Biochemical Pharmacology, Vol. 33, No. 12, pp. 1867–1872, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

DEOXYRIBONUCLEIC ACID CROSSLINKING BY 4-HYDROPEROXYCYCLOPHOSPHAMIDE IN CYCLOPHOSPHAMIDE-SENSITIVE AND -RESISTANT L1210 CELLS*

JOHN HILTON
Pharmacology Laboratory, The Johns Hopkins Oncology Center, Baltimore, MD 21205, U.S.A.

(*Received* 27 *May* 1983; *accepted* 27 *October* 1983)

Abstract—4-Hydroperoxycyclophosphamide, a synthetic, activated form of cyclophosphamide, has been used to study DNA crosslinking in L1210 cell lines sensitive and resistant to cyclophosphamide. The time course of crosslink appearance and the proportion of inter-strand to DNA–protein crosslinks support the belief that phosphoramide mustard is the ultimate alkylating agent derived from cyclophosphamide. Cell survival and DNA crosslinking studies with a cyclophosphamide-resistant L1210 cell line indicate that resistance is associated with a failure of 4-hydroperoxycyclophosphamide to produce DNA crosslinks. The ability to reverse this situation by exposure of resistant cells to disulfiram points to a role of aldehyde dehydrogenase in this mechanism of cyclophosphamide resistance.

Our knowledge of the enzymic and chemical conversions of cyclophosphamide indicate that the ultimate alkylating species is predominantly phosphoramide mustard [1]. A number of observations imply, however, that the antitumor properties and tissues selectivity of cyclophosphamide cannot be accounted for solely by the production of phosphoramide mustard. Cyclophosphamide has a much higher therapeutic index than phosphoramide mustard in a number of tumor systems [2, 3] and has more marked immunosuppressive activity than phosphoramide mustard [4].

Similarly, "activated cyclophosphamide", generated by *in vitro* incubation with mixed function oxidase, is many times more cytotoxic than an equivalent amount (chemical alkylating units) of phosphoramide mustard [2, 5]. The observations by Takamizawa *et al.* [6], Colvin [5] and Brock [2] that "activated cyclophosphamide" and 4-hydroxycyclophosphamide are approximately equitoxic are consistent with the hypothesis that 4-hydroxycyclophosphamide or a closely related compound may have superior transport properties over phosphoramide mustard, the latter being released intracellularly. Others have implicated that 4-hydroxycyclophosphamide and its open ring tautomer aldophosphamide occupy a pivotal position in the therapeutic action of cyclophosphamide. Although Sladek [7] and Cox *et al.* [3] have proposed that the facile enzymic oxidative detoxification of 4-hydroxycyclophosphamide and aldophosphamide leads to the tissue selectively of cyclophosphamide, the hypothesis has not been satisfactorily substantiated and has produced conflicting evidence [8]. Basic to the above considerations is the belief that the ultimate cytotoxic action of cyclophosphamide resides in the introduction of crosslinks into cellular DNA.

Cyclophosphamide is one of the most frequently used antitumor agents in man. There is an increasing number of circumstances emerging, both experimental and clinical, where the necessity of hepatic activation is undesirable. To circumvent this requirement a small number of activated forms of cyclophosphamide have been synthesized and evaluated [9–13]. The agent in greatest use at this time is 4-hydroperoxycyclophosphamide.

Cellular resistance to antitumor agents is believed to be a major cause of failure in cancer chemotherapy [14]. Resistance to cyclophosphamide in mouse L1210 leukemia cells was described in 1973 by DeWys [15] who isolated lines of L1210 cells which survived escalating cyclophosphamide challenge *in vivo*. The nature of this resistance has remained obscure but was investigated here in order to more easily approach the more significant problem of cyclophosphamide resistance in human tumors.

The present study sets out to describe the DNA crosslinking properties of 4-hydroperoxycyclophosphamide and related agents in L1210 cells and presents evidence for a mechanism of cyclophosphamide resistance.

MATERIALS AND METHODS

The cyclophosphamide-resistant line can be traced to an original cyclophosphamide-resistant line produced by DeWys [15]. The L1210 cells were established in culture in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol. The cell lines grow with a doubling time of 12 hr and are lethal when re-introduced into BDF_1 mice.

In preparation for alkaline elution studies, the cells were grown in the presence of [^{14}C]thymidine (0.05 μCi/ml, 10^{-6} M) for 24 hr and transferred to isotope-free medium for at least 1 hr before treat-

---

* Supported by National Cancer Institute Grants CA-28765, CA-16783, and CA-06973.

ment with chemical agents. Exposure to crosslinking agents was for 30 min at 37° followed by incubation in drug-free medium for a period of time (usually 3.5 hr) during which DNA crosslinking was maximally expressed. The alkaline elution procedure was based closely on that described by Kohn *et al.* [16]. The cells were prepared for alkaline elution by transferring them to saline–EDTA (0.075 M NaCl, 0.024 M EDTA, pH 7.4) at 0°. Each cell suspension was split into four equal portions which received 100, 200, 300, and 400 rad gamma irradiation (^{137}Cs source) at 0°. The irradiated cells were kept at 0° until their loading onto 2 μm porosity filters (Acropor; Gelman Sciences, Inc., Ann Arbor, MI). The cells were lysed with 0.2% Sarkosyl in 2 M NaCl, 0.02 M EDTA, pH 10 (30 min), the filters were washed with 0.02 M EDTA, pH 10 (30 min), and the DNA was eluted with tetrapropylammonium hydroxide, 0.02 M EDTA, pH 12.1 (7.5 hr) at a flow rate of 0.04 ml/min. The eluate was collected as one fraction. The radioactivity remained on the filter and that in the eluate were determined by liquid scintillation counting and used to calculate the fraction of DNA retained on the filter.

Treatment of cells with disulfiram (dissolved in ethanol or dimethyl sulfoxide) was carried out for 60 min prior to the addition of, and during the exposure to, the alkylating agents. 4-Hydroperoxycyclophosphamide was synthesized by the ozonation of cyclophosphamide in the presence of hydrogen peroxide by the method described by Hohorst *et al.* [9]. 4-Hydroxycyclophosphamide was prepared from 4-hydroperoxycyclophosphamide by reduction with sodium thiosulfate [17]. Phosphoramide mustard (NSC-69945) was obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute. Nornitrogen mustard [bis(2-chloroethyl)-amine] was of reagent grade.

The dilute agar colony method of Chu and Fischer [18] was used to determine cell viability following exposure to chemical agents.

RESULTS

The alkaline elution analysis of DNA crosslinking was performed according to the method of Kohn *et al.* [16] with some modifications. With presently available peristaltic pumps (Gilson Medical Electronics Inc., Middleton, WI; Ismatec/Brinkmann Instruments, Westburg, NY) which produce highly reproducible flow rates in each channel, the requirement for internal standardization with ^{3}H-labeled cells is eliminated. Additionally, rather than constructing a detailed time course of DNA elution at a given titrating radiation dose, we have made one measurement at 7.5 hr elution in cells exposed to four doses of titrating radiation and have expressed the results in a manner similar to that described by Kohn *et al.* [19] where the fraction of DNA retained was plotted versus the titrating radiation. The ratio of the slopes of these lines for untreated and crosslinked DNA is used to generate a crosslink factor identical to that described by Ross *et al.* [20]. At concentrations of alkylating agent which produce up to 3 to 4 logs cell kill, the relationship between exposure to crosslinking agent and the resultant crosslink factor is approximately linear.

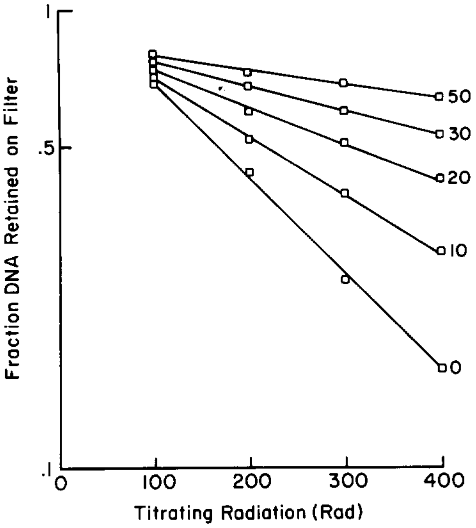

Fig. 1. Alkaline elution of DNA from L1210 cells exposed to various concentrations of 4-hydroperoxycyclophosphamide, for 30 min followed by 3.5 hr in fresh medium. Numbers adjacent to each curve indicate the concentration (μM) to which the cells were exposed.

Figure 1 depicts data typical of the alkaline elution analysis. Exposure of cells to increasing concentrations of 4-hydroperoxycyclophosphamide makes the DNA more refractory to elution, while increasing strand breakage through exposure to gamma radiation increases the elution. The elution of DNA from treated cells is conveniently expressed as a crosslink factor which is the ratio of DNA elution in untreated cells to elution in treated cells. Untreated cells thus have a nominal crosslink factor of 1.0.

Data from Fig. 1 expressed in terms of a crosslink factor (Fig. 2) demonstrate the near linearity of the response to increasing exposure to 4-hydroperoxycyclophosphamide over a 30-min period.

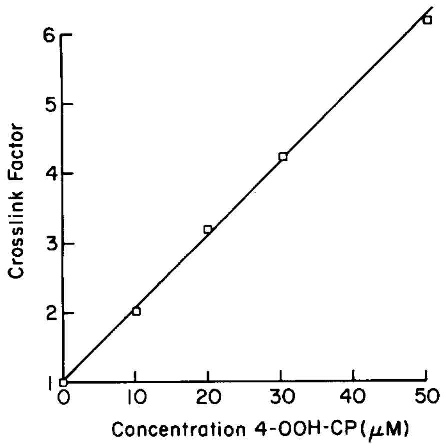

Fig. 2. Alkaline elution data from Fig. 1 expressed as crosslink factors produced at each concentration of 4-hydroperoxycyclophosphamide to which cells were exposed.

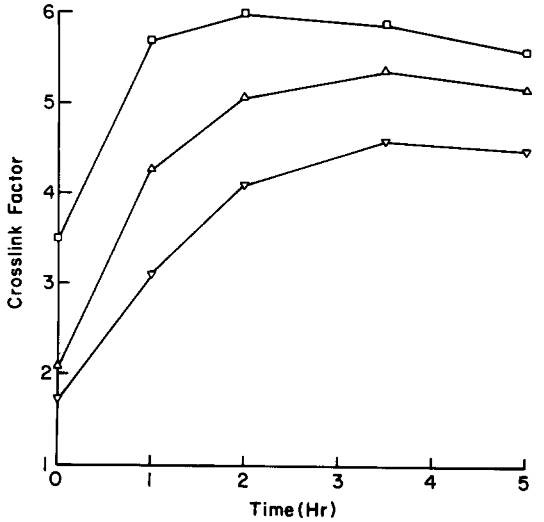

Fig. 3. Time course of appearance of crosslinks in DNA of L1210 cells exposed to 50 μM 4-hydroperoxycyclophosphamide (△), 200 μM phosphoramide mustard (▽), and 200 μM nornitrogen mustard (□) for 30 min. Cells were transferred to fresh medium and their DNA was analyzed for crosslinks after various time intervals.

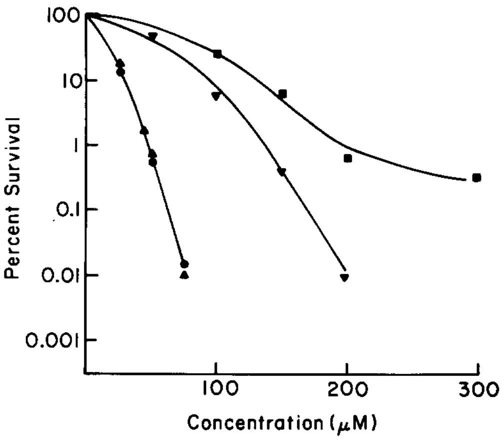

Fig. 4. Survival of L1210 (growth in soft agar) following a 30-min exposure to 4-hydroperoxycyclophosphamide (▲), 4-hydroxycyclophosphamide (●), phosphoramide mustard (▼), or nornitrogen mustard (■).

The time course of appearance of DNA crosslinks in L1210 cells exposed to a number of agents related to cyclophosphamide is shown in Fig. 3. In each case the cells were sampled at increasing time after a 30-min exposure to the alkylating agent. While cells exposed to phosphoramide mustard, 4-hydroperoxy- and 4-hydroxycyclophosphamide reached their maximal DNA crosslinking at 3 hr after removal of the agent, the cells exposed to nornitrogen mustard showed a conspicuously earlier maximum at about 2 hr. At longer times following a brief exposure to 4-hydroperoxycyclophosphamide, there was a gradual decline in DNA crosslinks (data not shown). Thus, cells exposed to 50 μM 4-hydroperoxycyclophosphamide for 30 min lose the greater part of their DNA crosslinks during the next 24 hr.

The introduction of a protease digestion step into the alkaline elution analysis allows the determination of the portion of DNA which is retained on the filter as a consequence of protein–DNA crosslinks. DNA which carriers covalently attached protein binds tenaciously to most filters used in the elution assay and is not eluted by the pH 12.1 buffer.

Table 1 contains crosslinking data from cells exposed to a variety of agents and analyzed by alkaline elution which include a protease K digestion step. The same cells were also analyzed without protease treatment. The DNA of cells pretreated with phosphoramide mustard, 4-hydroperoxy- and 4-hydroxycyclophosphamide exhibited similar behavior in the elution assay in that, when the protease digestion step was included, the crosslink factor fell to approximately half its original value, indicating that these agents produce a mixture of protein–DNA crosslinks and protease-insensitive interstrand crosslinks. In contrast, cells exposed to nornitrogen mustard exhibited no crosslinks when analyzed plus protease, indicating that all the nornitrogen mustard-induced crosslinks were of the protein–DNA variety.

In vitro cytotoxicity data for the crosslinking agents are shown in Fig. 4. The two activated

Table 1. Effect of digestion with protease K on alkaline elution of L1210 DNA from cells exposed to various alkylating agents*

Treatment	Crosslink factor	
	− Protease K	+
None	1.0	1.0
4-Hydroperoxycyclophosphamide (50 μM)	5.0	2.2
4-Hydroxycyclophosphamide (50 μM)	4.8	2.2
Phosphoramide mustard (200 μM)	5.3	2.5
Nornitrogen mustard (200 μM)	5.8	1.1

* L1210 cells were exposed to the agents at the concentrations shown for 30 min at 37° after which time they were transferred to drug-free medium at 37° for 3.5 hr. The extent of DNA crosslinking was determined by alkaline elution either with or without a 30-min digestion with protease K (0.5 mg/ml).

cyclophosphamide derivatives, 4-hydroperoxy- and 4-hydroxycyclophosphamide, had very similar toxicities and were about four times more cytotoxic than phosphoramide mustard. Nornitrogen mustard was the least toxic of the agents tested.

Up to this point the results describe crosslinking in L1210 cells sensitive to cyclophosphamide. Animals given 10^6 cyclophosphamide-resistant cells are incurable under any cyclophosphamide treatment schedule and die at the same time, or slightly before, tumor bearing animals which did not receive cyclophosphamide. For *in vitro* studies, resistant cells were established in long-term culture from cells passaged in mice. Survival studies *in vitro* reproduce the *in vivo* situation. Figure 5A which describes survival data for cyclophosphamide-sensitive and -resistant cells exposed to 4-hydroperoxycyclophosphamide indicates the sizeable difference in response of these two cell lines. The resistant cells required approximately four times the 4-hydroperoxycyclophosphamide exposure to produce equivalent toxicity found in sensitive cells. In the companion experiment to measure DNA crosslinking (Fig. 5B), the DNA of resistant cells showed little crosslinking even after a 30-min exposure to 200 μM 4-hydroperoxycyclophosphamide. The loss of viability in the resistant cells in the absence of extensive DNA crosslinking indicates the appearance of new toxicities unrelated to crosslinking at these unusually high exposures to 4-hydroperoxycyclophosphamide. In experiments similar to those in panels A and B of Fig. 5, 4-hydroxycyclophosphamide behaved similarly to the 4-hydroperoxy derivative in being much less toxic to cyclophosphamide-resistant cells than -sensitive cells and failing to produce DNA crosslinks in the DNA of resistant cells (data not shown).

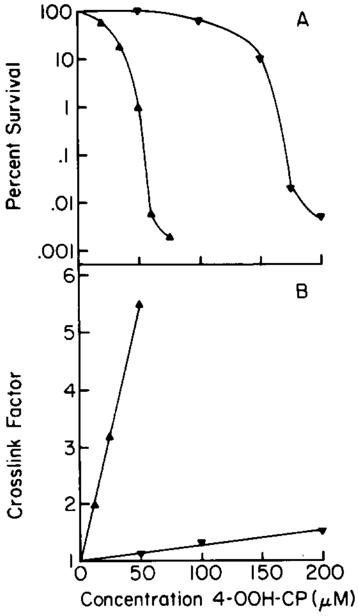

Fig. 5. Survival (A) and DNA crosslinking (B) in L1210 cells sensitive (▲) or resistant (▼) to cyclophosphamide following a 30-min treatment with 4-hydroperoxycyclophosphamide. Cells for crosslinking studies were returned to fresh medium for 3.5 hr before analysis by alkaline elution.

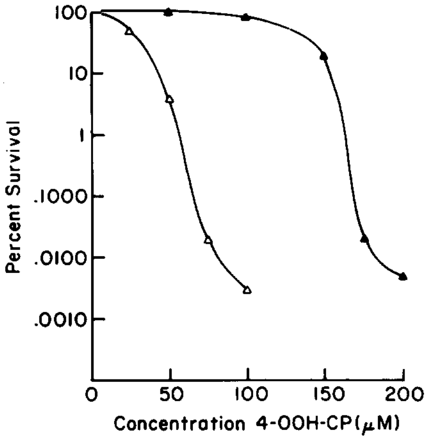

Fig. 6. Survival of cyclophosphamide-resistant L1210 cells following exposure to 4-hydroperoxycyclophosphamide (▲) for 30 min. One portion of the culture was pretreated with 10 μM disulfiram in dimethyl sulfoxide (DMSO) for 60 min before 4-hydroperoxycyclophosphamide treatment (△). The remaining half received an equal amount of DMSO.

A final series of experiments investigated the effect of the aldehyde dehydrogenase inhibitor disulfiram on the sensitivity of L1210 cells to activated cyclophosphamide (Fig. 6). Disulfiram was added to cultures for 60 min before 4-hydroperoxycyclophosphamide exposure (30 min) after which both agents were removed by washing and replacing the cells in fresh medium. Survival data revealed that a pretreatment of cyclophosphamide-resistant cells with 10 μM disulfiram was sufficient to render these cells sensitive to 4-hydroperoxycyclophosphamide. At this concentration, disulfiram alone showed no toxicity to either sensitive or resistant cells. Further control studies revealed no effect of this or higher concentrations of disulfiram on the response of cyclophosphamide-sensitive cells to 4-hydroperoxycyclophosphamide. Figure 6 indicates that disulfiram-treated cyclophosphamide-resistant cells are essentially indistinguishable from the sensitive cells.

DISCUSSION

The cyclophosphamide derivative 4-hydroperoxycyclophosphamide is in increasing use both experimentally and in a number of experimental clinical areas, especially in the purging of leukemic marrow prior to autologous marrow transplant [21]. A number of "activated cyclophosphamide" compounds based on 4-thioalkyl derivatives of cyclophosphamide have also been reported [10]. While these agents exhibit the major properties of activated cyclophosphamide in tissue culture cells, they are unusually toxic *in vivo* [11, 12]. 4-Hydroperoxycyclophosphamide is a relatively stable, non-alkylating, derivative of cyclophosphamide with a half-life in tissue culture medium at 37° of about 2 hr. It is actively transported into L1210 cells [22] and may be converted to 4-hydroxycyclophosphamide either by chemical reduction with sodium thiosulfate or triphenylphosphine or enzymically (unpublished observations). The product, 4-hydroxycyclophosphamide, is an aldehyde-positive compound with DNA crosslinking and cytotoxic properties indistinguishable from the 4-hydroperoxy derivative. 4-Hydroxycyclophosphamide is in equilibrium with the ring-opened aldophosphamide which spontaneously undergoes β-elimination to yield acrolein and phosphoramide mustard. Phosphoramide mustard is the only agent in this series with significant alkylating activity at physiological pH [1]. The role of nornitrogen mustard in the cytotoxic properties of cyclophosphamide is uncertain; it may be formed either by the hydrolysis of phosphoramide mustard or from 4-carboxyphosphamide [23].

The results in the present study describe DNA crosslinking and cell survival studies in mouse L1210 leukemia cells exposed to compounds related to activated cyclophosphamide. In general, there exists a correlation between the accumulation of DNA crosslinks and loss of cell viability. With occasional exceptions this correlation is typical of other studies with bifunctional alkylating agents [24]. Since the ultimate alkylating agent in the present experiments is believed to be phosphoramide mustard, the results may be interpreted in terms of factors affecting its intracellular levels.

Phosphoramide mustard itself, being charged at physiological pH, is likely to penetrate cell poorly and must thus be used in relatively high concentrations in order to produce DNA crosslinks or cell toxicity. 4-Hydroperoxycyclophosphamide, on the other hand, is actively transported into L1210 cells [22] and, compared to phosphoramide mustard, produces crosslinking and cell death at approximately one-fifth the concentration. Although the generation of an equal degree of DNA crosslinking requires a higher exposure to phosphoramide mustard than to 4-hydroperoxycyclophosphamide, the time course of crosslinking is the same for both agents. Similar responses to phosphoramide mustard and 4-sulfidocyclophosphamides have been reported [11] where maximum crosslinking was observed at about 4 hr following a brief exposure to the agents. It seems likely that the combination of active transport and facile conversion of 4-hydroperoxycyclophosphamide to the 4-hydroxy derivative by L1210 cells on the one hand, and the poor cellular entry of ionized phosphoramide mustard on the other, accounts for the 5-fold higher phosphoramide mustard concentrations required to produce a similar extent of crosslinking.

The origin of nornitrogen mustard is ill defined. It is found in small amounts in the plasma of patients receiving cyclophosphamide and is believed to be a product of hydrolysis of phosphoramide mustard and carboxyphosphamide. Its DNA crosslinking properties are unique amongst the nitrogen mustards thus far investigated in that it produces no interstrand DNA crosslinks. This inability is probably related to its low reactivity rather than to stereochemical limitations.

The equilibrium between 4-hydroxycyclophosphamide and aldophosphamide provides further opportunity to modulate intracellular phosphoramide mustard levels through the enzymic oxidation of potentially toxic aldophosphamide to inactive carboxyphosphamide. This mechanism has been proposed by Sladek [7] and Cox *et al.* [3] but has never been substantiated satisfactorily and has produced conflicting evidence for the role of aldehyde metabolism in the cellular toxicity of cyclophosphamide [8]. Our studies with the cyclophosphamide-resistant L1210 cells appear to be the first clear example of the role of aldehyde metabolism in the toxicity of cyclophosphamide.

Two features of the cyclophosphamide resistance in L1210 cells are noteworthy: the dimensions of the resistance and the low level of cross resistance with other alkylating agents [25]. *In vitro* the resistant cells require a 5-fold increase in 4-hydroperoxycyclophosphamide exposure to cause cell death equivalent to that in cyclophosphamide-sensitive cells. Furthermore, the shape of the survival curve is reminiscent of a titration phenomenon in that there exists a very large shoulder which, for the resistant cells, extends beyond the point where 5 logs of sensitive cells have been killed. Additionally, the low level of cross resistance with other alkylating agents suggests that neither transport, deactivation of the mustard moiety nor repair of DNA damage is likely to be a major determinant of cyclophosphamide resistance in these cells.

The studies with cells exposed to the aldehyde dehydrogenase inhibitor disulfiram suggest that the activity of aldehyde dehydrogenase between the cyclophosphamide-sensitive and -resistant cell lines is related to the cyclophosphamide resistance. In the absence of disulfiram it is proposed that the cyclophosphamide-resistant cells contain sufficient aldehyde dehydrogenase activity to continually channel aldophosphamide to carboxyphosphamide which is non-toxic. Only at very high aldophosphamide levels (high 4-hydroperoxycyclophosphamide exposures) would the capacity of the enzyme be exceeded and allow some aldophosphamide to β-eliminate to produce phosphoramide mustard. The sensitive cells which are proposed to have low aldehyde dehydrogenase activity are unable to divert aldophosphamide and thus suffer the consequences of phosphoramide mustard production. Inhibition of the aldehyde dehydrogenase by disulfiram makes the resistant cells become sensitive to 4-hydroperoxycyclophosphamide. While disulfiram and its reductive product diethyldithiocarbamate have a variety of effects related to their abilities to chelate transition metals and including their inhibition of carcinogenesis [26], the inhibition of aldehyde dehydrogenase remains the most likely mode of action relevant to the present studies.

Additional studies to determine the nature and activity of aldehyde metabolizing enzymes in cyclophosphamide-sensitive and -resistant tissues are in progress.

REFERENCES

1. M. Colvin, R. B. Brundrett, M-N. N. Kan, I. Jardine and C. Fenselau, *Cancer Res.* **36**, 1121 (1976).
2. N. Brock, *Cancer Treat. Rep.* **60**, 301 (1976).
3. P. J. Cox, B. J. Phillips and P. Thomas, *Cancer Res.* **35**, 3755 (1975).

4. L. L. Sensenbrenner, J. J. Marini and M. Colvin, *J. natn. Cancer Inst.* **62**, 975 (1979).
5. M. Colvin, *Proc. Am. Ass. Cancer Res.* **15**, 70 (1974)
6. A. Takamizawa, S. Matsumoto, T. Iwata, Y. Tochino, K. Katagiri, K. Yamaguchi and O. Shiratori, *J. med. Chem.* **18**, 376 (1975).
7. N. E. Sladek, *Cancer Res.* **33**, 1150 (1973).
8. J. H. Hipkens, R. F. Struck and H. L. Gurtoo, *Cancer Res.* **41**, 3571 (1981).
9. H-J. Hohorst, G. Peter and R. F. Struck, *Cancer Res.* **36**, 2278 (1976).
10. G. Peter and H-J. Hohorst, *Cancer Chemother. Pharmac.* **3**, 181 (1979).
11. L. C. Erickson, L. M. Romonas, D. S. Zaharko and K. W. Kohn, *Cancer Res.* **40**, 4216 (1980).
12. L. M. Romonas, L. C. Erickson, H. Ringsdorf and D. S. Zaharko, *Cancer Res.* **40**, 3704 (1980).
13. L. M. Romonas, L. C. Erickson, W. Kleese, K. W. Kohn and D. S. Zaharko, *Molec. Pharmac.* **19**, 331 (1981).
14. S. E. Salmon, A. W. Hamburger, B. Soehnlen, B. G. M. Durie, D. S. Alberts and T. E. Moon, *New Engl. J. Med.* **298**, 1321 (1978).
15. D. W. DeWys, *J. natn. Cancer Inst.* **50**, 783 (1973).
16. K. W. Kohn, L. C. Erickson, R. A. G. Ewig and C. A. Friedman, *Biochemistry* **15**, 4629 (1976).
17. A. Takamizawa, S. Matsumoto and T. Iwata, *Tetrahedron Lett.* 517 (1974).
18. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
19. K. W. Kohn, C. A. Friedman, R. A. G. Ewig and Z. M. Igbal, *Biochemistry* **13**, 4134 (1974).
20. W. E. Ross, R. A. G. Ewig and K. W. Kohn, *Cancer Res.* **30**, 1502 (1978).
21. M. Korbling, A. D. Hess, P. J. Tutschka, H. Kaizer, M. O. Colvin and G. W. Santos, *Br. J. Haemat.* **52,** 89 (1982).
22. W. R. Redwood, J. Hilton, M. Colvin and A. H. Owens, Jr., *Proc. Am. Ass. Cancer Res.* **23**, 169 (1982).
23. I. Jardine, C. Fenselau, M. Appler, M-N. Kan, R. B. Brundrett and M. Colvin, *Cancer Res.* **38**, 408 (1978).
24. K. W. Kohn, in *Methods in Cancer Research* (Eds. V. T. DeVita, Jr. and H. Busch), Vol. 16, p. 291. Academic Press, New York (1979).
25. F. M. Schabel, Jr., M. W. Trader, W. R. Laster, G. P. Wheeler and M. H. Witt, *Antibiot. Chemoth.* **23**, 200 (1978).
26. L. W. Wattenberg, *J. natn. Cancer Inst.* **60**, 11 (1978).