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Differential Cytotoxicity and DNA Cross-linking Produced by Polymeric and Monomeric Activated Analogues of Cyclophosphamide in Mouse L1210 Leukemia Cells

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SUMMARY

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4-*S*-(hexane-6-ol)-sulfidocyclophosphamide (monomer) and a compound composed of this derivative covalently bound to divinylether-maleic acid copolymer (DIVEMA) spontaneously hydrolyze *in vitro* with half-lives of hydrolysis of 9 and 48 min respectively, to form 4-hydroxycyclophosphamide. In turn, 4-hydroxycyclophosphamide further degrades to phosphoramide mustard with a half-life of 312 min. Sulfido-CP (cyclophosphamide) monomer, DIVEMA-sulfido-CP polymer, and phosphoramide mustard are qualitatively similar in producing DNA interstrand cross-links, as measured by alkaline elution, when incubated with mouse L1210 leukemia cells for 20 min. DNA interstrand cross-linking in L1210 cells increases for up to 6 hr following drug treatment and then decreases until it is completely removed within 18 hr for all three drugs. However, quantitatively more phosphoramide mustard (150 μM) is required than monomer (30 μM) to produce comparable amounts of cross-linking. An equivalent amount of monomer hydrolytically released by the polymer within 20 min is one-half as potent as monomer alone in producing cross-links. Similar concentration relationships are observed in cytotoxicity assays (L1210 soft agar colony formation). These *in vitro* data suggest that (a) the activated monomer or some degradation product prior to phosphoramide mustard is taken up by L1210 cells; (b) the polymer hydrolyzes extracellularly to a monomeric species before cellular uptake; and (c) tumor endocytotic uptake of polymer appears negligible in L1210 cells. *In vivo* antitumor studies with ascites L1210 in $B6D2F_1$ mice indicate that the monomer (200 mg/kg, LD_{10}) is cytotoxic to tumor cells (5 log tumor cell kill). However, the polymer at a dose equitoxic to the mice (100 mg/kg, LD_{10}) is less cytotoxic than monomer to tumor cells (1 log tumor cell kill).

INTRODUCTION

The binding of low molecular weight antitumor agents to polymers to enhance their anticancer action is a relatively novel approach in drug design that has recently met with some success (1–3). Ideally, the binding of a drug to a polymeric carrier may improve the therapeutic effect of the drug by (a) altering its pharmacological distribution (4); (b) providing sustained plasma levels of drug as a result of its slow release from the polymer (4); and (c) capitalizing upon selective endocytotic uptake of carrier-bound drug by tumor cells (5). Although biological macromolecules such as DNA and proteins may serve as drug carriers (6–8), synthetic copolymers such as divinylether-maleic acid offer the advantages of incorporating immunostimulating (9, 10) and antitumor properties (11, 12) into the carrier itself. The synthesis of a DIVEMA[1]-methotrexate drug has been reported (13) but, until recently, polymers consisting of an alkylating agent covalently linked to DIVEMA were unavailable.

Cyclophosphamide, a widely used drug in experimental and clinical cancer chemotherapy (14), has an undesir-

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[1] The abbreviations used are: DIVEMA, divinylether-maleic acid copolymer; monomer, 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide; CP, cyclophosphamide; polymer, DIVEMA-4-*S*-(hexane-6-ol)-sulfidocyclophosphamide; DMSO, dimethylsulfoxide.

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able side effect of being severely immunosuppressive. Because DIVEMA possesses immunostimulating as well as antitumor properties, it was reasoned that the covalent attachment of cyclophosphamide to DIVEMA might serve to minimize the immunosuppressiveness of the alkylating agent. However, cyclophosphamide requires metabolic activation to 4-hydroxycyclophosphamide by the liver before demonstrating antitumor activity (15). Since the 4-hydroxycyclophosphamide metabolite is a very unstable molecule, more stable 4-alkyl-sulfido-cyclophosphamide derivatives, which hydrolyze to 4-hydroxycyclophosphamide under physiological conditions, were used (16–18). These crystalline mercaptan derivatives lend themselves to covalent fixation to DIVEMA (19).

Although the mercaptan derivatives are potent antitumor agents (20), the enhanced toxicity *in vivo* (21) of these mercaptan derivatives linked to DIVEMA stimulated us to examine the hypothesis of tumor endocytotic polymer uptake. Therefore we have investigated the mechanism of action of 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide (monomer) and DIVEMA-4-*S*-(hexane-6-ol)-sulfidocyclophosphamide (polymer) *in vitro* at the cellular level on tumor cells. Both monomer and polymer spontaneously hydrolyze directly to 4-hydroxycyclophosphamide and subsequently to phosphoramide mustard; therefore they are expected to alkylate DNA *in vitro* without requiring metabolic activation. In these studies we examine the cytotoxicity and kinetics of DNA damage produced by the DIVEMA-sulfido-CP polymer, the sulfido-CP monomer, and phosphoramide mustard on mouse L1210 leukemia cells *in vitro*. The antitumor activity of these agents on ascites L1210 *in vivo* is also presented for comparative purposes.

MATERIALS AND METHODS

Cell culture and radioactive labeling. L1210 mouse leukemia cells were grown in spinner culture in Rosewell Park Memorial Institute 1630 medium supplemented with 20% heat-inactivated (56°, 30 min) fetal calf serum (Flow Laboratories, Rockville, Md.), 1 mM L-glutamine, penicillin, and streptomycin. Cultures utilized in drug experiments were maintained in an exponential growth phase at a density of 0.3–1.8 × 10^6 cells/ml. DNA was labeled by a 20-hr incubation at 37° of L1210 cells with either 0.02 μCi/ml of [^{14}C]thymidine (>56 m Ci/mmole, New England Nuclear Corporation, Boston, Mass.) or 0.05 μCi/ml of [^{3}H]thymidine (20 Ci/mmole, New England Nuclear Corporation), diluted with 10^{-6} M unlabeled thymidine. Fresh medium was added 0.5 to 1 hr prior to drug treatment by centrifuging the cells at 900 rpm at 37° and resuspending at a density of 1 × 10^6 cells/ml.

Drug treatments in vitro. Phosphoramide mustard (NSC-69945) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in sterile water immediately prior to treatment of cell cultures.

The activated CP analogs (Fig. 1) used in L1210 experiments, 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide, and the compound composed of this derivative covalently bound to divinylether-maleic acid copolymer, have half-lives of hydrolysis to 4-hydroxycyclophosphamide of 9

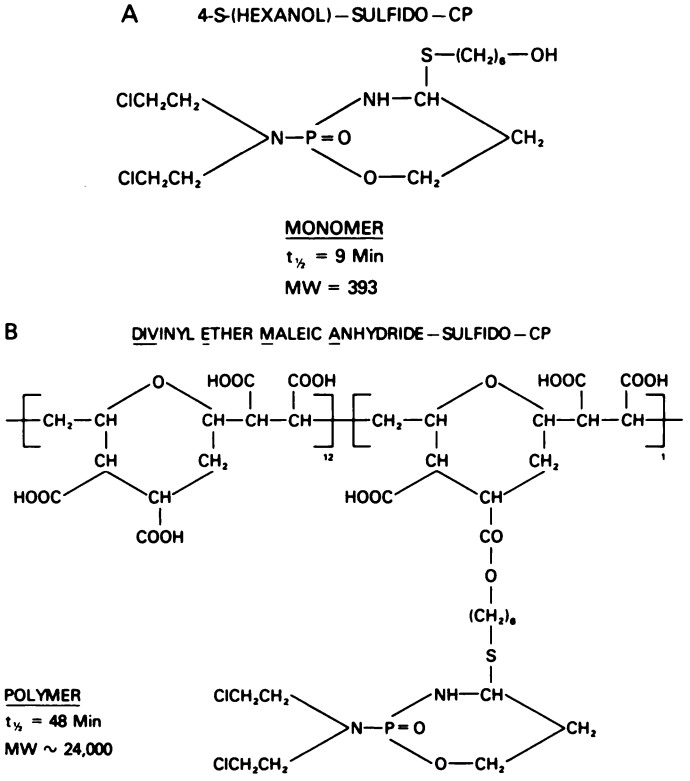

FIG. 1. *A, monomeric; B, polymeric derivatives of cyclophosphamide*

and 48 min, respectively. The drugs were kindly supplied by Drs. H. Ringsdorf and T. Hirano of the Institute of Organic Chemistry, Mainz University, Federal Republic of Germany. The DIVEMA-sulfido-CP polymer contained 10% by weight of sulfido-CP monomer. The DIVEMA (NSC-46015) used in the synthesis of the polymer was obtained from Dr. D. Breslow, Hercules Inc., Wilmington, Del., and had an average molecular weight of 2.3 × 10^4. Both monomer and polymer were first dissolved in sterile DMSO and then diluted 1:10 with sterile 0.1 M sodium phosphate buffer (pH 7.9) immediately prior to treatment of cell cultures. All drug treatments were carried out for 20 min at 37°. After treatment the drug was removed by washing the cells three times by centrifugation, and the cells were resuspended in fresh medium. Aliquots of cells were either placed in ice immediately and subsequently assayed by alkaline elution or kept in a dark incubator at 37° until aliquots were removed for assays.

Alkaline elution assays. The specific experimental procedures for the alkaline elution assay used in these experiments and the factors influencing DNA alkaline elution kinetics in general have been described in detail (22). The L1210 cells in this study were analyzed by a proteinase modification which minimizes the effect of DNA-protein cross-linking on the elution of DNA (23). A brief description of the alkaline elution method follows. Approximately 5 × 10^5 drug treated ^{14}C-labeled cells were mixed with a similar number of ^{3}H-labeled control cells and diluted with ice-cold 1630 medium. Prior to analysis, the cells on ice were irradiated with 300 R by two vertically opposed Philips RT-250 X-ray tubes, operating

at 250 Kev, to introduce a known frequency of DNA single-strand breaks. The cells were then diluted with 20 ml of ice-cold phosphate-buffered saline (0.15 M Nacl/0.014 M KH_2PO_4/0.086 K_2HPO_4), pH 7.4, and layered onto a polyvinyl chloride filter (pore size, 2 μm; diameter, 25 mm) (Millipore Corporation, Bedford, Mass.) using mild suction. Cells were immediately lysed with 5 ml of a solution containing 2% sodium lauryl sulfate, 0.1 M glycine, and 0.02 M EDTA, pH 10.0. The lysis solution was allowed to flow through by gravity, and, to increase deproteinization, 2 ml of lysis solution with proteinase K (0.5 mg/ml) were carefully layered on the filter with the flow stopped. Thereafter, 40 ml of tetrapropyl-ammonium hydroxide-0.02 M EDTA, pH 12.1, containing 0.1% sodium lauryl sulfate, were added and pumped through the filter, in the dark, at 0.035 ml/min. Eluted fractions were collected at 3-hr intervals and mixed with 10 ml of Aquasure (New England Nuclear Corporation) for scintillation counting. Filters were processed as previously described (22).

In these elution assays, ^{14}C-labeled cells receive 300 R X-ray immediately prior to elution to introduce a controlled number of random single-strand breaks. Other cells receive no X-rays and act as controls. As a result of interstrand cross-linking produced by drug treatment, there is an increased retention of labeled DNA on the filter of drug-treated cells resulting in an apparent reduction in the effect of the X-ray-induced breaks. ^{3}H-Labeled untreated cells are mixed with ^{14}C-labeled cells prior to X-ray. These tritiated cells act as an internal standard for the retention of DNA fragments on the filter and reduce quantitative variability due to differences between individual filters. In Fig. 2 the retention of [^{14}C]DNA on the filter is plotted against the retention of [^{3}H]DNA. The steepness of the negative slope indicates the severity of strand breaks. Therefore, the greater the number of interstrand drug-induced cross-links, the less steep is the slope.

Apparent DNA interstrand cross-link frequencies in rad equivalents induced by drugs were determined from the formula (24);

$$[\sqrt{(1 - r_0/(1 - r)} - 1] \times 300 \text{ rads} = \text{cross-link frequency}$$

where r is the fraction of [^{14}C]DNA retained on the filter relative to a constant fraction of [^{3}H]DNA retained for drug-treated cells and r_0 is this fraction for non-drug-treated cells.

Survival curves. Cells treated with polymer, monomer, or phosphoramide mustard for 20 min at 37° were washed three times in fresh medium and assayed for colony survival in soft agar by the method of Chu and Fisher (25). Colonies were counted after 10–14 days of incubation at 37°. Colony formation of control cells was >75%.

Drug treatments in vivo. Phosphoramide mustard (NSC-69945) and CP (NSC-26271), supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, were dissolved in 0.85% sodium chloride and immediately injected i.p. into mice. The polymer and DIVEMA were dissolved in 2% $NaHCO_3$ (pH 8.2) and administered i.p. to mice. The 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide monomer (sparingly water-soluble) was first dissolved in DMSO

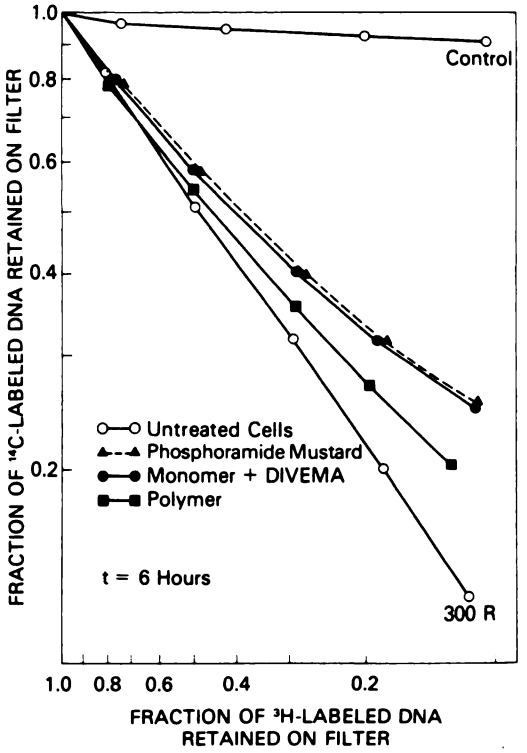

FIG. 2. *Alkaline elution profiles of L1210 cells 6 hr after 20-min treatment with 30 μM monomer, polymer (in hydrolytically released monomer equivalents), or 150 μM phosphoramide mustard*

All cells received 300 R of X-ray prior to alkaline elution except for control cells which received neither drug nor irradiation.

(Fischer Scientific Co., Fairlawn, N. J.), diluted with an equal amount of the polyoxyethylated vegetable oil Emulphor (GAF Corporation, New York, N. Y.), and made up to volume with 0.1 M sodium phosphate buffer (PO_4, pH 7.9) (1:1:4.7, DMSO:Emulphor:PO_4). Such a vehicle proved nontoxic to mice when given i.p. and allowed the sulfido-CP-monomer to be administered i.p. at high concentrations as a true solution rather than as an emulsion.

Animals and tumors. Mice were obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institute. They were housed in plastic cages in groups of 5–10, and given pelleted food and water ad libitum. Male B6D2F_1, mice, weighing 20–25 g, were used in toxicity and tumor experiments. L1210 leukemia was maintained by i.p. passage in male CD2F_1 mice and transplanted into the B6D2F_1 strain by i.p. injection of 0.1 ml of ascites fluid diluted with Hank's solution to contain 10^5 viable cells as determined by trypan blue dye exclusion.

Treatment always began 24 hr following tumor inoculation. Antitumor activity on L1210 was determined by comparing the median survival time of treated groups (T) with that of a control group (C) and expressed as a percentage of increase in median life-span (*ILS*).

$$\%ILS = (T/C - 1) \times 100$$

ILS calculations considered dying animals only. Thirty-day long-term survivors of L1210 were considered cured mice and were noted separately (26).

RESULTS

DNA interstrand cross-linking in vitro. An alkaline elution profile of L1210 cells exposed to polymer, monomer, or phosphoramide mustard for 20 min and incubated with drug free medium for 6 hr is shown in Fig. 2. Since previous work with L1210 cells exposed to monomer for 2 hr indicated that DNA interstrand cross-linking was maximal 6 hr after drug removal (27), we chose this time point for initial experiments involving the 20-min drug exposure. When cells receive only a 20-min exposure to monomer (30 μM), monomer (30 μM) plus DIVEMA, polymer (in monomer equivalents hydrolytically released from the polymer within 20 min), or phosphoramide mustard (150 μM), measurable amounts of DNA interstrand cross-links are observed 6 hr after drug removal. These drug concentrations were used in time course elution studies. Monomer alone or monomer plus DIVEMA give identical results. However, phosphoramide mustard (at 5 times the monomer concentration) or monomer plus DIVEMA produce approximately twice as many interstrand cross-links as the polymer. Separate experiments indicated that 120 μM polymer hydrolyzes to 30 μM monomer by a first-order kinetic rate within 20 min (21).

The formation and removal of DNA interstrand cross-links as a function of time following 20-min treatment and subsequent removal of extracellular monomer, polymer, or phosphoramide mustard is shown in Fig. 3. Apparent DNA interstrand cross-linking is maximal between 4 and 6 hr and completely removed by 18 hr. Although the kinetics of cross-link formation and removal are similar for the three drugs, the polymer, at all times investigated, produces approximately one-half the level of cross-links as the monomer. Phosphoramide mustard, at 5 times the monomer concentration, results in cross-linking frequencies and kinetics similar to those of the monomer.

Cytotoxicity in vitro. The survival of L1210 cells following a 20-min treatment with various doses of polymer, monomer, or phosphoramide mustard, as determined by colony formation in soft agar, is presented in Fig. 4. The D_0 values (dose increments that reduce survival by a factor of $1/e$ in the exponential phase of the curves) are 60 μM for polymer, 30 μM for monomer, and 150 μM for

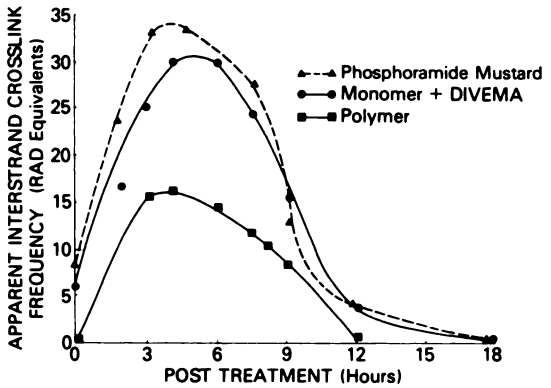

FIG. 3. *Apparent DNA interstrand cross-linking in L1210 cells exposed to 30 μM monomer, polymer (in hydrolytically released monomer equivalents), or 150 μM phosphoramide mustard following a 20-min. exposure*

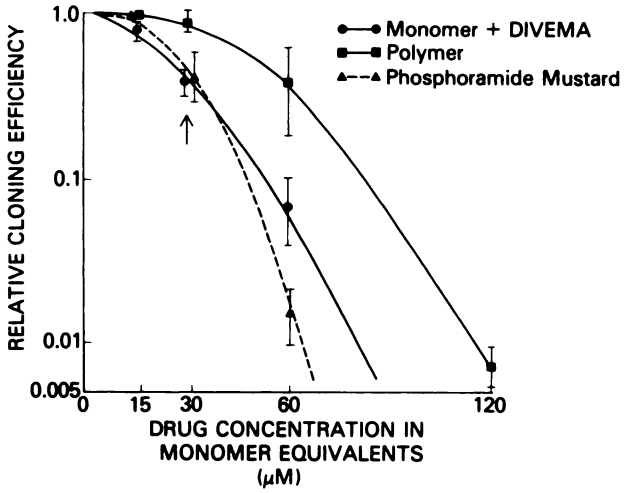

FIG. 4. *Survival of L1210 cells following 20-min treatment with monomer, polymer (in hydrolytically released monomer equivalents), or phosphoramide mustard (5× concentration indicated on abscissa)* *Arrow* indicates drug dose used in alkaline elution assays.

phosphoramide mustard. These doses indicate that the monomer is 5 times more cytotoxic than phosphoramide mustard and the monomer is twice as cytotoxic as the polymer. These cytotoxicity results correspond exactly with the cross-linking results. The drug concentrations used in cross-linking assays produce less than 1 log cell kill of L1210 cells and allow for 89%, 46%, and 42% survival of L1210 cells treated with polymer, monomer, or phosphoramide mustard. Cells treated with DIVEMA alone produce colonies similar to controls. DIVEMA added to the monomer does not influence the monomer's cytotoxicity.

Antitumor activity in vivo. Table 1 summarizes data from experiments comparing the antitumor activity of polymer, monomer, CP, and phosphoramide mustard administered i.p. CP, monomer, and phosphoramide mustard give a 5 log tumor cell kill and many long-term survivors. However, at an equitoxic dose (LD_{10}), the polymer gives a 1 log tumor cell kill and no long-term

TABLE 1

Antitumor activity of CP, phosphoramide mustard, monomer, or polymer in mice

L1210 cells (1×10^5) were inoculated intraperitoneally; drug treatment was given intraperitoneally 24 hr later.

Drug	Dose[a]	%*ILS*[b]	Cures[c]	Tumor cells surviving treatment
	mg/kg			
CP	250	160	6/10	<1
Monomer	200	—	5/5	<1
Phosphoramide mustard	250	167	6/10	<1
Polymer	100	35	0/10	~1×10^4
DIVEMA	90	23	0/10	~5×10^4
Monomer + DIVEMA	10, 90	35	0/10	~1×10^4

[a] LD_{10} except for DIVEMA and Monomer + DIVEMA. The doses for monomer + DIVEMA are equivalent to the content of monomer and DIVEMA in the polymer. This additive combination is less than an LD_{10}.

[b] *ILS*, Increase in median life-span.

[c] Thirty-day survivors of L1210.

survivors. Equivalent amounts of the monomer, when bound covalently to DIVEMA, are much more toxic to mice than is free monomer. A 100-mg/kg dose of polymer, containing only 10 mg/kg of bound monomer and 90 mg/kg of DIVEMA, is equitoxic (LD_{10}) to a 200-mg/kg dose of monomer. A single dose of DIVEMA (90 mg/kg) is nontoxic to mice, as is a single dose of monomer (10 mg/kg) or the combination.

DISCUSSION

Although the DIVEMA-sulfido-CP polymer was synthesized with tumor endocytotic uptake in mind, other behaviors are possible with respect to the interaction of the polymer with L1210 tumor cells. One possibility is that only the monomer freed by hydrolysis and not the monomer covalently bound to the polymer is taken up by L1210 cells. With a drug treatment time of 20 min the effective exposure (concentration × time) of L1210 cells to hydrolytically released equivalents of monomer, when cells are treated with polymer, would be one-half that of drug treatment with molar equivalents of monomer, because of the rate at which the monomer is hydrolyzed from the polymer. In agreement with this effective exposure concept, our data indicate both a cross-linking effect and a cytotoxic effect for monomer treatment which is twice as great as that for treatment with polymer (in hydrolytically released monomer equivalents). Since separate experiments indicated that DNA interstrand cross-linking is proportional to the (C × T) exposure of the monomer, these data suggest that extracellular hydrolytically freed monomer is responsible for the polymer's effect *in vitro*. These data do not support the concept that endocytosis or membrane binding of polymer is taking place in a significant manner.

DNA interstrand cross-linking for monomer and polymer correlates quantitatively with monomer and polymer cytotoxicity. This relationship suggests that DNA interstrand cross-linking in L1210 cells treated with monomer or polymer may be a lethal lesion resulting in cell death (27).

Phosphoramide mustard, the ultimate alkylating species derived from CP (28), is cytotoxic to L1210 cells and demonstrates cross-linking kinetics similar to those of the monomer and polymer. However, phosphoramide mustard requires concentrations 5 times those of the monomer to produce interstrand cross-links and cytotoxicity comparable to that of the monomer. Since the half-life of 4-hydroxycyclophosphamide degradation to phosphoramide mustard is 312 min (29), a drug treatment period of 20 min should allow only approximately 3% of the monomer to degrade to phosphoramide mustard. Since the monomer is 5 times as potent as the mustard, these data imply that the monomer, or some degradation product prior to phosphoramide mustard, is taken up by L1210 cells.

In vitro experiments consistently indicate that the polymer delivers only one-half the dose of alkylating activity to L1210 tumor cells as compared with the dose delivered by hydrolytic equivalents of monomer. If this finding is generalized to other cell types and tissues in the mouse, we might expect the polymer also to exhibit less toxicity than the monomer *in vivo*. However, this is not the case, since the polymer enhances toxicity *in vivo*. This observation is strong evidence that the covalent attachment of a low molecular weight drug to a negatively charged macromolecule can selectively alter the interaction of the drug with normal and tumor cells *in vivo*. DIVEMA, a polyanionic compound under physiological conditions (30), may create an unfavorable interaction with the L1210 tumor cells (31) but a favorable interaction with susceptible normal cells *in vivo*. This finding suggests an exploitable difference between normal cell membranes and L1210 tumor cell membranes. Future investigations will explore the effects of synthetic polymers with a variety of charge densities in similar biological systems.

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REFERENCES

1. Ryser, H. J. P., and W. C. Shen. Conjugation of methotrexate to poly(L-Lysine) increases drug transport and overcomes drug resistance in cultured cells. *Proc. Soc. Natl. Acad. Sci. U. S. A.* **75**:3867–3870 (1978).
2. Chu, C. F., and J. M. Whiteley. Control of solid tumor metastases with a high-molecular-weight derivative of methotrexate. *J. Natl. Cancer Inst.* **762**: 79–82 (1979).
3. Bernstein, A., E. Hurwitz, R. Maron, R. Arnon, M. Sela, and M. Wilchek. Higher anti-tumor efficacy of daunomycin when linked to dextran; *in vivo* and *in vitro* studies. *J. Natl. Cancer Inst.* **60**:379–384 (1978).
4. Ringsdorf, H. Structure and properties of pharmacologically active polymers. *J. Polym. Soc.* **51**:135–153 (1975).
5. De Duve, C., T. De Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoff. Lysosomotropic agents. *Biochem. Pharmacol.* **23**:2495–2531 (1974).
6. Rowland, G. F., G. J. O'Neill, and D. A. L. Davies. Suppression of tumor growth in mice by a drug-antibody conjugate using a novel approach to linkage. *Nature (Lond.)* **255**:487–488 (1975).
7. Trouet, A., D. D. Campeneere, and C. De Duve. Chemotherapy through lysosomes with a DNA-daunorubicin complex. *Nature [New Biol.]* **239**:110–112 (1972).
8. Jacobs, S. A., M. d'Urso-Scott, and J. R. Bertino. Some biochemical and pharmacologic properties of amethopterin-albumin. *Ann. N. Y. Acad. Sci.* **186**:284–286 (1971).
9. Merigan, T. C., and W. Regelson. Interferon induction in man by a synthetic polyanion of defined composition. *N. Engl. J. Med.* **277**:1283–1287 (1967).
10. Schultz, R. M., J. D. Papamatheakis, and M. A. Chirigos. Direct activation *in vitro* of mouse peritoneal macrophages by pyran copolymer (NSC-46015). *Cell Immunol.* **29**:403–409 (1977).
11. Kapila, K., C. Smith, and A. A. Rubin. Effect of pyran copolymer on phagocytosis and tumor growth. *Res J. Reticuloendothel. Soc.* **9**:447–450 (1971).
12. Morahan, P. S., J. A. Munson, L. G. Baird, A. M. Kaplan, and W. Regelson. Antitumor action of pyran copolymer and tilorone against Lewis lung carcinoma and B-16 melanoma. *Cancer Res.* **34**:506–511 (1974).
13. Przybylski, M., E. Fell, and H. Ringsdorf. Syntheses and characterization of polymeric derivatives of the antitumor agent methotrexate. *Makromol Chem.* **179**:1719–1733 (1978).
14. Hill, D. L. *A Review of Cyclophosphamide*. Charles C Thomas, Chicago, 3–7, (1975).
15. Brock, N., and H. J. Hohorst. Uber die aktivierung von cyclophosphamid *in vivo* und *in vitro*. *Arzneim. Forsch.* **13**:1021–1031 (1963).
16. Hirano, T., W. Klesse, J. H. Heusinger, G. Lamber, and H. Ringsdorf. Synthesis of activated cyclophosphamide derivatives bearing functional groups. *Tetrahedron Lett.* **10**:883–886 (1979).
17. Peter, G., and H. J. Hohorst. Synthesis and preliminary antitumor evaluation of 4-(SR)-sulfido-cyclophosphamides. *Cancer Chem. Pharmacol.* **3**:181–188 (1979).
18. Peter, G., T. Wagner, and H. J. Hohorst. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. *Cancer Treat. Rep.* **60**:429–435 (1976).
19. Hirano, T., W. Klesse, and H. Ringsdorf. Polymeric derivatives of activated cyclophosphamide as drug delivery systems in anti-tumor chemotherapy. *Makromol. Chem.* **180**:1125–1131 (1979).
20. Ramonas, L. M., L. C. Erickson, H. Ringsdorf, and D. S. Zaharko. Effect of

dose, schedule, and route of administration on the *in vivo* toxicity and antitumor activity of two activated sulfhydryl derivatives of cyclophosphamide. *Cancer Res.* **40**:3704–3708 (1980).
21. Hirano, T., H. Ringsdorf, and D. S. Zaharko. Anti-tumor activity of monomeric and polymeric cyclophosphamide derivatives compared with *in vitro* hydrolysis. *Cancer Res.* **40**:2263–2267 (1980).
22. Kohn, K. W., L. C. Erickson, R. A. G. Ewig, and C. A. Friedman. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**:4629–4637 (1976).
23. Ewig, R. A. G., and K. W. Kohn. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3,-bis (2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res.* **37**:2114–2122 (1977).
24. Ewig, R. A. G., and K. W. Kohn. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. *Cancer Res.* **38**:3197–3203 (1978).
25. Chu, M. Y., and G. A. Fisher. Incorporation of cytosine-^{3}H-arabinoside and its effects on the murine leukemia cells (L5178Y). *Biochem. Pharmacol.* **17**: 753–767 (1968).
26. Schabel F. M., Jr., D. P. Griswold, Jr., W. R. Laster, Jr., T. H. Corbet, and H. H. Lloyd. Quantitative evaluation of anticancer activity in experimental animals. *Pharmacol. Ther. Part A Chemother. Toxicol. Metab. Inhibitors* **1**: 411–435 (1977).
27. Erickson, L. C., L. M. Ramonas, D. S. Zaharko, and K. W. Kohn. Cytotoxicity and DNA crosslinking activity of 4-sulfido-cyclophosphamides in mouse leukemia cells *in vitro*. *Cancer Res.* **40**:4216–4220 (1980).
28. Brock, N. Comparative pharmacologic study in vitro and in vivo with cyclophosphamide (NSC-26271), cyclophosphamide metabolites, and plain nitrogen mustard compounds. *Cancer Treat. Rep.* **60**:301–308 (1976).
29. Hohorst, H. J., U. Draeger, G. Peter, and G. Voelcker. The problem of oncostatic specificity of cyclophosphamide (NSC-26271): studies on reactions that control the alkylating and cytotoxic activity. *Cancer Treat. Rep.* **60**:309–315 (1976).
30. Breslow, D. S. Biologically active synthetic polymers. *Pure Appl. Chem.* **46**: 103–113 (1976).
31. Olsnes, S., K. Refsnes, and A. Pihl. Mechanism of action of the toxic lectins abrin and ricin. *Nature* (*Lond.*) **249**:627–631 (1974).

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