Cancer Chemother Pharmacol (1986) 16: 85–90

Cancer Chemotherapy and Pharmacology

Cytotoxicity and metabolism of prednimustine, chlorambucil and prednisolone in a Chinese hamster cell line

Beryl Hartley-Asp, Per Olov Gunnarsson, and Jan Liljekvist

Aktiebolaget Leo, Research Laboratories, Box 941, S-251 09 Helsingborg, Sweden

Summary. Prednimustine and chlorambucil induce dose- and time-dependent cell death in V79 Chinese hamster cells in vitro. Prednimustine was found to be 3–4 times more potent than either chlorambucil or an equimolar mixture of its components chlorambucil and prednisolone after 24 h treatment.

Prednimustine was hydrolyzed to prednisolone and chlorambucil in the system, and the concentration of prednimustine was reduced by one half within 15 h. Prednisolone was not further metabolized, but chlorambucil was rapidly inactivated by dechlorination, the half-life being 2.5 h. No dechlorinated prednimustine was formed during the experiments. The higher stability of prednimustine than chlorambucil is probably due to protective binding to different serum proteins from those that bind chlorambucil.

Substitution of fetal calf serum by human serum albumin revealed that hydrolysis of prednimustine is catalyzed by esterases present in the serum. In similar substitution experiments cell survival studies indicated that prednimustine itself was not cytotoxic. Rather, cytotoxicity was found to correlate with hydrolysis to chlorambucil. Thus, it appears that the prolonged availability of chlorambucil is responsible for the increased potency of prednimustine in this system.

Introduction

Chlorambucil, a bifunctional alkylating agent, is active against several murine tumors [8]. This activity is thought to be due primarily to its reaction with DNA [16], but its binding to nuclear proteins [15] may also cause disturbances which lead to cell death. Prednimustine, a prednisolone-21 ester of chlorambucil, exhibits a similar profile to that of chlorambucil in murine tumors [6, 8], but also demonstrates activity against chlorambucil-resistant tumours [8]. Prednimustine is effective in the treatment of non-Hodgkin's lymphoma [3], and chronic lymphocytic leukemia [2] as is the combination chlorambucil and prednisolone [9]. However, in breast cancer a recent phase III trial showed that prednimustine produced a superior response rate to that obtained with the combination chlorambucil and prednisolone [10]. Thus, although prednimustine is known to be readily hydrolyzed to chlorambucil and prednisolone in the body [5, 13, 14], it appears that its efficacy in breast cancer cannot be mimicked by this combination. We have tried to find the reason for the higher efficacy of prednimustine by using cell survival experiments in combination with metabolism studies.

Offprint requests to: B. Hartley-Asp

Materials and methods

Chemicals. Chlorambucil [4-*p*-(bis-(2-chloroethyl)-amino)phenylbutyrate] was purchased from ISM, Italy. Prednisolone (11,17,21-trihydroxy pregna-1, 4-diene-3, 20-dione) was obtained from Roussel, Uclaf, France. Prednimustine, the 21-chlorambucil ester of prednisolone (Fig. 1) [4-*p*-(bis-(2-chloroethyl)-amino)phenylacetate; PAM] and 4-*p*-(bis-(2-hydroxyethyl)-amino)phenylbutyrate (Leo 1333) were synthesized at AB Leo, Helsingborg, Sweden. Other nonradioactive chemicals and reagents used were of highest available purity and were used without further purification.

[^{3}H]-Prednimustine (specific activity 80 μCi/mg [^{14}C]prednimustine (specific activity 22 μCi/mg) and [^{14}C]chlorambucil (specific activity 27 μCi/mg) were prepared at AB Leo, and [^{3}H]prednisolone (specific activity 147 mCi/mg) was bought from Amersham, England. The postitions of the labels are shown in Fig. 1. [^{3}H, ^{14}C]prednimustine was prepared by mixing equal amounts of [^{3}H]prednimustine and [^{14}C]prednimustine. Prior to use the radioactive compounds were purified by high-performance liquid chromatography (HPLC). The purity was at least 98%.

Acrylamide, N,N'-diallytartardiamide *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, periodic acid, and riboflavin were obtained from BIO-RAD, Richmond, Calif, USA.

All tissue culture media were purchased from Gibco Biocult, Scotland.

Cell cultivation and clonogenic assay. The Chinese hamster V79-4 cell line derives from the lung of a male Chinese hamster and orginates from Dr. E. H. Y. Chu, Oak Ridge National Laboratory, Tenn, USA. The cell line was maintained as a monolayer in Dulbecco's medium, supplemented with 15% fetal calf serum and the antibiotics penicillin and streptomycin. Under these conditions the cells grew logarithmically.

Cells from the day after subculture, which was carried out with 0.085 *M* sodium citrate solution, were used for all

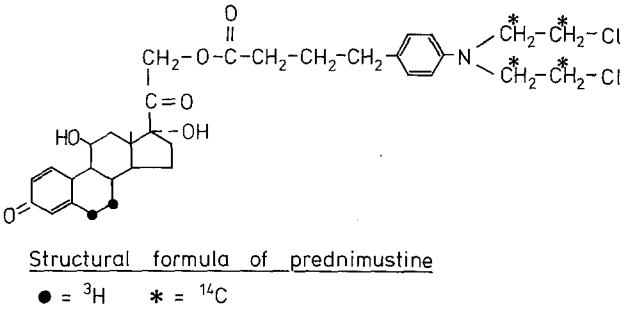

Fig. 1. Structural formula and position of radiolabel for prednimustine. *^{14}C; ●^{3}H

experiments. The cells were serially diluted to produce not more than 250 colonies per petri dish. The petri dishes were randomly divided into groups of five for treatment. A control group was present in every experiment. The cells were incubated at 37 °C in a 5% CO_2:95% air atmosphere for 24 h before treatment to eliminate the lag phase.

All substances were dissolved in ethanol and diluted with medium so that the final ethanol concentration did not exceed 0.2%. Approximate equimolar concentrations of chlorambucil and prednisolone were used in the combination studies.

Treatment was carried out and the cells rinsed with fresh medium to remove all traces of test substance. Plating efficiency was determined after 7 days by counting the number of methylene blue-stained colonies in an Artex counter, Model 880. The control colonies in each experiment served as reference for diameter settings and survival is given as a percentage of the control value.

Metabolism studies. Treatment with radiolabelled compounds was carried out as above, using 30 μg/ml prednimustine or a 15+15 μg/ml chlorambucil/prednisolone mixture. In these experiments drugs were dissolved in DMSO, to give a final concentration of 0.2%.

[^{14}C]Chlorambucil and [^{3}H]prednisolone were diluted with unlabelled substance to yield the same specific activity as [^{3}H, ^{14}C]prednimustine.

Extraction. Medium samples were diluted with the mobile phase and injected directly into the chromatograph. The cells were suspended with Na citrate, spun down, washed with Hank's balanced salt solution, and resuspended in 1 ml before extraction with 3×2 volumes of hexane containing 7% isoamyl alcohol. If necessary, emulsion was broken by freezing. The organic phases were pooled, dried under a gentle stream of nitrogen, and dissolved in the mobile phase before chromatography.

High-performance liquid chromatography. The liquid chromatograph consisted of a Model 6000 A pump, a guard column, a U6K injector and a Model 440 UV-detector, 254 nm (Waters Assoc., Milford, USA). The column was a C_{18} bonded-phase column, 3.9×300 mm (μ-Bondapak, Waters Assoc.). The chromatograms were recorded on a W+W TARKAN recorder 600, and the outlet of the detector was connected to a LKB 7000 Ultrorac fraction collector.

The mobile phase was methanol: 0.1 *M* acetic acid (80:20 by volume). The flow rate was 400 μl/min, and the volume of each fraction was 120 μl.

Authentic prednimustine, chlorambucil, prednisolone, PAM, and Leo 1333 were added to the samples before injection (5–10 μg dissolved in the mobile phase). The localization of each of the standard compounds was identified by their UV peaks.

Polyacrylamide gel electrophoresis. The double-gel discontinuous electrophoresis system of Davis [3] was used with the following modifications: (a) To solubilize the gels for liquid scintillation counting, the crosslinking agent *N,N'*-diallyltartardiamide [1] was used; and (b) to increase the resolution the separation gels were subjected to a 3-h pre-electrophoresis prior to the addition of sample gels [11].

Determination of radioactivity. HPLC and PAGE fractions were counted in 5 ml Aquassure in a Packard Tri-Carb Liquid Spectrometer model 2650, for at least 10 min or 8×10^5 counts. Corrections for quenching were made by the external standard channels ratio method.

Results

Toxicity studies

Cell death induced by chlorambucil and prednimustine is both dose- and time-dependent. The survival curves for chlorambucil and prednimustine after a 24-h treatment are given in Fig. 2. This shows that both substances cause dose-dependent cell death, and that prednimustine is at least 3 times as effective as chlorambucil throughout the dose range after a 24-h treatment. In these experiments 95% kill was achieved for prednimustine: higher concentrations could not be used due to solubility problems. The 24-h treatment period was required to obtain full expression of cell toxicity for prednimustine, whereas the maximum cell kill for chlorambucil was reached after 6 h

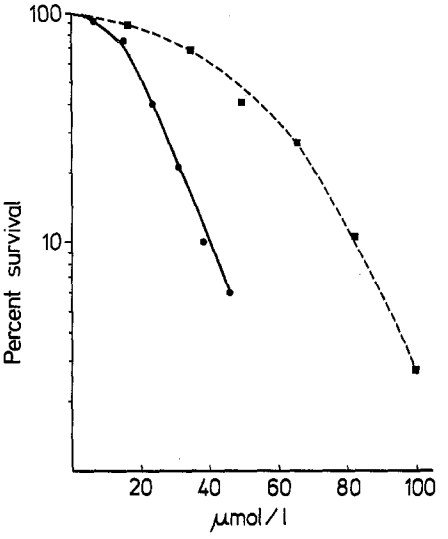

Fig. 2. The effect of 24-h treatments with chlorambucil (– – ■ – –) and prednimustine – ● –) on the survival of Chinese hamster V79 cells. Data are means of two separate experiments

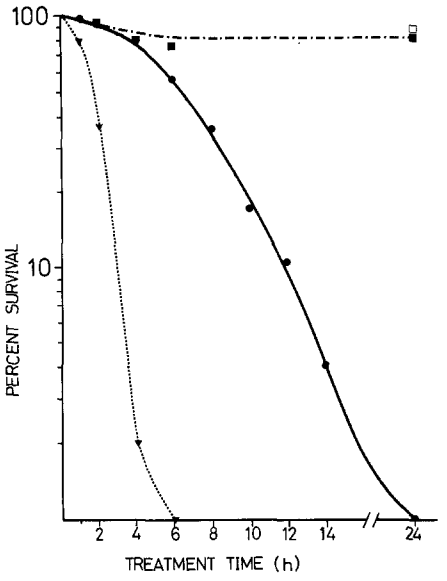

Fig. 3. The effect of different lengths of treatment with prednimustine 30 µg/ml (46.4 µmol) (– ● –), chlorambucil 15 µg/ml (49.3 µmol) (□) and 30 µg/ml (98.6 µmol (...▼...), and a combination of chlorambucil and prednisolone 15 + 15 µg/ml (49.3 + 54 µmol) (– · – ■ – · –) on Chinese hamster V79 cells. Data are means of two separate experiments

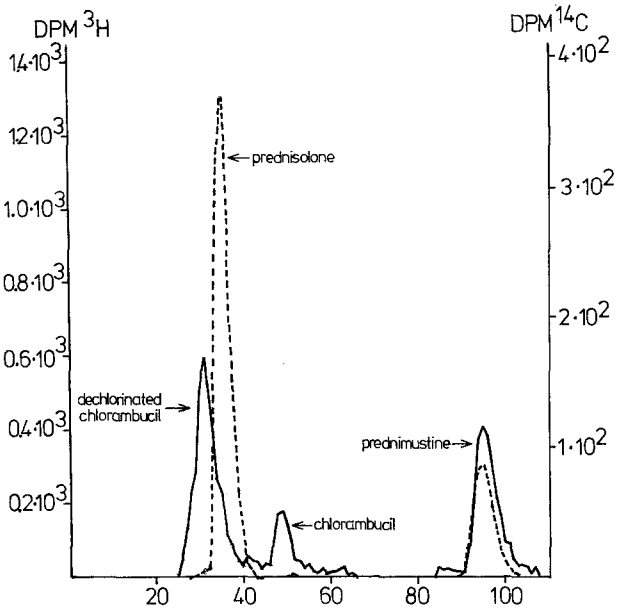

Fig. 5. Metabolism of prednimustine. Elution profile of radioactivity after high-performance liquid chromatography of a medium sample after 12 h incubation of [^{3}H, ^{14}C]prednimustine (30 µg/ml) with V79-4 – – –, ^{3}H; ———, ^{14}C

(Fig. 3). Figure 3 also shows that a one-to-one combination of chlorambucil plus prednisolone (15 + 15 µg/ml) only produced 18% cell death even after a 24-h incubation, whereas the corresponding concentration of prednimustine (30 µg/ml) caused 100% cell death. The combination was not superior to chlorambucil alone at any concentration, as is demonstrated in Fig. 3 for 15 µg/ml chlorambucil. Various other combination treatment schedules were tried, but no enhancement was found whether prednisolone was given before or after chlorambucil treatment. Increasing the concentration of prednisolone to a level which induced cell death still only produced an additive effect on combination with chlorambucil (Fig. 4).

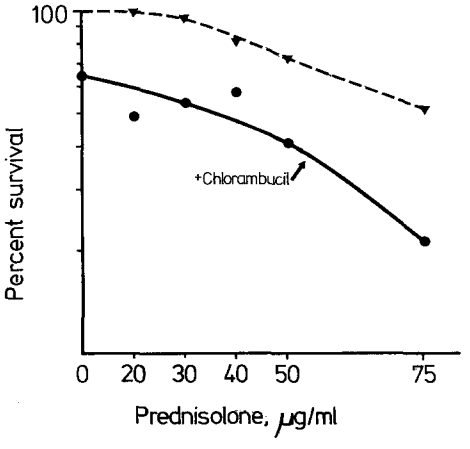

Fig. 4. The effect of 1 h 30 µg/ml chlorambucil in combination with a 24-h treatment with prednisolone (– ● –) at various concentrations and prednisolone alone (– – ▲ – –) on V79 cells.

Metabolism studies

To obtain further information on the biotransformation of prednimustine, [^{3}H, ^{14}C]prednimustine was incubated with V79 cells. The metabolite pattern in the medium is exemplified by a HPLCchromatogram obtained after a 24-h incubation period with prednimustine (Fig. 5). Besides prednimustine, prednisolone, chlorambucil, and dechlorinated chlorambucil were also present in the incubate, the latter via a chemical and not an enzymatic process. Although authentic dechlorinated prednimustine is not available, this compound would be much more hydrophilic and would therefore separate from prednimustine. Since no double-labelled compound other than prednimustine was found, it can be concluded that no dechlorinated prednimustine was formed in this system.

The kinetics of the metabolism of prednimustine, 30 µg/ml (Fig. 6a), reveal that the reaction is linear with time, which suggests zero-order kinetics. This is in agreement with an enzyme-catalyzed reaction with Michaelis-Menten kinetics under saturated conditions. The time needed to reduce the prednimustine concentration to one half was 15 h. As a comparison, the metabolite pattern after incubation with a combination of [^{14}C]chlorambucil and [^{3}H]prednisolone, 15 + 15 µg/ml, was studied (Fig. 6b). Prednisolone was quite stable under the conditions used, but chlorambucil was rapidly dechlorinated. The time course of chlorambucil degradation followed first-order kinetics; the half-life of the reaction was 2.5 h.

Extractions of cells treated with [^{3}H, ^{14}C]prednimustine showed that the intracellular metabolite pattern did not differ from that of the medium. The medium contains 10% fetal calf serum (FCS), and thus it is reasonable to assume that esterases present in serum are responsible for the hydrolysis of prednimustine, by analogy with previous studies using enzymes from plasma and serum [19]. To test this hypothesis we performed a series of experiments with me-

Table 1. Metabolism and survival of V79 Chinese hamster cells after treatment with [^{3}H, ^{14}C]+prednimustine, 30 μg/ml in the presence of 10% (v/v) fetal calf serum or 1% (w/v) human serum albumin

	Remaining prednimustine (%)						
Length of treatment (h)	0	2	4	6	8	12	24
10% FCS		94.8	90.8	83.8	81.2	71.7	37.5
1%HSA		98.3	97.2	95.5	90.7	89.6	76.5
				Survival (%)			
10% FCS	100	102	90.7	90.2	70.2	43.1	4.6
1% HSA	100	112	97.7	106	103	55.1	5.6

Plating efficiency: 10% FCS, 39%; 1% HSA, 30%. Results shown are means of two experiments

dium in the absence of hamster cells. The metabolite pattern and kinetics of the reactions were very much the same as in the presence of cells. Thus, our experiments strongly suggest that medium is responsible for the hydrolysis of prednimustine to chlorambucil.

To investigate whether hydrolysis of prednimustine to its components was connected with differences in cell death, experiments in which the 10% (v/v) FCS was substituted by 1% (w/v) human serum albumin (HSA) were carried out. The selection of 1% HSA was made because this amount of albumin held prednimustine in solution and gave a distinctly lower rate of hydrolysis than 10% FCS. The amount of albumin present on 10% FCS addition is approximately 2%. As seen in Table 1, hydrolysis of prednimustine takes place at a much slower rate in the presence of HSA than in the presence of FCS. The survival data show a similar pattern, cell death beginning to be evident between 6 and 8 h during treatment with FCS, whereas none occurs until 12 h during treatments with HSA. This indicates that prednimustine is inactive as an intact molecule, and that it is the release of chlorambucil which induces cell death.

The lack of cytotoxic activity of prednimustine itself might be due to protective binding in the incubation medium. Therefore, the binding pattern of prednimustine and the combination of chlorambucil and prednisolone was investigated by means of polyacrylamide electrophoresis. In both instances binding to the albumin band occurred. However, different patterns of binding to other proteins in the medium were found (Fig. 5). Prednimustine was found to bind prominently to proteins migrating slowly, which are found in the top slices of the gel column. Marked binding to the corticosteroid-binding globulin (CBG) migrating just behind albumin was also found. This kind of binding pattern was never seen in any medium containing the mixture of chlorambucil and prednisolone. The lack of binding of prednisolone to CBG is most probably due to a slow equilibration between prednisolone and CBG. This assumption is supported by the appearance of prednisolone

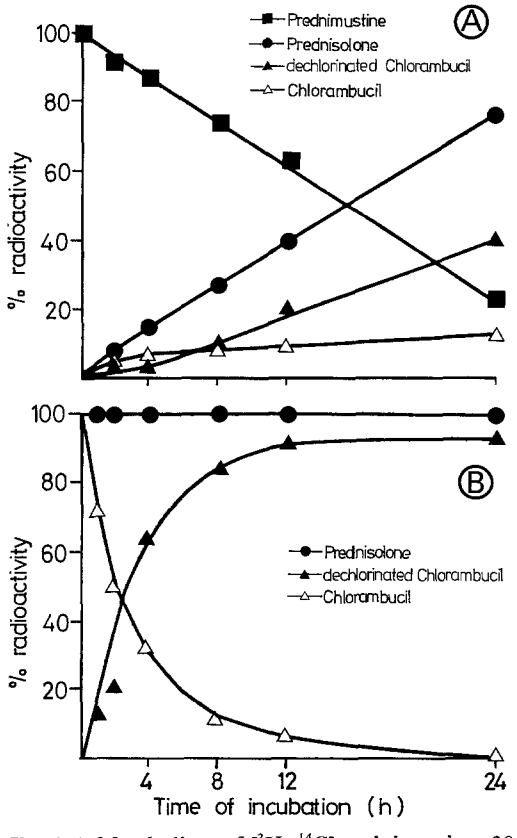

Fig. 6. A Metabolism of [^{3}H, ^{14}C]prednimustine, 30 μg/ml, in the V79 cell medium system. **B** Metabolism of chlorambucil and prednisolone, 15+15 μg/ml, in the V79 cell medium system. Percentage of radioactivity in the medium is presented. Data are means of two independent experiments

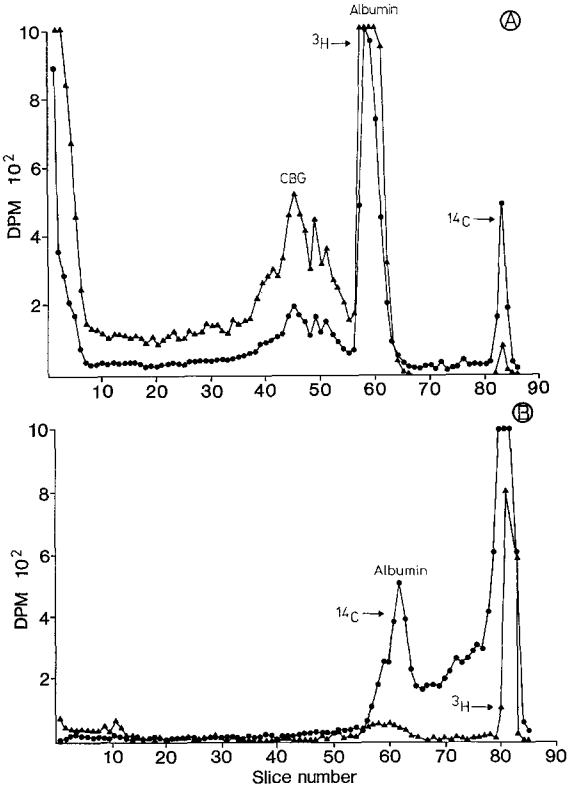

Fig. 7. A, B. Polyacrylamide electrophoresis of incubation medium after treatment with **A** ^{14}C, ^{3}H-prednimustine: ^{14}C activity, – ● –; ^{3}H-activity, – ▲ –; **B** a mixture of ^{14}C-chlorambucil (– ● –) and ^{3}H-prednisolone (– ▲ –)

at the position of CBG in samples taken at later times (results not shown). In the case of the mixture, radioactivity from chlorambucil is present between the albumin band and the marker dye.

Discussion

In Chinese hamster V79 cells prednimustine is 3–4 times as active as chlorambucil or an equimolar mixture of chlorambucil and prednisolone after 24 h treatment. Furthermore prednimustine-induced cell death is very time-dependent, being progressive during 24 h before the maximum effect is reached, whereas the maximum effect for chlorambucil is obtained after 6 h.

The dose-response data for chlorambucil agrees with that found by other authors for hamster cells [7, 16]; thus, the increased potency of prednimustine makes this a useful model for studying differences in action between the mixture and prednimustine. Preliminary data indicate similar differences for human breast cancer cells.

Prednimustine is hydrolyzed to prednisolone and chlorambucil by esterases present in the FCS supplement, but in this system prednisolone does not appear to play a contributory role in the enhanced action of prednimustine (see Figs. 3 and 4). Thus, we are left with the possibilities that prednimustine is active as an intact molecule or that an increased availability of chlorambucil after prednimustine is responsible for the higher efficacy. The latter hypothesis is more likely however, as previous experiments with Walker carcinoma and L1210 cells in vitro have shown that prednimustine cytotoxicity increases when a higher rate of hydrolysis is induced by addition of hog esterases [19]. However, to further substantiate this, FCS was substituted by HSA, which has a much lower level of esterase activity. These studies showed that the kinetics of cell death followed the hydrolysis of prednimustine to chlorambucil and thus indicate that prednimustine per se does not cause cell death. Rather, a prolonged availability of chlorambucil may be responsible for the difference in effect.

The metabolism data demonstrate that with the combination, although the cells are exposed to high levels of chlorambucil and prednisolone during the first 4 h of treatment, the concentrations of chlorambucil are very low at later time points. In fact, after 8 h the concentration was lower than that obtained upon treatment with prednimustine. Therefore, we suggest that the decrease in survival of cells treated with prednimustine is due to a continuous exposure to chlorambucil. Thus, cells which are in various phases of the cell cycle on passage into the S phase are exposed to a sufficient level of chlorambucil to cause cell death. This hypothesis is partially substantiated by results obtained by Newell et al. [12] in a Yoshida sarcoma resistant to chlorambucil. They demonstrated that the antitumor effect of a single SC injection of 40 mg/kg prednimustine could be mimicked by 20 doses of 1 mg/kg chlorambucil + prednisolone given at 2-h intervals.

The plasma protein binding of prednimustine is very high (unpublished results), and the present study shows that the binding of prednimustine to the protein fraction of the medium is more extensive than that of chlorambucil, and also qualitatively different. This sequestration of prednimustine probably makes the chloroethyl group of the molecule less vulnerable to dechlorination and thereby more stable than that of chlorambucil. Although sequestration is known to occur for other alkylating agents, such as nitrogen mustard and nitrosoureas [17, 18], this appears to be more pronounced for prednimustine. It is also reasonable to belive that the protein binding governs the rate of hydrolysis and thereby the cytotoxicity of prednimustine.

Pharmacokinetic studies in patients have indicated that the elimination of chlorambucil after administration of prednimustine is prolonged compared with that of chlorambucil per se [5], and it has been speculated that this finding indicates that the rate of elimination of chlorambucil is governed by the rate of formation, i.e., a flip-flop mechanism [14]. This, together with the results of this study, indicates that the increased clinical efficacy of prednimustine over that of its components is due to a prolonged availability of chlorambucil.

Acknowledgement. We would like to thank Eva Berggren, Leif Larsson, and Eva Sundberg for excellent technical assistance.

References

1. Anker HS (1970) A solubilizable acrylamide gel for electrophoresis. FEBS Lett 7: 293
2. Brandt L. Könyves I, Möller TR (1975) Therapeutic effect of Leo 1031, an alkylating corticosteroid ester in lymphoproliferative disorders: 1. Chronic lymphocytic leukemia. Acta Med Scand 197: 317
3. Cavallin-Ståhl E, Möller TR (1985) Prednimustine versus CVP in the treatment of non – Hodgkin's lymphoma with favourable histo-pathology – An update. Seminars in Oncology (in press)
4. Davis BJ (1964) Disc electrophoresis: II. Method and application to human serum proteins. Ann NY Acad Sci 121: 404
5. Ehrsson H, Wallin I, Nilsson SO, Johansson B (1983) Pharmacokinetics of chlorambucil in man after administration of the free drug and its prednisolone ester (Prednimustine, Leo 1031). Eur J Clin Pharmacol 24: 251
6. Fredholm B, Gunnarsson K, Jensen G, Müntzing J (1978) Mammary tumour inhibition and subacute toxicity in rats of prednimustine and its molecular components chlorambucil and prednisolone. Acta Pharmacol Toxicol 42: 159
7. Froese G, Hamade JF, Linford JH (1969) A comparison of the biological activities and of the chemical properties of chlorambucil and Trenimon. Cancer Res 29: 800
8. Harrap KR, Riches PG, Gilby ED, Sellwood SM, Wilkinson R, Könyves I (1977) Studies on the toxicity and antitumour activity of prednimustine, a prednisolone ester of chlorambucil. Eur J Cancer 13: 873
9. Ideström J, Kimby E, Björkholm M, Mellstedt H, Engstedt L, Garthon G, Johannson B, Killander D, Robérts KH, Stalfelt AM, Udén AM, Wadman B, Wählby S (1982) Treatment of chronic lymphocytic leukaemia and well-differentiated lymphotic lymphoma with continuous low- or intermittent high-dose prednimustine versus chlorambucil/prednisolone. Eur J Cancer Clin Oncol 18: 1117
10. Løber J, Mouridsen HT, Christiansen IE, Dombernovsky P, Mattson W, Rorth M (1983) A phase III trial comparing prednimustine (Leo 1031) and chlorambucil plus prednisolone in advanced breast cancer. Cancer 52: 1570
11. Maurer HR (1971) Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. Walter de Gruyter, Berlin, pp 59
12. Newell DR, Shepherd CR, Harrap KR (1981) The pharmacokinetics of prednimustine and chlorambucil in the rat. Cancer Chemother Pharmacol 6: 85
13. Newell DR, Calvert AH, Harrap KR, McElwain TJ (1983) Studies on the pharmacokinetics of chlorambucil and prednimustine in man. Br J Clin Pharmacol 15: 253

14. Plym Forshell G, Gunnarsson PO, Liljekvist J (1983) On the clinical pharmacology of prednimustine. Br J Pharmacol 16: 760
15. Riches PG, Harrap KR (1975) The binding of ^{3}H-chlorambucil to nuclear proteins of the Yoshida Acites Sarcoma. Chem Biol Interact 11: 291
16. Roberts JJ (1975) Inactivation of the DNA template in HeLa cells treated with chlorambucil. Int J Cancer 16: 91
17. Takahashi I, Ohnuma T, Kavy S, Bhardway S, Holland JF (1980) Interaction of human serum albumin with anticancer agents in vitro. Br J Cancer 41: 602
18. Weinkam RJ, Finn A, Levin VA, Kane JP (1980) Lipophilic drugs and lipoproteins: Partitioning effects on chloroethyl-nitrosourea reaction rates in serum. J Pharmacol Exp Ther 214: 318
19. Wilkinson R, Gunnarsson PO, Plym-Forshell G, Renshaw J, Harrap KR (1978) The hydrolysis of prednimustine by enzymes from normal and tumour tissues. Excerpta Medica, Amsterdam, p 260 (International congress series, vol 420)

Received July 25, 1985/Accepted July 5, 1985