypothesis is supported by the exclusive mitochondrial DNA incorporation of thymidine and 5-bromo-2′-deoxyuridine phosphorylated by mitochondrial thymidine kinase 2 [14,15]. Another possible explanation would be that araG is phosphorylated in the cytosolic/nuclear compartment by dCK, and that the phosphorylated araG subsequently was imported to the mitochondria. However, if araG-TP should have been present at high levels in the cytosolic/nuclear dNTP pool, it should have become incorporated into nuclear DNA as well. In summary, we believe that predominant incorporation of araG into mitochondrial DNA is due to the activity of dGK as the major araG phosphorylating enzyme in vivo.

AraG is selectively cytotoxic towards T-lymphoblasts compared to B-lymphoblasts whereas araC exhibits equal toxicity towards T- and B-cells [2–4]. The molecular basis of the different pharmacological profiles of araC and araG is not elucidated, but several studies show that T-cells accumulate higher levels of araG-TP than B-cells, whereas araC-TP levels are similar in cells of both B- and T-cell lineages [3,4]. These findings suggest that the metabolism of araG is different between T- and B-cells, and that this difference constitutes a possible explanation for the selective cytotoxicity. The total level of araG phosphorylating activity in crude cell extracts does however not differ between B- and T-cell lineages [4]. Based on these findings, it is tempting to speculate that the difference in araG-TP accumulation between T- and B-cells might be due to differences in mitochondrial uptake or intramitochondrial metabolism of araG.

The incorporation of araG into mitochondrial DNA suggests that its pharmacological actions may be caused by interference with mitochondrial DNA replication. However, we cannot exclude that a low level of araG is phosphorylated by dCK and incorporated into nuclear DNA, although we were not able to detect it by the autoradiography method used in the current study. Recently, Rodriguez and Gandhi reported a predominant incorporation of araG in S-phase CEM cells, and they showed that the S-phase cells exhibited highest sensitivity to araG-induced apoptosis [16]. These data suggest that at least part of the cytotoxic effects caused by araG are due to its incorporation into nuclear DNA during its replication in the S-phase. These data, however, do not contradict that araG may have effects on mitochondrial DNA as well. Overexpression of the mitochondrial dGK enhances the sensitivity of cancer cell lines to cytotoxic nucleoside analogs [12] and these data show that the intramitochondrial phosphorylation of araG can mediate cytotoxic effects. Furthermore, a mouse model of purine nucleoside phosphorylase deficiency has been shown to lose both dCK and dGK expression [17], suggesting that the phosphorylation of deoxyguanosine and its analogs can mediate toxic effects when phosphorylated either in the cytosolic/nuclear compartment by dCK or

when phosphorylated in the mitochondria by dGK. Several pyrimidine nucleoside analogs used in anti-viral therapy can damage mitochondrial DNA, cause mitochondrial dysfunction, and subsequent cell death [18]. Adverse effects associated with mitochondrial DNA damage range from neuropathies and myopathies to severe multi-organ failure. There are so far few clinical studies on the adverse effects of araG, but neuropathy is reported as the dose-limiting toxicity [7]. It will be important to find out whether this toxicity is due to mitochondrial dysfunction.

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References

[1] Kufe, D., Spriggs, D., Egan, E.M. and Munroe, D. (1984) Blood 64, 54–58.
[2] Cohen, A., Lee, J.W.W. and Gelfand, E.W. (1983) Blood 61, 660–666.
[3] Shewach, D.S. and Mitchell, B.S. (1989) Cancer Res. 45, 6498–6502.
[4] Verhoef, V. and Fridland, A. (1985) Cancer Res. 45, 3646–3650.
[5] Lambe, C.U., Averett, D.R., Paff, M.T., Reardon, J.E., Wilson, J.G. and Krenitsky, T.A. (1995) Cancer Res. 55, 3352–3356.
[6] Gandhi, V., Plunkett, W., Rodriguez, C.O., Nowak, B.J., Du, M., Ayres, M., Kisor, D.F., Mitchell, B.S., Kurtzberg, J. and Keating, M.J. (1998) J. Clin. Oncol. 16, 3607–3615.
[7] Aguayo, A., Cortes, J.E., Kantarjian, H.M., Beran, M., Gandhi, V., Plunkett, W., Kurtzberg, J. and Keating, M.J. (1999) Cancer 85, 58–64.
[8] Johansson, M. and Karlsson, A. (1995) Biochem. Pharmacol. 50, 163–168.
[9] Zhu, C., Johansson, M., Permert, J. and Karlsson, A. (1998) Biochem. Pharmacol. 56, 1035–1040.
[10] Johansson, M., Brismar, S. and Karlsson, A. (1997) Proc. Natl. Acad. Sci. USA 93, 11941–11945.
[11] Hatzis, P., Al-Madhoon, A.S., Jullig, M., Petrakis, T.G., Eriksson, S. and Talianidis, I. (1998) J. Biol. Chem. 273, 30239–30243.
[12] Zhu, C., Johansson, M., Permert, J. and Karlsson, A. (1998) J. Biol. Chem. 273, 14707–14711.
[13] Bestwick, R.K., Moffett, G.L. and Mathews, C.K. (1982) J. Biol. Chem. 257, 9300–9304.
[14] Berk, A.J. and Clayton, D.A. (1973) J. Biol. Chem. 248, 2722–2729.
[15] Davis, A.F. and Clayton, D.A. (1996) J. Cell Biol. 135, 883–893.
[16] Rodriguez Jr., C.O. and Gandhi, V. (1999) Cancer Res. 59, 4937–4943.
[17] Snyder, F.F., Jenuth, J.P., Dilay, J.E., Fung, E., Lightfoot, T. and Mably, E.R. (1994) Biochim. Biophys. Acta 1227, 33–40.
[18] Lewis, W. and Dalakas, M.C. (1995) Nat. Med. 1, 417–422.