Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 440-445

PHOSPHORYLATION OF NUCLEOSIDES CATALYZED BY A MAMMALIAN ENZYME OTHER THAN URIDINE-CYTIDINE KINASE

Nahed K. Ahmed, Glen S. Germain and Arnold D. Welch

Division of Biochemical and Clinical Pharmacology
St. Jude Children's Research Hospital
Memphis, Tennessee 38101 U.S.A.

and

Alan R. P. Paterson, John H. Paran and Suh-er Yang
Cancer Research Unit (McEachern Laboratory)
University of Alberta
Edmonton, Alberta, Canada T6G 2H7

Received May 23,1980

SUMMARY

Cells of a 3-deazauridine-resistant line of cultured lymphoblastoid cells, RPMI 6410,were found to be essentially devoid of uridine-cytidine kinase activity. The resistant cells tolerated high concentrations of 3-deazauridine in culture, but retained sensitivity, relative to the parent cells, to 6-azauridine and 5-azacytidine, compounds previously recognized as substrates for uridine-cytidine kinase. Intracellular formation of the 5'-monophosphate esters of these analogs is a necessary step in expression of their cytotoxicity. The sensitivity of 3-deazauridine-resistant cells to 6-azauridine and 5-azacytidine is interpreted as indicating that phosphorylation of these analogs by some enzymatic route other than uridine-cytidine kinase may occur in these cells.

INTRODUCTION

6-AzaUrd, 5-azaCyd and other analogs of the physiological pyrimidine ribonucleosides have been recognized as substrates for Urd-Cyd kinase (EC 2.7.1.48) (1-3). Consistent with the observation that DU is a substrate for the purified Urd-Cyd kinase from P815 mastocytoma cells (2)* is our earlier report that 2 variants of the RPMI 6410 cell line (human lymphoblastoid B-cells (5,6)), selected for resistance to DU, were deficient in Urd-Cyd kinase (7). We report here that in cells of one of these lines, 6410/MP/DU, the Urd-Cyd kinase activity is <1% of that in cells

Abbreviations: 6-azaUrd, 6-azauridine; 5-azaCyd, 5-azacytidine; DU, 3-deazauridine; MP, 6-mercaptopurine; 6-azaUMP, 6-azauridine 5'-monophosphate; Urd, uridine; Cyd, cytidine.

*Unpublished results of E.P. Anderson, cited by Brockman et al. (4).

0006-291X/80/130440-06$01.00/0

of the parent line, 6410/0, yet sensitivity to 6-azaUrd and to 5-azaCyd is retained. This result is interpreted to mean that phosphorylation of these analogs, evidently a necessary event in the manifestation of their cytotoxicity, may occur by some means other than catalysis by Urd-Cyd kinase.

METHODS

Cells were cultured in RPMI 1640 medium (supplemented with 10% dialyzed fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml)), using loosely capped containers incubated at 37° in humidified air containing 5% CO_2; cell concentrations were kept below 5 x 10^5/ml. Preparations for the assay of Urd-Cyd kinase activity were obtained from the soluble fraction of cell homogenates (after centrifugation at 100,000 x g) by fractionation with ammonium sulfate; the material precipitated by ammonium sulfate at concentrations between 30% and 50% of saturation was dialyzed twice before assay of kinase activity,** which employed [5-^{3}H]Urd and discs of DEAE-cellulose paper (Whatman DE-81) to bind phosphorylated products (8).

Assay mixtures contained, in a final volume of 0.2 ml, Tris-HCl buffer (pH 7.5 to 7.7), 50 mM; β-mercaptoethanol, 10 mM; $MgCl_2$, 12.5 mM; ATP, 3 mM; [5-^{3}H]Urd (0.1 μCi), 2.5 mM; and 100 μg of enzyme fraction. Incubation was carried out for 10 min at 39° and the reaction was stopped and processed in the same manner as that previously described (8). Under these conditions, substrate concentrations were 8-12 times in excess of their K_m values.

RESULTS AND DISCUSSION

Cells of the parent line, 6410/0, were rich in Urd-Cyd kinase activity (30 nmoles of nucleotides/min/mg of protein in the enzyme fraction). The Lineweaver-Burk plots in Fig 1 demonstrate the dependence of the reaction rate on the concentrations of both Urd and ATP; the apparent K_m values for these substrates were 19.8 x 10^{-5}M and 37.5 x 10^{-5}M, respectively, at saturating concentrations of the second substrate.

In contrast to the high specific activity of the Urd-Cyd kinase-containing fraction isolated from cells of the parent line, 6410/0, the kinase activity of the corresponding fraction prepared from cells of the DU-resistant clone, was approximately 0.2 nmoles of nucleotides/

**Using other cell lines, this fraction, high in Urd-Cyd kinase activity, has contained negligible amounts of Urd phosphorylase and 5'-nucleotidase activities.

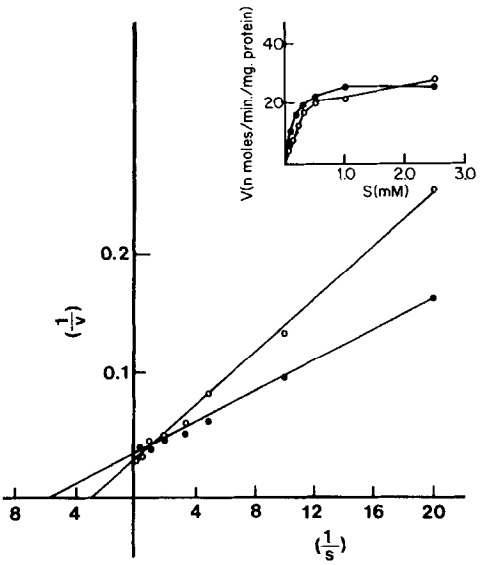

Fig. 1. Dependence of Urd-Cyd kinase activity on the concentration of Urd ● - ● and ATP o - o . The reaction was initiated by the addition of 100 µg of enzyme protein and incubated for 10 min at 39°. Ordinate: rate of Urd phosphorylation (nmole/min/mg protein); abscissa: mMolar concentrations of Urd or ATP. The results were subjected to linear regression analysis and the best-fit line was drawn for all points. The regression coefficient for K_mUrd was 0.998 and that for K_mATP was 0.997.

min/mg protein; this low activity was detectable only with high concentrations of the enzyme fraction (e.g., 1.0 to 1.5 mg of enzyme protein per assay).

Table 1 contrasts the response to DU of the DU-resistant cells, 6410/MP/DU, with that of the parental cell type, 6410/0; the IC_{50} value[+] for the inhibition by DU of the proliferation of the DU-resistant cells was about 1000-fold higher than that of the DU-sensitive cells (IC_{50}, 6 µM). This large difference in response of the 2 cell lines to DU was not seen when their responses to 6-azaUrd were compared. It is seen in Table 1 that the mutational change (deletion of Urd-Cyd kinase) that imparted high resistance to DU in 6410/MP/DU cells did not impart a correspondingly high resistance to 6-azaUrd or

[+]IC_{50}, the concentration of analog at which the cellular proliferation rate was 50% of that in the absence of the analog.

Table 1. DU-resistance in RPMI 6410/MP/DU cells*

	ID_{50}: concentration of analog (μM) at which the cellular proliferation rate was 50% of that in the absence of the analog+		
Cell Type	DU	6-AzaUrd	5-AzaCyd
6410/0	2.8	0.7	2.8
6410/MP/DU	3000	13	70

*Cell cultures in duplicate contained graded concentrations of the analogs indicated; concentrations of cells were determined daily.

+Proliferation rates (the number of doublings of the cell population in 72 hr of culture) in the absence of analogs ranged between 3.2 and 4.0.

to 5-azaCyd. Although 6410/MP/DU cells were distinctly less sensitive to 6-azaUrd than the parent cells, sensitivity to 6-azaUrd was about 230-fold greater than to DU. The fact that the mutation which altered so dramatically the cell response to DU did not cause parallel changes in response to 6-azaUrd was unexpected, because 6-azaUrd has long been recognized as a substrate for Urd-Cyd kinase (1-3,9). It is also apparent in Table 1, that relative to the parental cell type, 6410/MP/DU cells were more resistant to DU than to 5-azaCyd; this "retention" of 5-azaCyd-sensitivity in the Urd-Cyd kinase-deficient cells was also unexpected because the analog is a substrate for that enzyme (2,10,11).

Other experiments (data not shown) show that proliferation of 6410/MP/DU cells is inhibited by several pyrimidine ribonucleoside analogs, pseudoisocytidine, 5-hydroxyuridine and 5-aminouridine, which are not substrates for Urd-Cyd kinase.++

++Others (14,15) have suggested on the basis of studies with pseudoisocytidine-sensitive and -resistant lines of the murine mastocytoma P815, that the phosphorylation of pseudoisocytidine is catalyzed by Urd-Cyd kinase. This compound was not phosphorylated, however, by partially purified preparations of this enzyme from P815 cells, nor were 5-hydroxyuridine or 5-aminouridine phosphorylated by ATP and other preparations of Urd-Cyd kinase (N. K. Ahmed and A. D. Welch, unpublished results).

Table 2. Formation of 6-azaUrd 5'monophosphate (6-azaUMP) in RPMI 6410/MP/DU cells*

Compound	Cell content of 6-azaUrd and 6-azaUMP after these intervals of culture (pmoles/10^7 cells)			
	2 h	8 h	17 h	24 h
RPMI 6410/0 cells				
6-AzaUrd	0.6	1.7	0.9	0.6
6-AzaUMP	41.6	96.1	65.1	36.7
RPMI 6410/MP/DU cells				
6-AzaUrd	24.8	33.2	45.0	24.5
6-AzaUMP	247.2	77.9	64.5	50.4

*Cells were incubated for the intervals specified under culture conditions with [5-^{3}H]azaUrd (Moravek Biochemicals, City of Industry, Calif.); the 30-ml cultures contained 10^7 6410/0 or 6410/MP/DU cells and [5-^{3}H]6-azaUrd at 0.2 or 5.0 µM for the 6-azaUrd-inhibition of proliferation in cultures of these 2 cell types. To end incubation intervals, cultures were cooled in ice-water, cells were collected by centrifugation (800 x g, 5 min), washed once with cold 0.15 M NaCl and extracted with 0.4 M perchloric acid. Extracts were neutralized by the alamine-freon method of Khym (16), and concentrated by freeze-drying prior to thin-layer chromatography using a previously described system (polyethyleneimine cellulose thin layers developed with solvent system 1, ref. 12) to separate 6-azaUrd and its 5'-monophosphate. ^{3}H, which accompanied 6-azaUrd and 6-azaUrd 5'-monophosphate carriers, accounted for >95% of the ^{3}H-content of the samples applied to the chromatograms. The ^{3}H-content of chromatogram zones was determined by a combustion-liquid scintillation method employing a Packard Model 306 Sample Oxidizer.

The experiment summarized in Table 2 demonstrated that 6-azaUrd 5'-monophosphate was formed in both 6410/0 cells and 6410/MP/DU cells during intervals of culture with [5-^{3}H]6-azauridine at concentrations which would be expected to influence rates of cell proliferation. 6-AzaUMP was isolated from neutralized perchloric acid extracts of cells by chromatography on thin layers of polyethyleneimine cellulose (13) and was the only metabolite apparent in this analytical system.

Other experiments (data not shown) have indicated that the entry of uridine into 6510/MP/DU cells is mediated by a nitrobenzylthioinosine-sensitive transport system and the kinetic characteristics of the zero-

trans influx process are similar in both 6410/MP/DU and 6410/0 cell types.‡ DU, 6-azaUrd and 5-azaCyd evidently enter 6410 cells by way of the nucleoside-transport mechanism (12).

Taken together, these findings suggest the existence in 6410 cells of an enzyme activity other than Urd-Cyd kinase, which catalyzes the phosphorylation of (a) certain nucleosides that are substrates for the latter (6-azaUrd, 5-azaCyd), and (b) other nucleosides that do not appear to be substrates for Urd-Cyd kinase (pseudoisocytidine, 5-hydroxyuridine, 5-aminouridine). DU is evidently not a substrate for the unknown enzyme activity from 6410 cells.

Acknowledgements: This study was supported by the Whitehall Foundation, CA-21677 (N.C.I.), American Lebanese Syrian Associated Charities (ALSAC), and the National Cancer Institute of Canada (A.R.P.P.).

REFERENCES

1. Anderson, E. P. (1973) The Enzymes (Boyer, P. D., ed.), Vol. 9, pp. 49-96, Academic Press, New York.
2. Liacouras, A. S. and Anderson, E. P. (1975) Arch. Biochem. Biophys. 168, 66-73.
3. Čihák, A. and Rada, B. (1976) Neoplasma 23, 233-257.
4. Brockman, R. W., Shaddix, S. C., Williams, N., Nelson, J. A., Rose, L. M., and Schabel, F. M., Jr. (1975) Ann. N. Y. Acad. Sci. 255, 501-521.
5. Moore, G. E., Ito, E., Ulrich, K. and Sandberg, A. A. (1966) Cancer 19, 713-723.
6. Minowada, J., Nonoyama, M., Moore, G. E., Rauch, A. M. and Pagano, J. S. (1974) Cancer Res. 34, 1898-1903.
7. Mills-Yamamoto, C., Lauzon, G. J., and Paterson, A. R. P. (1978) Biochem. Pharmacol. 27, 181-186.
8. Ahmed, N. K. and Welch, A. D. (1979) Cancer Res. 39, 3102-3106.
9. Skold, O. (1960) J. Biol. Chem. 235, 3273-3279.
10. Lee, T., Karon, M. and Momparler, M. (1974) Cancer Res. 34, 2482-2488.
11. Drake, J. C., Stoller, R. G. and Chabner, B. A. (1977) Biochem. Pharmacol. 26, 64-66.
12. Paterson, A. R. P., Yang, S., Lau, E. Y. and Cass, C. E. (1979) Mol. Pharmacol. 16, 900-908.
13. Lauzon, G. J., Paran, J. H. and Paterson, A. R. P. (1978) Cancer Res. 38, 1723-1729.
14. Chou, T. C., Burchenal, J. H., Fox, J. J., Watanabe, K. A., Chu, C. K. and Philips, F. S. (1979) Cancer Res. 39, 720-728.
15. Zedeck, M. S. (1979) Biochem. Pharmacol. 28, 1440-1443.
16. Khym, J. X. (1975) Clin. Chem. 21, 1245-1252.

‡ E. Dahlig-Harley, C. E. Cass and A. R. P. Paterson, unpublished results.