Biochemical Pharmacology, Vol. 36, No. 19, pp. 3305–3307, 1987.
Printed in Great Britain.

0006-2952/87 $3.00+0.00

SHORT COMMUNICATIONS

Role of aldehyde dehydrogenase activity in cyclophosphamide metabolism in rat hepatoma cell lines*

(*Received* 14 *November* 1986; *accepted* 27 *March* 1987)

Cyclophosphamide (CP) is the most frequently used alkylating agent for treating a variety of hematologic and solid tumors [1–3]. However, the parent compound is inactive *in vitro*, indicating that biological activation is required for its therapeutic properties [4]. The activation process involves the formation of 4-hydroxycyclophosphamide (4-OH-CP) by hydroxylation of CP and further activation of 4-OH-CP via an aldehyde-containing intermediate, aldophosphamide, to phosphoramide mustard (PM) and acrolein, two putative cytotoxic metabolites of CP [5, 6]. It has been reported [4, 5, 7, 8] that both 4-OH-CP and aldophosphamide can be converted to carboxyphosphamide, a nontoxic and inactive CP metabolite. Thus, formation of carboxyphosphamide would result in less 4-OH-CP or aldophosphamide to be converted to PM and acrolein.

Because aldophosphamide contains an aldehyde group that can readily be oxidized to a carboxyl group, several investigators [4, 5, 7–9] have proposed that aldehyde-metabolizing enzymes, including aldehyde dehydrogenase (ALDH), and/or aldehyde oxidases may play a role in CP metabolism. Cox *et al.* [7] observed a qualitative correspondence between ALDH activity and CP deactivation ability in several rat tissues, including liver, kidney, gastrointestinal mucosa and spleen. This observation suggested a role of ALDH in CP metabolism. Hilton [10] reported that a strain of L1210 leukemic cells resistant to CP has approximately 200-fold higher NAD-dependent ALDH than an L1210-sensitive cell line. Sladek and Landkamer [11] showed that four ALDH inhibitors potentiate the cytotoxic action of the 4-hydroperoxy-CP against L1210/OAP and P388/CLA cells. In the presence of the inhibitors, sensitivity to CP can be fully restored in these two lines.

Regulation of ALDH activity during rat hepatocarcinogenesis is being studied intensively in our laboratory. A novel ALDH phenotype appearing in chemically-induced hepatocellular carcinomas is characterized by increased ALDH activity as judged by total enzyme activity determinations, histochemistry and immunochemistry. The tumor-specific ALDH activity is cytosolic and preferentially oxidizes aromatic aldehyde substrates using $NADP^+$ as coenzyme. The $NADP^+$-linked tumor ALDH also differs from the normal liver ALDH isozymes in a number of physical and functional properties [12, 13], including extreme sensitivity to the ALDH inhibitor disulfiram. In normal rat liver, ALDH activity is localized primarily to the mitochondrial and microsomal fractions, with little or no ALDH activity detectable in cytosol. At least four normal liver ALDH isozymes can be differentiated on the basis of substrate and coenzyme preference, substrate and coenzyme K_m, and sensitivity to inhibitors [14, 15]. Normal liver ALDHs are primarily NAD^+ dependent and prefer small aliphatic aldehydes as substrates. We have also shown that the $NADP^+$-linked ALDH activity of hepatocellular carcinomas is superimposed on the basal, normal liver ALDH phenotype [16].

A variety of hepatoma cell lines established from primary hepatocellular carcinomas produced in our laboratory (RLT-2M, RLT-3C, RLT-5G, RLT-9F) and established hepatoma cell lines obtained from other laboratories (HTC, H4-II-EC3, McA-RH 7777, JM_2) have been examined for their ALDH phenotype [17, 18]. HTC, JM_2, RLT-2M, RLT-3C, RLT-5G, and RLT-9F all express the tumor ALDH phenotype to varying degrees, whereas H4-II-EC3 and McA-RH 7777 do not. Thus, these eight cell lines offer a good model system to investigate the role of ALDH in CP metabolism. In this study, we attempted to establish a correlation between the ALDH activities of these hepatoma cell lines and their sensitivity to CP.

CP was obtained from the Sigma Chemical Co. (St. Louis, MO). 4-Hydroperoxy-CP was supplied by Dr R. F. Struck (Southern Research Institute, Birmingham, AL). CP was activated by incubation with rat liver S_9 fractions in the presence of an NADPH-generating system [NADP, 15 mg/ml; 50 μl of 0.1 M $MgCl_2$; glucose-6-phosphate dehydrogenase, 155 units/ml; glucose-6-phosphate, 15 mg/ml in 0.05 M Tris–KCl, 1.15% KCl (pH 7.4) buffer] [19]. The bioactivation mixture was incubated for 30 min at 37°, and the mixture was then filter-sterilized using a 0.22 μm Millipore filter. Aldophosphamide was prepared as described [20]. Briefly, 4-hydroperoxy-CP was dissolved in acetone and chemically treated with a 2-fold excess of triethylphosphite in −20°. The reaction produces 4-OH-CP which spontaneously becomes aldophosphamide. CP cytotoxicity was assessed by a colony formation efficiency assay [21]. Five hundred of the appropriate hepatoma cells were seeded into 60 mm petri dishes. Cells were incubated at 37° in an atmosphere of 5% CO_2 in air and maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-: streptomycin, 1% glutamine, and 1% non-essential amino acids [17]. After a 48-hr culture, bioactivated CP or 4-hydroperoxy CP was added to cells at final concentrations of from 10 to 50 μg/ml for CP or 0.1 to 1.0 μg/ml for 4-hydroperoxy-CP. Colony formation efficiency was scored after an additional 7 days of incubation. The dishes were rinsed three times with buffered saline, colonies were fixed with methanol, stained with 10% Giemsa, and counted. Aldehyde dehydrogenase activity was assayed at 25° by monitoring the change in A_{340} caused by NADH and NADPH production during the oxidation of aldehyde substrate in a modification of the assay described previously [22]. In this assay, activities measured with propionaldehyde and NAD in the range of 25–30 mIU/mg protein and 5–10 mIU/mg protein for benzaldehyde and NADP are indicative of the normal liver ALDH phenotype. Cell preparation for determination of ALDH activity was as reported [17].

All rat hepatoma cell lines were resistant to unactivated CP. There was no correlation between activated-CP cytotoxicity and the aldehyde dehydrogenase activity in the four hepatoma cell lines examined (Table 1). This was true using propionaldehyde, benzaldehyde or aldophosphamide as substrates with either NAD^+ or $NADP^+$ as coenzyme. The high ALDH activity lines, HTC and JM_2, were more

*Supported by Grant CA-21103 from the National Cancer Institute, Bethesda, MD.

Table 1. Inhibition of colony formation efficiency after cyclophosphamide treatment in rat hepatoma cell lines

Cyclophosphamide (μg/ml)	% Inhibition			
	HTC	McA-RH 7777	JM_2	RLT-9F
50	51*	13	25	84
40	42	ND†	0	64
30	5	14	0	53
20	11	12	0	33
10	0	0	0	10
Untreated control (colonies)‡	172	240	172	159
Substrate-coenzyme	Aldehyde dehydrogenase activity mIU/mg protein)			
Propionaldehyde-NAD†	112.5	14.0	155.0	27.1
Benzaldehyde-NADP†	466.6	2.5	680.7	316.7
Aldophosphamide-NAD†	6.8	5.2	17.2	10.3
Aldophosphamide-NADP†	3.2	0.2	2.7	1.9

*Data for at least three determinations. Values are expressed as the percent inhibition of colony formation compared to untreated controls.
†Not determined.
‡Data (100 % values) for at least three determinations.

sensitive to CP than the low ALDH activity line, McA-RH 7777. The apparent ID_{50} values were at least 2 to 4-fold lower for HTC and JM_2 than for McA-RH 7777 (data not shown). RLT-9F, a cell line that also possesses considerable $NADP^+$-linked ALDH activity, was extremely sensitive to CP.

To test the possibility that CP was not being completely bioactivated, we examined the cytotoxicity of 4-hydroperoxy-CP on the various hepatoma cell lines (Table 2). Again, among the lines examined, there was no correlation between ALDH actively determined with any of the three substrates and colony formation efficiency. The high $NADP^+$-dependent ALDH activity lines (HTC, RLT-2M, and RLT-9F) were extremely sensitive to 4-hydroperoxy-CP as were RLT-3C and RLT-5G, two lines of intermediate $NADP^+$-ALDH activity. McA-RH 7777 and H4-II-EC_3, the two lowest activity lines, as well as JM_2, a high activity line, were most resistant to 4-hydroperoxy-CP.

Interestingly, with aldophosphamide as substrate, JM_2, a line resistant to 4-hydroperoxy-CP, did have the highest ALDH activity. However, a cell line with barely detectable ALDH activity with aldophosphamide, H4-II-EC_3, was more resistant to 4-hydroperoxy-CP than five other cell lines with up to 5-fold more ALDH activity, all of which were extremely sensitive to 4-hydroperoxy-CP. Moreover, McA-RH 7777, the cell line most resistant to CP cytotoxicity, had only marginal ALDH activity with aldophosphamide as substrate.

Because we found no correlation between ALDH activity and CP cytotoxicity in the rat hepatoma cell lines tested, and because the line with the lowest ALDH activity, McA-RH 7777, was most resistant to CP, we examined the ALDH activity of the leukemia cell lines L1210R and L1210S. The NAD^+-linked ALDH activity of L1210R (18.8 mIU/mg protein with propionaldehyde) was approximately 6-fold greater than that of L1210S (2.7 mIU/mg protein), and

Table 2. Inhibition of colony formation efficiency after 4-hydroperoxy-cyclophosphamide treatment in rat hepatoma cell lines

4-Hydroperoxy-cyclophosphamide (μg/ml)	% Inhibition							
	HTC	McA-RH 7777	JM_2	H4-II-EC3	RLT-2M	RLT-3C	RLT-5G	RLT-9F
1	98*	60	11	82	100	100	100	100
0.5	89	37	0	44	98	98	99	100
0.1	19	3	0	20	0	21	28	63
Untreated control (colonies)†	102	56	118	252	131	198	318	133
	Aldehyde dehydrogenase activity mIU/mg protein)							
Propionaldehyde-NAD	112.5	14.0	155.0	10.2	38.7	10.5	8.1	27.1
Benzaldehyde-NADP	466.6	2.5	680.7	3.4	432.9	113.5	100.4	316.7
Aldophosphamide-NAD	6.8	5.2	17.2	2.2	5.9	6.4	5.4	10.2
Aldophosphamide-NADP	3.2	0.2	2.7	1.9	1.3	0.8	1.1	1.9

*Data for at least three determinations. Values are expressed as the percent inhibition of colony formation compared to untreated controls.
†Data (100 % values) for at least three determinations.

activated CP completely inhibited growth of L1210S without affecting the growth of L1210R. Interestingly, the NAD^+-dependent ALDH activity of L1210R was approximately equal to that of McA-RH 7777, the rat hepatoma cell line most resistant to CP. There were no differences in $NADP^+$-linked ALDH activity of L1210R and L1210S.

The lack of correlation between aldehyde dehydrogenase activity and CP cytotoxicity in rat hepatoma cell lines indicates that, at least for these cells, ALDH activity is not the major determining factor in CP cytotoxicity. Reports of correlations between aldehyde dehydrogenase activity and resistance to CP cytotoxicity have relied almost exclusively on the enhancing effects of putative ALDH inhibitors, especially disulfiram, on CP cytotoxicity. Among the several rat liver ALDH isozymes, only the micromolar K_m, NAD^+-dependent mitochondrial isozyme [12] and the various inducible cytosolic enzymes, including the $NADP^+$-linked isozyme [16], are disulfiram sensitive. In the present study, we have shown that there is no correlation between the NAD^+- or $NADP^+$-dependent ALDH activities with a variety of aldehyde substrates, including the putative active aldehyde-containing CP metabolite, aldophosphamide, and the resistance of these lines to CP cytotoxicity.

Hipkens *et al.* [23] have also presented data indicating that ALDH activity is not the determining factor in CP cytotoxicity. The survival rate of tumor-bearing C_3/StHa mice, in which a disulfiram-sensitive, NAD^+-dependent ALDH activity can be induced by phenobarbital, was no different after CP treatment with or without phenobarbital pretreatment.

While our data indicate that ALDH activity may not be the major factor in determining CP cytotoxicity for rat hepatoma cells, caution should be exercised in interpreting these results. It is possible that these eight cell lines differ in their abilities to bind and internalize CP or 4-hydroperoxy-CP [24]. A second possibility is that CP and 4-hydroperoxy-CP are differentially converted to 4-OH-CP in the various cell lines due to differences in glutathione peroxidase activity. An important role for glutathione peroxidase has been suggested for L12010R and L1210S cells [10]. A third possibility is that the differences in sensitivity of these cells to CP cytotoxicity are due to differences in the sensitivities of these cells to the active CP metabolites phosphoramide mustard or acrolein rather than the proportion of 4-OH-CP that becomes inactivated to carboxy-CP via aldophosphamide.

Any of these possibilities could result in differences in CP cytotoxicity irrespective of ALDH activity. However, the fact that seven of the eight cell lines examined differed less than 2-fold in their sensitivities to 4-hydroperoxy-CP but differed at least 5-fold in their ALDH activities measured with any substrate–coenzyme combination, including aldophosphamide and NAD^+, argues strongly against differential sensitivity to activated CP metabolites as a major confounding factor in interpretation of our results. The mechanism of JM_2 cell resistance to CP and especially 4-hydroperoxy-CP remains to be determined.

It is also possible that the ALDH activity of all of these hepatoma cell lines is high enough that the majority of 4-OH-CP is converted to carboxy-CP and that the differences in cytotoxicity are the result of secondary pathways of CP metabolism [25]. In this context it is interesting to note that, although liver is the major site of CP activation, there is little cytotoxicity to this organ [7], and hepatomas are generally refractive to CP treatment. Also, the tumors and tumor cell lines in which the best correlations between ALDH activity and CP cytotoxicity have been reported are of hematopoietic origin [10], a family of tissues which possess negligible aldehyde dehydrogenase relative to most other mammalian tissues.

In summary, the results reported here indicate that there is no correlation between the aldehyde dehydrogenase activity of rat hepatoma cell lines and their resistance to cyclophosphamide cytotoxicity. Therefore, at least for these cells, aldehyde dehydrogenase activity, *per se*, is not the determinant of the cytotoxicity of cyclophosphamide.

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REFERENCES

1. M. Colvin, in *Clinical Pharmacology of Antineoplastic Drugs* (Ed. H. M. Pinedo), p. 245. Elsevier/North-Holland, New York (1978).
2. R. B. Livingston and S. K. Carter, *Single Agents in Cancer Chemotherapy*, p. 25. Plenum Press, New York (1970).
3. S. K. Carter and R. B. Livingston, in *Principles of Cancer Treatment* (Eds. S. K. Carter, E. Glatstein and R. B. Livingston), p. 111. McGraw-Hill, New York (1982).
4. R. F. Struck, M. C. Kirk, M. H. Witt and W. R. Laster Jr., *Biomed. Mass Spectrom.* **2**, 46 (1975).
5. T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster and M. Jarman, *Biochem. Pharmac.* **23**, 115 (1974).
6. M. Colvin, R. B. Brundrett, M-N. Kan, I. Jardine and C. Fenselau, *Cancer Res.* **36**, 1121 (1976).
7. P. J. Cox, B. J. Phillips and P. Thomas, *Cancer Res.* **35**, 3755 (1975).
8. D. L. Hill, W. R. Laster and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
9. D. L. Hill, W. R. Laster, M. C. Kirk Jr., S. El Dareer and R. F. Struck, *Cancer Res.* **33**, 1016 (1973).
10. J. Hilton, *Cancer Res.* **44**, 5156 (1984).
11. N. E. Sladek and G. J. Landkamer, *Cancer Res.* **45**, 1549 (1985).
12. R. Lindahl, *Biochem. J.* **183**, 55 (1979).
13. R. Lindahl and R. N. Feinstein, *Biochim. biophys. Acta* **452**, 345 (1976).
14. R. Lindahl and S. Evces, *Biochem. Pharmac.* **33**, 3383 (1984).
15. S. O. C. Tottmar, H. Pettersson and K-H. Kiessling, *Biochem. J.* **135**, 577 (1973).
16. R. Lindahl and S. Evces, *J. biol. Chem.* **259**, 11991 (1984).
17. K-H. Lin, A. L. Winters and R. Lindahl, *Cancer Res.* **44**, 5219 (1984).
18. K-H. Lin, M. F. Leach, A. L. Winters and R. Lindahl, *In Vitro* **22**, 263 (1986).
19. D. S. Alberts, J. G. Einspahr, R. F. Struck, G. Bignami, L. Young, E. A. Surwit and S. E. Salmon, *Investl New Drugs* **2**, 141 (1984).
20. R. F. Struck, *Cancer Res.* **34**, 2933 (1974).
21. A. I. Grayzel and C. Beck, *Biochem. Pharmac.* **24**, 645 (1975).
22. R. Lindahl, *Biochem. J.* **164**, 119 (1977).
23. J. H. Hipkens, R. F. Struck and H. L. Gurtoo, *Cancer Res.* **41**, 3571 (1981).
24. I. Jardine, C. Fenselau, M. Appler, M-N. Kan, R. B. Brundrett and M. Colvin, *Cancer Res.* **38**, 408 (1978).
25. A. J. Marinello, S. K. Bansal, B. Paul, P. L. Koser, J. Love, R. F. Struck and H. L. Gurtoo, *Cancer Res.* **44**, 4615 (1984).

*This work was submitted by K-H. L. in partial fulfillment of the requirements for the Doctor of Philosophy degree in The Graduate School of The University of Alabama.

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