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QUARTERLY

Cytostatic and cytotoxic effects of (*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine on a solid tumor and a leukemia cell line✪★

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(*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine (FMdC), a deoxycytidine analog displaying a very high toxicity toward a variety of solid tumor cell lines and xenografts, is activated intracellularly by deoxycytidine kinase (dCK). We have compared cytotoxicity of FMdC towards a human promyeolocytic leukemia line HL-60 and a human colorectal carcinoma line COLO-205. Despite dCK activity being by far the highest in cells of lymphoid origin, the effects of FMdC were detectable at the lowest drug concentration only in a solid tumor cell line, and at higher concentrations they were qualitatively similar in the two tumor lines (increased cell protein content, cell cycle block and apoptosis). Apparently, low dCK activity in solid tumor cells sufficiently activates FMdC to yield cytotoxic effects, while high dCK activity in leukemia cells does not increase its cytotoxicity.

(*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine (FMdC, Fig. 1) is a pyrimidine nucleoside analog displaying very high toxicity toward a variety of solid tumor cell lines and xenografts. It inhibits growth and triggers apoptotic cell death of mammary, prostate, cervical and colorectal cancers, and gliomas [1–8]. When this compound was synthesized, its property of inhibiting ribonucleotide reductase (RR) activity in tumor cells of L1210 leukemia bearing mice has been recognized [9]. Later, its cytotoxicity was suggested to result from the

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Abbreviations: FMdC, (*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine; dCK, deoxycytidine kinase; DAPI, 4′,6′-diaminophenylindole; RR, ribonucleotide reductase; SAHH, *S*-adenosyl-L-homocysteine hydrolase; HL-60, human promyelocytic leukemia line; COLO-205, human colorectal carcinoma line.

action of FMdC triphosphate as a DNA chain terminator in the DNA polymerase-catalyzed DNA chain elongation [10]. There is some evidence that both mechanisms cooperate in mediating drug cytotoxicity [11], but their relative contributions are unknown.

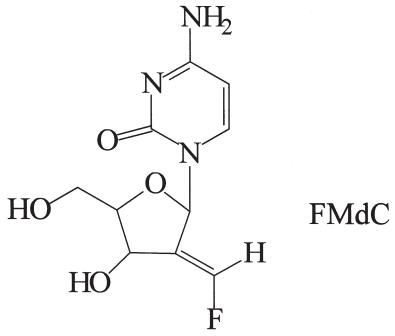

Figure 1. The structure of (*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine (FMdC).

Both RR inhibition and DNA chain termination by a nucleoside analog require its previous intracellular activation by phosphorylation, to diphosphate [12], and to triphosphate, respectively. The first step of FMdC phosphorylation is thought to be catalyzed by deoxycytidine kinase (dCK) [7], the enzyme known to phosphorylate a variety of cytotoxic and antiviral nucleoside analogs [13].

In contrast to FMdC, other dCK-phosphorylated cytotoxic nucleosides such as deoxyadenosine analogs cladribine (2-chloro-2′-deoxyadenosine, 2-CdA) and fludarabine (2-fluoro-arabinoadenosine), display toxicity almost exclusively restricted to cells of lymphoid origin [14]. Cladribine, for example, is highly toxic toward both normal and malignant lymphocytes [15], but appears to have no activity toward peripheral solid tumors *in vitro* [16], or *in vivo* [17]. This lymphoselectivity is explained by the uniquely high dCK activity in cells of lymphoid origin [18] enabling several hundred-fold accumulation of cladribine phosphates in susceptible cells [19].

Since FMdC is activated by the lymphospecific enzyme dCK, it could be expected to be particularly active towards cells of lymphoid origin. In the present study we compared the cytostatic and cytotoxic effects of this compound on a human lymphoid (promyeolocytic leukemia HL-60) and a human solid tumor (colorectal carcinoma COLO-205) cell lines, which supposedly vastly differ in the cellular dCK activity.

MATERIALS AND METHODS

Chemicals. FMdC was synthesized by Drs. M. Bretner and K. Felczak, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw) according to the slightly modified method of McCarthy *et al.* [9]. Chemical identity of the substance was confirmed by proton NMR by prof. L. Kozerski, Drug Institute (Warsaw). We used 4′,6-diamidino-2-phenylindole (DAPI), sulforhodamine 101 and propidium iodide from Molecular Probes (Eugene, OR, U.S.A.); PIPES disodium salt and Triton X-100 from Sigma-Aldrich (Poznań, Poland); Annexin V kit from Pharmingen (Torrey Pines, CA, U.S.A.); and culture media-MEM, RPMI 1640 and phosphate buffered saline (PBS) from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland); FCS from Gibco.

Cells and cultures. Cell lines were purchased from the American Type Culture Collection and were maintained routinely as a suspension culture (HL-60) in RPMI 1640 supplemented with 20% FCS or as a monolayer (COLO-205) in MEM supplemented with 10% FCS. The culture media contained also 1% L-glutamine, 0.5 g/l D-glucose, 1% non-essential amino acids and 1% antibiotic-antimycotic solution (all from Sigma- Aldrich). All experiments were performed on cultures in the exponential phase of growth.

The stock solution of FMdC (1 mg/ml in 0.9% aqueous NaCl) was diluted and added to the cell cultures in a constant volume of 10 μl per 1 ml of culture medium to obtain final concentrations of FMdC equal to 10, 100, 1000,

and 10 000 nM. The cultures were exposed to the drug for 6 and 24 h. All experiments were performed in triplicates.

Measurements. Flow cytometric determinations of DNA and protein content in the cells were performed using a FACS-Vantage flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.) equipped with a dual wavelength (UV and blue) argon ion laser (Innova Enterprise) and a set of dichroic mirrors and interference filters appropriate to measure blue, green, and red fluorescence. Data were acquired and analysed using a Cell-Quest software (Becton-Dickinson, San Jose, CA, U.S.A.). Cell cycle parameters were calculated from DNA histograms using Mac-Cycle software (Phoenix Flow Systems, San Diego, CA, U.S.A.).

RESULTS

Changes in cell cycle parameters for HL-60 leukemia cells are shown in Fig. 2. The cells responded with a concentration- and time-dependent cell-cycle block in G_1-phase. After longer exposition (24 h) the distortion of the DNA histogram shape was so heavy (due to the cell death) that the mathematical analysis of the histogram became unreliable (indicated by question marks on Fig. 2).

Figure 3 presents the cell-cycle changes for COLO-205 cells. It is evident that COLO cells are even more susceptible to FMdC than HL-60 cells. The G_1-phase block was visible even at a concentration of 0.01 μM (10-fold lower than the lowest FMdC concentration active for HL-60 cells).

Cell death was observed in both cell lines exposed to FMdC (data not shown). This process for COLO-205 was time-independent and only slightly concentration-dependent, and the fraction of dying cells (measured using the Annexin V/PI method) was approximately 50%. Death of HL-60 cells was time- and concentration-dependent, and following exposure to 10 μM for 24 h the fraction of dying cells reached 95%.

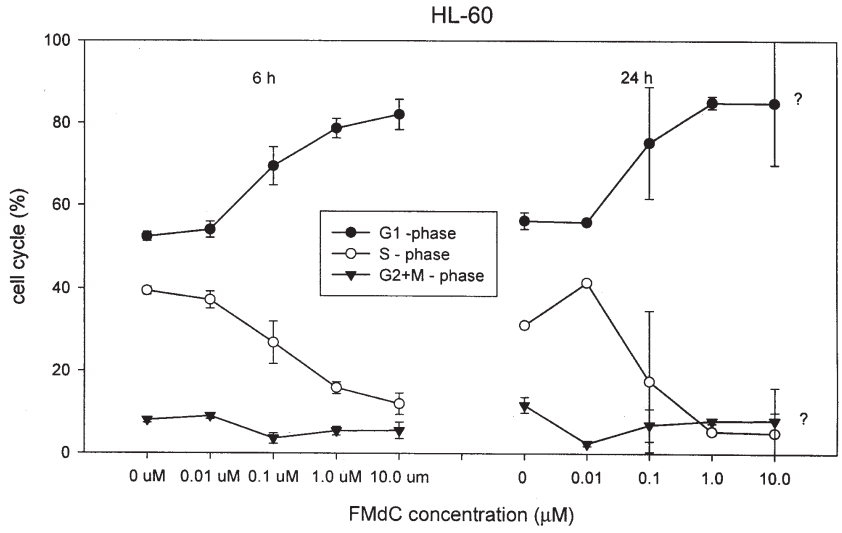

Figure 2. Changes in the cell cycle of HL-60 leukemia cell line.

Question marks denote values uncertain due to cell death.

We also found changes in the cellular protein content. For COLO-205 cells exposed to 0.1 μM and higher FMdC concentrations for 24 h it increased about two-fold. A similar picture was observed for HL-60 cells.

DISCUSSION

By far the highest dCK activity is encountered in cells of lymphoid origin. In solid tumors it is much lower, although usually somewhat higher than in normal cells [20]. Thus, the observation that cytostatic and cytotoxic

activity of FMdC at nanomolar concentrations toward a solid tumor is at least of the same magnitude as toward a leukemia line is a rather unexpected finding and it poses two questions. First, why FMdC is so active toward solid tumor cells supposedly containing relatively low dCK activity? Second, why high dCK activity in lymphoid cells does not accentuate cytotoxicity of this compound in the nanomolar concentration range?

The possibility to be considered is that the first step of FMdC phosphorylation is catalyzed not only by dCK, but also by other nucleoside kinase(s), e.g., one of the thymidine kinases. It is of note that FMdC is highly active against *Herpes simplex* viruses [21], presumably requiring activation by the viral thymidine kinase. Alternatively, it may be considered that FMdC is able to interfere with cell cycle and induce apoptosis without the prerequisite of phosphorylation. Van der Donk *et al.* [12] who studied inactivation of RR from *Escherichia coli* by FMdC diphosphate, considered a mechanism of inhibition analogous to that described by Yuan *et al.* [22] for the inhibition of *S*-adenosyl-L-homocysteine hydrolase (SAHH) by fluoride released from a sugar-fluorinated nucleoside (*Z*)-4′-5′-didehydro-5′-deoxy-5′-fluoroadenosine and inactivating this enzyme. Both SAHH and RR are NAD^+-requiring enzymes, and it is conceivable that the release of fluoride attached (directly or indirectly) to the sugar ring may constitute a common pattern of irreversible inhibition of NAD^+-requiring enzymes by some sugar-fluorinated nucleosides, which may be active irrespective whether these nucleosides are phosphorylated or not. Interestingly, both diphosphate and unphosphorylated forms of another RR inhibitor, gemcitabine (2′,2′-difluorodeoxycytidine) are active [23].

While none of the aforementioned mechanisms cannot be completly ruled out, their role in mediating FMdC cytotoxicity seems to be of minor importance. The excess of deoxycytidine has been shown to prevent FMdC cytotoxicity in human cervical carcinoma HeLa S3 cell line [7], and in our parallel study [24] the exposure to 0.1–10 μM FMdC in the presence of 1 mM deoxycytidine only slightly inhibited cell cycle progression of leukemia lines. More efficient catabolism of FMdC in lymphoid cells should also be ruled out, because this compound is resistant to cytidine deaminase [7]. Thus, the lack of cytotoxicity of the lowest doses of FMdC in a lymphoid tumor, compared with the response of a solid tumor with expected much lower dCK activity, requires another explanation.

Cladribine, a drug activated almost exclusively by dCK and active almost exclusively toward lymphoid tumors, slightly inhibits RR

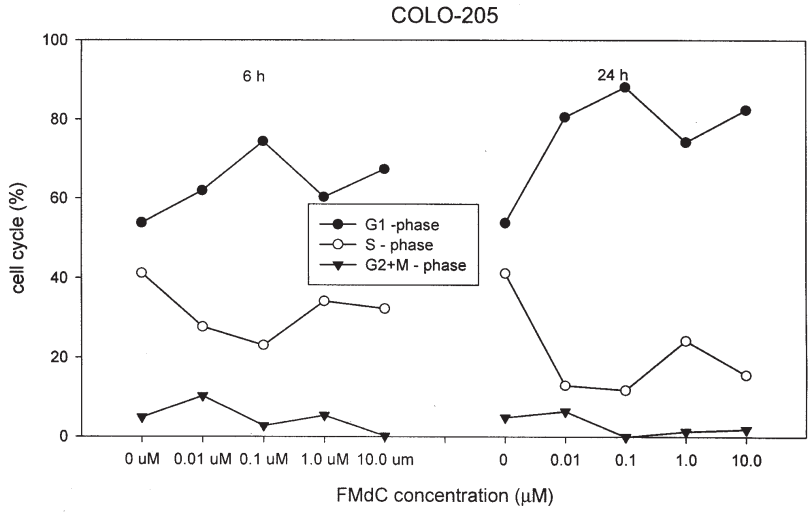

Figure 3. Changes in the cell cycle of COLO-205 colorectal carcinoma cell line.

[25]. It has been speculated that triphosphates of cladribine (as well as a related compound 2-chloro-2′-*arabino*-fluorodeoxyadenosine), inhibit RR by interacting with the dATP binding site on its regulatory subunit [26]. Another dCK-activated halogenopurine, fludarabine, which contains fluor attached not to sugar but to the purine ring, is also active exclusively against lymphoid malignancies [14]. In contrast, gemcitabine which contains two fluors on the sugar ring has a clinically useful activity against many non-lymphoid tumors [27] despite its being quickly deaminated. Furthermore, substitution of hydrogen with fluor in the 2′-*arabino* position of cladribine broadens its spectrum of cytotoxicity, and 2-chloro-2′-*arabino*-fluoro-deoxyadenosine is active against both leukemias [15, 28], and colon and mammary tumors [29, 30].

We conclude that the presence of fluor(s) on the sugar ring rather than very efficient phosphorylation mediated by high cellular dCK activity, is a prerequisite of cytotoxicity of sugar-fluorinated deoxynucleosides against solid tumors. In non-lymphoid cells, intracellular accumulation of these compounds appears to be sufficient to achieve cytotoxic effects even at relatively low dCK content.On the other hand, lymphoid cells may be less dependent on RR activity and, consequently, less affected by RR inhibition, possibly because they preferentially utilize salvaged nucleosides rather then those yielded from deoxyribonucleosides' reduction, as it has been shown in the case of deoxycytidine by Xu *et al.* [31].

REFERENCES

1. Bitonti, A., Dumont, J.A., Bush, T.L., Cashman, E.A., Cross-Doersen, D.E., Wright, P.S., Matthews, D.P., McCarthy, J.R. & Kaplan, D.A. (1994) Regression of human breast tumor xenografts in response to (*E*)-2′-deoxy-2′-(fluoromethylene) cytidine, an inhibitor of ribonucleoside diphosphate reductase. *Cancer Res.* **54**, 1485–1490.

2. Bitonti, A.J., Bush, T.L., Lewis, M.T. & Sunkara, P.S. (1995) Response of human colon and prostate xenografts to (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine, an inhibitor of ribonucleotide reductase. *Anticancer Res.* **15**, 179–182.

3. Kanazawa, J., Takahashi, T., Gomi, K. & Okabe, M. (1995) Antitumor activity of (*E*)-2′-deoxy- 2′-(fluoromethylene)cytidine, an inhibitor of ribonucleotide reductase (abstract). *Proc. AACR* **36**, A2416.

4. Piepmeier, J.M., Rabidou, N., Schold, S.C., Jr, Bitonti, A.J., Prakash, N.J. & Bush, T.L. (1996) *In vitro* and *in vivo* inhibition of glioblastoma and neuroblastoma with MDL-101731, a novel ribonucleoside diphosphate reductase inhibitor. *Cancer Res.* **56**, 359–361.

5. Ross, B.D., Boklye, K. & Chenevert, T.L. (1994) Assessment of the growth and treatment of experimental intracranial gliomas using magnetic resonance imaging and spectroscopy. *Sixth International Symposium on Pediatric Neuro-Oncology,* Houston, TX, p. 28.

6. Sun, L.Q., Li, Y.X., Guillou, L., Mirimanoff, R.O. & Coucke, P.A. (1997) Antitumor and radiosensitizing effects of (*E*)-2′-deoxy-2′-(fluoromethylene) cytidine, a novel inhibitor of ribonucleoside diphosphate reductase, on human colon carcinoma xenografts in nude mice. *Cancer Res.* **57**, 4023–4028.

7. Takahashi, T., Nakashima, A., Kanazawa, J., Yamaguchi, K., Akinaga, S., Tamaoki, T. & Okabe, M. (1998) Metabolism and ribonucleotide reductase inhibition of (*E*)-2′-deoxy-2′-2′-(fluoromethylene)cytidine, MDL 101,731, in human cervical carcinoma HeLa S3 cells. *Cancer Chemother. Pharmacol.* **41**, 268–274.

8. Wright, P.S., Cross-Doersen, D., Th'ng, J.P.H., Guo, X.W., Crissman, H.A., Bradbury, E.M., Montgomery, L.R., Thompson, F.Y., Loudy, D.E., Johnston, J.O. & Bitonti, A.J.

(1996) A ribonucleoside reductase inhibitor, MDL 101,731, induces apoptosis and elevates TRPM-2 mRNA levels in human prostate tumor xenografts. *Exp. Cell Res.* **222**, 54–60.

9. McCarthy, J.R., Matthews, D.P., Stemerick, D.M., Huber, E.W., Bey, P., Lippert, B.J., Snyder, R.D. & Sunkara, P. (1991) Stereospecific method to *E* and *Z* terminal fluoro olefins and its application to the synthesis of 2′-deoxy-2′-fluoromethylene nucleosides as potential inhibitors of ribonucleoside diphosphate reductase. *J. Am. Chem. Soc.* **113**, 7439–7440.

10. McCarthy, J.R. & Sunkara, P.S. (1995) Chemical and structural approaches to rational drug design (Weiner, D.B. & Williams, W.B., eds.) CRC Press, Boca Raton.

11. Yonetani, Y. & Mizukami, T. (1996) Kinetic analysis of inhibition of human ribonucleotide reductase and DNA polymerase alpha by a new anticancer agent (abstract). *Proc. AACR* **37**, A2764.

12. van der Donk, W.A., Yu, G., Silva, D., Stubbe, J., McCarthy, J.R., Jarvi, E.T., Matthews, D.P., Resvick, R.J. & Wagner, E. (1996) Inactivation of ribonucleotide reductase by (*E*)-2′-fluoromethylene-2′-deoxycytidine 5′-diphosphate: A paradigm for nucleotide mechanism-based inhibitors. *Biochemistry* **35**, 8381–8391.

13. Eriksson, S., Kiedraszuk, B., Munch-Petersen, B., Oberg, B. & Johansson, N.G. (1991) Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem. Biophys. Res. Commun.* **176**, 586–592.

14. Plunkett, W. & Gandhi, V. (1996) Pharmacology of purine nucleoside analogues. *Hematol. Cell Therapy* **38** (Suppl. 2), S67–S74.

15. Carson, D.A., Wasson, D.B., Esparza, L.M., Carrera, C.J., Kipps, T.J. & Cottam, H.B. (1992) Oral antilymphocytic activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2970–2974.

16. Larsson, R., Fridborg, H., Liliemark, J., Csoka, K., Kristensen, J., de la Torre, M. & Nygren, P. (1994) *In vitro* activity of 2-chlorodeoxyadenosine in primary cultures of human hematological and solid tumors. *Eur. J. Cancer* **30A**,1022–1026.

17. Weiss, G.R., Kuhn, J.G., Rizzo, J., Smith, L.S., Rodrigues, G.I., Eckardt, J.R., Burris, H.A., 3rd, Fields, S., VanDenBerg, K. & von Hoff, D.D. (1995) A phase I and pharmacokinetic study of 2-chlorodeoxyadenosine in patients with solid tumors. *Cancer Chemother. Pharmacol.* **35**, 397–402.

18. Arner, E.S. (1996) On the phosphorylation of 2-chlorodeoxyadenosine (CdA) and its correlation with clinical response in leukemia treatment. *Leuk. Lymph.* **21**, 225–231.

19. Liliemark, J. (1997) The clinical pharmacokinetics of cladribine. *Clin. Pharmacokin.* **32**, 120–131.

20. Eriksson, S., Arner, E., Spasokoukotskaja, T., Wang, L., Karlsson, A., Brosjo, O., Gunven, P., Juliusson, G. & Liliemark, J. (1994) Properties and levels of deoxynucleoside kinases in normal and tumor cells: Implications for chemotherapy. *Adv. Enzyme Regul.* **34**,13–25.

21. Bridges, C.G., Ahmed, S.P., Sunkara, P.S., McCarthy, J.R. & Tyms, A.S. (1995) The ribonucleotide reductase inhibitor (E)-2′(fluoromethylene)-2′-deoxycytidine (MDL 101,731): A potential topical therapy for herpes simplex virus infection. *Antiviral Res.* **27**, 325–334.

22. Yuan, C.-S., Yeh, J., Liu, S. & Borchardt, R.T. (1993) Mechanism of inactivation of *S*-adenosylhomocysteine hydrolase by (*Z*)-4′-5′-didehydro-5′-deoxy-5′-fluoroadenosine. *J. Biol. Chem.* **268**,17030–17037.

23. Baker, C.H., Banzon, J., Bollinger, J.M., Stubbe, J., Samano, V., Robins, M.J., Lippert, B., Jarvi, E. & Resvick, R. (1991) 2′-Deoxy-

2′,2′-difluorocytidine and 2′-deoxy-2′,2′-difluorocytidine-5′-diphosphates: Potent mechanism-based inhibitors of ribonucleotide reductase. *J. Med. Chem.* **34**, 1879–1884.

24. Skierski, J., Koronkiewicz, M. & Grieb, P. (1999) The effects of FMdC on cell cycle of some leukemia cell lines. *Cytometry* **37,** 1–6.

25. Griffig, J., Koob, R. & Blakley, L. (1989) Mechanisms of inhibition of DNA synthesis by 2-chlorodeoxyadenosine in human lymphoblastic cells. *Cancer Res.* **49**, 6923–6929.

26. Plunkett, W. & Saunders, P.P. (1991) Metabolism and action of purine nucleoside analogs. *Pharmacol. Ther.* **49**, 239–268.

27. Hansen, H.H. (1996) Gemcitabine — a review. *Ann. Oncol.* **7** (Suppl. 1), 29.

28. Xie, K.C. & Plunett, W. (1996) Deoxynucleotide pool depletion and sustained inhibition of ribonucleotide reductase and DNA synthesis after treatment of human lymphoblastoid cells with 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine. *Cancer Res.* **56**, 3030–3037.

29. Waud, W.R., Schmid, S.M., Harrison, S.D., Jr, Secrist, J.A., III & Montgomery, J.A. (1992) Preclinical and antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine. (Abstract) *Proc. AACR* **33**, 545.

30. Montgomery, J.A., Shortnacy-Fower, A.T., Clayton, S.D., Riordan, J.M. & Secrist, J.A., III (1992) Synthesis and biologic activity of 2′-fluoro-2-halo-derivatives of 9-β-D-arabinosylfluoroadenine. *J. Med. Chem.* **35**, 397–401.

31. Xu, Y.-Z., Huang, P. & Plunkett, W, (1995) Functional compartmentation of dCTP pools. Preferential utilization of salvaged deoxycytidine for DNA repair in human lymphoblasts. *J. Biol. Chem.* **270**, 631–637.